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Glucokinase and Glycemic Disease

From Basics to Novel Therapeutics

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Preface

This book was motivated by the desire of both the editors and other scientists who have had a lifelong interest in glucokinase to bring together, in one source, a condensed summary of our knowledge about this enzyme.

We were motivated to collect this information, at this point in time, by the discovery of glucokinase-activating compounds. We hope that this text will help others to translate this discovery into something of benefit for the many people who suffer from diabetes.

Our hope is that this text does not reflect the high watermark for the interest in glucokinase. Rather, we both believe that there is additional knowledge of fundamental importance that remains to be discovered. Indeed, the discovery of glucokinase-activating compounds opens a vast new arena of potential therapeutics, the actual impact of which is yet to unfold.

We thank Hoffman-LaRoche, Inc., for the financial support that made publishing this text possible, and Joe Grippo and Joe Grimsby for sharing the excitement of discovering the glucokinase-activating compound. We also thank Vesselina Panteva for assistance throughout this project.

Lastly, we also thank our wives, Elke Matschinsky and Lucile Houseworth, for their undying support of our academic interests and sometimes seemingly eccentric tendencies. Without their support, our accomplishments would have certainly been both more difficult and less satisfying.

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Mark A. Magnuson

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Glucokinase as a Glucose Sensor: Past, Present and Future

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Glucose is essential for life, but devastating consequences occur when the concentration of this sugar derivates from a narrow range. Insufficient glucose, or hypoglycemia, causes loss of consciousness, and eventually death, since the brain is dependent on it as an energy source. Conversely, sustained hyperglycemia, or diabetes mellitus, causes widespread metabolic derangements and eventually damages vital tissues via non-enzymatic glycosylation of proteins. If left unchecked, renal failure, blindness and cardiovascular disease occur. Fortunately, intricate homeostatic mechanisms that maintain the blood glucose concentration in a narrow physiological range have evolved and serve to assure both our health and well being as long as they are maintained.

Homeostatic feedback loops require both effectors and sensors. The two main effectors for regulating the blood glucose via a homeostatic feedback loop are insulin and glucagon. Other hormones such as catecholamines, glucocorticoids, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) also function as effectors, but serve largely to modulate either the secretion or action of insulin or glucagon. However, while the role of these hormonal effectors in regulating the blood glucose concentration has been apparent for many years, only over the past decade has the role of the enzyme glucokinase (GK) as a sensor component in the glucose homeostatic feedback loop become widely accepted. Indeed, what were largely hypothetical models about the role of this enzyme 10 years ago have progressed to the state where these concepts are generally beyond dispute.

The concept of a glucose sensor has been discussed for decades [1]. However, of the many different glucose-responsive cell types that have been

described only two of which, pancreatic β-cells and hepatocytes, have been sufficiently studied for the roles of specific molecules required for glucose sensing to be understood. The mechanisms whereby other glucose-responsive cell types, such as gut enterocytes and certain hypothalamic neurons, sense and respond to glucose remains less certain. Nonetheless, it is clear that the responses evoked by changes in glucose concentrations vary widely for different glucose sensitive cells. For instance, the response of neuroendocrine cells is hormone secretion (e.g. insulin, glucagon, GLP-1, and GIP) whereas neural cells respond by either hyperpolarization or depolarization of their plasma membrane. For neural/neuroendocrine cells, the cellular response can occur within seconds of the change in glucose concentration. In contrast, for hepatocytes, which are also glucose responsive, alterations in the glucose concentration invoke alterations in metabolic flux and, over a somewhat longer time period, the hepatic gene expression profile. This leads to marked changes in the hepatic metabolic pathways, particularly those that affect carbohydrate disposition.

Although the functions of the different glucose sensing cell types that exist within our bodies vary, together they form a complex glucose sensing network that is essential for maintaining glucose homeostasis, as is illustrated in figure 1. Indeed, glucose-sensitive cells are an essential part of the homeostatic feedback loops that assure that the blood glucose concentration is maintained within a safe and tolerable range, thereby avoiding the negative consequences of either too much or too little of this energetically valuable, but chemically reactive molecule.

Brief Historical Perspective

The discovery of the hexokinases occurred approximately 40 years ago and quickly led to the recognition that these enzymes were a cornerstone of glucose metabolism [2–4]. It was soon recognized that one of these enzymes, hexokinase IV or GK, had functional and structural features that set it apart from the others [5]. The key distinguishing features included a mass of approximately half that of hexokinase types I–III, a markedly lower affinity for glucose, and a lack of significant feedback inhibition by the end-product glucose-6-phosphate [6, 7]. GK was first thought to be restricted only to the liver, where it was regulated in a manner that suggested it had a major role in glucose uptake by this tissue [8]. However, the enzyme was found to also be expressed in mouse pancreatic β-cells, where it was immediately recognized to play a key role in glucose-stimulated insulin secretion [9]. At the same time it was discovered that intracellular glucose of β-cells was practically equal to plasma glucose and that glucose transport had a very high capacity showing that GK could

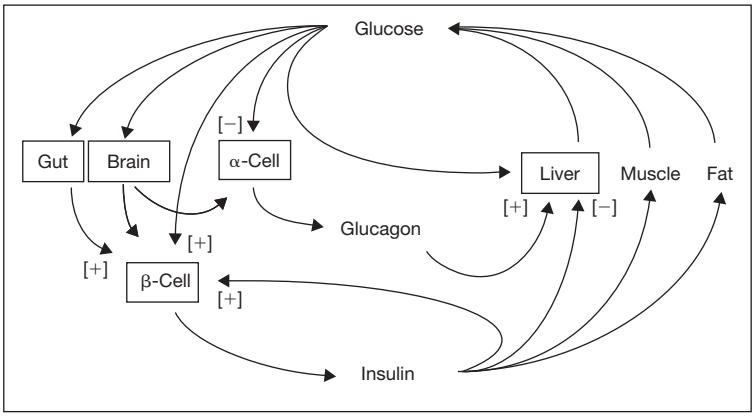

Fig. 1. Essential role for glucose sensors in the feedback loops that maintain glucose homeostasis. Both hormonal effectors (shaded) and glucose sensitive cells (boxed) are necessary for a closed feedback loop. Glucagon stimulates hepatic gluconeogenesis thereby increasing the plasma glucose concentration. Insulin promotes glucose utilization by liver, muscle and adipose tissues. Glucose stimulates insulin secretion (as indicated by the '+' sign) but inhibits glucagon secretion (hence the '−' sign). The responses of the liver are more complex than the neural/neuroendocrine glucose sensors since it is a major target for both insulin and glucagons. Nonetheless, it can also be considered as glucose sensor since the expression of key glycolytic and lipogenic enzymes is modulated by the concentration of glucose in the presence of insulin. The liver has the ability of being either a net producer or consumer of glucose, depending on the glucagon/insulin ratio. Insulin may stimulate its own secretion from the β-cell via a positive autocrine feedback loop. All glucose sensitive cells indicated in this figure express GK.

indeed serve as pacemaker of glycolysis and thus control insulin secretion [9]. GK enzymatic capacities similar to those found in rodents [9–11] were eventually demonstrated in human islet tissue [12], thereby justifying extension of the GK glucose sensor concept from rodents to man. This was made possible by the application of a very sensitive 'quantitative histochemical' method [13].

Studies that made use of transgenic mice and other sensitive genetic techniques have led to the realization that GK may serve as a marker for a network of glucose sensitive neural/endocrine cells [14], some of which may still remain to be identified due to the fact that the enzyme is expressed at very low levels in rare cell types. Nonetheless, the unique properties, particularly the much lower affinity for glucose and the tissue-specific expression control, have motivated many scientists to study the biochemistry, physiology and genetics of this enzyme.

Initially, the assertion that GK was a biochemical glucose sensor was based almost entirely on kinetic features of the enzyme [9]. However, many additional

lines of evidence have evolved that have further reinforced the concept that GK is the key molecular determinant for sensing changes in the glucose concentration to which the cell is exposed, both in pancreatic β-cells and other glucose-responsive cell types [15]. It was discovered, for example, that GK expression in pancreatic β-cells and in hepatocytes was differentially regulated [16]. This provided additional support for this enzyme serving in the unique role as a β-cell glucose sensor. Moreover, discovery of the molecular genetic corollary for differential tissue specific expression control in the form of two tissue-specific promoters in the single gene for GK was another important milestone [17, 18].

The most compelling evidence for the central role of GK in glucose homeostasis was provided by the discovery that mutations in GK cause lasting glycemic disorders in humans. Linkage of hyperglycemia to the GK gene was first reported in 1992 [19]. Since then, nearly 200 mutants of this gene have been observed worldwide that cause either hyperglycemia or hypoglycemia, depending on the nature of the mutation. During the last decade biochemical geneticists have worked intensively, and with great success, to interpret this most elaborate experiment of nature. Indeed, the understanding that has been gained about the role of GK in glucose homeostasis culminated in 2003 in the discovery of a class of compounds that are both specific and potent activators of this enzyme [20]. These agents have at least a dual mode of action in that they both enhance glucose stimulated insulin release from the β-cell, and stimulate hepatic glucose disposal. On account of this, they are widely viewed as being a promising new therapeutic approach to type 2 diabetes mellitus.

Glucose Sensor Concept

The GK glucose sensor concept, when applied to the β-cell, asserts that this enzyme, through a combination both of its kinetics and high control strength in the regulation of glycolysis, is the predominant determining factor for metabolism of glucose which then dictates insulin secretion. The concept is based on metabolic flux, although recent discoveries suggest that alterations in molecular conformation might also be a factor. The sensing of glucose requires a molecule with both a specificity and affinity for glucose that allows such a molecule to change its conformation or function in parallel with the plasma glucose concentration in the physiological range of 4–10 m*M*. Conceptually, the role of GK is little different from that of hormone receptors, which have affinities for the ligands they sense that, while at least a million times greater, also correspond to the concentrations of the hormones. Indeed, the principle that specific receptors are able to distinguish between different hormones and cytokines depends both on the nature of the ligand-binding site and on the

affinities of these receptors, which must be very high to detect molecules that are generally present only in nanomolar or even lower concentrations.

The GK glucose sensor paradigm is logically integrated with the threshold concept for glucose-stimulated insulin release. This threshold is close to 5 m*M* glucose for rodents and man and determines the set point for glucose homeostasis of these organisms. The ATP-dependent K^+ and voltage-dependent Ca^{2+} channels of the β-cell plasma membrane participate in setting this threshold, together with other mechanisms of neuroendocrine control affecting the intracellular concentrations of both Ca^{2+} and cAMP. The GK glucose sensor is thus coupled to diverse signaling pathways resulting in regulated insulin release when the β-cell is exposed to glucose levels exceeding 5 m*M*. On the basis of this knowledge, is has been possible to predict the altered glucose set points of patients with a wide variety of activating and inactivating GK mutations [21, 22].

Kinetics, Nomenclature and Bioenergetics

The unique biochemical kinetics of GK, compared to the other hexokinase isoforms, enable this enzyme to function as a glucose sensor. First, the enzyme has an $S_{0.5}$ for glucose of about 8 mmol/l and cooperativeness for binding of glucose (nH ~1.7). While many investigators describe GK as having a K_m for glucose, this is not entirely correct since the enzyme displays non-classical kinetics. Together, the $S_{0.5}$ and Hill coefficient extrapolate to an inflection point for the catalysis of glucose of ~4 mmol/l. This is the point at which GK is most sensitive to changes in the concentration of this enzymatic substrate. Indeed, the affinity of GK for glucose is precisely within the range that is necessary for catalytic flux to be dictated by the concentration of glucose. On the other hand, the affinity of the enzyme for MgATP (0.3–0.4 m*M*), its other substrate, is well below the intracellular concentration of this molecule (about 2.5 m*M*), further assuring that metabolic flux depends almost entirely on both the concentration of glucose and the amount of GK within the cell. Second, the lack of significant end product inhibition by glucose-6-phosphate is also important since catalysis is not diminished as the glucose concentration increases. The cooperativity of GK for glucose, as indicated by the nH ~1.7, although moderate contributes significantly to the graded change of glucose phosphorylation in the physiological range. Together, these kinetic features enable GK to dictate the rate at which glucose is phosphorylated. While other lower K_m and more ubiqitous hexokinases are generally co-expressed with GK, the flux through these enzymes is fixed at a lower level due to their lower K_ms for glucose, as well as their inhibition by glucose-6-phosphate.

It is worth noting here that GK is not specific for glucose, as the name falsely implies, since the enzyme also phosphorylates mannose and fructose. However, the affinities for these substrates are very low (about 25 and 300 m*M*, respectively) so that glucose is de facto the sole substrate under physiological conditions. For this reason, the name 'glucokinase' is used in preference to hexokinase IV or D since it is both biologically realistic and has proven practical in medical genetics (i.e. the different forms of GK disease) and pharmacology (i.e. GK activators).

For GK to determine the rate of glucose phosphorylation, and hence its metabolism, the entry of glucose into the cell, via one or more of the members of the glucose transporter gene family, must not be rate limiting. At least in rodents, where this issue has been most thoroughly studied, this does not appear to be the case [9, 23]. In fact, the high capacity glucose transporter GLUT2 is generally co-expressed with GK, although other glucose transporters, when highly expressed, seem to suffice. Nonetheless, the requirement for sufficient glucose transport has been demonstrated by the fact that GLUT2 null mice die early in life of severe diabetes, and that glucose-stimulated insulin secretion is restored when either GLUT1 or GLUT2 is expressed in β-cells [24].

Lastly, a necessary prerequisite that must be fulfilled for GK to serve as a metabolic glucose sensor within β-cells is that the transmission of the initial metabolic signal to (the phosphorylation of glucose by GK) to the final secretory event (insulin exocytosis) requires that metabolic flux changes originating from alterations of extracellular glucose must both be delivered to the plasma membrane, and that the exocytotic machinery must be able, in turn, to respond to these distal signals via mass action effects. Our current understanding of the principles and mechanisms of bioenergetics fully supports the validity of such a premise [15].

Structure

The functional and genetic characterization of GK has been greatly aided by model structures of GK that were developed on the basis of homology with yeast hexokinase B or mammalian brain hexokinase [25]. The substrate binding sites within the molecule have been delineated and the inactivating effect of several disease causing mutants have been traced to the disruption of substrate binding. Perhaps the most exiting development has been the identification of a novel allosteric activator site owing to the fact that several activating mutations (V62M, T65I, W99R, D158A, Y214A, Y214C, V455M and A456V) are clustered in and around a cleft area of the enzyme opposite to and remote from the substrate binding site. This observation has lead to the speculation that

GK-containing cells might generate an endogenous activator molecule that could regulate enzyme activity. This idea had gained indirect support from the striking discovery of a novel class of GK activator drugs that interact with GK at this particular site [20]. Indeed, it is interesting to note that mutations in and around the cleft area alter the kinetics of GK in a manner that is very similar to those that occur in presence of the pharmacological activator that binds to this site. Given that the crystal structure of the enzyme has now been established [20], it is likely that this newly gained structural knowledge will serve as a guide for future experimentation that seeks to align changes in enzyme kinetics with structural alterations of this enzyme. Such information is no doubt vital for a full understanding of how both normal and mutant forms of GK function within the cell.

Tissue Distribution

As already noted, GK is now known to be expressed in a variety of neural/neuroendocrine cells in addition to the pancreatic β-cell and heptocyte. The neural/neuroendocrine cells that express this enzyme include the pancreatic α-cell, L- and K-type gut enterocytes and certain rare neurons in the central nervous system, primarily in the hypothalamus [14, 26, 27]. Physiological studies have shown that many if not all of the cells that express GK are regulated in some manner by physiological changes in the concentration of glucose. For instance, glucagon secretion occurs in a manner that is reciprocal to that of insulin, thus pancreatic α-cells are also clearly glucose sensitive although they have not been studied in as much depth as have β-cells. Similarly, the secretion of GLP-1 and GIP by gut enteroendocrine cells is glucose-dependent, as is the hyperpolarization or depolarization of certain neurons in the brain. GK has been identified in all of these cell types, thereby further supporting the conviction that this enzyme is universally necessary for glucose sensing. Indeed, the presence of GK within a cell is now seen as strong evidence that it is glucose-sensitive.

The expression of the GK gene in all sites except the liver originates from the upstream promoter region, whereas the downstream promoter appears to be hepatocyte-specific. Thus, the upstream promoter, which was initially thought to be specific for the pancreatic β-cell, should instead be considered as being specific for glucose sensitive neural/neuroendocrine cells. Since only a few regulatory elements have been carefully mapped in either the upstream or downsteam promoter regions, a great deal of work remains to further characterize how the gene is regulated in all of these sites.

On account of alternate promoter usage that leads to use of alternate translation start sites, the physical properties of GK differ slightly for the neural/neuroendocrine isoform versus the liver isoform of the enzyme [17].

The differences, both in mass and pI, are minor, and they seem to have little or no effect on the kinetics [28]. The use of alternate promoters is of much greater impact, since it is provides a means for regulation the expression of GK mRNA in a tissue-specific manner [18, 29]. However, alternate promoter usage is not the only means for determining the amount of GK within the cell. Rather, the amount of activity within the cell is regulated by a variety of mechanisms that are cell-type-specific, as discussed in more depth in subsequent chapters.

Human Genetics

Genetic linkage studies have unequivocally demonstrated that mutations in GK cause three different disorders of glucose regulation, all of which should now be considered as GK diseases. The first is maturity onset diabetes of the young (MODY) [19, 30, 31]. Individuals with GK gene mutations (now termed MODY-2 or MODY-GK) have persistent elevations of their plasma glucose concentration due to haploinsufficiency of the enzyme, which leads to an increase in the set point for their blood glucose concentrations [30, 31]. The most frequent cause of MODY-2 is missense mutations. Kinetic studies of these mutants have shown that almost all have diminished catalytic ability that lowers the relative activity index of the enzyme [22, 32, 33].

The second GK-linked disease is persistent hyperinsulinemia hypoglycemia of infancy (PHHI-GK) [34]. While much less in number, the missense mutations that cause this disease lead to an enhancement in the relative activity of the enzyme. In the β-cell, this leads to a lowering of the threshold for glucose-stimulated insulin secretion. While the effects of these mutations remain uncharacterized in other cell types, it is likely that they also affect these cells. For instance, a more active GK would likely mimic transgenic mice that have increased GK gene expression, which has been shown to augment hepatic glucose usage [35–38].

The third GK disease that has been described is permanent neonatal diabetes (PNDM) [39]. This GK disease, whose phenotype was actually predicted from studies of mice, is much more pronounced than either of the other two and occurs when both GK alleles carry inactivating mutations. Infants with this disorder must be treated with insulin in order to survive, and are insulin dependent for life.

Mouse Genetics

Mouse models for both MODY-2 and PNDM have been created and found to closely mimic the human disease [40–43]. To date, these models have involved

the introduction of gene mutations that totally obliterate expression of the enzyme. Mice with one null GK allele have an elevated plasma glucose concentration whereas animals that totally lack GK due to two null alleles die shortly after birth from severe hyperglycemia [40, 41]. While mice with specific GK activating mutations have yet to be reported, transgenic mice that over express GK are characterized by mild hypoglycemia [35, 37, 38]. Thus, these animal models appear to fully reproduce the human diseases, and have served as the basis for studies in which the tissue-specific functions of the enzyme have been defined with greater clarity that can be achieved by studies in humans.

The tissue-specific roles of GK in the β-cell and liver have also been determined by use of the cre/loxP system [40]. These studies have shown that GK in β-cells is essential for life since mice with a β-cell-specific deletion of GK die within a week of birth of severe diabetes. Mice that lack GK in the liver are viable but exhibit marked impairments in both hepatic glucose usage and glycogen synthesis during hyperglycemia. Surprisingly, they also exhibit diminished glucose-stimulated insulin secretion despite having normal expression of GK in the β-cell. Thus, studies in mice have clearly demonstrated the functional important of GK for both of these tissues. Tissue-specific knockouts of GK in the brain and gut have not been reported, but will also be essential for clarifying the contribution of these cells to glucose homeostasis.

Glucose Sensing in the Pancreatic β-Cell

The pancreatic β-cell is the outstanding prototype for a glucose sensing cell, and consequently is the model by which other glucose responsive neuroendocrine cells are explored. The β-cell has long been known to be able to both sense and respond to changes in the ambient glucose concentration. The response of the cell, measured either by a change in plasma membrane potential, intracellular Ca^{2+} level or insulin secretion, is tightly coupled to changes in the glucose concentration. The secretion of insulin in response to glucose by the β-cell is a complex, multistep process in which there are at least two main pathways. One pathway, the so-called 'triggering pathway' depends on the ATP-dependent K^+ (K_{ATP}) channel, which responds to alteration in the ATP/ADP ratio in cells. In this pathway, increased cellular metabolism leads to an increased ATP/ADP ratio, closure of the K_{ATP} channel, depolarization of the cell, and insulin secretion. However, more recent studies also point to alternate pathways, or 'augmentation pathways', that do not require either the K_{ATP} channel or membrane depolarization for their effect [44]. A minimal sketch, as shown in figure 2, illustrates these pathways, and the essential role of GK, as glucose sensor, in modulating their activity. The stunning complexity of the

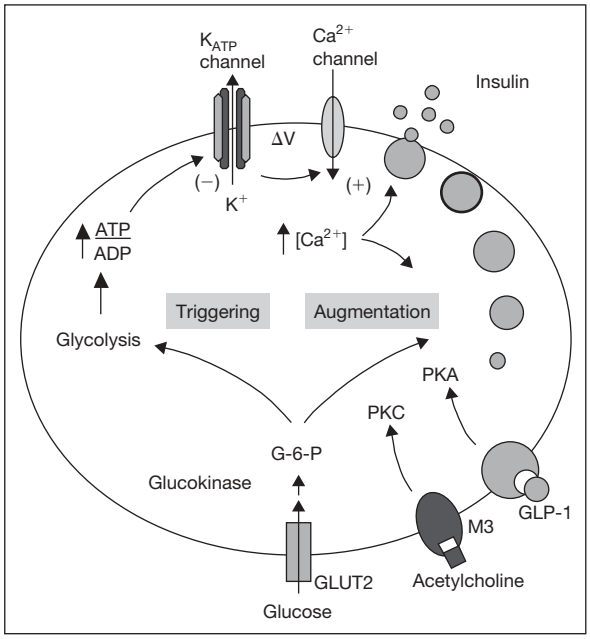

Fig. 2. Mechanisms of glucose stimulated insulin secretion. Glucose metabolism by the pancreatic β-cell, as determined by GK, leads to insulin secretion but the intervening mechanisms are complex and likely involve a large number of different signaling molecules and pathways, only some of which are shown here. A so-called 'triggering' pathway, which is activated by a rise in the ATP/ADP ratio, inhibits the K_{ATP} channel (a hetero-octomeric complex consisting of both ATP-sensitive K^+ channels and the type 1 sulfonylurea receptor) thereby activating voltage-dependent Ca^{2+} channel (or L type-channel). A rise in the intracellular concentration of Ca^{2+} then stimulates insulin exocytosis from granules docked at the plasma membrane. An 'augmentation' pathway is thought to act independently from membrane depolarization but requires that the intracellular Ca^{2+} concentration is elevated. These pathways are stimulated to varying degrees by neurotransmitters and hormones via activation of receptors on the pancreatic β-cell. For instance, acetylcholine, acting via the muscarinic receptor type 3 (M3) activates protein kinase C (PKC). Similarly, glucagons-like peptide-1 (GLP-1) stimulates a rise in cyclic AMP (cAMP) thereby activating protein kinase A (PKA).

proteins and processes involved in these pathways is explained in greater depth elsewhere [45, 46].

Regulation of GK in the Pancreatic β-Cell

Given the large mass of data pointing to a vital role of GK in glucose-stimulated insulin secretion, and that modeling studies suggest that even small

changes in the expression or activity of GK can have a significant effect on insulin secretion, it is not surprising that focus has shifted to studies pertaining to the regulation of GK in β-cells. Studies that have occurred over the past few years point to at least two major regulatory mechanisms. The first is regulation of GK gene transcription. Gene expression studies have shown that transcription is affected by a variety of factors, including insulin, placental lactogen, cAMP, biotin, and retinoic acid. While the location of the cis-regulatory elements conferring these changes remain to be precisely mapped, these finding suggest that all of these hormones and factors may modulate the amount of GK within the β-cell, thereby changing the response of the cell to glucose.

GK activity is also regulated within the β-cell at a post-translational level. High concentrations of glucose have long been known to induce GK activity, although the mechanisms by which this occurs have until recently been unclear [47]. Through application of a variety of microscopic techniques, some of which are newly developed, a model is beginning to emerge that may explain this regulation. First, several investigators have shown that GK within β-cells is associated with insulin granules [48–50]. Second, this association, which is dynamic, correlates with changes in both GK activity and conformational state. Third, the binding of GK to insulin granules, which is promoted by low glucose, is apparently blocked by inhibitors of insulin secretion [48, 51].

However, these findings, which have been obtained in studies of isolated islets, have yet to be fully reconciled with other data pertaining to insulin and IGF-1 signaling in β-cells. For instance, mice that lack insulin receptors in their β-cells develop glucose intolerance that is associated with diminished β-cell mass [52]. Moreover, insulin signaling in β-cells appears to be dependent on the specific insulin receptor isoform [53, 54], and the regulation of both insulin and GK genes may dependent on insulin signaling. Thus, further studies are certain to be forthcoming to investigate the possibility that linkage exists between insulin signaling and the regulation of GK, at both the transcriptional and post-translational levels. Nonetheless, evidence is accumulating that insulin signaling, via an autocrine feedback, may account for effects that were initially attributed only to glucose.

Glucose Sensing by the Hepatocyte

The liver has a complex response to changes in the glucose concentration, due in part to the fact that it is also a target for the action of both insulin and glucagon. The liver is also capable of alternating between being a site of glucose utilization to one of glucose production, depending on whether food has been recently ingested. During times of hyperglycemia or after feeding, when

GK is not bound to the hepatic GK regulatory protein (hGKRP) and thus is present in the cytoplasm, the enzyme facilitates hepatic glucose utilization and glycogen synthesis thereby helping to lower the blood glucose concentration.

GK has been shown to be essential for induction by glucose of key glycolytic and lipogenic enzymes, such as the L-type pyruvate kinase (L-PK), acetyl-CoA carboxylase, and fatty acid synthase, and repression of genes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase [55]. Thus, GK can be thought of as acting as a glucose sensor for the regulation of these glucose-responsive genes. Indeed, analysis of several glucose-responsive genes in the liver has led to the identification both of a specific DNA sequence, termed the glucose response element, as well as a novel transcription factor that binds to it, which are essential for glucose-stimulated changes in gene expression [56]. Consistent with GK also having a role as a glucose sensor in the liver, mice that totally lack hepatic GK do not appropriately regulate PEPCK, glucose-6-phosphatase and L-PK gene expression in response to a hyperglycemic challenge [unpubl. data]. Moreover, previous observations in cultured cell systems have clearly demonstrated that GK is necessary for transcriptional responses that are purely glucose-mediated [57].

While changes in glucose flux in both neural and neuroendocrine cells are coupled to changes in the plasma membrane potential via K_{ATP} channels, the mechanisms for the glucose-dependent hepatic responses are less clear. Indeed, glucose-6-phosphate, the pentose phosphate shunt, and the hexosamine biosynthetic pathway have all been suggested to be responsible for the effects of glucose on hepatic gene expression [55]. Moreover, it has been suggested that the factors that confer the glucose responsiveness to certain hepatic genes may themselves be affected by cAMP-dependent phosphorylation [56, 58]. Thus, it is no wonder why the glucose response of the liver has taken longer to understand compared to that of neural/neuroendocrine cells.

Regulation of GK in the Liver

Like the β-cell, GK gene regulation in the liver occurs at both a transcriptional and post-translational levels. However, the regulation of hepatic GK is unique for several reasons. First, transcription of GK mRNA in the liver is determined by a separate promoter, which is expressed only in the liver. Progress in characterizing the regulation of this gene has been slow, due in large part to the fact that permanent cell lines which express the hepatic GK gene do not exist, and since the elements necessary for expression of GK in the liver appear to be distributed over at least a 40-kb region of the gene [59, 60]. Moreover, the response of hepatic GK to insulin might not be direct since the

sterol regulatory element binding protein-1 (SREBP-1), has emerged as a major mediator of insulin action on both glycolytic and lipogenic gene expression in the liver [61]. Furthermore, the regulation of hepatic genes may not always involve direct interactions with discrete trans-acting factors given that interactions have been discovered between FOX-01 and PGC-1 (a transcriptional co-regulator) that modulate the expression of multiple gluconeogenic enzymes in the liver [62].

Greater success has been achieved in understanding the short term regulation of hepatic GK since the discovery of the hGKRP [63, 64]. The interaction between GK and hGKRP is stimulated by fructose-6-phosphate and inhibited by fructose-1-phosphate and inhibits GK [64]. Subsequently, it was discovered that when GK is bound to hGKRP the complex becomes sequestered in the nucleus of the hepatocyte [65]. This in turn has led to the unraveling of a nuclear-cytoplasmic shuttling cycle for hepatic GK. Interesting, GK contains a nuclear export signal that ensures, when fructose-1-phosphate levels rise and GK dissociates from hGKRP, that the enzyme is transported back to the cytoplasm [66]. The role of the hGKRP seems to be to serve as a reservoir for the enzyme. This is consistent with studies of mice that lack hGKRP which have lower levels of hepatic GK thereby suggesting that hGKRP acts to prevent the decay or destruction of GK during fasting [67].

GK Activators and Future Therapeutics

Given the clear role that GK has as a glucose sensor, it has been an obvious target for the development of therapeutics that might activate GK, thereby lowering of the blood glucose concentration. While it was not thought likely that a direct means of achieving this was possible, a novel and new class of potential pharmaceuticals has been found to do just that [20]. This exciting advance promises to open up a new field of GK activation as a powerful tool for research and a means of treating diabetes. When considered in light of the mutations that cause PHHI-GK, it becomes likely that there is more to the regulation of this enzyme than is known despite forty years of study. Indeed, the finding of a chemical activator of GK raises the intriguing possibility of a yet-to-be-identified endogenous activator for the enzyme.

Beyond Flux-Based Glucose Sensing

Lastly, while this chapter has been focused on glucose sensing mechanisms in mammals, and has presented the major arguments why GK is thought to

function as a glucose sensor, it is relevant to point out a recent study in plants indicating that a particular hexokinase (HKX1) appears to support various signaling functions in gene expression, cell proliferation and growth, leaf expansion and senescence, even when the enzyme has been mutated in a manner that it totally lacks any catalytic activity [68]. This raises the intriguing possibility that conformational changes in GK might also contribute to its function as a glucose sensor, as is the case in plants. Thus, while the GK glucose sensor model in mammals is based on regulation of metabolic flux, the possibility that conformational alternations are also involved remains to be explored. Indeed, given the finding of a chemical activator for the enzyme and the fact that the enzyme binds to insulin granules in β-cell and to GKRP in hepatocytes, make this possibility considerably less remote than a decade ago. Moreover, the fact that some of the mutations discovered as a result of human genetic studies do not induce the expected kinetic alterations when expressed in bacterial systems also suggests that there is more to be learned about the in vivo functions of GK [21]. Thus, future studies of GK ought to be directed toward exploring some of these possibilities.

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The Hexokinase Gene Family

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Metabolism of glucose is of major importance in virtually all organisms – microbial, plant, and animal. The initial step in most pathways of glucose metabolism (with the classic glycolytic pathway being of more general significance) is the ATP-dependent phosphorylation to form glucose-6-phosphate (G6P), the reaction catalyzed by hexokinase or glucokinase. In IUB nomenclature, the distinction between glucokinase and hexokinase is that the former is specific for glucose whereas hexokinase can phosphorylate other hexoses in addition to glucose. This distinction may be important in particular situations [1] but not in the present context, and thus we will use the term 'hexokinase' to include both hexokinases and glucokinases.

Consistent with their widespread metabolic significance, hexokinases are found in virtually all organisms. While the focus of the present volume is on the type IV isozyme of mammalian hexokinase, commonly called 'glucokinase' (a misnomer since, as emphasized elsewhere [1], the type IV isozyme is *not* specific for glucose and hence *not* a true glucokinase), we think it is useful to begin with a more general discussion of the hexokinase family, to provide perspective that is relevant not only to the evolutionary relationships between members of the hexokinase family but also to structural and functional features of these enzymes.

Based on molecular weights, hexokinases generally fall into one of three classes, with bacterial hexokinases having molecular weights of approximately 35,000, while hexokinases from other organisms have molecular weights of approximately 50,000 or 100,000 [1, 2]. Molecular weights alone obviously do not confirm sequence similarities and hence evolutionary relationships, but the latter observation led early on to speculation that the 100-kD hexokinases might have evolved by duplication and fusion of a gene encoding an ancestral 50-kD hexokinase [3]. This has been confirmed by subsequent work, as will be

discussed below. The somewhat smaller size of the bacterial hexokinases was problematic, and indeed it has been considered highly questionable whether these are homologous to the larger 50- and 100-kD enzymes [1]. As also discussed below, we believe there is a strong case to be made for an evolutionary relationship between the bacterial hexokinases and the 50- and 100-kD hexokinases from other organisms.

The cDNAs for hexokinases from a wide range of organisms have been cloned, and sequences available in the NCBI database at the time of this writing are indicated in table 1. The resulting deduced amino acid sequences are consistent with the distribution of the hexokinases into three classes based on size, with the bacterial enzymes generally being somewhat smaller. While it is true that the overall sequence similarity between the bacterial and other hexokinases is rather limited [1], there are key sequence motifs that are conserved between *all* of these hexokinases and mark them as members of a single protein family as well as providing information about how this family has evolved.

A key observation was made by Flaherty et al. [4], who noted the extraordinary structural similarity between yeast hexokinase and the ATP binding domain of the 70-kD bovine heat-shock cognate protein. This ATP-binding domain was also found in other proteins, including actin, and has come to be called the 'actin fold' [5, 6]. Bork et al. [5] identified five regions with characteristic sequence features within the actin fold structure. These five regions were called phosphate 1, connect 1, phosphate 2, adenosine, and connect 2, and were found at analogous positions within the sequence of the various actin fold proteins. These sequence motifs are present in yeast (*S. cerevisiae*) hexokinase, included for purposes of comparison in table 2, and other 50- and 100-kD hexokinases [1, 2, 5] *and* in the bacterial hexokinases (table 2). It thus seems very likely that the hexokinases and other actin fold proteins have evolved by divergence from a common primordial actin fold protein.

Comparison of the structures of various actin fold proteins [6] discloses that the principal differences are structural features located at the periphery of the molecule, surrounding a core represented by the actin fold. Functional differences between actin fold proteins, e.g. between actin, hexokinase, and glycerol kinase, are reasonably attributed to these unique structural regions. It follows that the smaller bacterial hexokinases represent a 'minimal hexokinase,' with ATP-binding capability provided by the actin fold and limited additional structure conferring the glucose-binding ability required for hexokinase function. Unfortunately, this minimal structural requirement for hexokinase function cannot be defined presently since there are no known structures for a bacterial hexokinase. The addition of further peripheral structural features presumably led to acquisition of functional properties seen with the 50-kD hexokinases but

Table 1. Based on size, the hexokinases from various organisms fall into three classes

Hexokinase from	Size (# of amino acid residues)	NCBI accession number(s)
Bacteria		
Zymomonas mobilis	327	M60615
Escherichia coli	321	U22490
Brucella abortus	348	U21919
Staphylococcus xylosus	328	X84332
Streptomyces coelicolor	317	X65932
Treponema pallidum	444	NP_218946
Plant		
Arabidopsis thaliana	435, 496, 502	U18754, S71205, AAC62130
Solamon tuberosum	496, 498	AAF14186, CAA63966
Brassica oleracea	499	AAL60584
Oryza sativa	507, 509	AAK51559, AAK51560
Lycopersicon esculentum	496, 498	AAG35735, AJ401153
Nicotiana tabacum	497	AF118133
Citrus sinensis	498	AAG28503
Zea mays	509	AAM80479
Fungi		
Saccharomyces cerevisiae	485, 486, 500	KIBYHA, KIBYHB, M24077
Schizosaccharomyces pombe	455, 484	S68693, S68694
Debaromyces (Schwanniomyces) occidentalis	478	S57203
Aspergillus niger	490, 495	CAA08922, CAA67949
Aspergillus oryzae	490	BAB12228
Kluyveromyces lactis	485	A48132
Yarrowia lipolytica	534	CAA09675
Pichia angusta (*Hansenula polymorpha*)	471	AY034434
Tuber borchii	497	AAG28789
Invertebrates		
Caenorhabditis elegans	500	NP_492475
Schistosoma mansoni	451	AAA29894
Entamoeba dispar	445	CAA72001
Entamoeba histolyticus	445	CAA57682
Haemonchus contortus	485	CAB40412
Plasmodium falciparum	493	A48457
Plasmodium yoelii yoelii	494	EAA21443
Trypanosoma brucei	471	CAC69958
Trypanosoma cruzi	471	CAD26835
Encephalitozoon cuniculi	475	CAD26064
Drosophila melanogaster	448, 453, 454, 465	AAG23063, AAG22916, AAG23113, AAG22917

Table 1 (continued)

Hexokinase from	Size (# of amino acid residues)	NCBI accession number(s)
Drosophila simulans	448, 453, 454, 465	AAG21970, AAG22968, AAG22942, AAG22967
Drosophila yakuba	453, 454, 458	AAG21973, AAG21971, AAG21972
Toxoplasma gondii	468	BAB55664
Vertebrates		
Sparus aurata	478	AAF40309
Cyprinus carpio	576	AAF28854
Xenopus laevis	458	X93494
Mammalian (rat) isozymes		
Type IV	465	A31810
Type I	918	C59226
Type II	917	NP_036867
Type III	924	P27926

Table 2. Actin fold sequence motifs are present in bacterial hexokinases

	Phosphate 1	Connect 1	Phosphate 2	Adenosine	Connect 2
S. cerevisiae	FLAI**D**L**GG**T	VALIN**D**TTGTL	GVIF**GT**GVNG	IAAD**G**SVYNR	DGSGA**G**AAVIA
Z. mobilis	IVAI**D**I**GGT**	HVLIN**D**FGAVA	ILGP**GT**GLGV	AAIE**G**VPFSL	TSVVI**G**GGVGL
E. coli	ALVG**D**V**GGT**	LEIIN**D**FTAVS	VYGA**GT**GLGV	VFIA**G**GIVPR	NPGLL**G**SGAHL
B. abortus	VLVG**D**I**GGT**	DLGFE**D**VTVLN	VLGP**GT**GLGV	AGLD**G**SNPQA	GVYLS**G**GIPVR
S. xylosus	ILAA**D**I**GGT**	DTDTS**D**TTGYL	AITL**GT**GLGG	ASAT**G**VVNLV	KYIVL**G**GGMST
S. coelicolor	TIGV**D**I**GGT**	VVVEN**D**ANAAA	CITL**GT**GLGG	WAGA**G**LADLA	SAFIV**G**GGLSD
T. pallidum	VIVI**D**A**GGT**	LVRFG**D**SGTPH	AEVI**GT**CVGA	GFIL**G**TGMNS	STTTL**G**ALLAP

not with the smaller bacterial enzymes. These might include broadened substrate specificity; Cárdenas et al. [1] have noted that most bacterial hexokinases exhibit a specificity for glucose (i.e. they are true glucokinases) that is not seen with hexokinases from higher organisms. Addition of other structural features may have led to acquisition of sensitivity to feedback inhibition by the product, G6P. The latter property is seen with the 50-kD hexokinases from some organisms (e.g. *S. mansoni)*, but not with all 50-kD hexokinases (e.g. not with the

yeast enzymes) [1, 2]. Sensitivity to inhibition by G6P is also a characteristic of the 100-kD mammalian isozymes, types I–III. These are thought to have evolved by duplication and fusion of a gene encoding an ancestral G6P-sensitive 50-kD hexokinase (see below), the latter also being the precursor for the G6P-sensitive enzyme of present-day *S. mansoni*. Indeed, the G6P-binding sites of the *S. mansoni* and mammalian type I isozyme are structurally equivalent [7].

Evolution of the Mammalian Isozymes of Hexokinase

Four isozymes of hexokinase are found in mammalian tissues. These are most commonly referred to as types I–IV, and have been discussed extensively elsewhere [1, 2]. The type IV isozyme ('glucokinase') is the central theme of the present volume and various aspects of it will be treated by other contributing authors. In the present context, we wish only to highlight major features of these isozymes that are pertinent to deducing their possible evolutionary relationships, both to each other and to other members of the hexokinase family.

The type I, II and III isozymes are frequently referred to as the 'low K_m' isozymes based on their relatively high affinity (sub-millimolar K_m) for glucose. These isozymes also share the property of being sensitive to inhibition by physiologically relevant concentrations of the product, G6P, and this is generally accepted as a major factor in their regulation. This is in marked contrast to the relatively low affinity of the type IV isozyme for substrate glucose, which is critical for its role as a 'glucose sensor' responsive to changes in plasma glucose levels within the physiological range, as well as the insensitivity of the type IV isozyme to product inhibition. The type I, II and III isozymes have molecular weights of approximately 100,000. They are comprised of N- and C-terminal halves with extensive sequence similarity, both to each other and to the 50-kD hexokinases found in other organisms, consistent with the view that the 100-kD mammalian isozymes have evolved by duplication and fusion of a gene encoding an ancestral 50-kD hexokinase similar to the G6P-sensitive hexokinase of *S. mansoni*.

Duplication and fusion of a gene encoding a 50-kD G6P-sensitive enzyme would be expected to lead to a 100-kD hexokinase in which both halves possess catalytic activity susceptible to product inhibition. That expectation is realized in the type II isozyme [8, 9]. In contrast, only the C-terminal halves of the type I [10] and type III [11] isozymes have retained catalytic function, with the N-terminal halves having evolved to serve regulatory functions [2, 11–13]. Thus, it has been proposed [9, 11] that the type II isozyme represents the more 'ancient' form, retaining the characteristics of the ancestral 100-kD hexokinase that first resulted from the gene duplication-fusion event, while the

type I and type III isozymes have resulted from further duplication of the gene encoding the ancestral 100-kD hexokinase, with subsequent mutations leading to loss of catalytic function but acquisition of regulatory functions in the N-terminal halves.

So where does the 50-kD type IV isozyme, glucokinase, fit into this? Based on sequence comparisons, the type IV isozyme is *much* more closely related to the 100-kD mammalian isozymes than it is to the 50-kD hexokinases found in other organisms. Moreover, as discussed below, the structure of the gene for the type IV isozyme clearly demonstrates its close relationship to the 100-kD mammalian isozymes. Yet, as noted above, the type IV isozyme is distinctly different from the other mammalian isozymes in its affinity for glucose and susceptibility to product inhibition, and in the latter property, it is also different from the ancestral 50-kD G6P-sensitive hexokinase thought to be the precursor for the 100-kD mammalian isozymes. How can these observations be reconciled, i.e. the close relationship between *all four* mammalian isozymes at the amino acid sequence and gene structure level, yet such notable differences in terms of kinetic and regulatory properties? We believe that the most plausible scenario is similar to that proposed by Cárdenas et al. [1] in which an initial duplication gave two copies of the gene encoding an ancestral 50-kD hexokinase (more specifically, a 50-kD hexokinase that was sensitive to inhibition by the product G6P). One of these was preserved as the gene encoding the 50-kD type IV isozyme, with subsequent mutations leading to decreased affinity for glucose and loss of sensitivity to G6P. The second gene resulting from this initial duplication event then gave rise, by further duplication and fusion, to the ancestral 100-kD hexokinase (≈present-day type II isozyme), with further duplication (but *not* fusion) and mutations leading to the type I and type III isozymes, as outlined above.

It should be noted that the evolutionary events described in the preceding paragraphs likely occurred prior to the emergence of mammals. Isozymes with kinetic properties similar to the mammalian type IV isozyme have been described in lower vertebrates [14], and a *partial* cDNA encoding 643 residues of a hexokinase with ≥80% sequence identity to the mammalian type I isozyme has been cloned from *Xenopus laevis* [15]. More extensive discussion of 50- and 100-kD hexokinases in various species and their possible evolutionary relationships may be found elsewhere [2], but it is interesting to note that, in addition to the 50-kD forms, 100-kD hexokinases occur in some plants and invertebrates. While sequence data is not yet available to confirm that these are indeed members of the hexokinase family discussed above, that seems very likely to be the case. Thus, gene duplication-fusion may have occurred more than once, giving rise to higher molecular weight hexokinases in different branches of the phylogenetic tree.

Structure of the Genes Encoding the Mammalian Isozymes of Hexokinase

The intron-exon structure has been determined for the genes encoding the rat [16] and human [17] type I isozyme, the rat [18], human [19, 20], and murine [21] type II isozyme, the human [22] type III isozyme, and the type IV isozyme from rat [23] and human [24]. The evolutionary relationships discussed above are reflected in the remarkable conservation of structure in these genes (table 3).

As noted previously [22], the most striking variation occurs in the first exon. Although the coding regions are of identical size in the first exons of the type I and type II isozymes, the coding region is much shorter in the first exon of the type IV isozyme. The difference is even greater with the type III isozyme in which the first exon, designated exon 1A, contains only noncoding sequence while the first exon with coding sequence, exon 1B, contains a coding sequence of 96 base pairs (bp), much longer than that in the first exons of the genes for the type I, type II, or type IV isozymes. It is not only the *length* of coding sequence but the sequences themselves which differ, with the result that there are remarkable differences in the N-terminal amino acid sequence of these isozymes [2], in contrast to the extensive similarity seen throughout the rest of the molecules. At least with the type I and type II isozymes, this N-terminal region is of major functional importance in that it is critical for binding of these isozymes to mitochondria [25, 26], a physical association that may lead to intimate metabolic interaction, with coupling of glucose phosphorylation to ATP production by mitochondrial oxidative phosphorylation [27].

Alternative first exons have been demonstrated for the type I isozyme (the exon represented in table 3 is for the form found in most mammalian tissues). These are associated with distinct promoters and, through alternative splicing of the transcripts, give rise to unique forms of the type I isozyme that are found in erythroid [17, 28, 29] or spermatogenic [29, 30] tissue. There are also alternative first exons for the type IV isozyme, each with its distinct promoter, leading to the pancreatic and liver forms of glucokinase discussed elsewhere in this volume.

As first noted by Printz et al. [18], the exon sizes (table 3) provide strong support for the view that the 100-kD isozymes arose by duplication and fusion of a gene encoding an ancestral hexokinase from which the present-day type IV isozyme is also derived. Thus, fusion of exon 10 (142 bp) with exon 2 (163 bp) of a duplicated gene for the 50-kD hexokinase would give rise to the 305-bp exon 10 of the 100-kD isozymes, and the identical (or nearly so) size of the analogous exons, exons 3–9 and 11–17, respectively, is also as expected.

Table 3. Comparison of exon sizes in genes encoding mammalian isozymes of hexokinase[a]

Isozyme	Size (bp) of exon																	
	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18[b]
Rat type I	63	163	149	120	96	100	184	156	234	305	149	120	96	100	184	156	234	145
Human type I	63	163	149	120	96	100	184	156	234	305	149	120	96	100	184	156	234	142
Rat Type II	63	163	149	120	96	100	184	156	234	305	149	120	96	100	184	156	234	142
Human type II	63	163	149	120	96	100	184	156	234	305	149	120	96	100	184	156	234	142
Mouse type II	63	163	149	120	96	100	184	156	234	305	149	120	96	100	184	156	234	145
Rat type IV	45	163	155	120	96	100	184	156	234	142[b]								
Human type IV	42	163	155	120	96	100	184	156	234	142[b]								
Human type III	96[c]	163	155	120	96	100	184	156	234	296	137	120	96	100	184	156	234	142

[a]References to work describing the structure of these genes are given in the text. Shaded areas indicate specific coding sequences discussed in the text.

[b]Coding sequence only.

[c]The first exon containing coding sequence is designated exon 1B; the first exon, designated 1A, contains only noncoding sequence.

The third exons for both the type III and type IV isozymes are six bp larger than exon 3 in the genes encoding the type I and type II isozymes. Initially, this might be interpreted as indicating some special relationship between the type IV isozyme and the N-terminal half of the type III isozyme but this is not the case. These inserts do *not* occur at the same position nor code for the same amino acids in these two isozymes, and therefore represent the results of distinct insertional events. Thus, the human type III isozyme contains two residues, Ala-Thr (residues 98 and 99) that are *not* present in the human type I, type II, or type IV isozymes, while the human type IV contains Lys-Thr at positions 101–102 but the type I, type II, and type III isozymes do *not* contain analogous residues. Also noted are deletions in exons 10 and 11 of the gene for type III hexokinase. These must have occurred selectively in the gene for the type III isozyme and after the duplication events giving rise to multiple genes for the 100-kD isozymes since corresponding deletions are *not* seen in exons 10 and 11 of the genes encoding the type I and type II isozymes.

Promoter Structure and Transcriptional Regulation

The type I isozyme is expressed in all mammalian tissues that have been examined and may be described as a 'housekeeping enzyme.' It is therefore not surprising that the promoter for the gene encoding the somatic form of the type I isozyme has characteristics frequently associated with promoters for housekeeping genes, namely, lack of a classical TATA sequence, multiple transcription initiation sites, and location in a GC-rich region [30, 31]. The promoter for the erythroid-specific form of type I hexokinase also lacks a canonical TATA element but contains a GATA sequence that is the functional equivalent of the TATA element in erythroid-specific genes [29]. In contrast to the promoter region for the somatic form of the type I isozyme, the promoter for the erythroid-specific type I isozyme has a single transcriptional start site and is not associated with a GC-rich region [28, 29]. The promoter region for the alternatively spliced type I isozyme of spermatogenic tissue has not been clearly identified [29, 30]. Consistent with the widespread expression of the somatic type I isozyme, the ubiquitously expressed Sp factors have been shown to function in transcriptional regulation [32]. Also consistent with a basal housekeeping role, expression of the type I isozyme in mammalian tissues seems remarkably unaffected by hormonal or other physiological changes that affect expression of other isozymes. With cultured cells, expression of type I hexokinase has been reported to be affected by insulin-like growth factor [33] and thyroid stimulating hormone [34]. The in vivo significance of these effects remains unclear but the effect of thyroid-stimulating hormone may be related to in vivo studies

demonstrating that expression of type I hexokinase in developing brain is affected by thyroid status [35].

The promoter for the gene encoding type II hexokinase has classical TATA and CAAT sequence elements [36–38]. While a single transcriptional start site appears to be predominant, its location may differ in the rat [18] and human [38] genes, and alternative start sites may also be functional [36, 38]. Expression of the type II isozyme has been reported to be influenced by hormonal factors or other changes in physiological status (e.g. hypoxia, exercise) too numerous to explicitly mention here. Two recent references [39, 40], and references therein, may be consulted for further information on this aspect.

As with the promoter for the type I isozyme, the promoters for the rat [41] and human [22] genes for type III hexokinase lack classical TATA and CAAT elements. A single transcriptional start site has been identified [41]. There is only limited information about factors regulating transcription of the type III isozyme, but the transcription factor, Oct-1, has been implicated [22, 41]. The latter may seem surprising in view of the fact that the type III isozyme is expressed rather selectively [2] while Oct-1 is ubiquitously expressed in mammalian tissues, but Oct-1 has been shown to be involved in tissue(cell)-specific expression of other genes [41]. Clearly, additional factors must be at work.

Transcriptional regulation of the type IV isozyme is discussed by other contributors to this volume.

Functional Importance of the 3′ Untranslated Region of the mRNAs for Isozymes of Hexokinase

We close this chapter by drawing attention to what is likely to be a significant factor governing the expression of these isozymes in mammalian tissues, but a factor that does not yet appear to have received explicit consideration. It is now well established that sequences within the 5′ and 3′ untranslated regions (UTR) can play a major role in post-transcriptional regulation [42, 43]. The 3′UTRs of the rat type I, II, III and IV isozymes are 808 [44], 1,114 or 2,142 (depending on which of two polyadenylation signals is used) [18], 821 [45], and 835 [46] nucleotides (nt) in length, more than twice the average length (457 nt) of 3′UTRs in rodent mRNAs [43]. Such extraordinary length clearly suggests the possibility that multiple functional elements might be present within these sequences, and it is reasonable to expect that sequence would be highly conserved in functionally important regions. A BLAST comparison of the 3′UTRs of the rat (808 nt) and human (748 nt [47]) type I isozyme disclosed four regions of high sequence identity. These regions contained 39 nt, 80 nt, 41 nt, and 50 nt,

were located at analogous positions within these 3′UTRs, and had sequence identities of 92, 80, 90, and 86%, respectively. Such regions surely merit attention in terms of assessing their possible functions, and with all of the 'tools' (cDNAs, expression systems [11–13]) available, this is now feasible.

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Comparative Biochemistry of Glucokinase

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Glucokinase (ATP: D-glucose 6-phosphotransferase, EC 2.7.1.1), also known as hexokinase D or hexokinase IV, is one of the four glucose-phosphorylating isoenzymes present in vertebrates and plays the role of a glucose sensor in pancreatic β-cells and in hepatocytes [1, 2]. It is the isoenzyme characteristic of liver, where it accounts for about 85% of the total glucose phosphorylating activity, though it is just one of four hexokinase isoenzymes in this organ. It represents about 95% of the total hexokinase activity of hepatocytes, and is absent from other liver cells [3]; the remaining activity in hepatocytes is due to hexokinases A, B, and C, the high-affinity hexokinases [3]. Glucokinase is affected markedly by dietary and hormonal manipulations [4–6]: its activity is considerably decreased in diabetic animals and animals subjected to starvation or a carbohydrate-free diet; however, it does not disappear completely, but reaches a final residual activity level of about 15–20% of normal values in hepatocytes [7]. The residual 'glucokinase' activity in conditions of starvation, diabetes or carbohydrate-free diet is definitely due to glucokinase and not, for example, to the glucose-phosphorylating activity of *N*-acetylglucosamine kinase.

The Reaction Catalyzed by Hexokinase

The term 'hexokinase' was introduced in 1927 by Meyerhof [8] as a name for the enzyme in a yeast autolysate that catalyzed the phosphorylation of glucose by ATP. Crane [9] subsequently found a similar enzyme activity in muscle tissue and other types of cells. He grouped the hexokinases into two classes according to whether they accepted just one or more than one hexose substrate.

All the mammalian hexokinases, including glucokinase, are in the second category, as they are all capable of phosphorylating a number of different acceptor molecules, binding others that can act as inhibitors even if they are not substrates. In every case, glucose is the principal physiological substrate [10] and has the highest phosphorylation coefficient; again, glucokinase is not an exception.

Unlike vertebrates, prokaryotes and lower eukaryotes typically contain a series of specific hexokinases, each of which acts on one hexose only, normally glucose, mannose or fructose. Various organisms have specific glucokinases (EC 2.7.1.2), including *Escherichia coli*, *Zymomonas mobilis*, *Bacillus stearothermophilus*, *Myxococcus coralloides*, *Streptococcus mutans*, *Aerobacter aerogenes*, *Brevibacterium fuscum*, *Dictyostelium discoideum* [11]. Some of these highly specific glucokinases, such as those of *S. mutans* [12], *A. aerogenes* [13] and *D. discoideum* [14], are not even inhibited by fructose or mannose, a further contrast with vertebrate 'glucokinase', as discussed elsewhere [1, 11, 15]. There is no evidence of homology between the prokaryote glucokinases and the hexokinases of yeast and vertebrates; if it exists the many mutations accumulated during the long period since the separation of prokaryotes and eukaryotes have eliminated any trace of it [11].

In terms of chemistry, hexokinases catalyze the phosphorylation of various hexoses by ATP according to the following equation:

$$R\text{-}CH_2OH + MgATP^{2-} \rightarrow R\text{-}CH_2O\text{-}PO_3^{2-} + MgADP^{-} + H^{+}$$

The acceptor molecule ($R\text{-}CH_2OH$) is the cyclic form of a hexose such as glucose, mannose, fructose, or 2-deoxyglucose. In general the nucleotide specificity is stricter than the sugar specificity, but some analogs of ATP, such as ITP, react slowly [16, 17].

Although the enzymes transfer phosphoryl and not phosphate groups, they continue to be called phosphotransferases (rather than phosphoryltransferases), in keeping with the standard principle that enzymes are named according to the complete reaction catalyzed, usually without taking account of mechanistic details.

Unlike hexokinases A and B, which follow Michaelis-Menten kinetics with respect to glucose, and hexokinase C, which is inhibited by excess of glucose, glucokinase shows cooperativity with this substrate, with a Hill coefficient of about 1.5 [18–20]. Of the other sugar substrates, mannose is slightly poorer than glucose, with essentially the same degree of cooperativity, but somewhat lower activity; 2-deoxyglucose is a significantly poorer substrate than mannose, and shows no cooperativity [21, 22]; galactose and *N*-acetylglucosamine are not substrates. The lack of reaction with *N*-acetylglucosamine is important for identifying glucokinase, because another enzyme, *N*-acetylglucosamine

kinase (EC 2.7.1.59), has many similar properties to glucokinase and its low affinity for glucose has sometimes caused it to be mistakenly identified as glucokinase [23–25]. Although the $K_{0.5}$ value of *N*-acetylglucosamine kinase for glucose is of the order of 300 mM, very much higher than the corresponding value for glucokinase, the apparent negative cooperativity with respect to glucose causes the $K_{0.5}$ obtained experimentally to vary with the range of glucose concentrations used: a range adequate for studying glucokinase could easily suggest a K_m of around 10 mM with *N*-acetylglucosamine kinase and mislead the researcher, especially one who is not fully aware of the positive cooperativity of glucokinase with respect to glucose [25].

Glucokinase Specificity

Hexokinase specificity has usually been taken to refer to comparison between glucose and fructose as substrates. Glucokinase was initially supposed to have much less activity than the other isoenzymes towards fructose relative to its activity towards glucose, as measured with only one sugar present. It is now widely recognized that meaningful assessment of specificity needs to be based on the capacity of an enzyme to discriminate between substrates when they are mixed together, and, at least for enzymes that obey simple Michaelis-Menten kinetics, this is independent of the concentrations at which the measurements are made. Comparison of the mammalian hexokinase isoenzymes is made more difficult by the fact that two of them do not obey Michaelis-Menten kinetics: hexokinase C is inhibited by excess substrate, and glucokinase is cooperative with respect to glucose. Nonetheless, an approximate assessment can be made on the basis of $V/K_{0.5}$ values, where V is the limiting rate at saturating substrate, and $K_{0.5}$ is the substrate concentration at which the rate is $0.5V$; for the four isoenzymes the ratio of values for fructose and glucose are of the same order of magnitude, indicating a similar degree of specificity [10]. The value of the ratio for glucokinase is 0.043, not the smallest value of the four as it is substantially greater than the value of 0.016 for hexokinase A, the brain isoenzyme. By this criterion, therefore, glucokinase is actually much less specific for glucose than hexokinase A. For hexokinase B, the muscle isoenzyme, the ratio is 0.052 and for hexokinase C it is 0.022.

Even if less appropriate criteria are applied the conclusion is the same: comparing ratios of $K_{0.5}$ values for glucose and fructose, glucokinase has a value between those of hexokinase A and hexokinase B; comparing V values glucokinase is actually by far the least specific isoenzyme for glucose. It may thus seem surprising that the misapprehension about the specificity of

glucokinase has survived so long. The point is that the original measurements were made at just one sugar concentration, 100 mM, at which hexokinases A, B and C are saturated with sugar, regardless of whether this is glucose or fructose; glucokinase, on the other hand, is far below saturation by fructose at this concentration, though it approaches saturation by glucose. It may be objected that the high activity of glucokinase at very high concentrations of fructose has no physiological significance, but this argument applies equally to the comparisons at 100 mM, far greater than any physiological concentration of either substrate. In any case proper assessments of specificity refer to discrimination between substrates when mixed together, and, as noted above, these do not depend on the concentration at which the measurements are made. Complications of cooperativity aside, the capacity of an enzyme to distinguish between two substrates depends only on the $V/K_{0.5}$ ratio and is the same whether the concentration is 1 μM or 100 mM.

In current practice the argument in favor of the name hexokinase D appears to have been lost, and it is clear that most researchers will continue to call it glucokinase regardless of the real specificity. For discussion of the role of the enzyme in physiology and medicine this probably does not matter very much, but in a broader context, such as discussion of evolutionary relationships [11], it does matter, because the incorrect implied specificity can easily lead to nonsensical conclusions, such as the notion that two hexokinase isoenzymes from thale cress are structurally similar to mammalian glucokinase and yeast hexokinase but not to mammalian hexokinases [26]. With diminishing hope of being followed, therefore, I continue to prefer the name hexokinase D and to hope that authors who call it glucokinase are at least aware of the real specificity [11], but for the sake of uniformity with the other chapters in this book I use the name of glucokinase here.

Pancreatic and Liver Glucokinase: One Gene Two Proteins

Although the glucokinases from pancreas and liver are similar in kinetic behavior and are coded by the same gene, their primary structures in the *N*-terminal regions are different because of different splicing of the RNA transcript. Thus, liver and islet enzymes from human and rat contain 465 amino acids [27, 28] with *N*-termini differing by 13–15 residues. Determination of the gene structures for the two rat enzymes showed that both cDNAs are coded by a single gene with 10 exons, but that exon 1 is different because of different promoter regions in the two tissues. The existence of alternative promoters allows tissue-specific regulation of the gene; no corresponding regulation has been described for the hexokinases of high affinity.

Molecular Mass and Evolutionary Considerations

Eukaryote hexokinases from different sources have molecular masses of 50 or 100 kDa, with subunits, when they exist, also of 50 or 100 kDa [29]. Fungal and invertebrate hexokinases have a monomer molecular mass of 50 kDa, although a few, such as yeast hexokinase, dimerize readily. Vertebrate hexokinases, on the other hand, are monomers of 100 kDa, apart from glucokinase, a monomer of about 50 kDa that does not dimerize. There is good evidence for gene duplication and fusion in the evolution of the vertebrate hexokinases, as the 100 kDa hexokinases are all dimer-like, with *N*- and *C*-terminal halves of the molecule that resemble one another; hexokinase D, with a molecular mass half those of the other three, resembles the *C*-terminal half of a 100-kDa hexokinase more than the *N*-terminal half. None of the enzymes shows any convincing evidence of a 25-kDa unit, and if there was ever a gene duplication and fusion of a 25-kDa molecule sufficient mutations have since accumulated to obliterate any evidence for it [11]. Glucokinase appears to have separated from the other vertebrate hexokinases before the gene duplication and fusion events that produced the 100-kDa molecules, and before the radiation of the different vertebrate classes [11].

Hexokinase Pattern in Vertebrates

When Niemeyer's group demonstrated that the phosphorylation of glucose in rat liver is catalyzed by four isoenzymes they named them hexokinases A, B, C and D according to their order of elution from chromatographic columns of DEAE-cellulose [30]. Their finding was soon confirmed on starch gel electrophoresis by Katzen et al. [31], who named them hexokinases I, II, III, and IV (fortunately respectively). With both methods the profile depends on the isoelectric point of each isoenzyme, which is sensitive to small alterations in primary structure, so it is not surprising that the order of elution may vary from one species to another. For example, the four isoenzymes are present in rat, mouse, hamster and guinea pig, but only the rat shows the electrophoretic order ABCD; in the mouse and guinea pig the order is ACBD, so that the choice of the rat as the basis for the 'standard' pattern has proved to be unfortunate.

The presence of the four hexokinase isoenzymes in rat and human liver is not a universal characteristic of vertebrate liver, however, or even of mammalian liver; in reality, the number of isoenzymes varies between the livers of different species of vertebrates, as some isoenzymes, sometimes even glucokinase, can be missing. The variations in hexokinase isoenzymes have been reviewed by Ureta and colleagues [32, 33] and a summary of the observations in mammals is given in figure 1.

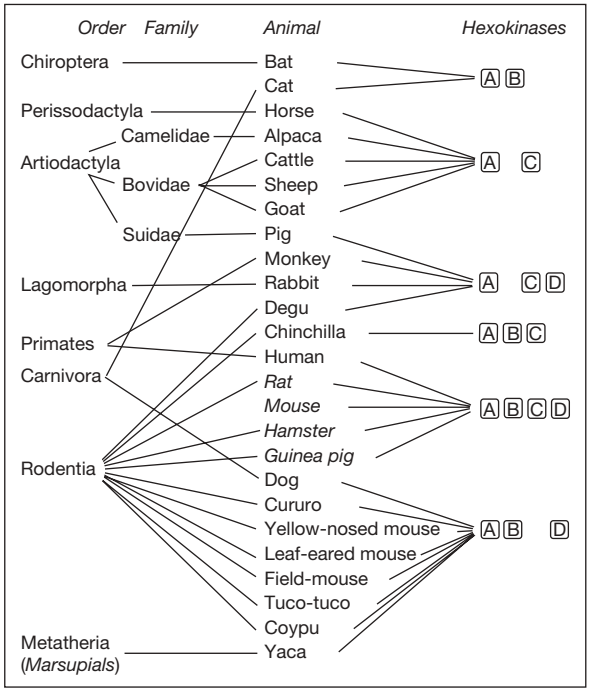

Fig. 1. Distribution of hexokinase isoenzymes in vertebrate species [32, 33].

The full complement of four hexokinases in liver is only observed in inbred laboratory rodents, and in human. A system of three hexokinases is more common, being observed not only in wild rodents but also in pig, rabbit, dog, monkey (*Saimiri scuirea*) and yaca (a marsupial); systems of only two isoenzymes are also found, for example the pattern AC in ruminants and horse, and AB in cat and bat. Some orders, such as rodents, show considerable variation in the profile; others, such as the ruminants, appear quite uniform.

Glucokinase is widely though not universally distributed in the livers of mammals (fig. 1), and also occurs in amphibians and turtles [33]. In all of these species it has similar kinetic characteristics, a high $K_{0.5}$ for glucose compared with the other isoenzymes, and cooperativity with respect to glucose [1, 21]. Glucokinase activity appears to be absent from the livers of 13 species from six orders of birds [34], and from 13 species of higher reptiles of the order Squamata [35]. Among the mammals it is absent from the livers of cat and bat. Although it was not initially found in fish, some authors have recently described the presence in several species of an enzyme that is induced by a diet rich in carbohydrates and that could correspond to glucokinase [36–39].

In animals where glucokinase has not been found it could, perhaps, be induced if they were given a carbohydrate-rich diet.

The absence of glucokinase from the livers of mammals like cat and bat raises the question of whether it could be present in the pancreas of these animals, because in rat and human, and presumably in most mammals, it appears to play an important role in insulin secretion. As the glucokinase gene has different promoters in liver and in pancreas, its absence from the liver does not necessarily imply its absence from the pancreas, where it appears to be constitutive in rat and human. However, there have been no comparative studies for the pancreas of different species. Apart from the existence of glucokinase in β-cells of rat [40], where it was first demonstrated unambiguously, and in human [41], it has also been reported in β-cells from rabbit [42] and mice [43]; there are also some indications that it may exist in the toad (*Bufo arenarum*) [44].

In the pancreas of birds and higher reptiles, the glucokinase gene may not be expressed as there are important differences between blood sugar levels in birds as compared to mammals, and the glucose sensing could be achieved by a different mechanism. If this is the case, what has become of the silenced gene? Is it now a pseudogene or is it merely permanently repressed? In fish the gene appears to be silent in wild conditions and glucose intolerance in carnivorous fish has been attributed to the lack of hepatic glucokinase activity. However, an experimental diet can induce expression of the gene, and the resulting production of a functional enzyme indicates that the gene is still present and has not become a pseudogene [39].

Cooperative Kinetics of Glucokinase with Glucose: A Well-Preserved Feature through Evolution

As glucokinase exists in the livers of many mammals, reptiles and amphibians, we explored whether the sigmoidal rate behavior of the rat enzyme with respect to glucose also applied to the enzyme from other species, and in particular if there was any species with a considerably higher degree of cooperativity. As illustrated in table 1, the enzyme from each vertebrate species studied has the same kinetic behavior as the rat enzyme [18, 21] with essentially the same Hill coefficient (1.4–1.7) throughout. Thus the sigmoidal saturation function appears to be a common characteristic of vertebrate hepatic glucokinase, and its preservation through evolution gives further support to the idea that it is of physiological importance.

The values of $K_{0.5}$ in different vertebrates seen in table 1 vary rather more than the Hill coefficients, with the amphibians consistently showing the lowest

Table 1. Kinetic characteristics at pH 7.5 of glucokinase from different species: the data are from ref. 21 apart from those for the human [45] and sea bream [39]

Animal	Hill coefficient	$K_{0.5}$ mM
Mammals		
Akodon (*Akodon olivaceus*)	1.4	3.0
Dog (*Canis familiaris*)	1.5	3.2
Pig (*Sus scrofa*)	1.5	3.8
Cururo (*Spalacopus cyanus*)	1.4	4.4
Coypu (*Myocastor coypus*)	1.4	6.2
Hamster (*Mesocricetus auratus*)	1.6	7.5
Rat (*Rattus norvegicus*)	1.6	7.5
Guinea pig (*Cavia porcellus*)	1.6	8.5
Human (*Homo sapiens*)	1.7	7.9
Fish		
Gilthead sea bream (*Sparus aurata*)	1.7	12.0
Reptiles		
Chilean turtle (*Geochelone chilensis*)	1.6	5.2
Amphibians		
Chilean frog (*Calyptocephalella caudiverbera*)	1.6	1.5
Clawed frog (*Xenopus laevis*)	1.7	2.6
Horned frog (*Ranita cornuda*)	1.7	2.6
Axolotl (*Ambistoma mexicanum*)	1.6	3.4

values. The Hill cofficient does not vary with pH, but $K_{0.5}$ decreases as the pH increases [21].

The evolutionary preservation of the cooperative kinetics of vertebrate glucokinase is paralleled by the presence of its regulatory protein in all the species where the enzyme itself exists, as described by Van Schaftingen in another chapter in this book.

Control by Gene Expression: Variation in Response to Diet

In contrast to the kinetic cooperativity of vertebrate glucokinase, which is a well conserved feature, its variation in response to diet is not well conserved, as it does not behave like an adaptive enzyme in all species. Thus, although glucokinase activity decreases after fasting in rat, mouse and hamster, or with a carbohydrate-free diet, it does not change significantly in other rodents, such as the guinea pig and the degu [46]. In addition, different strains of mouse respond

differently to diet: a carbohydrate-free diet (high protein, high fat) causes decreased glucokinase activity in mice of strain C57BL but not in those of the BALB type.

Among the other species with adaptive glucokinase are dog [47] and human [48]. By contrast, amphibians such as the toad *Bufo spinulosus* show no change of the glucokinase activity after several days of fasting [49]. In turtle, there are suggestions that the liver enzyme disappears during hibernation [21], but no systematic studies have been done.

In the chapter by Cornish-Bowden and Cárdenas elsewhere in this book, we argue that the kinetic properties of glucokinase are those appropriate for an enzyme that modulates a supply-regulated process, something relatively rare in metabolism, regulation by demand for end product being more usual. Several of the comparative aspects discussed in this chapter point in the same direction. The high degree of uniformity of kinetic properties across all the vertebrate species in which it has been characterized indicate that these properties fulfil a necessary regulatory role. The glucose transporter GLUT2 has a half-saturation concentration for glucose similar to that of glucokinase, and like glucokinase it is highly expressed in hepatocytes and pancreatic β-cells, allowing facilitated diffusion of glucose to respond sensitively to changes in the glucose concentration at physiological levels [50]. Finally, the genetic control of glucokinase expression in rat and human, with insulin acting to induce transcription of the RNA, is to be expected for an enzyme that responds to changes in substrate supply but would be difficult to explain for one that responded to demand for its product.

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Maturity Onset Diabetes of the Young Type 2

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The term MODY, for maturity onset diabetes of the young, describes a group of affections characterized by familial hyperglycemia with autosomal dominant inheritance [1, 2]. Hyperglycemia in MODY subjects usually develops at childhood, adolescence or young adulthood, and is associated with primary insulin-secretion defects. MODY is not a single entity but presents genetic, as well as metabolic and clinical heterogeneity. Mutations in six genes cause most of the MODY cases. These genes encode the enzyme glucokinase (associated with MODY2) [3] and the transcription factors hepatocyte nuclear factor 4-α, HNF-4α (MODY1) [4], HNF-1α (MODY3) [4], insulin promoter factor 1, IPF-1 (MODY4) [5], HNF-1β (MODY5) [6], and neurogenic differentiation factor 1 (MODY6) [7].

MODY seems to have a worldwide distribution, but its prevalence is still unknown. Although commonly thought to be a relatively rare form of diabetes, its frequency might have been underestimated, as the hyperglycemia can remain undiagnosed until adulthood. It has been estimated that 2–5% of patients with type 2 diabetes may in fact have MODY [8]. The relative prevalence of the different subtypes of MODY has been shown to vary greatly in studies of MODY families from different populations. MODY2 represents from 8 to 63% of cases and MODY3 from 21 to 64% of cases. The other MODY subtypes are rare disorders, having been described only in a few families, while additional unknown MODY locus or loci (MODY-X) represent 16–45% of the cases of MODY. These contrasting results may be due to differences in the genetic background

of these populations, or else, may reflect, at least partly, ascertainment bias in the recruitment of families.

Genetics of MODY2

Heterozygous mutations in the glucokinase gene are a common cause of MODY. One hundred ninety-one different mutations (table 1; fig. 1) have been reported in subjects of many different racial and ethnic backgrounds [9]. These mutations alter the protein sequence by changing one amino acid, or transform the sequence of a site of RNA splicing at an exon/intron junction resulting in the expression of abnormal species of messenger RNA, or still are responsible for the synthesis of a truncated protein by creation of a premature termination codon, either by a point mutation or by deletion or insertion of basepairs. The majority of the mutations identified to date have been described in only a single family suggesting that the present list is not exhaustive and that new mutations will continue to be found. Spontaneous mutations have been described but represent less than 3% of the mutations found [9]. It is noteworthy that homozygous or double heterozygous mutations in the glucokinase gene result in a different phenotype, leading to permanent neonatal diabetes mellitus [10] (see chapter by Harrison and Weber, pp. 135–144).

Pathophysiology of Glucokinase Mutations

Glucokinase plays a key role in the regulation and integration of glucose metabolism in pancreatic beta cells and hepatocytes. In pancreatic β-cells, glucose metabolism and insulin secretion are strongly dependent on the enzymatic activity of glucokinase. The study of patients with naturally occurring mutations has helped to give new insights into the role of glucokinase. Expression studies have shown that the enzymatic activities of the glucokinase mutants detected in MODY2 families were impaired [11–15], resulting in decreased glycolytic flux in pancreatic beta-cells. More details of the in vitro work and the modeling of results are given in chapter by Gloyn et al. [pp. 92–109].

β-Cell Dysfunction

The principal pathophysiological mechanism of hyperglycemia in patients with glucokinase mutations is β-cell dysfunction [16–18]. This is characterized by an increase in the blood glucose threshold that triggers insulin secretion, from a normal basal concentration of 4–5 m*M* to approximately 6–7 m*M* [16, 18], with a right shift in the dose-response curves relating insulin secretion

Table 1. Mutations in the glucokinase gene associated with hyperglycemia

Nucleotide change	Codon/sequence change	Protein effect/ mutation name	Population/ (number of families)	Primary references	Functional characterization
Exon 1a					
28delG	GCCdelG	A10fs (E9fsdelG)	Italian	[43]	
45+1G>T	IVS1A+1G>T	IVS1A+1G>T	Italian (2)	[43, 44]	
Exon 2					
76C > T	CAG > TAG	Q26X	Italian	[43]	
89T > C	CTG > CCG	L30P	Italian	[44]	
97_117dup	dupGTGATGAGA CGGATGCAGAAG	V33_K39dup (E40ins21)	UK (2)	[33, 35]	
106C > T	CGG > TGG	R36W	French	[45]	[15]
			Italian (2)	[43, 46]	
113A > C	CAG > CCG	Q38P	Italian (2)	[43, 44]	
118G > A	GAG > AAG	E40K	Czech (4)	[47]	
128G > A	CGC > CAC	R43H	German	[48]	
130G > A	GGC > AGC	G44S	Italian	[49]	[50]
			UK	[51]	
131G > A	GGC > GAC	G44D	Czech	[47]	
			UK (2)	[51]	
131delG	GGCdelG	G44fs (G44fsdelG)	UK	[51]	
148C > T	CAT > TAT	H50Y	Italian (2)	[43, 46]	
149A > G	CAT > CGT	H50R	Italian	[43]	
157G > T	GCC > TCC	A53S	French	[28]	[13, 15, 50]
184G > A	GTG > ATG	V62M	UK	[52]	[50, 52]
			Italian	[52]	
185T > C	GTG > GCG	V62A	Norwegian	[53]	[50]
208G > A	GAA > AAA	E70K	French	[3]	[12, 13, 50, 54, 55]
Exon 3					
214G > A	GGG > AGG	G72R	Scandinavian	[56]	[50]
			Canadian	[57]	
			Italian	[46]	
218A > G	GAC > GGC	D73G	Italian	[43]	
232G > C	GAC > CAC	D78H	Hungarian	[51]	

234C > G	GAC > GAG	D78E	UK	[51]	
238G > A	GGT > AGT	G80S	Italian	[58]	
239G > C	GGT > GCT	G80A	French	[28]	[13, 15]
241G > A	GGC > AGC	G81S	Italian	[43]	
264_273del	delGGTG AAGGTG	V89fs (L88fsdel10)	French	[45]	
*	*	E93del10	Italian	[43]	
292C > T	CAG > TAG	Q98X	French	[3]	
296G > A	TGG > TAG	W99X	German	[48]	
301G > A	GTG > ATG	V101M	Chinese	[59]	
308C > A	ACC > AAC	T103N (2)	Italian	[43, 46]	
311_314del	delAACA	K104fs (K104fsdel4)	Italian	[44]	
322T > C	TAC > CAC	Y108H	UK (2)	[35, 51]	
323A > G	TAC > TGC	Y108C	Spanish	[60]	[50]
323A > T	TAC > TTC	Y108F	African-American	[61]	
324C > A	TAC > TAA	Y108X	UK	[51]	
329T > C	ATC > ACC	I110T	Chinese	[62]	
332C > T	CCC > CTC	P111L	UK	[51]	
356C > A	GCT > GAT	A119D	Chinese	[62]	
363+1G>A	IVS3+1G>A	IVS3+1G>A	Scandinavian	[56]	
363+1G>T	IVS3+1G>T	IVS3+1G>T	Italian	[43]	
Exon 4					
364-2A > G	IVS3-2A > G	IVS3-2A > G	Scandinavian	[56]	
364-1G > T	IVS3-1G > T	IVS3-1G > T	French	[28]	
364C > G	CTC > GTC	L122V	Italian	[43]	
373_375del	delTAC	Y125del	Italian (2)	[43]	
373_394dup	dupTACATCTCTGA GTGCATCTCCGAC	Y125_D132dup	Scandinavian	[56]	
386G > A	TGC > TAC	C129Y	Italian	[43]	
			UK (2)	[51]	
389T > C	ATC > ACC	I130T	UK	[51]	
391T > C	TCC > CCC	S131P	Puerto-Rican	[31]	[12]
401T > C	CTG > CCG	L134P	Italian (2)	[43]	
410A > G	CAT > CGT	H137R	French	[28]	[13, 50]
413A > C	CAG > CCG	Q138P	Italian (2)	[43]	
437T > G	CTG > CGG	L146R	Norwegian	***	[50]

Table 1 (continued)

Nucleotide change	Codon/sequence change	Protein effect/ mutation name	Population/ (number of families)	Primary references	Functional characterization
445A > C	ACC > CCC	T149P	UK	[51]	
446C > T	ACC > ATC	T149I	Italian	[44]	
448_450del	delTTC	F150del	Italian	[43]	
449T > C	TTC > TCC	F150S	UK (2)	[33, 35]	
450C > A	TTC > TTA	F150L	Czech	[47]	
452_453del	TCCdelCC	S151fs (S151fsdelCC)	Italian	[43]	
461_462del	GTGdelTG	V154fs (V154fsdelTG)	German	[63]	
466C > T	CAC > TAC	H156Y	Italian	[51]	
469G > A	GAA > AAA	E157K	Italian	[43]	
483G > C	AAG > AAC	K161N	Italian	[46]	
483+2_483+11del	delTGGGCCGGGT	IVS4+2del10	French	[28]	
483+2_483+16del	delTGGGCCG	IVS4+2del15	French	[3]	
	GGTGGAGG		UK (2)	[33, 35]	
Exon 5					
485delG	GGCdelG	G162fs (G162fsdelG)	German	[48]	
491T > C	CTT > CCT	L164P	Korean	[64]	
502A > C	ACC > CCC	T168P	French	[28]	[13, 15, 50]
504delC	ACCdelC	K169fs	Argentine	[65]	
507G > ?*	AAG > AA?*	K169N	Italian	[43]	
511T > C	TTC > CTC	F171L	Italian	[46]	
517G > T	GCC > TCC	A173S	Italian (3)	[43, 66]	
523G > A	GGA > AGA	G175R	French	[3]	[11, 13, 50]
524G > A	GGA > GAA	G175E	Italian	[43]	
524G > T	GGA > GTA	G175V	Brazilian	[67]	
532G > C	GGG > CGG	G178R	Italian	[43]	
540T > G	AAT > AAG	N180K	UK (4)	[33, 51]	
542T > C	GTC > GCC	V181A	Italian	[43]	
544G > A	GTG > ATG	V182M	French (2)	[3]	[11, 13, 50, 68]
			Spanish	[69]	
			UK	[51]	
			American Caucasian	[51]	

556C > T	CGA > TGA	R186X	French (2)	[3, 28]	
			Japanese	[70]	
			Congolese	[45]	
			UK (4)	[51]	
562G > A	GCT > ACT	A188T	Japanese	[71]	[12]
			Sardinian	[72]	
			Spanish	[60]	
			UK	[51]	
[563C > A; 564T > A]	GCT > GAA	A188E	Spanish	[60]	[50]
571C > T	CGG > TGG	R191W	UK (2)	[33, 51]	
			Italian (4)	[43]	
572G > A	CGG > CAG	R191Q	Italian	[43]	
577G > C	GGG > CGG	G193R	Italian	[43]	
579+1_579+32del	delGTGAGGGGGC ACCTGTACCTGC CGGGGGGGCTG	IVS5+1del33	French	[45]	
Exon 6					
608T > C	GTG > GCG	V203A	French	[3]	[11, 13, 50, 55, 68]
			Swiss (4)	[73]	
617C > T	ACG > ATG	T206M	Sardinian	[72]	
			Italian (2)	[43, 46]	
617C > G	ACG > AGG	T206R	Canadian	[57]	
626C > T	ACG > ATG	T209M	French	[45]	[15]
629T > A	ATG > AAG	M210K	Norwegian**	[10]	[10, 50]
629T > C	ATG > ACG	M210T	French	[28]	[50]
637T > C	TGC > CGC	C213R	French	[28]	[13, 15, 50]
645C > A	TAC > TAA	Y215X	UK (2)	[33]	
646G > T	GAA > TAA	E216X	German	[48]	
660C > A	TGC > TGA	C220X	German	[48]	
			Czech	[47]	
661G > A	GAG > AAG	E221K	Italian (3)	[43, 46, 58]	
667G > A	GGC > AGC	G223S	Italian (2)	[43]	
			UK (2)	[51]	
671T > C	ATG > ACG	M224T	Italian	[43]	
673A > T	ATC > TTC	I225F	German	[48]	
675C > G	ATC > ATG	I225M	Italian (3)	[43, 44]	

Table 1 (continued)

Nucleotide change	Codon/sequence change	Protein effect/ mutation name	Population/ (number of families)	Primary references	Functional characterization
676G > A	GTG > ATG	V226M	French	[28]	[13, 15, 50]
			Czech (2)	[47]	
[677delT; 676_677insAA]	GTGdelTinsAA	V226fs	Spanish	[69]	
679G > T	GGC > TGC	G227C	Italian	[58]	
679+2T>A	IVS6+2T>A	IVS6+2T>A	Italian	[43]	
Exon 7					
680-2A > T	IVS6-2A > T	IVS6-2A > T	French	[3]	
682A > G	ACG > GCG	T228A	Italian	[46]	
683C > G	ACG > AGG	T228R	Italian	[46]	
683C > T	ACG > ATG	T228M	French	[74]	[11, 13]
			UK	[34]	
			Italian**	[10]	[10]
692delA	AATdelA	N231fs (N231fsdelA)	Italian	[43]	
704T > C	ATG > ACG	M235T	Italian	[44]	[50]
713delT	ATGdelT	M238fs (M238fsdelT)	Spanish (2)	[60, 69]	
716A > G	CAG > CGG	Q239R	Chinese (4)	[59]	
742G > T	GAG > TAG	E248X	Brazilian	[28]	
751A > G	ATG > GTG	M251V	Czech	[47]	
753G > ?*	ATG > AT?*	M251I	Italian	[43]	
754T > C	TGC > CGC	C252R	Czech	[47]	
755G > A	TGC > TAC	C252Y	Spanish	[60]	[50]
766G > A	GAG > AAG	E256K	Swedish	[11]	[11]
769T > C	TGG > CGG	W257R	French	[75]	[12]
772G > T	GGC > TGC	G258C	Italian	[46]	
775G > A	GCC > ACC	A259T	UK	[35]	
781G > A	GGG > AGG	G261R	French (3)	[28, 74]	[11, 13, 50]
			Japanese	[76]	
			Italian (3)	[43]	
			UK (2)	[51]	
782G > A	GGG > GAG	G261E	French	[45]	
787T > C	TCC > CCC	S263P	Canadian	[57]	[50]

790G > A	GGC > AGC	G264S	Italian	[43]	[50]
			Israeli Arabic**	[77]	
793G > T	GAG > TAG	E265X	American-Caucasian	[31]	
			Italian	[44]	
802G > T	GAG > TAG	E268X	Czech	[47]	
			Italian	[46]	
*	*	L271del22	Italian	[43]	
823C > T	CGC > TGC	R275C	Italian	[66]	[50]
834C > A	GAC > GAA	D278E	Italian	[44]	
835G > C	GAG > CAG	E279Q	African-American (2)	[11]	[11, 68]
835G > T	GAG > TAG	E279X	French	[78]	
842C > T	TCT > TTT	S281F	Italian	[43, 46]	
Exon 8					
864-1G > A	IVS7-1G > A	IVS7-1G > A	Italian	[44]	
			UK	[33]	
865C > T	CAG > TAG	Q286X	UK	[51]	
881G > A	GGT > GAT	G294D	Czech	[47]	
884delG	GGCdelG	G295fs (G295fsdelG)	UK	[51]	
893T > A	ATG > AAG	M298K	Spanish	[60]	[50]
895G > C	GGC > CGC	G299R	UK (3)	[32, 35, 79]	[11]
898G > C	GAG > CAG	E300Q	French	[3]	[11]
898G > A	GAG > AAG	E300K	French	[3]	[11, 13, 50, 54, 55]
911T > C	CTT > CCT	L304P	German	[48]	
926T > C	CTC > CCC	L309P	French	[3]	[11, 13]
928_929del	GTGdelGT	V310fs (V310fsdel)	UK	[51]	
952G > A	GGG > AGG	G318R	Czech	[47]	
			UK	[51]	
1000_1018del	delTTCGTGTCG CAGGTGGAGA	F334fs (F334fsdel19)	UK	[51]	
1007C > T	TCG > TTG	S336L	French	[28]	[13, 50]
1016A > G	GAG > GGG	E339G	Norwegian	***	[50]
1019+2T>G	IVS8+2T>G	IVS8+2T>G	Israeli Arabic**	[77]	
Exon 9					
1024A > C	ACG > CCG	T342P	UK	[51]	
1072C > T	CGA > TGA	R358X	Spanish	[69]	

Table 1 (continued)

Nucleotide change	Codon/sequence change	Protein effect/ mutation name	Population/ (number of families)	Primary references	Functional characterization
1079C > A	TCG > TAG	S360X	French	[28]	
1099G > A	GTG > ATG	V367M	French	[28]	[13, 15, 50]
1106G > C	CGC > CCC	R369P	Spanish	[60]	
1129C > T	CGC > TGC	R377C	Norwegian	***	[50]
1133C > T	GCT > GTT	A378V	Anatolian Turkish**	[77]	[50, 77]
1140C > A	CAC > CAA	H380Q	Italian	[66]	
1145G > A	TGC > TAC	C382Y	UK	[35]	
1148delC	TCGdelC	S383fs (S383fsdelC)	Italian	[43]	
1148C > T	TCG > TTG	S383L	German	[48]	[50]
			Spanish	[60]	
1148C > A	TCG > TAG	S383X	Italian	[46]	
1150G > A	GCG > ACG	A384T	UK	[35]	
1154G > T	GGG > GTG	G385V	Chinese	[62]	
1159G > A	GCG > ACG	A387T	UK	[51]	
1160C > T	GCG > GTG	A387V	UK	[51]	
1174C > T	CGC > TGC	R392C	UK	[35]	
1183_1209del	delGAGAGCC GCAGCGAGGA CGTAATGCGC	E395-R403del	Italian	[43]	
1201delG	GTAdelG	V401fs (V401fsdelG)	Brazilian	[28]	
1209delC	CGCdelC	I404fs (R403fsdelC)	UK	[35]	
			South Asian	[34]	
1211T > G	ATC > AGC	I404S	Italian	[66]	
1219G > A	GGC > AGC	G407S	Italian	[66]	
1232C > T	TCC > TTC	S411F	Spanish	[60]	[50]
1240A > G	AAG > GAG	K414E	French	[3]	[13, 50, 68]
1243C > G	CTG > GTG	L415V	Italian	[43]	
1253+1G>C	IVS9+1G>C	IVS9+1G>C	Italian	[43]	
1253+1G>T	IVS9+1G>T	IVS9+1G>T	Italian	[46]	

Exon 10				
1254-7_1257del	delTGCCCAGCTTC	IVS9-7del11	Spanish	[69]
1254-1G > C	IVS9-1G > C	IVS9-1G > C	French	[3]
1277_1278del	AGCdelGC	V427fs (S426fsdelGC)	German	[48]
1298_1309del	delGCTGC GAGATCA	S433_I436del	Italian	[46]
1301G > A	TGC > TAC	C434Y	Czech	[47]
1322C > G	TCG > TGG	S441W	Italian	[43]
1335_1345del	delTGGCC GGGGCG	S445fs (S445sdel11)	UK	[51]
1336G > C	GGC > CGC	G446R	UK	[51]
1340_1368del	delGGGGCGCGG CCCTGGTCTC GGCGGTGGCC	R447fs (R447del29)	German	[48]
1340G > A	CGG > CAG	R447Q	Portuguese	[51]
1358C > T	TCG > TTG	S453L	UK	[51]
1358C > A	TCG > TAG	S453X	Italian	[43]
1361C > T	GCG > GTG	A454V	UK	[51]
1379_1398+2del	delCCTGTATGCT GGGCCAGTGAGA	A460fs (A460fsdel22)	Italian	[44]

Mutations reported up to November 2003.

*Details not given in the original publication.

**Homozygous or double heterozygous mutations leading to permanent neonatal diabetes mellitus.

***Sagen JV, Bjorkhaug L, Odili S, Cuesta-Munoz A, Matschinsky FM, Molven A, Sovik O, Njolstad PR: Mutations in glucokinase and diabetes in Norway. Unpublished data.

All sequence information is based on Genbank reference sequence NM_000162.2.

The nomenclature for nucleotide and protein change is that recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/) [41]. The nomenclature used in the original publication is also given between parentheses when it differs from the recommended nomenclature.

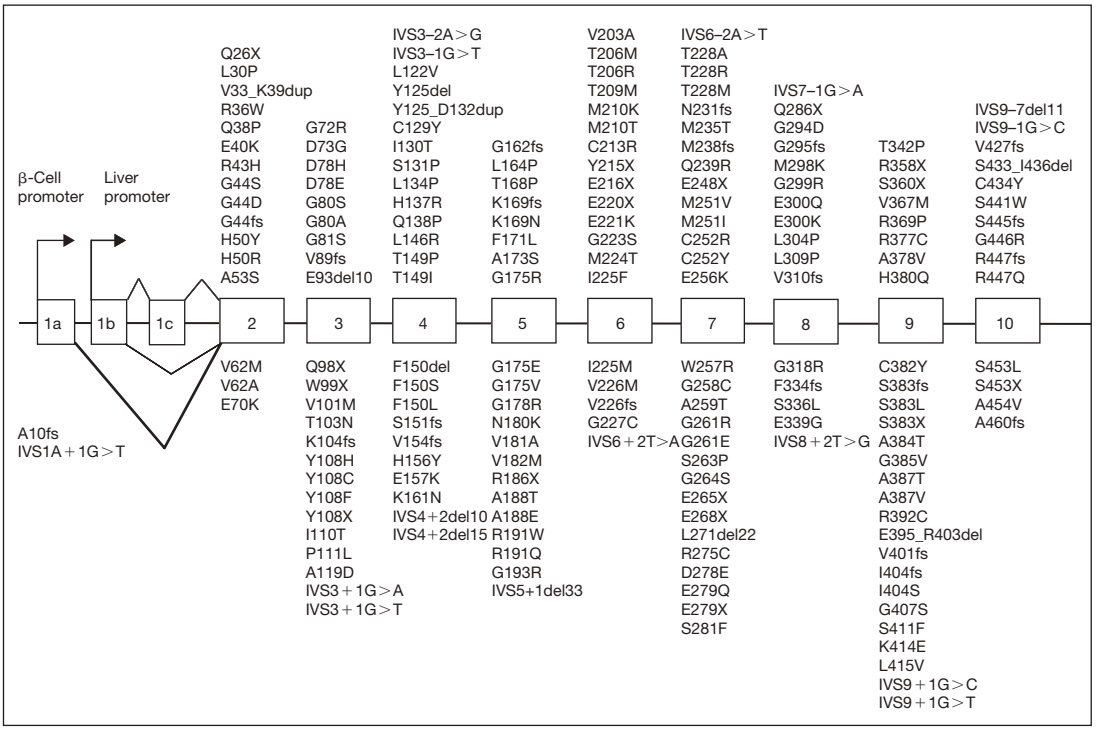

Fig. 1. Schematic representation of the glucokinase gene showing mutations associated with hyperglycemia reported up to November 2003. The nomenclature for nucleotide and protein change is that recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/) [41]. Adapted from Gloyn [9].

and β-cell responsiveness to glucose levels (fig. 2) [18]. This is consistent with a defect in glucose sensing by pancreatic beta-cells. Comparison of insulin secretion rates at a broad range of glucose levels demonstrated that glucokinase-deficient MODY2 subjects present an average 60% reduction in insulin secretion for a given glucose level [18]. Interestingly, insulin levels in MODY2 subjects are usually normal throughout the day, at the expense of the hyperglycemia [18]. The release of insulin in response to other secretagogues such as arginine is usually well preserved or only moderately decreased [19], which suggests that this secretory defect is indeed related to a relative glucose blindness of β-cells. Physiologic adaptation within the pancreatic β-cells limits the severity of the insulin secretion defect. This defect is proportional to the decrease in the glucokinase-mediated flux of glucose in subjects with glucokinase mutations associated with a small decrease in enzymatic activity

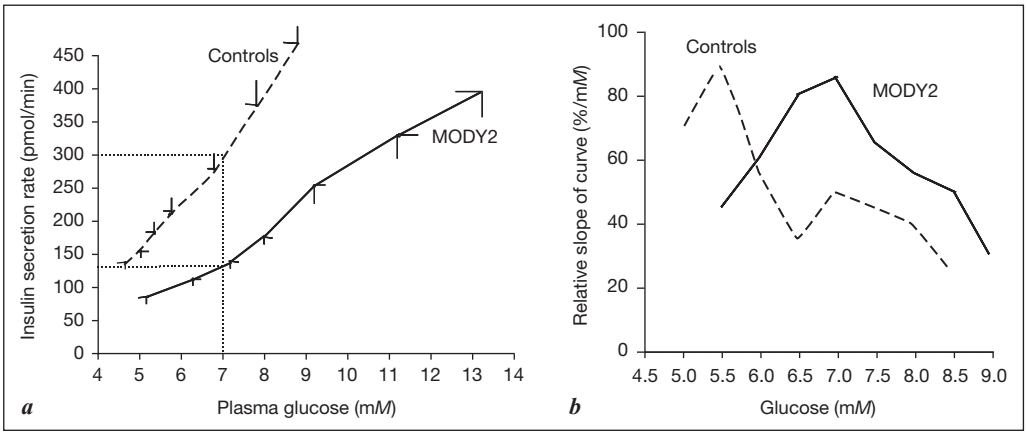

Fig. 2. a Relationship between average glucose concentrations and insulin secretion rates during graded intravenous glucose infusions in MODY2 (solid line) and control subjects (dashes). ***b*** β-Cell responsiveness to changes in plasma glucose concentration. The relative slope of insulin secretion rates (curve A) for each 0.5 m*M* increments in glucose was plotted against the glucose concentration. The data show that the increment in insulin secretion to a small change in glycemia is greatest for the 5.5–6.0 m*M* glucose interval for the controls (82%/m*M*; dashes). At this glycemic interval, the increase in insulin secretion is only 35%/m*M* for MODY2 subjects (solid line), with a maximal increase in insulin secretion (74%/m*M*) observed for the 6.5–7.5 m*M* glucose interval. In both groups, β-cell responsiveness is greatest slightly above the usual fasting glucose level of the group. Adapted from Byrne et al. [18].

[18, 20]. However, in subjects with glucokinase mutations that resulted in a severe decrease in enzymatic activity, the insulin secretion defect is less severe than predicted, suggesting the presence of compensatory mechanisms that increase the insulin-secretion response [18, 20]. Studies in animal models suggest that this compensatory mechanism is related to glucose-induced increased expression of the single wild-type glucokinase-gene allele [21].

Hepatic Glucose Metabolism Dysfunction

In addition to the altered beta cell function, abnormalities in liver glucose metabolism contribute to the pathogenesis of hyperglycemia in patients with MODY2 (fig. 3). Hepatic glycogen concentration was measured in MODY2 subjects using ^{13}C nuclear magnetic spectroscopy during a day in which three isocaloric mixed meals were ingested [22]. The relative fluxes of the direct (glucokinase-dependent) and indirect (gluconeogenic, non-glucokinase-dependent) pathways of hepatic glycogen synthesis were also assessed. The net increment in hepatic glycogen content following each meal was 30–60% lower in MODY2 subjects as compared to control subjects. Furthermore, the

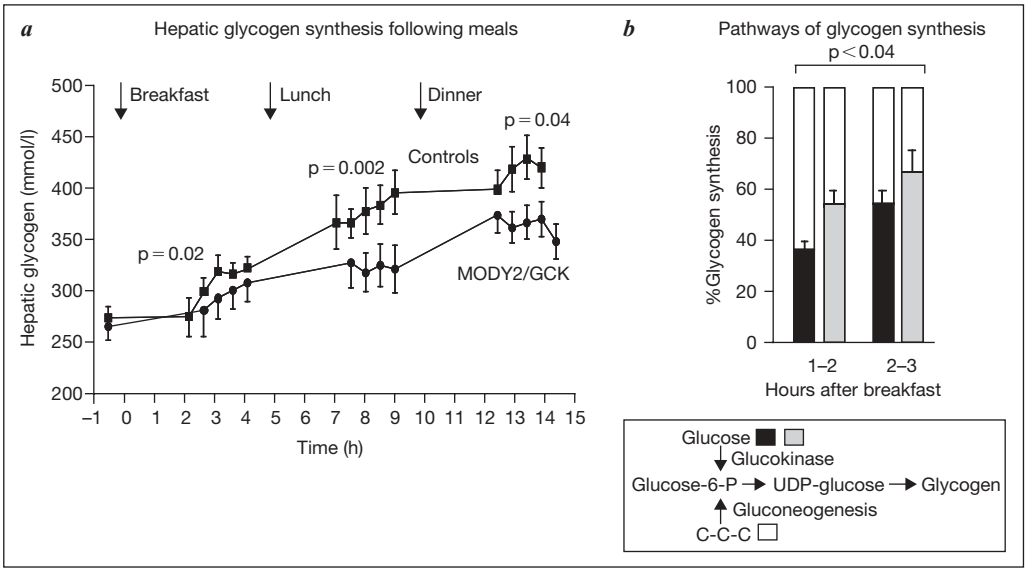

Fig. 3. ***a*** Time course for mean hepatic glycogen concentration following breakfast, lunch and dinner, assessed by nuclear magnetic resonance spectroscopy in MODY2 (circles) and control (squares) subjects. The increment in hepatic glycogen concentration after each meal was significantly less in patients than in controls. Data expressed as mean ± SEM. ***b*** Relative fluxes of the direct and indirect pathways of hepatic glycogen synthesis determined at 1-hour intervals after breakfast in MODY2 and control subjects. Closed and hatched bars represent the percentage of hepatic glycogen synthesized by the direct pathway in MODY2 and control subjects, respectively. Open bars represent the percentage of hepatic glycogen synthesized by the indirect pathway in each group. Adapted from Velho et al. [22].

gluconeogenic pathway was relatively more important for synthesizing hepatic glycogen in MODY2 subjects. In the first 3 h following breakfast more than 60% of the glycogen was derived from gluconeogenesis in MODY2 subjects compared to less than 50% in the control subjects. Because most of the glucose that is taken up by the liver after a meal is converted to hepatic glycogen [23], any decrease in net hepatic glycogen synthesis would be expected to exacerbate postprandial hyperglycemia. Normal glucose tolerance after a meal depends on normal suppression of hepatic glucose production [24]. The increased relative rates of gluconeogenesis observed following a meal is likely an important contributing factor to the moderate postprandial hyperglycemia seen in MODY2 subjects [25]. In support of this, abnormal suppression of hepatic glucose production by physiological levels of insulin has been observed in MODY2 subjects during an euglycemic clamp [26]. In addition MODY2 subjects have decreased hepatic

glucose cycling, and an endogenous glucose production that is abnormally high in relation to their plasma glucose levels and present a blunted suppression after oral glucose administration [27]. Taken together, these observations would suggest a role of the liver in the pathophysiology of hyperglycemia associated with glucokinase deficiency although it is likely to be less than the role of pancreatic β-cells. Although some MODY2 subjects may also present with peripheral insulin resistance, the decreased insulin sensitivity is probably unrelated to the glucokinase mutations [26].

Clinical Features of MODY2

Hyperglycemia and Presentation

The key clinical feature of patients with glucokinase mutations is that they have life long mild fasting hyperglycemia (typically 5.5–8.0 mmol/l) and their glucose is regulated at this elevated concentration. The phenotype is remarkably similar for all patients with glucokinase mutations despite considerable differences in the severity of the glucokinase mutations in vitro [25]. In a series of over 250 patients no subjects were found with a fasting glucose that was consistently less than 5.5 mmol/l [25]. The hyperglycemia is present from birth and may be diagnosed at any age from the neonatal period [28]. As the age of diagnosis is merely when the hyperglycemia is first tested, terms like penetrance are inappropriate in the context of MODY2. It is rare for patients to have symptomatic hyperglycemia and most patients are diagnosed incidentally or on routine or family screening [28]. Typically, there is little increase in blood glucose after food or an oral glucose load (fig. 4). In an oral glucose tolerance test (OGTT) the increase above fasting at 2 h is usually less than 3 mmol/l. This low increment explains why only a minority of patients have diabetes on WHO criteria and most patients have impaired fasting glucose and/or impaired glucose tolerance [25, 28]. In this regard, glucokinase patients are the only group of patients described in which diabetes is more likely to be diagnosed by fasting glucose than a 2-hour value [25]. In most MODY2 subjects, the increase in fasting glucose with age is only moderate (fig. 5), and probably similar to that seen in normal controls [25, 29]. However, some patients will have progressively increasing hyperglycemia, but this seems to be exceptional.

Diabetes-Related Complications

In keeping with the mild hyperglycemia found in these patients, there is a lower prevalence of diabetes microvascular complications (retinopathy and proteinuria) in MODY2 than in other subtypes of MODY and type 2 diabetes [28, 30]. Moreover, the well established association of type 2 diabetes or

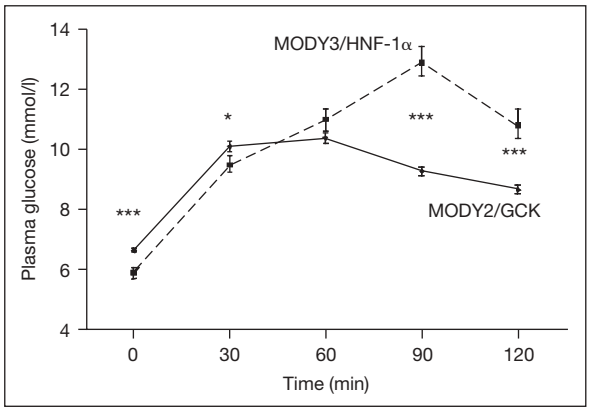

Fig. 4. Glucose concentration during an oral glucose tolerance test (OGTT) in subjects with MODY2 (glucokinase mutations, solid line) and MODY3 (HNF-1α mutations; dashes). Data expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.0001$. Adapted from Stride et al. [25].

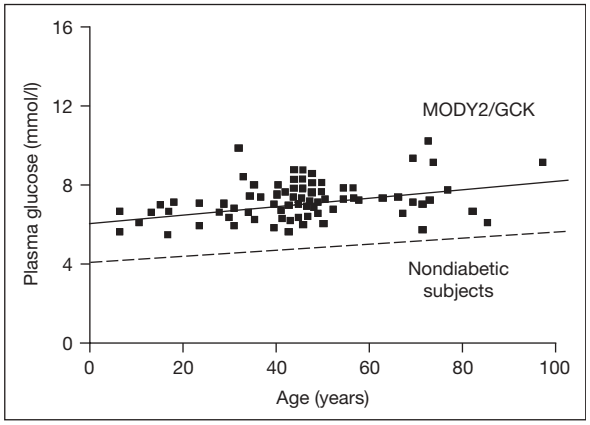

Fig. 5. Fasting glucose plotted against age of subjects with glucokinase mutations. The solid line (MODY2 subjects) and dashes (nondiabetic subjects, individual data not shown) represent the 'best fit' using the least squares method. Adapted from Pearson et al. [42].

impaired glucose tolerance with a cluster of risk factors for macrovascular disease including hypertension, obesity, and dyslipidemia is very rare in MODY2 subjects, which is consistent with the low frequency of coronary heart disease in these individuals [28].

Glucokinase Mutations and Pregnancy

Diagnosis in Pregnancy

Pregnant women are usually screened for hyperglycemia, especially when there is a family history of diabetes. As the mild, asymptomatic hyperglycemia associated with glucokinase mutations can be easily overlooked during childhood and young adulthood, the diagnosis of MODY2 is often made during pregnancy. Glucokinase mutations are found in 1–4% of women diagnosed with gestational diabetes [31–34]. The identification of MODY2 women is important because they have a different clinical course both within and outside pregnancy compared to most other women with gestational diabetes. Specific criteria favoring a diagnosis of a glucokinase mutation in women presenting with gestational diabetes have been shown to be persistent fasting hyperglycemia, a small increment on the oral glucose tolerance test (less than 3 mmol/l) and a family history of mild hyperglycemia [33].

Fetal Growth in Glucokinase Pregnancies

A fascinating observation is that glucokinase mutations not only results in hyperglycemia after birth, but also in reduced fetal growth and decreased birth weight [35, 36]. This effect might be due to a reduction in fetal insulin secretion [37]. Despite the reduced birth weight, no differences in height, weight or BMI are observed in pre-adolescent, adolescent and adult MODY2 subjects as compared to their unaffected sibs [36].

As glucokinase mutations cause mild fasting hyperglycemia, it would be predicted that the offspring of mothers with mutations would be large for gestational age. This is was found with offspring, on average, 601 g heavier than babies born to normal mothers [35]. However, in glucokinase families, fetal growth is dependent on the mutation status of the fetus as well as that of the mother [35, 36]. If a fetus inherits a mutation there is a reduction of birth weight by 521 g [35]. This is because reduced sensing of the maternal glucose by the fetal pancreas will lead to reduced fetal insulin secretion and hence reduced fetal mediated intra-uterine growth, with a reduction in birth weight. The outcome of pregnancy is therefore an additive effect of a maternal mutation increasing birth weight and a fetal mutation reducing birth weight (fig. 6). So, a fetus who inherits a glucokinase mutation from the father, and who is born to a normal mother is small. A fetus who does not inherit the mutation, and who is born to a mother with a mutation is large. If both mother and fetus have a glucokinase mutation, the two opposing effects are cancelled out and the baby is of normal weight.

These observations have led to the ‘fetal insulin hypothesis’ [37] which proposes that genetics defects that alter either fetal insulin secretion or fetal insulin

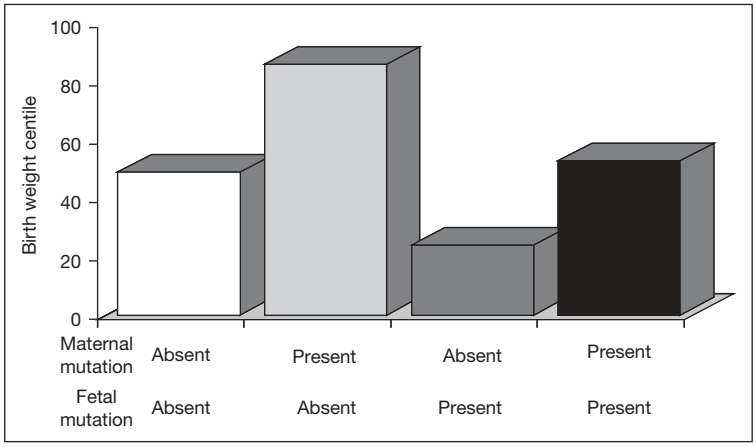

Fig. 6. Birth weight centile distribution in 58 offspring of parents with glucokinase mutations according to maternal and fetal genotype. Adapted from Hattersley et al. [37].

action could, by reducing insulin-mediated growth, reduce birth weight. This therefore provides an explanation of the association seen between low birth weight and diabetes in adult life. This association had previously been attributed to intra-uterine programming as the result of intra-uterine malnutrition [38].

Treatment of MODY2

Treatment of Hyperglycemia

Treatment of hyperglycemia is rarely needed in patients with glucokinase mutations as they are not symptomatic, HbA1c is close to the upper limit of normality and the risk of complications is very low. In some series, as many as 2–30% of patients are treated with oral hypoglycemic agents or even insulin. However, therapy, in most cases, does not alter the glycemic control. As insulin is regulated in these patients, giving exogenous insulin will just result in reduced endogenous insulin secretion, unless a full replacement dose is given (0.6–0.9 U insulin/kg). The role of sulphonylureas is also limited. MODY2 subjects have an increased threshold of hypoglycemic counter-regulation [39; Spyer, Hattersley and MacLeod, pers. commun.] that makes chronic reduction of blood glucose very difficult. In the majority of drug-treated MODY2 patients, discontinuing insulin or other oral agents does not increase the HbA1c. Even dietary recommendations might have a limited role, considering that the exertion in blood glucose is small in MODY2 subjects taking an oral load of 75 grams of glucose [25]. Therefore normal healthy eating advice is the

most appropriate. It is likely that only a small percentage of MODY2 patients will ever need pharmacological treatment. We might speculate that these subjects also carry other susceptibility alleles to diabetes, frequent in the general population. The follow-up of MODY2 patients does not need to be as intensive as in other forms of diabetes, and a yearly review with HbA1c is probably appropriate. Further work is needed to assess the morbidity associated with glucokinase mutations and the role of other genetic and epigenetic factors so that decisions on treatment and follow up can be more strongly evidence-based.

Treatment of MODY2 Pregnancy

Women with glucokinase mutations are often treated with insulin during pregnancy, in an attempt to correct the fasting hyperglycemia. However, fetal genotype but not treatment of the MODY2 mother alters birth weight [Spyer and Hattersley, unpubl. data]. This probably reflects the difficulty in lowering the blood glucose in glucokinase patients due to the increased threshold of hypoglycemic counterregulation [39; Spyer, Hattersley and MacLeod, unpubl. data]. In some cases where the baby has inherited the mutation, intensive insulin treatment has resulted in a low-birth-weight child [40]. This is to be expected, as a small baby is seen when the fetus inherits a mutation from the father and is born to a normoglycemic mother [35, 36]. Treatment decisions in glucokinase gestational diabetes should therefore be related to fetal growth, with insulin only used when there is evidence of macrosomia. If insulin is used to lower glucose, full replacement doses will be required. A mother with a glucokinase mutation will need no pharmacological treatment postdelivery, even if she has received very high insulin doses in pregnancy. This is in contrast to subjects with non-glucokinase gestational diabetes where deterioration to type 2 diabetes is usually seen over the following 5–10 years.

Role of Molecular Genetics in Clinical Care

In this chapter we have shown that making a diagnosis of MODY2 is crucially important for appropriate patient care, as well as being of scientific interest. Patients with glucokinase mutations have a different clinical course from those with type 2 diabetes, regarding the progression of hyperglycemia, treatment requirements, complication risk and pregnancy outcome. Molecular genetic testing for glucokinase mutations is now available as a routine diagnostic service in most European countries. Most centers use sequencing, which is very accurate but still expensive, and time consuming. Patients should be carefully selected for testing as this can greatly increase the positive detection rate [33]. We would recommend limiting testing to those patients and families who

show the appropriate phenotypical characteristics, mainly a mild fasting hyperglycemia that increases little after food or an oral glucose load. However, it is important to realize that the absence of family history of diabetes is not an absolute exclusion criterion, as parents are often not tested for diabetes and a mild fasting hyperglycemia may remain undetected for several decades. As the finding of a glucokinase mutation will have a big impact for patients, especially in young children, we believe the cost of selective testing is easily justified.

Summary

The identification of the glucokinase gene as a MODY gene has allowed us to characterize a specific subtype of familial diabetes. Heterozygous mutations in the glucokinase gene lead to a form of mild and stable chronic hyperglycemia, in which complications are unusual and for which treatment is rarely needed. MODY2 patients are often detected by screening during pregnancy. Recent work has suggested that while maternal mutations increase birth weight as a result of maternal hyperglycemia, the presence of fetal mutations reduce insulin-mediated fetal growth and birth weight. Molecular genetic testing for glucokinase mutations is now used in routine clinical practice. This allows a firm diagnosis of MODY2 to be made. Differences in prognosis and treatment strongly support the increased use of molecular genetic testing for glucokinase mutations.

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Permanent Neonatal Diabetes mellitus due to Glucokinase Deficiency

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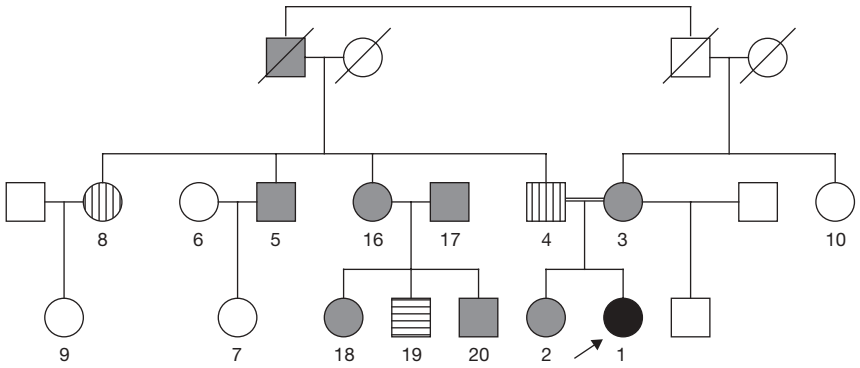
Neonatal diabetes mellitus is defined as insulin-requiring hyperglycemia occurring within the first month of life. It is a rare form of diabetes with an incidence of about 1 in 500,000 live births [1, 2]. Although neonatal diabetes can present with other congenital abnormalities (e.g. macroglossia, umbilical hernia and situs inversus), it is an isolated finding in most cases. Neonatal diabetes is often associated with intrauterine growth retardation. However, insulin treatment leads to catch-up growth suggesting that intrauterine growth retardation is not the cause of neonatal diabetes but rather a consequence of low fetal insulin levels.

Neonatal diabetes can be either transient or permanent [3]. Transient neonatal diabetes mellitus (TNDM) accounts for about 50% of the cases with recovery after 4–60 weeks of age [1, 2]. Some cases of TNDM result from abnormalities of chromosome 6 including paternal uniparental isodisomy and paternal inherited duplications of 6q24 with loss of imprinting [4]. Two imprinted genes, *ZAC* (zinc finger protein associated with apoptosis and cell cycle arrest) and *HYMAI* (imprinted in hydatidiform mole) have been suggested as candidates for TNDM [5, 6]. In many cases, however, the etiology remains unknown. TNDM is a risk factor for development of early-onset type 2 diabetes [1, 2].

Permanent neonatal diabetes mellitus (PNDM) represents about 50% of cases of neonatal diabetes and is characterized by a life long requirement for insulin therapy. PNDM can result from complete deficiency of the β-cell transcription factor insulin promoter factor 1 (IPF-1) (IPF-1-related PNDM or PNDM1) or



Family member no	1	2	3	4	5	6	7	8	9	10	16	17	18	19	20
Glucokinase M210K genotype	MM	NM	NM	NM	NM	NN	NN	NM	NN	NN	NM	NN	NM	NM	NM
Age at diagnosis/ present age, Years	0/17	7/20	25/51	60/65	47/58	–/50	–/23	–/55	–/24	–/56	34/63	62/65	24/28	13/22	22/26
Birth weigtht, g	1,670	3,810	3,500	3,500	3,500	3,500	3,490	4,000	3,400	3,250	1,800	4,500	3,250	3,780	3,790
BMI, kg/m^2	25.6	30.5	32	17.5	23.4	33.1	25.7	25.1	21.5	–	23.7	32.1	18.4	21.8	23.5
Treatment	Insulin	Insulin	Insulin	Diet	OHA	–	–	None	–	–	OHA	Insulin	Insulin	OHA	Insulin
Insulin dose, U/kg/d	1.10	0.90	0.86	–	–	–	–	–	–	–	–	0.42	0.06	–	0.16
Fasting blood glucose, mmol/l	19.1	14.9	12.5	6.6	7.0	5.6	5.0	6.1	3.9	4.1	9.4	9.0	7.1	6.4	7.0
2-h blood glucose, mmol/l	–	–	–	6.2	–	–	6.6	6.8	–	–	20.6	10.9	9.7	8.2	8.6
HbA_{1c}, %	10.7	9.4	8.6	6.8	6.4	5.2	5.2	6.5	4.9	–	7.6	7.5	6.0	6.0	6.9
Other	Ab– RE	Ab+	Ab–	Ab– HC	–	–	HT	HC	–	–	Ab– HC MD	Ab– HT MD	Ab–	Ab– HT	Ab– HT

Fig. 1. Pedigree and clinical characteristics of a Norwegian family (N17) with glucokinase-related PNDM and MODY due to the mutation M210K [10]. The subjects who were studied are numbered and their clinical features are described in the insert. The proband, N17–1, with PNDM and situs inversus (black) is indicated by the arrow. Diabetes mellitus (grey), impaired fasting glucose (horizontal stripes) and impaired glucose tolerance (vertical stripes) were defined using the diagnostic criteria of the WHO. The family has been

the glycolytic enzyme glucokinase (glucokinase-related PNDM or PNDM2). Autoimmune destruction of the pancreatic β-cells is a rare cause of PNDM.

IPF-1 plays a key role in pancreatic development and the regulation of gene expression in the pancreatic β-cell and complete deficiency as a consequence of mutations in both alleles results in pancreatic agenesis with a complete absence of both exocrine and endocrine pancreatic cells [7]. PNDM1 is a very rare disorder with only two individuals with this form of diabetes reported to date. Individuals with a partial deficiency of IPF-1 (i.e. heterozygous carriers) have the type 4 form of maturity-onset diabetes of the young (MODY4, OMIM #606392) [8]. MODY is a form of diabetes mellitus characterized by diabetes with onset usually before 25 years of age and often in childhood or adolescence, autosomal-dominant inheritance and abnormal β-cell function and insulin secretion [9].

Complete deficiency of the glycolytic enzyme glucokinase can also cause PNDM (OMIM #606176) [10, 11]. The parents of these children have one mutant glucokinase allele and have the type 2 form of MODY (MODY2 or glucokinase-related MODY) (OMIM #125851) which is characterized by a mild elevation in fasting glucose levels due to rightward shift in the dose-response curve relating glucose and insulin secretion [9]. They rarely require treatment with oral agents or insulin or develop late-diabetic complications [12]. A fraction of infants with only one mutant glucokinase allele may present with a neonatal diabetes-like disorder that differs from both TNDM and PNDM in that these patients have neonatal hyperglycemia and a transient insulin requirement occurring 15 days to 5 years after birth [13].

Clinical Features of Subjects with Glucokinase-Related PNDM

The infants with glucokinase-related PNDM studied to date had a similar presentation including low birth weight, marked hyperglycemia (>12 mmol/l) within a few days of birth requiring insulin treatment, and parents with various degrees of glucose intolerance including frank diabetes as well as impaired glucose tolerance, impaired fasting glycemia and gestational diabetes (fig. 1; table 1) [10, 11, 14, 15]. An interesting feature is the presence of intrauterine growth

followed for 17 years, and the recent investigations were done during the years 1998–2000. Abbreviations: genotype: N indicates normal M210 allele, and M the mutant K210 allele; Ab−, antibodies against GAD and IA-2 not detectable; Ab+, elevated values for antibodies against GAD and IA-2; BMI = body mass index; HC = hypercholesterolemia; HT = hypertriglyceridemia; OHA = oral hypoglycemic agents; MD = micro- or macrovascular disease; RE = retinopathy; – = no data or not applicable. None had nephropathy. Normal range for HbA_{1c} is 3.0–6.2%.

Table 1. Clinical characteristics and genotypes of subjects with glucokinase-related PNDM

Case No.	Genotype			Parents			Neonatal history					Insulin treatment		Ref.
	proband	mother	father	mother	father	relation-ship	BW/centile g	sex	gestational age weeks	age at diagnosis days	blood glucose* mmol/l	initial U/kg/day	present U/kg/day	
1	M210K/ M210K	M210K/N	M210K/N	DM	IGT	first cousins	1,670 (<3)	F	36	1	16.8	0.8	1.1	10
2	T228M/ T228M	T228M/N	T228M/N	IGT	IFG	#	1,650 (<3)	F	38	1	39.6	2	1.4	10
3	A378V/ A378V	A378V/N	A378V/N	IGT	DM	first cousins	1,550 (10)	F	33	1	13.4	2.4	0.6	11
4	IVS8/IVS8	IVS8/N	IVS8/N	DM	DM	second cousins	1,900 (<3)	M	40	11	57.0	1.2	TDU	11
5	IVS8/ G264S	IVS8/N	†	DM	†	first cousins	1,870 (<3)	M	38	2	12.0	TDU	0.9	11

BW = Birth weight; DM = diabetes mellitus; F = female; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; IVS8 = the intron 8 mutation is a mutation of the splice donor site, IVS8nt+2T>G; M = male; N = normal allele; TDU = treated, but dose unknown.

*First measurement.

†Deceased, unavailable for analysis.

#Not known to be related, but share same chromosomal segment.

Table 2. Effect of glucokinase mutation carrier status of mother and/or child on child's birth weight

Feature	Mother	Child	Mother	Child	Mother	Child	Mother	Child
Genotype	NN	NM	NM	NN	NM	NM	NM	MM
Birth weight	~0.5 kg ↓		~0.5 kg ↑		normal		~1.5 kg ↓	

M = Mutant allele; N = normal allele.

retardation even when the mother is diabetic, and thus imposing her hyperglycemia on the fetus (table 2) [16]. This is indirect evidence that fetal β-cells lacking glucokinase are unresponsive to maternal hyperglycemia.

The hyperglycemia in infants with glucokinase-related PNDM appears to be due to a profound defect in glucose-stimulated insulin secretion (GSIR). C-peptide levels were nearly undetectable from birth in the three cases where this was investigated [Barbetti and Sarici, pers. commun.; 10]. This is not unexpected since glucokinase plays a key role in the regulation of insulin secretion as shown in studies of humans with MODY2 and mice that lack one or both glucokinase alleles [12, 16–19]. Other mammalian hexokinases, such as hexokinase I, II, and III, which are characterized by a high affinity for glucose and unlike glucokinase are subject to feedback control, cannot substitute for glucokinase, primarily because they are kinetically unsuited to serve as a glucose sensor for pancreatic β-cells [20]. In contrast to patients with MODY2, who have a partial deficiency of glucokinase resulting in mild fasting hyperglycemia, the subjects with glucokinase-related PNDM have severe hyperglycemia and require insulin treatment soon after birth. Mice lacking glucokinase also present with growth retardation and hyperglycemia at birth and die soon thereafter if untreated. These mice also have hypertriglyceridemia, hepatic steatosis, and reduced stores of hepatic glycogen, abnormalities that have not been observed in the patients with glucokinase-related PNDM studied to date. Although glucokinase is also expressed in the liver and in glucose-sensing neurons in the gut and central nervous system, there is no evidence for extrapancreatic manifestations of complete glucokinase deficiency in humans. In this regard, a glucagon challenge in 1 patient led to an increase in blood glucose levels indicating that she was able to mobilize glucose from hepatic glycogen stores [10]. The other tissues in which glucokinase is expressed appear able to compensate for its absence whereas the β-cell cannot. The total absence of basal insulin release in subjects with glucokinase-related PNDM is unexplained. Why does basal insulin release, which normally contributes as much as half of the daily insulin output, cease even though it could be stimulated by other fuels (e.g. amino acids and fatty acids) and potentiated by hormones and

Table 3. Kinetic characteristics of wild-type and mutant forms of human glucokinase

Kinetic parameter	Wild-type	M210K	T228M	G264S	A378V
k_{cat}, s^{-1}	64.2 ± 3.06	16.7 ± 1.6	0.004 ± 0.0004	63.5 ± 2.70	55.4 ± 4.69
$S_{0.5}$, mmol/l	7.56 ± 0.31	38.7 ± 1.9	5.5 ± 1.5	9.76 ± 0.74	576 ± 12.1
nH	1.77 ± 0.04	1.63 ± 0.04	0.71 ± 0.07	1.57 ± 0.05	0.94 ± 0.02
K_m ATP, mmol/l	0.37 ± 0.01	1.38 ± 0.11	0.62 ± 0.16	0.48 ± 0.05	9.92 ± 0.31
Relative activity index	1	0.0016	0.0005	0.86	0.0006

Data are mean ± SE. The results are the means of the kinetic analysis of four independent expressions of wild-type and mutant GST-glucokinase. Note that nH and relative activity index are unitless.

neurotransmitters (e.g. glucagon-like peptide 1, gastric inhibitory peptide, and acetylcholine) [20]? Systematic studies of the insulin secretory response in these subjects may provide a better understanding of the role of glucose and other secretagogues in the regulation of insulin secretion.

Biochemical and Cell Biological Considerations

Marked reduction or total blockade of β-cell glucose metabolism provides a plausible explanation for all clinical observations. Biochemical studies of wild-type and mutant forms of glucokinase have shown that three of the disease-causing mutations (M210K, T228M and A378V) lead to the synthesis of a glucokinase molecule that is enzymatically inactive (table 3). The splice-site mutation IVS8nt+2T>G is also predicted to lead to the synthesis of an inactive protein. The missense mutation G264S found on one allele in case 5 (table 1) has only a small effect on glucokinase activity (table 3). Since the kinetic properties are near normal, we believe that the G264S mutation affects glucokinase function in a manner not detectable by the routine kinetic analysis such as altered interaction with other proteins that may be involved in the regulation of glucokinase function in the β-cell. The association of G264S with MODY2 in the family of case 5 and the description of this mutation in an unrelated subject with MODY2 from Italy [21] suggests that it is pathogenic. Further studies of this mutation may reveal new mechanisms of glucokinase regulation in pancreatic β-cells.

The genetic defect in cases 1–4 (table 1) is predicted to result in a complete absence of GSIR (fig. 2). In case 5, the threshold for GSIR is predicted to increase from 5 to about 10 mmol/l. We believe that the profound defect in glucose metabolism has other effects on β-cell function including synthesis of

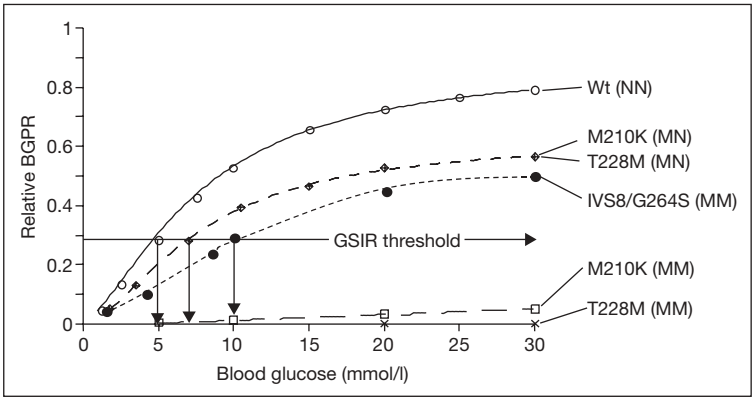


Fig. 2. Comparison of the functional properties of wild-type and mutant forms of glucokinase. The panel shows the results of mathematical model predicting the effect of the wild type (Wt) glucokinase and glucokinase mutations in the heterozygous (MN) and homozygous (MM) state on the β-cell glucose phosphorylation rate (BGPR) and glucose-stimulated rate of insulin release (GSIR). In the case of M210K and T228M in the homozygous state (M210K MM and T228M MM), the threshold for GSIR cannot be achieved in either case at the blood glucose concentrations during insulin treatment (i.e. based on adaptation of glucokinase to a fasting blood glucose concentration of 20 mmol/l), thus leading to a total failure of GSIR. M210K and T228M in the heterozygous state (M210K MN and T228M MN), however, led to the predicted rightward shift of GSIR from 5 to 7 mmol/l. The effect of the mutations IVS8nt+2T>G and G264S in the compound heterozygous state (IVS8/G264S MM) is less pronounced compared to homozygous M210K and T228M as the GSIR is increased from 5 to only 10 mmol/l. Modified from Njølstad et al. [10] with permission from The Massachusetts Medical Society, Boston, Mass., USA.

insulin and other proteins as well as β-cell development and replication. If β-cell mass is reduced, this might explain why non-glucose nutrients, hormones and even sulfonylurea compounds are not able to stimulate insulin release in these patients [10, 22; Søvik and Njølstad, unpubl.].

Treatment of Glucokinase-Related PNDM

These infants have no endogeneous insulin secretion, and need replacement therapy from the first day of life. The initial insulin requirement seems to vary (0.8–2.4 U/kg/day; table 1). Insulin treatment in small neonates is a demanding task requiring diluted insulin preparations and insulin pumps. The doses needed are minute and insulin 100 U/ml is not recommended. We have used insulin 40 U/ml in insulin pumps that have pre-set programs for this

insulin dilution and not other dilutions to avoid possible overdoses that could be fatal. With adequate insulin therapy there is rapid catch-up growth and normal development.

Mathematical modeling predicts that endogenous insulin secretion is not possible at physiological blood glucose levels in patients with glucokinase-related PNDM [10, 11]. In theory, these patients should respond to glucokinase independent insulin secretagogues, such as arginine and sulfonylurea. However, in preliminary studies none of these agents have proved effective [10, 22; Søvik and Njølstad, unpubl.]. Other interesting therapeutic candidates are glucagon-like peptide-1 [23] and glucokinase activators [24] which remain to be tested. Eliciting some endogenous insulin secretion could provide a rationale for adjuvant treatment and accordingly improve the metabolic control in the patients.

Genetic Screening for Glucokinase Mutations in PNDM

Which cases of neonatal diabetes should be screened for glucokinase mutations? We would advocate testing patients who present with neonatal diabetes, intrauterine growth retardation and parents showing some degree of glucose intolerance. Testing patients who do not fulfill these criteria is unlikely to reveal any with glucokinase mutations (table 1) [13, 25, 26].

Summary

Permanent neonatal diabetes due to glucokinase deficiency (PNDM2) is a genetic disorder (OMIM #606176) in which the probands are homozygous or compound heterozygotes for an inactivating mutation in the glucokinase gene leading to complete glucokinase deficiency and subsequent insulin deficiency resulting in growth retardation and permanent diabetes. The parents are heterozygous for the glucokinase mutation and have MODY2. Permanent neonatal diabetes due to glucokinase deficiency can be regarded as an inborn error of the glucose-insulin signaling pathway.

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Glucokinase-Linked Hypoglycemia

Clinical Aspects of Activating Glucokinase Mutations

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Congenital hyperinsulinism caused by activating mutations in the gene encoding for glucokinase, GCK; gene name *GCK* (CHI-GCK) is a rare subtype of the heterogeneous clinical disorder of hyperinsulinemic hypoglycemia, also known as persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [1].

Most frequently, inactivating mutations in the sulfonylurea receptor (SUR1; gene name *ABCC8*) gene are found [2–5]. Other rare causes of CHI are inactivating mutations in the potassium channel inward rectifier Kir6.2 (*KCNJ11*) gene [6–8], or activating mutations in the glutamate dehydrogenase (GDH; gene name *GLUD1*) gene [9, 10]. In addition, CHI may occur in short chain acetyl-CoA deficiency and in the related gene syndrome of Beckwith-Wiedemann.

In contrast to the numerous inactivating *GCK* mutations which cause maturity-onset diabetes of the young subtype 2 (MODY2) in the heterozygous form, or permanent neonatal diabetes mellitus (PNDM) in the homozygous form, activating *GCK* mutations (CHI-GCK) have only been reported in five families to date. Each family has their own private heterozygous mutation; V455M in exon 10, A456V in exon 10, T65I in exon 2, W99R in exon 3, and Y214C in exon 6 (preliminary report) [11–14].

The mutations are inherited as an autosomal-dominant trait with affected individuals in each generation. In two of the families, a spontaneous mutation has been documented one generation above the proband.

Functional in vitro studies have shown that these mutations (V455M, A456V, T65I, W99R and Y214C) enhance GCK activity by increasing the

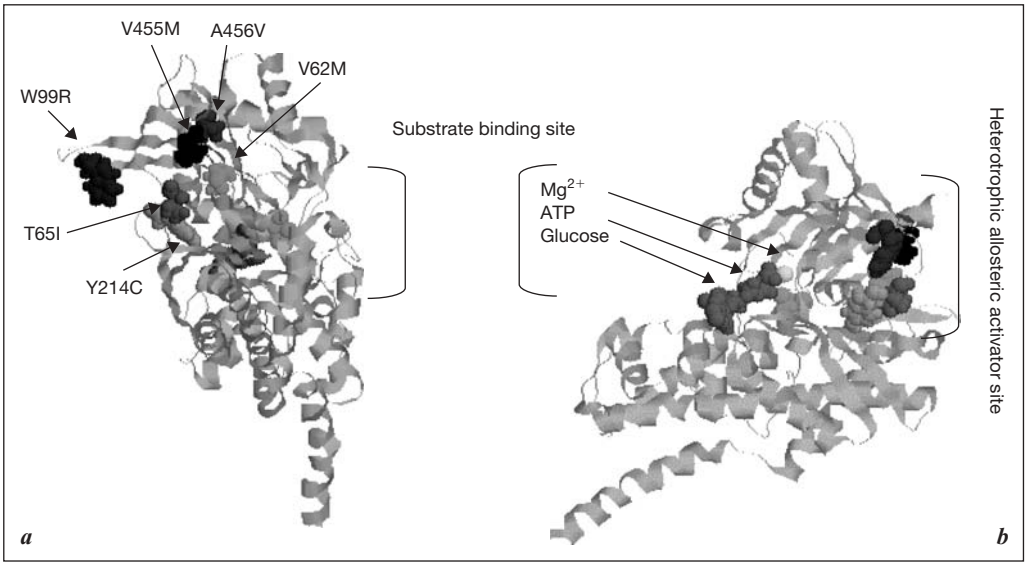

Fig. 1. 'Allosteric' activation domain of GCK. The structural model of GCK shows: ***a*** the location of the activating mutations V62M, T65I, W99R, D158A, Y214A, Y214C, V455M and A456V in a circumscribed 'allosteric' domain, distinct from the binding cleft for glucose and MgATP. ***b*** This particular orientation emphasizes the large molecular distance of the putative activator region from the substrate binding site.

affinity for glucose, indicated by a 2- to 6-fold increase in the affinity of glucose seen by the decrease in the glucose $S_{0.5}$. Additional features were an increased maximal activity, K_{cat} (V455M, A456V, W99R), or lowered K_{cat} (T65I), a lowered Hill coefficient (A456V, T65I), and an increased ATP K_m (W99R); the relative activity indices varying from 6.36 (W99R) to 34 (A456V). Mathematical modeling predicted a threshold for glucose stimulated insulin release, GSIR, at 1.40 mmol/l (A456V), 2.45 mmol/l (V455M), 2.8 mmol/l (W99R), and 3.1 mmol/l (T65I).

The close genetic proximity of the two first mutations, V455M and A456V, in adjacent codons, suggested an area of critical importance in the GCK enzyme. In the structural model of the GCK enzyme of Mahalingam et al. [15], the two synthetic activating *GCK* mutations D158A and Y214A [16, 17], the paradoxical V62M mutation with activating properties in vitro, but with the phenotype of MODY2 [17–19], the T65I and W99R and the latest Y214C mutation are all located in the same, well-circumscribed domain of the enzyme, opposite and spatially remote from the binding sites of the substrates glucose and MgATP (fig. 1).

It has been hypothesized that this area of activating mutations represents a heterotrophic, allosteric activator site in the normal GCK enzyme [12, 19]. The impact of this hypothesis is wide. Firstly, it predicts the possibility of acute or chronic dual control by as yet unidentified endogenous activators and inhibitors. Accordingly, any impairment of such control could be of significance for glucose homeostasis. Secondly, the novel GCK activator drugs [21–23] have been shown to exert their action by altering the GCK structure at this site mimicking the activating mutations. In this light, the clinical details on the few patients with activating *GCK* mutations become a valuable source of information about the clinical consequences of long term GCK activation.

Central Clinical Feature

The central clinical feature of CHI-GCK patients is an intact glucose regulated insulin release, but from a lowered glucose threshold, in accordance with the predictions from the in vitro studies. This has been confirmed by several clinical investigations, which are described below.

24-Hour Insulin Secretory Rates

To characterize the insulin secretory abnormalities, proband 1 and his sister underwent a 24-hour study of insulin secretory rates (ISR) while consuming a standard diet of 30 kcal/kg consisting of 50% carbohydrate, 15% protein, and 35% fat. The ISRs were similar to normal control subjects during this study [24]. In fact, when the glucose levels fell overnight, the ISRs also declined. The only significant abnormality was the low fasting and post-prandial glucose levels, suggesting that insulin release was glucose sensitive, but from a lowered glucose threshold.

Short and Prolonged Fasting

Short and prolonged fasting showed variable results with respect to suppression of insulin at low blood glucose, most probably due to a stabilization of the blood glucose just above, and sometimes just below, the threshold for glucose stimulated insulin release (GSIR).

Fasting p-insulin and C-peptide were suppressed at low blood glucose in patients I, II-2; 2-III-1; and 4,III-1 (table 1).

In a 19-hour, and a 25-hour fast, respectively, the hypoglycemia of proband 1 and his sister were in similar ranges of 2.4–2.7 mmol/l. Hypoglycemia did not continue to decrease as occurs in insulinomas and in the severe forms of CHI-SUR1 or CHI-KIR6.2, where insulin secretion is totally uncoupled from glucose metabolism. Insulin and C-peptide were, however, not suppressed during the fast.

Table 1. Clinical characteristics of CHI-GCK patients in family 1–4. Probands in **bold**. tGSIR, predicted threshold for glucose stimulated insulin release; rel.a.index, relative activity index

Mutation (tGSIR; rel.a.index)	Family, patient*	Gender	Birth weight g	Age	BMI kg/m^2	Symptoms and signs	Fasting blood glucose mmol/l	Plasma insulin pmol/l	Plasma C-peptide pmol/l	Plasma proinsulin pmol/l	Subsequent treatment
V455M	1, I-1	male	–	childhood	–	'hypoglycemic symptoms'	–	–	–	–	frequent meals
(2.45; 15.7)				48 years+	–	diabetes	–	–	650	–	insulin
	1, II-2	female	–	15 years+	–	–	'hypoglycemia'	–	–	–	none
				36 years	21.5	postprandial chest tightness	2.1–2.8	3–35	138–350	–	none
	1, II-3	male	3,490	30 years		weakness, shakiness, pallor	–	–	–	–	none
				31 years	28.7	attack of unconsciousness	2.0–2.6	119–210	620–1,120	–	diazoxide, responsive
	1, III-3	male	3,608	4½ years	–	shakiness, seizures	1.3	40	–	–	diazoxide, responsive
				13 years	26.0	diazoxide controlled	–	–	–	–	diazoxide, responsive
	1, III-4	female	2,926	1 year	–	shakiness, seizures	–	–	–	–	none
				2 years	–	–	1.56	34.3	–	–	diazoxide, responsive
				11 years	21.0	diazoxide controlled	–	–	–	–	diazoxide, responsive
				12 years	27.0	no aggravation	–	–	–	–	diazoxide, responsive
A456V	2, II-2	female	3,750	<3 weeks	–	cyanotic attacks, jitterness	–	–	–	–	none
(1.40; 34)				42–45 y	23.3	almost asymptomatic	2.9–3.5	23–31	515–544	5	none
	2, III-1	male	2,400	1st day	–	jitterness, dysmature	0.8	–	–	–	i.v. glucose, steroids
				14 days+	–	asymptomatic	2.5–2.7	<12–103	–	–	diazoxide
				8 months–4 years	11–17.6	asymptomatic	2.4–3.0	–	–	–	frequent meals
				14 years	34.0	seizures, unconsciousness	2.6–3.1	206–224	1,508–1,574	29–88	diazoxide, responsive

T65I	3, II-2	female	–	15–20 years	–	seizures	2.2	93	–	–	partial pancreatectomy
(3.1; 9.81)				35 years	–	–	–	–	–	–	diazoxide, responsive
	3, III-1	male	3,130	<1 months	–	asymptomatic	2.0–2.6	–	–	–	none
				15 years	17.8	seizures	2.3	56	–	–	diazoxide, responsive
W99R	4, II-2	male	–	33 years	–	asymptomatic	2.7	16	613	–	none
				33 years	–	asymptomatic	2.7–3.1	–	–	–	none
(2.8; 6.38)	**4, III-1**	male	3,080	1st day	–	–	1.8	–	–	–	i.v. glucose
				28 days	–	–	2.0–3.5	29–68	–	–	diazoxide, side effects
				16 weeks	–	–	2.9	29	610	–	diazoxide/chlorothiazide, partially responsive
				10 months	–	–	1.2–2.4	<14–31	<50–800	–	diazx./chlth./ nifedipine/octreotide, responsive

Probands in **bold**. tGSIR = Predicted threshold for glucose stimulated insulin release; rel.a.index = relative activity index.

In proband 1, extended fasting provoked an increase in blood glucose to 3.2 mmol/l, followed by a gradual decrease to 2.2 mmol/l after 45 h fasting, at which time he developed lightheadedness and the fast was terminated.

In the children of proband 1, prolonged fasting for 16 to 18 hours resulted in a fall in blood glucose to 1.3 and 1.56 mmol/l, respectively, without provoking seizures or loss of consciousness. Their insulin levels were not suppressed (40 and 34.3 pmol/l, respectively).

In the mother of proband 3, a prolonged fast showed a gradual decrease in blood glucose from 3.2 to 2.2 mmol/l after 48 h, but a spontaneous rise in blood glucose to 2.9 mmol/l after 60 h. The insulin concentration was 78 pmol/l at 48 h, and 114 pmol/l at 60 h, indicating inappropriate hyperinsulinemia, but preserved glucose sensitivity.

In the father of proband 4, a 48-hour fast showed unchanged blood glucose values of 2.7–3.0 mmol/l, while insulin levels were suppressed from 16 to less than 10 pmol/l and C-peptide fell from 613 to 301 pmol/l.

C-Peptide Suppression Test

In a C-peptide suppression test in proband 1 and his sister, both began the study with hyperinsulinemic hypoglycemia, figure 2. In response to the insulin infusion and further hypoglycemia, however, the levels of C-peptide fell significantly. In the proband, plasma glucose at time zero was 2.4 mmol/l, C-peptide 620 pmol/l, insulin 119 pmol/l. Following administration of insulin, the glucose level fell to 2.0 mmol/l and the C-peptide decreased by 61% in 35 min. In the sister, glucose fell from 2.8 to 1.9 mmol/l by 30 min and she developed palpitations and peripheral numbness. Glucose was administered and the glucose level rose to 5.9 pmol/l at 40 min. Her C-peptide decreased by 55% from 400 to 180 pmol/l before glucose administration.

The C-peptide suppression tests confirmed that insulin secretion was glucose sensitive and that insulin was suppressible also in these patients, when glucose was taken below 2.4 mmol/l. This corresponded well to the computed threshold 2.45 mmol/l for the V455M mutation. The fact that insulin was not suppressed in the children of proband 1 at blood glucose levels of 1.3 and 1.56 mmol/l requires further studies in infants and young children with CHI-GCK to fully elucidate the reason for this.

Variation in Phenotype

As shown in table 1, the phenotype of the affected members of the four first families was highly variable, even within each family (for the fifth mutation, Y214C, no other family members were affected), with regard to the age at

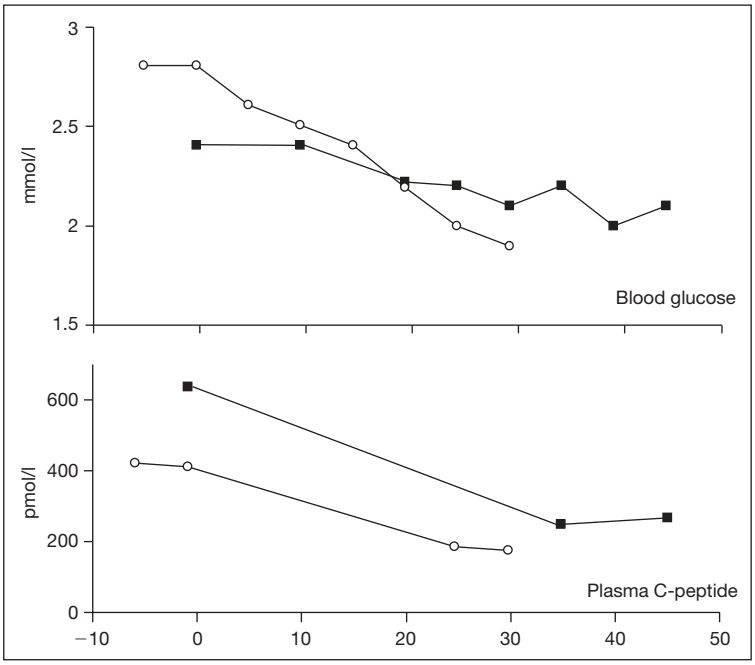

Fig. 2. Suppression of C-peptide with insulin-induced hypoglycemia in proband 1 (■) and his sister (○). Insulin dose 0.125 U/kg/h.

onset of symptoms, the severity of the hyperinsulinism, and the clinical course of each patient. During hypoglycemia, absolute hyperinsulinism with supranormal insulin levels as well as relative hyperinsulinism with inappropriate p-insulin in the normal range were seen. Moreover, the clinical spectrum was wider than the logistic limits of the term 'hyperinsulinemic hypoglycemia', ranging from attacks of severe hypoglycemia with seizures and unconsciousness, over asymptomatic hypoglycemia, to diabetes.

The in vitro differences in glucose sensitivity and maximal activity of the four *GCK* mutations were not readily mirrored in the phenotypes. All four genotypes presented in some, but not all, of the family members in the neonatal period or early childhood. The adult fasting glucose level ranged from 2.0 mmol/l to diabetic levels without relation to the differences in the computed thresholds of GSIR. Indeed, the most severe *GCK* mutation predicted, A456V, presented in the mother of proband 2 as asymptomatic hypoglycemia in adulthood.

Perhaps mutations which have higher relative activity indices and more extreme predicted thresholds for GSIR may expand the phenotypic spectrum of

activating *GCK* mutations in the future and reveal evidence of a genotype-phenotype correlation. For example, the artificial mutation Y214A has a very high relative activity index of 164, predicting that a naturally occurring Y214A mutation at this site may result in a more severe phenotype than the today known. In the preliminary report of a patient with a Y214C *GCK* mutation, a 95% pancreatectomy and subsequent diazoxide treatment was not efficient to prevent hypoglycemia [14]. However, conclusions about diazoxide response must await publication of the details of the dose of diazoxide administered and patient compliance.

Onset and Birth Weight

The reported onset of hypoglycemia varied from day 1 of life to adulthood. It is hypothesized that hypoglycemia in CHI-GCK patients is present from birth as is the case for hyperglycemia in MODY2 , but often unrecognized due to lack of symptoms for many years, in some patients into adulthood.

Delay of diagnosis was strongly suggested in the mother of proband 2, in whom hypoglycemic symptoms with attacks of cyanosis and jitteriness were present in the neonatal period, but diagnosis was delayed until the age of 42 years.

In patients with a maternally inherited CHI-GCK mutation, the low blood glucose of the mother and fetus during pregnancy may cause a lower birth weight of the neonate, which in combination with increased glucose sensitivity, may result in a more pronounced hypoglycemia.

The average birth weight of patients with a maternally inherited mutation was 2,765 g compared to 3,276 g in patients with a non-maternally inherited mutation. Neonatal onset hypoglycemia occurred however, not only in both patients with a maternally inherited mutation, but in 1 patient with a paternally inherited mutation too.

Data from more CHI-GCK patients may elucidate, whether CHI-GCK patients with maternally inherited mutations tend to have a lower birth weight compared to CHI-GCK patients with paternal or de novo mutations, analogous to the documented opposite effect in MODY2, where inactivating *GCK* mutations of maternal origin are associated with a higher birth weight compared to MODY2 patients with a paternally inherited mutation [25].

None of the CHI-GCK patients had a very high birth weight, resembling diabetic fetopathy. This is in contrast to the vast majority of CHI patients with inhibiting potassium channel (SUR1 and Kir6.2) mutations resulting in glucose-insensitive, severe hyperinsulinemic hypoglycemia of neonatal onset.

Accommodation to Hypoglycemia

The counterregulatory hormones were not elevated during hypoglycemia in proband 1 (normal glucagon, epinephrine, cortisol and growth hormone) and

proband 2 and 3 (normal cortisol and IGF_1), and in the neonatal period in proband 4 (normal cortisol and growth hormone).

This is consistent with previously reported responses to insulin-induced hypoglycemia in normal adults [26]. In the probands, it may reflect accommodation to the hypoglycemia analogous to the loss of counterregulatory responses seen in insulin treated diabetic patients with frequent hypoglycemia.

Another similarity between the adult CHI-GCK patients and insulin treated diabetic patients with frequent hypoglycemia was the markedly lowered glucose threshold for neuroglycopenic symptoms.

Accommodation to hypoglycemia was, however, not always sufficient to avoid neuroglycopenic symptoms and even seizures. Which leaves us with an unanswered question: What triggers the severe hypoglycemic attacks in CHI-GCK?

Severe Hypoglycemic Attacks

The severe hypoglycemic attacks of proband 1, 2 and 3 could not be explained by a lower fasting blood glucose compared to their adult family members 1,II-2, 2,II-2 and 3,II-2. Even prolonged fasting did not result in severe hypoglycemic attacks in any of the children or adults. The preserved glucose sensitivity in CHI-GCK with suppression of insulin release around the blood glucose threshold of GSIR prevented the severe hypoglycemic attacks in fasting.

At several occasions, reactive hypoglycemia was more severe than fasting hypoglycemia, especially in obese patients. The severe hypoglycemic attacks were only seen in adolescences or adults with obesity and/or insulin resistance. It is hypothesized that reactive hypoglycemia in patients with some degree of insulin resistance may be responsible for the severe hypoglycemic attacks with seizures and loss of consciousness.

Obesity and Insulin Resistance

The probands in family 1 and 2 had a higher BMI and much higher fasting levels of p-insulin and C-peptide compared to the sister in family 1 (II-2) and the mother in family 2 (II-2) (table 1). The plasma insulin and C-peptide concentrations correlated positively with the BMI with the highest levels in proband 2 having a BMI of 34.0 kg/m^2.

The association between obesity and aggravation in the hyperinsulinism could in part be explained by augmentation of β-cell volume, a well-known effect of obesity [27–30]. In addition, factors related to adipose tissue mass may increase pancreatic *GCK* expression with enforced consequences in CHI-GCK patients [12]. Obesity-induced increase in pancreatic *GCK* expression may also explain why obese MODY2 patients surprisingly have a better glucose tolerance by OGTT compared to lean ones, as reported by Massa et al. [31].

The fact that the lean and obese CHI-GCK patients had fasting blood glucose values in largely the same range suggested that some degree of insulin resistance occurred in the obese patients. The obese CHI-GCK probands had, however, a strong tendency to hypoglycemic attacks in contrast to their lean family members, suggesting that the aggravated hyperinsulinism at some occasions let to a further lowering in the blood glucose.

In proband 3, hypoglycemic seizures occurred at the age of 15 years when his BMI was only 17.8 mg/m^2.

At a blood glucose of 2.3 mmol/l, his p-insulin was not suppressed (56 pmol/l), and at higher glucose levels of 3.3–5.8 mmol/l he had apparently some degree of insulin resistance with a p-insulin level of 230–323 pmol/l. The insulin resistance was, however, not associated to obesity.

OGTT and Reactive Hypoglycemia

In the adolescent or adult members of family 1 and 2, a 75-gram OGTT was performed during no medication (fig. 3). The increase in blood glucose was followed by a much higher β-cell response in the obese probands compared to the lean sister 1,II-2 and the lean mother 2,II-2. In the probands, plasma insulin peaked at 60 min with a concentration of 2,483 and 1,297 pmol/l, respectively, far above normal peak values. Correspondingly, C-peptide peaked at 60 min with huge values of 5,100 pmol/l, and 4,816 pmol/l, respectively.

In contrast, the lean individuals had 60-min β-cell peptide values below the normal range. The peak values were subnormal or delayed in both lean individuals.

Irrespective of the differences in the β-cell response, reactive hypoglycemia occurred at 2.5 to 3 h in probands 1 and 2, in individual 1,II-2, and in the father of proband 4. The blood glucose fell to 1.6; 1.8 and 2.1 mmol/l, which was 0.6–1.0 mmol/l lower than their time zero values. In proband 2, the only symptom or sign was mild sweating of his hands and he did not report any postprandial hypoglycemia symptoms. Postprandial chest tightness was, however, the primary complaint in the sister to proband 1. The tendency to reactive hypoglycemia was retrospectively confirmed by an intramuscular glucagon test in proband 2 at 26 days of age. From a basal blood glucose level of 2.8 mmol/l, a normal maximal glucose response was followed by rebound hypoglycemia with blood glucose of 2.3 mmol/l at 60 min.

Postprandial hypoglycemia probably contributed to the need for frequent meals triggering obesity. The severe hypoglycemic attacks of the lean or obese probands were not, however, provoked by the OGTTs. A higher oral glucose load, or perhaps exogenous substances lowering blood glucose, may be necessary to provoke a hypoglycemic seizure. In addition, the threshold for neuroglycopenia may be altered by certain conditions. The dramatic increase in BMI

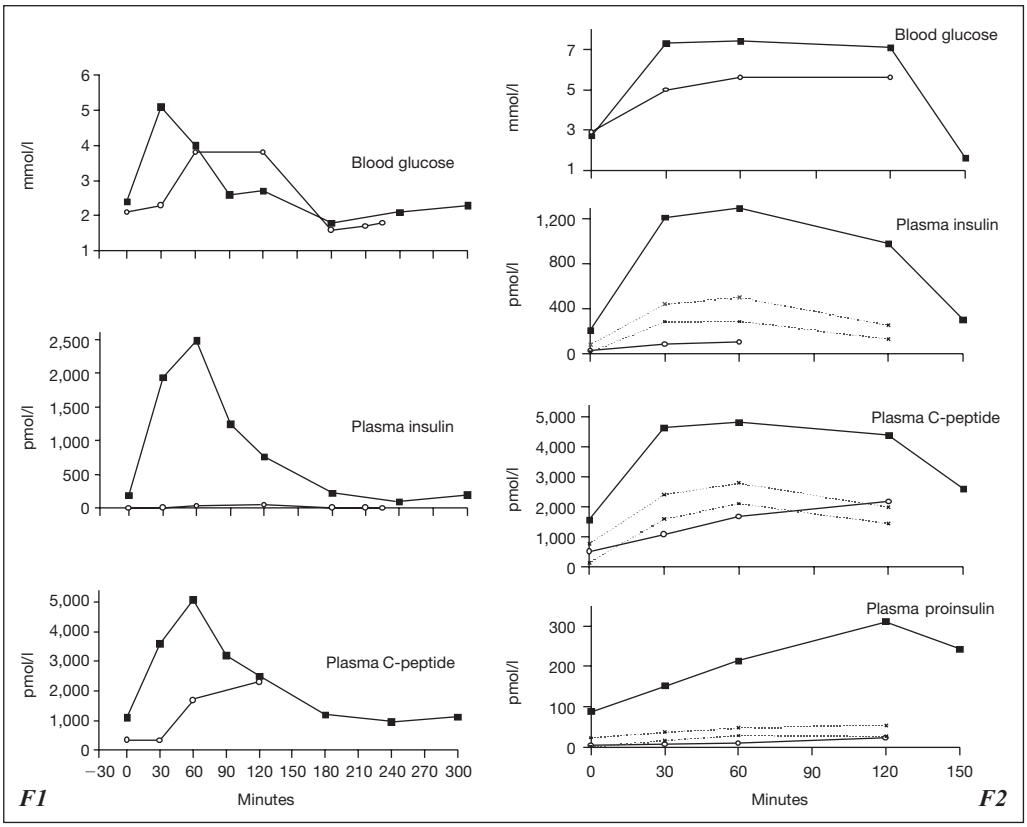

Fig. 3. OGTT in family 1 (F1), the proband (■), his sister (○); and family 2 (F2), the proband (■) and his mother (○). Upper and lower normal limits ±2 SD are represented by dotted lines in F2.

in proband 2 prior to the onset of neuroglycopenic attacks may have been too fast to ensure brain adaptation to a blood glucose level lower than provoked by the OGTT.

Intravenous Glucagon Test

Glucagon is a direct stimulus of β-cell insulin secretion. A short, intravenous glucagon tests was performed in the affected individuals of family 2 with early determination of blood glucose, plasma C-peptide, proinsulin and insulin according to Faber and Binder [32]. In proband 2, a pronounced β-cell response with large, early increases in plasma insulin, C-peptide and proinsulin was seen at 2–4 min, compared to the moderate increases in the mother.

In the proband, the early increase in insulin and C-peptide was comparable to the increase seen in the OGTT at time 30 min, although the increase in blood glucose was much lower by the i.v. glucagon test, an indication of the direct action of glucagon on the β-cell. The early β-cell peptide increase in the mother was not accompanied by any change in blood glucose. Reactive hypoglycemia was not seen in the patients in this short test terminated at 14 and 10 min, respectively.

Clinical Course

The clinical course of CHI may be transient, recurrent, or permanent, varying with the subtype of this disorder. The major clinical differences are shown in table 2.

Transient, neonatal onset CHI is associated with a single, paternal SUR1 mutation and a microscopic, focal loss of maternal chromosome 15p in the pancreas including loss of maternal SUR1, Kir6.2 and a maternally imprinted tumor suppressor gene [33]. However, the majority of mild transient CHI cases remains, etiologically unexplained, even once transient hyperinsulinism secondary to maternal diabetes and other factors have been excluded.

Recurrent CHI is seen in the protein sensitive CHI-GDH, formerly known as leucine-sensitive hypoglycemia. Permanent CHI is also seen in severe, homozygous or compound heterozygous patients with SUR1 or Kir6.2 mutations and a diffuse β-cell hypertrophy, indicative of permanent insulin hypersecretion. Several studies have shown that hyperinsulinism tend to fade out and may end up with a type 2 diabetic state. Conservatively treated patients with CHI-SUR1 enter clinical remission and some develop impaired glucose tolerance and type 2 diabetes (T2DM) [33–36]. In the dominant CHI-SUR1 mutation E1506K, a seemingly progressive loss of β-cell function is seen from mild hyperinsulinemic hypoglycemia to frank T2DM at the age of 39–60 years [36]. In transgenic mice with a Kir6.2 mutation and hyperinsulinemia, β-cells undergo apoptosis [37], perhaps initiated by the increased intracellular calcium concentration [38, 39], which could provide a mechanism for the development of T2DM.

In CHI-GCK, the islet histology of the mother to proband 3 was normal, indicative of a state of hypoglycemia without permanent, absolute hypersecretion of insulin, predicted to be permanent throughout life.

However, the development of insulin resistance especially in the obese CHI-GCK patients, the blunted or delayed insulin and C-peptide responses in the β-cell stimulation tests of the 36-year-old sister in family 1 and the 42-year-old mother in family 2, and the development of diabetes in the oldest known patient 1, I-1, challenge the prediction of a stable phenotype throughout life in CHI-GCK. The diabetes in patient 1,I-1 from the age of 48 y did not respond to

Table 2. Comparison of clinical characteristics of 4 types of CHI

	CHI-GCK	CHI-SUR1/Kir6.2	CHI-GDH
Inheritance (autosomal)	dominant	mostly recessive, rarely dominant	dominant
Beta cell histology	normal	diffuse hypertrophy, focal hyperplasia, or mixed	normal
Birth weight	normal	mostly increased	normal
Onset	variable. always neonatal, but often asymptomatic?	neonatal	variable
Blood glucose	fasting level around threshold for the lowered GSIR; reactive hypoglycemia more severe.	mild to very severe hypoglycemia	mild-to-moderate intermittent hypoglycemia
Insulin level	glucose regulated, may be inappropriately high	inappropriately high, no or poor glucose sensitivity	intermittent inappropriately high, protein hypersensitive
Treatment	SUR agonist responsive (high doses, more than one drug may be needed)	SUR agonist responsive; partially or totally irresponsive. subtotal pancreatectomy	SUR agonist responsive. diet
Course	permanent hypoglycemia, but often asymptomatic? may progress to insulin resistance, T2DM	transient or permanent. may burn out with progression to T2DM	proposed permanent

oral antidiabetic drugs. A random p-C-peptide was 650 pmol/l. β-Cell autoantibodies were not measured. More clinical data from CHI-GCK patients are needed, before the variable spectrum and the clinical course of the disease can be fully described.

The proband 3 and his mother had seizures in adult life even after achieving glucose control. As in other diseases with recurrent hypoglycemia, brain damage with epilepsy may follow.

Treatment

Treatment of CHI-GCK patients with the sulfonylurea receptor agonists diazoxide and octreotide is expected to be successful. In contrast, CHI-SUR1 and CHI-Kir6.2 patients may be irresponsive, partially responsive or responsive to these drugs, depending of the genotype, although a close genotype-phenotype correlation is not seen.

In the CHI-GCK patients of family 1–4, the hypoglycemia was responsive to sulfonylurea receptor agonists. In family 1, 2 and 3, monotherapy with diazoxide, or diazoxide-chlorothiazide treatment, were effective. In proband 4, only a partial response to a maximal dose of diazoxide plus chlorothiazide was seen. Additional treatment with octreotide was effective, as was, in part, nifedipine. The proband with the Y214C mutation may have been medically controlled on medical treatment, but underwent an early 95% pancreatectomy.

The putative role of obesity as a cause of aggravation in CHI-GCK stresses the need of glucose control in CHI-GCK subjects with an increasing BMI towards obesity. The lean adults with CHI-GCK did seemingly not suffer from their hypoglycemia and were not medically treated. The problem of brain damage from hypoglycemia was not in question in these individuals, probably because they did not suffer from intermittent attacks of very low blood glucose.

As for children with CHI-GCK, diazoxide should be given irrespectively of symptoms when blood glucose is repeatedly low to avoid potential brain damage in the developing brain and aggravation by obesity from overeating to escape mild hypoglycemic symptoms.

With the present knowledge today, adults should be tested for the potential risk of developing impaired glucose tolerance and diabetes.

GCK Activator Drugs

The novel GCK activator drugs may have a role in the treatment of T2DM [23]. The safety of GCK activation in humans has not been studied, but CHI-GCK individuals represent nature's own trial of long-term GCK activation. In normal rats, overexpression of *GCK* in the liver was associated with a marked increase of blood lipids, which would greatly limit the usefulness of GCK activator drugs [40]. The lipid profile of proband 2 during diazoxide treatment showed an elevated fasting plasma triglyceride of 2.64 mmol/l (normal range 0.46–1.86 mmol/l) at the beginning of treatment, followed by a gradual decline to 1.37 mmol/l at repeated measurements. His fasting p-cholesterol, LDL-cholesterol and direct HDL-cholesterol were repeatedly within normal range. His mother had a marginally high *p*-HDL-cholesterol of 2.15–2.27 mmol/l (normal range 0.78–1.74 mmol/l), whereas *p*-LDL-cholesterol, *p*-cholesterol and *p*-triglyceride were repeatedly normal.

None of the 4 affected members of family 3 and 4 had an adverse lipid profiles.

These data suggest that long-term GCK activation does not have marked adverse effects on the lipid profile in humans.

In rodents, the GCK activator drug RO-28–1675 has an activating effect on liver glucokinase resulting in an increase of glucose utilization [23]. The effect of activating *GCK* mutations on liver glucokinase remains to be studied.

Acknowledgements

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Glucokinase and the Regulation of Blood Sugar

A Mathematical Model Predicts the Threshold for Glucose Stimulated Insulin Release for *GCK* Gene Mutations that Cause Hyper- and Hypoglycemia

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Glucokinase (GCK) is regarded as the pancreatic β-cell glucose sensor on account of its kinetic characteristics, which allow it to alter the rates of cellular phosphorylation and the ensuing metabolism of glucose as a function of blood glucose thereby regulating insulin release. These kinetic characteristics are the enzyme's low affinity for the substrate glucose ($S_{0.5}$ 7–9 mmol/l), its near saturation physiologically with the second substrate MgATP (K_m 0.2–0.4 mmol/l), its co-cooperativeness with glucose (Hill number of ~1.7) and the lack of inhibition by its product glucose-6-phosphate. Although glucokinase is expressed in the pancreatic β-cells, the liver and several other tissues, it is its critical function in the β-cells, which governs blood glucose concentrations. Given the central role of glucokinase it is understandable that mutations in the gene encoding this enzyme (*GCK*) cause hyper- and hypoglycemia. Over 190 mutations in the *GCK* gene have been described, and these are distributed throughout the gene [1]. The majority are heterozygous inactivating mutations, which cause the type 2 form of maturity-onset diabetes of the young (MODY2/GCK-MODY), but five homozygous or compound heterozygous inactivating mutations have been described which result in a more severe phenotype presenting at birth as

permanent neonatal diabetes mellitus (PNDM) [2, 3]. To date only a handful of heterozygous activating *GCK* mutations have been reported which result, as expected, in the opposite phenotype persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [4–7].

The clinical consequences of these mutations can be explained by the 'GCK-glucose-sensor' concept, which states that the capacity of GCK in β-cells as defined by its K_{cat} (maximal specific activity or enzyme turnover) and the affinities for its substrates glucose and ATP (the enzyme's glucose $S_{0.5}$ and ATP K_m, respectively) determine the threshold for glucose stimulated insulin release (GSIR). In humans and most laboratory rodents the β-cell threshold for GSIR in precisely maintained at close to 5 mmol/l. The biophysical characteristics of crucial ion channels for K^+ and Ca^{2+} codetermine with GCK this well defined threshold for GSIR in accordance with a general design principle that several sequential signalling steps of stimulus secretion coupling are interdependent. Therefore, in subjects with GCK-MODY, where inactivating mutations of one allele lead to decreases in the affinity of the enzyme for glucose (an increase in the $S_{0.5}$) and/or decrease in the K_{cat}, the threshold is moderately increased to around 7 mmol/l, whilst in GCK-HI, also autosomal-dominant, the opposite it seen in that the threshold for GSIR is reduced and may be as low as 1.5 mmol/l due to reductions in the glucose $S_{0.5}$ and/or increases in the K_{cat}. In the case of PNDM where both alleles are inactivated it may be infinitely high because of very marked or total inactivation of GCK. Non-sense, splice site, frameshift mutations are predicted to result in truncation of the protein and abolish enzyme activity. Such heterozygous mutations will thus result in haploinsufficiency and lead to elevated glucose levels.

A Mathematical Model to Predict the Threshold for GSIR

A simple mathematical model has been created which can be used to predict the β-cell thresholds for GSIR for individuals with wild-type and mutant *GCK* [8, 9]. This model uses the kinetic characteristics of the normal or mutated enzyme and is based on the following assumptions:

(i) The physiological threshold for GSIR for wild type GCK is 5 mmol/l.

(ii) GSIR is the result of enhanced glucose metabolism and the β-cell glucokinase serves as glucose sensor by tightly controlling glucose metabolism.

(iii) The glucose dependency of β-cell glucose phosphorylation rate is defined by the Hill equation. The ATP dependency of β-cell glucose phosphorylation rate is defined by an expression based on Michaelis-Menten kinetics. The two equations can be combined to describe two-substrate kinetics of glucokinase.

(iv) There is altered expression of both the mutant and the wild type GCK alleles due to adaptation to the prevailing blood glucose concentration [9–11].

The Minimal Mathematical Model

$$BGPR = 2\frac{G^{hw} \times K_{cat}{}^{w}}{G^{hw} + S_{0.5}{}^{w\,hw}} \times \frac{ATP}{ATP + {}^{ATP}K_{m_1}^{w}} \times GCKB^{w} \times E^{w} \times S^{w} \quad (1)$$

Equation 1 is normalized by dividing it by $2GKB^{w}K_{cat}^{w}E^{w}S^{w}$

$$\frac{BGPR}{2GCKB^{w}K_{cat}E^{w}S^{w}} = \frac{G^{hw}}{G^{hw} + S_{0.5}{}^{w\,hw}} \times \frac{2.5}{2.5 + {}^{ATP}K_{m}^{w}} \quad (2)$$

Using the kinetic data in table 1 for wild-type GCK this gives a BGRP of ~28% at 5 mmol/l glucose.

The β-cell glucose phosphorylation rate (BGPR) is expressed in terms of mole of glucose used per gram of β-cell tissue per second (mol/g tissue/s) and it is a function of the basic kinetic constants of wild-type (w) GCK as shown in equation 1. The basal GCK content of the β-cell (GCKB) is defined to be unity and has the dimension of mol/g. Two coefficients govern GCKB: E an enzyme expression co-efficient (defined below) and S a stability coefficient (see below) which is defined as unity. The basic kinetic characteristics included in the model are: the K_{cat}, the maximal specific activity of the enzyme or its rate of turnover when saturated with both substrates (s^{-1}); the $S_{0.5}$, the glucose concentration at half maximal activity of GCK (mmol/l), the Hill number (h) an expression of sigmoidal glucose dependency (unit less) and the ${}^{ATP}K_m$, the affinity constant for ATP (mmol/l). At the threshold for GSIR the extra- and intracellular concentrations of glucose (G) are practically the same and equal to 5 mmol/l and the intracellular $MgATP^{2-}$ concentration (ATP) is equal to 2.5 mmol/l. It is stipulated that the ATP concentration is not markedly influenced by glucose, which is a reasonable approximation because cellular ATP levels are precisely maintained physiologically. At the threshold concentrations the β-cell glucose usage reaches the rate of about 28% of normal capacity.

The Enzyme Expression Co-Efficient (E)

The expression co-efficient (E) takes into account the effect of blood glucose concentrations on the expression of glucokinase. There is considerable evidence that high glucose induces β-cell GCK by a post-translational mechanism which allows the wild type allele to compensate for the mutant allele [9–11]. This adaptive process needs to be incorporated into the mathematical model and the contribution of both the wild type and the mutant alleles to the total

Table 1. Kinetic characteristics of wild-type GCK and 41 GCK mutants

Mutant	$S_{0.5}$ mmol/l	Hill number h	ATP K_m mmol/l	[a]K_{cat}, s^{-1}	[b]K_{cat}, s^{-1}
WT[†]	7.55 ± 0.23	1.74 ± 0.04	0.41 ± 0.03	61.6 ± 7.07	63.00 ± 8.75
G44S**	32.2 ± 3.14	1.59 ± 0.05	0.23 ± 0.03	37.2 ± 2.47	41.5 ± 6.38
A53S*	6.96 ± 1.05	1.8 ± 0.09	0.33 ± 0.01	50.9 ± 1.85	52.7 ± 0.85
V62A*	27.4 ± 2.71	1.51 ± 0.00	0.20 ± 0.00	48.8 ± 0.85	37.30 ± 1.7
V62M[‡]	4.88 ± 0.25	1.49 ± 0.12	0.46 ± 0.03	54.9 ± 5.47	52.30 ± 2.79
T65I*	1.84 ± 0.08	1.30 ± 0.05	0.64 ± 0.09	16.30 ± 3.3	22.10 ± 2.05
E70K*	14.1 ± 0.00	1.48 ± 0.03	0.40 ± 0.01	65.30 ± 5.9	61.00 ± 4.15
G72R*	5.5 ± 0.07	1.50 ± 0.01	0.77 ± 0.11	25.50 ± 3.15	26.80 ± 3.0
W99R*	4.49 ± 0.16	1.39 ± 0.02	1.35 ± 0.13	97.3 ± 16.7	114.00 ± 22.7
Y108C*	13.6 ± 0.25	1.52 ± 0.07	0.28 ± 0.025	44.0 ± 17.6	41.2 ± 3.5
H137R*	10.4 ± 0.81	1.62 ± 0.04	0.38 ± 0.02	60.20 ± 5.4	56.70 ± 0.85
L146R**	123.0 ± 1.87	0.95 ± 0.03	0.11 ± 0.01	0.23 ± 0.09	0.21 ± 0.01
D158A*	3.29 ± 0.23	1.72 ± 0.19	0.33 ± 0.01	58.50 ± 4.0	65.10 ± 4.65
T168P*	186.0 ± 3.0	1.00 ± 0.00	16.3 ± 4.15	0.98 ± 0.07	1.80 ± 0.7
G175R*	38.6 ± 0.4	1.37 ± 0.01	0.5 ± 0.01	34.30 ± 1.4	37.30 ± 4.95
V182M*	83.9 ± 9.6	1.32 ± 0.06	0.10 ± 0.05	23.50 ± 0.25	24.70 ± 0.85
A188E*	305 ± 15.0	1.00 ± 0.00	0.23 ± 0.02	12.8 ± 0.3	9.95 ± 1.05
V203A*	54.2 ± 20.9	1.72 ± 0.33	0.56 ± 0.05	7.28 ± 1.43	9.90 ± 0.6
M210K*	42.3 ± 10.1	1.51 ± 0.13	1.00 ± 0.58	18.40 ± 4.95	22.80 ± 3.95
M210T*	127.0 ± 21.5	1.24 ± 0.07	0.41 ± 0.02	32.90 ± 7.4	29.90 ± 2.25
C213R*	27.1 ± 4.25	1.5 ± 0.07	0.79 ± 0.21	18.30 ± 2.45	23.90 ± 2.85
Y214C*	1.39 ± 0.00	1.41 ± 0.06	1.25 ± 0.03	54.90 ± 0.85	68.90 ± 4.9
V226M*	32.0 ± 8.0	1.1 ± 0.03	2.23 ± 0.14	26.60 ± 2.25	54.40 ± 2.25
M235T*	9.82 ± 0.28	1.59 ± 0.06	0.26 ± 0.01	14.50 ± 2.3	14.20 ± 2.45
C252Y*	24.5 ± 1.25	1.85 ± 0.18	0.56 ± 0.00	13.20 ± 0.65	17.80 ± 0.45
G261R*	65.3 ± 15.1	1.51 ± 0.11	0.62 ± 0.10	19.60 ± 2.3	19.20 ± 1.2
S263P*	12.3 ± 0.05	1.55 ± 0.04	0.57 ± 0.06	61.10 ± 5.4	62.10 ± 2.6
G264S[‡]	9.76 ± 0.74	1.57 ± 0.05	0.48 ± 0.05	60.7 ± 3.04	65.3 ± 2.36
R275C*	7.81 ± 0.3	1.66 ± 0.03	0.46 ± 0.02	77.20 ± 3.6	63.60 ± 2.65
M298K**	15.9 ± 3.05	1.25 ± 0.13	3.70 ± 0.25	24.60 ± 5.36	55.70 ± 3.84
E300K**	9.17 ± 0.15	1.65 ± 0.15	0.7 ± 0.02	59.5 ± 11.4	52.4 ± 4.77
L309P*	12.5 ± 1.5	1.23 ± 0.17	0.62 ± 0.05	2.46 ± 1.03	2.67 ± 1.37
S336L*	7.81 ± 1.1	0.85 ± 0.04	9.50 ± 0.9	0.98 ± 0.06	2.70 ± 0.16
E339G*	26.3 ± 5.8	1.44 ± 0.09	2.17 ± 0.3	38.30 ± 3.4	63.60 ± 3.35
V367M*	8.39 ± 0.41	1.68 ± 0.07	0.45 ± 0.03	85.00 ± 1.35	94.40 ± 0.75
R377C*	42.8 ± 9.55	1.75 ± 0.10	0.37 ± 0.08	7.49 ± 1.62	8.11 ± 2.19
A378V*	584 ± 0.5	0.93 ± 0.04	8.08 ± 0.03	41.00 ± 0.4	72.50 ± 5.75
S383L*	13 ± 0.25	1.47 ± 0.00	0.78 ± 0.13	24.00 ± 4.0	17.90 ± 4.25
S411F*	3.61 ± 0.51	1.00 ± 0.00	9.58 ± 0.55	0.09 ± 0.00	0.4 ± 0.07

Table 1 (continued)

Mutant	$S_{0.5}$ mmol/l	Hill number h	ATP K_m mmol/l	[a]K_{cat}, s^{-1}	[b]K_{cat}, s^{-1}
K414E*	7.17 ± 0.07	1.81 ± 0.10	0.93 ± 0.11	36.20 ± 1.3	48.60 ± 1.45
V455M*	3.02 ± 0.04	1.62 ± 0.01	0.37 ± 0.08	53.00 ± 10.45	57.80 ± 2.2
A456V**	2.03 ± 0.11	1.4 ± 0.02	0.25 ± 0.00	80.30 ± 3.8	82.40 ± 5.13

Data are means ±SEM. The results are the means of the kinetic analysis of 2*, 3**, 4‡, 5† independent expressions of GST-GCK. The assay conditions were as used previously (5, 6, 8). $^{ATP}K_m$ was measured at a glucose concentration of 10 times the enzymes $S_{0.5}$, but usually not exceeding 500 mmol/l. The glucose S0.5 was determined with 5.0 mmol/l ATP in the majority of cases. However, the ATP was 10 mmol/l in the case of V378A and 25 mmol/l in several other cases (L146R, A188E, V226M, G261R, M298K, S336L, R377C, and S411F).

[a]Protocol A using glucose as variable substrate.

[b]Protocol B using ATP as variable substrate [8].

Note that this table is based entirely on the most recent and detailed kinetic studies, which were performed by the authors during 2001–2003. It was necessary to re-examine also previously published data on many of these mutants to establish an optimal and uniform data base for the present comprehensive modelling study of blood glucose regulation in glucokinase (GCK) disease.

β-cell GCK content at a given glucose concentration needs to be considered. The following expression co-efficient is used to describe the relationship between basal blood glucose and GCK expression: $E = (G^h \times 2)/(G^h + S_{0.5}{}^h)$ where G refers to the glucose concentration (mmol/l) at threshold, h is the Hill number for cooperativity with glucose, the numerical value of 2 indicates that half maximal induction is reached at glucose $S_{0.5}$ and $S_{0.5}$ refers to the concentration of glucose needed to achieve half maximal rate of phosphorylation [9]. Using the data in table 1 for wild type, $E = 0.66$ at the glucose threshold of 5 mmol/l.

The Enzyme Stability Co-Efficient (S)

The exploration of more than 40 recombinant enzymes with missense mutations in *GCK* has uncovered several mutants that are thermally labile (H137R, S263P, M298K, E300K and V367M) [9, 10]. These mutations may have additional kinetic defects (M298K) or appear kinetically close to normal or entirely normal (H137R, S263P, E300K, V367M). The stability of only one recombinant thermo lability mutant, i.e. GST-E300K-GCK, has been studied extensively in a cell biological assessment of enzyme expression and stability in addition to in vitro thermolability assays providing evidence that instability is indeed pathogenic in this case [10, 12]. It is possible that in addition to their

kinetic changes other mutant GCK enzymes are also instability mutants but because the alteration of their kinetic features can explain their effect on a patient's phenotype, thermolability has usually not been investigated. To illustrate the effect of severe enzyme instability on the threshold for GSIR we can assume that the steady state level of mutant E300K GCK is 10% of wild type GCK. Therefore, we use an enzyme stability co-efficient of 0.1 rather than 1 which gives predicted GSIR thresholds of ~7 mmol/l for the heterozygous MODY case caused by E300K. These considerations show that it may be necessary to consider instability in the mathematical model in order to explain the increase in the threshold for GSIR and the associated phenotype.

Using the Model to Predict the Threshold for GSIR

Table 1 gives the kinetic characteristics of the 41 missense mutations that we have functionally characterized for the purpose of this comprehensive modelling study. This table illustrates that many kinetic characteristics can be altered and that this usually happens in combinations. A useful way of comparing the severity of the mutations in vitro is to calculate the activity index of the mutant relative to wild type (Ia). This index accounts for practically all factors that determine the enzymatic function of β-cell GCK. A list of all relative activity indices is shown in table 2 and expressions 3 and 4 illustrate how the relative activity index is calculated.

The Relative Activity Index

$$Ia = \frac{K_{cat}}{{}^{G}S_{0.5}{}^{h}} \times \frac{ATP}{ATP \times {}^{ATP}K_{m}} \quad (3)$$

The activity index is therefore expressed in terms of $s^{-1} \times mmol/l^{-1}$. The calculated activity index for each mutant is then divided by the value obtained from wild type to give a relative value, which is unit less.

$$\text{Relative Ia}^{*} = \frac{\text{Mutant Ia}}{\text{Wild-type Ia}} \quad (4)$$

*Note that sometimes Ia is normalized to 5 mmol/l glucose by multiplying with the expression $(5^{h} \times 2)/(5^{h} + S_{0.5}{}^{h})$ and in cases where instability is a known factor the index should be multiplied by $S << 1.0$.

Predicting the Threshold for GSIR for GCK Mutations

The mathematical model can be used to predict the threshold for GSIR for the mutant GCK enzymes. Using our database of kinetic characteristics

Table 2. Predicted threshold for glucose stimulated insulin release (GSIR) calculated from the kinetic characteristics in table 1 using the mathematical model

Mutant	Threshold for GSIR, mmol/l adapted	Non-adapted	Calculated relative activity index
WT	5.00	5.00	1.00
G44S	6.90	9.0	0.08
A53S	5.0	5.2	0.88
V62A	6.80	9.0	0.15
V62M	4.00	4.5	2.59
T65I	3.10	6.0	3.11
E70K	6.10	6.4	0.65
G72R	5.30	5.65	1.01
W99R	2.80	3.0	4.14
Y108C	6.30	7.1	0.37
H137R*	5.75	6.0	0.69
L146R	7.00	11.0	0.00
D158A	2.75	3.0	4.62
T168P	6.90	11.0	0.00
G175R	6.90	9.0	0.13
V182M	6.90	10.0	0.04
A188E	6.90	10.5	0.01
V203A	6.90	10.5	0.02
M210K	6.90	10.0	0.04
M210T	6.90	10.5	0.04
C213R	7.00	9.5	0.08
Y214C	1.40	2.15	17.98
V226M	6.70	8.5	0.40
M235T	6.40	8.5	0.21
C252Y	6.90	9.75	0.02
G261R	6.90	7.5	0.02
S263P*	6.00	6.2	0.64
G264S	5.40	5.65	0.95
R275C	5.00	5.65	1.10
M298K*	6.50	8.0	0.44
E300K*	5.80	6.0	0.66
L309P	6.90	10.5	0.06
S336L	6.90	8.75	0.06
E339G	6.80	8.5	0.19
V367M*	4.75	4.4	1.40
R377C	6.90	10.75	0.01
A378V	6.90	10.75	0.03

Table 2 (continued)

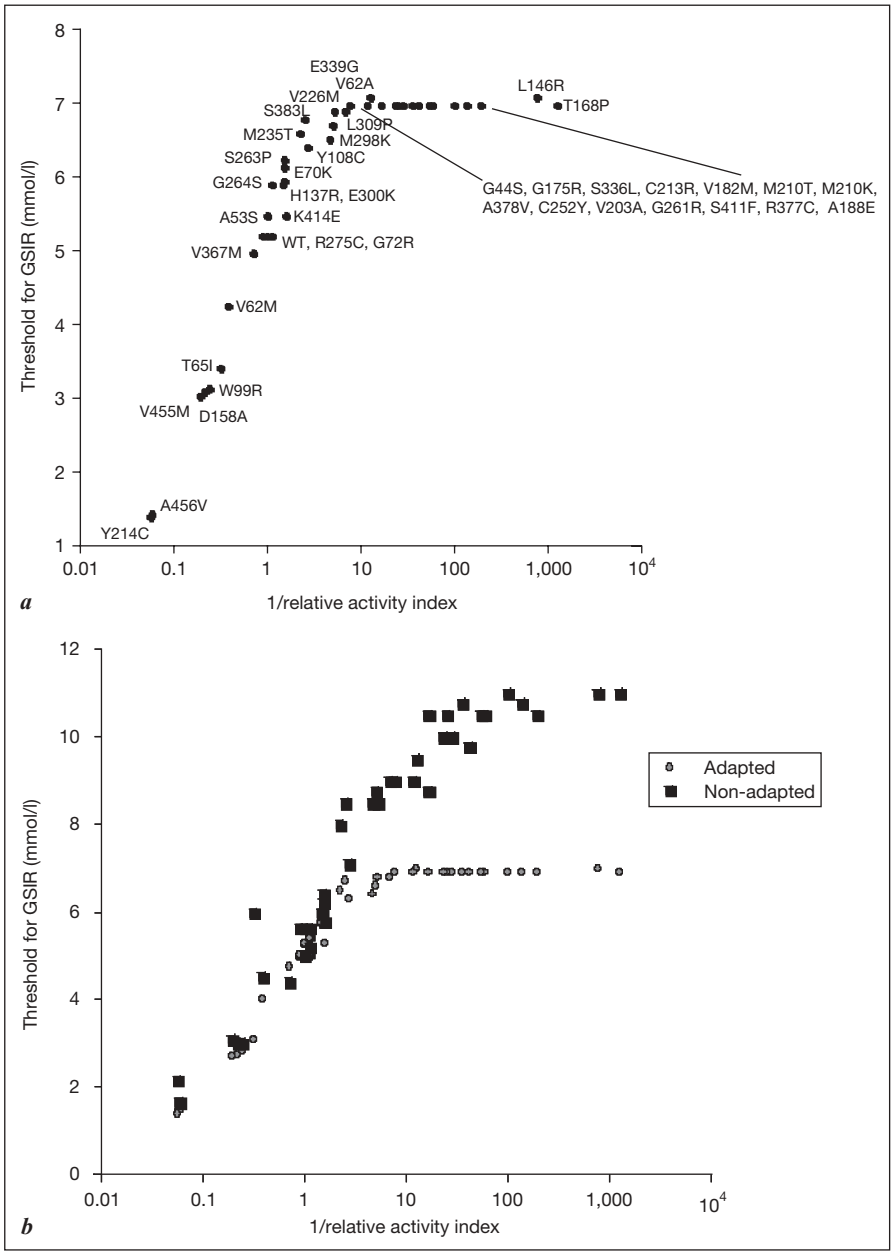
Mutant	Threshold for GSIR, mmol/l adapted	Non-adapted	Calculated relative activity index
S383L	6.60	8.75	0.20
S411F	6.90	11.0	0.00
K414E	5.30	5.8	0.62
V455M	2.70	3.1	5.23
A456V	1.45	1.65	17.06

The relative activity index for each GCK mutant was calculated using equations 3 and 4. The predicted threshold for GSIR was calculated using equation 6. *Note that thermolability was ignored in these calculations. If it had been included the corresponding thresholds for GSIR would be about 7.0 mmol/l.

of recombinant wild-type GCK, mutant GCK from GCK-MODY, GCK-PNDM and GCK-HI syndromes together with the corresponding clinical data from the literature we can assess the mathematical model. The database was obtained from the kinetic and thermolability studies of human recombinant wild type GCK, one incidental GCK mutant (D158A) previously used as a control enzyme by others [13] and 39 spontaneous mutants causing MODY, PNDM or HI linked to GCK. Equations 5 and 6 illustrate how the threshold for GSIR for a homozygous mutant and a heterozygous mutant are calculated, respectively. The predicted values of the activity indices for the 42 enzymes (41 mutants and wild type) are listed in table 2. Figure 1a shows the relationship between the relative activity index of the enzyme and the predicted threshold for GSIR when adaptation is included in the calculations. As one might predict, the threshold for GSIR decreases as the relative activity index increases. The relationship has the shape of a hyperbola (note that in the semi log plot which is employed here it appears to be sigmoidal). This mirrors the clinical situation where an increase in the relative activity index extrapolates to a marked enhancement of β-cell glucose usage and a decrease in the threshold for GSIR and hypoglycemia with the reverse being the case for inactivating mutations.

Using the model to predict the threshold for GSIR for a homozygous GCK mutant

$$28\% = \frac{G^{hm}}{G^{hm} + S_{0.5}{}^{m\,hm}} \times \frac{K_{cat}{}^{m}}{K_{cat}{}^{w}} \times \frac{2.5}{2.5 + {}^{ATP}K_{m}^{m}} \times \frac{E^{m(adapt)}}{0.66} \qquad (5)$$


Threshold for GSIR (mmol/l)
1/relative activity index
E339G
V62A
V226M
S383L
L309R
M235T
M298K
S263P
Y108C
E70K
G264S
H137R, E300K
A53S
K414E
WT, R275C, G72R
V367M
V62M
T65I
W99R
V455M
D158A
A456V
Y214C
L146R
T168P
G44S, G175R, S336L, C213R, V182M, M210T, M210K,
A378V, C252Y, V203A, G261R, S411F, R377C, A188E
a
Adapted
Non-adapted
b

Using the Model to Predict the Threshold for GSIR for a Heterozygous GCK Mutant

$$28\% = \frac{0.5\,G^{hw}}{G^{wh} + S_{0.5}{}^{wh\,w}} \times \frac{2.5}{2.5 + {}^{ATP}K_m^{w}} \times \frac{E^{w(adapt)}}{0.66} + \frac{0.5\,G^{hm}}{G^{hm} + S_{0.5}{}^{m\,hm}} \times \frac{2.5}{2.5 + {}^{ATP}K_m^{m}} \times \frac{K_{cat}{}^{m}}{K_{cat}{}^{w}} \times \frac{E^{m(adapt)}}{0.66} \quad (6)$$

We can compare the predicted values for GSIR with the basal blood glucose values published in the literature for patients with GCK-MODY, GCK-PNDM and GCK-HI. Table 3 summaries the published data and the predicted thresholds calculated using the mathematical model. The clinical picture in GCK-MODY differs very little and usually shows moderate hyperglycemia despite the different severities of the mutations, whilst in GCK-PNDM the phenotype is always severe. It is evident that in GCK-MODY increased blood glucose levels favor increased expression of the wild-type allele (and to some extent the mutant allele), which then compensates for the decreased relative activity of the mutant allele. However, in GCK-HI the decreased blood glucose concentration favors adaptation of the mutant allele. If this model genuinely reflects what is happening in vivo it is possible that unlike GCK-MODY mutations, which have a predicted threshold for GSIR of ~7 mmol/l and patients have a very narrow range of fasting plasma glucose values (6–8 mmol/l), we will see greater heterogeneity with GCK-HI mutations. There is already some evidence for this with the different clinical presentation and clinical course of the patient with the GCK-Y214C and A456V mutations compared to those with other less active GCK-HI mutations (4; 7). The predicted thresholds for GSIR for these mutations are 1.40 and 1.45 mmol/l, respectively. One of these patients (Y214C) did not seem to respond well to treatment with diazoxide. For GCK-PNDM there is no compensation by the wild-type allele. The severity of the

Fig. 1. ***a*** The relationship between the enzyme's relative activity index and the threshold for glucose-stimulated insulin secretion. The GCK kinetic database in table 1 and expressions 3, 4 and equation 6 were used to calculate the relative activity index and the predicted threshold for GSIR for each GCK mutant. Note that the mutants A53S, H137R, R275C and V367M cluster around an activity index of 1 (wild-type) and a predicted threshold for GSIR of 5 mmol/l. If severe instability were included in these latter cases the thresholds would be 6.90. ***b*** Critical role of adaptation: The effect of adaptation of GCK to glucose concentrations on the threshold for glucose-stimulated insulin release. For the nonadapted state, equation 6 was used without the expression coefficients (E).

Table 3. A comparison of the published clinical basal blood glucose levels in GCK-MODY, GCK-PNDM and GCK-HI and the mathematical model predictions

	Fasting blood glucose, mmol/l			
	Controls	GCK-MODY	GCK-HI	GCK-PNDM
Mathematical model	5 (by definition)	6.4 ± 0.97 (n = 41) (predicted)	2.37 ± 0.74 (n = 5) (predicted)	55.0 (M210K/M210K) †9.5 (G264S/IVS8 + 2T > G) >55 (A378V/A378V) (all predicted)
Velho et al. [21]	5.0 ± 0.5* (n = 341)	7.0 ± 1.1* (n = 260)		
Page et al. [22]	4.9 ± 0.4* (n = 12)	7.3 ± 1.8* (n = 17)		
Byrne et al. [23]	5.0 ± 0.02** (n = 6)	6.7 ± 0.1** (n = 6)		
Glaser et al. [5]	5.1 ± 0.2**	6.8 ± 0.4**	2.25 (n = 2)	
Christesen et al. [6]	(n = 8)	(n = 6)	2.9–3.5 (n = 2)	
Gloyn et al. [4]			2.6 (n = 4)	
Njølstad et al. [2, 3]				16.8 (n = 1) M210K 24.6 (n = 1) A378V ‡77 (n = 1) IVS8 + 2T > G 12.0 (n = 1) G264S/ IVS8 + 2T > G

*Data represent mean and SD.

**Data represent means and SEM.

†Functional data are not available on the homozygous IVS8 + 2T > G but since the mutation is predicted to result in failure to remove intron 8 producing an in-frame stop codon giving rise to an inactive mutant protein we have modelled the homozygous mutant and compound heterozygous (G264S/IVS8 + 2T > G) mutant using the functional data from the A378V mutant as this mutation results in an enzyme with virtually no activity.

‡Highest measured glucose level prior to insulin treatment.

mutation plays therefore a more important role in the phenotype. For homozygous M210K mutations the model predicts a threshold for GSIR of ~55 mmol/l whilst for homozygous A378V mutations a threshold for GSIR of >55 mmol/l is predicted. This point is also illustrated by the recently reported compound heterozygous mutant G264S/IVS8 + 2G > T [3]. Figure 3 shows a graphical representation of the different functional properties of wild-type, heterozygous G264S, homozygous G264S, compound heterozygous G264S/IVS8 + 2G > T. Mathematical modelling of a heterozygous G264S mutant predicts a threshold

for GSIR of 5.5 mmol/l and for a homozygous mutation the threshold is only slightly increased to 6.1 mmol/l, indistinguishable from many GCK-MODY mutations. However, the compound heterozygous mutation (G264S/IVS8 + 2G > T) detected in the patient with PNDM is predicted to have a threshold for GSIR of 9.0 mmol/l. These examples demonstrate that the variability in clinical severity of cases with PNDM results from corresponding differences in severity of GCK activity losses.

Effect of Adaptation on the Threshold for GSIR

Using the mathematical model the compensatory effect of the wild type allele can be quantitatively assessed. Figure 1b shows a graphic representation of the effect of adaptation on the predicted threshold for GSIR and from comparing figure 1a with figure 1b, it is clear that a model ignoring GCK induction by the prevailing ambient glucose would fail critically in predicting the actual basal or threshold glucose levels. Using a model that lacks the expression co-efficient E, the GCK-MODY mutants would be predicted to have thresholds ranging from 7 to 11 mmol/l with the vast majority approaching 11 mmol/l. Whereas for the GCK-HI mutant T65I, without adaptation, the predicted threshold would be ~6 mmol/l and the patient would therefore not have hypoglycemia. The relative contribution of the mutant allele's kinetics to expression levels of the enzyme results in a decreased threshold for GSIR and hypoglycemia.

An Exploration of the Contributions of the Individual Kinetic Parameters to the Threshold for GSIR

The relative impact of the various kinetic parameters of mutant GCK on the activity index and the predicted threshold for GSIR needs to be appreciated and was therefore investigated. The results of altering only one of the parameters are shown graphically in figure 2. The graph illustrates that it is the enzyme's affinity for the substrate glucose ($S_{0.5}$) and its turnover (K_{cat}) that have the greatest influence over the control of insulin secretion in the physiological range of 4–7 mmol/l. The impact of the Hill coefficient is small and is apparent only when it decreases. The enzyme's affinity for ATP only influences the system when the $^{ATP}K_m$ increases above the normal level of 0.41 mmol/l and then it overlaps with that of equivalent changes in K_{cat}. It is worthy to note in this context that the model largely ignores the influences that certain well known

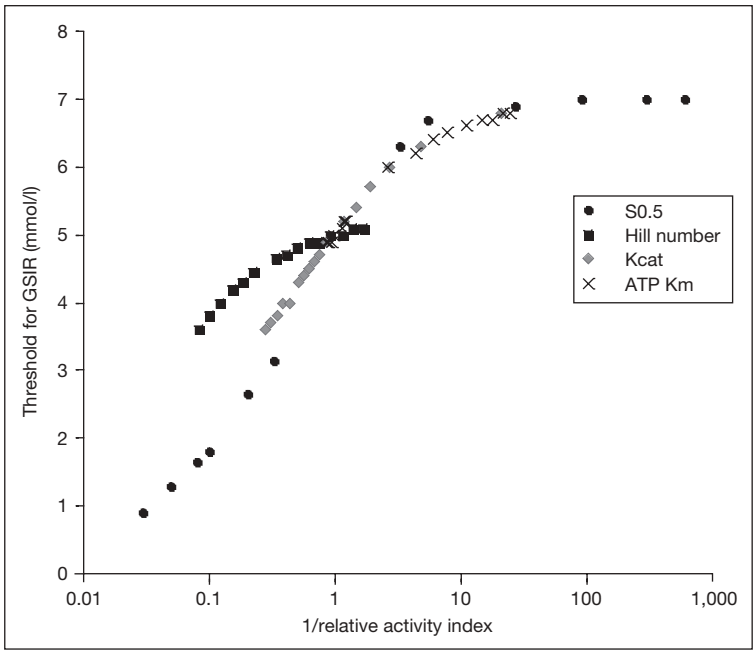


Fig. 2. Impact of individual kinetic parameters of the mutant GCK activity index on the β-cell threshold for glucose-stimulated insulin secretion. Each kinetic parameter was varied in turn for one allele whilst maintaining all other parameters as those measured for wild type GCK. For each change in value the relative activity index and threshold for GSIR was calculated using expressions 3, 4 and equation 6 as described previously. This graph illustrates that the control strengths of K_{cat} and glucose $S_{0.5}$ are comparable in the physiological range of 3–7mmol/l. It also shows that ATP only influences the system when the $^{ATP}K_m$ increases above the normal level of 0.41 mmol/l and that then the effect overlaps with that of equivalent K_{cat} changes. The impact of the Hill number is small and only apparent when it decreases.

but subtle alterations of the $^{ATP}k_m$ might have on the threshold for GSIR. This approach is justified because GCK operates physiologically at near saturation with this co-substrate. For example, a fivefold increase of the $^{ATP}K_m$ would lower the enzyme's actual in vivo rates by only one third. We have used here the $^{ATP}k_m$, which was obtained at near saturation with glucose. This results in a small error because the $^{ATP}K_m$ rises slightly with increasing glucose. The impact of this subtle ATP/glucose interaction is however minimal, i.e. $<5\%$. It is also noteworthy that in some mutants high glucose lowers an abnormally high $^{ATP}K_m$ (M210K, C213R and G261R) again with little impact on the threshold calculations [Matschinsky et al., unpubl.; 14].

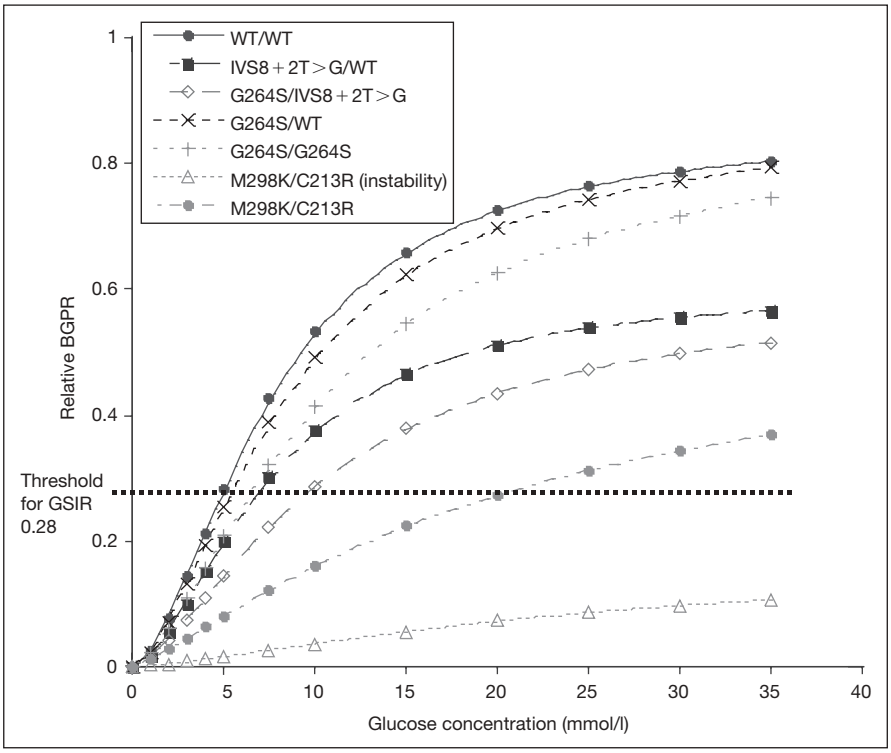


Fig. 3. Comparison of the functional properties of wild-type (WT) and heterozygous G264S, homozygous G264S, compound heterozygous G264S/IV8 + 2T < G and the hypothetical compound heterozygous M298K/C213R (with and without an expression for instability). This figure illustrates the range of phenotypes possible when both alleles are affected differently. G264S in the homozygous state is still only a mild mutation with a subtle shift of the curve to the right. In contrast with the compound heterozygous mutation (G264S/IVS8 + 2T > G) the threshold for GSIR is shifted even further to the right with a predicted threshold for GSIR of 9.0 mmol/l. A hypothetical compound heterozygous mutant M298K/C213R is also illustrated. This mutant is predicted to have a threshold for GSIR of ~20 mmol/l, and if the instability of M298K is taken into account a threshold for GSIR may never be reached.

Missense Mutations in GCK that Appear Kinetically Normal but Cause Disease

The careful kinetic analysis of 41 mutant recombinant enzymes has uncovered six mutants that show practically normal kinetic characteristics, A53S, V62M, H137R, G264S, R275C and V367M. It is possible that these are functionally silent

polymorphisms rather than pathogenic mutants but co-segregation with GCK-MODY in families and the fact that these variants have not been detected in over 100 normal chromosomes would argue against this. It is possible that there are other mechanisms by which these mutations result in GCK-MODY (besides instability which was discussed earlier). V62M is a case point, and this mutation has now been identified in two families, one from the UK and one from Italy [15]. The mutation has been shown to co-segregate with GCK-MODY in these families arguing against this variant being a silent polymorphism. Moreover, this variant has been shown to be totally refractory to the recently reported novel GCK activator drug [15, 16]. These results have been interpreted to mean that a critical as yet undiscovered natural endogenous ligand is required for optimal GCK activity and that mutant GCK-V62M does not respond to this endogenous activator. This inability to respond to a putative endogenous activator has been hypothesized as the molecular basis for GCK-MODY in these families [15]. It is likely that the continued careful study of the naturally occurring GCK mutants will provide greater insight into the regulation of glucokinase. With this improved knowledge will come a greater understanding of how blood glucose levels are regulated by GCK allowing evolution of the mathematical model to incorporate any new information.

Discussion

These and previous studies using the mathematical model have shown that it accurately predicts the clinical picture in GCK-MODY, GCK-HI and GCK-PNDM and illustrates the critical role of β-cell GCK in the regulation of blood sugar. It is demonstrated again how relatively small changes in the kinetic parameters can have pronounced effects on the threshold for GSIR and basal blood glucose levels as predicted many years ago [17]. These small kinetic changes that have significant biological effects also illustrate why GCK is an attractive drug target for the treatment of hyperglycemia. Indeed, recent work has shown that pharmacological agents can activate glucokinase in a way similar to that described for the activating hypoglycemia mutations, suggesting that they may be acting through a common site and mechanism [16].

It should be stressed that what has been presented is a minimal mathematical model and that other factors might be involved and could be taken into account. To illustrate this point, recent studies by Leibiger et al. [18] have provided evidence that in addition to the direct effect of glucose and of its metabolism [19] it is conceivable that insulin has a direct positive feedback effect and may play a role in the adaptation of the GCK sensor to ambient glucose levels. As these leads are further pursued and as the molecular mechanisms of

GCK induction are elucidated in greater depth the current mathematical model might have to be modified to include an expression co-efficient which in addition to the role of the glucose $S_{0.5}$ and the blood glucose concentration takes into account the possible effects of intermediary metabolism and neuroendocrine factors. However, since the mathematical model outlined in this chapter accurately predicts the basal glucose level of individuals with GCK disease without involving altered intermediary metabolism and/or insulin signalling it seems that current evidence favoring the simple model remains on balance scientifically sound and realistic. The newly proposed role of insulin is not convincing because GCK induction/adaptation in GCK-MODY seems to occur at insulin levels that are clearly indistinguishable from those in control subjects.

The mathematical model is solely based on the contribution of β-cell GCK to glucose metabolism and homeostasis. Although it has been demonstrated that GCK-MODY patients have a hepatocellular glucose phosphorylation deficit [20] it is not known whether this defect is necessary to explain the hyperglycemia seen in GCK-MODY. Unlike the β-cell, the hepatocyte does not seem to have a threshold response to glucose, which could explain the predominant impact of the former on the glucose set point in an organism. However, GCK based glucose sensor cells have been found in the gut mucosa, in hepatic vascular tissue and in the brain and may influence the glucose set point of the β-cell in various ways still to be explored.

In conclusion, we have outlined and discussed a minimal mathematical model that can be used to predict the threshold for GSIR of activating and inactivating mutations of *GCK* in man. The impressive power of this model to accurately predict the basal blood glucose or threshold for GSIR in glucokinase disease attests again to theoretical soundness and medical significance of the GCK glucose sensor paradigm. The modelling studies also add in a significant way to the scientific foundation for the proposal to use a novel class of GCK activators in the drug treatment of T2DM.

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Glucokinase/Glutamate Dehydrogenase Interactions in the GDH form of Congenital Hyperinsulinism

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Fuels that stimulate β-cell insulin secretion include amino acids, in addition to glucose. Stimulation of insulin secretion by amino acids provides a closed loop regulatory system, since as part of its role in controlling the switch between the fed and fasted states, insulin controls amino acid metabolism by promoting protein synthesis while suppressing protein breakdown. The importance of amino acids as insulin secretogogues was originally appreciated through the identification of infants with leucine-sensitive hypoglycemia. Indeed, one of the first applications of the insulin radioimmunoassay was to demonstrate that these children failed to suppress plasma insulin concentrations when hypoglycemic. Subsequently, in vitro studies of perfused pancreas confirmed that leucine and many other amino acids could stimulate insulin secretion.

Unlike glucose-mediated insulin secretion, amino acid-stimulated insulin secretion is conditional. Most amino acids stimulate insulin release only in the presence of glucose. Leucine is an exception to this rule, since glucose inhibits leucine-stimulated insulin secretion. The observation that pre-treatment with tolbutamide can induce leucine-sensitive hypoglycemia in normal individuals provides an additional example of the conditional nature of amino acid-stimulated insulin secretion. The purpose of this chapter is to review new insights into the links between the pathways of glucose- and amino acid-mediated insulin secretion. These insights come from observations in children with hyperinsulinism due to glutamate dehydrogenase (GDH) mutations, which disrupt normal regulation of leucine-stimulated insulin secretion. Extension of

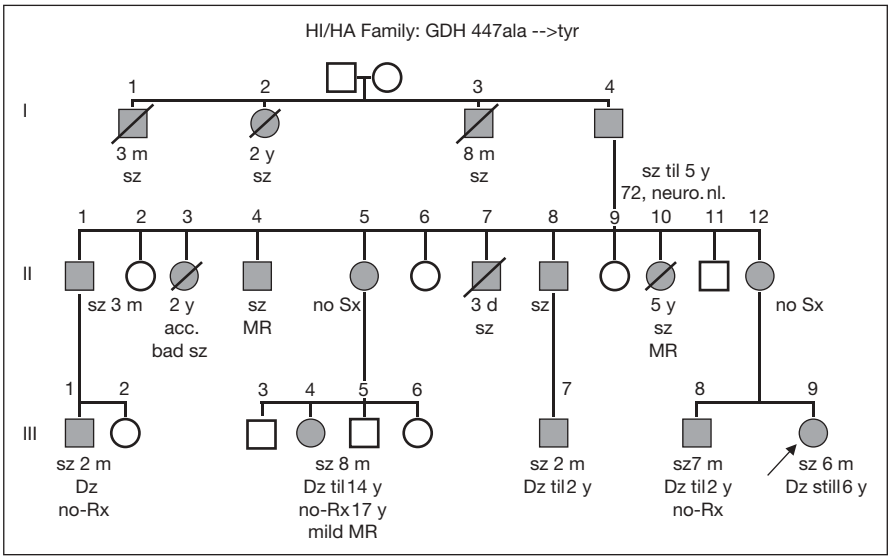

Fig. 1. Pedigree of HI/HA family. Three generations are known to be affected. Affected subjects are shown in filled squares/circles. (Family histories courtesy of Dr. Constantine Polychronakos, Montreal.)

these clinical observations to in vitro studies in isolated rat islets indicates that both the phosphate-dependent glutaminase (PDG) and GDH enzymatic steps of glutaminolysis mediate the interactions between glucose- and amino acid-stimulated insulin release.

Hyperinsulinism/Hyperammonemia Syndrome in Children with Gain of Function Mutations of Glutamate Dehydrogenase

Hypoglycemia in the HI/HA Syndrome

The major features of the HI/HA syndrome are symptomatic fasting and postprandial hypoglycemia and a persistent, mild but asymptomatic hyperammonemia [1]. The range of hypoglycemic manifestations is illustrated in a family with three generations of affected individuals (fig. 1). The pedigree clearly shows that the disorder is autosomal-dominantly inherited, although, as noted below, 80% of HI/HA cases are de novo. As seen in this family, many affected individuals presented with symptoms of hypoglycemia, such as seizures, during the first few years of life. Neonatal hypoglycemia and large for gestational age

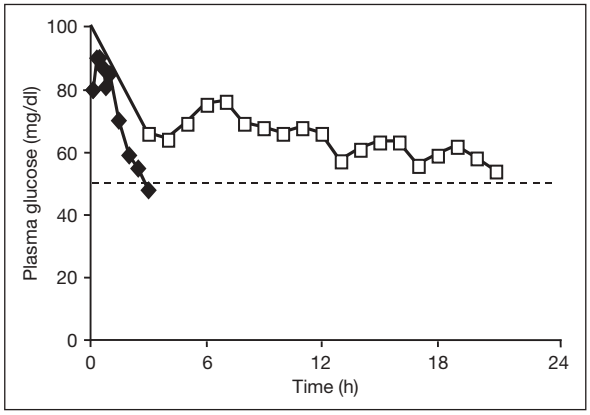

Fig. 2. Blood glucose responses to fasting and a protein feeding in HI/HA. Shown are the blood glucose concentrations during fasting (open squares) and a protein meal (solid squares) in a teenage girl with HI/HA. This subject was able to fast approximately 14 h before blood glucose decreased to less than 60 mg/dl. However, blood glucose decreased to <50 mg/dl within 2.5 h of a protein feed (1.5 g/kg).

birth weight, which are features of a more severe form of hyperinsulinism due to K_{ATP} channel mutations, are not common in the HI/HA syndrome. Nonetheless, as shown by this family, hypoglycemia in HI/HA children can lead to permanent brain damage and mental retardation. Several children shown in figure 1 died in early infancy, apparently from complications of hypoglycemic seizures and brain damage. Before the hypoglycemia in this family was detected, the children with recurrent seizures were assumed to have epilepsy. Note that some individuals completely escaped recognition, such as the two mothers, II-5 and II-12, both of whom had severely symptomatic children. The grandfather in this family, I-4, had seizures in infancy, but unlike three of his affected siblings, survived to father a large family. In childhood, the grandfather was noted to have 'spells' that seemed to be triggered by one of his favorite foods, smoked meat, a delicacy similar to pastrami. This complaint emphasizes the importance of protein-sensitive hypoglycemia as a feature of HI/HA. Fasting hypoglycemia, which is typically associated with HI in general, is present but frequently less pronounced than post-prandial hypoglycemia (fig. 2) [2]. Random exposure to fasting and protein feeding are probably the major 'environmental' factors that explain the apparent variability in phenotype of the disorder in this family. Note that in the children in the last generation, hypoglycemia was controlled with diazoxide, a K_{ATP} channel agonist. This feature is different from the usual diazoxide-unresponsiveness found in children with K_{ATP} channel hyperinsulinism.

Hyperammonemia in the HI/HA Syndrome

A distinctive feature of the HI/HA syndrome is hyperammonemia, an abnormality not found in other forms of hyperinsulinism. Serum ammonium concentrations in affected individuals of all ages are persistently elevated to 2–10 times normal (median = 135 vs. <35 μmol/l in normals) [3]. The hyperammonemia is unlike that associated with the urea cycle enzyme defects (UCED), such as ornithine transcarbamylase deficiency [4]. In contrast to individuals with UCED, patients with HI/HA appear to be asymptomatic from their hyperammonemia. They also lack the abnormalities in plasma and urinary amino acids that characterize UCED. In particular, HI/HA syndrome patients do not have elevations of plasma glutamine, which always accompany hyperammonemia in the UCED. Also, unlike in the UCED, plasma ammonia levels are extremely stable in HI/HA patients; they do not rise with protein feeding and do not fall with protein restriction [2, 5–7]. Benzoate and phenylacetate, drugs used to treat hyperammonemia by increasing alternate pathways of ammonia disposal, are ineffective in children with HI/HA [6, 8, 9]. In 2 patients, N-carbamylglutamate, an analogue of N-acetylglutamate (NAG), which allosterically activates the first step in ureagenesis, reduced serum ammonium concentrations by 50%, but did not produce any clinical benefit [7, 8]. A more recent study found no effect of N-carbamylglutamate on serum ammonia concentrations [10].

GDH Mutations in the HI/HA Syndrome

All patients with HI/HA syndrome have been found to have missense mutations of GDH (GLUD1 on 10q). Enzymatic studies of GDH from patient lymphoblasts and of mutant GDH expressed in *Escherichia coli* reveal decreased enzyme sensitivity to inhibition by GTP and ATP [1, 3, 5, 9–12]. This loss of sensitivity to allosteric inhibition makes the HI/HA syndrome one of the very few disorders of intermediary metabolism in which mutations cause a gain of enzyme function. As shown in figure 3, mutations occur within the pocket on the enzyme surface that binds the allosteric inhibitors GTP and ATP. Mutations also occur in the pivot helix and antenna regions of the enzyme, which are presumed to mediate cooperative interactions with other subunits in the GDH homohexamer. GDH mutations causing HI/HA have been identified in approximately 70 families (table 1) [1, 3, 5, 9–12]. Familial transmission in an autosomal-dominant fashion accounts for only 20% of cases. The remaining 80% represent post zygotic, de novo mutations.

The presumed mechanism by which GDH mutations cause hyperinsulinism and hyperammonemia is shown in figure 4. The impairment in sensitivity to GTP inhibition results in a gain of GDH enzyme function. Oxidative deamination of glutamate by GDH supplies α-ketoglutarate to the Krebs Cycle,

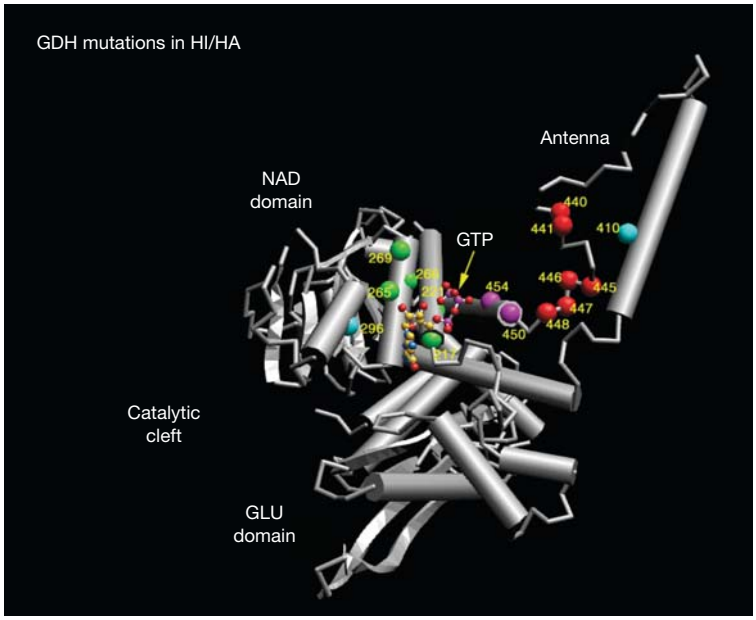

Fig. 3. X-ray crystal structure of GDH and HI/HA mutation locations. Shown is one subunit of the bovine form of GDH. Mutations are found in the GTP-binding site (exons 6 and 7) and the antenna and hinge regions (exons 11 and 12).

hence, generating ATP. This increase in the ATP/ADP ratio triggers insulin release through the K_{ATP} channel pathway [13]. Overactivity of GDH in the direction of α-KG, as is proposed to occur in HI/HA, thus, drives unregulated insulin secretion. Enhanced GDH activity in the direction of glutamate oxidation in the liver would also account for the hyperammonemia associated with the HI/HA syndrome. Figure 3 demonstrates the dual roles that glutamate performs in hepatic ammonia detoxification: (1) NAG synthesis, and (2) ammonia generation. As shown, excessive GDH activity depletes glutamate, thereby reducing NAG synthesis. Since NAG is a necessary allosteric activator in the first step of ureagenesis, impaired NAG production would impede ammonia detoxification. In addition, increased oxidation of glutamate via GDH produces ammonia, further contributing to hyperammonemia.

Leucine and Protein Sensitivity in the HI/HA Syndrome

In 1956, Cochrane et al. [14] reported a father and his two daughters who suffered from hypoglycemia, which was aggravated by a high protein diet or ingestion of leucine. Subsequently, 'leucine-sensitive hypoglycemia' was used

Table 1. Mutations of GDH in HI/HA patients

Exon	Mutation	Amino acid	Sporadic	Familial
6	C822G	S217C		1
6	C833T	R221C	7	3
7	G966C	R265T	2	
7	G966A	R265K	1	
7	G966C	R265T	2	
7	A969G	Y266C	1	
7	C977T	R269C		1
7	G978A	R269H	10	1
7	A1059C	E296A	1	
10	A1400T	N410Y	1	
10	T1409G	L413V	1	
11	C1492A	F440L	1	
11	A1494G	Q441R		1
11	C1506T	S445L	20	1
12	G1508C	G446R	1	
12	G1508A	G446S	2	
12	G1509A	G446D	3	
12	G1509T	G446V	1	
12	G1511A	A447T	1	
12	T1514C	S448P		3
12	A1522G	K450E	2	
12	C1532T	H454Y	1	1

synonymously with congenital hyperinsulinism and affected children were instructed to avoid protein. Therefore, we investigated whether the impairment in GDH inhibitory control in HI/HA children would be associated with enhanced insulin responses to leucine stimulation and whether leucine-sensitivity was specific for this form of HI. To avoid inducing dangerous hypoglycemia, we modified the traditional leucine tolerance test by giving a smaller dose of leucine by intravenous bolus and measuring the acute insulin response, rather than the fall in blood glucose [16]. As shown in figure 5, HI/HA patients have a brisk insulin response to a leucine infusion, whereas normal controls have no response. Unrestrained allosteric activation of GDH by leucine in the HI/HA patients probably accounts for their characteristic protein-induced hypoglycemia. As shown in figure 6, hypersensitivity to leucine is not present in all forms of hyperinsulinism, such as the more common form of the disorder due to recessive mutations of the K_{ATP} channel [16]. Interestingly, a subset of patients with hyperinsulinism not due to GDH mutations is leucine-sensitive; the genetic defect(s) in these patients are not yet clear.

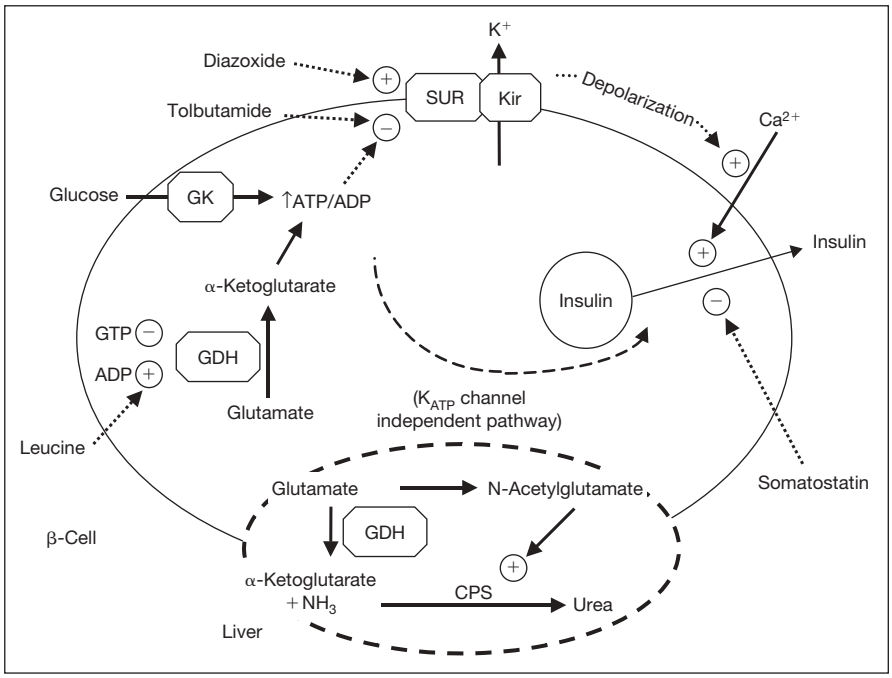

Fig. 4. Role of GDH in β-cell insulin secretion and hepatic ureagenesis. In the β-cell, leucine activates GDH to stimulate insulin secretion. Upon entry of glucose into the β-cell, glucokinase (GK) initiates metabolism of glucose, generating ATP. The increased ATP to ADP ratio leads to closure of the K_{ATP} channel, cell membrane depolarization, calcium influx, and ultimately, insulin secretion. GDH stimulates insulin secretion by oxidation of glutamate to αKG. αKG fuels the Krebs cycle, generating ATP and activating the same cascade of insulin release as glucose. GDH is allosterically activated by leucine and ADP and is allosterically inhibited by GTP. In the hepatocyte, GDH activity generates ammonia through oxidative deamination of glutamate. Glutamate is necessary for production of N-acetylglutamate, a necessary allosteric activator of carbamoyl phophate synthetase, the rate-controlling first step of the urea cycle.

Because of the long-standing observation that glucose inhibits leucine mediated insulin secretion [17], we tested whether pre-loading with glucose to raise the β-cell phosphate potential might improve the inhibitory control of GDH and prevent leucine hypersensitivity in HI/HA patients. As shown in figure 7, the exaggerated acute insulin response to leucine is partly suppressed by high blood glucose [16]. The therapeutic corollary of this observation is to recommend that patients with HI/HA eat a serving of carbohydrate prior to eating protein. Limited observations suggest that this maneuver is helpful in preventing protein-induced hypoglycemia in these children.

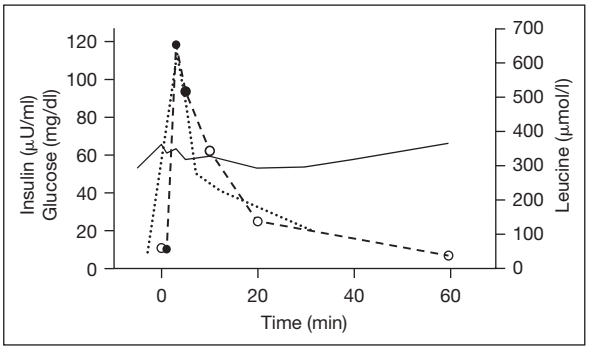

Fig. 5. Acute insulin response to leucine in HI/HA. The acute insulin response to leucine is defined as the delta increase in insulin at 1 and 3 min following an intravenous bolus of leucine (15 mg/kg). A dramatic increase in serum insulin occurred (circles) concomitantly with an increase in serum leucine concentrations (dashed line) following the intravenous leucine bolus in this HI/HA patient. Blood glucose (line) remained stable which distinguishes this study of leucine sensitivity from previous studies which required the development of hypoglycemia to diagnose leucine sensitivity.

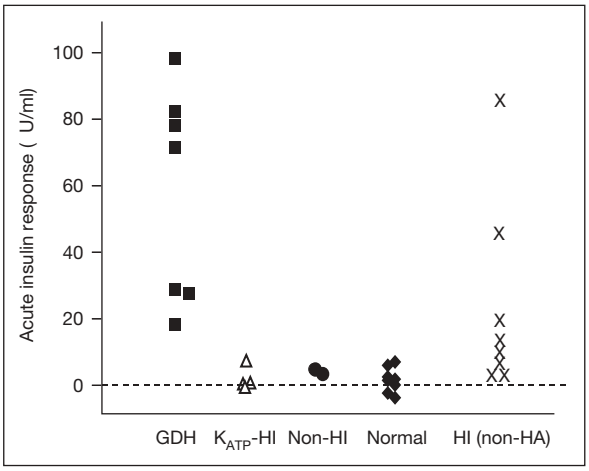

Fig. 6. Acute insulin responses in HI/HA and controls. All patients with HI/HA were leucine sensitive as demonstrated by their acute insulin responses to leucine unlike subjects with recessive K_{ATP}-HI, hypoglycemia not due to HI, and normal controls. A subset of patients with HI whose genetic defects have yet to be identified were leucine sensitive.

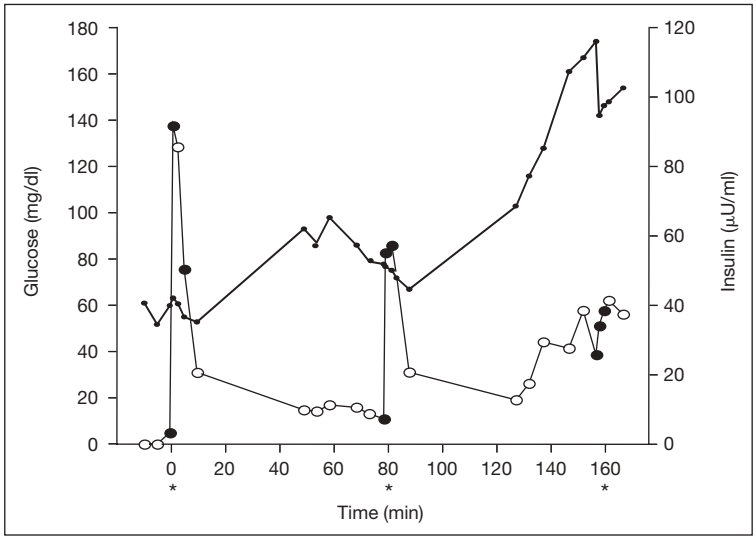

Fig. 7. Effect of glucose on leucine-stimulated insulin secretion in a patient with HI/HA. Blood glucose (points) was maintained in the 50–60, 70–80, and 160–180 mg/dl ranges with intravenous dextrose. Acute insulin response to leucine studies were performed at 0, 80 and 160 min. With increasing blood glucose, the acute insulin response to leucine was suppressed. Closed circles represent the 0-, 1- and 3-minute insulin concentrations for the acute insulin response test. Open circles represent other insulin measurements.

Studies of Glucose-Leucine Interactions in Rat Islets

Effects of Glucose on Leucine-Stimulated Insulin Secretion: The 'Run-Down' Phenomenon

To investigate the inter-connections between glucose and leucine observed in HI/HA patients, we considered that two pathways exist for leucine to stimulate insulin release. The first, direct, mechanism involves leucine oxidation in mitochondria via transamination of leucine to a-ketoisocaproate (KIC) and then subsequent oxidation. The second, more indirect, mechanism involves the allosteric activation of GDH by leucine, which accelerates the rate of glutamate oxidation to α-ketoglutarate (α-KG) and ammonia. Since other allosteric regulators, such as inhibition by GTP and ATP, can affect the GDH activity, we examined the effect of glucose exposure upon islet responsiveness to leucine and KIC and upon rates of glutamine flux to glutamate and then to α-KG plus ammonia [18].

As shown in figure 8a, when isolated rat islets, cultured for 3 days in 10 m*M* glucose-containing media, are removed from glucose for 120 min,

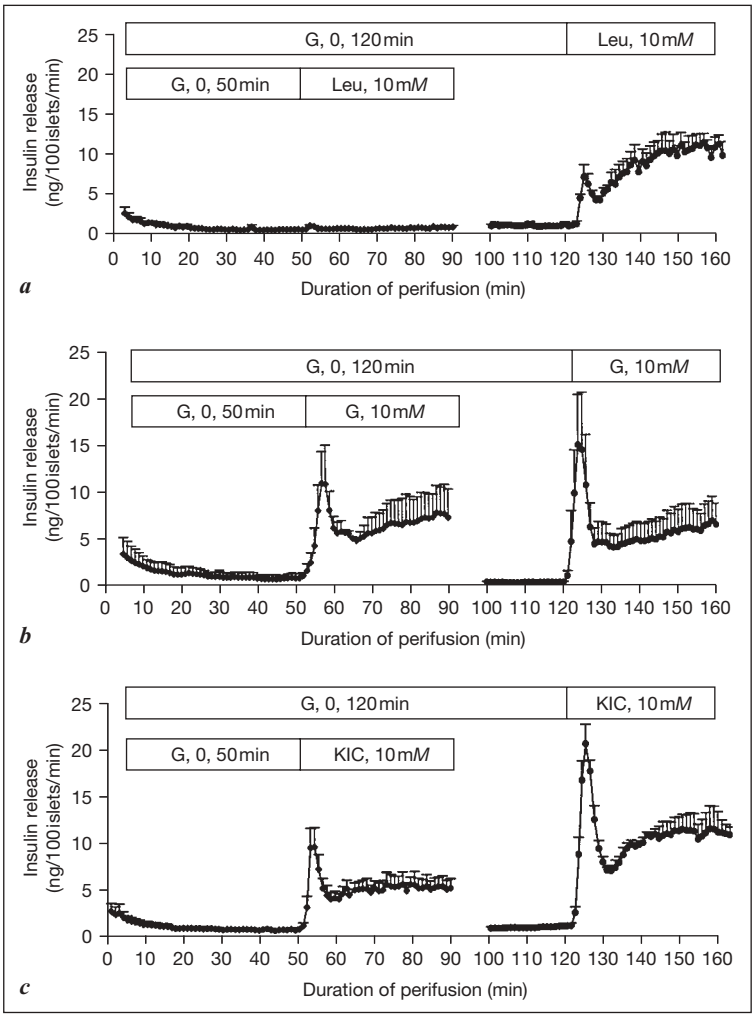

Fig. 8. Effect of run-down duration on islet responsiveness. Isolated rat islets were cultured with 10 m*M* glucose for 3 days and then perifused with 2 m*M* glutamine in the absence of glucose for run-down periods of 50 min (diamonds) or 120 min (circles) prior to stimulation with 10 m*M* leucine (***a***), 10 m*M* glucose (***b***) and 10 m*M* KIC (***c***). Values represent the means ± SE for 100 islets from 3 separate perifusions.

10 m*M* leucine provokes insulin release. In contrast, after removal from glucose for only 50 min, islets are refractory to leucine stimulation. In experiments not shown, the islet phosphate potential declines during the period of glucose withdrawal. Thus, removal of glucose allows islet phosphate energy to 'run

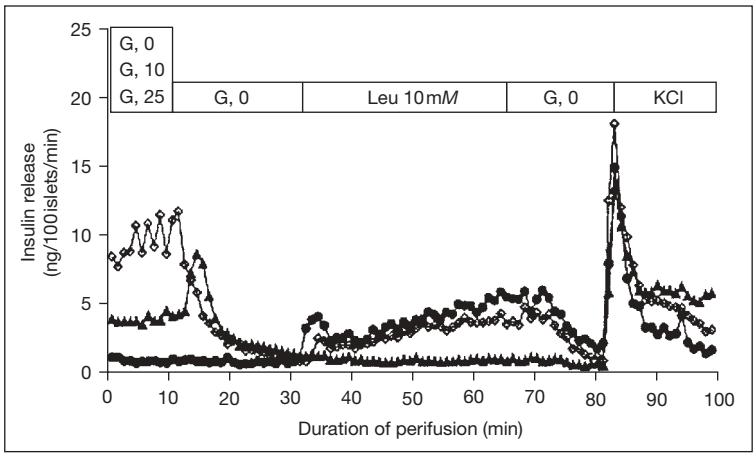

Fig. 9. Effect of glucose concentration on islet responsiveness to leucine. Isolated rat islets were cultured with 10 m*M* glucose for 3 days and then perifused with 2 m*M* glutamine in the presence of different concentration of glucose for 120 min prior to stimulation with 10 m*M* leucine. Circles represent 0 m*M* glucose, diamonds 10 m*M* glucose, triangles 25 m*M* glucose. At the end of the experiment, after removal of leucine for 10 min (glucose 0 m*M*), islets were stimulated with 30 m*M* KCl.

down', thereby, reducing inhibition of GDH by GTP and ATP. This effect of islet 'run down' does not alter the 'direct' pathway for leucine stimulation of insulin secretion, since glucose withdrawal does not alter the response to KIC, the first intermediate in the 'direct' pathway of leucine stimulated insulin release (fig. 8b). Similarly, glucose stimulated insulin release is unaffected by islet run down (fig. 8c).

The inhibitory effect of glucose on the sensitivity of cultured isolated rat islets to leucine stimulation during prolonged energy run-down is concentration dependent. As shown in figure 9, a prolonged period of glucose withdrawal enhanced sensitivity to 10 m*M* leucine stimulation. In contrast, islet responsiveness to leucine stimulation was completely abolished by perifusion in 25 m*M* glucose prior to the 120 min energy run-down period. Insulin release in response to leucine was partially inhibited by prior perifusion in 10 m*M* glucose. Islet responsiveness to 30 m*M* potassium chloride depolarization remained unchanged under all three conditions.

Interactions of Glucose and Leucine with the Glutaminolysis Pathway

In order to evaluate the mechanism of the islet run-down phenomenon in more detail, we carried out measurements of flux from glutamine through

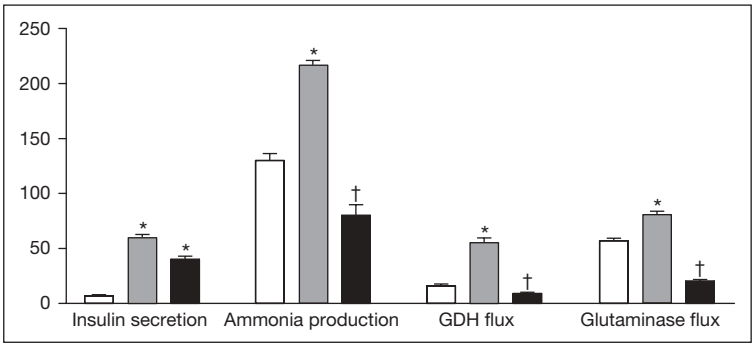

Fig. 10. Effects of leucine and glucose on GDH and glutaminase fluxes and insulin secretion. Islets were cultured with 10 m*M* glucose for 3–4 days. Batches of 1,000 islets were first preincubated with unlabeled 10 m*M* glutamine for 90 min. The islets were then incubated under following conditions for another 120 min: 10 m*M* [2–^{15}N]-glutamine as control (γ), in addition to 10 m*M* [2–^{15}N]-glutamine, 10 m*M* leuicne (▒) and combination of 10 m*M* leucine and 25 m*M* glucose (■). The flux through GDH and glutaminase were calculated depends on the isotopic enrichment as well as the metabolites concentration. Both leucine and the leucine plus glucose stimulated insulin release (10 times, μg/1,000 islets/2 h). 10 m*M* leucine stimulated total ammonia production (nmol/1,000 islets/2 h), GDH and glutaminase flux (nmol/1,000 islets/2 h), this stimulation effect was inhibited by high glucose. Compared with control, *$p < 0.01$, compared with 10 m*M* leucine, †$p < 0.01$.

glutamate to α-KG and ammonia using ^{15}N stable isotope methods. For these studies, islets were incubated in batches for 2 h in 10 m*M* glutamine labeled with ^{15}N at the amino nitrogen. In this way, total ammonia generation via glutaminase and GDH, as well as the specific release of the amino ^{15}N in the GDH step could be determined by measuring the isotopic enrichment of ammonia. As shown in figure 10, leucine-stimulated insulin release was accompanied by a nearly twofold increase in total ammonia production. The addition of glucose significantly lowered the rate of total ammonia production. Leucine increased flux through phosphate-dependent glutaminase (PDG) by 50% and tripled flux through GDH. Glucose markedly suppressed flux through both enzymes whether in the presence or absence of leucine.

Figures 11a and b summarize the interactions between glucose and leucine stimulated insulin secretion revealed in the above experiments. In the absence of glucose (fig. 11a), the phosphate potential is low and the inhibitory control of both PDG and GDH is minimal. Under this condition, leucine potently stimulates GDH activity and secondarily promotes flux through PDG. As a result, glutamine flows through glutamate into the TCA cycle, generating ATP to activate insulin release. In the presence of a limited acetyl-CoA supply, the carbon skeleton of glutamate flowing into alpha-KG is ultimately converted to

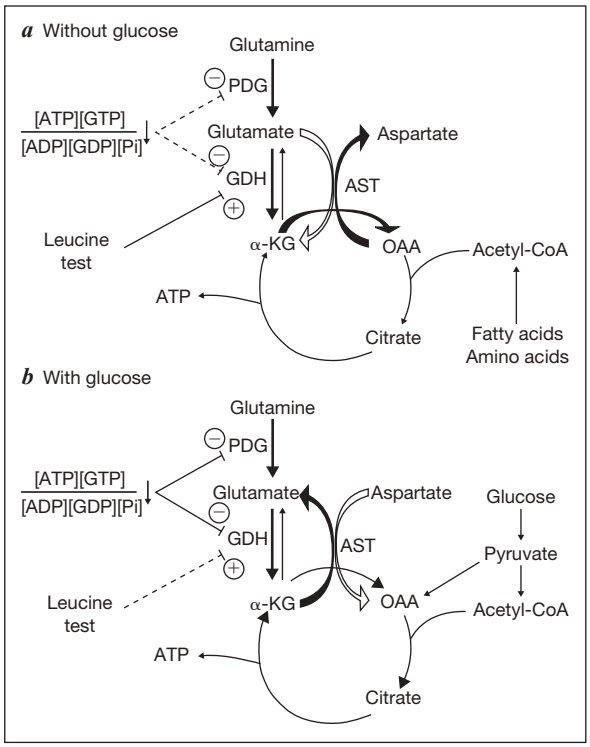

Fig. 11. Effects of glucose on the pathways of glutaminolysis and leucine stimulated insulin secretion in pancreatic β-cells. ***a*** In the absence of glucose, the islet phosphate potential decreases, leading to de-inhibition of both phosphate-dependent glutaminase (PDG) and of glutamate dehydrogenase (GDH), as shown by the dashed line. In this state, GDH is responsive to allosteric stimulation by leucine, as shown in solid line. Flux is increased from glutamine to glutamate and into the TCA cycle intermediate, α-ketoglutarate. When the supply of acetyl-CoA is limited, the TCA cycle stalls at oxaloacetate leading, by transamination with glutamate, to an accumulation of aspartate. ***b*** In contrast, in the presence of glucose, elevation of the phosphate potential leads to inhibition of both PDG and GDH and, as shown by the dashed line, renders GDH insensitive to stimulation by leucine. The pathway of glutaminolysis from glutamine to glutamate and to α-ketoglutarate is suppressed. The end products of glycolysis, pyruvate and acetyl-CoA, sustain a complete TCA cycle, shifting the flow of aspartate toward glutamate formation by transamination.

aspartate via transamination of oxaloacetate. In the presence of glucose, the phosphate potential is elevated, resulting in inhibition of both PDG and GDH. In this condition, leucine is unable to overcome inhibition of GDH and, thus, unable to promote insulin release.

Summary and Conclusions

The sensitivity of islets to leucine stimulation is tightly controlled by the intracellular phosphate potential, to which the main contributor is glucose metabolism. Glucose regulates leucine-stimulated insulin secretion by inhibiting glutamine flux through both the glutaminase and GDH enzymatic steps through the effects of Pi and ATP on these two enzymes. Together, GDH and glutaminase serve as intracellular energy sensors, which modulate islet responsiveness to leucine stimulation.

Acknowledgement

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Glucokinase: A Monomeric Enzyme with Positive Cooperativity

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Long before the relationship of glucokinase with MODY was established, its unusual structural and kinetic properties attracted the attention of several groups, the enzyme from rat liver showing mild positive cooperativity with respect to its substrate glucose, with a Hill coefficient of about 1.5 [1, 2]. Other cooperative enzymes were already well known, but most showed stronger cooperativity, with Hill coefficients between 3 and 4; glucokinase also lacked other typical characteristics of regulatory enzymes, such as allosteric inhibition by a downstream metabolite and a number of subunits at least as large as the Hill coefficient. Although the mild cooperativity seen with glucokinase suggested that it might be a dimeric enzyme, there was no direct evidence of this: it appeared to be monomeric in all conditions, including those of the assay in which the cooperativity had been found [3, 4]. Models for cooperativity in monomeric enzyme were known [5, 6], but these appeared not to be needed to explain the positive cooperativity of any enzyme with its natural substrate, and in practice were thought to be mainly of theoretical interest. The mechanism of the kinetic cooperativity of glucokinase therefore presented a problem to be understood, and forms the main topic of this chapter. Although a few other cases of cooperativity in monomeric enzymes have been reported, glucokinase remains the unique example of an important enzyme of primary metabolism for which the kinetic origin of the cooperativity has been clearly established [7].

Terminology

In our two chapters we have maintained consistency with the rest of the book by calling the enzyme glucokinase rather than hexokinase D, the name we

prefer, as it accords better with the specificity. The name glucokinase (EC 2.7.1.2) properly refers to enzymes of bacteria and other microorganisms that are specific for glucose as sugar substrate [8, 9]; the liver isoenzyme of hexokinase belongs with the other mammalian hexokinases as EC 2.7.1.1, and calling it glucokinase reflects a historical misapprehension of its specificity (one derived from measurements with liver extracts [10] made long before it was isolated). Although older editions of Enzyme Nomenclature listed the liver isoenzyme as EC 2.7.1.2 this was changed in the 1984 and later editions [11], and it is now listed correctly under EC 2.7.1.1.

Cooperativity with Respect to the Sugar Substrate

Rat-liver glucokinase, the most thoroughly studied form, follows strict Michaelis-Menten kinetics with respect to some sugar substrates, such as fructose and 2-deoxyglucose. There is, however, recent evidence that human glucokinase shows positive cooperativity with fructose [12], but the reasons for this discrepancy are not yet understood.

With the principal substrate glucose, and also with mannose, plots of rate against sugar concentration are slightly sigmoid (fig. 1), thus showing positive cooperativity, with a Hill coefficient of about 1.6. This value is large enough for the sigmoidicity to be perceptible in a plot of rate against substrate concentration (and easily detectable as a lack of straightness in a typical kinetic plot, as seen in inset ***a*** of the figure), but small enough to be overlooked if insufficient care is taken. In consequence, even though the existence of cooperativity has been clear for nearly 30 years there has been much confusion and misleading information about it in textbooks [13–16]. Even books that give a basically correct account of the properties of glucokinase often confuse it by referring to a K_m value for glucose [17]. However, as K_m is, by definition, a parameter of the Michaelis-Menten equation it has no clear meaning in the context of an enzyme that does not follow Michaelis-Menten kinetics: what is probably meant by K_m in such sources is the half-saturation concentration, or $K_{0.5}$.

The Hill equation can be written in a way that resembles the Michaelis-Menten equation as follows:

$$v = \frac{V[\mathrm{Glc}]^h}{K_{0.5}^h + [\mathrm{Glc}]^h} \qquad (1)$$

In reality no enzyme obeys this equation exactly (except, trivially, with $h = 1$, when it degenerates to the Michaelis-Menten equation), but many enzymes obey it fairly well over a wide range of substrate concentrations. This is not sufficient for detailed mechanistic investigation, but it is normally quite

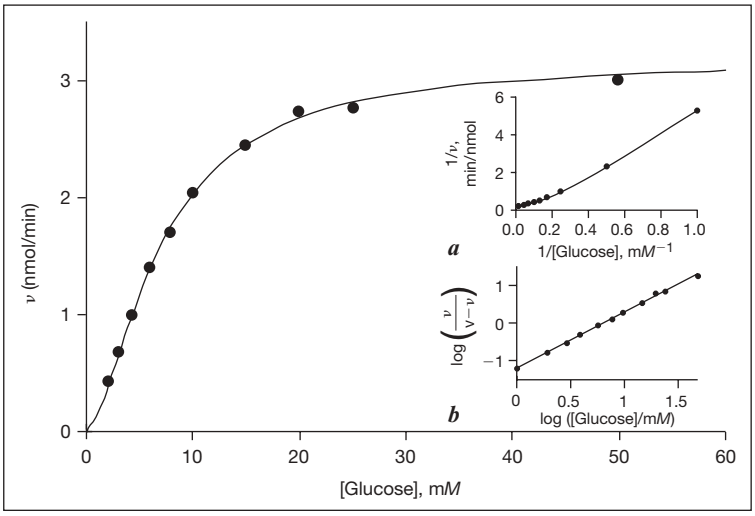

Fig. 1. Sigmoidal kinetics of glucokinase. Data for the rat-liver enzyme measured at pH 7.5 and 30°C [1]. Inset A shows the same points in double-reciprocal coordinates, and inset B shows them in the form of a Hill plot.

satisfactory for physiological characterization of an enzyme. Mutant forms of glucokinase, for example, can be adequately compared in terms of the three parameters of equation (1), the limiting rate V (or, better, the catalytic constant $k_{cat} = V/e_0$ if values of the enzyme molarity e_0 are available), the half-saturation concentration $K_{0.5}$ and the Hill coefficient h.

To do this correctly it is essential to obtain all three parameters from a single fitting to the Hill equation, and computer programs capable of doing this are now widely available. One must not, for example, obtain V and K_m from a preliminary fit to the Michaelis-Menten equation and then use the value of V to construct a Hill plot of log[v/(V–v)] against log[Glc] to obtain the value of h. Figure 2 illustrates the sort of results likely with this sort of approach. Both steps involve an element of arbitrariness, as the initial fitting to the Michaelis-Menten equation (inset ***a***) involves ignoring the manifest failure of the equation to fit the data, and estimating the slope of the Hill plot (inset ***b***) requires a decision about which points lie on the 'straight' part of the plot and which ones are at the extremes where curvature is to be expected. Inevitably, therefore, different people may obtain quite different sets of parameters from the same operations; in all cases they are likely to fit the data as badly as the line drawn in the main part of figure 2 from the parameters obtained in the insets, even though a parameter set exists that defines an excellent fit to the data. It may be tempting to attribute the faults in this example to the use of graphical methods, but this

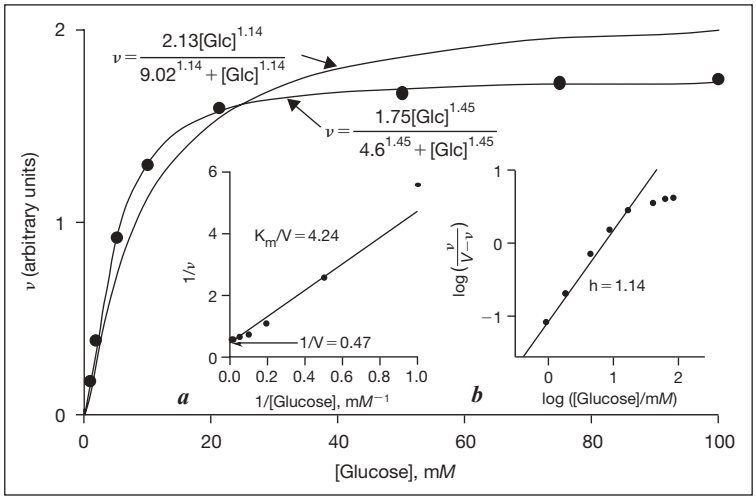

Fig. 2. Consequences of using an inappropriate method to define the kinetic parameters. If values of K_m and V are obtained from a double-reciprocal plot (inset A), ignoring the non-linearity in the data, and the value of V is then used to construct a Hill plot (inset B), the value of h obtained from the slope should not be combined with the values of K_m and V obtained initially. If it is, the fit will be very poor. The correct procedure is to obtain all three parameters simultaneously by non-linear regression.

is an error: exactly the same problems arise when the whole operation is done by computer [18]; the graphs simply illustrate points that are likely to pass unnoticed in computer analysis. Unfortunately one of the earliest comparisons between mutants of glucokinase associated with diabetes mellitus [19, 20] was rendered virtually meaningless by misanalysis even more bizarre than what is illustrated in figure 2, as discussed elsewhere [8]. An additional important point for the study of mutant forms of enzymes is that they are often less stable than wild-type forms, so particular care is necessary to check the stability of the enzyme in assay conditions, to avoid artefactual indications of cooperativity.

Michaelis-Menten Kinetics with Respect to the Nucleotide Substrate

The glucokinase-catalyzed rate follows strict Michaelis-Menten kinetics with respect to the concentration of $MgATP^{2-}$, within the limits of detection. Nonetheless, analysis of the kinetics with respect to $MgATP^{2-}$ requires proper care to avoid a different potential pitfall. $MgATP^{2-}$ exists in equilibrium with several other species, notably Mg^{2+}, ATP^{4-} and Mg_2ATP, all in various states of protonation, any of them capable of interfering as inhibitors or activators of glucokinase or of auxiliary enzymes used for the assay.

To ensure that effects attributed to variations in the concentration of $MgATP^{2-}$ are not in reality due to changes in the concentration of ATP^{4-} or another of the species mentioned, the concentration of interest should be varied in a way that minimizes changes in other concentrations. This is most easily achieved by varying the total ATP concentration at will and maintaining the total $MgCl_2$ concentration in a constant 1 m*M* excess (though this can be increased to about 5 m*M* if the assay uses a coupling enzyme such as pyruvate kinase that requires free Mg^{2+} for activity). Fuller discussion of this design, as well as others that are in common use but give much worse results, may be found elsewhere [8].

Models to Account for the Cooperativity with Respect to Glucose

The classical models for positive cooperativity [21–23] involve interactions between multiple subunits, and cannot be applied to monomeric enzymes unless these have multiple active sites on a single polypeptide chain. The possibility that a single hexokinase polypeptide chain might contain more than one active site was long discounted, but it proved to be a reality for the muscle isoenzyme hexokinase B [24, 25]. However, although this observation was both surprising and interesting it did not imply a new view of the structure of glucokinase, because hexokinase B had long been known to be 'dimer-like', i.e. to have a single polypeptide chain of about 100 kD consisting of two similar halves, each resembling the complete molecule of glucokinase [9]. Thus, extrapolation from the two active sites on the 100 kD hexokinase B molecule implies just one active site on the 50 kD glucokinase molecule, in agreement with the original conclusion [26].

Positive cooperativity without interacting sites can only be a kinetic property that cannot occur at thermodynamic equilibrium. It thus requires a kinetic mechanism in which binding of substrates is not necessarily at equilibrium in the steady state. Care must be taken in formulating a model to ensure conformity with thermodynamic principles, and in particular with the principle of microscopic reversibility: simply put, this means that the product of ratios of forward and reverse rate constants around any cycle must be equal to the equilibrium constant of any transformation accomplished by the cycle, or to 1 if it accomplishes no net transformation. Two models that appeared particularly appropriate for glucokinase are the 'mnemonical' model [27] and the slow-transition model [28], first used to explain complex kinetics observed with hexokinases from plants and yeast, respectively. These models are shown in figure 3 in the forms that were applied to glucokinase [31, 32].

Several features in the mnemonical model (fig. 3a) are noteworthy. The enzyme exists in two distinct forms, E and E′, that are relatively slowly interconverted; of these, the more stable form E′ that predominates in the absence

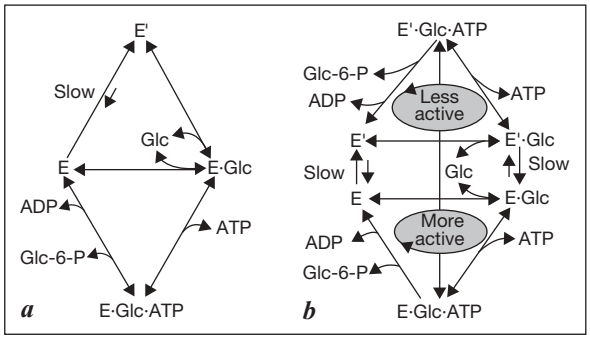

Fig. 3. Mnemonical (***a***) and (***b***) slow-transition models as applied to glucokinase.

of substrates is not the one released at the end of the catalytic cycle, though both can bind glucose to form the same enzyme-glucose complex. This parallel binding of glucose in two different steps implies, by the normal principles of steady state kinetics [29] that the full rate equation contains terms in the squared concentration of glucose, thereby allowing deviations from Michaelis-Menten kinetics. However, the other substrate, $MgATP^{2-}$, binds in a unique step, and therefore should not display such deviations.

Because the isomerization of E to E′ is slow, equilibrium in the conversion can be unbalanced by the catalytic reaction if this is fast enough; this can only be at high $MgATP^{2-}$ concentrations, because in the limit at low concentrations it must be possible for equilibrium to be established between glucose and the two forms of free enzyme more quickly than the enzyme-glucose complex can be removed by $MgATP^{2-}$. The model thus makes a number of predictions that fit the behavior of glucokinase: cooperativity with respect to glucose, but not with respect to $MgATP^{2-}$, with a Hill coefficient for glucose that increases with the $MgATP^{2-}$, and tends to a value of 1 (Michaelis-Menten kinetics) when the $MgATP^{2-}$ concentration is very low. Moreover, given that the explanation of the glucose cooperativity depends absolutely on the ability of the enzyme-glucose complex to react rapidly with $MgATP^{2-}$, the model also predicts that a poor nucleotide substrate should fail to induce the glucose cooperativity, and this has been confirmed in studies with $MgITP^{2-}$ [30].

Although the mnemonical model thus accounts satisfactorily for the main features of glucokinase kinetics, it is in general almost impossible to rule out other explanations, because models of cooperativity typically include more adjustable parameters than one can hope to define accurately. The slow-transition model (fig. 3b) is somewhat more complicated, but gives a better account of some of the fine details of the kinetics of glucokinase [8, 32]. The main

additional feature is that now both conformations can accomplish a catalytic cycle, but with different kinetic parameters for glucose, and the steady state rate is the sum of the rates for the two cycles; the cooperativity derives from the fact that the steady-state distribution of glucokinase between the two forms E′ and E varies with the glucose concentration: as this increases, a higher proportion of the enzyme exists in the more active form. Recently a model combining features of these two has been proposed [12]. Other models and fuller discussion, including evidence for glucose-induced conformational changes, is given elsewhere [8].

As drawn in figure 3 both models show the products released in the opposite order from what one might expect, glucose-6-phosphate before $MgADP^-$. This order was suggested long ago [31] on the basis of product inhibition, but the interpretation remained questionable on account of the glucose cooperativity, which rendered analysis of the inhibition difficult and dubious. This difficulty is avoided, however, in recent product inhibition studies with the non-cooperative substrate 2-deoxyglucose instead of glucose [33]. This work provided new evidence not only of the order of product release shown in figure 3, but also of the presence of only one sugar-binding site in the enzyme.

Physiological Role of Glucokinase Cooperativity

Cooperative responses of enzymes to feedback inhibitors constitute a major and essential feature of biochemical regulation, as has been recognized for many years [34]. They allow regulation of a biosynthetic pathway to be regulated by demand for the end-product rather than by supply of the starting materials [35], and, indeed, allow elementary principles of economics to work much better in the cell than they do in human economies. Substrate cooperativity is a less obviously useful property, however, and in most systems is most easily explained as a side effect of the evolution of inhibitor cooperativity without any particular physiological importance. The cooperative binding of oxygen to hemoglobin, an example often used to illustrate the physiological role of substrate cooperativity, is irrelevant because hemoglobin is not an enzyme. The well-known regulatory enzymes, such as phosphofructokinase, typically have very low flux control coefficients for the flux through the pathways in which they occur [36]: this means that genetic manipulations of their activity in vivo typically have little or no effect [37]. Such observations initially surprised many investigators, but are now well understood [35, 36]. They have the additional implication that the fluxes through such enzymes in vivo should be resistant to variations in the concentrations of their substrates, and little affected by any substrate cooperativity that they may have.

Glucokinase is an exception to all of these generalizations; its unusual combination of structural and kinetic properties, producing cooperativity in a

monomeric enzyme, is paralleled by non-typical physiological properties. It has a very high flux control coefficient in glycogen synthesis [38], and also appears to control the glycolytic flux in pancreatic β cells [39]: together with its cooperativity with respect to glucose this confirms that this is not a typical demand-regulated pathway but a supply-driven one. Any small demand for the products of glucose phosphorylation that the mammalian liver has is satisfied by the basal level of glucokinase and the small amounts of high-affinity hexokinase isoenzymes in hepatocytes [40]; glucokinase is primarily present to catalyze the major glucose-phosphorylating activity of the liver, not to meet its demand for glucose-6-phosphate but to maintain homeostasis of blood glucose. For this purpose a high flux control coefficient is essential, and also a high sensitivity to the glucose concentration. The major function of the cooperativity is then to bring the concentration of greatest sensitivity from zero, where it would not be helpful, to within a small factor of the physiological concentration.

The view that the regulatory properties of glucokinase are those to be expected in a supply-regulated enzyme also agree with the initially puzzling observation that MODY is characterized by genetic dominance [41]. In general dominance is observed far more often in humans than in other organisms, but this does not reflect a genuine physiological difference between humans and other organisms, but a difference in the way they are studied: in humans reported gene effects often reflect detailed biochemical studies, for example measurements of blood-glucose concentrations, whereas in other organisms they reflect gross phenotypic effects. Such gross effects normally reflect differences in metabolic fluxes, and it is now clear that at this level most mutations are 'silent', i.e. they can be deleted without obvious effects, but they do normally have effects on metabolite concentrations [42, 43]. As MODY is a symptomless condition, at least when initially detected, it fits this categorization well, but it does not remain symptomless indefinitely. This again confirms that the role of glucokinase in liver and in pancreas is to modulate a supply-regulated process: failure to respond correctly to variations in the blood glucose concentration imply failure to regulate it properly, and hence leads eventually to clinical manifestations.

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Molecular Models of Human Glucokinase and the Implications for Glycemic Diseases

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Nearly 200 glucokinase mutations with distinct enzymatic properties have been found in three different glycemic diseases [1, 2]. Many inactivating mutations have been found in patients with the MODY2 form of diabetes type II where only one allele is defective [3]. Inactivating mutations on both glucokinase alleles have been associated with severe permanent neonatal diabetes [4]. The kinetic defects include reduced catalytic activity, increased affinity for the substrates glucose and ATP, and lowered Hill coefficient [5, 6]. Recently, patients with hypoglycemia were shown to have two mutations that increase the catalytic activity of glucokinase [7, 8]. The activation results from the separate or combined effects of increased catalytic activity, decreased affinity for substrates, and lowered Hill coefficient. The kinetic effects of these mutations have been interpreted using structural models of glucokinase in complexes with its substrates [5, 9, 10].

Two sets of molecular models have been built for human glucokinase and its substrates. Initially, the structure of glucokinase with glucose was modeled by analogy to the crystal structure of yeast hexokinase [9]. Yeast hexokinase is a paradigm for catalysis by induced fit. Glucose binding induces a large conformational change, as shown by comparison of crystal structures of yeast hexokinase A with glucose and hexokinase B with an inhibitor [11–13]. Glucokinase is expected to undergo similar conformational changes when glucose binds. The accuracy of the model was limited by the relatively low similarity of 30% identical amino acid residues and presence of 10 gaps in the aligned sequences of human glucokinase and yeast hexokinase. However, the high conservation of active site residues resulted in accurate prediction for the glucose-binding site

of glucokinase. This model proved valuable for understanding the activity of mutations associated with hyperglycemia [5, 9].

Crystal structures of human brain hexokinase I have provided the basis for more accurate structural predictions of glucokinase with its substrates. Human brain hexokinase I has similar N- and C-terminal halves that each resemble glucokinase. Several crystal structures have been determined of complexes of hexokinase I: an inhibited complex with glucose and the glucose-6-phosphate inhibitor [14], an active complex with glucose and phosphate [15], and a complex with glucose and ADP [16]. The N- and C-terminal halves were all in the closed conformation, except for the C-terminal half with phosphate that resembled the open conformation of yeast hexokinase. Structural divergence increases with sequence divergence [17]. Therefore, more accurate structure prediction is expected due to the higher sequence similarity of 53% for glucokinase with brain hexokinase compared to 31% with yeast hexokinase. Moreover, the sequence alignment of glucokinase with brain hexokinase has only one two-residue insertion located on the protein surface far from the substrate binding sites. In contrast, the sequence alignment of glucokinase with yeast hexokinase had 10 insertions or deletions, including one six-residue insertion in glucokinase, which will decrease the accuracy of the model since the structure of longer insertions cannot be predicted reliably [18]. Therefore, the glucokinase model based on brain hexokinase will be significantly more accurate than that of the earlier model based on the more distantly related yeast hexokinase [10]. However, this model of glucokinase with glucose and ATP had two disadvantages. Firstly, it was necessary to position the ATP manually since no nucleotide was present in the brain hexokinase crystal structures. Secondly, the model was based on an inhibited structure of brain hexokinase with glucose-6-phosphate. However, the predicted ATP binding site had good agreement with the mutations that show changes in K_m for ATP.

Recently, brain hexokinase was crystallized in complex with glucose and ADP [16]. The new structure has been used to obtain a better model of glucokinase in order to analyze the binding sites for its substrates and the new activators.

Research Design and Methods

The crystal structure of human brain hexokinase I in complex with glucose and ADP [16] was used to model the closed conformation of human glucokinase. The catalytically active C-terminal half was taken as the starting model; it shares 53% sequence identity with human glucokinase with a single two-residue insertion in glucokinase. The insertion of Gly 97 and Gln 98 was placed

at the turn between the third and fourth beta strands in the C-terminal domain of hexokinase. The amino acid residues of hexokinase were replaced by the residues 8 to 461 of glucokinase. The termini were not modeled. The C-terminal residues were not visible in the crystal structure, and the N-terminal residues may have different conformations in the two enzymes since the equivalent residues of brain hexokinase connect with the N-terminal regulatory half. The glucose and ADP were included. The positions of all new atoms were generated with the program AMMP [18] using the sp4 potential set [19] and a randomized analytical procedure [20]. The ADP was converted to ATP and an Mg^{2+} ion was added. The ATP was moved to position the gamma-phosphate oxygens about 5 Å from the glucose-6-hydroxyl group to mimic a reaction intermediate. Then, the whole complex was minimized using conjugate gradients. All computations were performed on a 1-GHz PC under the LINUX operating system. The open conformation of glucokinase was built using the C-terminal half of the hexokinase I structure [15], as described above. Models were examined with AMMP and RasMol [21].

Results and Discussion

Conformational Changes in Glucokinase and Hexokinase

The crystal structures of hexokinases show different conformations. Similar open conformations were observed for yeast hexokinase with inhibitor [12] and the C-terminal domain of brain hexokinase with glucose and phosphate [15]. The closed conformations of yeast and brain hexokinases are more different. The brain hexokinase C-terminal domain with glucose and ADP [16] is in a more closed conformation than observed for yeast hexokinase with glucose [11, 13]. The more closed conformation has the advantage of providing more interactions with the ATP, as shown in the glucokinase model [10]. The enzymes may exist in more than two different conformational states during substrate binding and catalysis.

The glucokinase structure was modeled in the open conformation without substrates and in the closed conformation with glucose and ATP. The new models will more accurately show the conformational changes due to the binding of glucose and ATP, since the closed conformation is based on the similar conformation of brain hexokinase with glucose and ADP. Comparison of the two models illustrates the conformational change that is expected to occur on binding of glucose (fig. 1). Glucose is bound in a deep cleft between the two domains. The residues that show distinct differences in position in the two models are mostly in the small domain: residues 77–86, 96–98, 105–139, 142–144, 150–155, 164–179, 181, 183–198, 341–344, 346, 442–446 and 456–461. The structure of

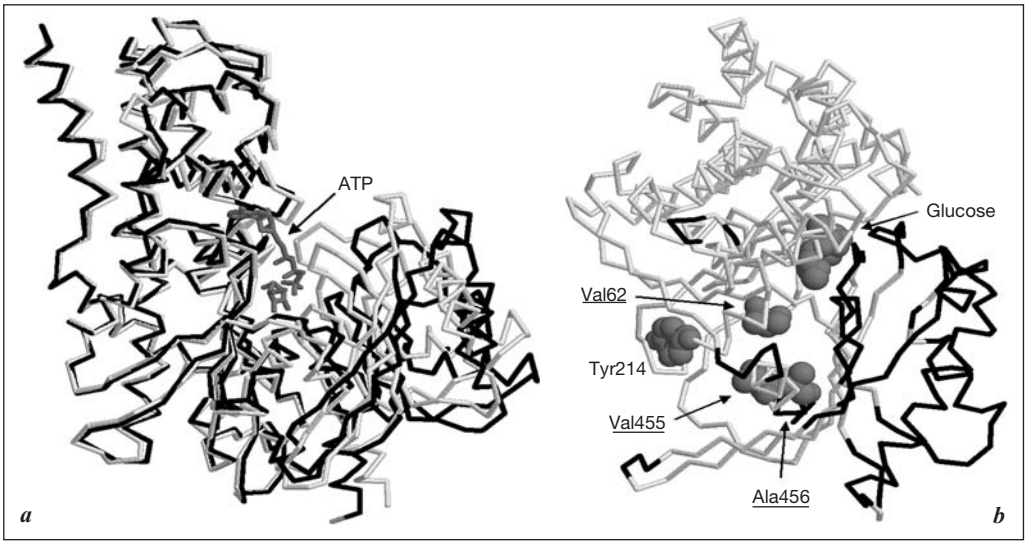

Fig. 1. Models of glucokinase structure. ***a*** The open conformation without substrates (black) is superimposed on the closed conformation (grey). The α-carbon atoms of the backbone are shown, with glucose and ATP in grey stick representation. ***b*** Glucokinase in the open conformation is viewed down the C-terminal helix. Grey indicates backbone atoms that do not change position in the two conformations. Black indicates the backbone atoms that move to a new position in the closed conformation. The sites of activating mutations near the hinge are indicated. Underlined residues are the sites of mutations associated with MODY2 (V62M) or hypoglycemia (V455M and A456V).

the larger domain can be considered as remaining constant, while the small domain rotates to provide better interactions with ATP. The conformational change moves the loop containing residues 80–85 into position to interact with ATP (fig. 1a). The C-terminal helix (residues 447–458) appears to provide a hinge for the closing of the two domains (fig. 1b). This helix includes several small residues, glycine and 4 alanines, which may assist with the movement about the hinge. Concomitant with the closing of the two domains, the distal region at the back of the hinge opens slightly. A small pocket is opened next to the terminal C-helix, where the V455M and A456V mutations associated with hypoglycemia are found [7, 8]. This pocket is formed by residues 62–65, 159, 210–211, 214, 220, 451–452, 455 and 456. Mutations of several residues in this pocket, V62M, Y214A, V455M and A456V, were shown to activate glucokinase [8]. Therefore, this region was postulated to be the binding site for the novel activators and an unknown physiological activator of glucokinase [1, 8]. However, the inactivating mutation M210T is located in this pocket, while the

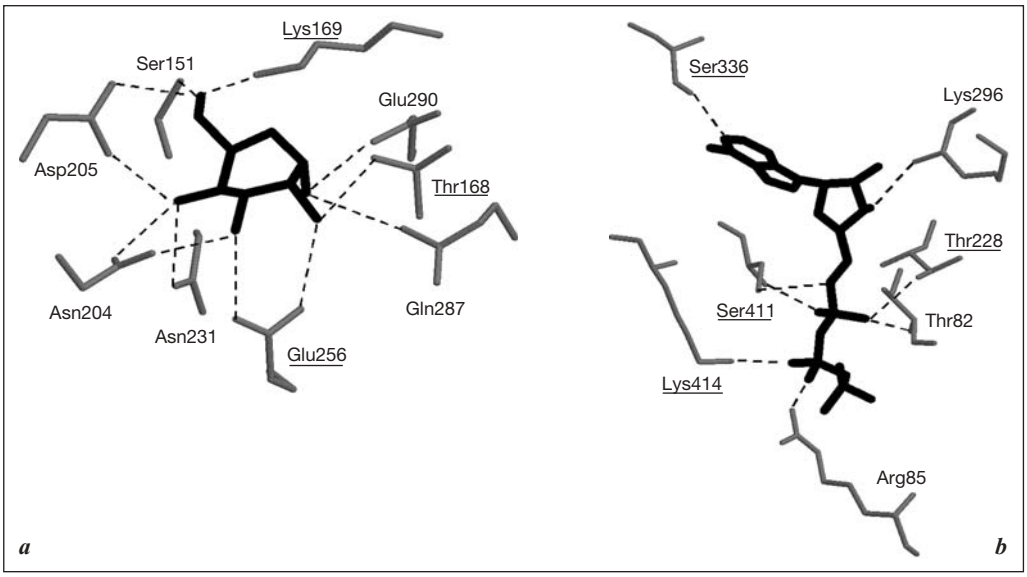

Fig. 2. Predicted interactions of glucokinase with its substrates. Hydrogen bond interactions are indicated by dashed lines. ***a*** Interactions with glucose. ***b*** Interactions with ATP. Underlined residues are the sites of mutations in MODY2 patients.

inactivating mutation C213R is adjacent to C220 in the pocket. Glucokinase activity is very sensitive to mutation of residues in this pocket near the hinge for conformational change.

Glucose-Binding Site in the Closed Conformation of Glucokinase

The glucose-binding site is essentially unchanged from previous models [9, 10] and is in good agreement with kinetic effects of mutations of glucose-binding residues. Glucose binds between the two domains in a cleft formed by residues 151–153, 166–169, 204–206, 225–231, 254–258, 287 and 290. Most of the residues that form the binding site for glucose are identical in both glucokinase and brain hexokinase. The only exceptions are glucokinase residues Asn 166 and Cys 230, which are Thr and Ser, respectively in brain hexokinase. All the oxygen atoms of glucose form hydrogen bond interactions with the side chains of glucokinase residues Ser 151, Thr 168, Lys 169, Asn 204, Asp 205, Asn 231, Glu 256, and Glu 290 (fig. 2a). Mutational analysis has confirmed the importance of several of these residues [5, 22]. Ala substitutions of Asn 204, Glu 256, and Glu 290 resulted in more than 100-fold decrease in k_{cat}/K_m values compared to those of the wild type enzyme [22]. The T168P mutant had nearly

20-fold increase in S0.5 for glucose [5]. Mutation of Ser 151 to Gly resulted in lowering of the K_m for glucose by 40-fold [23]. Ser 151 is particularly sensitive to mutation, since in the open conformation it does not form a hydrogen bond interaction with glucose [10]. The kinetic changes of the mutants are consistent with elimination of the predicted hydrogen bond interactions with glucose.

ATP-Binding Site

ATP is predicted to bind on the outside of the active site cleft and form interactions with both domains. The ATP-binding site is formed by residues: 80–85, 227–230, 295–296, 332–333, 336, 409–416 (fig. 2b). The Mg^{2+} ion coordinates with the gamma-phosphate oxygen atoms and the carboxyl group of the catalytic Asp 205. The phosphate oxygens of ATP form several hydrogen bond and ionic interactions with glucokinase residues. The β-phosphate oxygens interact with the side chains of Arg 85 and Lys 414. The α-phosphate oxygens interact with the hydroxyl side chains of Thr 228 and Ser 411, and with the amides of Thr 82 and Ser 411. The ribose 3′hydroxyl interacts with the amide of Lys 296. The predicted interactions with the ribose and phosphates differ slightly from those of the previous model [10]. The ionic interaction of Lys 414 with ATP was not predicted in the previous model, while Ser 411 formed a different interaction. The new model is expected to be more accurate since it is based on the ADP-bound form of hexokinase. The aromatic adenine ring is packed between the hydrophobic side chains of Val 412 and Leu 415 on one side and Thr 332 and Arg 333 on the opposite side. The adenine ring forms a hydrogen bond interaction between the N1 atom and the hydroxyl of Ser 336. This interaction was predicted by our previous model [10] and has been confirmed by the crystal structure of brain hexokinase with glucose and ADP [16]. The predicted binding site for ATP has been verified by kinetic data for mutants. Mutants G80A, V226M, T228M, S336L and K414E showed increased K_m for ATP, consistent with their location in the predicted ATP binding site [10]. The side chains of Thr 228, Ser 336 and Lys 414 are predicted to form hydrogen bond or ionic interactions with ATP, which would be eliminated in the T228M, S336L and K414E mutants, in agreement with the lowered activity. S336L showed the largest increase in K_m for ATP, while there was little change in the S0.5 for glucose. These effects are consistent with the interaction of Ser 336 with N1 of the adenine ring of ATP and distant from the glucose.

Possible Allosteric Sites

The crystal structures of brain hexokinase I show binding sites for the inhibitor glucose-6-phosphate [14] and a second allosteric site for ADP [16]. Mammalian hexokinase I is inhibited by glucose-6-phosphate, unlike glucokinase or yeast hexokinase. The glucose-6-phosphate binding site of brain

hexokinase is separate from the glucose binding site and partially overlaps with the ATP-binding site. The residues forming the two glucose-6-phosphate binding sites of brain hexokinase are identical in glucokinase with one exception. Ser 88 in the N-terminal half of brain hexokinase is equivalent to Ala 532 in the second half and Thr 82 in glucokinase, and also forms part of the ATP-binding site. This residue is a candidate for the difference in effect of glucose-6-phosphate. Alternatively, the different effects of glucose-6-phosphate on the two enzymes could arise from the more complex tertiary and quarternary structural interactions of the larger dimeric brain hexokinase I [14].

The monomeric form of brain hexokinase I was observed to bind ADP at a second site in the N-terminal half that is remote from the glucose-binding site [16]. However, only 2 of the 10 hexokinase residues that form the second ADP-binding site are conserved at the equivalent positions of glucokinase, or in both halves of brain hexokinase. Therefore, it is unlikely that glucokinase shares this allosteric ADP-binding site.

Hyperglycemia Arises from Defective Glucokinase

The effects of the missense mutations that are observed in patients with glycemia diseases can be interpreted using the molecular models of glucokinase. Mutations that eliminate interactions with substrates (fig. 2) have been shown to produce larger kinetic defects [10]. Mutations that are predicted to indirectly alter the structure of the binding sites also show kinetic defects. Missense mutations of residues 150, 168, 169, 203, 206, 225, 227, 228, 256, 257, and 259 that are found in patients with hyperglycemia [1] are predicted to alter the glucose-binding site. Mutations of residues 80, 81, 227, 228, 336, 411, 414 and 415 are predicted to alter the ATP-binding site. These mutations are predicted to cause hyperglycemia by reducing the glucokinase activity.

Several clinical mutations introduce the helix-breaking residue proline into helices in the glucokinase structure. These mutations are predicted to distort the secondary structure and reduce activity, as observed for L309P [10]. Mutations L30P, Q38P, L134P, and R369P are also predicted to reduce activity by distorting the secondary structure. Other mutations may cause structural distortions that are more difficult to interpret and must be examined individually.

Activating Mutations and Hypoglycemia

Activating mutations V62M, Y214A, V455M and A456V have been proposed to indicate the allosteric binding site of an unknown physiological activator [8]. These mutations are located around the molecular hinge for the conformational change on binding of glucose (fig. 1b). The model of the closed form of glucokinase shows an internal space in this region with potential for binding a small planar molecule. The space is reduced in the open conformation.

This difference is consistent with an activator that binds preferentially to the closed conformation and promotes ATP-binding and catalysis. This mode of action is likely for the novel activating drugs. The mutations V455M and A456V, which are found in hypoglycemic patients, are predicted to promote the closed conformation by the addition of large side chains that better fill the space in the closed conformation. These two mutations are located on the C-terminal helix that lies in the middle of the hinge. However, another activating mutation, V62M, was observed in patients with MODY2. Therefore, the clinical effects of activating mutations are difficult to predict, except that the two known mutations associated with hypoglycemia both introduce larger side chains on the C-terminal helix.

Conclusions

The predicted glucokinase structure is expected to be very similar to that of brain hexokinase, due to the 53% sequence identity and presence of only one two-residue insertion. The glucokinase was modeled in a closed conformation with glucose and ATP and in an open conformation without substrates. The models are in excellent agreement with the kinetic defects observed for mutations of residues predicted to form the binding sites for glucose or ATP. Sugar phosphorylation by glucokinase is very sensitive to the exact conformation of the glucose and ATP binding sites. Mutations that cause kinetic defects by distorting the protein secondary structure, or the ATP and glucose binding sites are found in patients with hyperglycemia. The glucokinase activity is also sensitive to mutations or activators that alter the space formed near the hinge during the conformational change. Two activating mutations, which are located on the C-terminal helix at the hinge, have been found in patients with hypoglycemia.

Summary

Molecular models of human glucokinase with its substrates have been used to understand the effects of the missense mutations that are implicated in the development of diabetes and hypoglycemia. Human glucokinase shares 54% identical amino acid residues with human brain hexokinase I. This similarity was used to build a structural model of glucokinase by analogy to the crystal structure of brain hexokinase. Glucokinase was modeled in the open conformation without substrates and in the closed conformation with glucose and ATP, in order to understand the effect of mutations on activity and conformational change. The glucose is predicted to form hydrogen bond interactions with the side chains of glucokinase residues Ser 151, Thr 168, Lys 169, Asn 204, Asp 205, Asn 231 and Glu 290, similar to those observed for brain hexokinase I. ATP is predicted to form hydrogen bond interactions with Thr 82, Arg 85, Thr 228, Lys 296, Ser 336, Ser 411 and Lys 414. Mutations of residues close to the substrate binding sites produced decreases in the glucokinase activity and resulted in

hyperglycemia, in agreement with our model. Glucokinase can be activated by mutation of residues near the hinge for the conformational change, and these mutations are associated with hypoglycemia. These models help rationalize the potential effects of mutations in diabetes and hypoglycemia, and may facilitate the discovery of pharmacological glucokinase activators and inhibitors.

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Crystal Structure of Human Liver Glucokinase Bound to a Small Molecule Allosteric Activator

Insights into the Activating Mutations

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Islet and hepatic glucokinase (GK) play an important role in whole body glucose homeostasis by catalyzing the ATP-dependent phosphorylation of glucose to glucose-6-phosphate (G-6-P) [1, 2]. Glucose-stimulated insulin release (GSIR) is tightly regulated by islet GK which acts as a molecular sensor to couple glucose metabolism to insulin release. This is best illustrated by the naturally occurring inactivating and activating mutations identified in humans that cause maturity onset diabetes of the young type 2 (MODY-2) [3, 4] and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [5–8], respectively. Furthermore, GK transgenic knockout and overexpression experiments in rodents confirm GK's preeminent role in glucose homeostasis [9–18].

GK, also known as hexokinase IV or D, is one of a family of four mammalian isozymes (hexokinases I–IV) catalyzing the phosphorylation of glucose. GK is unique with respect to its molecular mass, tissue distribution, structure, kinetic properties and function. Two structural models for GK have been built that accurately predicted the interactions critical for glucose binding and provided important structure/function analysis for conceptualizing the effects of MODY-2 mutations on GK enzymatic activity [19–21].

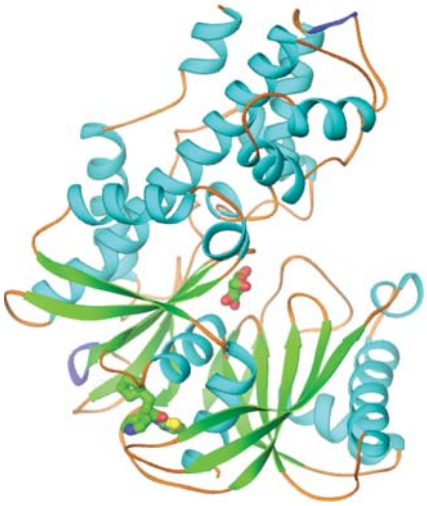

Fig. 1. Ribbon drawing of GK with the activator and glucose shown in ball-and-stick form. The atom colors are green for C, blue for N, red for O, and yellow for S.

As described previously, we discovered a small molecule that acts as a non-essential mixed type GK activator (GKA) [22]. GKAs lower the $[S]_{0.5}$ for glucose and increase the V_{max} of the phosphorylation reaction. We have now obtained the X-ray crystal structure of GK in complex with an activator, and report the discovery of an allosteric regulatory site 20 Å distant from the glucose-binding site.

Results

GK-GKA Co-Crystal Structure

Crystals of GK were obtained in the presence of glucose and the activator RO0275145. The structure was solved via MAD phasing with data extending to 2.7 Å. One molecule of glucose and one molecule of the activator RO0275145 are bound to GK in the crystal structure. The activator, RO0275145, is bound to a site 20 Å removed from the active-site, at the interface between the large and small domains (fig. 1). This allosteric site is located on the back of the structure, with respect to the location of the glucose binding site. The activator binding site is present and empty in a crystal structure of GK obtained in the absence of the GKA, and an overall comparison of the two structures shows they are very similar. The rms deviation calculated for 441 Cα atoms is only 0.4 Å after alignment of the X-ray structures with and without the GKA bound. This result suggests the mechanism of allosteric activation may be more subtle than a direct coupling between the allosteric and active sites.

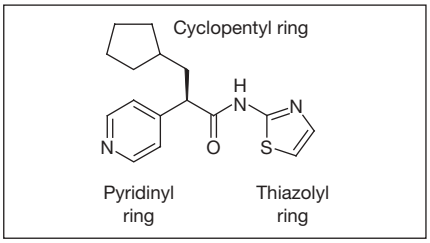

Fig. 2. Chemical structure of GKA RO0275145.

The top of the activator binding site is formed by residues 65–68. These residues are part of the first connection between the two domains of GK; however, they do not pack against any other part of the structure. In all other reported hexokinase (HK) structures, the corresponding residues pack closely against neighboring structural elements, leaving no cavity suitable for binding small molecule regulators. This structural difference between GK and the hexokinases is consistent with the biochemical data showing the small molecule activators have no effect on HK activity [22].

The chemical structure of the small molecule activator includes three arms decorating an aliphatic amide (fig. 2). Each arm consists of a cyclic moiety (cyclopentyl, thiazolyl, and pyridinyl groups) and the three cyclic groups are joined in a shape resembling the letter Y. Along the stem of the Y, the amide NH and thiazole N donate and accept hydrogen-bonds to the Arg63 backbone carbonyl O and NH, respectively. In addition to these specific hydrogen-bonds, a large number of van der Waals packing interactions contribute favorably to the binding energetics (fig. 3).

A hydrophobic surface formed by the side-chains of residues Met235, Met210, Ile211, Val62, Val452, and Ile159 covers the floor of the allosteric binding site. The aliphatic cyclopentyl group of the activator on one arm of the Y nestles between the two Met side-chains and the side-chain of Tyr214. The pyridinyl group which forms the second branch of the Y sits between Tyr214 and Pro66. And, finally, at the other end of the hydrophobic surface, the thiazolyl group lies on top of Val62, Val452, and Ile159, and underneath Val455 and Ala456.

Activating Mutations

Interestingly, mutations of some of the residues forming the allosteric site have been associated with familial hypoglycemia in humans (V455M, A456V, Y214C, and T65I as shown in fig. 3) [5, 6, 8, 25]. All of these mutations lower the $[S]_{0.5}$ for glucose, achieving the same effect as the small molecule activators. An additional activating mutation located in the allosteric activator site has been discovered in a site-directed mutagenesis study (Y214A) [26]. Finally, two activating mutations, W99R and D158A [7, 8] map on the structure nearby

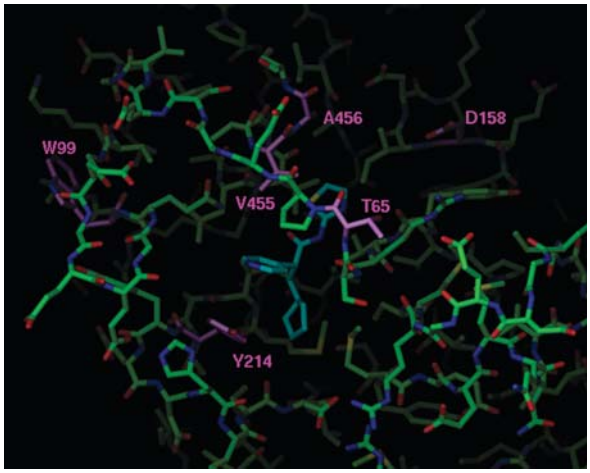

Fig. 3. Close-up of GKA binding site. The activating mutations T65I, Y214C, V455M, and A456V form a cluster around the GK activator (sites of mutation are colored purple, the GKA is colored light blue). The activating mutation W99R on the far left and D158A on the far right are more distant from the GK activator.

the allosteric activator site. A much larger set of mutations associated with the MODY-2 phenotype inactivate GK, and these mutations map all over the structure, consistent with the idea that it is relatively easy to compromise GK activity via mutation.

The activating mutations T65I, V455M and A456V replace small side-chains with larger ones, and would fill part of the space in the allosteric site independently of the binding of a small molecule activator. The activating mutations Y214A and Y214C replace a large hydrophobic side-chain with a smaller residue. The structure of GK with RO0275145 bound shows that two arms of the activator pack against the Y214 side-chain and in effect bury the side-chain in the structure. A common feature of these five activating mutations is that they change the hydrophobic surface area exposed to solvent, either by partially filling the allosteric site or by replacing a large hydrophobic residue with a smaller one. The mutations W99R and D158A alter residues potentially involved in domain-domain interactions. The mutation W99R introduces a positively charged residue on the small domain, which could form a salt bridge to either Glu216 or Glu442 on the large domain. A salt bridge between domains would stabilize the glucose-bound form of GK relative to more open forms, and thereby lower the $[S]_{0.5}$ for glucose. The mutation D158A may also activate GK via modulation of domain-domain interactions. Asp158, on the large domain, has the potential to interact with residues at the C-terminus of GK in the small

Table 1. Responsiveness of GK mutants to GKA

Mutation	Activity index	Activation factor	EC_{50} μM
WT	1.53	15.3	6.36
T65I	5.91	3.82	7.23
W99R	5.02	38.2	5.95
D158A	6.90	13.9	8.69
Y214C	23.7	0.97	n.a.
V455M	6.85	2.92	10.5
A456V	24.0	4.58	4.21

The kinetic properties of wild-type GK and activated mutants were characterized in an in vitro phosphorylation assay as described. The activity index is a combined metric sensitive to changes in kcat and the $S_{[0.5]}$ for glucose. The activation factor is the factor by which the activity index increases in the presence of a saturating concentration of GKA.

domain when the protein is in the open form. One published model of an open form of GK with no glucose bound shows the side-chain of Asp158 hydrogen-bonding to the side-chain of Gln465 [19]. A salt bridge with Lys459 in the same region of the structure is also possible. The effect of the mutation D158A would be to destabilize the open form of GK by eliminating the favorable interaction(s) the Asp158 side-chain makes with the small domain in the open form. Analysis of the X-ray structure suggests there could be multiple mechanisms for activation of GK, with the common theme of favoring one conformation of GK over others.

Effect of GKA on GK-Activating Mutations

We also examined whether the small molecule activators and activating mutations were synergistic. For the mutants T65I, Y214C, V455M, and A456V with residue changes in the activator binding site, addition of the small molecule activator leads to a less than fivefold increase in GK activity (table 1). For comparison, wild-type GK is roughly fifteen-fold more active in the presence of saturating amounts (30 μM) of small molecule activator. The mutations which have the most severe effects on the activator binding site (V455M and Y214C) are those which are least susceptible to further activation by the GKA. However, for the mutations involved in domain-domain interactions, activation factors of approximately fifteenfold (D158A) and fortyfold (W99R) were observed. In these two mutants, the activator binding site must be present and functional. These results showing synergy between some activating mutations

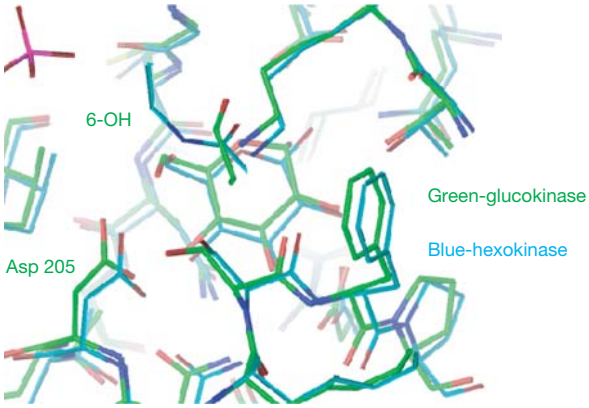

Fig. 4. Comparison of GK and HK crystal structures, showing the glucose-binding site of GK superposed on the glucose-binding site of HK I. The hydroxyl group of glucose which becomes phosphorylated is labeled.

and the small molecule activators lend support to the idea that there are multiple mechanisms of stabilizing a more active conformation of GK.

Comparison of the GK and Hexokinase I Structures

The GK structure with glucose bound shows the glucose binding site is nearly identical to that of hexokinase I (fig. 4). The residues involved in direct interactions with glucose are conserved. There are no significant differences in the interactions with glucose that would explain the comparatively high $S_{[0.5]}$ value of GK for glucose. Although it is possible a higher resolution structural analysis would reveal differences between GK and HK I at the glucose-binding site, we favor another explanation for GK's higher $S_{[0.5]}$. The GKA site on GK is not present in HK I, and the GKA site exposes several hydrophobic residues to solvent in the glucose-bound form of GK observed in the crystal structures. If the domain closure associated with glucose binding were to expose the hydrophobic residues in the GKA site to solvent, then glucose binding would be associated with an energetic penalty and a high $S_{[0.5]}$ value would be the result. Model-building studies support the suggestion that the residues in the GKA site are not exposed to solvent prior to glucose binding, and work in other systems has shown that differential exposure of hydrophobic residues upon ligand binding can have a strong influence on the energetics of binding [19, 27].

Glucose-6-Phosphate Sensitivity

In contrast to HK I and the HK from *Schistosoma mansoni*, GK is insensitive to inhibition by G-6-P. G-6-P is a competitive inhibitor with respect to

ATP for HKs, and its mode of binding to HK I has been visualized in the crystal structure of HK I complexed with both glucose and G-6-P. A comparison of the GK and HK structures shows there are significant differences in the area where G-6-P binds (the Protein Data Bank entries for the HKs are 1BG3 and 1BDG). In GK, a salt-bridge between Arg85 and Glu443 occludes part of the G-6-P-binding site. Glu443 aligns structurally with a glycine in HK I and the HK from *S. mansoni*, which precludes formation of a salt bridge to the conserved Arg residue. Glu443 also organizes the start of the α-helix spanning residues 443 to 461 in GK, via formation of a hydrogen-bond between the side-chain carboxylate and the backbone NH of Gly446. The corresponding α-helix in the HKs starts two residues later in the sequence, and the early start of the α-helix in GK places Ser445 at the beginning of the helix in a position where steric clash with G-6-P would occur. Hence small, local differences in the GK and HK structures in the vicinity of the G-6-P binding site explain the differential sensitivity of the enzymes to inhibition by G-6-P. Replacement of Glu443 with a smaller residue such as alanine via site-directed mutagenesis could increase the sensitivity of GK to G-6-P inhibition given the high degree of sequence conservation of the other residues involved in G-6-P binding.

Discussion

The glucose binding sites of hexokinases and glucokinase appear identical. The obvious structural difference in comparing GK with hexokinases is the presence in GK of a hydrophobic allosteric activator binding site. As yet, there are no experimentally determined structures of GK available in which the glucose-binding site is accessible from solvent, representing a form of GK early in the catalytic cycle prior to glucose binding or late in the cycle at the step of G-6-P release. A model of GK based upon an open form of the yeast hexokinase B inhibited with *o*-toluyl glucosamine has been published and is available from the Protein Data Bank as entry 1 glk [19]. In a structural alignment, 243 residues of the model can be superimposed with the corresponding residues from the X-ray structure with an rmsd of 1.2 Å. The superposition aligns the large domains reasonably well but leaves the small domains displaced from one another. Interestingly, in the model, the loop which forms the top of the allosteric activator binding site and the C-terminal α-helix which forms one wall of the site have both moved, essentially collapsing into the allosteric site. The space occupied by the thiazolyl and pyridinyl rings of the small-molecule activator in the crystal structure is not accessible in the model of the open form of GK. The model places Pro66 of the connecting loop in contact with the side-chain of Tyr214, consistent with the suggestion that binding of glucose and closure of

the domains exposes the non-polar surface of the Tyr214 side-chain to solvent. The penalty for exposing hydrophobic surfaces enters into the binding equilibrium constant as an entropic penalty. Interestingly, a thermodynamic study of glucose binding to yeast HK has shown that glucose binding is entropy driven [28]. As glucose binds to HK a number of water molecules are expelled from the active site, and freeing these waters results in an entropic gain. Further study of the energetics of glucose binding to GK and the activating mutants could prove rewarding. The crystal structure of GK thus helps us understand this fascinating enzyme while at the same time posing new questions to be answered.

Methods

Structural analysis

Detailed procedures for expression, purification, and crystallization of GK have been described [23]. Briefly, GK was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli*. After purification of the GST fusion protein on glutathione-Sepharose, the GST fusion tag can be removed with factor Xa protease, which also removes five residues from the N-terminus of GK. The tag was removed prior to crystallization of GK. Enzyme assays were performed with GST-GK fusion proteins. The crystal structure was solved via MAD phasing with Se-Met rather than by using the molecular replacement (MR) technique starting from one of the known hexokinase structures. Subsequent trials of MR with hexokinase I (pdb entry 1HKC residues 16–459) yielded a solution, but following this route would have made interpretation of the activator binding site problematic. The structure was solved at 2.7 Å with data measured at Brookhaven beamline X8C. The program SnB located the Se atoms [24]. The structural model includes GK residues 14–157. Although the activator present during the crystallization was a racemic mixture of R and S stereoisomers, the best fit to the observed electron density is with the R isomer.

Kinetic Analysis

The activity assays of WT GK and the mutants were performed as described in [7].

Summary

The 2.7 Å crystal structure of human liver up GK is reported together with an analysis of the known activating mutations of GK. The structure provides a snapshot of GK bound to glucose and one of the recently described small molecule GK activators. The GK activator

binds to a newly discovered allosteric site 20 Å away from the bound glucose. Several activating mutations map to the GKA-binding site, highlighting again the importance of allosteric regulation for GK activity.

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Molecular Biology of Glucokinase Regulation

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The single most notable feature in the molecular biology of the glucokinase gene is the presence of two promoters. The identification of two promoters in the glucokinase gene arose from the discovery of glucokinase mRNAs with different 5′ ends in rat liver and pancreatic islets of Langerhans [1, 2]. The glucokinase mRNA from the islets was traced to the promoter at the 5′ end of the locus, and the mRNA in liver was traced to the downstream promoter [2]. Each tissue was shown to contain only one mRNA type, suggesting that the alternative promoters were active in a mutually exclusive manner. The two promoters are separated by a very long intervening sequence of 30, 35 and 27 kbp in the human, mouse and rat genes, respectively. The identification of two widely separated promoters led to the hypothesis that transcription from the two promoters would be governed by distinct sets of tissue-specific regulatory factors present either in pancreatic endocrine cells or in hepatocytes. The second prediction was that the dual promoter arrangement could provide a molecular basis for differential ontogeny and hormonal regulation of glucokinase expression in liver and islets of Langerhans. In general, these predictions have been validated experimentally, although it is now realized that many transactivators that were once regarded as ‘cell-type’ specific, for example ‘hepatocyte nuclear factors’ (HNFs), have indeed a rather broad tissue distribution. The purpose of this chapter is to summarize our current knowledge on gene regulatory regions, transcription factors and hormonal effectors that control the expression of glucokinase in various cell-types.

Transcriptional Regulation of Glucokinase in Endocrine and Neural Cells

Regulatory Region of Neuroendocrine Promoter

The first step in the functional characterization of the upstream promoter in the rat gene was the finding from transient transfection experiments that cis-acting elements within a short DNA segment of 300 bp flanking the transcriptional start sites were capable to elicit the expression of a reporter gene preferentially in insulinoma cells compared to fibroblasts [3]. This finding led Magnuson and collaborators to examine whether this short promoter fragment would drive transcription of a reporter gene, in this case the structural gene for human growth hormone (hGH), in restricted tissues of transgenic mice [4]. As could be anticipated, transgene expression was readily demonstrated in β-cells of the islets of Langerhans. More provocative, however, was the expression of the hGH transgene in a number of additional cell types for which no evidence of glucokinase mRNA and protein expression based on conventional Northern [1] and Western blotting [5] had been found. These cell types included α and δ cells of the pancreatic islets, as well as some pancreatic duct cells. Outside of the pancreas, positive cells were noted among enteroendocrine cells of the stomach and small intestine, accessory cells of the thyroid gland, rare epithelial cells of the respiratory tract and, last but not least, neurons of the hypothalamus [4]. Corticotrope cells of the anterior pituitary were also positive, in accordance with the previous identification of islet-type glucokinase mRNA in pituitary tumor cells [6]. In pituitary cells, glucokinase mRNA had been shown to be alternatively spliced and not to encode full-length glucokinase [7].

The results obtained in transgenic mice suggested, but did not prove, that the upstream glucokinase promoter could be active in a wider range of cell types than had been previously realized. A careful search for authentic glucokinase mRNA and protein in these cell types led to the unambiguous detection by in situ hybridization and RT-PCR of endogenous glucokinase mRNA in hypothalamic neurons and enteroendocrine cells of stomach and intestine. Immunocytochemical assays confirmed the presence of glucokinase in these cell types [4]. Subsequently, glucokinase mRNA and protein were positively identified in α-cells of the islets as well [8]. The upstream promoter, previously designated as the β promoter, was renamed the neuroendocrine promoter after these findings. The implications of glucokinase in the CNS and in a variety of endocrine cells besides the β-cells for the physiology of glucose sensing are discussed elsewhere in this book.

Transacting Factors

Pdx-1. In transient transfection of insulinoma cells, the function of the neuroendocrine promoters from the rat and human glucokinase genes was dependent

on conserved DNA elements in the 300-bp proximal region [3, 9]. A conserved cis-acting element named UPE3 at −102 to −93 bp of the human gene (−104 to −95 bp in the rat gene) was shown to serve as binding site for the homeodomain transcription factor Pdx-1, a transactivator involved in the differentiation of the pancreas. Co-expression of Pdx-1 stimulated the reporter activity of a upstream glucokinase promoter/luciferase plasmid containing the UPE3 element in chinese hamster ovary (CHO) cells [10]. However, a role for Pdx-1 at the neuroendocrine glucokinase promoter has not been definitively proven. Conflicting results as to the binding of Pdx-1 to the promoter in the natural chromatin environment of the β-cell were obtained in different laboratories using chromatin immunoprecipitation (ChIP) assays [11, 12]. Moreover, the level of glucokinase appeared to be unaffected in the islets of mice with postnatal inactivation of the Pdx-1 gene, suggesting that Pdx-1 is dispensable for the maintenance of glucokinase gene transcription [13].

Beta2. A functional cis-acting element of the E box type was identified at positions −221 to −216 bp of the rat neuroendocrine promoter. Using the electrophoretic mobility shift assay (EMSA), the β-cell-enriched dimer Beta2/E47, belonging the basic helix-loop-helix (bHLH) class of factors, was shown to bind specifically to this E box. Co-transfection of expression plasmids for Beta2 and E47 in baby hamster kidney (BHK) cells stimulated the activity of a luciferase reporter plasmid containing repeats of the E box from the glucokinase neuroendocrine promoter [14]. Evidence for the binding of Beta2/E47 to the promoter in chromatin from MIN6 insulinoma cells was obtained by ChIP assay.

Other Factors of Pancreatic Differentiation. Recently, the ChIP assay was used to examine whether the neuroendocrine glucokinase promoter was occupied in insulinoma cells by factors of differentiation, which are known to transactivate the insulin and other genes expressed in β-cells. In addition to Pdx-1 and Beta2, positive signals were obtained for binding of Pax6 and Nkx2.2 [12]. Consistent with a direct or indirect role of Nkx2.2 at the glucokinase promoter, glucokinase immunoreactivity was markedly decreased in the pancreatic islets of Nkx2.2 knock-out mice at embryonic day 18.5 [15].

PPARγ. A putative binding site for the nuclear receptor PPARγ has been described just downstream of the neuroendocrine transcriptional start (+47 to +68 bp) in the rat gene. This sequence region is highly conserved at an equivalent position in the mouse and human glucokinase genes. Glucokinase promoter/luciferase plasmids with this site intact, but not with the site mutated, were activated by troglitazone and 9-cis retinoic acid in CV-1 monkey kidney cells and HIT-T15 hamster insulinoma cells co-transfected with expression plasmids for both PPAR-gamma and RXR. The physiologic significance of these findings was supported by showing that glucokinase mRNA, and to a

lesser extent, glucokinase activity were increased in mouse MIN-6 and rat INS-1 insulinoma cells cultured with troglitazone plus 9-cis retinoic acid [16].

Regulation by Hormones and Related Effectors

A number of hormonal agents were reported to regulate the level of glucokinase mRNA in pancreatic islets or insulinoma cells. These studies are reviewed in detail elsewhere in this book. Here, the evidence relating specifically to transcriptional activation at the neuroendocrine promoter is briefly discussed.

Cyclic AMP. In fetal rat islets maintained in culture, the addition of 8-bromo-cyclic AMP induced an approximately 2-fold increase in glucokinase mRNA. This was accompanied by a 50% increase in reporter activity of a 1,000 bp glucokinase neuroendocrine promoter plasmid, compatible with a transcriptional mechanism of induction. Glucokinase activity and mRNA levels were proportionately increased in bromo-cyclic AMP-treated islets, but surprisingly the time-course of enzyme activity exhibited essentially no lag with respect to the time-course of mRNA induction [17]. This apparent inconsistency would deserve clarification, as would the question to know if GLP-1, a major hormone acting via cyclic AMP in the β cell, would also induce glucokinase.

Glucose and Insulin. Experiments suggesting a transcriptional induction of glucokinase in rat islets and HIT insulinoma cells during brief exposure to high glucose were recently published [18]. The glucose effect was abolished when glucose-stimulated insulin secretion was prevented. Direct addition of exogenous insulin to the culture medium mimicked the glucose effect, suggesting that the response to glucose was mediated by an autocrine effect of released insulin. Insulin signaling was ascribed to protein kinase B (PKB, also named cAKT), based on co-transfection experiments with a glucokinase promoter reporter plasmid and effector plasmids for PKB or the upstream activating protein kinase PDK1 [18]. Because data for the actual levels of the overexpressed kinases, or for downstream phosphorylation of protein substrates are not available in this type of experiments, the evidence for signaling via PKB should be considered tentative at best. More generally, the reported effects of glucose or insulin on endogenous glucokinase gene transcription, mRNA accumulation, as well as on promoter-dependent reporter gene expression, were very small, making their detection itself something of a tour de force. Independent confirmation is required, especially in view of multiple evidence against a glucose effect on glucokinase gene expression in islets and insulinoma cells [see note 1 added in proofs].

Rat islets cultured for a few days at a high glucose concentration did exhibit increased glucokinase activity and protein level, but virtually without any change in glucokinase mRNA, suggesting an effect at the translational or protein stability levels [19]. Islets from the transgenic mice harboring the glucokinase promoter/hGH did not increase hGH production in response to glucose elevation [20].

No effects of elevated glucose concentration or insulin were apparent in MIN6 insulinoma cells expressing a luciferase reporter gene under the direction of the neuroendocrine glucokinase promoter [21]. In my laboratory, we could detect no effect of glucose on glucokinase gene transcription and mRNA in insulinoma INS-1 cells, whereas the L-type pyruvate kinase gene was strongly induced in the same cells [22]. Finally, and most importantly, glucokinase mRNA levels, and glucokinase enzyme assayed by quantitative immunoblotting, were unchanged in rat islets of Langerhans during the fasting-glucose refeeding transition [1]. In sharp contrast, a dramatic induction of glucokinase at the transcriptional level occurred in the livers of fasted-refed rats, as will be discussed below.

Transcriptional Regulation of Glucokinase in Hepatocytes

Regulatory Regions of Liver Promoter

Transcription at the downstream promoter in the glucokinase locus, termed the liver promoter, is known to occur at a significant level only in parenchymal cells of the liver. Thus, the activity of the liver promoter appears to be more stringently restricted than that of the neuroendocrine promoter. So far, experiments in transgenic mice have yielded little information on the control of glucokinase gene transcription in the liver. Transgenic mice with extra copies of the entire locus as a transgene displayed correctly initiated glucokinase mRNA in their livers. In addition, glucokinase mRNA of transgene origin was properly induced in liver during the fasting-refeeding transition, indicating the presence of physiologically relevant hormone-response elements within the 80 kb transgene [23]. However, the transgenesis approach has failed so far to pinpoint precise DNA regulatory region(s) that would confer liver specific expression to a reporter gene in the whole animal.

In transient transfection experiments, the proximal promoter region from nucleotides −123 to +79 bp with respect to the liver transcription start site in the rat gene was sufficient to drive luciferase gene expression preferentially in hepatocytes and hepatoma cells. An upstream genomic region, between −1,017 and −587 bp, increased luciferase activity about 3-fold, acting as a true enhancer in an orientation and, to some extent, position independent manner [24]. The enhancer was extremely cell selective for primary hepatocytes. No enhancer effect was noted in hepatoma cells, which are known not to express the glucokinase gene. When analyzed using the in vitro DNAse I protection assay, the enhancer fragment displayed seven protected DNA elements, at least three of which were selectively protected by nuclear extracts from rat liver. In addition, a DNAse I hypersensitive site mapping to the exact position of the enhancer was revealed in the nuclei of primary hepatocytes, but not in the

nuclei of other cell-types including hepatoma cells. The above data suggest an important role of the −1,017 to −587 bp enhancer for hepatic glucokinase expression. A DNA fragment with 75% sequence identity to the rat enhancer sequence is found in the human glucokinase gene, at an equivalent position in the locus. The human sequence acted as an enhancer in transfection experiments when placed upstream of the liver promoter of the rat gene [24].

Two additional DNAse I hypersensitive sites, mapping 2,300 bp upstream and 1,300 bp downstream of the liver promoter respectively in the rat gene, were specifically detected in primary hepatocytes. These sites were absent from nonhepatic cells and hepatoma cells which are deficient for glucokinase expression. Thus, they might mark areas of disturbed chromatin structure that are critical for liver specific glucokinase expression. However, because the inclusion of the cognate DNA regions in luciferase reporter constructs did not result in enhancement of promoter activity in transfected rat hepatocytes, their regulatory significance remains uncertain [24]. Likewise, the role of several other DNAse I hypersensitive sites at longer distance from the liver promoter in rat hepatoma or mouse liver nuclei has not been elucidated [25].

Transacting Factors

Hepatic Nuclear Factor 4-Alpha. Hepatic nuclear factor 4α (HNF4α) is an orphan nuclear receptor important in the transcription of genes for many regulatory enzymes of lipid and carbohydrate metabolism. In cultured primary hepatocytes, transfection of an expression vector for HNF4 resulted in increased luciferase activity driven by a −101 to +79 bp liver glucokinase/luciferase (GK-180 Luc) plasmid [26]. In this promoter fragment, an element from −54 to −35 bp termed P1 was footprinted by liver nuclear extract using the in vitro DNAse I protection assay [27]. This sequence element was shown by electrophoretic mobility shift assay (EMSA) to harbor a binding site (from −61 to −38 bp) for HNF4. Point mutations within this element which abolished binding of HNF4, also supressed the stimulation by HNF4 of reporter activity in the hepatocyte co-transfection system [26]. Together, the preceding data provide good evidence for an activator role of HNF4 at the liver glucokinase promoter.

Upstream Stimulatory Factor. Upstream stimulatory factors 1 and 2 (collectively USF) are members of the family of basic helix-loop-helix zipper (bHLHz) transactivators which have been incriminated in the transcriptional regulation of genes for regulatory enzymes of metabolism such as L-type pyruvate kinase and fatty acid synthase. The core binding site for bHLHz factors is designated the E box. A canonical E box sequence 5′-CACGTG-3′ is present at positions −87 to −82 bp of the rat liver promoter. This element is localized within a footprint termed P2 that was displayed in a DNAse I protection assay performed with nuclear extract from rat liver [26]. Binding of USF to this sequence was confirmed

by EMSA using nuclear extracts from rat liver or HepG2 human hepatoma cells. The equivalent human sequence containing the core 5′-CACATG-3′ was also capable of binding USF. In transient transfection of primary hepatocytes, the activity of the GK-180 Luc plasmid was reduced 50% when the E box was mutated. Co-expression of USF1 with the reporter plasmid in HepG2 hepatoma cells stimulated luciferase activity, dependent on an intact E box element.

The above findings indicate that USF can bind to and activate the liver glucokinase promoter. However, all bHLHz factors recognize very similar binding sequences on DNA and it is therefore possible that several of them could target the E box in the liver glucokinase promoter. Of particular interest is the sterol-response-element binding protein 1c (SREBP1c), a transcription factor of the bHLHz class which has been proposed as a major transcriptional regulator of liver glucokinase transcription (see other chapter in this volume) [28]. Whether SREBP1c binds to the E box in the liver glucokinase promoter, or to other unidentified binding site(s), has not yet been addressed [see notes 2 and 3 added in proofs].

Hepatocyte Nuclear Factor-6. The liver-enriched transactivator hepatocyte nuclear factor6 (HNF6), the prototype of the one-cut class of homeodomain transcriptional regulators, plays a role in the differentiation of the liver and pancreas and contributes to the transcriptional regulation of the hepatic gluconeogenic enzymes PEPCK and glucose-6-phosphatase. A DNA element capable of binding HNF6 has been identified at positions −877 to −868 bp upstream of the liver start of transcription in the mouse gene. This element is conserved at 9 out of 10 positions in the rat gene and 8 of 10 positions in the human gene, and maps close to a footprinted area within the hepatocyte-specific enhancer discussed above. Experimental evidence from transfection experiments using FTO-2B rat hepatoma cells and knock-out mice homozygous for HNF6 inactivation is compatible with a role for HNF6 as a transactivator of liver glucokinase transcription [29] (see chapter by Postic et al., in this book).

Signal Transducer and Activator of Transcription 5. The signal transducers and activators of transcription (STAT) are signaling phosphoproteins that, in response to the binding of cytokines and other polypeptide hormones to cell membrane receptors, migrate as dimers to the nucleus and transactivate specific target genes. A putative cis-acting element for STAT5 has been identified by EMSA at nucleotides −1,368 to −1,360 bp upstream of the liver start site of transcription in the human glucokinase gene. Following transfection of a liver glucokinase promoter/luciferase plasmid containing this element into HepG2 cells, a small increase in luciferase expression was noted in the presence of insulin [30]. However, luciferase activity in these experiments was barely above the background seen with a promoterless plasmid. Furthermore, a STAT5 binding element is not conserved at equivalent positions in the mouse and rat genes.

Finally, we recently showed that STAT5 activation was neither necessary nor sufficient for transcriptional induction of the endogenous glucokinase gene by insulin in primary cultures of hepatocytes [31].

Hormonal Regulation

Nutritional and Hormonal Effects in the Whole Animal. During ontogeny in the rat, glucokinase is present in fetal islets of Langerhans at the end of gestation [32], whereas glucokinase mRNA and protein do not appear in the liver until weaning time, concomitant with a nutritional shift from a fat-rich to a carbohydrate-rich diet [33]. In adult life, glucokinase mRNA was undetectable in the livers of fasted rats and briskly accumulated upon glucose ingestion [33]. Similarly, glucokinase mRNA was absent from the livers of rats with severe streptozotocin diabetes and rose rapidly following insulin injection [34]. Compared to the mRNA level in healthy animals with unrestricted access to food, a massive overshoot of the mRNA occurred both in the glucose refeeding and the insulin administration situations. Using nuclear run-on assays, the induction of glucokinase mRNA was shown to result from stimulated gene transcription. The induction of liver glucokinase gene transcription during the fasting-refeeding transition was blunted in hypothyroid rats and restored to normal after short-term treatment with triiodothyronine (T3) [35].

Insulin Action in Cultured Hepatocytes. As previously mentioned, lines of hepatoma cells established in culture do not express the glucokinase gene. Therefore, studies on the regulation of liver glucokinase gene expression at the cellular level had to be carried out using primary cultures of hepatocytes. When nanomolar concentrations of insulin were added to rat hepatocytes that had been cultured overnight in hormone-free medium, the cellular amount of glucokinase mRNA rose 20- to 30-fold above the starting level in 6–8 h and remained at a plateau thereafter. Induction was at the transcriptional level, as assessed by nuclear run-on assay. Insulin was active in the virtual absence of extracellular glucose, indicating that the build-up of an intracellular glucose metabolite was unlikely to account for the insulin effect [36]. The effect of insulin was suppressed in hepatocytes challenged with inhibitors of protein synthesis prior to insulin [37]. Whether the inhibitors of protein synthesis acted by blocking the synthesis of a short-lived protein involved at some step of the transcriptional induction mechanism, or whether they acted via activation of a c-Jun NH2-terminal kinase/p38 MAP kinase [38] that would antagonize insulin signaling, has not been determined. A robust induction of glucokinase mRNA by insulin was also noted in cultured human hepatocytes [39].

The extent of induction by submaximal concentrations of insulin was found to be inversely related to the partial pressure of O_2 during culture. Stronger induction of glucokinase mRNA occurred in rat hepatocytes cultured in an

atmosphere of 8% O_2, mimicking venous blood conditions, than in 16% O_2 like in arterial blood [40]. The effect of relative hypoxia in hepatocyte culture is interesting in relation to the heterogeneous distribution of glucokinase in the intact liver: higher levels of enzyme are known to occur in the perivenous area than in the periportal area of the hepatic lobule. More generally, the decreasing oxygen tension from the periportal to the perivenous region has been regarded as a crucial factor underlying the 'metabolic zonation' of hormone receptors, enzymes and transcriptional regulators in the liver [41].

After insulin binds to its receptor at the plasma membrane, two major signaling modules are activated in the cell: the classical extracellular signal regulated kinases (ERK1/2) cascade, and the phosphoinositide 3-kinase (PI3-K) cascade. The inhibitors of PI3-K, wortmannin and LY294002, completely prevented the insulin-dependent build-up of glucokinase mRNA in cultured hepatocytes. Thus, activation of PI3-K is a requisite for insulin induction of hepatic glucokinase [42]. Downstream of PI3-K, the protein kinase termed mammalian target of rapamycin (mTOR) appeared to mediate part of the insulin effect, since the glucokinase mRNA response was blunted by approximately 50% in presence of the inhibitor rapamycin. Finally, the MEK inhibitor PD98059, which interferes with the activation of ERK1/2 kinase cascade, reduced the extent of glucokinase mRNA induction by 50%, suggesting that full induction requires the participation of the ERK1/2 cascade [42].

The 3-phosphoinositides synthesized at the plasma membrane following activation of PI3-K are responsible for the activation of protein kinase B (PKB), also known as cAKT, and other related kinases. Protein kinase B, either directly or via downstream kinases such as mTOR, is thought to mediate at least some of the metabolic effects of insulin. To address the question of a possible role for PKB in insulin induction of hepatic glucokinase, we made use of an estrogen receptor-PKB chimeric protein that is activated on tamoxifen binding. Specifically, rat hepatocytes in culture were transduced with an adenoviral vector encoding the chimeric PKB protein and subsequently challenged with tamoxifen. The addition of this ligand led to an increase in cellular PKB activity, as evidenced by phosphorylation of downstream substrates such as glycogen synthase kinase 3β (GSK3β), and to glucokinase mRNA induction, both types of effects mimicking the action of insulin [42]. Thus, acute activation of PKB by non-natural means was sufficient to elicit an insulin-like effect on glucokinase expression.

Studies in other cell and gene systems have identified a number of transactivators whose activity is altered, directly or indirectly, following stimulation of PKB. Among these transactivators, the factor termed FOXO1 has several features which make it an interesting potential candidate for the insulin regulation of liver glucokinase transcription. First, FOXO1 is a substrate for direct phosphorylation by PKB. Second, phosphorylation of FOXO1 by PKB results in

well-documented physiological effects, namely nuclear export and cytoplasmic retention of the factor [43]. Third, PKB-induced nuclear exclusion of FOXO1 appears to play a crucial role in the negative effect of insulin on transcription of the gene for the catalytic subunit of glucose 6-phosphatase [44]. Finally, and most interestingly, FOXO1 can function not only as transactivator, but also as co-repressor when associated with transactivators such as the glucocorticoid receptor and other nuclear receptors [see note 4 added in proofs] [45]. If FOXO1 acted as co-repressor at the liver glucokinase promoter, insulin-induced nuclear exclusion of FOXO1 could induce the gene by relieving co-repression.

Recently, glucokinase mRNA induction by insulin was found to be unimpaired in hepatocytes expressing a 'dominant negative' mutant of PKB [46]. How can we reconcile the studies using the tamoxifen-activatable form of PKB on the one hand and the 'dominant negative' mutant of PKB on the other? For one thing, insulin-dependent activation of PKB might have been only partially blunted in the cells expressing 'dominant negative' PKB. In our hands, various interfering mutants of PKB proved very inefficient at blocking insulin-stimulated PKB signaling in primary hepatocytes [see note 5 added in proofs]. Alternatively, it could be that, downstream of PI3-K, a protein kinase distinct from, and to some extent redundant with, PKB might act to induce the glucokinase gene. Further investigations are required to resolve this issue.

Glucocorticoids and Thyroid Hormones. A requisite for the induction of hepatic glucokinase mRNA by insulin in primary cultures of hepatocytes is the presence of a glucocorticoid such as dexamethasone [36]. The glucocorticoid effect is permissive [47], in that dexamethasone by itself did not induce glucokinase mRNA. In cultures of newborn rat hepatocytes, the thyroid hormone T3, as well as retinoic acid, were shown to trigger de novo accumulation of glucokinase mRNA [48]. Insulin and T3 had additive effects in this system.

Glucagon. Glucagon and its second messenger cyclic AMP have a key negative role in the regulation of liver glucokinase. When hepatocytes were challenged with glucagon prior to the addition of a maximal concentration of insulin, the induction of glucokinase mRNA was inhibited [36]. The effect of insulin was likewise prevented by cell permeable analogs of cyclic AMP or inhibitors of cyclic AMP phosphodiesterase [37]. In hepatocytes actively transcribing the glucokinase gene during culture with insulin, the subsequent addition of cAMP repressed glucokinase gene transcription within 15 min, in spite of the continued presence of insulin. What is the role of such a stringent mechanism for gene repression? After the cessation of gene transcription, glucokinase mRNA decayed with a half-life of approximately 45 min [36]. Repression of the gene by glucagon would thus result in rapid cessation not only of glucokinase mRNA transcription, but also of glucokinase protein synthesis, thus minimizing energy expenditure at times of food restriction.

Concluding Remarks

Perhaps the most outstanding aspect of the molecular biology of the mammalian glucokinase gene is its transcriptional induction in the liver by insulin. Glucokinase induction can be viewed as the archetypal response of the hepatocyte to insulin, just as the translocation of GLUT4 to the plasma membrane is the archetypal insulin response in the adipocyte. If studies of insulin-stimulated glucose transport in the adipose cell teach us something, that is that multiple signaling events in diverse cascades cooperate to produce a physiological response [49]. Therefore, coordinated changes in the activity of multiple signaling pathways, impacting more than one transcriptional regulator, rather than the firing of a single linear cascade, are likely to be implicated in insulin induction of the liver glucokinase gene. Beyond the traditional protein kinase cascades, a possible link between insulin-dependent alterations in actin dynamics and hepatic glucokinase gene induction would deserve to be explored, given interesting precedents in the areas of insulin-stimulated glucose transport and transcriptional regulation in other systems [50].

Notes Added in Proof

1. A clonal line of MIN6 insulinoma cells was reported to respond to a rise in the medium glucose concentration from 3 to 30 m*M* by a somewhat variable, but significant increase in glucokinase mRNA level. This response was absent in cells in which the insulin receptor had been depleted by small interfering RNA, suggesting that the glucose effect on glucokinase mRNA could be secondary to the release of endogenous insulin acting via its receptor in an autocrine manner [51].

2. Another candidate factor for transactivation of the liver glucokinase promoter via the −87 bp E box is hypoxia-inducible factor-1α (HIF-1). This factor was shown to stimulate glucokinase promoter activity in hepatocytes, dependent on an intact sequence in the E box region. Stimulation by HIF-1 was enhanced by co-expression with HNF4 and the co-activator p300. It was suggested that a synergy between HIF-1, HNF4 and p300 might play a role in the stimulation of the liver glucokinase promoter by insulin [52].

3. Evidence for the binding of PPARγ-RXR to the rat liver glucokinase promoter between −116 and −104 bp was published recently. The regulation of endogenous glucokinase mRNA in hepatocytes by ligands for the above nuclear receptors was not convincingly established in this report [53].

4. Protein-protein interactions between HNF4 and FOXO1, and between PPARγ and FOXO1, have recently been demonstrated. In both cases, FOXO1 repressed the transcriptional stimulatory activity of the nuclear receptor transactivators [54, 55].

5. The ability of the sphingolipid ceramide to inhibit insulin-induced activation PKB, and simultaneously glucokinase gene induction, in hepatocytes was shown in a recent paper from our laboratory. The same study showed that different forms of so-called ‘dominant negative’ mutants of PKB did not significantly inhibit insulin stimulation of PKB, or induction of glucokinase, in these cells [56].

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Role of the Transcription Factor Sterol Regulatory Element Binding Protein-1c in Hepatic Glucokinase Gene Regulation

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When a meal that contains carbohydrate is absorbed, it induces several metabolic events aimed at decreasing endogenous glucose production by the liver (glycogenolysis and gluconeogenesis) and increasing glucose uptake and storage in the form of glycogen in the liver and muscle. If glucose is delivered into the portal vein in large quantities and once the hepatic glycogen stores are repleted, glucose can be converted in the liver into lipids (lipogenesis) which are exported as VLDL and ultimately stored as triglycerides in adipose tissue. Regulation of the activity of enzymes of glucose utilization and production involves short-term effects on proteins but also long-term effects on their expression. Indeed, the expression of several key glycolytic and lipogenic enzymes is induced by a high-carbohydrate diet in the liver [1]. In vitro studies on cultured hepatocytes have shown that the transcription of *L*-pyruvate kinase, fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase genes require both an increased insulin and glucose concentration in order to be induced.

Regulation of Glucokinase Transcription

Hepatic and β-pancreatic glucokinases play a key role in glucose metabolism and β-cell insulin secretion as underlined by the diabetes mellitus associated to glucokinase mutations or by the consequences of tissue specific knock-outs [2–5]. In addition to short-term regulations which are described elsewhere in this book, glucokinase is regulated at a transcriptional level. Two different promoters direct glucokinase transcription in the liver or in pancreatic β-cells [5, 6]. The downstream promoter determines the expression of the hepatic GK. In the liver, glucokinase transcription is activated by insulin and is

repressed by glucagon via cAMP [6–8]. In contrast to the glycolytic/lipogenic genes mentioned above, a high glucose concentration is not necessary for the maximal induction of glucokinase. Transgenic mice containing extra copies of a mouse GK gene locus indicate that the region of the gene from −55 to +28 kb contains all the regulatory elements necessary to confer tissue specificity as well as hormonal regulation [9]. However, despite intensive studies, the *cis* acting sequences that determine hepatocyte-specific expression as well as insulin-responsive sequences have not yet been identified.

In the last years, important progresses have been made in the identification of the partners involved in the events following insulin binding to its receptor. In contrast, the factors involved in the transcriptional effects of insulin were until recently largely unknown. We will review here briefly the reasons why we think that the transcription factor sterol regulatory element binding protein (SREBP)-1c can fulfill this role for specific genes.

The SREBP Family of Transcription Factors

SREBP-1c belongs to a family of transcription factors originally involved in the regulation of genes by the cellular availability in cholesterol [10]. Three members of the SREBP family have been described in several mammalian species (mice, rat, hamster and human). SREBP-1a and SREBP-1c are encoded by a single gene through the use of alternative transcription start sites and differ by their first exon [11]. The other exons are common to both isoforms. In mice, the first exon of SREBP-1a is composed of 29 amino acids of which 8 are acidic whereas the first exon of SREBP-1c is composed of 5 amino acids of which only one is acidic. In vitro or in vivo experiments expressing identical levels of both isoforms have shown that SREBP-1a is a much more potent activator than SREBP-1c [12]. Another major difference between the 1a and 1c isoforms is their tissue distribution. SREBP-1c is mainly expressed in most of the tissues of mice and human with especially high levels in the liver, white adipose tissue, adrenal gland and brain [13]. SREBP-1c is also expressed in various muscles in adult rats and humans at appreciable levels [14, 15]. By contrast, SREBP-1a is mainly expressed in cell lines and in tissues with a high capacity of cell proliferation such as spleen and intestine [13]. The third member of the family, SREBP-2 is derived from a different gene and presents 50% homology with the SREBP-1 amino acid sequence. The three isoforms have a common structure: (i) an amino-terminal fragment of 480 amino acids which is in fact a transcription factor of the basic domain-helix loop helix, leucine zipper family, and (ii) a region of 80 amino acids containing two transmembrane domains separated by 31 amino acids and a regulatory C-terminal domain of 590 amino acids. These transcription factors are synthesized as a precursor form bound to the endoplasmic reticulum and nuclear membranes. Brown and Goldstein have

elegantly unraveled the mechanisms by which the transcriptionally active fragment of SREBP-2 and 1a is liberated [16–18]. When the concentration of cholesterol decreases in the membranes, the precursor form of SREBP-2 and SREBP-1a is increased through an enhanced gene transcription. Then this precursor form is cleaved by a complex mechanism involving two proteolytic cleavages catalyzed by two distinct proteases (S1P and S2P), a protein 'sensor' for the cholesterol concentration (SCAP) and an anchoring protein (INSIG). The mature form migrates inside the nucleus where it activates the promoter of genes involved in cholesterol uptake such as the low density lipoprotein receptor, or in cholesterol synthesis, such as the cytoplasmic hydroxymethylglutaryl-CoA synthase or the hydroxymethylglutaryl-CoA reductase. SREBP factors bind as homodimers both to E-boxes (5′-CANNTG-3′) and to sterol response elements (SRE) 5′-ATCACCCAC-3′, due to the presence of a tyrosine in place of an arginine (as in other members of the basic domain-helix loop helix family) in their basic domain [19].

SREBP-1c Expression Is Controlled by the Nutritional Environment in the Liver

In contrast to SREBP-2 and SREBP-1a, SREBP-1c expression and nuclear abundance is not increased in case of low cholesterol availability. A nutritional protocol aimed at increasing the demand for cholesterol induces in the hamster liver a clear-cut increase in the expression and nuclear abundance of SREBP-2, whereas SREBP-1c expression and nuclear abundance are rather decreased [20].

In fact, SREBP-1c expression and transcriptional effects seem to be related to carbohydrate and lipid metabolism. In mouse and rat liver, the expression and the presence of the mature form of SREBP-1c in nuclei are increased when starved animals are fed with a high carbohydrate diet [21]. In contrast, this experimental protocol has only a modest effect on SREBP-2 expression [21]. Several studies have shown that overexpression of SREBP-1c is concomitant with the induction of most of the lipogenesis-related genes such as fatty acid synthase, acetyl-CoA carboxylase, ATP-citrate lyase, glucose-6-phosphate dehydrogenase [22]. It must be pointed out that lipogenesis is a classical target of insulin stimulatory action.

SREBP-1c Expression Is Controlled by Insulin and Glucagon

The decisive argument allowing to involve SREBP-1c in insulin signalling comes from studies performed in primary cultured hepatocytes in which insulin is able to strongly activate SREBP-1c expression at concentrations compatible with an effect through the insulin receptor [23] (fig. 1a). This effect is transcriptional as shown by run-on assays. Glucagon which antagonizes the action of insulin on many metabolic hepatic processes opposes the effects of insulin

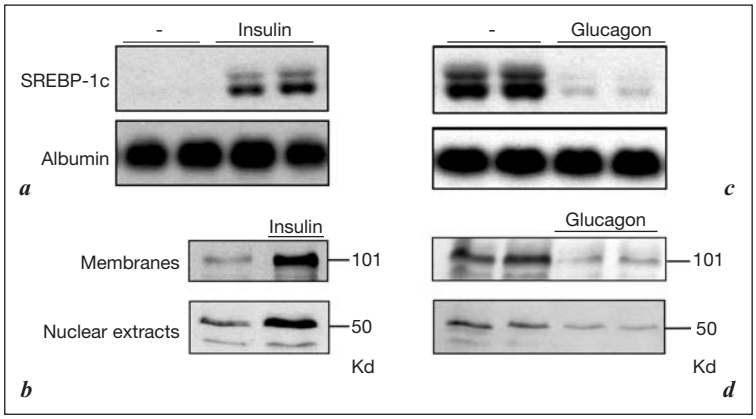

Fig. 1. Effects of insulin and glucagon on SREBP-1c mRNA and protein concentrations in rat hepatocytes. Rat hepatocytes were cultured in the absence or presence of 100 n*M* insulin for 6 h and mRNA of SREBP-1c and albumin analyzed by Northern-blot (**a**) or the abundance of the precursor and mature form of SREBP-1c protein determined by Western blot from, respectively, the membrane and nuclear protein fractions (**b**). Hepatocytes were cultured for 16 h in the presence of 100 n*M* insulin. Cells were then incubated for 24 h with 100 n*M* insulin in the absence or in the presence of 1 μ*M* glucagon and mRNA of SREBP-1c and albumin analyzed by Northern blot (**c**) or the abundance of the precursor and mature form of SREBP-1c protein determined by Western blot from, respectively, the membrane and nuclear protein fractions (**d**).

on SREBP-1c expression (fig. 1c) via its second messenger cAMP [23]. At the protein level, the induction of SREBP-1c expression by insulin is followed by an increase in the precursor form of SREBP-1c and a concomitant increase in the nuclear mature form [24] (fig. 1b). These effects of insulin on the SREBP-1c proteins are antagonized by glucagon (fig. 1d). The effect of insulin on SREBP-1c is corroborated by in vivo studies showing that SREBP-1c expression and nuclear abundance are low in the liver of diabetic rats and increase markedly after an insulin treatment [25]. The effect of insulin on the SREBP-1c transcript is highly specific in that no changes in levels of the mRNAs encoding SREBP-1a or SREBP-2 are detected [25].

SREBP-1c Is the Mediator of Insulin Action on the Hepatic Glucokinase Gene

The fact that insulin strongly activates SREBP-1c gene expression [23], whereas glucagon opposes the effect of insulin was consistent with the known effects of these hormones on the regulation of the glucokinase gene expression in the liver.

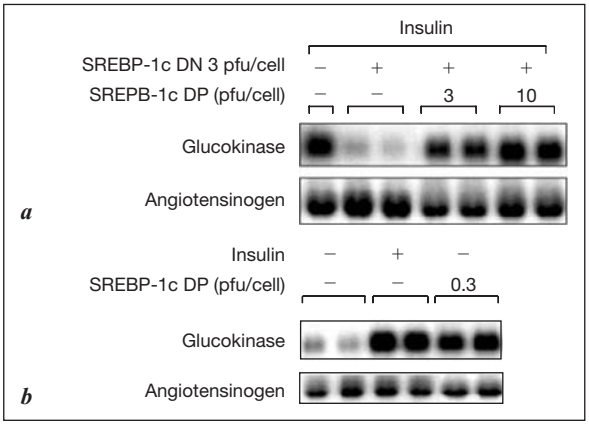

Fig. 2. Expression of the glucokinase gene can be manipulated by dominant negative and positive forms of SREBP-1c. ***a*** Rat hepatocytes were cultured in the presence or absence of 100 n*M* insulin either without adenovirus, or with the SREBP-1c DN adenovirus in the absence or presence of increasing titers of the SREBP-1c DP adenovirus. After 18 h, total RNAs were extracted and analyzed for the expression of glucokinase and angiotensinogen genes. ***b*** Rat hepatocytes were cultured with or without 100 n*M* insulin, or with the SREBP-1c-DP adenovirus. After 18 h, total RNAs were extracted and analyzed for the expression of glucokinase and angiotensinogen genes.

In order to involve SREBP-1c in the effects of insulin on hepatic glucokinase gene expression, two different forms of SREBP-1c were overexpressed in hepatocytes using an adenoviral vector: a dominant positive version of SREBP-1c (SREBP-1c DP) which corresponds to the mature nuclear form of SREBP-1c, directly imported into the nucleus and a dominant negative form of SREBP-1c (SREBP-1c DN) which is the mature form of SREBP-1c containing an alanine mutation at amino acid 320 in the basic domain. This mutation abolishes the binding of SREBP-1c to both SRE and E-boxes, but still allows dimerization leading to a decreased availability of endogenous SREBP-1c [19].

Rat hepatocytes cultured in the presence of insulin express glucokinase mRNA. Adenovirus-mediated expression of the SREBP-1c DN totally and selectively prevents the expression of glucokinase in the presence of insulin (fig. 2a). This impairment of glucokinase expression by the SREBP-1c DN is counteracted by the concomitant expression of the SREBP-1c DP showing that it was specifically due to the lack of transcriptionally active SREBP-1c (fig. 2a). This series of experiments demonstrates that the presence of an active form of SREBP-1c is necessary in the nucleus for glucokinase expression. If SREBP-1c is a major mediator of insulin action on glucokinase gene expression it should be possible to mimic the effect of insulin by expressing SREBP-1c DP in

hepatocyte nuclei. Indeed, hepatocytes infected with the adenovirus containing the SREBP-1c DP express glucokinase mRNA at a high level in the absence of insulin (fig. 2b). In other words, a transcriptionally active form of SREBP-1c mimics the effect of insulin on glucokinase gene expression.

In order to confirm the finding that SREBP-1c is involved in insulin action on glucokinase expression we took advantage of a physiological model in which transcription of the glucokinase gene occurs for the first time in rat liver, the suckling/weaning transition [26]. During the suckling period, which lasts about 2 weeks in rats, insulin concentrations are low and glucagon concentrations are high due to the ingestion of milk, which is a high-fat, low-carbohydrate diet and the glucokinase gene is not transcribed [27]. Glucokinase is expressed for the first time after transition from a milk diet to a high-carbohydrate diet after weaning, which is concomitant with an increase in insulin level. When insulin is added to cultured suckling rat hepatocytes it induces between 6 and 12 h the appearance of SREBP-1c mRNA [28]. Glucokinase mRNA is not detectable until a high expression of SREBP-1c is apparent, i.e. between 12 and 24 h after the addition of insulin. In order to functionally relate the appearance of SREBP-1c and glucokinase expression, the same experiment was repeated in the presence of SREBP-1c DN. In these conditions insulin induces the normal appearance of endogenous SREBP-1c mRNA but the appearance of glucokinase mRNA is markedly blunted [28].

The importance of SREBP-1c for the expression of the glucokinase gene was also demonstrated in vivo. In streptozotocin diabetic mice, adenovirus-mediated overexpression of SREBP-1c in the liver resulted in an increase of glucokinase gene expression and activity as well as an increase in the hepatic glycogen content [29], mimicing the effect of an insulin injection. A number of recent studies have involved mice with selective deficiencies in the SREBP system. In a mouse line deficient for the SREBP-1c isoform, glucokinase expression in fed mice is reduced by 60% [30]. Refeeding induces a normal increase in glucokinase expression. However, in these mice refeeding is also concomitant with an increase of both SREBP-1a and SREBP-2 isoforms (a feature which is absent in wild-type mice). When mouse livers lack all forms of nuclear SREBP (SCAP or S1P deficient mice) the response of glucokinase to refeeding is abolished [30]. SREBP-1c expression (but not SREBP-1a or SREBP-2) can be also increased in the liver by LXR agonists [31]. The endogenous LXR ligands are oxysterols which are metabolic derivatives from cholesterol [32]. When SREBP-1c expression and nuclear abundance are increased by an LXR agonist to an extent which is similar to the refed situation, glucokinase expression is increased 2- to 3-fold in wild-type mice but not in SREBP-1c null mice.

The bulk of these studies demonstrated that SREBP-1c is essential for glucokinase expression and that it is a mediator of insulin action on the hepatic gene expression.

Stoeckman et al. [33] in a study using an adenovirus expressing a doxycycline-inducible mature form of SREBP-1c were unable to demonstrate an effect of SREBP-1c on the expression of glucokinase. However, it must be pointed out that in this study on primary cultured hepatocytes, overexpression of the nuclear mature form of SREBP-1c was also unable to robustly activate lipogenic-related genes whereas this has been repeatedly shown in numerous studies (see [22] and [34] for reviews).

Insulin Signalling Pathway for SREBP-1c Expression

How does insulin stimulate SREBP-1c expression and thus glucokinase in the liver? This effect could be mediated through IRS-1 since animal models of insulin resistance presenting a reduced or absent expression of IRS-2 (ob/ob mice, lipodystrophic mice and $IRS2^{-/-}$ mice) are still able to strongly express SREBP-1c and its target genes [35]. In addition, inhibition of insulin-induced IRS-1 phosphorylation abolishes the induction of SREBP-1c gene expression by insulin in cultured rat hepatocytes [36]. Studies using inhibitors of various branches of the insulin signalling pathway have shown that the effect of insulin on SREBP-1c expression and synthesis involves mainly the PI-3-kinase pathway [24]. The identity of the downstream effector of PI-3-kinase is more controversial. On one hand it was shown that acute activation of PKB/Akt is sufficient to induce SREBP-1c mRNA accumulation in primary hepatocytes [37] whereas on another hand a dominant negative form of PKB/Akt promotes the accumulation of SREBP-1c mRNA [36] probably through an increased insulin-induced phosphorylation of IRS-1. It must be pointed out that in the studies in which insulin signalling was manipulated, a striking correlation between SREBP-1c and glucokinase expression was found [24, 36].

At present, the mechanism by which glucagon reduces the expression of SREBP-1c and thus of glucokinase is totally unknown except that it occurs through the usual cAMP system [23].

Insulin Action on the Mature Form of SREBP-1c

Insulin action on gene expression is a rapid process. For instance, the glucokinase gene transcription is activated by the hormone in less than one hour in adult rat hepatocytes [8, 38]. When comparing the timing of the insulin-induced expression of the glucokinase gene and of the nuclear form of SREBP-1c, it is clear that the former is detectable before the latter [24]. Although one cannot rule out a problem of sensitivity of the SREBP-1c Western-blot (detection of the endogenous SREBP-1c is a rather difficult task!) these results argue in favor of additional actions of insulin in order to explain its rapid effect on gene transcription. These effects could involve an activation of the residual amount of the mature form of SREBP-1c in the nucleus by a phosphorylation

or dephosphorylation process. A number of arguments indeed suggest that insulin could stimulate the mature form of SREBP-1c. When the mature form of SREBP-1c is overexpressed in 3T3-L1 adipocytes, its transcriptional activity is further enhanced by insulin [39]. It has been shown that in HepG2 cells, insulin stimulates the transcriptional activity of SREBP-2 and SREBP-1a by a mitogen-activated protein kinase (MAP kinase) pathway [40]. However, in cultured hepatocytes, inhibitors of the MAP kinase pathway do not antagonize the effect of insulin on SREBP-1c expression [24] as well as the effect of insulin on SREBP-1c-target genes [41] suggesting that SREBP-1c could be rather phosphorylated by downstream kinases of PI-3 kinase. Thus, although the main activating action of insulin on SREBP-1c is at the transcription level, additional regulations could exist at post-translational steps. Alternatively, in order to explain the rapid effects of insulin on glucokinase expression, insulin could act on a yet unidentified SREBP-1c partner.

Physiological Significance of the Regulation of the Glucokinase Gene by SREBP-1c

SREBP-1c is induced only in conditions of carbohydrate availability since it is strongly dependent upon high insulin concentrations. Activation of the SREBP-1c pathway will then increase glucokinase activity and thus glucose-6-phosphate synthesis. This will contribute to replenish the hepatic glycogen stores [42, 43], an important actor of body glucose homeostasis. Synthesis of glucose-6-phosphate will also activate carbohydrate-responsive transcription factors [1] which in conjunction with SREBP-1c will increase the lipogenic capacity by inducing the expression of lipogenic enzymes. Glucose carbons will then be transformed into lipids ultimately stored in adipose tissue. It is probably not a coincidence if SREBP-1c is also an adipocyte differentiation factor [44]. SREBP-1c can be then considered as a real 'thrifty' gene, i.e. a gene which has allowed the species survival in the context of successive episodes of food availability and restriction.

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Regulation of Hepatic Glucokinase Gene Expression

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Hepatic glucokinase (GK) was first discovered in liver [1] where its higher specificity and lower affinity for glucose distinguished it from three lower K_m hexokinases. Studies in the following 40 years since glucokinase was discovered have shown that the unique catalytic properties of the enzyme have important consequences for glucose homeostasis. This was clearly illustrated by the association of the GK gene mutations with early onset type 2 diabetes (MODY-2) [2].

While much was learned about the physiological role of hepatic GK, the mechanisms that determine its cell-specific expression in liver is complex and features of the gene have made several detailed studies difficult. Indeed, in contrast to the advances made in studying the β-cell expression of GK, studies of the hepatic promoter have been more problematic. At least two reasons seem to account for this lack of progress. First, although we have succeeded in cloning the entire mouse GK gene locus [3], the exact DNA sequences necessary for liver-specific expression and hormonal regulation of GK remains to be determined. Second, established liver cell lines do not express hepatic GK. Indeed, in most hepatoma cells the high K_m Glut2/glucokinase proteins are replaced by the ubiquitous low-K_m Glut1/hexokinase type 1 proteins. The loss of GK gene expression in these cell lines may be due to the loss of specific hepatic transcription factors like members of the hepatocyte nuclear factor (HNF) family [4, 5], as discussed below. However, in the mhAT3F hepatoma cell line, which expresses Glut2 but no GK, a high K_m hexokinase of 30 kD that shares some common characteristics with GK was identified [6].

This chapter will review the developmental and hormonal regulation of glucokinase in liver as well as some of the recent progress made on its transcriptional control.

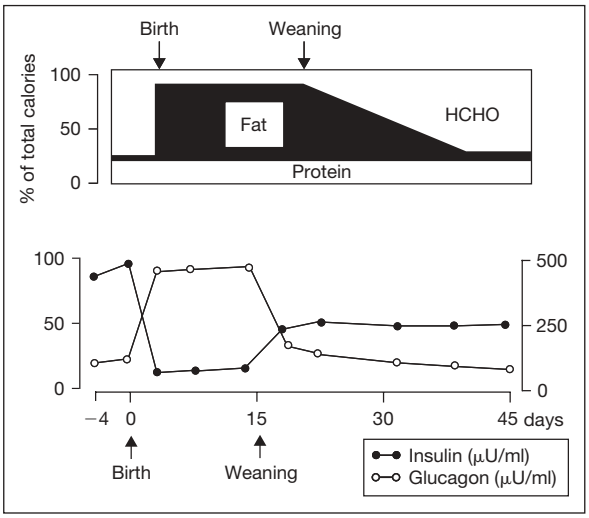

Fig. 1. Changes in nutrition and hormone concentrations during the perinatal period and the suckling-weaning transition. HCHO = High-carbohydrate diet.

Regulation of Hepatic GK during Development

Hepatic GK activity has been described as first appearing at the time of weaning (during the third postnatal week) in rats [7]. The transition from maternal milk (high-fat, low-carbohydrate diet) to laboratory chow (high-carbohydrate, low-fat diet) is accompanied by an increase in insulin and a decrease in glucagon concentrations, this change in the insulin/glucagon molar ratio is thought to be the GK inducing stimuli (fig. 1) [8]. We have been in the past 10 years particularly interested in determining the molecular mechanisms involved in hepatic GK gene expression at weaning. GK mRNA appears at approximately 15 days after birth in the rat, i.e. 24 h before GK activity can be measured, and it rises progressively to adult levels by the end of the weaning period, at about 30 days after birth (fig. 2). In order to determine the exact time course of the increase in GK mRNA that occurs after weaning to a high-carbohydrate diet, suckling rats were artificially weaned at 21 days of age to a high-carbohydrate, low-fat diet [8]. Since suckling rats begin to nibble the mother's food from 15 days of age, the mothers were fed a high-fat low-carbohydrate diet. Under these conditions, GK mRNA does not appear in liver of 15- and 21-day-old pups nibbling the high-fat diet of the mother. Abruptly weaning 21-day-old rats to a high-carbohydrate low-fat diet results in a rise of plasma insulin and a fall of plasma glucagon concentrations. GK mRNA that

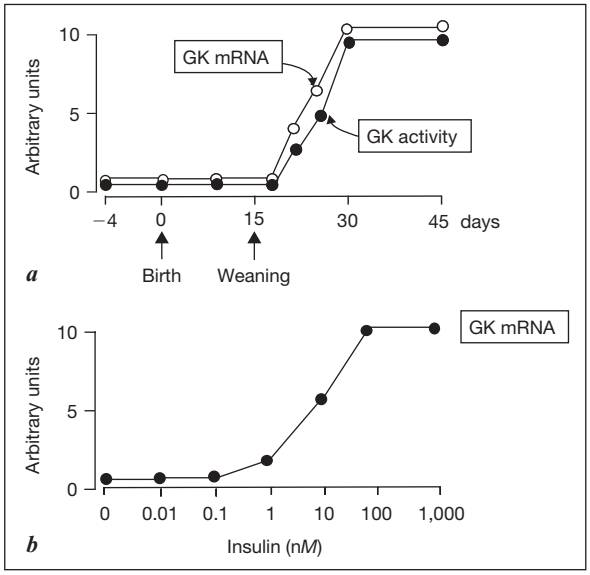

Fig. 2. ***a*** Hepatic glucokinase expression during the suckling-weaning transition in rats. During the suckling period, the expression of hepatic GK is low and gradually increases after weaning on a high-carbohydrate low fat diet. ***b*** Effect of insulin on GK gene expression in hepatocytes from suckling rats. Hepatocytes from 12-day-old suckling rats were isolated and cultured in the presence of increasing insulin concentrations. Insulin induces GK gene expression in a dose-dependent manner. Insulin is able to mimic changes observed at weaning for GK mRNA, showing that during this period, the hormone is crucial for the metabolic adaptations of glucose metabolism. Adapted from Girard et al. [8].

was undetectable before weaning markedly increases and reaches its maximal value within the first 24 h following weaning to a high-carbohydrate diet.

Factors responsible for the appearance of liver GK after weaning act by turning on the transcription of the gene. Indeed, GK gene transcription is dramatically increased one hour after carbohydrate feeding and its mRNA begins to accumulate from two hours onward [8]. The molecular mechanisms involved in the initial transcription of the GK gene in response to insulin are however not fully elucidated. Indeed, despite the presence of high plasma insulin concentration in fetal rat liver, GK mRNA is not detectable (fig. 1) [9]. Several experimental attempts had been made in the past to advance or delay the age at which GK activity first appears. Premature induction of GK activity to 30% of adult levels can be obtained by oral glucose administration to 13-day-old rats, and it is prevented by simultaneous injection of mannoheptulose which inhibited insulin secretion [10]. We showed that the GK gene transcription can be prematurely induced in vivo by modification of the hormonal environment.

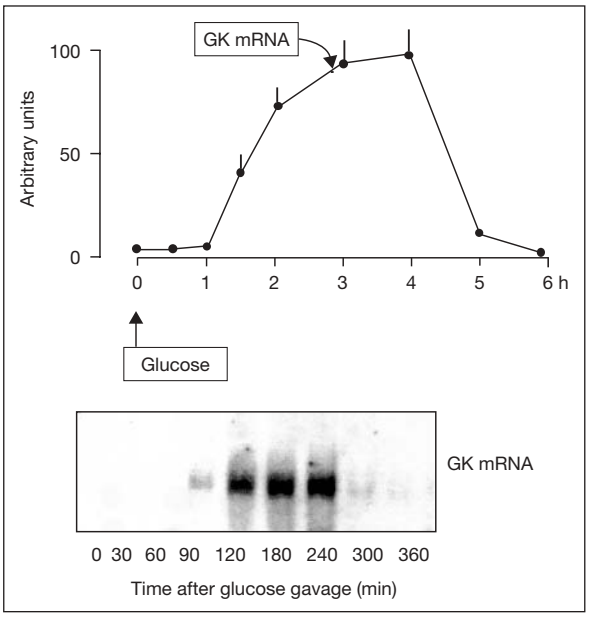

Fig. 3. Glucose administration induces the premature expression of liver GK in 10-day-old rats. Ten-day-old rats were force-fed with a *D*-glucose solution (5 g/kg body weight) via a gastric catheter. GK mRNA concentrations were then measured at different times following the glucose load. Adapted from Bossard et al. [9].

Oral administration of glucose to 1- to 10-day-old suckling rats causes an increase in plasma insulin levels and a fall in plasma glucagon concentrations allowing a rapid accumulation of hepatic GK mRNA, secondarily to a stimulation of the gene transcription (fig. 3) [9]. Similarly, when an increase in fetal plasma insulin is artificially induced with a glucose infusion for 36 h to late pregnant rats, GK expression becomes detectable in fetal liver. This level of detection is however low, 100-fold lower than that observed in liver of adult rats [9]. Studies of the chromatin structure of the GK gene by identification of DNase I hypersensitive sites (Hss) have indicated the presence of 5 liver-specific hypersensitive sites in the rat promoter region (fig. 5). These sites are already present in term fetus but the intensity of the two proximal sites located upstream of the transcription start site increases markedly at birth [9]. These sites, however, do not play a role in the regulation of GK gene expression by insulin [11]. The normal development of GK activity can also be delayed by various treatments that prevent the post-weaning increase of plasma insulin: weaning rats to carbohydrate-free, high-fat or high-protein diets [12]. The developmental regulation of hepatic GK has not been extensively studied in mouse. We showed

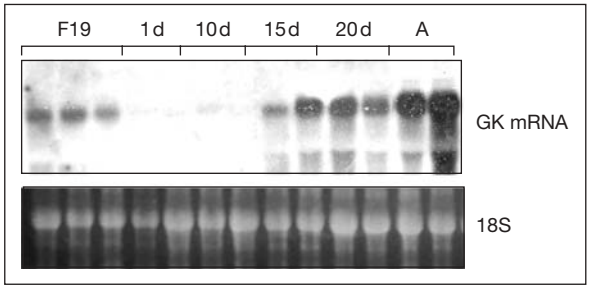

Fig. 4. Developmental expression of hepatic GK in mouse liver. F19 = 19-day-old mouse fetuses; 1–10–15–20 d = 1-, 10-, 15-, 20-day-old mice; A = 8-week-old mice fed on a high-carbohydrate low-fat diet.

that, in contrast to what is observed in the rat, GK expression is detected in liver of 19-day-old mouse fetuses (fig. 4). GK mRNA levels drop to undetectable levels during the suckling period to rise progressively to adult levels by the end of weaning (fig. 4). Liver-specific GK knockout mice (hGK-KO mice) are viable [13]. Interestingly, the cell-specific deletion of the GK gene by the albumin-cre transgene occurs progressively during development being only complete after weaning [14]. It is therefore possible that GK expression be intact in liver of hGK-KO fetuses. The fact that GK is expressed at such early stages of development in mouse liver is interesting and merits further studies.

Nutritional and Hormonal Regulation

Problems encountered in characterizing the expression and regulation of hepatic GK have stifled progress in understanding what is, undoubtedly, an important model of nutritional and hormonal regulation of gene transcription in liver. Hepatic GK activity is determined by both transcriptional and post-transcriptional regulatory mechanisms. The amount of functionally active GK is controlled at a post-transcriptional level by the binding of GK to a GK regulatory protein which determines both the activity [15] and the location of GK in hepatocyte [16]. Hepatic GK activity and mRNA levels are decreased by fasting and diabetes [17–19] and are restored by carbohydrate feeding or insulin treatment [17–19]. Studies have shown that insulin [18], triiodothyronine [20] and biotin [21] increase hepatic GK gene transcription whereas glucagon, acting via cyclic AMP (cAMP), decrease GK transcription [18]. The response to insulin is dramatic, a 20-fold increase in the rate of gene transcription that has been shown to occur within 45 min of treatment. Equally important is the dominant effect of glucagon or cAMP which is able to fully suppress the stimulatory effect of insulin.

We have used primary cultures of hepatocytes from newborn rats to study the de novo induction of GK mRNA by hormones [20]. Addition of insulin or triiodothyronine results in the induction of GK mRNA in a dose-dependent manner (fig. 2) [20]. Triiodothyronine acts only at the transcriptional level of the gene because no modification of GK mRNA stability is observed in the presence or in the absence of the hormone [22]. Dexamethasone alone does not induce GK mRNA but potentiates the response to insulin [20]. The permissive effect of glucocorticoids on insulin-induced GK mRNA accumulation in suckling rat hepatocytes could be due to the stabilizing effects of glucocorticoids on GK mRNA. We also showed that the induction of GK mRNA by insulin is not affected when the medium glucose concentration is varied between 5 and 15 m*M*, nor when culture is conducted in glucose-free medium supplemented with lactate and pyruvate or galactose [20]. Indeed, in contrast to other glycolytic genes such as *L*-pyruvate kinase or lipogenic genes such as fatty acid synthase or acetyl-CoA carboxylase that require both increased glucose and insulin concentrations in order to be induced [23], hepatic GK is exclusively stimulated by insulin.

An interesting finding is that a delay of 18–24 h is observed in the initial accumulation of GK mRNA in response to insulin in primary culture of hepatocytes from 12-day-old suckling rats [24]. Reexposure of these hepatocytes to the hormone allows a rapid accumulation (less than 8 h) of GK mRNA, as in adult rat hepatocytes [24]. This delay, that does not exist in hepatocytes from adult rats, is not due to elevated levels of cAMP or to a defect in insulin signaling. In contrast, it is markedly dependent upon the fact that GK has already been expressed in vivo or not. Hepatocytes from rats that have already expressed GK in vivo (forced-fed suckling rats for example) show no delay in their response to insulin in culture, whereas hepatocytes from rats that have never expressed GK in vivo (suckling rats) showed a delay of 24 hours. Inhibitors of protein synthesis (cycloheximide and puromycine) prevent the initial accumulation of GK in response to the first exposure to insulin but not the secondary accumulation of GK mRNA in response to reexposure to insulin. The synthesis of one or several insulin-dependent proteins is therefore necessary for the first activation of GK gene transcription in response to the first exposure to insulin [24]. The identity of such protein(s) is still unknown. In addition, despite the importance of insulin in hepatic gene regulation, and particularly on GK regulation, little is know about hepatic transcription factors that mediate insulin action.

Transcriptional Regulation

GK is encoded by a single gene in mice [3], rats [19] and humans [25] and contains two widely separated cell-specific promoters (fig. 5). Transcription of

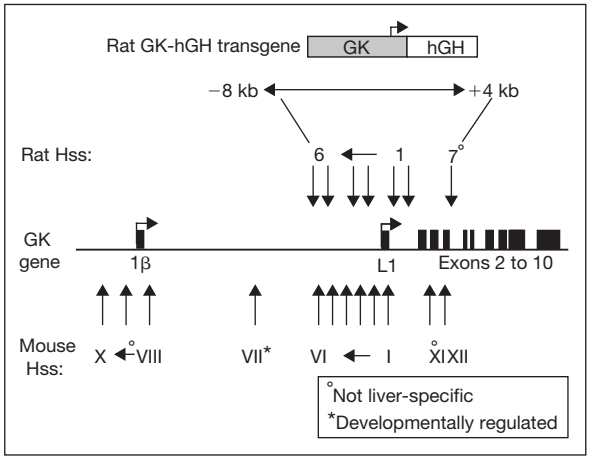

Fig. 5. Mouse GK gene locus. The exon organization of the mouse GK gene is shown (black boxes). The start site of the two major transcription units of the GK gene is indicated by 1β or L1. The DNase I hypersensitive sites identified in both the rat and the mouse GK genes are represented by arrows.

the GK gene is initiated in the liver at a liver-specific (downstream) promoter used uniquely in this tissue, whereas transcription in all extra-hepatic cells expressing GK is initiated at another promoter localized far upstream in the locus (fig. 5). The distance between these two promoters, that was previously estimated as being over 20 kb based on mapping data in the rat, is 35 kb in the mouse gene [3]. Using DNA probes derived from the mouse GK gene locus, we have examined the chromatin structure of a 48-kb region, containing both downstream and upstream promoters. Actively transcribed genes reside in domains of chromatin that have an 'open configuration' that can be detected by the increased sensitivity of surrounding DNA cleavage by DNase I hypersensitive sites. These domains mark sites where the chromatin is accessible to binding by nuclear factors that are necessary for transcription. The identification of DNase I hypersensitive sites is therefore essential to identify important control elements when they are located at large distances away from the transcription start site. We had first obtained information about the chromatin structure of the rat GK gene [9]. Five DNase I hypersensitive sites were identified in a region contained between −8 kb upstream and +4 kb downstream from the liver transcription start site, including the first exon (fig. 5). Four of the five hypersensitive sites identified were liver-specific but none of them were altered by changes in the nutritional and hormonal status [9]. Hss 1 and 2 were however not detected in hepatoma cells such as FAO and H4IIEC3

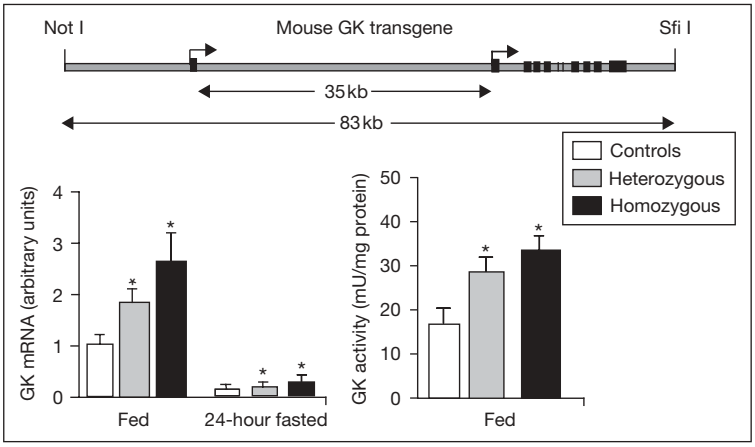

Fig. 6. Expression and regulation of the GK gene locus transgene in liver. A diagram of the mouse GK gene fragment used to generate transgenic mice is shown. Hepatic GK mRNA and activities were measured in either fed or 36 h fasted transgenic mice over-expressing the mouse GK transgene (line 37). Adapted from Niswender et al. [26].

cells, in which GK is not expressed. Two other liver-specific DNase I hypersensitive sites were later identified and located at 2.5 and 3.5 kb, respectively, upstream of the liver transcription start site [11]. These additional sites did not display an increased sensitivity to DNase I after exposure to insulin [11]. We also used a rat DNA fragment containing sequences from −7.5 to +18 kb (relative to the transcription start site in liver) linked to the human growth hormone (hGH) gene to generate transgenic mice [3] (fig. 5). Only 2 of 10 founder mice expressed the GK-hGH transgene in liver, suggesting that sequences necessary for liver-specific expression of GK lie outside of the 7.5-kb fragment tested. Unfortunately, sites upstream of −8 kb were not studied in the rat because of the lack the necessary genomic clones. In fact, attempts by several laboratories to clone this DNA region in the rat gene failed. The cloning of the mouse GK gene in 1995 defined the entire locus as well as provided DNA probes to all of the DNA that lies between the two promoter regions, in turn allowing twelve DNase I hypersensitive sites to be identified [3] (fig. 5). We also generated transgenic mice that expressed an 83-kb GK transgene containing the entire GK gene locus and showed that it was nutritionally regulated in liver (fig. 6) [26]. Moreover, we thought that knowledge of the exact mapping of all liver-specific DNase I hypersensitive sites in the GK gene would help locating the *cis*-acting factors necessary for liver-specific gene expression. Therefore, some of those newly identified liver-specific

hypersensitive sites in the mouse GK gene were examined for function in transgenic mice in the context of mini-gene constructs [Postic and Magnuson, unpubl. data]. Hss VI which lies 20 kb away from the liver transcription start site appeared to be developmentally regulated in liver, its expression being concomitant with the expression of GK at weaning [Decaux, unpubl. data]. Hss VI was cloned upstream of a minimal GK promoter fragment (from −1,000 to +18 bp) linked the β-galactosidase reporter gene. However, this transgene failed to be expressed in a liver specific manner in transgenic mice [Postic and Magnuson, unpubl. data].

Analysis of microsatellites within the mouse GK gene locus have revealed regulatory sequences predictive of either high or low hepatic GK gene expression [27]. Variations in hepatic GK activities in different strains of mice were first identified almost 30 years ago. Based on cross-breeding experiments in which a representative high GK activity strain was mated with a low activity strain and then crossed back to the parental strains, Coleman first suggested the existence of at least two different *Gk activity* alleles [28]. After finding no differences in the thermal stability, electrophoretic mobility, kinetic or antigenic properties of hepatic GK from representative high and low activity strains, James et al. [29] concluded that the *Gk activity* alleles were likely to be a regulatory locus that affected GK gene expression. We identified and analyzed microsatellites within the mouse GK gene and showed that variations in 3 simple sequence repeats were associated with differences in the amount of hepatic GK mRNA in different strains of mice [27]. This tetranucleotide repeats is located in the first exon of the hepatic transcription unit and lies close to Hss XI (fig. 5), suggesting the potential functional importance of this site in regulating GK expression in vivo. We are currently investigating the functional importance of Hss XI in both transient transfection assays and transgenic mice.

Role of Hepatocyte Nuclear Factors in Regulating GK Transcription

GK gene expression in the liver is controlled largely at the transcriptional level and depends upon specific DNA-protein interactions. Functional analysis of numerous hepatocyte-specific promoter and enhancer regions reveals that they are composed of multiple *cis*-acting DNA sequences that bind different families of hepatocyte nuclear factors. These include the HNF-1, HNF-3, HNF-4, CCAAT/enhancer binding protein (C/EBP), and HNF-6 transcription factor families [30]. Although none of these transcriptional regulatory proteins is entirely liver-specific, the requirement for combinatorial

protein interactions among them in order to achieve abundant transcriptional activity plays an important role in maintaining hepatocyte-specific gene expression.

Recent studies by Rausa et al. [31] have assessed the role of HNF-3β in hepatocyte-specific gene regulation. Elevated expression of HNF-3β in liver of transgenic mice leads to growth retardation, diminished liver glycogen storage and elevated levels serum bile acid concentration. A significant increased in postnatal expression of hepatic GK is observed in liver of 7-day-old HNF-3β transgenic mice, suggesting that GK transcription may be regulated by HNF-3β or by the HNF-3β transcription network. Interestingly, GK expression in liver of HNF-3β transgenic mice does not remain elevated throughout development. In fact, GK expression in liver of 6 week-old HNF-3β transgenic mice is similar to the one of wild type of the same age, suggesting that increased expression of HNF-3β leads to a premature and transitory expression of hepatic GK in liver of transgenic mice. Consistent with the possible involvement of HNF-3β in regulating GK gene expression, the human hepatic GK promoter contains a consensus HNF-3β binding site (AcTATTGACTgA) located between −797 and −784 bp relative to the transcription start site.

Examination of the mouse hepatic GK promoter also shows that it contains two putative binding sites for HNF-6. A proximal sequence (TGATCAATCG) is located on the sense strand at the position −877 to −868 bp and a distal on the antisense strand (−7,613/−7,622, AAATCAATAT), both being compatible with the 10-bp consensus for binding HNF-6 [32]. These sequences are highly conserved in the rat and the mouse suggesting their functional importance. HNF-6 binds to the hepatic GK promoter and stimulates the activity of a minimal mouse hepatic GK promoter (from −1,000 to +19 bp, containing the first putative HNF-6 binding site) in transiently transfected FTO-2B hepatoma cells [32]. An additive effect is observed when a fragment containing the second HNF-6 binding site (−7,800 to −6,000 bp) is added to the minimal promoter sequence. Although the inactivation of the *hnf6* gene in mice (hnf6$^{-/-}$) does not alter the pattern of DNase I hypersensitive sites in the mouse GK promoter it is associated with a 50% decrease in liver GK gene expression [32].

HNF-4α is important for glucose and lipid homeostasis since mutations in the HNF-4α gene cause maturity onset diabetes of the young (MODY-1). Hepatic GK gene expression was recently shown to be activated by HNF-4α via the sequence −52/−39 bp of the rat GK promoter [33]. HNF-4α was also shown to be involved in the transcriptional control of liver genes such as the carnitine palmitoylransferase I gene, e.g. by glucagon via cAMP [34]. In addition, the impairment in glucose utilization observed in MODY-1 patients could be due to a reduced hepatic GK expression due to defective HNF-4.

Transcriptional Regulation by Insulin

In liver, the transcription of the most the genes encoding metabolic enzymes is induced by insulin. In the last few years, although important progress has been made in the identification of the partners involved in the events following insulin binding to its receptor, factors involved in the transcriptional effects of insulin are largely unknown. Although insulin response elements (IREs) have been identified in some hepatic genes, it has been impossible until now to delineate an IRE on the hepatic GK promoter either by classical transfection experiments or by using transgenic mice. The recent finding that SREBP-1c, a transcription factor that can mediate the effect of insulin on the hepatic GK gene as well as on other hepatic insulin/glucose responsive genes [35], may help identify the IRE on the hepatic GK promoter. Indeed, it seems clear now at least for two lipogenic genes, fatty acid synthase [36, 37] and Spot 14 [38], that there is a co-localization of an IRE and a functional SREBP-1c binding sequence within their promoter sequence.

Concluding Remarks

Hepatic GK plays a key role in glucose metabolism as underlined by the diabetes mellitus associated with glucokinase mutations or the consequences of tissue-specific knock-outs [13]. As reviewed here, despite numerous efforts, the exact DNA sequences responsible for the liver-specific and hormonal regulation of the hepatic GK gene have not yet been determined. Classical transgenic approaches have failed in identifying sequences involved in the liver-specific expression of the hepatic GK gene, probably because key regulatory elements are located far upstream or far downstream of the transcription start site, as suggested by the presence of DNase I hypersensitive sites throughout the GK locus. Other approaches, in which the structure and/or the conformation of the endogenous GK gene locus is kept intact, such as knock-in experiments or point mutations of potential regulatory sequences, should help unravel the cell-specific and hormonal control of hepatic GK expression.

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Discovery and Role of Glucokinase Regulatory Protein

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Glucokinase is well known for its low affinity for glucose, its high sensitivity to this sugar and its lack of sensitivity to physiological concentrations of glucose-6-phosphate, which make it a unique glucose sensor. In addition to this, a remarkably conserved property of this enzyme is its sensitivity to inhibition by a ≈68-kD regulatory protein, which also serves to control the subcellular localization and the amount of glucokinase in liver. The purpose of this chapter is to review the discovery, the properties and the physiological role of this protein.

Discovery of the Regulatory Protein

Glucose phosphorylation in intact cells can be measured through the release of tritiated water from [2-^{3}H]glucose. This is because phosphoglucose isomerase partially exchanges the hydrogen bound to C_2 with protons from the medium. Using this technique, Bontemps et al. [1] first showed that the apparent affinity of glucokinase for glucose was about twofold lower in intact isolated hepatocytes than it was in cell-free extracts. Intriguingly, the affinity of glucokinase for glucose was increased in hepatocytes when the standard, Na^+-rich Krebs-Henseleit medium was replaced by a K^+-rich medium. Since glucose transport in hepatocytes is not rate-limiting, these effects indicated that glucokinase activity could be modulated on a short-term basis by something else than the glucose concentration.

Soon later, Clark et al. [2] reported that fructose, at a concentration of 2.5 m*M*, stimulated by two- to threefold the release of tritium from [2-^{3}H]glucose. Further investigations [3] showed that fructose had an effect analogous to that

of a K^+-rich medium, in that it increased the apparent affinity of glucokinase for glucose without changing V_{max}. Furthermore, fructose acted at quite low concentrations, a half-maximal effect being reached at ≈0.05 m*M*, and very rapidly, since its effect was maximal in less than 5 min. Furthermore, the effect was lost once the cells had been washed free of fructose, indicating its reversibility. Taken together these results suggested that glucokinase activity in isolated hepatocytes was inhibited competitively with respect to glucose and that fructose, or one of its metabolites, antagonized this inhibition. As stimulation of detritiation of [2-^{3}H]glucose was also observed with sorbitol and *D*-glyceraldehyde, but not with dihydroxyacetone, glycerol, lactate or pyruvate, it was tentatively concluded that the effect of fructose was mediated by fructose-1-phosphate (Fru-1-P) [3]. The latter is formed by phosphorylation of fructose by ketohexokinase and metabolized to *D*-glyceraldehyde and dihydroxyacetone-phosphate by aldolase B. It also transiently appears in the presence of *D*-glyceraldehyde through reversal of the aldolase B reaction [4].

Experiments with cell-free extracts showed indeed that Fru-1-P stimulated the phosphorylation of glucose in liver high-speed supernatants, but that it was devoid of effect on purified glucokinase [5]. Fractionation of liver extracts on an anion exchanger was found to separate glucokinase from a protein responsible for its sensitivity to Fru-1-P. This protein, termed *glucokinase regulatory protein* (*GKRP*), behaved as an inhibitor in the absence but not in the presence of Fru-1-P. The inhibition exerted by this protein was reinforced by fructose-6-phosphate (Fru-6-P), which acted competitively with respect to Fru-1-P. The concentrations of these two phosphate esters required to affect glucokinase kinetics were in the micromolar range, which pleaded for the physiological significance of these effects [5].

Sequence

GKRP was purified to homogeneity, identified as a ≈62-kD protein [6] and partially sequenced. Its cDNA was cloned and shown to encode a protein of 626 residues [7, 8]. Human [9] and *Xenopus* GCKR [10] share 89 and 57% sequence identity with rat GKRP. There is no close GKRP homolog in the genomes of *Drosophila*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. By contrast, many bacterial genomes encode a protein of ≈300 residues homologous to the N- and C-terminal half of GKRP, which is therefore the result of an ancient gene duplication event, followed by independent evolution of the N- and C-terminal halves. These show indeed a much lower degree of identity with each other than each of them shows with the homologous bacterial proteins [11]. The function of these bacterial proteins is presently unknown. The presence of

their gene in the same operon as a phosphotransferase and a penicillin-binding protein in some bacteria suggests that it is involved in the metabolism of a phosphate ester resulting from the degradation of the bacterial wall.

The gene encoding human GKRP contains 19 exons and is located on chromosome 2p23 at about ≈400 kb from the gene encoding ketohexokinase [12]. The presence of these two genes at a distance corresponding to about 0.015% of the whole genome size is probably coincidental, as the intervening genes have no evident functional relationship with GKRP or with ketohexokinase.

Distribution

Using an assay based on the inhibition of rat liver glucokinase, GKRP could be detected in the livers of all tested species that have hepatic glucokinase, i.e. rat, man, pig, dog, rabbit, turtle, frog and *Xenopus laevis*. Livers from species that do not express glucokinase such as cat and goat do not have detectable GKRP [13]. This is consistent with the concept that the two proteins form a functional unit. Of interest is the fact that the proteins from lower vertebrates, such as turtle, frog and *X. laevis* are unaffected by Fru-6-P and Fru-1-P.

GKRP appears in rat liver a few days before birth, and its concentration increases progressively during the first month of extrauterine life [13]. GKRP is therefore present in liver when glucokinase starts to appear at the third week, in keeping with the idea that it is necessary for the proper functioning of this enzyme. Diabetes and starvation, which are known to reduce markedly the expression of glucokinase, also cause a reduction in the concentration of GKRP, though the decrease is slower and smaller in relative terms than in the case of glucokinase [13]. Of interest is the fact that the molar GKRP/glucokinase ratio appears to be always higher than unity, and is about 3 in fed rats [14].

The glucokinase inhibition assay did not disclose the presence of GKRP in tissues other than liver [13]. Northern blots confirmed that the liver is the major site of GKRP expression, though a faint, but clearly detectable signal was also recorded in lungs [7, 15], a finding that was confirmed by RNAse protection assays and by western blotting [Veiga da Cunha, unpubl. results]. The presence of GKRP in neurons of the hypothalamus, mainly in the paraventricular nucleus, was detected by in situ hybridization and by immunohistochemistry [16]. The identity of the immunoreactive protein was confirmed by showing that it forms a complex with glucokinase in the presence of Fru-6-P but much less so in the presence of Fru-1-P.

The occurrence of GKRP in pancreatic islets was indicated by the findings that glucose phosphorylation is stimulated by Fru-1-P (by about 20%) in cell free extracts and that *D*-glyceraldehyde, which is partially converted to Fru-1-P,

exerts a modest stimulation on the detritation of [5-^{3}H] glucose [17]. Fructose exerts no detectable stimulation, possibly because ketohexokinase, though present in pancreatic islets [18], is much less active than in liver (about 1,000-fold less on a per weight basis). Though RT-PCR indicated the presence of GKRP mRNA in islets [19], this was not confirmed by Northern or Western blotting [15; Veiga da Cunha, unpubl. results]. Furthermore, the sensitivity of insulin secretion to glucose does not appear to be affected in GKRP$^{-/-}$ mice [20]. The physiological significance of GKRP in islets is therefore questionable.

Effects on Glucokinase Kinetics

The inhibition exerted by GKRP on glucokinase is competitive with respect to glucose. Dixon plots extrapolate to infinity, indicating that the glucokinase/GKRP complex is devoid of activity [21]. Elevated concentrations of GKRP tend to decrease the apparent cooperativity of glucokinase, as is observed with other competitive inhibitors [22].

The effects of GKRP are best observed at low salt concentrations and at a pH close to neutral. The customary use of assaying glucokinase at a higher pH (e.g. 8.0) and in the presence of high KCl concentrations is probably one of the reasons why GKRP escaped detection for so long. Anions release the inhibition exerted by GKRP with the following order of potency: acetate $<$ F^- $<$ Cl^- $<$ NO_3^- $<$ Br^- $<$ I^- $<$ inorganic phosphate [21]. Of note is the fact that at neutral pH, GKRP is about 5 times more potent as an inhibitor of glucokinase in the presence of a physiological intracellular concentration of Cl^- (25 m*M*) than with 100 m*M* KCl. This effect of Cl^- probably explains the stimulation exerted by a K^+-rich medium on glucokinase activity in intact hepatocytes [1]. The high [K^+] results in cell swelling and in a ≈3-fold increase in the intracellular concentration of chloride [23].

The inhibition exerted by GKRP is modulated by Fru-6-P and Fru-1-P, which act competitively with each other. In the presence of Fru-6-P, GKRP behaves as a partial inhibitor of glucokinase, the maximal inhibition reached being dependent on the concentration of GKRP [24]. It must be underlined that GKRP exerts some inhibition on glucokinase even in the absence of Fru-6-P. In the case of rat GKRP, a saturating concentration of this phosphate ester increases the apparent affinity of the glucokinase/GKRP complex by a factor of about 15 [5]. GKRP from guinea pig [Van Schaftingen, unpubl. results] is less dependent on Fru-6-P, whereas, as mentioned above, the protein from lower vertebrates is insensitive to this phosphate ester.

Fru-1-P suppresses the inhibition exerted by GKRP in the absence of Fru-6-P and antagonizes the effect of Fru-6-P [5, 24]. The competition

observed between these phosphate esters is due to the fact that they both bind to one single binding site onto GKRP [11]. Remarkably, the effects of low concentrations of Fru-1-P take minutes to appear at 30°C [6] and this is apparently due to slow binding of this phosphate ester to GKRP [25].

Graphical methods were developed to estimate the dissociation constant of GKRP for ligands that modify the inhibition [24]. Among a series of compounds that were tested, sorbitol-6-phosphate and its C2 epimer mannitol-6-phosphate were found to mimic the effect of Fru-6-P at 5- and 2.5-fold lower concentrations, respectively. This result suggests that Fru-6-P binds to GKRP in its open conformation. In marked contrast, allitol-6-phosphate and ribitol-5-phosphate, which have a D-configuration in C3, behave like Fru-1-P. This is true also for most other phosphate esters that have been tested including epimers of Fru-1-P, mannose 6-phosphate (though not glucose-6-phosphate), intermediates of the pentose-phosphate cycle and inorganic phosphate. Interestingly, two compounds, arabinose-5-phosphate and arabitol-5-phosphate display effects intermediate between those of Fru-6-P and Fru-1-P, i.e. they reinforce the inhibition exerted by GKRP in the absence of Fru-6-P, but partially suppress the inhibition exerted by Fru-6-P. These compounds most likely bind to GKRP in a conformation that is intermediate between the 'Fru-6-P conformation' and the 'Fru-1-P conformation' (see below). From a physiological standpoint, only Fru-6-P, Fru-1-P and possibly inorganic phosphate need to be considered.

Mechanism of Action

That GKRP forms a one-to-one complex with glucokinase in the presence of Fru-6-P, but not in the presence of Fru-1-P, was first demonstrated by ultracentrifugation experiments [6]. Binding of the two proteins to each other was confirmed with the yeast two-hybrid approach, as well as by coprecipitation experiments [26]. These results indicate that GKRP inhibits glucokinase by forming a complex with this enzyme, a conclusion that is consistent with kinetic data [6], including the effects of the concentration of GKRP on the apparent affinities for Fru-6-P and Fru-1-P [24].

Fru-6-P and Fru-1-P act by binding in a competitive way to GKRP with a stoichiometry of 1 mole per mole of protein [25]. The opposite effects that these phosphate esters exert are best explained by assuming that Fru-6-P and Fru-1-P bind to (or induce by binding) two different conformations of GKRP, only one of which is able to bind to glucokinase (fig. 1). Until now, no direct evidence has been provided for the existence of these two conformations.

The competition between glucose and GKRP indicate that the latter cannot bind simultaneously with the glucose to glucokinase. Intriguingly, competitive

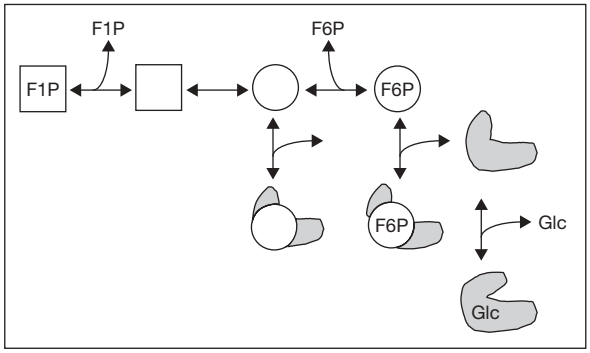

Fig. 1. Model for the action of the regulatory protein on glucokinase. Fructose-6-phosphate (F6P) and fructose-1-phosphate (F1P) bind to two differents conformations of GKRP symbolized by circles and squares. Only one of the two conformations is able to bind to glucokinase. This binding is competitive with glucose Glc.

inhibitors that, in all likelihood, bind to the catalytic site, show effects that are either independent (N-acetylglucosamine) or 'competitive' (mannoheptulose) with those of GKRP, indicating that the former, though not the latter binds to glucokinase simultaneously with GKRP [21, 27]. This paradoxical behavior is probably due to preferential binding of glucose and mannoheptulose on the one hand, and N-acetylglucosamine on the other hand, to different conformations of the enzyme. Yeast hexokinase, which has basically the same 3D structure as glucokinase [28] and other hexokinases, is indeed known to close its catalytic cleft when binding glucose [29] and this conformational change appears to be a prerequisite for catalysis. Closure of the catalytic cleft does probably not take place with N-acetylglucosamine, as indicated by studies on brain hexokinase [30], but must occur with glucosamine and mannoheptulose, which are substrates for glucokinase, though pour ones [31, 32]. It is therefore likely that GKRP can bind simultaneously with N-acetylglucosamine to glucokinase because both show preference for the same conformation of glucokinase.

Mutations of 'glucokinase-specific' residues in *X. laevis* glucokinase as well as the study of MODY human glucokinase mutants allowed the identification of amino acids playing a role in the binding of GKRP to glucokinase [33, 34]. When positioned in three-dimensional models of glucokinase [28], these residues cluster at the tip of the smaller domain and in the hinge region of the enzyme. Most of these residues have no function in substrate binding, and their conservation reflects therefore the importance of the role played by the interaction of GKRP with glucokinase. In addition to this, the glucose-binding residue Asn204 and the neighboring residue Val203 may also participate in the binding [34]. Additional residues are probably also involved in this binding as

indicated by a study of glucokinase/hexokinase II chimeras [35]. The location of the GKRP-binding site on the smaller domain and the hinge region of glucokinase is consistent with a mechanism whereby GKRP freezes this enzyme in an open conformation.

GKRP residues involved in binding Fru-6-P and Fru-1-P have been identified thanks to the distant homology of GKRP with the isomerase domain of glucosamine-6-phosphate synthase, a protein for which Fru-6-P is a substrate and for which the crystal structure has been solved [36]. Both proteins belong to the SIS (sugar isomerase) protein family [37] and contain two SIS domains between which the substrate or effector binding site is located. Mutations of three residues of rat GKRP predicted to interact with Fru-6-P resulted in proteins that were ≈5-fold (S110A) and 50-fold (S179A and K514A) less potent as inhibitors of glucokinase and had an at least 100-fold decreased affinity for both Fru-6-P and Fru-1-P [11]. These results indicate the presence of a common binding site for both effectors. Interestingly, another mutation (G107C) close to the putative Fru-6-P binding site resulted in a 20-fold increase in the affinity for Fru-6-P and a 40-fold decrease in the affinity for Fru-1-P. This indicates that a point mutation of GKRP can result in a protein variant acting as a 'super-inhibitor' of glucokinase.

Some progress has also been made in the identification of the glucokinase-binding site on GKRP. By using a peptide library displayed by M13 phages, Baltrusch et al. [38] identified a glucokinase-binding peptide (EYLSAIVAGPWP) showing 6 identities (underlined) with a conserved sequence in GKRP (SVGLSAPFVAGQMD). This region is close to one of the identified Fru-6-P-binding sites mentioned above. Interestingly, one of the mutations in this motif (S179A) resulted in a marked decrease in the affinity of GKRP for glucokinase (which may, however, partly result from a decreased affinity for Fru-6-P) [11].

Control of the Subcellular Localization of Glucokinase

Reversible translocation of glucokinase inside liver cells was first demonstrated using a digitonin permeabilization technique [39]. If hepatocytes that have been incubated in the presence of a low concentration of glucose (5 m*M*) are permeabilized with digitonin in a medium containing 5 m*M* $MgCl_2$, only minimal glucokinase release occurs. When in contrast the cells have been incubated in the presence of fructose, sorbitol, mannitol, an elevated concentration of glucose or mannoheptulose, a much larger release of glucokinase is observed. These effects are counteracted by ethanol, glycerol and glucosamine [39–41]. In the presence of $MgCl_2$, GKRP was also only minimally released

from hepatocytes by digitonin treatment, though with no apparent effect of pretreatment of the cells with fructose or an elevated glucose concentration [42].

Immunohistochemistry experiments showed that these changes correspond to a transfer of glucokinase from the nucleus to the cytoplasm and that GKRP is a nuclear protein [43, 44], leading first to the hypothesis that GKRP serves as an anchor that maintains glucokinase in the nucleus under conditions where the two proteins have strong affinities for each other (low glucose concentration, no fructose), but allows it to be transferred to the cytoplasm when dissociation of the complex is favored.

This dissociation is stimulated by compounds acting on GKRP (Fru-1-P), explaining the excellent correlation between the effects of fructose and sorbitol on glucokinase translocation and their ability to increase the Fru-1-P concentration [27]. It is also promoted by compounds that favor dissociation of the complex by binding to glucokinase, such as glucose and mannoheptulose. It should be mentioned that the effect of glucose appears to involve also an increase in the Fru-1-P concentration [27]. On the contrary, translocation of glucokinase is counteracted when the Fru-6-P analog, mannitol-1-phosphate, accumulates [45] or if GKRP is overexpressed [46] in hepatocytes. These findings point therefore to GKRP playing a critical role in the control of glucokinase translocation.

This role is further indicated by the observation that glucokinase mutants that have little or no affinity for GKRP do not concentrate in the nucleus when transfected in hepatocytes [47]. Furthermore, cotransfection of glucokinase or glucokinase mutants and GKRP in cells that do not naturally express GKRP (Hela cells, human embryonic kidney cells) led to the conclusion that the nuclear localization of glucokinase is critically dependent on the presence of GKRP and on the affinity of glucokinase for this protein, and that the catalytic activity of glucokinase plays no role in this process [35, 48]. As glucokinase has no nuclear localization signal, the current view is that it enters the nucleus as a complex with GKRP through a 'piggy-back' mechanism. The complex, unlike the dissociated forms of glucokinase and GKRP, would be unable to be exported.

Active export of free glucokinase is indicated by the finding that upon stimulation by high glucose concentration and/or sorbitol or fructose, the nucleus is 'emptied' of glucokinase, even when the latter is present as a fusion protein with GFP and is therefore clearly too big to cross the nuclear pores by mere diffusion [47]. Although the exit of glucokinase is insensitive to leptomycin B [35, 49], evidence has been provided for the presence of a nuclear export signal corresponding to amino acid 300 to 310 [35]. Intriguingly, two of the critical leucines in this motif are not conserved in *X. laevis* glucokinase, being replaced by an isoleucine (Leu307) and a methionine (Leu309), respectively.

GKRP is evenly distributed between the nucleus and the cytoplasm when transfected in cells that do not express glucokinase, but is predominantly located in the nucleus if leptomycin B is added or if the cells are co-transfected with glucokinase [35, 48]. These results indicate that free GKRP shuttles between the nucleus and the cytoplasm and that its exit from the nucleus is blocked by the formation of a complex with glucokinase.

In the context of this scenario, one expects GKRP to be partly present in the cytosol and that agents promoting dissociation of the glucokinase/GKRP complex would cause translocation of GKRP, though maybe to a different extent than glucokinase. There are conflicting data in the literature on this point, some authors reporting no detectable translocation of GKRP [42, 44, 50], and others modest but significant export [49] or even substantial translocation [50] upon addition of high concentrations of glucose or fructose. The technical difficulties encountered in such investigations, particularly to discriminate between specific GKRP signal and background signal in the cytoplasm, is probably one of the explanations for these discrepancies.

Effect of GKRP Ablation

The knock-out of the mouse GKRP gene (see chapter x) revealed an additional role for this protein. It was indeed found that the total activity of glucokinase was reduced in heterozygous and homozygous knockouts to 85 and 50–60% of the control level in ad libitum fed mice. After 16 h of starvation, these levels amounted to 62 and 15%, respectively [20, 52]. Similar decreases in the amount of immunoreactive glucokinase were also observed, whereas the corresponding mRNA was unchanged, or slightly increased, leading to the conclusion that the positive effect of GKRP on glucokinase expression is mediated by a post-translational mechanism.

As expected, glucokinase was found exclusively in the cytoplasm in hepatocytes of homozygous knock-out mice [20]. The availability of glucokinase in reduced amount, but uninhibited, is probably the reason why no change in the blood glucose or insulin level was observed in fed mice. However, the total glycogen concentration was reduced by 33% in the homozygous knock out. These mice were also found to display some degree of glucose intolerance when challenged with a glucose load after 16 h of starvation, presumably because of the reduced glucokinase amount that was available under these conditions. Furthermore, they developed hyperglycemia and hyperinsulinemia if maintained on a sucrose- and fat-rich diet for 8 weeks after weaning. Taken together, these data indicate that one of the roles of GKRP is to maintain in the nucleus a functional reserve of glucokinase that can be quickly mobilized after a meal [20, 52].

A mutation screening was carried out in the GKRP gene, because it is located in a region that is linked to obesity. This search resulted in the identification of several mutations. One of these (R227X) introduces a premature stop codon and certainly results in an inactive protein. No significant linkage was however observed between this mutation (in the heterozygous state) and obesity or diabetes [53].

Other Potential Glucokinase Regulatory Proteins

Screening approaches based on the yeast two-hybrid system and the phage display technique have led to the identification of three other proteins that interact with glucokinase. The techniques used to identify these three glucokinase-binding proteins are extremely powerful and it remains to be shown that these interactions take place physiologically.

PropionylCoA carboxylase has been identified by screening a RIN5 cDNA library [54]. At concentrations of the order of $\approx$10–30 μg/ml (i.e. about 100-fold higher than the dissociation constant of the glucokinase/GKRP complex), this enzyme stimulates glucokinase activity by up to 20%. Since propionylCoA carboxylase is a mitochondrial enzyme, the physiological significance of this effect is doubtful unless a significant part of the enzyme resides in the cytosol before being transferred to the mitochondrion.

A new dual-specificity phosphoprotein phosphatase was identified by screening a liver cDNA library with glucokinase as bait [55]. This phosphatase was shown to be a cytosolic protein and to dephosphorylate glucokinase that had been phosphorylated by protein kinase A and to increase the V_{max} of glucokinase by up to 2.5-fold. The tissular distribution of this protein phosphatase is quite broad and it must certainly have other functions than the regulation of glucokinase.

A phage display approach led to the identification of peptides sharing identities not only with GKRP (see above) but also with the bifunctional enzyme PFK2-FBPase 2, the enzyme that forms and degrades the potent glycolytic effector fructose-2,6-bisphosphate [38]. Both liver and islet isoforms of this enzyme were shown to interact with glucokinase through the yeast two-hybrid approach, but in vitro assays did not indicate any effect of the interaction on the activity of glucokinase.

Physiological Significance

GKRP makes that glucokinase in liver and possibly in other cells types can be regulated by Fru-1-P and Fru-6-P. Together with ketohexokinase and

glucokinase, GKRP forms a 'fructose sensing system', which is quite sensitive to this sugar due to the elevated affinity of GKRP for Fru-1-P. Fructose can therefore act as a nutritional signal that favors glucose utilization when mixed carbohydrates are present in the diet [14]. Stimulation of hepatic glucose utilization by fructose has been observed in hepatocytes [2, 3, 56, 57], in perfused liver [58], and in vivo [59, 60], leading to the suggestion that small amounts of dietary fructose may be beneficial in type 2 diabetes [61]. One may also wonder if the complete 'fructose sensing system' is present in the hypothalamus and if it plays a role in the control of appetite.

The role of the regulation by Fru-6-P is to decrease glucose phosphorylation when glycogenolysis and/or gluconeogenesis are stimulated. Under such conditions, the concentrations of both glucose-6-phosphate and Fru-6-P are increased, leading to a coordinate regulation of glucose-6-phosphatase which is stimulated through an increase in substrate concentration, and glucokinase, which is inhibited. It should be stressed that this inhibition is obscured by the exchange reaction that glucose-6-phosphatase catalyses between glucose and glucose-6-phosphate [62].

Another important aspect of the physiological role of GKRP is related to glucokinase translocation. What is probably a wrong interpretation is that import of glucokinase in the nucleus would decrease its activity by separating it from its substrates. Glucose and ATP are small enough to diffuse readily through the nuclear pores (and ATP is indeed necessary for many nuclear processes). Furthermore, GKRP is an authentic inhibitor of glucokinase and there is therefore no need to postulate any limitation of access to the substrates.

A potential advantage of the translocation phenomenon is that it may increase the sensitivity of glucokinase to glucose. We have seen that, when tested in cell free system, GKRP does not increase, but rather slightly decreases, the Hill coefficient of glucokinase for glucose. Mere inhibition by GKRP does therefore not account for the rather elevated ($\approx$2.0) Hill coefficient of glucokinase in isolated hepatocytes [1]. One potential explanation is that the effect of raising the glucose concentration is not only to compete GKRP away through a pure phenomenon of competitive inhibition, but also to promote the translocation of glucokinase from a compartment where the GKRP concentration is elevated to one in which this concentration is low. This is quite an original way for adding a level of control to make a system more sensitive to an effector. The reason why the nucleus has been chosen as a glucokinase reservoir is probably that it is the only compartment where proteins can be both imported and exported.

Another facet of the translocation problem is that GKRP appears to stabilize glucokinase, which can therefore be in sufficient amount to cope with sudden increases in the blood glucose concentration after a short period of fasting. It is likely, although not proven at this stage, that the sequestration in the

nucleus participates in this protection, possibly by separating glucokinase from proteases that could destroy it. Answering this question will require the production of glucokinase and GKRP mutants that are still able to interact normally with each other, but that are unable to be concentrated in the nucleus, or, alternatively, that permanently reside in the nucleus.

Whatever the precise role of GKRP, the elaboration of such a sophisticated mechanism during evolution but pleads for the importance of glucokinase in liver. This is in keeping with results of liver-specific glucokinase knock-out, which indicate that the absence of this enzyme in hepatic tissue results in glucose intolerance [63].

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GKRP/GK: Control of Metabolic Fluxes in Hepatocytes

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Glucokinase (hexokinase IV) is the predominant hexokinase expressed in the insulin secretory cells of the pancreas and in the liver of most mammals [1, 2]. It is also expressed in neuroendocrine cells in the hypothalamus and in the gut [3]. On account of its low affinity for glucose and sigmoidal kinetics [1], glucokinase confers on cells where it is the predominant hexokinase, a glucose-sensory mechanism that enables the rate of glucose metabolism to respond to changes in blood glucose concentration.

In liver cells, but not in insulin secretory cells, glucokinase is regulated by a 68-kDa regulatory protein that decreases the affinity of glucokinase for glucose [4]. This protein allows both long-term and short-term adaptive changes in the affinity of glucose phosphorylation for glucose. Accordingly, changes in the ratio of glucokinase/regulatory protein, as occur during endocrine control of glucokinase gene expression [5], lead to changes in the affinity of glucose phosphorylation for glucose. Similarly binding of low-molecular-weight ligands to the regulatory protein that alter its affinity for glucokinase can affect rapid changes in the rate of glucose phosphorylation [4]. This protein thus enables an additional level of control of glucose phosphorylation than can be achieved by glucokinase alone.

Two observations on liver glucose metabolism were the stimulus for the studies by Van Schaftingen [4] that led to the discovery of the regulatory protein. These were the rapid stimulation of glucose phosphorylation by low concentrations of fructose [5] and the lower affinity of glucose phosphorylation for glucose in intact hepatocytes relative to the kinetics of purified glucokinase [6]. The kinetic properties of glucokinase during interaction with the rat liver regulatory protein are very well characterized [8–10]. The regulatory protein acts as a competitive inhibitor with respect to glucose. Its binding to glucokinase is

potentiated by fructose-6-P and counteracted by fructose-1-P. These properties account for the low affinity for glucose phosphorylation in hepatocytes and the stimulation by fructose.

Sub-Cellular Compartmentation of Glucokinase and the Regulatory Protein

In addition to its role as a competitive inhibitor of glucokinase, the regulatory protein also determines the subcellular location of glucokinase within the liver cell. The regulatory protein is present predominantly but not exclusively in the nucleus of hepatocytes both in vivo and in vitro [11–13]. Glucokinase translocates between the nucleus and the cytoplasm depending on the metabolic state of the cells [11–15]. The role of the regulatory protein in sequestering glucokinase in the nucleus is supported by studies on regulatory protein knock-out mice, where glucokinase is present exclusively in the cytoplasm [16, 17]. Whether the regulatory protein has a more complex role in shuttling glucokinase between the nucleus and the cytoplasm is still open to debate [18, 19].

The sub-cellular location and substrate-induced translocation of glucokinase in hepatocytes has been studied by indirect immunofluorescence which measures the relative staining intensity in the nucleus and the cytoplasm [13, 20] and by a cell permeabilization assay which measures the distribution of glucokinase between free and bound states [21]. Indirect immunofluoresence staining and quantitative imaging is a useful technique to measure relative changes in the nuclear content of glucokinase (or its regulatory protein) in different metabolic conditions. However, it does not allow the direct determination of the amount of protein in the cytoplasm. The nuclear/cytoplasmic staining intensity for glucokinase is highest when hepatocytes are incubated at low glucose. Ratios ranging from 3 to 12 have been reported using different antibodies and staining methods. Because the cytoplasmic staining includes non-specific fluorescence, which cannot be accurately corrected for, the cytoplasmic glucokinase concentration cannot be determined directly. Since the nuclear volume in hepatocytes is about 10% of cell volume [22], a nuclear/cytoplasmic staining ratio of 10 may be indicative of sequestration of 50% of the glucokinase in the nucleus or of a higher proportion (up to 100%) if the cytoplasmic staining is largely due to non-specific fluorescence.

An increase in glucose concentration (10–25 m*M*) or micromolar concentrations of precursors of fructose-1-P (such as fructose or sorbitol) cause translocation of glucokinase from the nucleus to the cytoplasm (fig. 1). This is associated with partial translocation of the regulatory protein [13]. Whether this has a role in shuttling or in binding to glucokinase in the cytoplasm is not established.

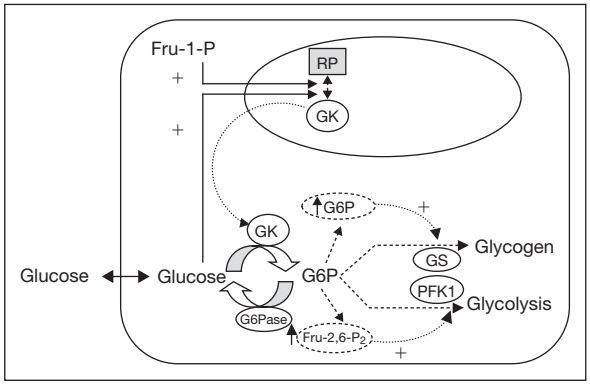

Fig. 1. Translocation of glucokinase from the nucleus to the cytoplasm by glucose and fructose-1-P. Glucokinase is sequestered in the nucleus bound to the regulatory protein at 5 m*M* glucose. An increase in glucose concentration causes translocation of glucokinase to the cytoplasm. This effect of glucose is markedly potentiated by fructose-1-P, which acts synergistically with glucose [24]. The increase in cytoplasmic glucokinase results in increased glucose phosphorylation and an increase in the cell content of glucose-6-P and fructose-2, 6-bisphosphate [26] which are major allosteric regulators of enzymes involved in glycogen metabolism and glycolysis, respectively.

The distribution of glucokinase between free and bound states has been measured by permeabilization of hepatocyte monolayers with low concentrations of digitonin (<0.1 mg/mg cell protein), in medium of low ionic strength containing millimolar concentrations of Mg^{2+} [21, 23]. Digitonin binds to the cholesterol in the plasma membrane and permeabilizes the cell to metabolites and cytoplasmic proteins. The fraction of glucokinase that is released (designated as 'free glucokinase') ranges from about 20 to 80% depending on the preincubation conditions, with minimal release from cells pre-incubated with 5 m*M* glucose and maximal release from cells pre-incubated with high glucose (35 m*M*) and precursors of fructose-1-P [24, 25]. The residual bound glucokinase can then be released by elution with 150 m*M* KCl (in the absence of Mg^{2+}) which dissociates glucokinase from the regulatory protein [9].

Since metabolic conditions that cause translocation of glucokinase from the nucleus to the cytoplasm (high glucose and precursors of fructose-1-P) also cause an increase in the proportion of free glucokinase activity it has been generally assumed that the free glucokinase activity corresponds to the cytoplasmic fraction [25]. The correlation between the rate of glucose phosphorylation and the free glucokinase activity in a wide range of experimental conditions including incubation with precursors of fructose-1-P [25, 26] and overexpression of the regulatory protein [27] supports the hypothesis that the enzyme released in

the digitonin assay represents the glucokinase that is not bound to the regulatory protein. It remains possible, however, that only part of the cytoplasmic glucokinase is unbound from the regulatory protein and that the permeabilization assay measures only the free enzyme in the cytoplasm. Part of the glucokinase that translocates to the cytoplasm may remain bound to the regulatory protein. Such a hypothesis would be consistent with the observations that the regulatory protein translocates to the cytoplasm during incubation with high glucose concentration or sorbitol [13] but is not released in the digitonin permeabilization assay [28]. It might be bound to the microfilament cytoskeleton as has been suggested from filament cross-linking studies [29].

Short-Term Control of Glucose Metabolism by Translocation of Glucokinase to the Cytoplasm and Dissociation from the Regulatory Protein

Two classes of compounds cause translocation of glucokinase to the cytoplasm and an increase in the free glucokinase activity. These are precursors of fructose-1-P (such as fructose and sorbitol) and glucose analogues including glucose and glucokinase inhibitors [24]. The effect of sorbitol and fructose has been explained by the lower affinity of the regulatory protein for glucokinase in the presence of fructose-1-P, as shown by the correlation between the free glucokinase activity and the cell content of fructose-1-P in the studies by Niculescu et al. [23]. The effect of glucose and glucose analogues is presumed to be due to dissociation of glucokinase from the regulatory protein. The effect of most but not all glucose analogues on translocation is sigmoidal and synergistic with respect to fructose-1-P [24]. Whether dissociation of glucokinase from the regulatory protein occurs within the nucleus before translocation [18, 19] or within the cytoplasm after translocation of glucokinase complexed to the regulatory protein is not established. Time course studies suggest that dissociation is slower than translocation [Mukhtar et al., unpubl. results] which supports the latter hypothesis.

Precursors of fructose-1-P stimulate glucose phosphorylation, glycolysis and glycogen synthesis and there is a good correlation between the stimulation of the rate of metabolic flux and the increase in free glucokinase activity [25–27]. This indicates that the free fraction of glucokinase is metabolically active in glycolysis and glycogen synthesis. Whether these two metabolic pathways occur in different cytoplasmic compartments or involve distinct glucokinase pools is uncertain. Jetton and colleagues showed that during substrate-induced translocation from the nucleus to the cytoplasm, glucokinase accumulates in the cell periphery [20], which is also the initial site of glycogen storage [30]. Since

glycogen synthase also translocates to the periphery at high glucose [30] there may be colocalization of these proteins at the sites of glycogen synthesis. Metabolic studies using [2-^{3}H]glucose have also provided suggestive evidence for channelling of intermediates between glucokinase and glycogen [21], which would be consistent with colocalisation of glucokinase and glycogen synthase at the sites of glycogen storage.

Adaptive Changes in Glucose Phosphorylation Affinity during Fasting and Feeding

The ratio of glucokinase/regulatory protein shows marked changes during fasting and refeeding or during streptozotocin-induced diabetes [5], because the concentration of glucokinase changes more rapidly and to a greater extent than that of the regulatory protein [5]. This is explained by the greater responsiveness of glucokinase gene expression [31] to insulin compared with the regulatory protein [16]. The effect of changes in the ratio of glucokinase/regulatory protein on the affinity of glucose phosphorylation for glucose in hepatocytes can be shown by adenovirus-mediated overexpression of the regulatory protein in hepatocytes expressing endogenous glucokinase [27]. An increase in regulatory protein expression by 2-fold relative to endogenous levels causes an increase in the $S_{0.5}$ for glucose from 25 to 36 m*M* [27]. Accordingly, the physiological changes in ratio during fasting or induction of diabetes [5] are predicted to be associated with correspondingly large changes in affinity for glucose [27].

Adaptive Changes in Glucokinase Expression and the High Control Coefficient of Glucokinase on Glycolysis and Glycogen Synthesis

The contribution of glucokinase to the control of flux through glucose metabolism can be determined by titrated protein overexpression using recombinant adenoviruses and application of Metabolic Control Analysis [32, 33]. This is a quantitative method that can be used to describe how control of flux in a metabolic pathway is distributed between component enzymes in the metabolic system or to determine the sensitivity of metabolic flux to small fractional changes in the concentration of specific proteins or metabolic intermediates. The relation between the rate of flux through a metabolic pathway and the concentration of a protein can be expressed as the flux control coefficient (or control strength) of the protein (C_{E}^{J}). This is defined as the fractional change in pathway

flux (J) that results from a small fractional change in protein concentration (E). It can be positive or negative depending on whether an increase in protein concentration causes an increase or decrease in pathway flux. Most enzymes have flux control coefficients that are much smaller than unity [32, 33]. However, enzymes that exert a high degree of control have coefficients approaching and sometimes exceeding unity. Flux control coefficients are properties of the metabolic system and are dependent on the activities of all other enzymes in the system. Accordingly, when the concentration of a particular enzyme changes, both its flux control coefficient and those of other enzymes in the system change. For most enzymes the flux control coefficient decreases with increasing enzyme concentration. However, enzymes that exert a high degree of control on pathway flux may show sustained high coefficients with increasing protein concentration [34]. Experimentally, the flux control coefficient of a protein can be determined in cells overexpressing the protein by varying degrees relative to the endogenous level, from the initial slope of a double log plot of the rate of metabolic flux (measured in steady state conditions) against the enzyme concentration or activity as shown in figure 2a [35].

The flux control coefficients of glucokinase on glycolysis and glycogen synthesis in hepatocytes are markedly dependent on the glucose concentration and are greater than unity at low glucose (fig. 2b). In the absence of subcellular compartmentation or binding of an enzyme to a receptor the flux control coefficient is expected to be less than unity. The high flux control coefficients at low glucose, can be explained by the glucose-dependence of the sub-cellular compartmentation of glucokinase [24, 35]. Overexpression of glucokinase in the absence of expression of the regulatory protein results in a greater fractional increase in free glucokinase (not bound to the regulatory protein) than in total enzyme activity at low glucose concentration because of saturation of the regulatory protein which is present in less than 2-fold molar excess relative to glucokinase. The fractional change in flux thus reflects the fractional change in free glucokinase (rather than total) activity and this accounts for coefficients greater that unity. Conversely, at high glucose concentrations which cause dissociation of glucokinase from the regulatory protein, overexpression of glucokinase is associated with a similar fractional change in free as in total glucokinase activity and accordingly with lower control coefficients [35].

Precursors of fructose-1-P, which like glucose, cause dissociation of glucokinase from the regulatory protein also decrease the flux control coefficient of glucokinase on glycogen synthesis, whereas incubation conditions which stimulate glycogen synthesis without causing dissociation of glucokinase from the regulatory protein, such as high concentrations of proline, do not decrease the flux control coefficients of glucokinase. This supports the hypothesis that

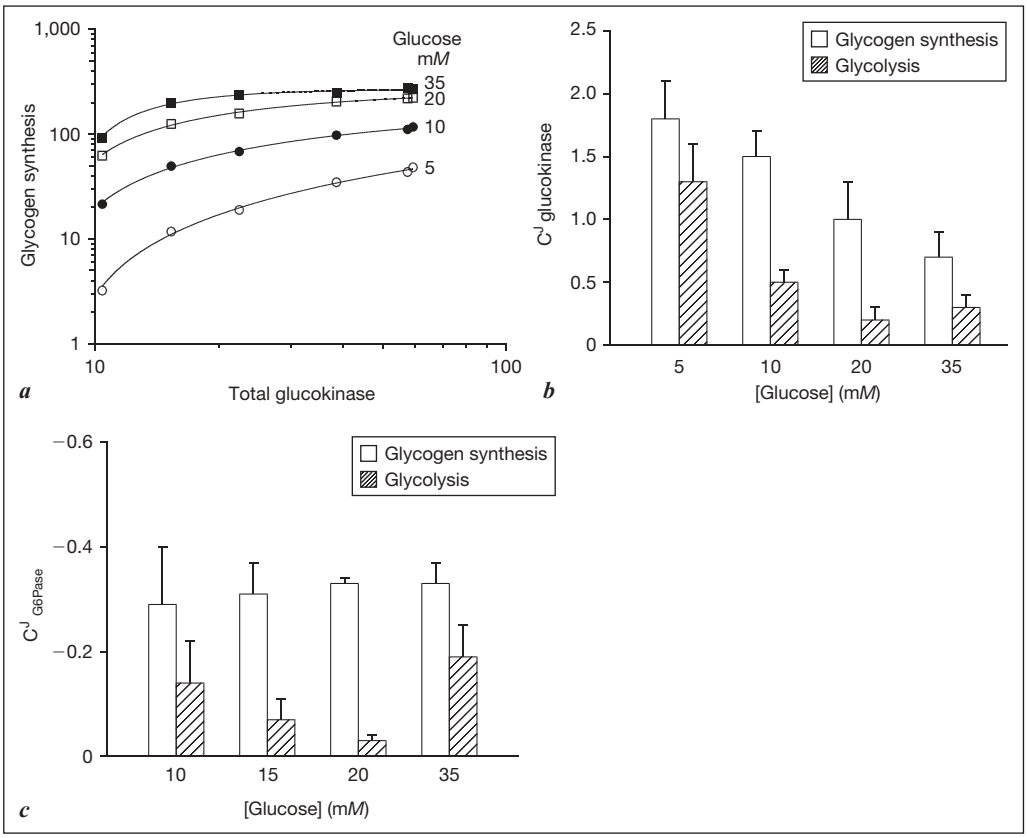

Fig. 2. The flux control coefficients of glucokinase but not of glucose-6-phosphatase on glycogen synthesis and glycolysis are glucose-dependent. Hepatocytes were treated with varying titres of recombinant adenoviruses encoding rat liver glucokinase or glucose-6-phosphatase for overexpression of the enzymes by up to 6-fold above endogenous activity [35, 39]. Rates of glycogen synthesis (incorporation of [U-^{14}C]glucose into glycogen) and glycolysis (detritiation of [3-^{3}H]glucose) were determined at the glucose concentrations shown and the flux control coefficients (C^J_E) of glucokinase (***b***) and glucose-6-phosphatase (***c***) were determined from initial slope of the double log plots of metabolic flux (glycogen synthesis open bar, glycolysis, hatched bar) against total enzyme activity. ***a*** A representative double log plot of glycogen synthesis against total glucokinase activity from which the flux control coefficients were determined from the initial slope at the endogenous activity of glucokinase. Mean ± SE for 4 experiments. For experimental details, see [35, 39].

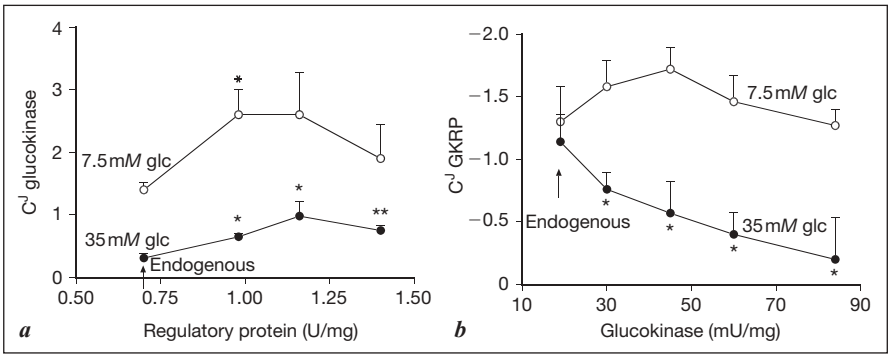

Fig. 3. Interdependence of the flux control coefficients of glucokinase and the regulatory protein at varying levels of expression of these proteins. Glucokinase and the regulatory protein were overexpressed in hepatocytes using recombinant adenoviruses and the flux control coefficients of these proteins on glycogen synthesis were determined at 7.5 or 30 m*M* glucose. ***a*** Flux control coefficients of glucokinase at endogenous levels of regulatory protein or overexpression up to 2-fold. ***b*** Flux control coefficients of the regulatory protein at varying levels of glucokinase overexpression. Reproduced from J Biol Chem [27], with permission.

binding of glucokinase to the regulatory protein is a component of the high control coefficient of glucokinase on glucose metabolism.

The Control Coefficients of Glucokinase and the Regulatory Protein are Balanced

The role of the regulatory protein as a major determinant of the high flux control coefficient of glucokinase is supported by two sets of observations. First, that overexpression of the regulatory protein in hepatocytes by about 50% above endogenous levels in the fed state causes an increase in the flux control coefficient of glucokinase by 50–80% at both low and high glucose concentration (fig. 3a) [27]. Second, that the regulatory protein itself has a high negative flux control coefficient on glucose metabolism (fig. 3b), indicating that glucokinase and the regulatory protein exert reciprocal control [27]. The glucose-dependence of the flux control coefficient of the regulatory protein is manifested at increasing glucokinase overexpression but not at endogenous levels. Thus, at endogenous levels of both proteins as are expressed in the fed state, the regulatory protein has a high control strength on glycogen metabolism at both low and high glucose concentration. The interdependence of the flux control coefficients of glucokinase and the regulatory protein on the concentrations of these proteins shows that the molar ratio of these proteins (which is between 1.4 and 2 in the fed state) is finely balanced to achieve maximum or near

maximum control by both proteins over a wide range of glucokinase/regulatory protein ratios as occur during the fasting and refeeding [5].

The Role of Glucose-6-P in Explaining the Higher Control Coefficient of Glucokinase on Glycogen Synthesis Relative to Glycolysis

Although binding to the regulatory protein is a major contributing factor to the high control coefficient of glucokinase on glycogen synthesis or glycolysis particularly at low glucose concentration, additional factors must be involved to explain the higher control coefficient on glycogen synthesis relative to glycolysis (fig. 2b). Stimulation of glycogen synthesis by glucokinase overexpression is associated with activation of glycogen synthase (covalent modification) and with translocation of the enzyme from a soluble state to the particulate fraction [36]. Both these effects can be explained (at least in part) by the increase in glucose-6-P, which occurs with glucokinase overexpression [37]. The higher flux control coefficient on glycogen synthesis compared with glycolysis could be explained by a greater sensitivity of glycogen synthesis to glucose-6-P than glycolysis [38]. Support for this hypothesis was obtained from experiments involving overexpression of glucose-6-phosphatase, which lowers the hepatocyte content of glucose-6-P [39] without affecting the rate of glucose phosphorylation (fig. 4). Overexpression of glucose-6-phosphatase by 4-fold relative to endogenous levels, had negligible effect on the flux control coefficient of glucokinase on glycolysis but it lowered the flux control coefficient on glycogen synthesis (fig. 4c). The latter effect cannot be explained by substrate depletion because the concentration of UDP-glucose is not affected by glucose-6-phosphatase overexpression [40]. Although an increase in glucose-6-P is expected to increase glycolysis by inhibition of the phosphatase activity of the bifunctional protein that generates and degrades fructose 2,6-bisphosphate, a potent activator of phosphofructokinase-1 [41], the higher sensitivity of glycogen synthesis to changes in glucose-6-P concentration may be explained by the multiplicity of mechanisms by which glucose-6-P activates glycogen synthase. Glucose-6-P is an allosteric activator of glycogen synthase [37] and it promotes the dephosphorylation of glycogen synthase by a substrate-directed mechanism [37]. It also stimulates translocation of glycogen synthase from a soluble to particulate fraction [42] and it promotes the inactivation of phosphorylase by a substrate mediated activation of phosphorylase phosphatase and/or inhibition of phosphorylase kinase, which releases the inhibition of synthase phosphatase by its allosteric inhibitor phosphorylase-a [43]. The concerted effect of these four mechanisms accounts for the sensitivity of glycogen synthesis to glucose-6-P [38].

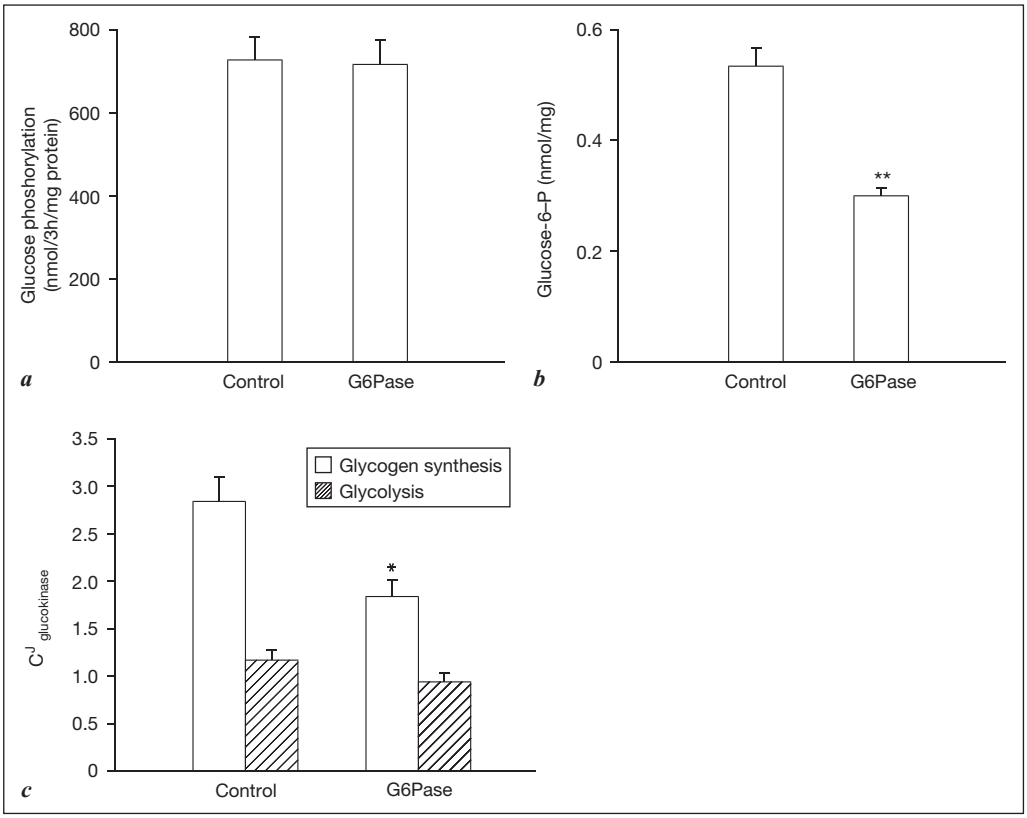

Fig. 4. Overexpression of glucose-6-phosphatase lowers the flux control coefficient of glucokinase on glycogen synthesis but not on glycolysis. Hepatocytes were either untreated (control) or treated with recombinant adenovirus for overexpression of glucose-6-phosphatase by 4-fold relative to endogenous levels. The rate of glucose phosphorylation (***a***) and the cell content of glucose-6-P (***b***) were determined after a 3-hour incubation with 25 m*M* glucose. ***c*** The flux control coefficient of glucokinase on glycogen synthesis (open bars) and glycolysis (hatched bars) was determined from the double log plot of flux against free glucokinase activity in incubations with varying sorbitol concentration. Mean ± SE for 5 experiments. For experimental details, see Aiston et al. [39].

Glucose-6-Phosphatase Has a Negative Control Coefficient on Glycogen Synthesis and Glycolysis

By analogy with the stimulation of glycogen synthesis and glycolysis that results in metabolic conditions associated with an increase in glucose-6-P, proteins that lower glucose-6-P are predicted to have negative control coefficients on glycogen synthesis and glycolysis. Glucose-6-phosphatase overexpression

lowers the hepatocyte content of glucose-6-P [40] and accordingly inhibits these pathways [39]. Two points are of interest when comparing the flux control coefficients of glucose-6-phosphatase and glucokinase (fig. 2). First, the control coefficients of glucose-6-phosphatase are smaller in magnitude than for glucokinase and are not glucose-dependent (fig. 2b, c). This supports the hypothesis that the glucose-dependence in the case of glucokinase is due to binding to the regulatory protein. Second, similar to glucokinase, the control coefficient of glucose-6-phosphatase on glycogen synthesis is greater than for glycolysis. This is consistent with a greater sensitivity of glycogen synthesis compared with glycolysis to glucose-6-P.

Lack of Feedback Inhibition of Glucose Phosphorylation by Glucose-6-P in Hepatocytes

In tissues where glucose utilization is determined by metabolic demand the rate of glucose phosphorylation by the low K_m hexokinases (I and II) is regulated by feed-back inhibition by glucose-6-P [44]. Glucokinase, unlike the low K_m hexokinase isoforms is not inhibited by physiological concentrations of glucose-6-P [44]. However, since binding of the regulatory protein to glucokinase is enhanced by fructose-6-P [4], which is in equilibrium with glucose-6-P, there is the theoretical possibility that an increase in glucose-6-P indirectly inhibits hepatic glucose phosphorylation by increasing the binding of glucokinase to its regulatory protein. If changes in glucose-6-P within the physiological range have a role in feedback inhibition of glucokinase, then a lowering of the glucose-6-P content by glucose-6-phosphatase overexpression would be expected to increase the dissociation of glucokinase from the regulatory protein caused by glucose or precursors of fructose-1-P. However, no evidence for this was found in cells overexpressing glucose-6-phosphatase by 4-fold relative to endogenous activity, which results in a lowers glucose-6-P by 50% [39]. This does not support a role for feed-back inhibition of glucokinase activity by increased binding to the regulatory protein at high concentrations of glucose-6-P.

The conclusion that can be drawn is that the hepatocyte content of hexose-6-P is sufficient to maintain binding of glucokinase to the regulatory protein in most metabolic conditions and that a 50% decrease in hexose-6-P does nor promote dissociation of glucokinase from the regulatory protein regardless of concentration of glucose or fructose-1-P. It can be inferred that whilst binding of glucokinase to the regulatory protein in vitro is determined by fructose-6-P and fructose-1-P which promote binding and dissociation, respectively [4], the main regulators in vivo are fructose-1-P [23] and glucose but not fructose-6-P.

Conclusion: The High-Flux Control Coefficient of Glucokinase Is Explained by Feed-Forward Activation of Hepatic Glucose Metabolism by Glucose

The high control coefficient of hepatic glucokinase on glucose utilization and particularly on glycogen synthesis [35] can be explained by: (i) the regulatory protein which sequesters glucokinase in an inactive pool in the nucleus at low glucose [4]; (ii) the accumulation of glucose-6-P that results from the increased rate of glucose phosphorylation when glucokinase is released from the regulatory protein [26, 40]; (iii) the multiplicity of mechanisms by which glucose-6-P activates glycogen synthase [37] and inactivates phosphorylase [43], and (iv) the lack of feed-back inhibition of glucose phosphorylation by glucose-6-P [39].

The concerted effect of these four mechanisms is the feed-forward activation of glycogen synthesis by the rise in glucose-6-P concentration that results from glucose-induced translocation of glucokinase to the cytoplasm and dissociation from its regulatory protein [21].

Protein movement within the hepatocyte in response to a rise in blood glucose concentration is not confined to glucokinase. Glycogen synthase also translocates to the cell periphery [30] and may colocalize with glucokinase [20]. Migration of proteins to the sites of glycogen synthesis may involve the organized assembly of a 'glycogen-metabolon' with the intent of facilitating the channelling of intermediates between glucose and glycogen: a concept first proposed by Srere [45] for mitochondrial metabolism almost two decades ago.

Acknowledgements

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This paper is dedicated to the late Paul Srere for his inspiring and passionate teaching that the cell is not a bag of enzymes.

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Regulation of Glucokinase as Islets Adapt to Pregnancy

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Pregnancy is an occasion in the life history of the β-cell where there is an increased need for insulin that occurs over a relatively short period of time. This amounts to days in rodents and months in humans. The need for enhanced islet function emerges as a consequence of an increase in peripheral insulin resistance at the same time as the placenta, a major target organ for insulin, develops. To accommodate this increase in insulin demand, the islet must undergo changes that lead to increased insulin secretion under normal glucose conditions.

The primary short-term regulation of insulin secretion is achieved by elevating the glucose concentration. However, if this were the primary adaptive mechanisms during pregnancy, there would be a need for persistent hyperglycemia – a condition deleterious to the developing embryo, fetus and mother. Thus, in the face of this increased demand for insulin, islets must undergo structural and functional changes. The outcome of this long term upregulation of islets must be enhanced insulin secretion at normal glucose levels. Failure of this long-term adaptive process can lead to gestational diabetes.

Evidence for functional changes in islets during pregnancy first appeared in the 1960s shortly after the development of a sensitive radioimmunoassay for insulin. Spellacy and coworkers [1–3] reported that there was a progressive increase in both fasting and glucose-stimulated insulin secretion throughout the course of pregnancy. These and subsequent studies led to the characterization of pregnancy as a condition of elevated serum insulin levels, slightly lower blood glucose levels and peripheral insulin resistance (see reviews [4, 5]).

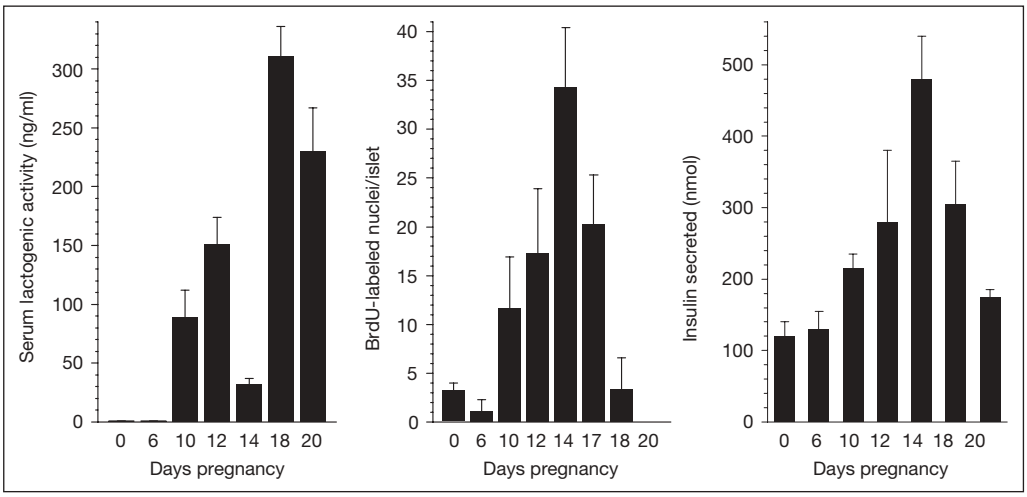
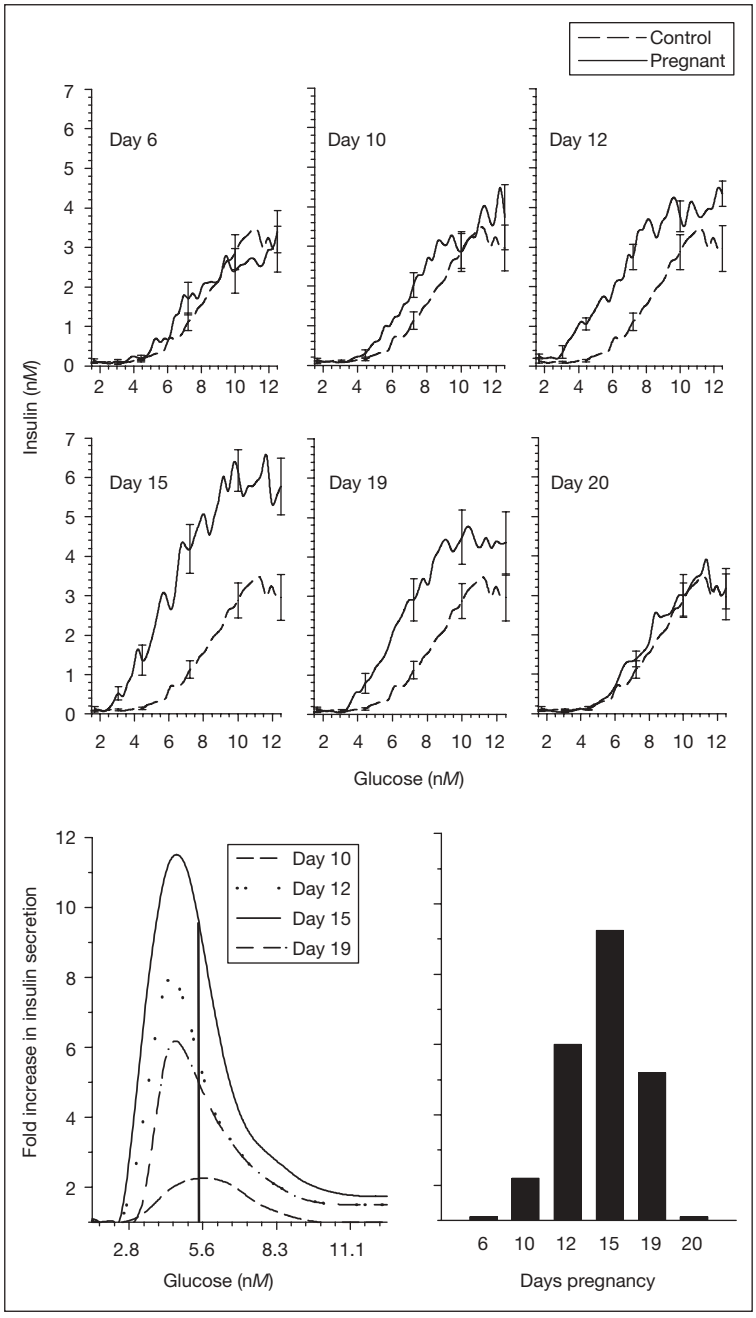


Fig. 1. Effect of pregnancy on BrdU labeling of islet nuclei (center panel) and extent of glucose stimulated insulin secretion in rats (right panel) [9]. The increase in islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion (left panel). The two peaks of serum lactogen activity correspond to the secretion of PL-I and PL-II during rat pregnancy.

Islet β-Cell Proliferation and Hypertrophy during Pregnancy

Islets can respond to an increased demand for insulin by increasing islet mass and the functionality of the β-cells. In fact, suggestions that islets undergo changes during pregnancy was suggested as early as the 1930s when it was first reported that there was an increase in total islet volume. Subsequently, a number of studies have shown that the increase in islet mass is due to both hypertrophy and hyperplasia [see review, 6]. When we examined DNA content/islet on day 20 and protein content on days 15 and 20 of pregnancy, there was a 25% increase in DNA and a 50 and 100% increase in protein, respectively.

Islet cell proliferation during pregnancy in rats has been examined by tritiated thymidine incorporation and bromodeoxyuridine (BrdU) labeling. Tritiated thymidine incorporation was increased 2- to 3-fold on day 12–14 of pregnancy and then approached control levels by day 19 [7, 8]. Similarly, there was a progressive increase in BrdU-labeled nuclei from day 10 to 14 of pregnancy at which the labeling was 10-fold greater than in controls (fig. 1). Subsequently, the number of labeled nuclei/islet declined to control levels by day 18 of pregnancy [9]. These rates of BrdU labeling are consistent with a 30% increase in cell mass during pregnancy and correspond with the 25% increase in DNA that was


Control
Pregnant
Day 6
Day 10
Day 12
Day 15
Day 19
Day 20
Insulin (nM)
Glucose (nM)
Day 10
Day 12
Day 15
Day 19
Fold increase in insulin secretion
2.8
5.6
8.3
11.1
Glucose (nM)
6
10
12
15
19
20
Days pregnancy

observed. A study designed to determine the number of islets and islet volume during pregnancy indicated that the increased islet volume results from growth of pre-existing islets and not from neo-formation of islets [10].

Thus, there is uniform agreement that pregnancy results in an increase in total islet mass. This growth is due to both β-cell hyperplasia and hypertrophy. The increase in β-cell proliferation is first observed around day 10 and corresponds to the observed increase in placental lactogen [9]. The β-cell proliferation peaks around day 14 of pregnancy and then returns to control levels by the end of pregnancy. From this data one can surmise that the capacity for insulin secretion, based on islet mass, is increased about 2-fold during pregnancy.

Insulin Secretion during Pregnancy

During pregnancy in rodents, fasting serum insulin levels are increased about 75% and glucose levels are decreased by about 25% [11–13]. Glucose tolerance tests are normal, but show enhanced insulin secretion [11]. A similar situation is observed in humans during pregnancy [1–5]. There have been relatively few studies examining insulin secretion from islets isolated during pregnancy. These studies showed that pregnancy results in enhanced glucose-stimulated insulin secretion from these islets [8, 11, 14]. Green et al. [14, 15] also noted that there was a leftward shift in the glucose response curves for insulin secretion and insulin synthesis. This was the first report indicating that the threshold for glucose-stimulated insulin secretion could be lowered below that observed under normal, non-pregnant conditions. That is, an increase in the islet's sensitivity to glucose stimulation.

Although these studies indicated that there was an increase in insulin secretion during pregnancy, there were no studies examining the correlation of secretion parameters with changes in placental lactogen (PL) secretion throughout pregnancy. To investigate the temporal profile of changes in islets during pregnancy, we examined insulin secretion during gestation in rats (fig. 2) [9]. Differences in both the threshold of glucose-stimulated insulin secretion and the amount of insulin released above this threshold could be detected by day 10

Fig. 2. The top six panels show glucose-dependent insulin secretion from perfused pancreases during the course of pregnancy in comparison to the controls [9]. The shift in the glucose-stimulation threshold and above threshold insulin secretion is first detected on day 10. The differences increase until day 15 and then return to control levels by day 20 of pregnancy. The bottom left panel shows the ratio of insulin secretion during pregnancy compared to controls for each glucose concentration. The bottom right panel shows the fold increase in insulin secretion compared to control at a normal blood glucose concentration of 5.6 m*M*.

(fig. 2). By day 12, the threshold was lowered from 5.7 m*M* glucose to 3.3 m*M*, remained at this level through day 15, and returned towards normal by day 20. Concomitant with the increased sensitivity of β-cells to glucose, the amount of insulin released above the threshold was increased by day 12, peaked on day 15, and returned to control levels by day 20. This lowering of the threshold for glucose-stimulated insulin secretion is an important feature of islets as they adapt to pregnancy. It is only by this maneuver that a large increase in insulin secretion can be achieved at normal blood glucose levels. The magnitude of this effect can be seen by comparing the rates of insulin secretion at 5.6 m*M* glucose from pregnant animals *versus* controls (fig. 2).

The onset of the changes in islet cell proliferation and insulin secretion correlate with the appearance of circulating PL [9]. However, PL remains elevated until the end of pregnancy when islet cell proliferation and insulin secretion have returned to control levels. Since islets cultured in the presence of PRL or PL show persistent increases in islet cell division and insulin secretion, it is likely that the increase in steroids or other effectors during the later stages of pregnancy are counteracting the effects of PL. This hypothesis is supported by experiments in vitro where islets are cultured with both PRL and progesterone [16]. In this case, insulin secretion and BrdU labeling increased during the first 4 days, but subsequently returned to control levels by day 8. This temporal pattern of changes in islet function closely mimics that observed in islets during pregnancy [9]. Similarly, increased plasma glucocorticoid levels during the later part of pregnancy could effectively reverse the lactogen-induced upregulation of islet function by inhibiting insulin secretion and cell proliferation while increasing apoptosis [17].

The summary of the evidence that the adaptation of islets to pregnancy is mediated by way of lactogenic activity (i.e. PRL or PL) is shown in table 1. In addition, the magnitude of the effects observed with the homologous PRL or PL in vitro is comparable to those observed during pregnancy.

It should also be mentioned that the physiological relevance of PL secretion has been questioned because placentas of many species do not produce a distinct PL (e.g. rabbits, pigs, dogs, and cats) [18]. In addition, the occurrence of normal pregnancies in women with undetectable serum concentrations of human PL, because of a gene deletion, has been reported [19–21]. Unfortunately, insulin secretion during pregnancy has not been examined in either of these conditions. An elevation in insulin secretion may still occur because other mechanisms may compensate for an absence of a distinct PL. First, the decreased capacity for glucose disposal from the induction of peripheral insulin resistance may be sufficient to indirectly stimulate an increase in islet function. Second, an increase in serum levels of PRL may compensate for the absence of a distinct PL during pregnancy. An increase in serum levels of PRL during pregnancy has been shown in dogs [22] and humans [23–25].

Table 1. Comparison of the effects of pregnancy with species homologous lactogenic hormones (prolactin and or placental lactogen)

Islet structure and function	Pregnancy	PRL/PL
β-Cell proliferation	+++	+++
Islet volume	+++	+++
Glucose-stimulated insulin secretion	+++	+++
Lower threshold for insulin secretion	+++	+++
Insulin synthesis	+++	+++
Insulin content	+++	+++
β-Cell junctional coupling	+++	+++
Glucose utilization	+++	+++
Glucose oxidation	+++	+++
Glucokinase activity	+++	+++
Glucokinase protein/DNA	+++	+++
Glucose transporter 2 levels	+++	+++
c-AMP metabolism	+++	+++

Furthermore, the extent of the PRL receptor expression in target tissues may also be important because the binding of PRL to the mammary gland is much greater in species without a distinct PL [18]. Until additional evidence is available, these studies should not be interpreted as disproving that an increase in islet function is an essential component of the alterations of maternal metabolism during pregnancy in mammalian species.

Role of Lactogens in the Regulation of Islets during Pregnancy

The long form of the PRL receptor is present in islets [26–31]. Within islets, the PRL receptor is only present on β-cells and not α-cells or δ-cells [32] (fig. 3). The intensity of immunohistochemical staining in control islets is quite variable among β-cells, with many showing a low level of PRL receptor expression. By day 14 of pregnancy in rats, this cellular heterogeneity for PRL receptors is markedly decreased with nearly all cells having a uniformly high level of staining [31]. Also, an increase in PRL receptor mRNA has been reported in islets during pregnancy and after treatment with PRL in vitro [33]. These studies suggest that lactogens induce the expression of the PRL receptor and this likely has a role in the islet's adaptation to pregnancy. The long form of the GH

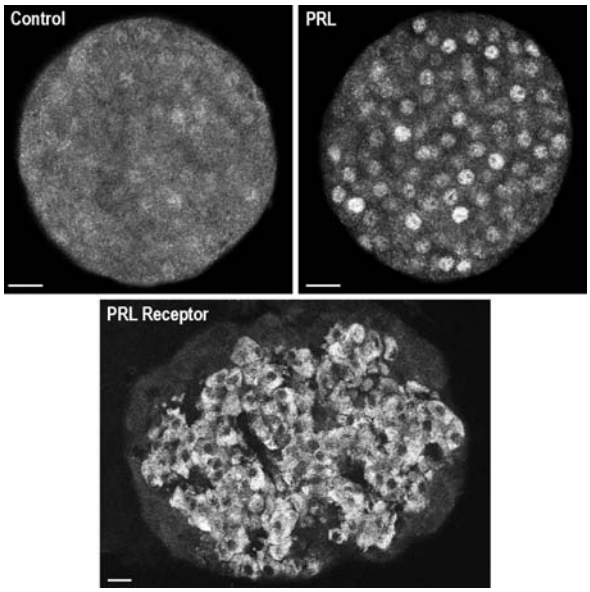


Fig. 3. Immunohistochemical detection of STAT5 in isolated islets (top panels). The left panel is a control islet and the right panel shows cytoplasm to nuclear translocation of STAT5 after 30 min of prolactin treatment. The bottom panel is an immunohistochemical demonstration of prolactin receptors in islet β-cells. Note the absence of prolactin receptors in the mantel containing α- and δ-cells.

receptor is also present in islets [27, 29]. However, in contrast to the PRL receptor, GH has no effect on the expression of the GH receptor [33].

PRL and GH receptors belong to the cytokine superfamily of receptors, which do not have intrinsic tyrosine kinase activity but interact with members of the JAK (Janus kinase) family of tyrosine kinases [34]. The activated JAK kinases phosphorylate a member of the STAT (Signal Transduction and Activators of Transcription) family of transcription factors, which then dimerize and translocate to the nucleus to regulate the expression of specific genes [35]. The principal components involved in PRL and GH receptor signaling are JAK-2 and STAT5, although others may be involved. In recent immunohistochemical studies, we have identified JAK2 in rat islets and INS-1 β-cell line and have observed the translocation of STAT5 to the nucleus after treatment with PRL [32, 36]. This translocation of STAT5 is a rapid event, detectable within a few minutes, and is maximal within 30 min (fig. 3). Interestingly, the dose/response relationship between PRL concentration and STAT5 activation (translocation from cytoplasm to nucleus) parallels the dose response relationship between PRL and glucose-stimulated insulin secretion and BrdU labeling of islet β-cells [32].

Mechanisms of Increased Glucose-Stimulated Insulin Secretion during Pregnancy

With the demonstration that lactogens induce the changes in islets during pregnancy, it was important to identify the cellular mechanisms involved. In particular, the lowering of the threshold of glucose-stimulated insulin secretion is an essential feature of the successful adaptation of islets to pregnancy. Although conditions have been identified with an increase in the glucose-stimulation threshold, for example somatostatin, fasting and reduced glucokinase expression [37–39], few studies have examined insulin secretion when there is a lowering of the threshold [14, 40, 41]. Therefore, we undertook a study to investigate whether the threshold can change in vivo after PRL or GH infusion, glucose loading or fasting, or in vitro after treatment with forskolin, glucagon, cholecystokinin, carbamylcholine or gastric inhibitory peptide [42]. Unexpectedly, all treatments that enhance insulin secretion lowered the threshold of glucose stimulation into the range of 3.0–4.0 m*M* glucose. This suggests that the metabolism of approximately 3.0 m*M* glucose activates all of the pathways necessary for glucose-stimulated insulin secretion. Further increases in glucose metabolism would increase the activity of these pathways and/or stimulate additional regulatory pathways. This model of insulin secretion is supported by the observation that most secretagogues require stimulatory or near stimulatory concentrations of glucose to have an effect on insulin secretion.

Glucose metabolism is central to regulated insulin secretion and glucokinase is regarded as the critical glucose sensor. Moreover, a reduction in glucokinase activity increases the threshold of glucose-stimulated insulin secretion [37]. Therefore, we examined whether changes in glucose metabolism, glucokinase activity and expression, and the expression of the glucose transporter Glut2 correlated with the changes in insulin secretion observed in islets during pregnancy or after culture with PRL in vitro [43]. The rates of glucose oxidation and utilization in islets were elevated during pregnancy. This enhanced glucose sensitivity results in a shift of the glucose metabolism profiles to lower concentrations. A similar increase in glucose metabolism was found in islets treated with PRL in vitro. This increase in glucose metabolism correlates with a 50% increase in glucokinase activity observed in these islets. This change also correlates with the increase in glucokinase protein (fig. 4). The glucose transporter Glut2 expression was also increased more than 2-fold in these islets. These changes support the hypothesis that the upregulation of key components in glucose metabolism has a primary role in the long-term adaptation of islets to pregnancy. The best candidate for a central role in this process is glucokinase, which is the rate limiting step in islet glucose metabolism [44]. The observed 50% increase in glucokinase activity is close to the predicted amount [45]

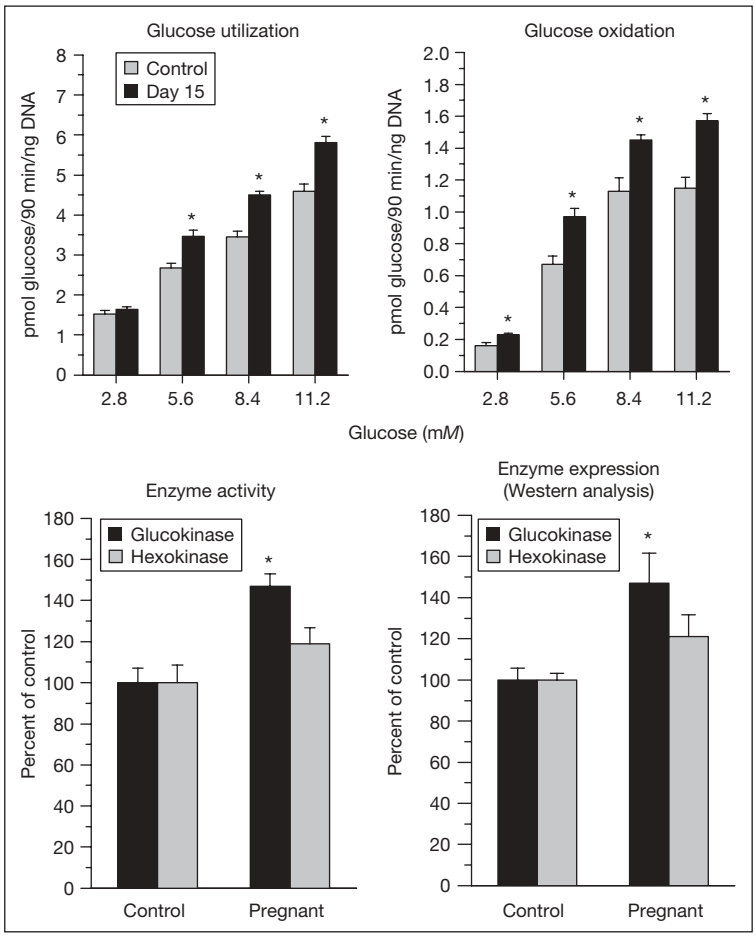


Fig. 4. Glucose oxidation, utilization, glucokinase and hexokinase during pregnancy in rat islets. On day 15 of pregnancy, when glucose stimulated insulin secretion is at its greatest, there is an increase in glucose metabolism as demonstrated by measuring glucose utilization and glucose oxidation. At this time there is also an increase in glucokinase enzyme activity and protein as demonstrated by Western analysis.

needed to lower the glucose-stimulation threshold to that observed during pregnancy and in islets treated with PRL in vitro. Since hexokinase could also potentially contribute to changes in the glucose-stimulated insulin secretion threshold, it was also examined. In mid-pregnancy, when the greatest change in insulin secretion occurs, there were no changes in hexokinase activity or expression levels. Also, in the in vitro experiments using PRL-treated islets, hexokinase is barely detectable and again no changes were observed. Thus, it

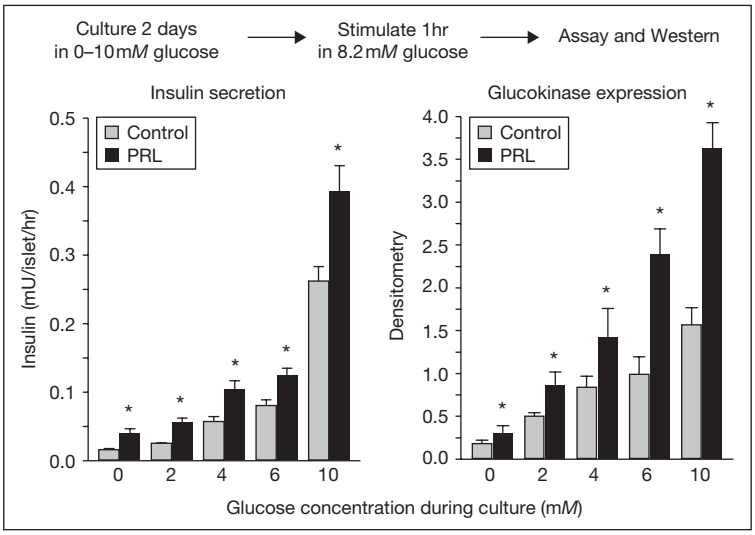


Fig. 5. Effect of glucose and prolactin treatment on subsequent glucose-stimulated insulin secretion and expression of glucokinase in isolated islets. Culturing in increasing concentrations of glucose for 2 days leads to a subsequent increase in glucose-stimulated insulin secretion and glucokinase expression. At all concentrations of glucose there was an incremental increase in insulin secretion and glucokinase expression in the islets treated with prolactin.

appears that hexokinase is not required for the changes in insulin secretion that occur during pregnancy.

The above studies strongly implicated glucokinase in the changes in insulin secretion that occur during pregnancy. Therefore, additional studies were done to further characterize its role and the mechanisms by which this occurs. Initial experiments were done using INS-1 cells examining the effect of PRL on glucose metabolism, insulin secretion and glucokinase expression by Western blots. These experiments showed glucose-dependent increases in each of these parameters with an additional increase with PRL treatment at all glucose concentrations. Similar results were observed with isolated islets (fig. 5). These experiments demonstrate that both glucose and PRL can regulate the expression of glucokinase in islets. Furthermore, this increase in glucokinase expression resulted in a lowering of the threshold for glucose-stimulated insulin secretion and an overall increase in the extent of insulin release.

Because these changes with PRL were observed even in the absence of glucose, PRL appeared to be able to alter glucokinase expression independent of glucose metabolism. Consequently, additional experiments were done with islets cultured in the absence of glucose and then examined for glucose-stimulated

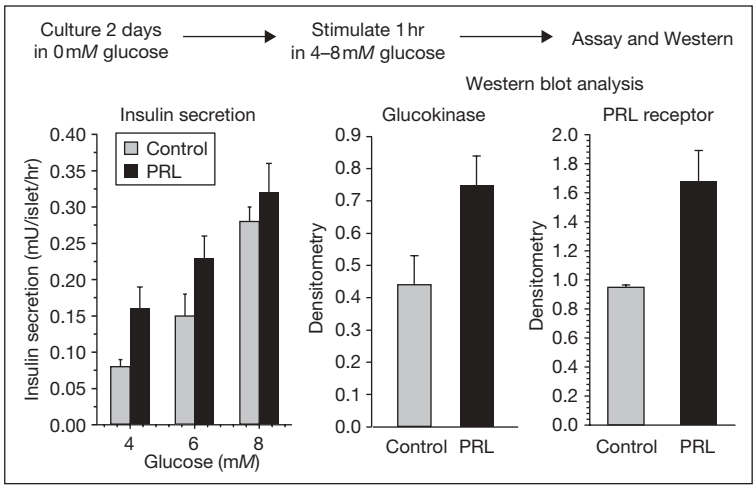


Fig. 6. Effect of prolactin treatment on islets cultured for two days in the absence of glucose. PRL treatment resulted in an increase glucokinase expression (middle panel) and a coordinate increase in glucose stimulated insulin secretion (left panel). An increase in prolactin receptor expression was also observed (right panel).

insulin secretion and glucokinase expression. PRL receptor expression was also examined as this had previously been demonstrated as being under the direct regulation by the PRL/JAK/STAT5 pathway [30]. These results demonstrate that glucokinase expression could be induced by PRL treatment and that it did not require glucose or its metabolism for the effect to be observed (fig. 6). These experiments also show that the increase in glucokinase induced by PRL in the absence of glucose is sufficient to lower the threshold of glucose-stimulated insulin secretion. This raised the possibility that glucokinase, like the PRL receptor, may be directly regulated by a STAT5 mediated mechanism.

STAT5 binds to DNA by way of a GAS related sequence (GRE) which consists of the consensus motif TTC(n = 1–4)GAA. Six of these putative STAT5 binding sites can be found in the rat β-cell glucokinase promoter [46]. To examine the possibility that glucokinase is regulated by a STAT5 mechanism, experiments were done to characterize the putative interaction between STAT5 and GRE containing oligonucleotides from the glucokinase promoter. The initial experiments were southwestern analysis and consisted of treating INS-1 cells with PRL. The nuclei were extracted and electrophoresis and first examined by Western blots, which demonstrated the presence of activated (phosphorylated) STAT5. Probing with a GAS containing oligonucleotide from the glucokinase promoter revealed binding to STAT5. Similar results were obtained when examining GRE containing oligonucleotide probes from the PRL receptor promoter

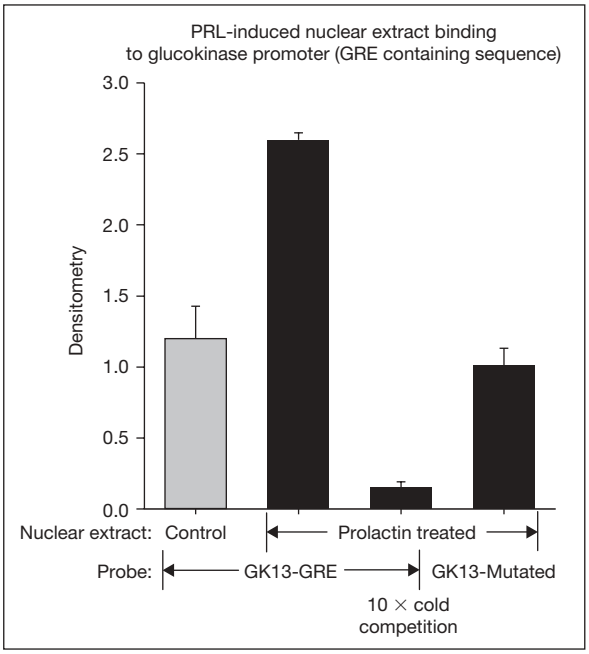


Fig. 7. Effect of prolactin treatment on nuclear extract protein binding to a GRE containing oligonucleotide sequence (GK13-GRE) from the glucokinase promoter. The EMSA shows that prolactin treatment induces a protein which is capable of binding GK13-GRE, but not when its STAT5 binding motif is mutated (GK13-mutated). The putative STAT5 binding of labeled GK13-GRE is competitively inhibited by incubating with a 10-fold excess of unlabeled probe.

and the insulin promoter. To further characterize the interaction between STAT5 and the glucokinase promoter, electromobility shift assays (EMSA) were done. In these experiments, a GRE-containing oligonucleotide probe of the glucokinase promoter was incubated with nuclear extracts from INS-1 cells that were treated with or without PRL for 30 min and then separated by electrophoresis. Nuclear extract protein that bound to the probe was identified as a retarded band. The retarded band from the PRL treated cells had a density 2.5-fold greater than that observed for the control cells. The band density was reduced to control levels by competition with a cold probe. Similarly a mutated probe was unable to bind greater than that observed for the control (fig. 7). Evidence that the retarded band was STAT5 was demonstrated in supershift experiments. A supershifted band was produced by the binding of anti-STAT5b, but not anti-STAT5a (fig. 8). These data demonstrate that PRL-treatment induces the binding of STAT5b to GRE sequences in the glucokinase promoter and is evidence that glucokinase levels are increased in PRL treated islet cells by way of a STAT5b mediated mechanism.

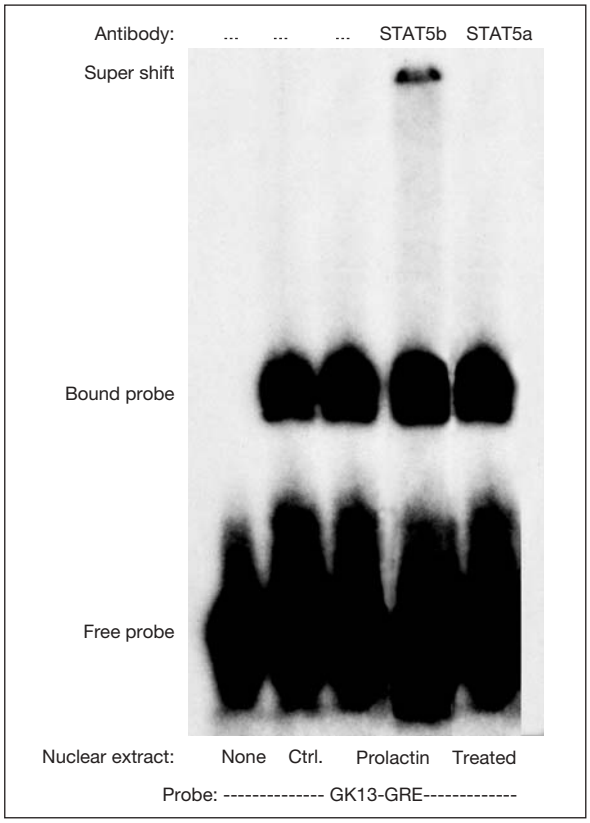


Fig. 8. Supershift experiment of prolactin induced binding of the GRE containing oligonucleotide sequence (GK13-GRE) from the glucokinase promoter. Inclusion of anti-STATb, but not anti-STATa, in the incubation media results in a supershift of the bound probe and is evidence that STAT5b contributes to protein retardation in the EMSA experiments.

Previous studies have also reported an increase in c-AMP metabolism during pregnancy and proposed that it was responsible for the increase in glucose-stimulated insulin secretion [40, 41]. An increase in c-AMP levels has been shown to potentiate insulin secretion and lower the threshold for glucose-stimulated insulin secretion in control islets [42, 47–49]. We re-examined this issue by studying c-AMP metabolism in islets during pregnancy and after treatment with PRL in vitro [50]. This study indicated that these changes c-AMP metabolism are most likely a consequence of the increased glucose metabolism and not a primary event in altering the pattern of insulin secretion. Support for this idea is that under all conditions studied, whether pregnancy, PRL treated or

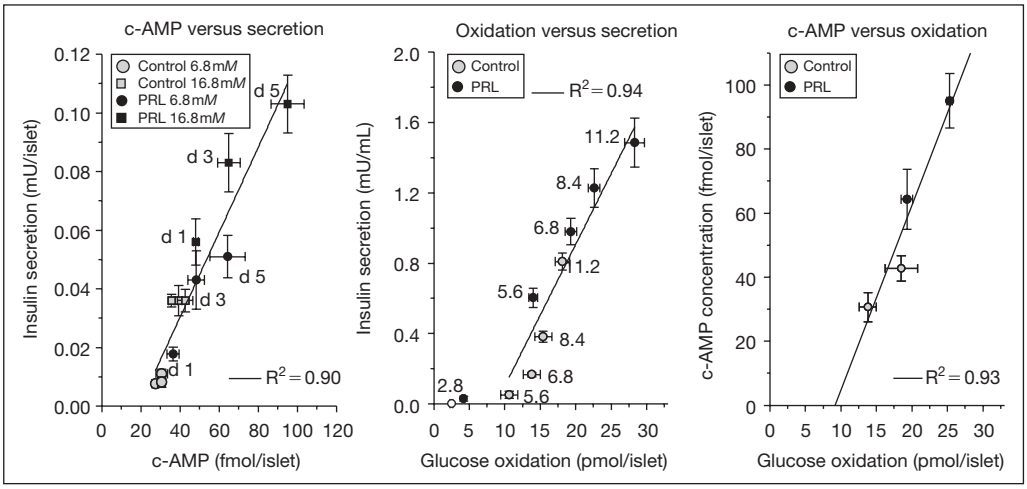


Fig. 9. Summary of data on the relationships among PRL treatment and glucose metabolism, c-AMP metabolism and insulin secretion. Under all treatment conditions, with and without PRL and with different concentrations of glucose, the levels of c-AMP are comparable for similar rates of glucose metabolism. The results suggest that the changes in c-AMP metabolism are most likely a consequence of the increased glucose metabolism and not a primary event in pregnancy or PRL induced alterations of insulin secretion.

with different glucose concentrations, the levels of c-AMP are comparable for similar rates of glucose metabolism (fig. 9).

Although the amount of insulin secretion in pregnancy or PRL-treated islets is higher than that in control islets at the same glucose concentration, it is appropriate for the amount of glucose oxidation observed in control islets at a higher glucose concentration [50]. This is strong evidence that the increased insulin secretion seen in pregnancy or PRL-treated islets in vitro is primarily due to an increase in glucose oxidation because of the increase in glucokinase activity. Thus, it is expected that in addition to c-AMP, all pathways normally influenced by glucose metabolism in islets will be upregulated in parallel to the enhanced glucose metabolism that occurs as a consequence of the increase in glucokinase protein and its activity.

Model of the Mechanisms by which PRL and PL Induce Changes in β-Cells during Pregnancy

Based on the available information, we have developed a model for lactogen regulation of islets (fig. 10). During pregnancy, islets undergo a number of

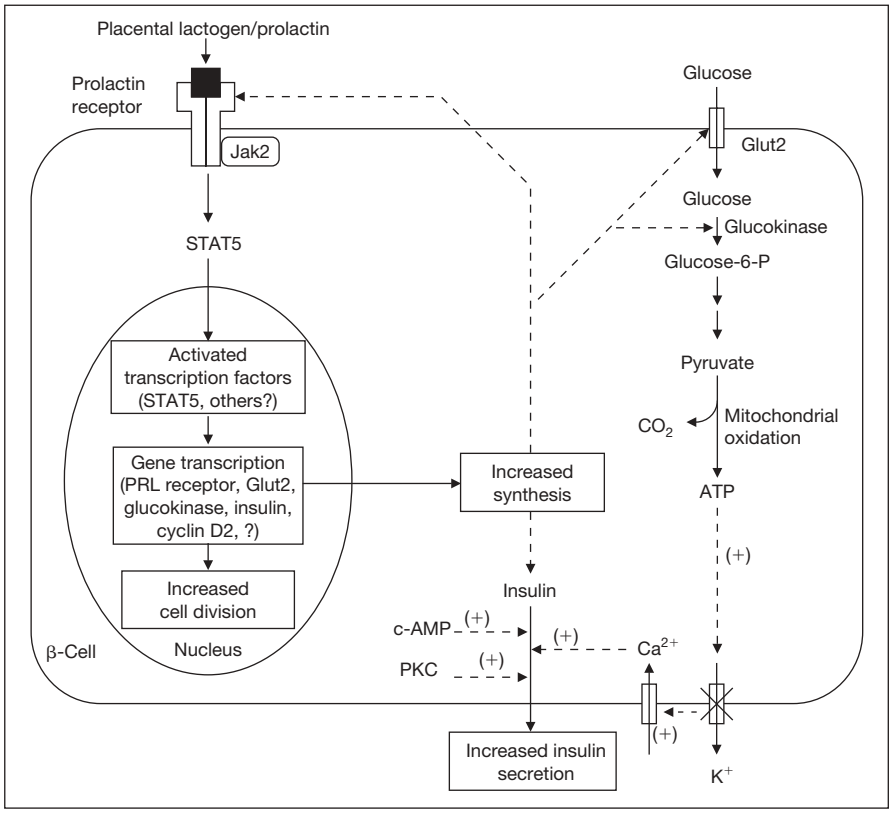


Fig. 10. A model of the mechanisms whereby prolactin/placental lactogen bring about changes in islets characteristic of those observed during the adaptation of islets to pregnancy.

changes to adapt to the increased demand for insulin. These changes occur under direct influence of lactogenic hormones and under normal blood glucose conditions. This is not an acute short-term regulatory process, but rather occurs over an extended period of time. The most significant outcomes are an increase in islet mass and enhanced insulin secretion with a lowering of the threshold of glucose-stimulated insulin secretion. These are two quite diverse cellular processes, one which is common to many cells (mitosis) and one which is highly differentiated (insulin secretion).

Binding of PL or PRL to the PRL receptor on β-cells leads to the activation of tyrosine kinase JAK2 and the transcription factor STAT5. With translocation of activated STAT5 to the nucleus, an increase in the expression of several genes required for the upregulation of islet function occurs. An increase in the expression of the PRL receptor leads to an increased sensitivity to lactogens.

The increased expression of glucokinase results in enhanced glucose metabolism. This increase in glucose metabolism is the primary event responsible for the lowering of the threshold of glucose-stimulated insulin secretion. As a consequence of this increase in glucose metabolism, all other regulatory pathways, such as cAMP and intracellular calcium concentration [42, 51, 52], are activated at higher levels than normally observed for a comparable concentration of glucose in normal (i.e., non-pregnant) islets. The combination of this lowering of the threshold and increased activity of regulatory pathways results in a marked enhancement of insulin secretion at normal blood glucose levels. An increase in the expression of the cell cycle regulator cyclin D2 should lead to an increase in β-cell proliferation [53, 54]. This effect will be further increased due to the stimulation of β-cell proliferation by the enhanced glucose metabolism. Although this increase in islet mass contributes to the increased insulin secretion during pregnancy, it is only by the lowering of the threshold that islets can secrete large amounts of insulin without the need for prolonged hyperglycemia.

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Regulation of Glucokinase by Vitamins and Hormones

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All cells regulate gene expression in response to changes in the environment. Multicellular organisms report these environmental changes to the cell through nutritional and hormonal signals. As a critical regulator of glucose and insulin homeostasis, glucokinase activity must respond to these nutrient and hormonal signals, but this response needs to vary depending on the tissue. Early studies by Bedoya et al. [1], before the development of molecular biology techniques, suggested that tissue specific regulation might be achieved through the expression of different glucokinase isoenzymes in the liver and pancreatic β-cell. The discovery of alternate promoters in a single glucokinase gene [2–4] strengthened the concept that distinct signals regulate glucokinase expression in the two tissues and provided a conceptual framework for understanding how the dual regulation of glucokinase might occur.

Molecular Mechanisms of Glucokinase Regulation

Initial Induction of Glucokinase Expression

The critical importance of nutrient and hormonal signals is first manifested by the timing of expression of the hepatic isoenzyme. In rats, in contrast to the early fetal expression of glucokinase in pancreatic β-cells [5], glucokinase activity in the liver cannot be detected until 2 weeks after birth [6] and then rises rapidly over 10–12 days to the adult level [6, 7]. The appearance of the hepatic isoenzyme at this comparatively late stage of development represents

one of the final steps of liver maturation and coincides with the weaning from milk to a solid diet, and the resulting changes in the hormonal milieu. No single signal induces glucokinase expression in the neonatal liver: the increased glucose content in solid food, elevated adrenocortical secretions [8], a rising portal insulin/glucagon ratio [9], and the presence of thyroid hormone all are required to initiate expression of the enzyme in the liver [10].

Precocious induction of hepatic glucokinase can be promoted in vivo and in vitro by several nutrient and hormonal signals. Studies in vivo have shown that insulin [6], glucose [11], glucocorticoids [7] and thyroid hormone [10], promote the precocious expression of the liver isoenzyme. In vitro, insulin [12, 13], thyroid hormone [13] and retinoic acid [14], but not glucose [13], accelerate glucokinase expression. The normal developmental accumulation of glucokinase can be delayed by pharmacological doses of estradiol [7], as well as a several physiological signals. Galactose, which along with glucose forms the disaccharide lactose found in milk, inhibits expression of glucokinase in the liver, as do epinephrine, glucagon, and cyclic AMP agonists [11].

Regulation of Glucokinase in the Adult Liver

The activity of the hepatic isoenzyme is regulated at the level of gene transcription in response to fasting and refeeding [3, 15], with insulin and glucagon serving as the mediators of this response [16]. In addition, other hormones involved in energy homeostasis, as well as metabolites and vitamins, further modulate hepatic glucokinase gene expression (table 1).

Insulin. Several studies in different experimental systems have demonstrated that insulin increases glucokinase activity and expression. In vivo studies in streptozotocin-diabetic rats have shown that insulin stimulates glucokinase enzyme activity [17] and mRNA expression [2, 18, 19]. A decrease in the turnover of glucokinase protein also has been observed in response to insulin [20].

In isolated hepatocytes, insulin induces glucokinase activity, protein synthesis and mRNA levels [21, 22], and these effects are not dependent upon the concentration of glucose [22]. Nuclear run-on analyses have shown that the increase in mRNA levels is due, at least in part, to a direct effect on gene transcription [22].

Early attempts to identify cis-acting elements regulated by insulin in the hepatic glucokinase promoter were unsuccessful [23, 24], but more recently it has been discovered that the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) acts as a mediator of glucokinase induction by insulin [25]. Insulin stimulates SREBP-1c and glucokinase gene transcription

Table 1. Hormones and vitamines involved in modulation of glucokinase gene expression

	Immature hepatocyte		Adult hepatocyte		Pancreatic β-cell	
	effect	ref.	effect	ref.	effect	ref.
Insulin	↑ activity ↑ mRNA levels ↑ mRNA synthesis	6, 12 13 13	↑ activity ↑ protein synthesis ↑ mRNA levels ↑ mRNA synthesis	17, 21, 18 20, 21 2, 16, 18, 19, 22 22, 19	↑ mRNA levels ↑ promoter activity	see chapter by Leibiger, pp. 249–261
Glucagon (cAMP)	↓ activity	11	↓ activity ↓ protein synthesis ↓ mRNA levels ↓ mRNA synthesis	17 20 22, 16 22	↑ activity ↑ mRNA levels ↑ promoter activity	47 47 47
Glucocorticoids	↑ activity	7	↑ activity ↑ protein synthesis ↑ mRNA levels	15, 28, 29, 30, 31 15, 29, 31 15	↑ activity ↑ mRNA levels	48 48
Thyroid hormone	↑ activity ↑ mRNA levels ↑ mRNA synthesis	10 13, 14 14	↑ activity ↑ protein synthesis ↑ mRNA levels ↑ mRNA synthesis	15, 32 15, 32 15, 33 33	no effect: activity no effect: protein mass ↓ mRNA levels	48 49 48, 49
Pregnancy hormones	↓ activity	7			↑ activity	chapter by Sorenson, pp. 222–239
Growth hormone					↓ protein mass* no effect: mRNA levels	49 49
Exercise					↓ mRNA levels	50
Fatty acids			↑ activity	34, 35		
Biotin			↑ activity ↑ mRNA levels ↑ mRNA synthesis	37, 38, 39, 41 40, 41 40	↑ activity ↑ mRNA levels	51 51, 52
Retinoic acid	↑ mRNA levels ↑ mRNA synthesis	14 14	↑ activity ↑ mRNA levels	42 42	↑ activity ↑ mRNA levels ↑ activity promoter	48, 55 48, 55 55

*In fetal islets but not in adult islets.

through the insulin receptor-phosphoinositol 3-kinase signaling cascade [22, 26], via the protein kinase B/cAkt [27].

Glucagon. In the liver, glucokinase activity and mRNA levels are decreased by glucagon [16, 22]. This effect is consistent with the role of glucagon in stimulating gluconeogenesis and glycogenolysis, while suppressing glycolysis and glycogen synthesis, pathways that utilize glucokinase. Glucagon binds to a G-protein-linked receptor and increases intracellular levels of cyclic AMP (cAMP). Nuclear run-on assays demonstrate that elevated cAMP levels can completely shut off hepatic glucokinase gene transcription within 30 min [16, 22]. When insulin and glucagon both are present, the negative effect of glucagon prevails and the glucokinase gene is repressed.

Glucocorticoids. Studies in vivo have shown that the synthesis of glucokinase induced by glucose refeeding is significantly enhanced by the presence of glucocorticoids, whereas the steroid hormone per se has no effect [29], suggesting a permissive role for the hormone in glucokinase synthesis. Furthermore, in adrenalectomized rats, refeeding results only in a small increase in glucokinase activity, synthesis and mRNA levels [15]. Glucocorticoids also stabilize the glucokinase mRNA [15]. These positive permissive effects on glucokinase induction are countered by the enhancement of glucokinase degradation by glucocorticoids [29]. The permissive effect of glucocorticoids on glucokinase activity has also been observed in cultured hepatocytes [30]; however, Spence and Pitot [31] found that dexamethasone also increased glucokinase activity and mass in the absence of insulin, suggesting a direct effect of the glucocorticoids in the regulation of the enzyme.

Thyroid Hormone. Tri-iodothyronine [T3] also plays a permissive role in hepatic glucokinase expression. In rats, T3 enhances glucokinase induction by refeeding, but has no effect in a starved animal [32, 33]. Thyroid replacement in either thyroidectomized rats [32, 15] or in the media of cultured hepatocytes isolated from thyroidectomized rats [31] increases hepatic glucokinase activity. T3 enhancement results from increased transcription from the glucokinase gene, without any effect on mRNA half-life [15, 33].

Fatty Acids. Rat liver glucokinase activity is inhibited specifically by long chain acyl-CoA [34, 35]. The inhibition is competitive with both ATP and glucose and does not affect the positive cooperation that glucokinase normally displays with glucose (Hill coefficient).

Biotin. In addition to its role as a carboxylase cofactor, a growing body of evidence supports a role for the vitamin in development and gene expression [36]. Several lines of evidence demonstrate that biotin regulates hepatic glucokinase gene expression. In biotin-deficient rats, hepatic glucokinase levels are decreased [37], and biotin supplementation can increase hepatic glucokinase levels in both normal and biotin-deficient rats [38]. In alloxan diabetic

rats, biotin treatment can increase glucokinase activity to values similar to those observed in nondiabetic rats [39]. Biotin stimulates glucokinase synthesis at the level of gene transcription [40]. The effect of biotin on glucokinase is also observed in cultured rat hepatocytes [41] and appears to be produced through biotin-induced increases in cyclic GMP [41].

Retinoic Acid. It is now well established that the pleiotropic effects of vitamin A, with exception of the vision process, are mediated by its acid derivatives. In addition to retinoic acid prematurely inducing hepatic glucokinase mRNA levels in neonatal rat hepatocytes [14], it can also increase glucokinase activity in mature fully-differentiated hepatocytes, as well as in neonatal hepatocytes already expressing glucokinase [42].

Regulation of Glucokinase in the β-Cell

In the pancreatic β-cells, glucose plays an important role in the modulation of glucokinase activity [1, 43–45]. Until recently, most evidence suggested that glucose regulates glucokinase at the posttranscriptional level [45, 46]. However, recent studies suggest that glucose affects several steps of pancreatic glucokinase expression including transcription. Some of these effects are mediated via insulin secreted in response to glucose. Metabolic regulation of glucokinase expression is reviewed in other chapters of this book.

Cyclic AMP. One of the main physiological differences between the hormonal regulation of glucokinase in the liver and pancreas lies in the response to cAMP. Pancreatic glucokinase activity, mRNA levels and promoter activity increase in response to cAMP [47], in contrast to the repressive effect of cAMP on hepatic glucokinase activity and expression [16, 22]. This difference fits with the distinct roles of cAMP in these two tissues. In the liver, cAMP mediates the effects of the counter-regulatory hormone glucagon, which stimulates glucose production by the liver, and therefore opposes the glucose catabolic pathways involving glucokinase. In contrast, in the β-cell, cAMP mediates the effects of glucagon-like peptide 1 (GLP1), the meal stimulated incretin hormone which amplifies glucose-stimulated insulin secretion.

Thyroid Hormone. The protein levels and activity of pancreatic glucokinase are not affected by T3 despite the negative effect of this hormone on glucokinase mRNA levels in the pancreas [48, 49]. Together, these data suggest that additional mechanism(s) must increase glucokinase synthesis or decrease protein turnover in response to T3.

Glucocorticoids. Similar to its effects on the hepatic isoenzyme, dexamethasone increases activity and mRNA levels of the pancreatic glucokinase isoenzyme in the pancreatic β-cell line RIN5mF [48].

Growth Hormone. Growth hormone treatment does not alter glucokinase mRNA levels in either fetal or adult cultured pancreatic islets. In contrast, treatment with the hormone in vitro significantly reduces glucokinase protein levels in fetal islets, but not in adult islets [49].

Pregnancy Hormones. In order to adapt to the increased demand of insulin that occurs during pregnancy, pancreatic islets undergo structural and functional changes. As part of these changes, glucokinase expression and activity increase. The effect appears to be orchestrated by prolactin. This issue is discussed in detail in another chapter in this book.

Exercise. Exercise training enhances insulin sensitivity and decreases insulin secretion. In exercise-trained rats, glucokinase mRNA levels are decreased [50]. Furthermore, a significant correlation ($r = 0.95$) was found between the decreases in glucokinase and proinsulin mRNA levels, suggesting that the expression of these genes is regulated in parallel. The signals that mediate the effects of exercise on pancreatic glucokinase have not yet been identified.

Biotin. As in the liver, biotin also stimulates the expression of glucokinase in the pancreas. Increases on mRNA levels and enzyme activity have been observed in response to biotin treatment in cultured islets [51] and in the pancreatic RIN 1046–38 cell line [52]. In biotin-deficient rats, glucokinase activity and mRNA levels are 50% less than in normal rats [51]. Interestingly, it has been found that the precursor of the β-subunit of propionyl-CoA carboxylase, a biotin-dependent enzyme, interacts with β-cell glucokinase and augments its activity [53].

Retinoic Acid. A functional role for retinoic acid in pancreatic β-cells was suggested by the observation that impaired insulin secretion in cases of vitamin A deficiency is restored to normal with the administration of retinoic acid [54]. Studies using the RINm5F pancreatic β-cell line have shown that retinoic acid increases glucokinase activity and mRNA levels [48]. This stimulatory effect is also observed in mature, fully differentiated pancreatic islets in culture, as well as in immature fetal islets [55], suggesting that retinoic acid regulates glucokinase independent of the stage of β-cell maturity and that retinoic acid plays a physiological role in glucose metabolism. Retinoic acid achieves its effects on pancreatic glucokinase, at least in part, through the stimulation of the β-cell glucokinase promoter [55].

Conclusions and Perspectives

The importance of glucokinase in the regulation of glucose and insulin homeostasis makes this enzyme a promising target in the development of therapeutic strategies for the treatment of type 2 diabetes mellitus. Our increasing

knowledge of the role of hormones and vitamins in the tissue-specific regulation of glucokinase, and understanding of the underlying mechanisms by which these factors act provide an intellectual basis for the development of novel pharmacological regulators of glucose metabolism that act through their effects on glucokinase expression and function.

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Regulation of β-Cell GK Gene Transcription by Insulin

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Although glucokinase is encoded by a single-copy gene it exists as two major transcription units that provide the two major isoforms of the enzyme, i.e. the liver isoform and the pancreatic β-cell isoform (BGK). The β-cell differs from its counterpart in the liver only in the first 15 amino acids of the N-terminus [1, 2], which are encoded by two alternative first exons [3, 4]. Two different promoters are responsible for the expression of the respective isoforms in liver and pancreatic islets [3, 5, 6]. The β-cell active (upstream) promoter of the rat glucokinase gene (rBGK) shows no significant sequence homology with the liver (downstream) promoter, which indicates differences in the use of *cis*- and *trans*-acting elements and implies different modes in the nutrient- and hormone-dependent transcriptional regulation. The characterization of the 5′-flanking region of the β-cell-specific transcription unit of the rat glucokinase gene by us [7–9] and others [10–17] revealed the presence of several *cis*-elements that are similar or even identical with those found in insulin gene promoters suggesting common mechanisms in transcriptional regulation. Although glucokinase plays a central role in the stimulus-secretion coupling of the pancreatic β-cell and its dysfunction leads to the development of the MODY2 type of diabetes mellitus, the regulation of its expression in general, and by nutrients and hormones in particular, is poorly understood. Studies concerning a glucose-dependent transcriptional regulation of BGK gene expression led to contradictory results [4, 18–20]. Consequently, the mechanisms and regulatory elements, which link the glucose stimulus with the transcriptional machinery, remain unknown.

Glucose Activates β-Cell GK Gene Transcription Immediately

Because of the above mentioned presence of many similar *cis*-elements in BGK and insulin promoters, i.e. A-box and E-box elements, we speculated that the β-cell transcription unit of the GK gene might be regulated by glucose in a similar, if not identical, way as the insulin gene. We have shown that glucose upregulates insulin gene transcription fast, with the dynamics of an immediate early gene [21, reviewed in 22]. Indeed, stimulation of pancreatic islets (rat and mouse) or insulin-producing HIT T15 cells for only 15 min with 16.7 m*M* glucose resulted in a 2- to 3-fold increase in BGK mRNA levels within 60 min after the start of stimulation [23]. Two processes regulate steady-state mRNA levels: the generation of mRNA, i.e. gene transcription, and mRNA degradation. Employing actinomycin D as a tool that abolishes the synthesis of new mRNA, we found that glucose stimulation does not stabilize BGK mRNA. Moreover, this approach revealed that the BGK mRNA half-life is relatively short, i.e. approximately 60 min. Employing a nuclear run-off assay which reflects the transcription initiation rate, we were able to demonstrate that glucose increases BGK gene transcription in HIT T15 cells as early as 15 min after start of stimulation reaching a maximum 30 min after start of stimulation (fig. 1a) [23]. These data showed that glucose exhibited its effect at the level of BGK gene transcription initiation rather than stabilizing the existing BGK mRNA. In order to analyze the mechanism(s) that link the glucose stimulus with the transcriptional machinery, we established a reporter gene assay by fusing the rat BGK promoter (−278/+123 bp) with the cDNA encoding the enhanced version of the green fluorescent protein (GFP). We used the BGK promoter fragment up to −278 bp because it has been shown to contain all *cis*-elements responsible for both glucose-dependent and β-cell-specific transcriptional control [13, 16]. Employing GFP as the reporter gene allowed us to overcome the limitations set by the amount of tissue when working with primary islet cells due to the possibility to monitor gene expression at the single cell level. Moreover, this approach gave also the advantage to monitor stimulation-dependent BGK promoter-driven GFP expression on-line [23, 24]. Stimulation with 16.7 m*M* glucose resulted in elevated BGK-promoter driven GFP expression and fluorescence in transfected HIT cells, primary islet cells, and in adenovirus-transduced pancreatic islets (fig. 1b) [23].

Secreted Insulin is a Key Factor in Glucose-Stimulated β-Cell GK Transcription

In order to understand the nature of the signal which triggers glucose-stimulated BGK gene transcription initiation, we wanted to know where in the

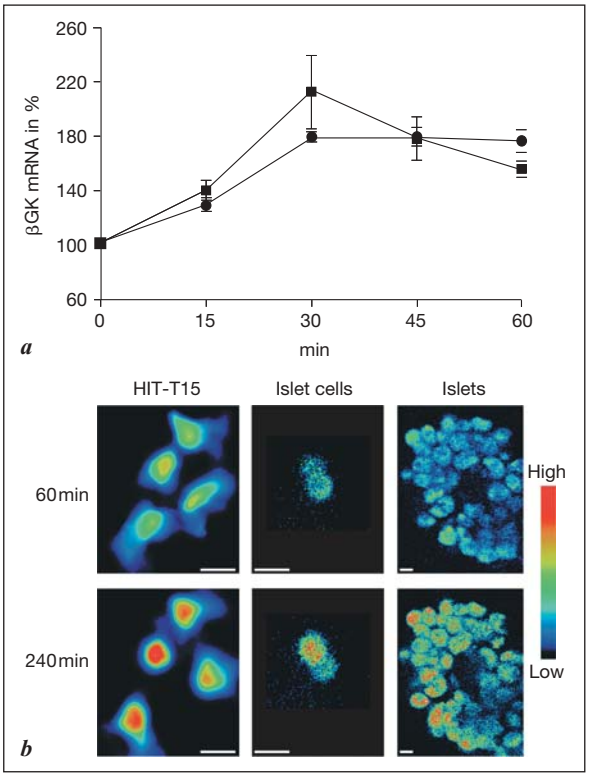

Fig. 1. ***a*** Dynamics of transcription initiation (squares) and cytoplasmic BGK mRNA amounts (circles) in response to short-term glucose stimulation in HIT cells. Transcription initiation was studied by nuclear run-off analysis in nuclei of cells stimulated for 15 min with 16.7 m*M* glucose. Amounts of cytoplasmic BGK mRNA were determined by comparative RT-PCR. Elevation of RNA levels in stimulated cells is shown as percentage of RNA levels of the nonstimulated control (given as 100%). The values of BGK mRNA were normalized to amounts of β-actin mRNA. Data are shown as mean ± SE from three experiments. ***b*** On-line monitoring of glucose-stimulated BGK promoter-driven GFP expression in transfected HIT-T15 cells, islet cells and in whole islets. Representative images of HIT cells (n = 40), islet cells (n = 40) and islets (n = 3) are shown 60 and 240 min after start of glucose stimulation. The pseudo-color images were created by converting the original 'gray-scale' data using ISee software; the fluorescence increases from blue to red. The scale bars represent 10 μm. Data taken from Leibiger et al. [23].

'stimulus-secretion coupling' the signal originates from. So we asked whether enhanced glucose metabolism and ATP-production per se is an absolute requirement for the upregulation of BGK gene transcription or whether the stimulatory signal rather originates from downstream events, linked to the process of insulin secretion. To answer this question we stimulated exocytosis of insulin by either

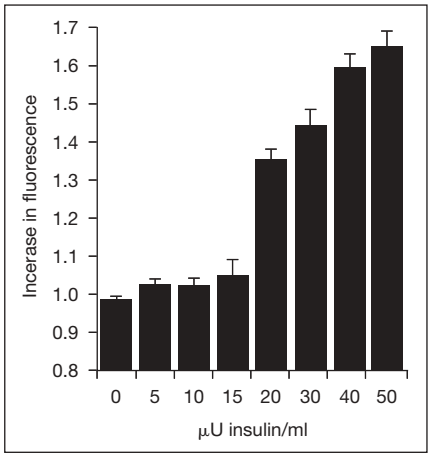

Fig. 2. Effect of increasing concentrations of insulin added to the culture medium for 5 min on BGK promoter-driven GFP expression in transfected HIT cells. On-line monitoring data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± SE (n = 7). Data taken from Leibiger et al. [23].

glucose (16.7 m*M*), thus stimulating glucose metabolism and augmented ATP production, or by depolarizing concentrations of KCl (50 m*M*) or stimulating concentrations of the sulfonylurea compound glibenclamide (1 μ*M*), both at substimulatory glucose concentrations (5.6 m*M* for islets and islet cells and 0.1 m*M* for HIT cells), i.e. depolarizing the β-cell plasma membrane and thereby opening the voltage-gated L-type Ca^{2+} channels without triggering augmented ATP production. Treatment of isolated rat pancreatic islets, primary islet cells and HIT cells with either 16.7 m*M* glucose, 50 m*M* KCl or 1 μ*M* glibenclamide resulted in an enhanced insulin secretion (data not shown) and in an elevation of endogenous BGK mRNA levels as well as in BGK promoter-driven GFP expression. Vice versa, inhibiting insulin secretion by using the L-type Ca^{2+} channel blocker nifedipine (10 μ*M*) abolished stimulus-dependent upregulation of BGK gene transcription/promoter activity [23].

In order to test, whether similar to the insulin gene [25], secreted insulin is a key-factor in glucose-stimulated BGK transcription, we stimulated HIT cells, islets cells or islets with exogenous insulin at substimulatory glucose concentrations. Indeed, addition of only 20 μU insulin per ml of fully supplemented culture medium was sufficient to elevate endogenous BGK mRNA levels as well as BGK promoter-driven GFP expression (fig.2) [23].

Signaling via Insulin Receptor B-Type Is Involved in the Upregulation of BGK Gene Transcription

To test whether insulin activates BGK gene transcription by signal transduction via the insulin receptor we took advantage of the βIRKO mouse, a knock-out

model that lacks the expression of insulin receptors specifically in the pancreatic β-cell [26]. Similar to the insulin gene, stimulation with either 16.7 m*M* glucose or 5 mU insulin/ml led to an increase in endogenous BGK-mRNA levels in islets of wild type mice. No increase in insulin and BGK-mRNA levels was observed in islets prepared from βIRKO mice [23]. These data suggested that signaling through insulin receptors is an absolute requirement to gain the stimulatory effect by insulin on both insulin and GK gene expression and that signaling via IGF-I receptors is unlikely to be involved.

The insulin receptor exists in two isoforms as a result of alternative splicing of the 11th exon [reviewed in 27]. The A-type (Ex11−) lacks the 12 amino acids encoded by exon 11, whereas the B-type (Ex11+) contains the respective amino acid sequence, which is located extracellulary at the C-terminus of the α-subunit of the receptor. Pancreatic β-cells as well as β-cell lines do express both isoforms in an almost one-to-one ratio [Leibiger et al., unpubl. data]. Overexpression of the A-type isoform led to a pronounced effect of insulin stimulation on insulin promoter activation but had no additional effect on BGK promoter activation, while overexpression of the B-type isoform stimulated further the BGK promoter but not the insulin promoter (fig. 3) [23, 25]. More interestingly, selective blocking of B-type receptor signaling using a blocking antibody raised against the 12 amino acids encoded by exon 11 abolished insulin-stimulated upregulation of the BGK promoter (fig. 3) but had no effect on insulin-stimulated upregulation of the insulin promoter in the same cell [23]. This led us to suggest that insulin activates the transcription of its own gene by signaling through the A-type receptor while it activates the BGK gene through the B-type insulin receptor.

Signaling via Insulin Receptor B-Type, PI3 Kinase Class II-Like Activity, PDK1 and Possibly PKB/cAkt Is Required for Glucose/Insulin-Stimulated Activation of BGK Gene Transcription

Insulin, secreted in response to glucose stimulation, upregulates the transcription of its own gene by signaling via the A-type insulin receptor, PI3 kinase class Ia, p70 s6 kinase and CaM kinase II pathways [22, 23, 25]. Interestingly, analysis of the effector proteins involved in glucose/insulin-stimulated activation of BGK gene transcription that are downstream the insulin receptor revealed clear differences from those involved in the upregulation of the insulin gene. Employing pharmacological inhibitors as a tool in cell experiments, we found neither PI3 kinase Ia (25 μ*M* LY294002 or 50 n*M* wortmannin) and p70 s6 kinase (10 n*M* rapamycin) as in the case of the insulin gene, nor other classical insulin-activated effector proteins such as MAP kinases Erk1/2

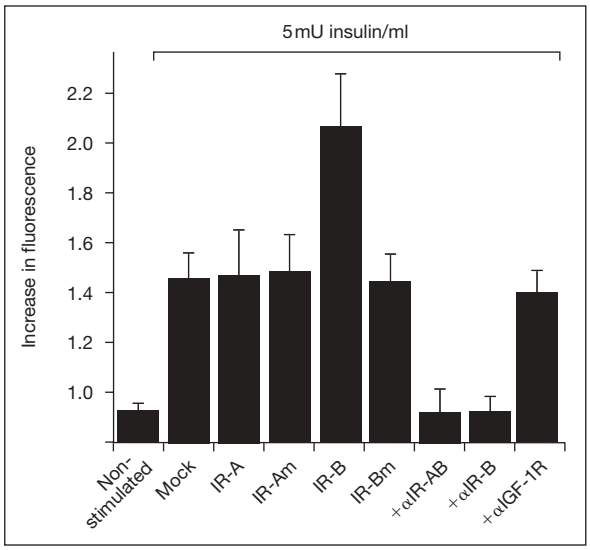

Fig. 3. The role of insulin receptors in insulin-stimulated BGK gene transcription. On-line monitoring of BGK promoter-driven GFP expression in transfected islet cells that were either co-transfected with IR variants or pre-incubated with antibodies against IR or IGF-1R. Cells were co-transfected with prβGK.GFP and either expression constructs for wild type isoforms of IR-A (IR-A), IR-B (IR-B) or the M1153I-mutant of the respective receptor isoform (IR-Am and IR-Bm). Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± SE (n = 10). Effect of antibodies that block signaling through IR-A and IR-B (αIR-AB), through IR-B (αIR-B) and through IGF-I receptors (αIGF-1R) on insulin-stimulated BGK promoter-driven GFP expression in transfected islet cells. Cells were incubated with 0.67 μg/ml of the respective antibodies 30 min prior to stimulation and throughout stimulation. All data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± SE (n = 10). Data taken from Leibiger et al. [23].

(20 μ*M* PD98059), JNK/SAPK1 (10 μ*M* PD169316), p38/SAPK2a (20 μ*M* SB203580) to be involved in insulin-dependent upregulation of BGK promoter activity (fig. 4) [23].

Besides signaling via the MAP kinase and the PI3 kinase Ia/mTOR/p70s6k pathways, insulin has been shown to exert its effect via the activation of PKB (c-Akt) [28]. In insulin-producing HIT cells, PKB activation was observed 5 min following stimulation with 16.7 m*M* glucose and 2 min following stimulation with 5 mU insulin/ml, at substimulatory glucose concentrations. Because of the lack of a selective pharmacological inhibitor of PKB, we tested its involvement in insulin-stimulated BGK gene transcription by transiently overexpressing PKBα/c-Akt1. Whereas overexpression of PKBα had no effect on insulin-stimulated

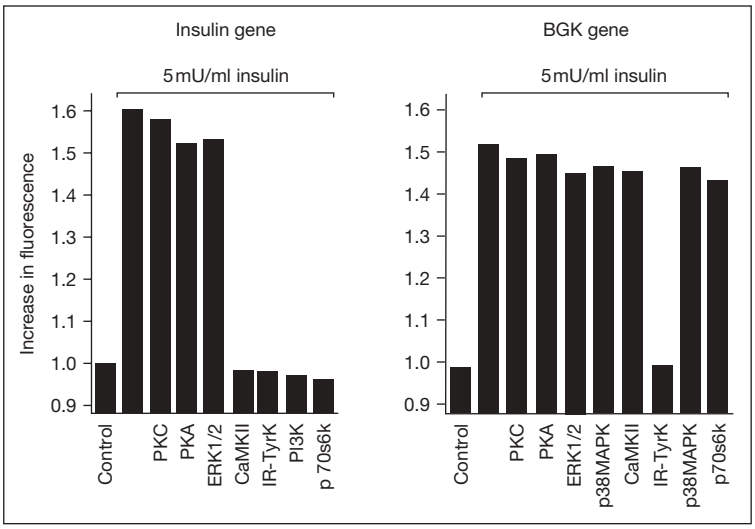

Fig. 4. Effect of various protein kinase inhibitors on insulin-stimulated insulin- and BGK promoter-activity. On-line monitoring of insulin promoter-driven and BGK promoter-driven GFP expression in transfected islet cells. Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± SE (n = 10). Cells were incubated with pharmacological inhibitors for the indicated protein kinases (see text) 30 min prior to stimulation and throughout stimulation. Data taken from Leibiger et al. [23].

insulin gene transcription, it led to a more pronounced effect on insulin-stimulated BGK promoter-driven GFP expression [23]. Vice versa, overexpression of the dominant negative acting PKB-CAAX [29] abolished the insulin-dependent up-regulation of the BGK promoter.

According to the current view, insulin-stimulated PKB activation involves the phosphorylation of PKB by the phosphoinositol-dependent kinase 1, PDK1 [30]. Indeed, transient overexpression of PDK1 led to a pronounced stimulation of insulin-triggered BGK promoter activity, whereas overexpression of the antisense transcript of PDK1 abolished the stimulatory effect of insulin on insulin-triggered BGK promoter activity [23].

The activation of PKB has so far been shown to be dependent on the activity of PI3 kinase [30] and therefore to be sensitive to the independent pharmacological inhibitors wortmannin and LY294002. Whereas treatment of insulin-producing cells with 25 μM LY294002 clearly abolished insulin-stimulated rat insulin-I gene promoter activity, it did not block insulin-stimulated rat BGK promoter activity. When analyzing the effect of LY294002 on insulin-stimulated insulin- and BGK-promoter activity in a dose-dependent manner in cells co-transfected with prBGK.GFP and prIns1.DsRed, we observed that

LY294002 inhibited the two promoters at different concentrations. Whereas 25 μ*M* LY294002 blocked insulin-stimulated insulin promoter activity, 100 μ*M* LY294002 was needed to completely abolish insulin-stimulated BGK promoter activity. The effect of wortmannin was similarly concentration dependent. Treatment of cells with 50 n*M* wortmannin was sufficient to inhibit insulin-stimulated insulin promoter activity, whereas 150 n*M* wortmannin was necessary to block insulin-stimulated BGK promoter activity [23].

In agreement with the above data, we found that the two insulin receptor isoforms do utilize different classes of PI3 kinases. We overexpressed separately A- and B-type receptors that were tagged with GFP and studied the sensitivity of PI3 kinase activity to wortmannin in vitro following immunoprecipitation with GFP-antibodies. Whereas the PI3 kinase activity in the A-type immunoprecipitate was inhibited by wortmannin in the lower nanomolar range, as typical for PI3 kinase class I and III, the PI3 kinase activity in the B-type immunoprecipitate was only inhibited at higher concentrations, as described for PI3 kinase class II [reviewed in 31]. The member of the class II PI3 kinase family that can be activated by insulin and comes closest to the pharmacological profile of inhibiting concentrations of wortmannin and LY294002 is PI3K-C2α [32–34]. It is noteworthy that PI3K-C2α is expressed in pancreatic islets and insulin-producing cell lines [own unpubl. data].

Detection of a Glucose/Insulin-Sensitive *cis*-Element Contributing to Glucose/Insulin-Stimulated BGK Gene Transcription

Next we wanted to evaluate the involvement of characterized *cis*-elements of the BGK promoter in the insulin-dependent transcriptional up-regulation. Although a multitude of genes have been described to be regulated by insulin at the transcriptional level [35] no conserved transcriptional regulatory element has yet been reported. The characterization of the 5′-flanking region of the β-cell-specific transcription unit of the rat glucokinase gene by us [7–9] and others [10–17] revealed similar *cis*-elements with insulin gene promoters suggesting common mechanisms in transcriptional regulation. Our studies on glucose/insulin-stimulated rat insulin-I gene regulation suggest that the insulin signal is mediated via an E-box element because mutations of either the E1-box or the E2-box in that promoter abolished the up-regulatory effect of insulin [25]. Although the BGK proximal promoter also harbors E-box motifs (at −131 and −221 bp), data obtained when using a deletional analysis approach suggested that the glucose/insulin-sensitive *cis*-element is located between nucleotides −121 and −97 (fig. 5a). This implied the possible involvement of an A-box

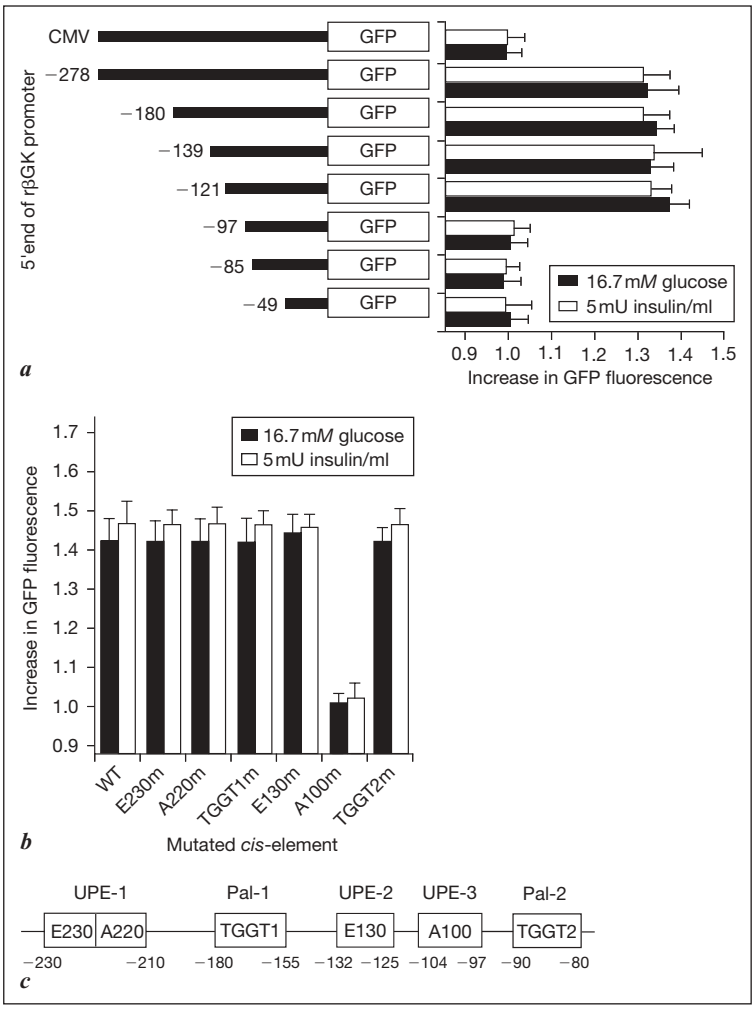

Fig. 5. Role of BGK promoter *cis*-elements in glucose/insulin-stimulated rBGK.GFP expression. ***a*** 5′Deletion analysis of the rat BGK (rBGK) promoter. Islet cells were transfected with GFP-expression constructs containing rBGK promoters with the indicated 5′end. ***b*** Effect of site-directed mutagenesis of individual *cis*-elements within the −278rBGK promoter fragment in transfected islet cells. Transfected cells were stimulated with either 16.7 m*M* glucose for 15 min (black bars) or with 5 mU/ml insulin for 5 min (white bars). On-line monitoring was performed from minutes 60 to 240, following start of stimulation. Data are presented as the ratio of GFP-fluorescence values obtained at minutes 240 and 60 and represent mean values ± SE for at least nine monitored cells. ***c*** Schematic illustration of *cis*-elements within the proximal part of the rat (upstream) BGK promoter up to −278 bp. The nomenclature used by Magnuson et al. [10, 11] is given in the top line.

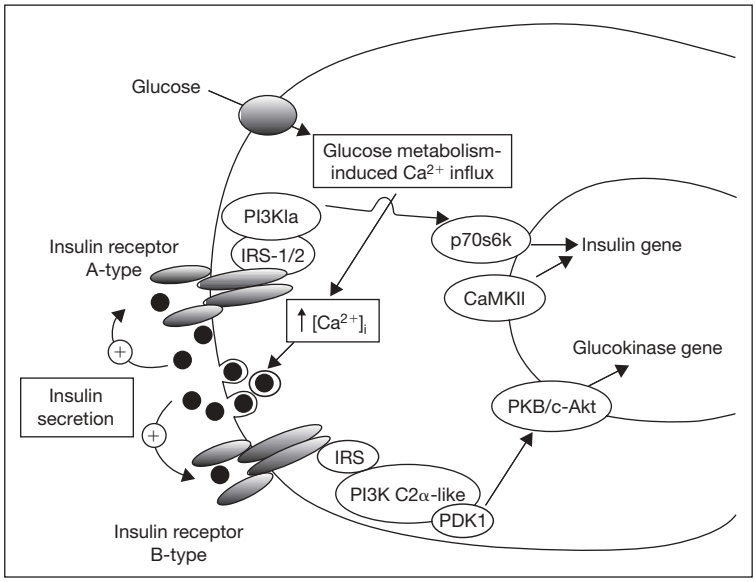

Fig. 6. Activation of BGK and insulin gene transcription by selective insulin signaling via A and B type insulin receptors. The scheme illustrates the coupling between insulin exocytosis and the activation of transcription of BGK and insulin genes [23].

motif at −104 bp (A100 in fig. 5c). In order to corroborate this data, we separately mutated the so far characterized *cis*-elements in prBGK.GFP, i.e. the E-box at −230 bp (E230), the A-box at −220 bp (A220), the TGGT1-box at −180 bp, the E-like motif at −132 bp (E130), the A-box at −104 bp (A100) and the TGGT2-box at −90 bp (TGGT2). Whereas mutation of all other *cis*-elements except A100 had no effect on glucose/insulin-stimulated up-regulation of BGK promoter activity, mutation of the A-box motive at −104 bp (TCTAAT to TgTAcT) abolished the stimulatory effect of glucose and insulin (fig. 5b, A100 m).

The nature of the responsible transcription factor remains to be disclosed. A potential candidate is the homeobox containing transcription factor PDX-1/IPF-1, which has been demonstrated to interact with a BGK promoter fragment containing the discussed A-box [36] and which has been reported to exhibit an increased binding activity towards insulin promoter A-box motifs [37].

Conclusion

Taken together, our data demonstrate that like the insulin gene, the transcription unit of the GK gene is also positively regulated by insulin that is

secreted upon glucose stimulation. However, the activation of the two transcription units involves signaling pathways that differ already at the level of the target receptor for insulin. While insulin upregulates the transcription of its own gene by signaling via insulin receptor A-type/PI3 kinase class Ia/p70 s6 kinase and CaM kinase II pathways, it needs signal transduction via insulin receptor B-type/PI3 kinase C2α-like activity/PDK1 and probably PKB(c-Akt) to upregulate the upstream promoter of the GK gene in pancreatic β-cells (fig. 6). To what extent upregulated GK transcription contributes to an immediate increase in GK protein levels and to the recently reported insulin-dependent recruitment of active GK molecules [38] remains to be elucidated.

Acknowledgments

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Interaction of GK with the Bifunctional Enzyme 6-Phosphofructo-2-Kinase/ Fructose-2,6-Bisphosphatase (6PF2K/F26P_2ase)

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Glucokinase (GK) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/F26P_2ase), also known as the bifunctional enzyme, are key enzymes of carbohydrate metabolism [1–8]. These enzymes are important for the control of glucose homeostasis in both the liver and pancreas. By catalyzing the phosphorylation of glucose, GK acts as the glucose sensor and thereby controls the glucose-stimulated insulin release in β-cells and, in liver acts as a determinant of hepatic glucose output. 6PF2K/F26P_2ase is a regulatory enzyme that synthesizes and breaks down fructose-2,6-bisphosphate (F-2,6-P_2) [7, 8]. High levels of F-2,6-P_2 then dictate the flow of carbon through the glycolytic (β-cell and liver) and gluconeogenic (liver only) pathways by exerting a positive allosteric effect on the 6-phosphofructo-1-kinase (liver and β-cell), and a negative allosteric effect on the fructose-1,6-bisphosphatase. Low levels decrease glycolysis and increase gluconeogenesis [7, 8].

Because of their roles in carbohydrate metabolism, both of these enzymes are important in diabetes. In fact, both have been considered as targets for antidiabetic therapies and have been subject to transgenic and adenovirus-mediated

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overexpression studies [9–13]. Generally, strategies involve increasing GK activity to increase sensing and glycolysis and in the case of 6PF2K/$F26P_2$ase, increasing F-2,6-P_2 concentration to decrease hepatic glucose output [12, 14].

A regulatory nexus has been established between these important enzymes involved in the regulation of carbohydrate metabolism. These enzymes interact in at least two different ways: (1) by effects of F-2,6-P_2, the product of 6PF2K/$F26P_2$ase, on GK gene expression, and (2) by protein:protein interactions between GK and 6PF2K/$F26P_2$ase. Here we describe how these interactions were discovered and their physiological relevance to the regulation of carbon flux, insulin release and diabetes.

Regulation of GK Gene Expression by F-2,6-P_2

An in vivo adenovirus-mediated bifunctional enzyme overexpression system was developed manipulate hepatic F-2,6-P_2 for the purpose of lowering blood glucose levels in diabetic mouse models. It was thought that high levels of F-2,6-P_2 would increase glycolysis and decrease gluconeogenesis and thereby decrease blood glucose levels. This was demonstrated in both type I (STZ-treated) and type II (KK/H1J mouse) diabetic mouse models, where blood glucose levels could be corrected to near control levels [12, 14]. In the STZ-treated mouse, where there is no detectable insulin, GK, which requires insulin for gene expression, was not present [14]. This begged the question of how we were able to see a stimulatory allosteric effect of F-2,6-P_2 on the glycolytic enzyme, 6-phosphofructo-1-kinase, in the absence of the glycolytic gateway enzyme, GK, which is obligate to get flux through glycolysis in the liver.

It was determined that, in the absence of insulin, high levels of F-2,6-P_2 produced by adenovirus overexpression alone could lead to a robust increase in the expression of GK (see fig. 1 for Northern and Western blot analysis of both STZ-treated (fig. 1a) and KK mice (fig. 1b)). STZ treatment caused a dramatic decrease in hepatic F-2,6-P_2 content, accompanied by a 14-fold decrease in glucokinase and a 3-fold increase in glucose-6-phosphatase (G-6-Pase) proteins [15]. These observations are consistent with the loss of insulin action on the bifunctional enzyme, GK, and G-6-Pase. Upon adenoviral overexpression of a bisphosphatase-deficient 6PF2K/$F26P_2$ase (Ad-Bif-DM), hepatic F-2,6-P_2 content was elevated. In response to the elevation, the protein level of glucokinase was increased 13-fold compared to diabetic control mice.

A time course showed that increase in GK message closely follows the increase in F-2,6-P_2 and lactate concentration, that is, increased glycolytic flux. After restoration of hepatic GK protein, hyperglycemia was reduced to near

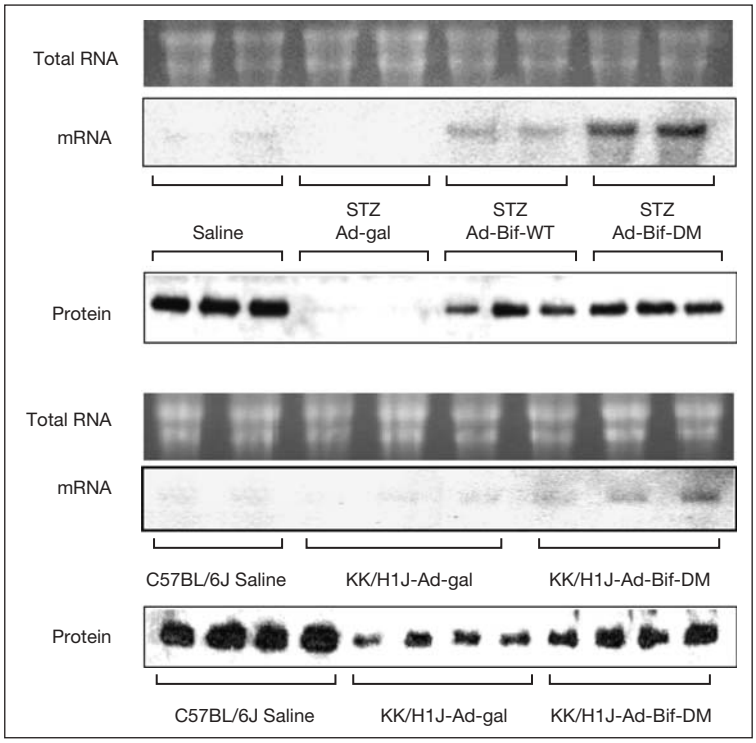

Fig. 1. Effects of F-2,6-P_2 on hepatic glucokinase gene expression. STZ-treated 129J mice and KK/H1J mice were used as type 1 and type 2 diabetic model, respectively. 129J or C57BL/6J mice treated with saline were used as normal control. Diabetic mice were treated with adenovirus encoding a mutant of 6PF-2-K/F-2,6-P_2ase (Ad-Bif-DM) or control virus Ad-gal for 7 days. Hepatic gene expression of glucokinase was analyzed at levels of message and protein.

normal levels. High F-2,6-P_2-induced repression of G-6-Pase is a later event which we attribute to the glucose-lowering effects of high F-2,6-P_2. Lowering the blood glucose would lead to a decrease in the glucose stimulation of G-6-Pase expression. Glucose presumably does this by antagonizing the negative insulin-mediated effects on G-6-Pase gene expression. Since HNF-1α plays a role as an accessory factor for inhibitory action of insulin on G-6-Pase gene expression [16], the finding that upon increasing F-2,6-P_2content HNF-1α protein is increased is consistent with an involvement of HNF-1α in the downregulation of G-6-Pase expression. Interestingly, an elevation in HNF-1α in protein amount was observed. These data argue for a more direct effect of F-2,6-P_2 content on GK gene expression than that on G-6-Pase.

In KK/H1J mice, high levels of F-2,6-P_2 also stimulated GK gene expression by 4.3-fold with respect to message and 1.8-fold with respect to protein when compared animals treated with control virus. This effect was not as dramatic as in the STZ-treated mice due to the presence of insulin in these mice. In contrast to STZ-treated mice these mice are hyperinsulinemic. They are also insulin resistant, which could explain the inability of insulin to fully induce the GK gene expression. By stimulating GK gene expression, high F-2,6-P_2 may be circumventing this insulin resistance.

Intriguingly, the magnitude of the response of the GK gene is dependent on the change in F-2,6-P_2 concentration and not on the absolute amount of F-2,6-P_2. This is illustrated in a comparison of the type 1 and type 2 diabetic models, minus and plus Ad-Bif-DM, where virus-treatment increases the F-2,6-P_2 to the same level, 13.5 nmol/g, in both models. Due to the higher basal level of F-2,6-P_2 in the KK/H1J type 2 diabetic mice, the magnitude of the increase was much less than that in the STZ-treated mice. In the type 2 (KK/H1J) diabetic mouse model the 2.2-fold increase in F-2,6-P_2 level corresponds to a 1.8-fold increase in GK protein, and in the type 1 (STZ-treated) model a 15-fold increase lead to a 13-fold increase in GK protein. This indicates that the effect of high F-2,6-P_2 on GK gene expression is likely metabolic, where the change in concentration has produced a metabolite that has a direct effect on GK transcription. This means that it may be the rate of flux through the 6-phosphofructo-1-kinase/fructose-1,6-bisphosphatase cycle that is proportional to the increase in GK gene expression. Given the allosteric effects of F-2,6-P_2 on 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase, we postulate that a metabolite past fructose-6-phosphate is elevated in the glycolytic direction or a metabolite past fructose-1,6-bisphosphate is decreased in the gluconeogenic direction, to promote the stimulatory effect on GK gene expression. However, in vivo NMR data show that the hexose-6-phosphate pool is close to equilibrium [17].

Since GK gene expression is thought to be under the control of insulin [11, 18–20], it was reasonable to postulate that the effect of high F-2,6-P_2 was mediated through an insulin-signaling pathway and that the effect of high F-2,6-P_2 feeds into that pathway downstream of insulin. Therefore, the level and phosphorylation state of proteins in the insulin-signaling pathway were examined in liver extracts of the diabetic mouse models. While no differences in amount or phosphorylation state were seen in the insulin receptor or IRS1 and IRS 2, changes the amount and phosphorylation state of Akt2 were observed upon elevation of F-2,6-P_2. Akt2 is a key downstream component of the insulin-signaling pathway. The activation of Akt2 by phosphorylation mediates many aspects of insulin action. In the livers of type 1 (STZ) diabetic mice having a very low concentration of hepatic F-2,6-P_2, phosphorylation of Akt

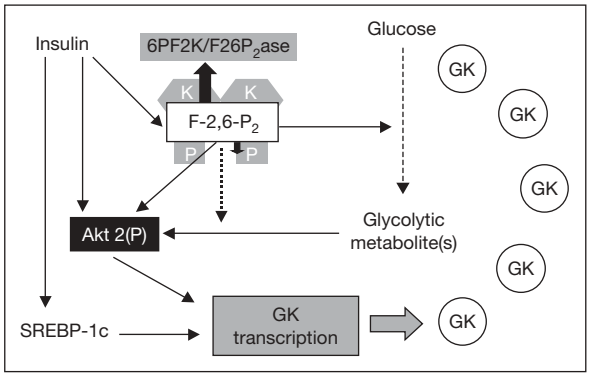

Fig. 2. The scheme for the regulation of hepatic gene expression of GK. See text for explanation.

(ser473) was decreased, presumably due to a lack of insulin. Upon increasing hepatic F-2,6-P_2, both amount of Akt2 protein and phosphorylation were up-regulated (see fig. 2 for schematic representation). These changes would lead to the downstream activation of GK gene expression. Identification of Akt2 as a F-2,6-P_2-responsive signaling intermediate gives us a toe-hold in mapping the F-2,6-P_2-driven signaling pathway. Hepatocyte systems are currently being used to map and to determine similarities between this pathway and the insulin-signaling pathway.

The stimulatory effect of high F-2,6-P_2 on GK expression does not appear to be dependent on SREBP-1c. SREBP-1c may mediate the effect of insulin on induction of hepatic GK gene expression [11, 21, 22]; however, no change in SREBP-1c was detected upon increasing hepatic F-2,6-P_2 [15]. This suggests that SREBP-1c is not involved and that there is an SREBP-1c-independent pathway for induction of hepatic GK gene expression. This possibility was supported by a recent study in primary hepatocytes, which showed that SREBP-1c failed to induce GK gene expression [23].

GK-6PF2K/F26P_2ase Protein Interaction: A Novel Mechanism of Posttranslational Regulation of GK

Posttranslational mechanisms are of crucial importance for the regulation of GK activity both in pancreatic β-cells and liver [3, 24]. In liver the glucokinase regulatory protein (GRP) acts as a short-term regulator of GK activity [13, 24, 25]. The GRP functions as a shuttling protein binding tightly to the GK

and translocating the enzyme between the nuclear and cytoplasmic compartment in response to intracellular changes of glucose and fructose metabolites [for details see chapters by Agius and Van Schaftingen, this vol.]. The GRP is an example of a protein, which specifically interacts with the GK and competitively inhibits the activity of the enzyme [26, 27]. Notably the GRP is not expressed in pancreatic β-cells as shown by Northern blot analyses and functional assays [28]. However, there is strong experimental evidence that the modulation of GK enzyme activity through interaction with specific binding partners is also a regulatory principle in pancreatic β-cells [28]. Permeabilization studies using insulin-producing cell-lines revealed that the GK interacts with matrix proteins [28] and insulin granules [29], which prevented diffusion of the enzyme to the outside of a digitonin-permeabilized cell. This model is comprised of a freely diffusible portion of the GK protein in RINm5F cells with high intrinsic activity and a matrix-bound portion with low intrinsic activity [28]. Heat inactivation and protease treatment of cellular extracts clearly indicated that the factor binding to GK is a protein, which was subsequently identified [28]. To identify this protein, we followed a systematic approach for the identification of specific binding epitopes of the GK protein – a random peptide phage display screening [30]. For this purpose, recombinant human β-cell GK protein was fixed to a solid support through a molecular spacer. The immobilized GK protein was exposed to a phage library which randomly displayed 12mer peptides on their surface. Through a stringent selection process we identified consensus sequences for binding epitopes, which were further analyzed for homologous proteins in protein databases. As expected from the GK-GRP interaction in liver we first identified the -LSA-XX-VAG- motif as the binding epitope of the GRP (swissnew Q14397 GCKR human glucokinase regulatory protein, swissnew Q07071 GCKR rat glucokinase regulatory protein). This result served as an internal validation of the methodology because we found a sequence motif of a well-known GK interaction partner.

In addition to the GRP binding sequence we identified a second consensus motif – SLKVWT – which showed a homology to the bisphosphatase domain of the bifunctional enzyme 6PF2K/F26P_2ase (swissprot P0793 F261 rat 6PF2K/F26P_2ase; swissprot P16118 F26L human 6PF2K/F26P_2ase). In binding assays and yeast-two hybrid experiments, the interaction of the GK protein with 6PF2K/F26P_2ase proved to be one third as strong as the interaction with the inhibitory GRP [30].

6PF2K/F26P_2ase proteins are expressed from at least four different genes designated PFKFB1–PFKFB4 [8]. The binding epitope within the bisphosphatase domain of 6PF2K/F26P_2ase is conserved among the different isoforms of this regulatory enzyme [30]. The well-characterized liver 6PF2K/F26P_2ase

enzyme is coded by the PFKFB1 gene locus and is modulated in the kinase/bisphosphatase ratio by a cAMP-dependent protein kinase A [8]. Pancreatic islets express a brain isoform which is coded by another gene locus, the PFKFB2 [8]. Interestingly, the brain isoform of 6PF2K/F26P_2ase shows various splice variants within the linker region between the kinase and bisphosphatase domain of the enzyme [31]. Sequence analysis of 6PF2K/F26P_2ase cDNA from rat pancreatic islets revealed a previously unknown islet-specific variant of this enzyme with 8 additional amino acids in exon 9 [30]. The functional relevance of this splice variant in endocrine cells is not clear at the moment but analogous to the 6PF2K/F26P_2ase splice variants in other tissues it may affect the kinetic properties, susceptibility to post-translational modification and/or the stability of the enzyme [31, 32]. Yeast two-hybrid experiments clearly show that the liver isoform and the islet/brain isoform interact with the GK at comparable binding affinities [30]. Thus, the GK-6PF2K/F26P_2ase interaction could be a principle dictating the coordination of glucose phosphorylation and glycolysis in liver as well as in pancreatic β-cells.

To address the physiological relevance of the GK-6PF2K/F26P_2ase interaction we overexpressed the islet and liver isoform of 6PF2K/F26P_2ase in insulin-producing RINm5F-GK and INS-1 cells [33]. Surprisingly, the overexpression of 6PF2K/F26P_2ase significantly increased the intrinsic activity of GK by 70–110% in both insulin-producing cell lines. Thus, 6PF2K/F26P_2ase proved not to be the expected inhibitory matrix protein from the permeabilization studies [28], rather it appears to be an activator of GK. The activation of GK by 6PF2K/F26P_2ase was achieved by overexpression of the islet isoform, the liver isoform and a mutant liver isoform which lacked the phosphorylation sites of the cAMP-dependent protein kinase A and bisphosphatase activity [34, 35]. This fact is of crucial importance for the mechanism of GK activation because the three isoforms of 6PF2K/F26P_2ase have different kinetic properties with respect to the kinase/bisphosphatase ratio and the generation/consumption of F-2,6-P_2 [8]. Our recent studies provide evidence that the activation of GK is not dependent upon the generation of F-2,6-P_2 because this phenomenon was observed at high intracellular levels (overexpression of bisphosphatase-deficient liver 6PF2K/F26P_2ase, 31 pmol/mg DNA), intermediate (overexpression of islet/brain 6PF2K/F26P_2ase, 20 pmol/mg DNA) and low intracellular levels (overexpression of liver 6PF2K/F26P_2ase, 6 pmol/mg DNA) of this metabolite [33]. Furthermore F-2,6-P_2 did not increase the intrinsic activity of recombinant GK protein which is activated upon direct interaction with 6PF2K/F26P_2ase protein. We also observed that only the complete 6PF2K/F26P_2ase protein but not the F26P_2ase domain was able to activate the GK protein so that an occupation of the binding epitope is apparently not sufficient to induce an increase of GK activity [Baltrusch, unpubl. obs.].

The most feasible mechanism by which the interaction of 6PF2K/F26P_2ase stimulates GK activity is the stabilization of a catalytically favorable conformation of the enzyme protein comparable to the function of a chaperone. Two aspects of GK activity regulation support the postulate of a conformational stabilization:

Glucose is a potent – and probably the most potent – activator of GK activity. Glucose acts in a dual way inducing a release of the GK protein from matrix proteins or secretory granules and, through a slow transition, increases the intrinsic activity by conformational changes induced by the substrate glucose [28, 29, 36, 37]. Recent studies of fluorescent GK proteins support this concept of a glucose-induced conformational change of the enzyme protein [29]. This mechanism likely explains the posttranslational upregulation of glucokinase activity which was observed in pancreatic islets at high glucose concentrations [38–42]. Fructose and fructose metabolites do not act directly upon the GK activity but significantly affect the interaction with the GRP which indirectly determines the accessibility of free GK protein for interaction with 6PF2K/F26P2_2ase in liver [25, 43, 44].

The glucokinase protein shows an extraordinary sensitivity towards SH-group oxidation [44–47]. This oxidation occurs readily when reducing agents are omitted from the buffer and results in a significant decrease of the catalytic activity [48]. The oxidized GK molecule is locked by the multiple intramolecular disulfide bridges which block the conformational changes required for a proper catalytic process [47]. The interaction with 6PF2K/F26P_2ase may protect the GK protein against SH-group oxidation either through stabilization of an enzyme conformation that is less prone to attack of the cysteine residues or through direct protection of free SH-groups.

The recent identification of compounds which allosterically activate GK has led the development of a novel class of antidiabetic drugs which improve the glucose sensor function of GK in pancreatic β-cells [49–51] [see chapter by Grimsby, this vol.]. It remains to be clarified in future studies whether 6PF2K/F26P_2ase is an endogenous activator of GK that acts by a similar mechanism. This knowledge may give also new insights into the mechanism of the GK activation by these chemical compounds.

General Conclusions on GK Regulation

F-2,6-P_2 Activates GK Gene Expression in Liver

In summary, high F-2,6-P_2 levels stimulate hepatic GK gene expression, which is related to the effect of F-2,6-P_2 on promotion of glycolytic flux and activation of signaling through Akt2 phosphorylation. The restoration of GK is

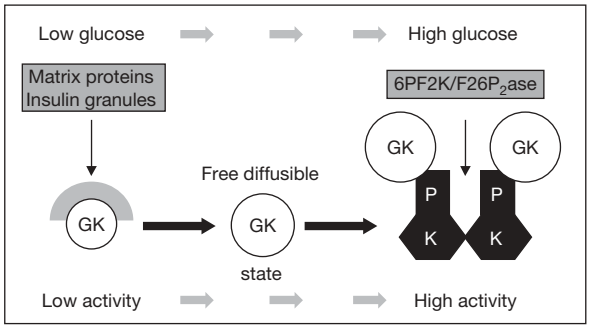

Fig. 3. Activation of GK enzyme activity through interaction with the 6PF2K/F26P_2ase protein in insulin-producing cells. Glucokinase is regulated through interaction with specific proteins which inhibit (matrix proteins, insulin granules) or activate (6PF2K/F26P_2ase) the catalytic function of the enzyme. Glucose confers the interchange from the inhibitory to the activating binding partner.

necessary to lower plasma glucose demonstrating that F-2,6-P_2 coordinates glucose phosphorylation (GK) to glycolysis (6-phosphofructo-1-kinase/fructose-1,6-bisphosphatase cycle). The effect of high F-2,6-P_2 on GK gene expression is shown schematically in figure 2.

The ability to elevate F-2,6-P_2 concentrations in the absence of insulin by adenovirus-mediated overexpression of a bisphosphatase-deficient liver 6PF2K/F26P_2ase has revealed a novel hepatic GK regulating system. Because the high F-2,6-P_2/no-insulin state is not seen in nature, it is difficult to assess the contribution of a high F-2,6-P_2 state to GK gene expression under normal conditions. It may function as a primary signaling molecule for GK gene expression or it may be the downstream target of insulin in its signaling of increased GK gene expression. In either case we have revealed a physiologically important signaling pathway.

GK-6PF2K/F26P_2ase – An Endogenous Posttranslational Activator of GK Activity

While F-2,6-P_2, the enzymatic product of 6PF2K/F26P_2ase, is a key activator of GK gene expression in liver, the interaction between GK and 6PF2K/F26P_2ase is effective on the posttranslational level of GK regulation (fig. 3, and see below). The activation of GK seems to be independent of the intracellular levels of F-2,6-P_2 and relies upon a chaperone function of 6PF2K/F26P_2ase requiring the whole bifunctional enzyme. While the GK interaction is mediated by the bisphosphatase domain, the kinase domain confers the dimerization of the 6PF2K/F26P2$_2$ase. In the liver, the GK switches between

the interaction with the inhibitory GRP and the activating 6PF2K/F26P_2ase. Glucose and fructose-1-phosphate induce a translocation of the GK protein from the nucleus and dissociation from the GK/GRP complex. The dissociated GK interacts with the 6PF2K/F26P2$_2$ase in the cytoplasm, which increases the GK activity. In pancreatic β-cells the regulation of GK is different from that in liver. Under basal conditions of low substimulatory glucose concentrations the GK protein is bound to matrix proteins and/or to secretory granules. In β-cells, the GK protein is always located outside the nucleus. Millimolar glucose concentrations lead to a dissociation of the GK protein from the matrix complex making the GK protein freely diffusible. In this state 6PF2K/F26P2$_2$ase can bind to the GK protein and activate the enzyme. Thus the GK activity is regulated by interaction with inhibitory and activating proteins, which determine the pivotal glucose sensor function for the initiation of glucose-induced insulin secretion and glucose metabolism in liver.

Liver versus Islet GK Regulation

The two regulatory systems described herein are significant and may not be exclusive to the systems in which they have been described, liver for gene expression and islet for protein:protein interaction. The posttranslational mechanism of GK activation by interaction with 6PF2K/F26P_2ase has been demonstrated, to date, only in insulin-producing cells. However, the regulatory principle outlined in figure 3 may also be of relevance for GK activity regulation in liver as both proteins are abundantly expressed in hepatocytes. In contrast to insulin-producing cells, the binding of GK to 6PF2K/F26P_2ase in liver is dependent upon the transcriptional activation of the GK gene and the release from the GRP. In this scenario an increase of blood glucose stimulates GK gene/protein expression via insulin and/or high F-2,6-P_2, and the translocation of GK from the nucleus to the cytoplasm. At this point, the interaction with 6PF2K/F26P_2ase contributes to the increase of GK activity, which is observed under conditions of refeeding in liver. In pancreatic β-cells, the interaction of GK and 6PF2K/F26P_2ase may be crucial element to ensure the glucose sensor function for the metabolic-stimulus-secretion coupling of insulin secretion. Because in pancreatic islets GK protein levels are not significantly affected by the nutritional status, posttranslational mechanisms of GK regulation play a pivotal role for modulation of enzyme activity. The 6PF2K/F26P_2ase could be an integral part of this posttranslational regulation as an endogenous activator of GK. Future studies will address the tissue specific aspects of 6PF2K/F26P_2ase for the regulation of GK and the molecular mechanism of gene activation and protein interaction.

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Role of Sulfhydril Groups in GK Catalysis for GK Function

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Glucokinase – The Most Sensitive Protein of the Hexokinase Enzyme Family

The instability of the glucokinase (GK; hexokinase type IV) enzyme is an important aspect which is easily underestimated in experimental work upon cellular extracts from insulin-producing cells and to a lesser extent also from liver [1–4]. The term 'instability' characterizes a constant loss of glucokinase activity while at the same time the activity levels of the high affinity hexokinase isoforms (types I-III) remain stable. This characteristic of GK has been exemplified in a study using the insulin-producing RINm5F-GK cell line generated through a stable overexpression of human β-cell glucokinase [5]. RINm5F-GK cells, like pancreatic β-cells, express glucokinase and hexokinase enzymes at comparable activity ratios [5]. Cell extracts after sonication show a constant decrease of glucokinase activity by 90% after a 2-hour incubation at 37°C (fig. 1). The hexokinase activities, however, were not significantly altered over a period of 4 h. On the other hand, RINm5F-GK extracts which were incubated on ice at 0°C preserved 90–100% of the glucokinase activity over several hours. Notably, the glucokinase protein levels remained constant at both temperatures indicating a functional loss of enzyme activity rather than proteolytic degradation (fig. 1). These data raise the question which factors are able to stabilize, preserve or activate the function of the glucokinase protein? Analysis of the methodologies in glucokinase research reveals three common supplements to preserve the enzyme activity both in cellular extracts and of purified protein: (1) glycerol; (2) glucose at millimolar concentrations, and (3) thiol reagents such as mercaptoethanol or dithiotreitol. The integrative rationale for these

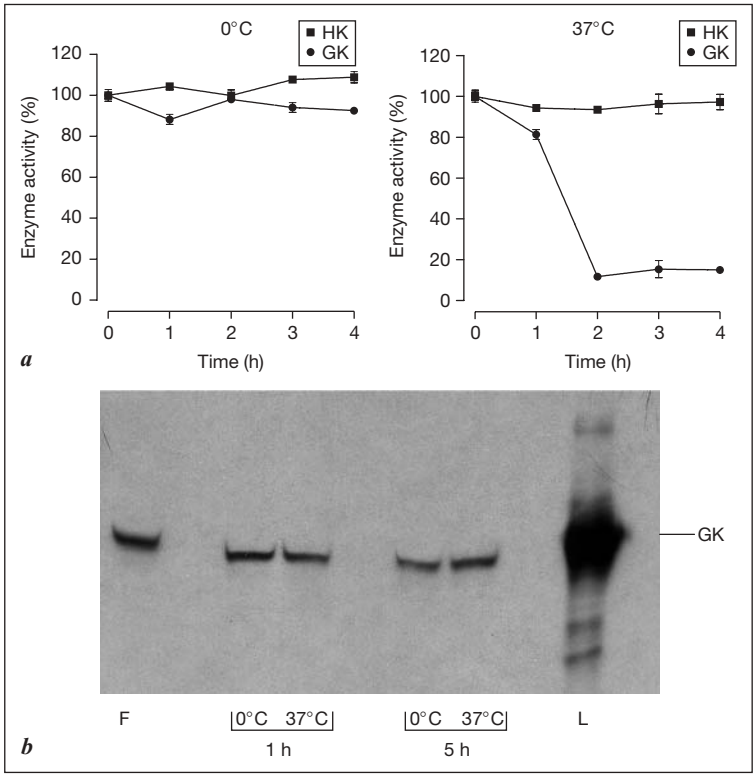

Fig. 1. Stability of hexokinase (HK) and glucokinase (GK) enzyme activities in RINm5F-GK cells. ***a*** RINm5F-GK cells [5] were homogenized by sonication and kept at 0 or 37°C for a period of up to 4 h. GK and HK activities were measured at the indicated time points by an enzyme-coupled spectrophotometric assay. ***b*** GK protein of cellular extracts was quantified by Western blot analysis 1 and 5 h after homogenization in comparison to a freshly prepared extract (F) and 20 μg rat liver cytosolic protein (L).

three protective supplements is to prevent the oxidation of free sulfhydril groups within the glucokinase protein. Sulfhydril group oxidation is favored when the enzyme protein undergoes a conformational change during catalysis or the so-called 'slow transition' induced by the substrate glucose [4, 6–8]. Because several cysteine residues are located in the vicinity of the substrate binding site glucose may protect these sulfhydril groups through spatial blockade [9, 10]. Glycerol significantly slows down the velocity of conformational changes of the GK protein [11] thus minimizing the risk of sulfhydril group oxidation, as these groups must be in close spatial vicinity to allow the formation of disulfide bridges. As elaborated below in detail, thiol reagents provide a

highly effective protection against sulfhydril group attack of glucokinase. Furthermore, the redox status of the sulfhydril groups may have important implications for glucokinase function under conditions of cellular stress and in type 2 diabetes mellitus.

Cysteine Pattern of Glucokinase and Hexokinase

A different sulfhydril group susceptibility of glucokinase and hexokinase can be explained by the spatial localization of reactive sulfhydril side groups of the GK enzyme in comparison with published models of the high affinity yeast hexokinase [12–14]. The 50-kD yeast hexokinase, originating from a 'monomeric' ancestral hexose phosphorylating enzyme [15, 16], differs from the mammalian 50-kD glucokinase protein in the total number of cysteine residues [17]. While the yeast hexokinase protein comprises only four cysteine residues mammalian GK proteins contain 12 cysteine residues [18, 19]. A 3D model of human β-cell GK could be generated by St. Charles et al. [9] through structural alignment on the basis of the open conformation of yeast hexokinase B and proved to be a valuable tool to locate the cysteine residues in the GK enzyme protein. Interestingly the cysteine residues Cys 213, Cys 220, Cys 230, Cys 233 and Cys 252 appear as a ring motif which surrounds the substrate binding cleft of the GK enzyme molecule (fig. 2 upper panel, adapted from the 1 glk PDB model, Protein Data Bank, Brookhaven National Laboratory [20]). In particular, these cysteine residues may be prone to form disulfide bridges. The pyrimidine derivative alloxan proved to be a valuable tool to clarify the molecular mechanism of sulfhydril group oxidation of the GK enzyme [3, 10, 21].

Alloxan – A Helpful Tool to Characterize the Sulfhydril Group Sensitivity of GK

Alloxan is a pyrimidine derivative which shares a structural similarity with glucose [21]. This compound is widely used in experimental diabetes research because it induces a selective β-cell destruction in rodents [21]. Alloxan is highly reactive towards thiols oxidizing protein bound thiol groups [22, 23]. The GK protein proved to be an extremely sensitive target for alloxan with EC_{50} values in the range of 2–5 μmol/l for extracts from rodent islets and liver [24, 25]. Glucose and mannose, the physiological substrates of the GK, protected the enzyme against the inhibition by alloxan. Notably the alpha anomer of glucose provided significantly greater protection than the β anomer which corresponded to the anomer preference of GK [25–27]. Other protective sugars

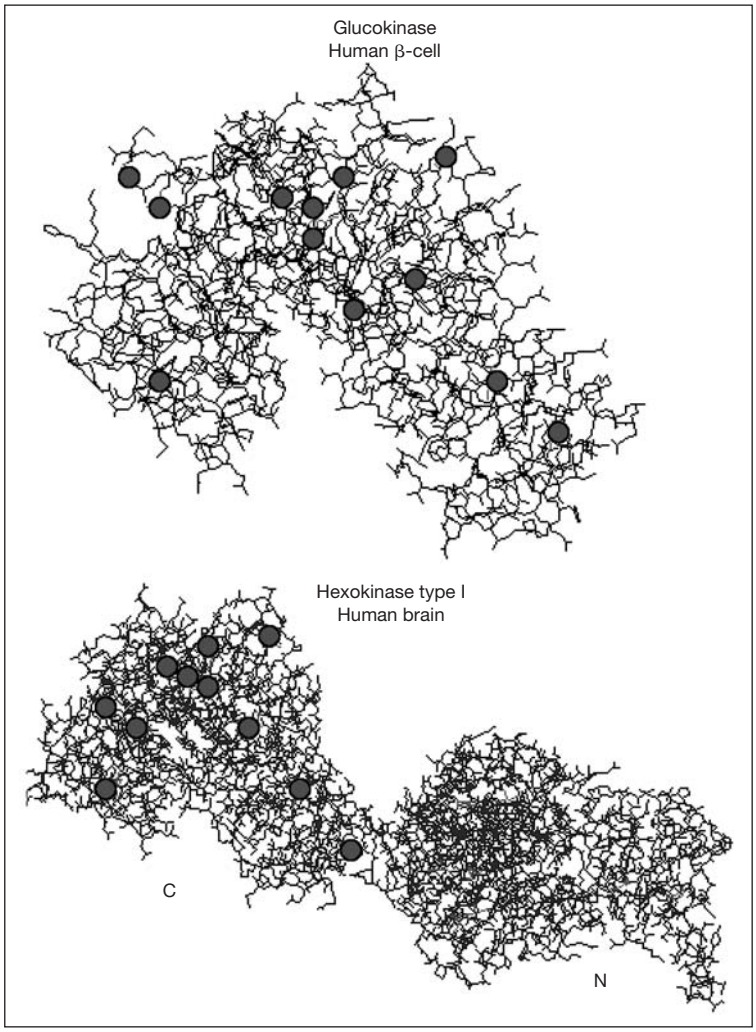

Fig. 2. Pattern of cysteine residues in human β-cell GK and human brain hexokinase type I. The GK model [9] (PDB code: 1 glk) and the hexokinase type I model [49, 50] (PDB code: 1HKB) were derived from the Protein Data Bank (Brookhaven National Laboratory). The cysteine residues are circled. N = NH_2-terminal half of the hexokinase type I protein; C = C-terminal half of the hexokinase type I protein.

against alloxan-mediated inhibition of GK were the competitive inhibitors mannoheptulose, glucosamine, and N-acetylglucosamine indicating an interaction of alloxan with the substrate binding site of the enzyme protein [28]. It should be emphasised at this point that the inhibition of GK by alloxan accounts for the

selective inhibition of glucose-induced insulin secretion [21] but does not account for the phenomenon of the selective pancreatic β-cell destruction responsible for induction of alloxan diabetes which from our current knowledge requires the attack of other β-cell structures resulting in a necrotic destruction [21, 29, 30]. While the EC_{50} values for the inhibition of GK were around 5 μmol/l, the EC_{50} values for the inhibition of hexokinase were around 500 μ*M* [23]. Thus, there is a hundredfold higher sensitivity of the GK enzyme towards an inhibition by alloxan. To address the sulfhydril group sensitivity of the human GK we expressed and purified His-tagged liver and β-cell isoforms from the heterologous *E. coli* system [3]. The purified GK protein allowed detailed studies upon the kinetics of alloxan-induced inhibition without interferences due to the presence of contaminating cytoplasmic compounds and high affinity hexokinases. In contrast to the competitive inhibitor mannoheptulose alloxan caused a noncompetitive type of enzyme inhibition of the human GK isoforms with K_i values between 1.0 and 1.5 μ*M* [3]. As expected from the high degree of homology there were no significant differences between human β-cell and liver isoenzymes and between human and rat GK, in accordance with the overall identity of the kinetic enzyme characteristics [3]. Although the type of inhibition was clearly noncompetitive, glucose protected the GK enzyme against inhibition by alloxan with half maximal protective concentrations between 11–16 m*M* [3]. Notably, the protection by glucose was incomplete conserving maximally 60% of the enzyme activity. Thus, the noncompetitive mechanism of enzyme inhibition reduced the catalytic activity of the enzyme but not the affinity for the substrate glucose through an oxidation of free sulfhydril groups [3].

The inhibitory effects of alloxan on GK enzyme activity can be prevented and reversed by sulfhydril group reducing reagents. In particular the dithiol 1,4-dithiothreitol (DTT) protected against and reversed the inhibition of GK by alloxan after inhibition of GK by 50 μ*M* alloxan [3, 31]. Interestingly the protective effect of dithiols was dependent upon an intermediate spacing between the sulfhydril groups. Thus 1,3-dimercaptopropane, 1,4-dimercaptobutane, 1,4-dithioerythritol, and 1,4-DTT, provided a protection against as well as a reversal of the inhibition of GK by alloxan [31]. Dithiols with two vicinal sulfhydril groups such as 1,2-dimercaptoethane and 2,3-dimercaptopropanol (BAL) were ineffective in the same way as dithiols with more widely spaced SH groups such as 1,5-dimercaptopentane and 1,6-dimercaptohexane [31]. The monothiol cysteine protected against alloxan but could not reverse its inhibitory effect. In contrast the physiological monothiol glutathione had no protective effect against alloxan inhibition of GK [31]. These studies indicate that the spatial distance between two or more free sulfhydril groups of the GK is of crucial importance for the protection or stabilization of the enzyme function.

Conformational Hindrance of GK by Sulfhydril Group Oxidation

Purified recombinant human GK proteins proved to be valuable tools to elucidate conformational changes of the enzyme protein in dependence on the sulfhydril group oxidation status. When human recombinant GK proteins were subjected to SDS-polyacrylamide gel electrophoresis under non reducing conditions, two protein bands could be observed whose molecular sizes were 49 and 58 kD [3]. Western blot analysis revealed that both bands were GK proteins, however, with a different electrophoretic mobility. The intensity of the 49 kDa GK band significantly increased after incubation of the purified human pancreatic β-cell GK with alloxan as compared to the 58-kD GK band. Thus, the appearance of the 49-kD GK band could be most likely explained by disulfide bond formation after oxidation of sulfhydril groups of the protein [3]. The formation of intracellular disulfide bridges results in a compact oxidized GK protein which is incompletely unfolded by SDS and shows a faster migration in the polyacrylamide gel [3]. Importantly freshly purified recombinant GK protein also exhibits an electrophoretic double band pattern indicating that the native enzyme already exists in different conformations. We could exclude that the low-molecular 49-kD band might represent a spliced protein because the incubation with the reducing reagent dithiotreitol after alloxan exposure shifted the GK bands again towards the 58-kD species [3]. On the other hand, the monothiol reagent iodoacetamide, which reacts with free sulfhydril groups to yield S-carboxamidomethyl cysteine, could not counteract the effects of alloxan upon sulfhydril group oxidation. These studies clearly show that conformational changes of the GK protein occur only after reaction with two specific sulfhydril groups in a distinct spatial localization of the native enzyme which are accessible only to dithiol compounds. The redox status of the sulfhydril groups crucially determines the conformation of the GK and the intrinsic activity of the enzyme.

In an elegant study upon purified liver GK protein Tippett and Neet [4] could demonstrate a constant decay of GK activity when thiol compounds were absent in the buffer, a process which was described as ‘sulfhydril-related reversible kinetic transitions’.

Conformational changes induced by glucose have been suggested as a key mechanism for the cooperativity of a monomeric GK enzyme either by a ‘mnemonical’ mechanism [32, 33] or a kinetic ‘slow transition’ mechanism [6, 7]. Both models assume two different conformations of the enzyme which are modulated by the binding of the substrate glucose. Thus, the occupation of the substrate-binding site by glucose protects sulfhydril groups of the GK enzyme against an attack by oxidizing agents or spontaneous oxidation of cysteine side groups which get into close vicinity.

Dissecting the kinetics of sulfhydril-related changes of GK enzyme activity Tippet and Neet [4] showed that liver GK protein undergoes a 'kappa' activity decay in the presence of glucose but in the absence of reducing agents. Interestingly the 'kappa' decay increased the K_m value for glucose but resulted in a loss of kinetic cooperativity. Different from this situation the absence of glucose and reducing agents resulted in progressive decrease of the V_{max} value, the so-called 'mu' decay of GK activity [4]. The rationale for both decays is the 'lock' of the GK protein which becomes particularly apparent in the absence of glucose.

The electrophoretic mobility data with the interconvertible 49- and 58-kD bands correspond well to the 'kappa' and 'mu' decay of rat liver GK activity. They result from sulfhydril group interconversions in dependence on changes of the enzyme conformation by alloxan which shares a high degree of spatial homology to glucose [3, 4, 28]. When the conformational flexibility of the GK is the prerequisite for the sigmoidal phosphorylation kinetics in a millimolar concentration range for the substrate glucose, the sulfhydril group instability may be the price which had to be paid for the glucose sensor function of this hexokinase isoform.

Multiple Cysteine Residues Participate in the Conformational Lock of GK

Free sulfhydril groups of the native and unfolded GK proteins can be quantified through titration with Ellman's reagent [34]. This method revealed that three sulfhydril groups were accessible per GK molecule under native conditions and five per enzyme molecule after unfolding by 2% SDS [3]. Alloxan (10 m*M*) decreased the number of free sulfhydril groups by 1.3 per enzyme molecule in the native state and 3.1 in the unfolded state. Site directed mutation of cysteine residues of human β-cell GK allowed the identification of distinct mutants with significantly lower alloxan-induced decay of free sulfhydril groups both in the native and denatured state [10]. The Cys 230 and the Cys 382 GK mutants showed virtually no decrease of free sulfhydril groups after incubation with 10 m*M* alloxan [10]. Thus, the cysteine residues of the GK protein showed a different susceptibility towards an oxidation of the sulfhydril groups which cannot be simply explained by an association with the substrate binding site. In the GK model, the cysteine residue Cys 230 is located in the vicinity of the substrate-binding site whereas the cysteine residue Cys 382 is located in distance from the catalytic cleft [9, 35].

These data raised the question whether alloxan might induce the formation of more than one intramolecular disulfide bridge. Controlled proteolysis of

human β-cell GK by proteinase K provided evidence for the existence of multiple intramolecular disulfide bridges after alloxan treatment as demonstrated by the increased number of low molecular weight fragments after reduction by dithiotreitol [10]. Furthermore, the electrophoretic 49/58 kD ratio of the cysteine mutants Cys 213, Cys 220, Cys 230, Cys 233, Cys 252, Cys 364 , Cys 371 and Cys 382 under reducing and non-reducing conditions was not different from the wild-type protein [10]. Thus, it can be excluded that the inhibitory effects of alloxan and apparently also the spontaneous sulfhydril group oxidation of the GK protein can be allocated to distinct cysteine residues. It should be emphasized that the GK is constitutively susceptible to sulfhydril group attack already in the absence of protective thiol reagents [3, 4]. Native recombinant human β-cell GK protein taken from a stock stabilized with 1 m*M* dithiotreitol showed the characteristic electrophoretic double-band pattern within minutes after a hundredfold dilution of dithiotreitol [3]. Thus, sulfhydril group stabilization can be regarded as a prerequisite to maintain the catalytic function of the GK enzyme in particular in pancreatic β-cells which are poorly equipped with free radical scavenging enzymes [36–38].

Cysteine Residues, Catalytic Function and Sulfhydril Group Susceptibility of GK

The human GK protein contains 12 cysteine residues (SwissProt P35557, PDB code: 1 glk, Protein Data Bank, Brookhaven National Laboratory [20]) which are located at a high density in the region of the protein which forms the catalytic cleft and also at the C-terminal end of the enzyme protein [9, 18, 19] (fig. 2). Interestingly the cysteine pattern of the GK model [9], deduced from crystal structure of yeast hexokinase [12, 13] which contains only four cysteine residues, resembles the C-terminal half of the 100 kDa hexokinase type I [39] (fig. 2). Thus this pattern has been conserved during evolution of mammalian hexokinases irrespective of the kinetic properties of the enzymes. Mutations of the cysteine residues Cys 213, Cys 252 and Cys 382 have been reported to be associated with MODY-2 diabetes mellitus due to impaired GK kinetics of the mutated proteins which alter the glucose sensor function [40, 41]. Replacement of cysteine residues by serine or phenylalanine had significant effects upon the kinetic characteristics, the susceptibility to alloxan and the stability of the recombinant human β-cell GK proteins [10]. In particular the mutation of the cysteine residues Cys 233, Cys 252 and Cys 382 resulted in catalytically inactive proteins with low expression levels in *E. coli* bacteria which are an indicator for an incorrect folding of the recombinant proteins [10]. The Cys 213, Cys 220, Cys 364 and Cys 371 mutants showed a significant decrease of the V_{max}

Table 1. Kinetic characteristics and susceptibility to alloxan inhibition of human wild-type β-cell GK and cysteine mutants

Mutants		Glucose	ATP	IC_{50} Alloxan μmol/l
Wild-type	$S_{0.5}$, m*M*	6.4 ± 0.3 (4)	0.14 ± 0.09 (4)	1.4 ± 0.2 (4)
	V_{max}, U/mg	47 ± 2.2 (4)	36.2 ± 4.2 (4)	
Cys 213	$S_{0.5}$, m*M*	5.2 ± 0.1 (4)	0.15 ± 0.8 (4)	5.8 ± 1.0 (4)
	V_{max}, U/mg	23 ± 2.2 (4)*	21 ± 0.7 (4)*	
Cys 220	$S_{0.5}$, m*M*	5.0 ± 0.4 (4)	0.32 ± 0.02 (4)*	2.8 ± 0.5 (4)*
	V_{max}, U/mg	16 ± 0.9 (4)*	15 ± 4.2 (4)*	
Cys 230	$S_{0.5}$, m*M*	7.5 ± 0.2 (4)	0.29 ± 0.03 (4)*	17.7 ± 1.5 (4)*
	V_{max}, U/mg	46 ± 1.0 (4)	47 ± 3.6 (4)	
Cys 364	$S_{0.5}$, m*M*	6.9 ± 0.5 (4)	0.19 ± 0.01 (4)*	15.4 ± 1.2 (4)*
	V_{max}, U/mg	27 ± 1.8 (4)*	32 ± 3.8 (4)	
Cys 371	$S_{0.5}$, m*M*	6.8 ± 0.5 (4)	0.21 ± 0.01 (4)*	7.8 ± 1.5 (4)*
	V_{max}, U/mg	21 ± 1.2 (4)*	22 ± 0.9 (4)*	

The data are summarized from Tiedge et al. [10]. GK enzyme activity was measured by an enzyme-coupled spectrophotometric assay. The GK mutants Cys 233, Cys 252 and Cys 382 completely lacked catalytic activities. Results shown are means ± SEM. Statistical analyses were performed with ANOVA followed by Dunnett's test for multiple comparisons.

*$p < 0.01$ compared with wild-type GK.

values in the range of 43–65% for glucose whereas the affinity for this substrate was not affected (table 1). The co-operative behavior for glucose was not altered in any of the cysteine mutants [10]. Thus, the cysteine residues apparently play an important role for the catalytic process itself rather than for the substrate binding of the GK. Overall cysteine residues positively affect the stability of the GK proteins as demonstrated by a decrease of the activity after exposure to temperatures between 37 and 55°C [10]. The Cys 213, Cys 220, Cys 230 and Cys 364 mutants showed a higher susceptibility to heat inactivation whereas the Cys 371 residue conferred higher stability to the enzyme against heat denaturation [10].

Alloxan irreversibly inhibited enzyme activity of wild-type GK with an IC_{50} value of 1.4 μ*M* (table 1). The Cys 220 mutant showed a comparable sensitivity with an IC_{50} value of 2.4 μ*M* (table 1). With the exception of the Cys 220 mutant the exchange of all other cysteine residues resulted in a significantly lower susceptibility to alloxan with particular emphasis upon the Cys 230 and Cys 364 mutants (table 1). Notably, none of the cysteine mutations was completely resistant against the inhibitory effects of alloxan corresponding to the data obtained by controlled proteolysis of alloxan-treated GK protein

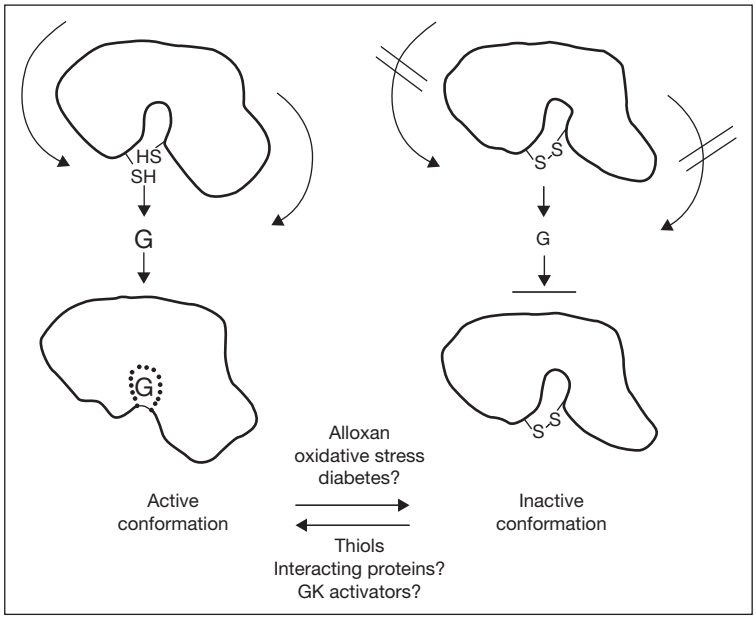

Fig. 3. Regulation of the GK enzyme by the sulfhydril group Redox status.

which provides evidence for the existence of multiple disulfide bridges in the molecule.

Conclusions and Perspectives

The studies upon purified GK protein provide cumulative evidence that free sulfhydril groups can be regarded as the Achilles heel of the enzyme. Oxidation of SH groups and reduction of disulfide bridges determine the ratio between active and inactive GK enzyme (fig. 3). The pancreatic β-cell shows a low enzymatic antioxidative defense status which favors the oxidation of cysteine side groups under conditions of oxidative cellular stress [36–38]. With the exception of the Cys 230 residue all other cysteine side groups are essential for the catalytic process and participate in the formation of multiple intracellular disulfide bridges. It will be the challenge of future studies to clarify by which mechanism GK enzyme activity is preserved in β-cells of the pancreas and in liver. Glutathione may serve as the physiologically relevant candidate for protection of GK although monothiols proved to be significantly less efficient to keep the sulfhydril groups in a reduced state [31]. A different

strategy for maintenance of high intrinsic GK activities might evolve from the interaction with β-cell matrix proteins [5, 42], the interaction with insulin granules [43, 44] and newly identified specific binding partners [45, 46]. These endogenous activators or more precisely stabilizers may keep the GK protein in a conformation which is less sensitive to sulfhydril group oxidation. In recent studies it could be demonstrated that the bifunctional regulatory protein 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is a binding partner of GK [46] which increased the enzyme activity by a so-far unknown mechanism [see chapter 'Interaction of GK with PFK-2/FBPase-2']. A reduced accessibility of free sulfhydril groups towards oxidation could contribute to this effect. Thus PFK-2/FBPase-2 may serve as an endogenous stabilizer of GK activity. This stabilization might be of crucial importance in situations of β-cell dysfunction such as in type 2 diabetes mellitus. The potency of novel pharmacological GK activators to lower the blood glucose concentration in rodent models of type 2 diabetes emphasises the critical role of this enzyme for β-cell function [47, 48]. β-Cell dysfunction in type 2 diabetes mellitus may lead to a failure of the endogenous GK stabilization to preserve the catalytic function of the enzyme thereby hampering the glucose sensor function and concomitantly also insulin secretion. A better understanding of the mechanism which GK activity in pancreatic β-cells and liver can be maintained in an optimal range will open the perspective to develop new strategies to modulate the GK enzyme activity according to the requirements of a metabolic glucose sensor.

Summary

The glucose sensor glucokinase (GK; hexokinase type IV) is crucially dependent upon a proper catalytic function of the enzyme both in pancreatic β-cells and in liver. In contrast to the high affinity hexokinases type I-III the GK protein shows an extraordinary sensitivity towards sulfhydril group oxidizing compounds such as alloxan and ninhydrin. Importantly, the spatial distribution of cysteine residues is similar in the GK and the high affinity hexokinases type I–III and cannot explain the differential sulfhydril group sensitivity. These cysteine residues are indispensable for the catalytic function of GK, in particular those in the vicinity of the glucose binding site. Alloxan induces the formation of multiple intramolecular disulfide bridges within the GK molecule which significantly reduce the intrinsic activity of the enzyme. This sulfhydril oxidation can be efficiently prevented by dithiol compounds. Apparently, the conformational flexibility of GK favors the disulfide bond formation in dependence upon the intracellular redox status. This makes the GK a sensitive component within the metabolic stimulus-secretion coupling of the pancreatic β-cell, a cell with a low antioxidative defense status. Perspectively, the preservation of GK activity may become a key aspect in future therapeutic strategies aiming at an improvement of the secretory responsiveness of pancreatic β-cells under hyperglycemic conditions in type 2 diabetes mellitus.

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Mouse Models of Altered Glucokinase Gene Expression

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Naturally occurring mutations in the human glucokinase (GK) gene cause three different glycemic diseases. First, mutations of a single GK gene allele that impair either the function or expression of the enzyme cause maturity onset diabetes of the young, type 2 (MODY-2) [1–3]. Second, activating mutations of GK, only several of which have been identified, cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [4, 5]. Third, inheritance of mutations that impair either the function or expression of both GK gene alleles causes persistent neonatal diabetes (PND), a life-threatening disease that requires immediate insulin therapy [6]. While the existence of these related genetic diseases in humans clearly indicates that GK plays a crucial role in the mechanisms that assure a normal blood glucose concentration, they do not readily reveal how alterations in either the activity or expression of this particular hexokinase isoform are able to cause such profound and lasting alterations in the plasma glucose concentration.

GK is expressed in a network of glucose sensing cell types, including the liver, pancreatic β-cells, gut endocrine cells, and specific regions of the brain, that either by their own actions, or the effects of the different neuronal and hormonal factors they secrete, have potent effects on the blood glucose concentration. Indeed, the combined interactions of these different glucose sensing cell types is the basis whereby glucose homeostasis is generally maintained. However, the multitude of potential and real interactions that occur between various cell types to assure a normal glucose concentration makes it difficult to be certain about the specific contributions of each individual cell type. For this reason, a full understanding of the functional roles of GK in vivo, and how changes in net activity of this enzyme perturb glucose homeostasis, has

Table 1. Summary of transgene over expression and gene knockout experiments that have been performed to augment or attenuate the expression of GK in mice

Type of manipulation	Summary of phenotype	Reference
Models of GK overexpression		
Transgenic mice that overexpress hepatic GK	mild hypoglycemia; diminished insulin	[7, 8]
Transgenic mice that overexpress both islet and hepatic GK	mild hypoglycemia; β-cell GK is downregulated; no change in basal insulin concentrations	[34]
Adenoviral GK gene transfer	normalization of fasting blood glucose and diminished insulin in diabetic mice on a high fat diet	[15]
Models of GK deficiency		
Homozygous null global GK knock-out mice	perinatal death from severe diabetes	[16, 18]
Heterozygous null global GK knock-out mice	mild diabetes; impaired insulin secretion	[16, 18]
Homozygous β-cell-specific knock-out mice	severe hyperglycemia and death by postnatal day 4	[18, 20]
Heterozygous β-cell-specific knock-out mice	mild diabetes; diminished insulin secretory response	[18, 20]
Total liver-specific knock-out mice	moderate hyperglycemia; impaired insulin secretory response to glucose; impaired glycogen synthesis	[18]

required generation and characterization of a variety of novel animal models. This chapter summarizes the different mouse models that have been produced (table 1), and how this information has been useful for defining the role of GK in specific cell types. Together, the sum of knowledge gained from these animals has provided an increasingly clear picture of how alterations in GK gene expression or activity in humans causes alteration in the blood glucose concentration.

Models of Glucokinase Overexpression

The effect of increased GK gene expression has been examined by several groups of investigators. Stable genetic models of increased GK have been produced through the generation of mice that express either tissue-specific transgenes or the entire GK gene locus. Transient models of increased GK were achieved by use of recombinant adenoviruses that, when injected into an animal, lead to increased GK gene expression in the liver.

Liver-Specific Transgenes

Transgenic mice that stably overexpress GK in the liver were generated by two groups [7, 8]. Both lines of transgenic mice contain fusion genes that linked promoter sequences active in the liver to GK cDNA sequences. Both lines exhibited only small increases in the amount of hepatic GK, which led to lower blood glucose and insulin concentrations in both lines. Animals with increased hepatic GK exhibited an increased rate of both glucose clearance and glycogen synthesis, consistent with an increased rate in hepatic glucose disposal. In both lines of mice, the increase glucose metabolism occurred without hypertriglyceridemia, as was observed when the enzyme was over expressed to a much higher degree in liver using a recombinant adenoviral strategy [9].

GK Gene Locus Transgene

The effects of increased GK gene expression on blood glucose homeostasis was also examined by the generation of mice that contained additional copies of the entire mouse GK gene locus [10]. These studies made use of the entire GK gene locus, which was contained in an 83-kb fragment of mouse GK DNA. Identification of a line of that contained a single extra copy of this large transgene, which contained both the neural/neuroendocrine and liver-specific promoters as well as all coding exons, enabled the effect of increased GK gene copy number to be explored.

Hepatic GK mRNA was increased by ~1.5 fold in heterozygous GK gene locus transgenic animals and by ~2-fold in homozygous animals [11]. This led to a lowering of the plasma glucose concentration by 25 and 37%, respectively, for mice that had three and four functional copies of the GK gene (fig. 1). Both basal and hyperglycemic clamp studies were performed to assess the effect of increasing the expression of GK on whole body homeostasis [11]. Animals with one additional copy of the GK gene locus transgene had a 21% increase in glucose clearance rate under basal conditions in the absence of any difference in basal insulin concentrations [11]. Under hyperglycemic conditions, these mice had glucose turnover and clearance rates similar to controls, but secreted ~50% less insulin. Also hepatic glycogen content was markedly increased after the 2-hour glucose infusion even though insulin levels did not rise [11] (table 2). Interestingly, the amount of GK detected in islets by immunostaining was decreased, despite the presence of a transgene that, due to the presence of the upstream GK promoter, should have also been expressed in β-cells.

Besides exhibiting a reduction of the blood glucose concentration, mice with a greater than normal amount of GK also exhibited a dramatic resistance to the development of hyperglycemia and hyperinsulinemia normally brought on by consumption of a high fat diet [12]. These data suggest that the

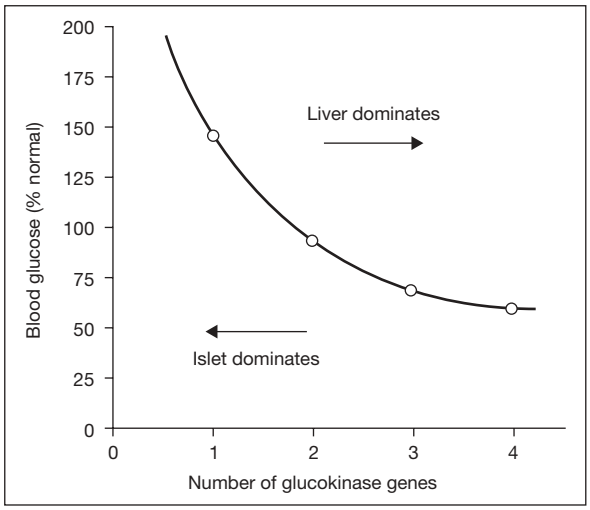

Fig. 1. Relationship between GK gene copy number and the blood glucose concentration. This figure shows the effect on the blood glucose concentration of changes in the GK gene copy number, obtained from GK over-expressing transgenic mice and heterozygous global GK knockout mice. Mice that totally lack GK have blood glucose concentration greater than 500 mg/dl and do not survive the neonatal period.

enhancement of GK activity may have a therapeutic potential for the treatment of type 2 diabetes.

Viral Studies

Recombinant adenoviruses have also been used to overexpress GK in primary hepatocytes [13, 14], the liver of rats [9] and mice [15]. In all cases, these studies indicate that hepatic GK determines the rate of hepatic glucose uptake and consequently lowers the basal plasma glucose concentration. However, the ability to precisely titrate the amount of GK produced from recombinant adenoviruses is difficult, thereby making it difficult to achieve increases in gene expression that are reflective of those that might normally occur. This may explain the failure to observe any effect on the fasting blood glucose levels when efforts were made to achieve a low-level adenovirus-mediated GK expression. In contrast, recombinant adenoviruses enabled the effect of very high levels of hepatic GK gene expression to be assessed within an adult animal. Interestingly, while only mild hypoglycemia was observed when rats were treated with a GK-expressing adenovirus, there was a marked increase in both plasma free fatty acid and triglyceride levels [9]. However, when mice were

Table 2. Summary of metabolic parameters from various GK altered mouse models (adapted from Postic [35])

	Basal[1]				Hyperglycemic clamp[2]			
Mouse model[3]	gk^{3}	$gk^{del/w}$	$gk^{lox/w}$ + *Ins2-Cre*	$gk^{lox/lox}$ + *Alb-Cre*	gk^{3}	$gk^{del/w}$	$gk^{lox/w}$ + *Ins2-Cre*	$gk^{lox/lox}$ + *Alb-Cre*
Blood glucose	−12%	+38%*	+23%*	+40%*	raised to ~300 mg/dl			
Plasma insulin	n.d.	n.d.	n.d.	n.d.	−40%*	−70%**	−70%**	−70%**
Glucose turnover	n.d.	−50%*	n.d.	n.d.	n.d.	−67%*	−56%*	−60%*
Glycogen synthesis	–	–	–	–	+360%**	−90%**	−55%*	−88%**

[1]For basal studies, saline solution was infused during a 120-min experimental period. Each group of mice was compared to its appropriate control and differences between parameters are expressed as percent of controls. Only significant values are indicated in the table. n.d. = Not statistically different. *$p < 0.05$.

[2]For the hyperglycemic clamp studies, blood glucose concentrations were raised to ~300 mg/dl. Each group of mice was compared to its appropriate control and differences between parameters are expressed as percent of controls. Only significant values are indicated in the table. n.d. = Not statistically different. *$p < 0.05$; **$p < 0.001$.

[3]The following mouse models were used: gk^{3}: Transgenic mice that overexpress the entire GK gene locus and thus have three functional GK alleles in all tissues. $gk^{del/w}$: Heterozygous GK knockout mice that have a single functional GK gene in all tissues. $gk^{lox/w}$ + *Ins2-Cre*: Heterozygous β-cell-specific GK knockout mice. These mice have a single functional GK allele in the pancreatic β-cell and the normal complement of two functional alleles elsewhere. $gk^{lox/lox}$ + *Alb-Cre*: Liver-specific GK knockout mice. These mice totally lack hepatic GK but have two functional genes in all other tissues.

treated in a similar manner, no significant increase in plasma or hepatic triglycerides, or plasma free fatty acids, were observed [15].

Models of Glucokinase Deficiency

While mice that have increased GK gene expression were useful in demonstrating a relationship between hepatic GK activity, hepatic glucose uptake, and the basal blood glucose concentration, the studies provided only a partial picture of the role of this enzyme in the whole animal. Thus, a variety of different gene knockout studies were also performed that have led to a better appreciation of the role of this enzyme in other cell types, particularly the pancreatic β cell.

Global Gene Knockouts

Information complementary to the GK loss of function studies was obtained by three groups who used gene targeting studies to totally eliminate GK gene expression [16–18]. In all cases, mice with one null allele were hyperglycemic, thereby indicating that the mouse was able to serve as a good model

for humans with MODY-2. These studies all showed that a 50% reduction in GK gene expression leads to an impairment in glucose-stimulated insulin secretion (GSIS), as was generally expected given the proximal and rate-determining role of this enzyme in glucose metabolism within the β-cell.

By interbreeding mice of animals that had single allele GK gene knockouts, animals were obtained that were globally deficient in GK. These mice appeared normal at birth, but died within several days of birth due to severe hyperglycemia. A similar phenotype has subsequently been demonstrated in human infants with PND that are also born with little if any GK activity [6]. Mouse pups that lack GK have marked hepatic steatosis and decreased glycogen content, due to hypoinsulinemia brought on by the lack of GSIS by the β-cells, despite a markedly elevated plasma glucose concentration. Indeed, the hypoinsulinemic phenotype in the global GK knockout animals is indistinguishable from that observed in mice that have null mutations of both insulin genes [19]. While one report described a phenotype of embryonic lethality at day 9.5, this now appears due to the inadvertent knock-out of a second gene whose function is necessary during early embryogenesis [17].

Since mice with a global knockout of GK die shortly after birth, studies of the roles of GK in specific tissues in adult animals required both the development of a conditional GK allele by gene targeting in mouse embryonic stem (ES) cells and the generation of transgenic mice that express *Cre* recombinase under control of control of different tissue-specific promoters.

Neural/Neuroendocrine-Specific Knockout

To assess the role of GK in both the pancreatic β cell and other neural/neuroendocrine cell types, Terauchi et al. [20] performed gene targeting in ES cells to eliminate GK gene expression from exon 1β. Mice with this mutation lack expression of the β-cell GK isoform while retaining normal expression of the hepatic GK isoform. These mice exhibit a phenotype that is essentially identical to mice with a global GK gene knockout. Heterozygous mutant mice showed early-onset mild diabetes whereas mice that were homozygous for the mutation developed severe diabetes and died within a week. However, while these results clearly demonstrate a vital role for the β-cell GK isoform in glucose homeostasis, the model does not truly represent a true β-cell-specific knockout of GK, as was advocated [20]. This is due to the fact that the β-cell GK isoform is expressed in a variety of different neural/neuroendocrine cells in the brain and gut in addition to pancreatic β-cells.

β-Cell-Specific Knockout

To better assess the cell-specific roles of GK, Postic et al. made use of a Cre-loxP gene targeting strategy [18] which allows the deletion of GK to be

more precisely restricted. To determine the role of GK in pancreatic β-cells, mice with a conditional (or loxed) GK allele were generated then interbred with animals that expressed Cre under the control of the insulin 2-promoter (Ins2-Cre). Homozygous β-cell-specific knockout pups exhibited steatosis, a depletion of hepatic glycogen content, a ~70% decrease in plasma insulin concentrations, and died within a few days of birth as a result of severe diabetes. Thus, animals that lack GK only in the pancreatic β-cell exhibit a phenotype that is very similar to animals with either a global or neural/neuroendocrine-specific knockout of GK.

Studies of the heterozygous β-cell-specific GK knockout mice revealed fasting hyperglycemia (+25%) without measurable difference in basal insulin levels. Moreover, during a hyperglycemic clamp study, glucose turnover rates and insulin secretion were reduced by 56 and 70%, respectively, consistent with a shift in the threshold point for GSIS (table 2). It should be pointed out that subsequent analysis of the Ins2-Cre mice using a cre-inducible LacZ reporter mouse line [21] has shown that besides being expressed in pancreatic β-cells, Cre is expressed within the ventral cerebral cortex, particularly in hypothalamic neurons [22]. Thus, an element of uncertainty still remains as to whether the phenotype observed in these mice is due solely to the lack of GK in β-cells, or is also due also to the lack of the enzyme in the hypothalamus where GK has also been found to be expressed. Until brain-specific GK knockout animals are generated and characterized, this issue is likely to remain unsettled.

Liver-Specific Knockouts

Although the studies described previously have clearly demonstrated that hepatic GK plays a key role in regulating hepatic glucose disposal, the liver has generally not been thought to contribute very much to the hyperglycemia that occurs in MODY-2. Rather, a shift in the threshold for GSIS, brought on by diminished expression of GK in the pancreatic β cell, has been thought to be the major if not sole cause of hyperglycemia in this genetic disorder. To determine whether this generally held assumption was true, mice with a liver-specific knockout of GK were also generated using a Cre-loxP strategy. In this case, animals bearing the loxed GK allele were interbred with animals that expressed Cre under control of the albumin promoter/enhancer. The albumin-Cre mice that were used for this purpose, when interbred with the ROSA26 LacZ reporter mice, exhibited complete recombination within the liver by 6–8 weeks of age [23].

In contrast to the perinatal lethality that occurs in animals that lack GK only in β-cells, mice that totally lack hepatic GK are viable. Moreover, the fed blood glucose concentration of mice without any GK in their livers is only ~10% higher than their littermate controls. However, the effect of the lack of

hepatic GK became fully apparent during both basal and hyperglycemic clamp studies. First, the blood glucose concentrations were found to up to 40% higher after an 8-hour fast in the absence of any difference in either the plasma insulin concentrations or glucose turnover rates. Second, the whole body glucose turnover rate of mice lacking hepatic GK was markedly reduced during hyperglycemia, and glycogen synthesis was also reduced by ~90% (table 2). Third, and most surprising, mice that lacked hepatic GK secreted about 70% less insulin in response to the glucose challenge.

These results demonstrate that hepatic GK contributes to glucose homeostasis in two ways. First, the enzyme determines rates of hepatic glucose utilization. In the absence of the enzyme, there is a marked reduction in hepatic glucose metabolism. This leads to a small but significant elevation in blood glucose concentration that is most pronounced when the mouse is fasted. Second, the elevation in the blood glucose concentration, although seemingly small, impairs islet function, as indicated by the marked impairment of GSIS. While it is surprising that such a small elevation in the plasma glucose concentration has such a marked impact on islet function, Laybutt et al. [24] have recently shown that mild but chronic hyperglycemia causes marked alteration in the expression of genes involved in multiple metabolic pathways. Thus, there may be an interplay between hepatic glucose utilization, as determined by GK, and GSIS. This interplay is not well understood and needs to be further characterized.

The phenotype of the liver-specific GK knock-out mice is similar to that of animals that lack the GK regulatory protein (GKRP) [25, 26]. GKRP regulates hepatic GK activity by both allosteric inhibition and by translocating GK into the nucleus, and the absence of GKRP was predicted to result in higher GK enzymatic activity in the liver. However, contrary to this prediction, GK activity is diminished in the GKRP-mutant mouse liver, so like the liver-specific GK knockouts, these animals exhibited both lower glycogen levels and higher glucose levels after a glucose challenge. These results have led to the suggestion that GKRP may function to maintain a pool of GK during the fasting state that is ready for release upon carbohydrate ingestion [27].

Physiological Integration

Studies of animal models with altered GK expression have clearly indicated that both hepatic and β-cell GK play both important and complimentary roles in determining the blood glucose concentration. Moreover, they indicate that there is a direct, but inversely proportional relationship between the amount of GK and the blood glucose concentration, as shown in figure 1. Even small

alterations in the amount of GK, by alternate rates of glucose metabolism, are sufficient to cause changes in the plasma glucose concentration. While both the liver and pancreatic β-cells play key roles in glucose homeostasis, their relative contributions change depending on whether there is too much or too little GK activity. In the situation of too much GK, the liver appears to play the more dominant role in lowering the plasma glucose concentration since the expression of islet GK is impaired. Conversely, in the situation of diminished GK, as occurs in both MODY-2 and PND, it appears that the lack of GK activity in the β cell, by impairing GSIS, is the dominant physiological effect.

Unanswered Questions and Next Steps

While the various mouse models and physiological studies have provided essential information for understanding the impact of the different types of GK gene mutations that occur in humans, important questions still remain.

First, the role of GK in other neural/neuroendocrine cells, besides the pancreatic β-cell, remains to be determined. Like pancreatic β-cells, selected neurons, primarily in hypothalamic nuclei, possess the capacity to detect variation in glucose. The available evidence, albeit indirect, suggests that GK may also functions as glucose sensor in these hypothalamic neurons [28–30]. Glucose-sensing by hypothalamic neurons may contribute to nutrient homeostasis by affecting feeding behavior or by influencing pancreatic hormone release via direct innervation pathways. However, despite identification of GK in both the ventromedial hypothalamus and arcuate nucleus [31], both the mechanism of action and role of GK in the brain are still poorly understood. Thus, the generation and characterization of brain-specific GK knockout mice, in addition to the pancreatic β-cell- and liver-specific gene knockouts that were described, will help answer this question.

Second, the effect of specific GK gene mutations needs to be assessed in new mouse models. To date, a total of 195 different mutations of GK have been reported in 285 MODY-2, PND and PHHI pedigrees [32]. The effects of these mutations on the kinetics of the GK have largely been determined by expression of the mutant enzymes in *Escherichia coli*, most often as fusion proteins with *glutathione-S-transferase* (GST) from *Schistosoma japonicum* [33]. Interestingly, while most MODY-2 mutations appear to impair the function of GK by altering either the affinity of the enzyme for glucose or diminishing the reaction velocity (V_{max}), some mutations have been found that do not show the expected kinetic impairment when expressed in vitro. V62M mutation is one such example. This mutation was independently identified in two different MODY-2 pedigrees. Surprisingly, when expressed in bacteria, this mutant

exhibits increased, not decreased, GK activity. Thus, the generation of mice that contain specific GK mutations may be essential for assessing why some mutations result in phenotypes that cannot be explained solely on the basis of changes in enzyme kinetics. Our knowledge of the tissue-specific roles of GK, and how they change depending on whether there is more or less GK activity, needs to be better defined in order to gain of full understanding of the role of glucose sensing in the regulation of blood glucose homeostasis. Indeed, the generation of additional mouse models that contain specific mutations of this enzyme will likely be the only means to settle some of the uncertainties about the role of GK in glucose homeostatic regulation.

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Anatomy, Physiology and Regulation of Glucokinase as a Brain Glucosensor

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The brain has evolved specialized glucosensing neurons which participate in glucose and overall energy homeostasis in the body. Glucosensing neurons utilize glucose as a signaling molecule to alter their firing rate, as opposed to the vast majority of neurons which primarily utilize glucose to fuel their metabolic needs [1]. Oomura [2] and Anand [3] and their co-workers first demonstrated such neurons in both the fore- and hindbrain in 1964. Although these neurons can respond directly or indirectly to either the complete absence of glucose or to levels as high as 20 m*M* [4–11], it is likely that their primary range is between 0.5–3.5 m*M* under physiologic conditions [12, 13]. Mounting evidence supports a role for glucokinase (GK) as the gatekeeper for neuronal glucosensing within this physiologic range [11, 14]. Importantly, glucosensing neurons reside in brain areas known to play critical regulatory roles in energy homeostasis and neuroendocrine and autonomic function [15–18].

Glucose sensing neurons are either excited (glucose excited; GE) or inhibited (glucose inhibited; GI) by rising ambient glucose levels [6, 7, 9, 10]. However, early studies used extracellular recording techniques to monitor neuronal activity at glucose levels virtually never seen by the living brain, i.e. 0 vs. 10–20 m*M* [6, 10]. Neurophysiologic studies have provided direct evidence that GE neurons function much like the pancreatic β-cell where elevated glucose levels increase the ATP/ADP ratio and inactivate (close) an inwardly rectifying K^+ pore-forming unit (Kir6.2) of the ATP-sensitive K^+ (K_{ATP}) channel [4, 5, 9, 10, 19]. This channel on GE neurons is similar, but not completely identical to

the K_{ATP} channel found on β-cells [4, 5, 9, 10, 19]. But, while the K_{ATP} channel appears to be necessary for neuronal glucosensing in GE neurons [20], it is clearly not sufficient for all glucosensing since most neurons probably contain the K_{ATP} channel [21, 22] and since GI neurons clearly do not utilize the K_{ATP} channel to sense glucose [10].

The Case for GK as the Neuronal Glucosensor

To establish a definitive role for GK as the regulator of neuronal glucosensing, GK must be present in glucosensing neurons. Also, conditions which modify GK activity should alter the ability of glucosensing neurons to utilize glucose as a signaling molecule. Thus, removal or attenuation of GK activity in glucosensing neurons should prevent or impair their ability to sense glucose. Stimulation of GK activity in glucosensing neurons should enhance their ability to detect glucose. As summarized below, we currently have good molecular and pharmacologic evidence for the presence of GK in both GE and GI neurons. However, eventual generation of animals with a specific neuronal GK deletion should go a long way to establish GK as the rate-limiting step in neuronal glucosensing.

GK Is Present in Brain Areas Involved in Glucosensing (fig. 1). In 1994, Jetton et al. [23] provided the first clear demonstration of GK in cellular elements within the hypothalamus, cerebellum, cerebral cortex and brainstem. GK was also found in the ependymal cells lining the IIIrd cerebral ventricle (ependymocytes), small cells adjacent to ventral hypothalamic perivascular spaces and anterior pituitary cells. Roncero et al. [24] demonstrated immunoreactive GK protein in immunoblots of the hypothalamus. They provided evidence for both high (8.9 m*M*) and low (0.07 m*M*) K_m glucose phosphorylating activity in this brain area. The high K_m (presumably GK) activity accounted for 40% of hypothalamic and cerebral cortical, 36% of thalamic and brain stem and 19% of amygdala and cerebellar glucose phosphorylation. However, these results are open to some question since GK protein is highly restricted [25], or not demonstrable at all in the forebrain by immunocytochemistry (except ependymocytes) [26] and GK mRNA is expressed only at very low levels or not at all in some of these areas [11, 14]. This low level of GK expression is not surprising since glucosensing neurons comprise no more than 20–40% of the total neuronal population, even in known glucosensing areas [6, 8, 10, 27–29]. Thus, we await confirmatory studies before accepting the idea that GK activity provides such high levels of glucose phosphorylation, even in known glucosensing brain areas.

Regardless of our ability to measure the glucose phosphorylating activity of brain GK, there is a high correlation between GK expression and the

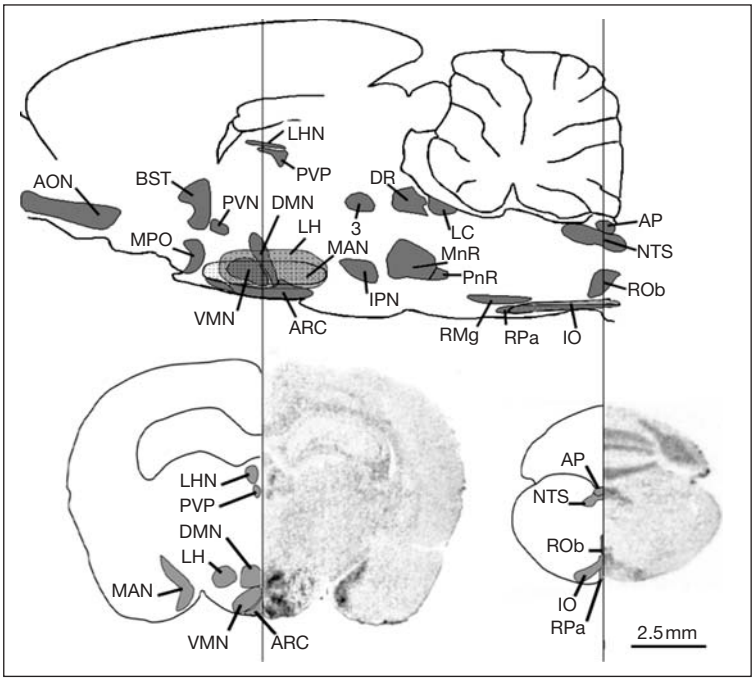

Fig. 1. Location of cells expressing GK mRNA or immunoreactive protein in sagittal and coronal sections of the rat brain [8, 11, 23, 24, 26, 82–84]. In situ hybridization autoradiographs of GK mRNA are included with the corresponding coronal sections through the hypothalamus and medulla. Abbreviations: hypothalamus: arcuate n. (ARC), dorsomedial n. (DMN), lateral hypothalamus (LH), paraventricular n. (PVN), ventromedial n. (VMN); thalamus: paraventricular n, posterior part (PVP); Extended amygdala: bed n. stria terminalis (BST), interpeduncular n. (IPN), lateral habenula n. (LHN), medial amygdalar n. (MAN), medial preoptic area (MPO); serotonin nuclei: dorsal raphe (DR), median raphe (MnR), ponine raphe (PnR), raphe magnus (RMg), raphe obscurus (ROb), raphe pallidus (RPa); noradrenergic: locus coeruleus (LC), n. tractus solitarius (NTS); other: anterior olfactory n. (AON), area postrema (AP), oculomotor n. (3), inferior olive (IO).

presence of glucosensing neurons in specific brain areas. The number of areas containing documented glucosensing neurons is limited [30], as is the distribution of GK (fig. 1) [8, 14, 26, 31]. Overlapping populations of glucosensing neurons and cells expressing GK are found in the hypothalamic paraventricular, dorsomedial, ventromedial (VMN) and arcuate (ARC) nuclei [8, 14, 25], as well as the nucleus tractus solitarius and area postrema in the medulla (fig. 2) [11]. However, GK mRNA is also expressed in areas not previously assessed for the presence of glucosensing neurons such as the lateral habenula, bed nucleus of the stria terminalis, inferior olive, retrochiasmatic and medial preoptic areas

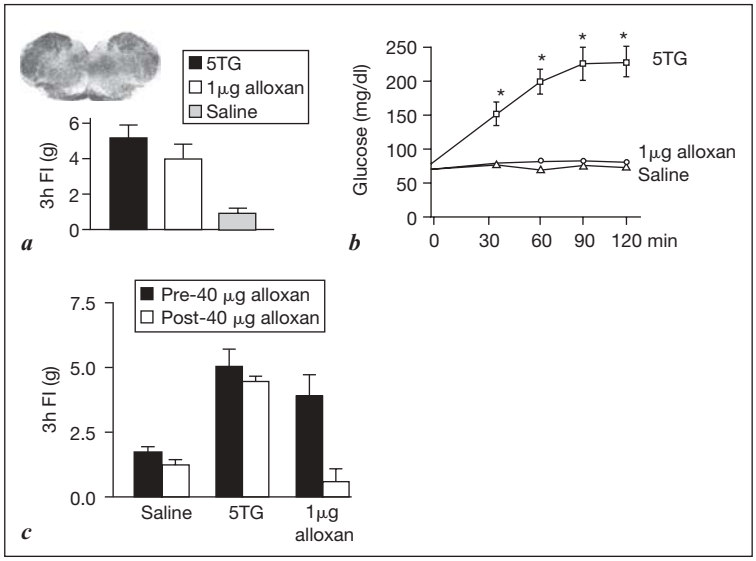

Fig. 2. Injections of 200 nl of 5-thioglucose (5TG), pharmacologic dose of alloxan (1 μg) or saline through cannula into raphe pallidus/obscurus region of hindbrain (upper right diagram in ***a***). Injection of 5TG and alloxan stimulates food intake (FI) in overnight-fasted rat. ***b*** Injection of 5TG but not alloxan stimulates hyperglycemia. ***c*** Injection of toxic dose (40 μg) of alloxan inhibits alloxan- but not 5TG-induced food intake.

and thalamic posterior paraventricular, interpeduncular, oculomotor, and anterior olfactory nuclei [14]. Since glucose-induced alteration of neuronal activity correlates well with the presence of GK [11, 32], we propose that GK expression in these additional areas suggests the presence of glucosensing neurons. Finally, aside from ependymocytes, which have no known glucosensing function, GK appears to be predominantly a neuronal marker. GK mRNA is present in neuropeptide Y (NPY) pro-opiomelanocortin (POMC) and γ-aminobutyric acid expressing neurons in the ARC, as well as in noradrenergic locus coeruleus [11, 14] and hindbrain raphe serotonergic neurons [26]. Many of these neurons and brain areas have well established roles in energy homeostasis, neuroendocrine and autonomic function [15, 16, 18, 33–35]. However, others are involved in motivation, reward, stress [36, 37], arousal [38, 39], respiratory and cardiovascular function [40, 41].

Evidence for GK as a Physiologic Regulator of Neuronal Glucosensing. Combined pharmacological and electrophysiological studies have provided the best evidence of GK as the gatekeeper for neuronal glucosensing. Before accepting such studies at face value, it is important to establish specific criteria

for a glucosensing neuron. Most importantly, a glucosensing neuron must respond directly (i.e. not via a presynaptic input) to changes in ambient glucose that lie within physiologic brain glucose levels. The direct response of glucosensing neurons to changes in ambient glucose from 0.5 to 3 m*M* involves alterations in the biophysical properties of the neuron such as changes in action potential frequency, membrane potential and/or membrane resistance. These glucose-induced changes might also alter the responsiveness of a glucosensing neuron to other neurohumoral inputs. It is likely that many prior estimates of the frequency of glucosensing neurons has been overestimated because of the failure to take presynaptic input to such neurons into account. For example, in VMN slice preparations only 14% of neurons were directly excited (GE) and only 3% were directly inhibited (GI) by altered glucose levels [10]. On the other hand, changing extracellular glucose concentrations changed the firing rate of an additional 33% of neurons. But this effect was abolished by blocking presynaptic input to these neurons [10]. When we use these strict criteria for a GE neuron, selective inhibition of GK activity with alloxan [42, 43] increases the membrane conductance (suggesting opening of an ion channel) and inhibits neuronal firing in VMN neurons in slice preparation (unpublished data) and depolarization-induced Ca^{2+} entry in isolated neurons [11, 44]. A rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) follows inactivation of the K_{ATP} channel in GE neurons and can be used as a surrogate for glucose-responsive changes in neuronal activity in dissociated VMN neurons, free of presynaptic input. Using this technique [11, 44], we showed that 71–100% of GE neurons reduced or ceased their Ca^{2+} spiking at 2.5 m*M* in the presence of the GK inhibitors, alloxan, mannoheptulose and glucosamine [11, 42–45]. On the other hand, 72–100% of GI neurons increased their Ca^{2+} spiking at 2.5 m*M* when alloxan, mannoheptulose or glucosamine were applied to the bath [11, 44]. These data suggest that reduction of intracellular ATP by inhibition of GK activity inhibits the firing of GE neurons, but stimulates the firing of GI neurons held at 2.5 m*M*. In addition, our data using single cell reverse transcriptase-polymerase chain reaction (RT-PCR) in dissociated VMN neurons suggest that GE and GI neurons both express GK mRNA, while only 8% of non-glucosensing neurons contain GK mRNA [44]. Importantly, all neurons tested to date, regardless of their glucosensing capacity, also express hexokinase I [44]. This is similar to the pancreatic β-cells [46]. Further, while β-cells are activated by increasing glucose concentrations, glucagon-producing α-cells are inhibited by increasing glucose and both express GK [47]. These data suggest that both GE and GI neurons, like the pancreatic β- and α-cells, employ GK as the gatekeeper for regulating their glucosensing function. The low K_m hexokinase I, on the other hand, might serve to regulate glycolysis under conditions such as hypoglycemia where glucose becomes limiting.

Physiologic Functions of Putative GK-Containing Glucosensing Neurons

Alloxan has proven to be an informative compound with regard to its interactions with GK. At low doses, it is an inhibitor of β-cell GK enzymatic activity with an IC_{50} of ~2–5 μ*M* [42, 43]. In dissociated VMN neurons, alloxan has an IC_{50} of ~4 m*M* for the pharmacologic (but not toxic) alteration of $[Ca^{2+}]_i$ spiking in both GE and GI neurons [11]. The tenfold lower sensitivity of VMN glucosensing neurons compared to β-cells may be due to the fact that alloxan appears to be transported into cells best by GLUT2 [48] and hypothalamic neurons do not appear to contain GLUT2 [49]. This is supported by our single cell PCR results showing that most VMN neurons contain the ubiquitous neuronal glucose transporter GLUT3, but little GLUT2 [44]. At higher concentrations, alloxan kills β-cells, presumably by the formation of free radicals and DNA damage through activation of poly(ADP-ribose) synthetase and depletion of $NADH^+$ [50]. When administered intracerebroventricularly (i.c.v.) to rats, low doses of alloxan stimulate food intake, while high doses attenuate or completely block the feeding responses to low dose alloxan or to glucoprivic agents such as 2-deoxyglucose or 5-thioglucose (5TG) [51–56]. Interestingly, high dose i.c.v. alloxan has no effect on the hyperglycemia associated with the counterregulatory response to systemic 2-DG [52–55]. These data suggest that alloxan acts to inhibit GK enzymatic activity or actually destroys GK-containing glucosensing neurons involved in glucoprivic feeding but that a separate set of non-GK-containing neurons mediates the glucoprivic stimulation of the counterregulatory mechanism responsible for producing hyperglycemia to 2DG.

Further support for this idea comes from our recent, unpublished studies using microinjections of alloxan into the GK-expressing serotonin neurons of the raphe pallidus/obscurus in the medulla (fig. 2). Injections of the glucoprivic agent, 5-thioglucose (5TG) into this area elicits both glucoprivic feeding and counterregulatory hyperglycemia [57]. However, low dose alloxan (1 μg) microinjected into this area evokes only food intake but not hyperglycemia. Pretreatment with a high, presumably toxic dose, of alloxan (40 μg) completely blocks the feeding response to low dose alloxan but has no effect on either the feeding or hyperglycemic effect of 5TG injected into this area (fig. 2). Interestingly, glucose but not 5TG can block the cytotoxic effect of alloxan on β-cells [58]. Taken together, these data suggest that alloxan and 5TG might act on different glucosensing cell populations in this brain area, one dependent upon GK and the other not. In fact, the serotonin neurons in this brain area are involved in the regulation of respiratory and cardiovascular function [40] and pancreatic insulin and glucagon release [35]. While some are sensitive to changes in plasma glucose [59], others respond primarily to altered oxygen

availability or pH [60, 61]. Thus, some non-GK neurons may be sensitive to lowering of intracellular ATP utilizing a GK-independent pathway.

Brain GK in Health and Disease

GK is expressed in brain areas and specific neurons involved in the regulation of energy homeostasis and neuroendocrine function such as the ventromedial hypothalamus (ARC + VMN) [8, 11, 14, 18]. Neurons in this brain area play critical roles in the maintenance of energy homeostasis [18]. These neurons can be considered metabolic sensors since they not only respond to glucose and express GK [11, 14, 44], but also respond to metabolic signals such as leptin [62–64] and insulin [65].

The ventromedial hypothalamus plays a prominent role in both energy homeostasis in obesity [18] and in the counterregulatory response to insulin-induced hypoglycemia [66–68]. GK expression is altered in animal models of both. Obesity-prone rats have a number of defects in their ability to detect and respond to glucose within the ventromedial hypothalamus [10, 69, 70–72]. In this same hypothalamic area, obesity-prone rats also overexpress GK mRNA, perhaps as a compensatory response to the multiple glucosensing abnormalities in these animals [11]. Another example of a possible compensatory rise in GK expression comes from the model of recurrent hypoglycemia. A single bout of insulin-induced hypoglycemia blunts the counterregulatory response to a subsequent bout of hypoglycemia [73–77]. In the ventromedial hypothalamus of rats, this counterregulatory blunting is associated with apparent apoptosis of cells and reduced expression of neuropeptides involved in neuroendocrine and autonomic function [75]. However, GK mRNA expression is increased in this same area [11]. Thus, ventromedial hypothalamic GK expression is increased in both the obesity-prone rat, with its multiple defects in glucosensing, and in rats with a defective ability to mount a counterregulatory response to hypoglycemia, These data supports the hypothesis that GK expression can increase when glucosensing function is impaired.

Summary and Future Directions

GK is an attractive candidate as the gatekeeper for neuronal glucosensing. It is located primarily in neurons which are located in hypothalamic and hindbrain areas involved in the regulation of neuroendocrine function and energy homeostasis [8, 11, 14, 18, 26, 44]. Inhibition of GK enzymatic activity reduces the activity of VMN GE neurons and increases the activity of VMN GI glucosensing neurons [8, 11, 44]. Inhibition of GK activity in the behaving animals with alloxan stimulates feeding in a manner which may be independent of conventional

glucoprivic stimulation of food intake [56]. Finally, GK mRNA expression is elevated as a possible compensatory mechanism where glucosensing function is impaired [11].

But several issues remain unresolved. First, it is unclear how GK, with a K_m for glucose phosphorylation of 8–10 m*M* [24, 78–80] might serve as a regulator of neuronal glucosensing when brain glucose levels generally range between 0.5–3.5 m*M* [12, 13]. This is barely at the inflection point of GK activity [10, 80]. Quite possibly neurons in the ARC may be exposed to plasma glucose levels because of their proximity to the incompetent blood-brain barrier in the median eminence [81]. But glucosensing neurons in the VMN almost certainly 'see' and function primarily at the much lower brain glucose levels [10]. Yet they appear to utilize GK to regulate their glucosensing function at these lower ambient glucose levels [11, 44]. Could the presence of GK regulatory protein in these brain areas [24, 82] somehow lower the functional K_m of GK? Are there undiscovered intracellular mechanisms for altering the availability and/or enzymatic activity of GK such that it can function at the very low levels of brain glucose? These critical questions are challenges for future research in this area.

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A Glucokinase/AP-1 Glucose Transduction Mechanism in the Ventromedial Hypothalamic Satiety Center

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A major goal of metabolic research is to identify causes of obesity. The problem of identifying variables that account for the considerable variance in human adiposity has attracted increasing concern because obesity is a major risk factor for morbidity and mortality in human populations, and, in contrast to most chronic pathologies, the incidence of obesity is increasing rapidly, especially in developing societies. Since nutrition-sensitive feedback signals normally act to maintain relatively stable food intake and adiposity, it is plausible that failure in one or more of these feedback signals could contribute to obesity. Therefore, the nature of these nutritional signals and the mechanisms by which these signals reduce food intake and adiposity have been the subject of intense investigation. The present review examines evidence that glucose conveys an important nutritional signal to neuroendocrine systems regulating food intake and body weight, and that failure of the neuroendocrine glucose sensing system involving glucokinase could contribute to obese phenotypes.

Glucose as a Hypothalamic Nutritional Feedback Signal

As early at 1916, Carlson [1] suggested that meal-related changes in blood glucose could serve as a signal for meal initiation (when glucose falls before

a meal) and meal termination (when glucose rises after a meal). However, Carlson's hypothesis focused on the role of the stomach as the site of transduction of the glucose signals, and a series of studies (especially by Sherington) appeared to rule out an important role for the stomach in the regulation of food intake. In addition, a major criticism of the hypothesis was that diabetic humans and animals, while exhibiting elevated plasma glucose, are in fact hyperphagic. Thus, for about 30 years the hypothesis that glucose plays an important role to regulate food intake was largely abandoned.

A more nuanced version of the hypothesis was formulated by Mayer and Bates [2] in 1952. A major stimulus for Mayer was the serendipitous discovery that a single injection of the glucose analog gold-thio-glucose could lead to permanent obesity in mice [3], and the developing appreciation during the 1940s that hypothalamic lesions could also reliably produce obesity [4]. While it had been generally appreciated that insulin-induced hypoglycemia could robustly increase food intake, whether this mechanism could be activated by normal periprandial dips in blood glucose level remained unclear. Mayer and Bates therefore undertook a series of careful studies (within the limits of the techniques available at the time) to assess if manipulating glucose within the physiological range could reduce food intake compared with carefully matched control treatments. These studies indicated that subcutaneous injections of 3 calories per day of glucose (split over two injections per day) would reduce food intake of normal rats by about 10 calories (from about 80 to about 70 calories per day food consumed), whereas the equivalent injection of sucrose (which cannot be converted to glucose after s.c. injection) or calorically equivalent injection of fat emulsion had no effect on food intake. Furthermore, insulin injections that produced fasting levels (but not lower) of blood glucose stimulated caloric consumption from about 80 to about 100 calories per day. These results were even more robust in hypophysectomized alloxan-treated rats, which, while not overtly diabetic, were unable to regulate blood glucose so that after subcutaneous injection of glucose blood glucose remained elevated for many hours. In this preparation injection of glucose produced over a 50% reduction in daily food intake; strikingly, when the injections were spread over three injections per day rather than two, 6 of the 8 injected rats starved to death. To explain these results, Mayer and Bates proposed that glucose serves to regulate appetite by acting on hypothalamic centers through the production of 'metabolites' and 'high-energy phosphate bonds'. A key feature of this 'glucostat' hypothesis was the neuronal metabolism of glucose, rather than glucose per se, as the essential neuroendocrine satiety signal. This distinction was emphasized to explain why diabetic individuals are hyperphagic: in the glucostat scheme, it was not glucose per se, but rather the (insulin-stimulated) metabolism of glucose in hypothalamic neurons, that serves at the signal for nutritional state.

In his first complete elaboration of the glucostatic hypothesis [5], Mayer followed this logic to its natural conclusion: 'For variations of blood glucose levels to influence hypothalamic glucoreceptors, glucose has to cross the membranes of these cells. This presumably implies phosphorylation through the hexokinase reaction. If phosphorylation is impaired, "effective sugar levels" will be in fact lower than absolute values as measured.' To the extent that glucose sensing plays a role in regulating body weight, this implies that impairments in the 'hexokinase' reaction could cause obesity. It is now clear that the relevant hexokinase is in fact the pancreatic form of glucokinase, haploinsufficiency in which produces hyperphagia and predisposes to obesity, as described below.

The glucostatic mechanism generated considerable interest during the two decades after Mayer's first description, but interest waned after several papers reported that acute elevation of plasma glucose had no short-term effect on food intake [6–8]. Indeed, in a 1986 review, Grossman commented that 'Many investigators in this field are indeed, disenchanted and dissatisfied with a model that has failed to produce more compelling support in spite of 30 years of intense empirical investigation', though it should be noted that Grossman also concluded that 'As yet, none of these [9] hypotheses has attracted sufficiently consistent and persuasive empirical support to warrant dismissal of the glucostatic theory' [10]. Of particular interest have been refined studies reporting that reductions in blood glucose do in fact predict the onset of hunger [11–16], leading Bray to recast the 'glucostat' mechanism as a 'glucodynamic' mechanism [17]. Infusion of glucose directly into the brain has also been reported to activate the sympathetic nervous system [18], increase metabolic rate [19], and decrease appetite [20] in lean, but not obese, rats. Conversely, a large literature has supported a key role for glucose metabolism as a satiety signal, since attenuation of brain glucose metabolism produces a prompt and robust stimulation of food intake [21–23]. Similarly, attenuation of glucose metabolism in the ventromedial hypothalamus activates peripheral sympathetic counterregulatory responses [24], and infusions of glucose into the hypothalamus block counterregulatory responses to peripheral hypoglycemia [25]. Furthermore, numerous papers have corroborated Mayer's initial report [5], that, whatever effects of elevating glucose may be on short-term food intake (still a controversial topic) at least over 24 h or longer elevating plasma glucose consistently reduces food intake [26, 27]. The recent paper by Gilbert et al. [27] is particularly of interest since this study clearly ruled out leptin as a mediator of the satiety effect of glucose, and corroborated previous studies that elevation of brain glucose concentrations, without influencing blood glucose concentrations, is sufficient to reduce food intake.

Early support for the importance of glucose-sensitive hypothalamic neurons in the regulation of body weight was that the glucose derivative gold-thio-glucose

(GTG) produces characteristic hypothalamic lesions leading to obesity, and that these GTG-induced lesions and subsequent obesity absolutely require the glucose-moiety of GTG and insulin and are blocked by inhibition of glucose transport suggesting that GTG causes obesity by targeting specific glucose-sensitive hypothalamic neurons [28]. The GTG lesion, though often thought of as 'VMH' lesion, is in fact centered on the cell-poor area between the ventromedial nucleus and the arcuate nucleus, though producing partial lesions of each of these nuclei. It is of particular interest that whereas glucose injections reduce food intake, and 2-deoxyglucose stimulates food intake, in normal mice, these treatments have no effect in GTG-treated mice; in contrast, CCK is as effective in reducing food intake in GTG-treated mice as in normal mice [29]. Consistent with these observations, i.p. injections of glucose induce jun-b mRNA (an immediate-early gene that complexes with c-fos to produce the transcription factor AP-1) primarily in the hypothalamic field destroyed by GTG [30]. Furthermore, the GTG lesion almost precisely overlaps the area expressing hypothalamic pro-opiomelanocortin (POMC) [31] and it is now clear that impairments in the hypothalamic POMC system can play a key role in causing obesity in rodents and humans [32]. We have now also shown that feeding induces c-fos in POMC neurons, independent of leptin [Shu et al., submitted], further implicating AP-1 in the glucose transduction mechanism in hypothalamic neurons that regulate satiety. Similarly, fasting downregulates hypothalamic POMC independent of leptin or insulin [33], also consistent with a direct effect of glucose to activate hypothalamic POMC neurons. Taken together these data support that glucose activates hypothalamic POMC neurons through a mechanism entailing activation of the AP-1 transcription factor. In turn, POMC neurons play an essential role in regulating metabolic homeostasis, including body weight, food intake, and, possibly most importantly, peripheral insulin sensitivity, since these metabolic phenotypes are largely reversed by transgenic restoration of POMC tone in genetically obese mice [34].

Hypothalamic Neurons Are Electrically Activated or Inhibited by Elevated Glucose

Considering the evidence that hypothalamic neurons play a role in maintaining glucose homeostasis, it is of particular interest that in vivo sub-populations of hypothalamic neurons become gradually and increasingly active or increasingly silent concomitant with a gradual rise in blood glucose from 3.6 to 17 m*M* [35]. In contrast, neurons from other brain areas including cortex and hippocampus do not exhibit any change in activity during the transition from 3.6 to 17 m*M* glucose, although neurons throughout the brain become inhibited when blood

glucose falls below about 2 m*M* [36]. Similarly, elevation of blood glucose induces c-fos activation primarily in hypothalamus [30], even when the brain blood glucose is elevated by carotid infusion without elevating peripheral blood levels [37]. Furthermore, this hypothalamic response to blood glucose was not observed in rats genetically predisposed to obesity [38]. The physiological relevance of glucose-sensitive hypothalamic neurons is suggested by the observation that these neurons cease firing just before a meal begins, and then begin to fire again as the meal progresses [39], and that these neurons are stimulated by other agents which decrease feeding behavior [40]. Subpopulations of hypothalamic neurons in in vitro slice preparations also become increasingly active or increasingly silent as bath glucose concentrations increase from 5 to 10 m*M* [40–45] and as with in vivo studies, such neurons are not observed in other brain areas such as cortex [41, 45]; neurons that are active at very low glucose concentrations but silent at higher glucose concentrations may represent hypothalamic neurons activated by hypoglycemia. Recent in vivo studies in humans [46, 47] and rats [48] using functional MRI have corroborated that ingestion of glucose causes a rapid change in hypothalamic neuronal activity that appears to be related to changes in plasma insulin [46]. Consistent with results in rats [20, 49] this hypothalamic response to glucose is impaired in obese humans [47].

Glucose-Sensing Mechanisms in Pancreatic β-Cells: The Metabolic Fuel Hypothesis

In view of the apparent importance of glucose-sensitive hypothalamic neurons in metabolic control, the mechanisms mediating effects of glucose on these neurons are of considerable interest. Most cells are insensitive to physiological changes in plasma glucose, but pancreatic cells are, like hypothalamic neurons, secretory cells whose excitation is modulated by changes in glucose concentrations in the physiological range (3–10 m*M*). Therefore, a guiding hypothesis has been that the glucose-sensing mechanisms of hypothalamic neurons and pancreatic endocrine cells may share common features. It is now well-established that glucose stimulates insulin secretion through a mechanism involving glucose metabolism in the β-cell, a mechanism referred to as the metabolic fuel hypothesis [50, 51]. The metabolic fuel hypothesis was based on the observation that glycolytic intermediates (though not pyruvate) can mimic the effect of glucose on pancreatic β-cells, whereas inhibitors of glycolysis would block the effects of glucose on pancreatic β-cells [52]. However, this mechanism posed a mechanistic problem, since in general the rate of intracellular glucose metabolism is not sensitive to plasma glucose concentration because hexokinase is such a high-affinity enzyme that its capacity is saturated at levels far below plasma levels

of glucose, and also hexokinase is back-inhibited by ATP. Through a series of remarkable studies, Matschinsky resolved this problem by demonstrating that pancreatic β-cells express an unusual form of hexokinse, called glucokinase. Unlike other hexokinases whose properties are such that glucose metabolism is maximum at about 0.2 m*M* so that higher levels of glucose do not produce higher levels of metabolism, the specific properties of glucokinase allow cells that express glucokinase, including pancreatic β-cells, to metabolize glucose in proportion to plasma levels of glucose when plasma glucose is in the physiological (5–20 m*M*) range. Key support for the hypothesis that glucokinase serves as a 'glucosensor' was provided by detailed studies which demonstrated that β-cell responses to glucose were in inverse proportion to the degree of inhibition of glucokinase by the high affinity glucokinase inhibitor mannoheptulose [51, 53–56]. Similarly, inhibition of glucokinase activity with the low affinity glucokinase inhibitor glucosamine in β-cells also inhibited insulin secretion [57, 58]. Though compelling on their own, these studies were dramatically corroborated by studies demonstrating that pancreatic β-cells with a disrupted glucokinase gene are completely unresponsive to glucose [59–61]. Indeed, homozygous glucokinase knockout mice die within days of birth due to insulin insufficiency.

Glucose Sensing in the Hypothalamus: A Metabolic Fuel Mechanism Dependent on Glucokinase

These studies led us to assess if the hypothalamic glucose sensing system entailed a metabolic fuel sensor. We originally hypothesized that glucokinase might mediate effects of glucose on hypothalamic neurons to account for apparent glucose toxicity in these neurons, analogous with glucose toxicity in pancreatic β-cells [62]. This hypothesis was supported by the observation that the pancreatic form of glucokinase was expressed in the hypothalamus [30, 45, 63–66]. We therefore undertook to examine the metabolic fuel hypothesis in much greater detail. Since hypothalamic neurons do not secrete insulin, it was necessary to use electrical activity as the marker of hypothalamic responses to changing glucose concentrations. Therefore, we followed the design used by Dean et al. [52], who studied the role of metabolism in regulating pancreatic β-cell electrical activity. These studies required a major design decision: since brain tissue levels of glucose are generally much lower than plasma levels [36], it was not clear whether to focus on responses to glucose concentrations at these low levels, or to focus on responses to the higher concentrations of glucose typically observed in the plasma. Some earlier studies had examined the mechanism by which neurons are electrically inhibited by the transition from 20 to 0 m*M*

glucose [67, 68]. However, 0 m*M* glucose inhibits electrical activity in most if not all neurons, whereas we observed that hypothalamic neurons appear to be unusual, if not unique, in their ability to respond to changes in glucose concentrations in the plasma concentration range between 5 and 20 m*M* [45], consistent with observations that peripheral elevations of plasma glucose induced jun-b primarily or exclusively in the hypothalamus [30]. Furthermore, while the presence of glucokinase in the hypothalamus strongly suggested an important role of glucokinase in mediating hypothalamic responses to glucose, responses to glucose, the high K_m glucokinase activity would be expected to be maximally active between 5 and 10 m*M* [69] would not be appreciably active at the much lower glucose concentrations of brain parenchyma. We therefore decided to focus on plasma, rather than brain, levels of glucose, on the assumption that glucose-sensing hypothalamic neurons may be specifically exposed to these concentrations, possibly via a unique glucose transporter that might also account for the susceptibility of these neurons to GTG.

We observed that about 17% of neurons in the ventromedial hypothalamic area were stimulated (as assessed by single-unit extracellular recording) by the transition from 5 to 20 m*M* glucose, but we never observed cortical neurons to respond to this transition [45]. However, almost all neurons in the hypothalamus and cortex became silent at 0–1 m*M* glucose. Focusing on neurons stimulated by the transition from 5 to 20 m*M*, we observed that glucokinase inhibitor glucosamine, reversibly blocked the responses of these neurons to the transition from 5 to 20 m*M* glucose; at the concentrations used, glucosamine had no effect on spontaneous electrical activity of neurons that were insensitive to glucose. Together with subsequent studies corroborating that inhibition of glucokinase attenuates hypothalamic responses to glucose [70], these data supported the hypothesis that glucokinase mediates hypothalamic responses to plasma concentrations of glucose, although as with any inhibitor studies other effects cannot be ruled out. Interestingly, these studies were also consistent with a previous report in which glucosamine infused into the third ventricle stimulated food intake [71]. We further observed that another inhibitor of glycolysis, iodoacetic acid, also profoundly inhibited responses of glucose-stimulated neurons; indeed, this inhibition was so profound that the inhibition was reversed only very slowly, if at all. We noted that the step inhibited by iodoacetic acid was the only step by which glycolysis produces NADH.

Having shown that inhibition of glycolysis could block effects of glucose on glucose-stimulated hypothalamic neurons, we conversely assessed if glycolytic intermediates could mimic effects of glucose. For these studies, we identified hypothalamic neurons whose electrical activity was stimulated by the transition from 5 to 20 m*M* glucose. Glucose was restored to 5 m*M*, then 15 m*M* mannose, galactose, glyceraldehyde, glycerol, pyruvate, or lactate was added to the bath.

All these glycolytic intermediates were quite effective to mimic effects of glucose on glucose-stimulated neurons except one: pyruvate failed to stimulate glucose-stimulated neurons, just as pyruvate fails to stimulate pancreatic β-cells [72]. However, we did demonstrate that pyruvate could be metabolized to support electrical activity, when, for example, glucose levels were lowered to 0 m*M*. Thus, the failure of pyruvate to mimic glucose seemed to be specific to the neuroendocrine sensing mechanism. Since pyruvate failed to mimic glucose, yet pyruvate is the final intermediate before oxidative metabolism (where the vast amount of ATP is produced), these data did not appear to support an important role for production of ATP in mediating the neuroendocrine effects of glucose. However, the observation that lactate did mimic effect of glucose, yet lactate is converted directly to pyruvate, suggested another intermediate was the signal: NADH, which is produced when lactate is converted to pyruvate. As indicated above, this observation is consistent with the observation that iodoacetic acid is a particularly effective inhibitor of the neuroendocrine effects of glucose, since iodacetic acid blocks the only glycolytic step at which NADH is produced.

The hypothesis that NADH, rather than ATP, might be a key metabolic intermediate mediating effects of glucose on hypothalamic neurons, while entirely consistent with the glucokinase metabolic fuel hypothesis, was met with considerable skepticism because previous studies had focused on the hypothesis that glucose influences hypothalamic function by altering ATP-dependent potassium channel function [67]. We had addressed the role of K-ATP channels in our own studies and, broadly consistent with previous reports, had found that indeed inhibition of these channels do activate glucose-stimulated neurons [45]. However, consistent with the observation that K-ATP channels are expressed ubiquitously throughout the brain, we observed that inhibition of K-ATP channels would activate most neurons throughout the brain at low (brain-physiological) glucose concentrations, suggesting a general, rather than neuroendocrine, role for K-ATP channels (possibly to generally protect neurons at low glucose concentrations). Furthermore, we observed that diazoxide, which antagonizes the effects of ATP on K-ATP channels and thus inhibits insulin secretion, blocked effects of glucose in fewer than half of the glucose-stimulated hypothalamic neurons examined [45], consistent with our observation that pyruvate, though metabolizable to ATP in hypothalamic neurons, could not substitute to mimic glucose in glucose-stimulated hypothalamic neurons. Furthermore, leptin activates K-ATP channels and inhibits electrical activity, even though, as logic might dictate, we and several other laboratories have observed that leptin activates glucose-stimulated neurons [73–75], apparently ruling out an important role of K-ATP channels in regulating glucose-stimulated hypothalamic neurons. Indeed, the inability of pyruvate to stimulate insulin secretion, as well as other anomalies

[76], suggests that the role of ATP production even in insulin secretion is not as clear as had been previously supposed. Furthermore, a recent study also indicated that NADH, acting through the mitochondrial NADH shuttle system, constitutes an obligatory component of the pancreatic β-cell glucose-sensing mechanism [77]. These observations suggested to us that while K-ATP channels might participate in hypothalamic glucose sensing in a general sense, NADH likely constituted the more specific metabolic signal in neuroendocrine glucose signaling.

The NADH hypothesis was strongly supported by a series of remarkable studies representing a technical tour de force [78]. In these studies, luciferase, whose activity is ATP-dependent, was transfected into hypothalamic and cerebellar neurons and glia. The authors observed that elevating glucose concentrations had no effect on ATP concentrations in hypothalamic neurons, but increased ATP concentrations in hypothalamic glia and cerebellar neurons and glia. Furthermore, the authors observed that reduction of glucose inhibited hypothalamic neuronal activity even when ATP concentrations were fixed at a high intracellular concentration. Of particular interest, lactate, but not pyruvate, could mimic effects of glucose. Taken together with our results, these studies strongly support the hypothesis that NADH may be more important than ATP in mediated neuroendocrine effects of glucose on hypothalamic neurons.

In contrast to the obvious mechanism by which ATP might depolarize cells (by blocking the K-ATP channel), the mechanisms by which NADH might depolarize cells are not as obvious. It should be emphasized that NADH might in fact also act by blocking the K-ATP channel, which is a very complex structure regulated by many factors other that ATP. In view of reports that ablation of the K-ATP channel impairs hypothalamic glucose sensing [79], this is an attractive hypothesis. On the other hand, based on evidence that NADH signaling required transport to the mitochondria [77], we hypothesized that elevation of NADH might influence hypothalamic function by increasing neuronal levels of malonyl CoA [45]. Subsequently, Loftus et al. [80] serendipitously discovered that fatty acid synthase inhibitors produce profound inhibition of food intake and body weight, apparently by elevating hypothalamic malonyl CoA. Even more strikingly, Gilbert et al. [27] have recently demonstrated sustained elevation of blood or brain glucose concentrations produce profound reduction in food intake independent of leptin, but apparently dependent on elevation of hypothalamic malonyl CoA.

Role of Glucokinase in Appetite and Body Weight Regulation

Taken together these data strongly support, but do not yet prove, that glucose acts through a glucokinase-dependent mechanism to activate hypothalamic neurons

responsible to maintain stable body weight and food intake. At the moment neither the role of glucokinase in regulating hypothalamic responses to glucose, nor the role of hypothalamic glucose sensing in the regulation of body weight, can be taken to be securely established. To address these questions more definitively, it would be desirable to examine the neuroendocrine phenotypes of mice in which glucokinase has been ablated, preferably specifically in neurons. Unfortunately it has not been possible to carry out such studies because complete ablation of the glucokinase gene leads to early neonatal death due to insulin insufficiency. Although Grupe et al. [60] reported successful rescue of glucokinase mice by expressing glucokinase under control of the insulin promoter, using the same construct we have never been able to do so. In our hands glucokinase knockout mice always die even if expressing the insulin-promoter-glucokinase construct. We therefore hypothesize that since the insulin promoter can sometimes drive neuronal expression, the viable line obtained by Grupe et al. [60] in fact expressed glucokinase in the hypothalamus, and that glucokinase must be expressed in the hypothalamus as well as in β-cells for viability. Nevertheless, we have been able to characterize two different lines of heterozygous glucokinase knockout mice, originally developed by Efrat [61] and the line described by Grupe et al. [60]. Although the phenotypes of these lines differ in some respects (notably that the line produced by Grupe et al. [60] are more hyperglycemic than the line produced by Bali and coworkers), we have observed that both lines exhibit higher food intake than wild-type controls. The hyperphagia appears to be independent of either insulin or leptin. Indeed, on a high-fat diet the heterozygous glucokinase knockout mice are not only more obese than wild-type mice, they exhibit higher levels of leptin and insulin than wild-type controls. These phenotypes are plausibly mediated by impaired hypothalamic sensitivity to glucose, since the heterozygous glucokinase knockout mice exhibit enhanced hypothalamic sensitivity to hypoglycemia, as reflected by enhanced hypoglycemia-induced food intake and hypoglycemia-induced expression of immediate-early genes as well as GLUT-1. We hypothesize that complete ablation of glucokinase in the hypothalamus would produce a much more profound hyperphagia and obese-prone phenotype, possibly comparable to that observed in leptin-receptor-deficient mice. In any case, we believe these studies strongly support the hypothesis that the hypothalamic glucose-sensing mechanism specifically involving glucokinase represents a system failure which could constitute an important cause of obesity.

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The Hepatoportal Glucose Sensor

Mechanisms of Glucose Sensing and Signal Transduction

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Glucose sensors located in different anatomical sites constantly monitor blood glucose levels and send nervous or hormonal signals to coordinate different physiological functions controlling whole body glucose homeostasis and energy balance. As described in the preceding chapters, the signalling pathway controlling glucose-induced insulin secretion by pancreatic β-cells is under the control of glucokinase enzymatic activity. This is demonstrated, in particular, by the superimposible dose-response curves of glucose phosphorylation, glucose oxidation and insulin secretion and by the fact that glucokinase mutations lead to dysregulated insulin secretion. Many recent studies have also described the presence of glucokinase in hypothalamic and brain stem nuclei and it is widely thought that its expression in specific neurons is required for glucose sensing in the central nervous system. Besides pancreatic β-cells and central neurons, glucose sensors have also been described in other location such as the hepatoportal vein region. Here, I will review some of the data pertaining to the description of the hepatoportal glucose sensor and recent studies performed using genetically modified mice to identify the mechanisms of glucose sensing and of signal transduction to peripheral tissue and which stimulate glucose utilization.

The Hepatoportal Sensor

The hepatoportal vein glucose sensor is activated when a portal-arterial glucose gradient is established [1–3], a situation which occurs during meal

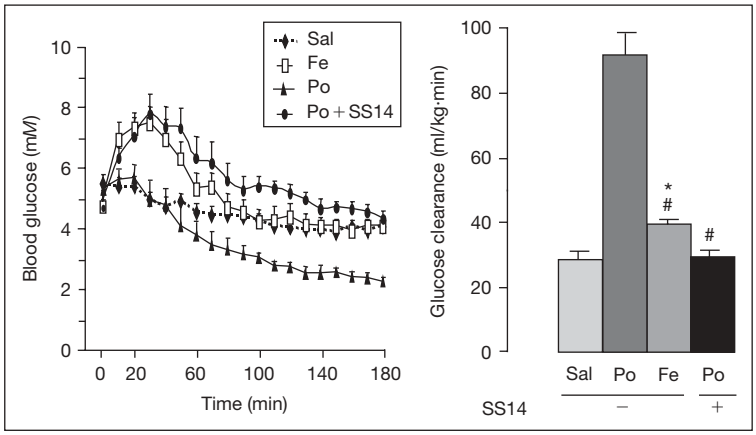

Fig. 1. Development of hypoglycemia and increased glucose clearance by portal vein glucose clearance infusion. Left: Glycemic profiles over the 3 h of the glucose or saline (Sal) infusions, either in the portal (Po) or femoral (Fe) veins and the effect of somatostatin co-infusion with glucose (Po + SS14). Portal vein glucose infusion induces progressive development of hypoglycemia, an effect blocked by co-infusion of somatostatin. Femoral glucose infusion induces a transient hyperglycemia followed by a return to normoglycemia. Right: Glucose clearance is strongly increased by portal vein glucose infusion and only moderately by femoral glucose infusion. Reproduced with permission from Burcelin et al. [9].

absorption. Activation of the portal sensor in the dog and the rat by direct portal glucose infusion results in a stimulation of glucose utilization in the liver and its storage as glycogen [4]. The activated portal sensor also blocks counterregulation induced by peripheral, insulin-induced hypoglycemia [5, 6]. A role of this sensor in inducing anorexia has also been demonstrated [7, 8]. More recently, in studies in mice, Burcelin et al. [9] demonstrated that glucose infusion in the hepatoportal vein at a rate equivalent to that of hepatic glucose production, led to development of hypoglycemia (fig. 1). This was caused by a stimulation of glucose clearance by peripheral tissues, as assessed by glucose turnover measurements. Quantification of ^{14}C-deoxy-glucose storage as ^{14}C-deoxy-glucose-6-phosphate in different tissues, indicated that uptake was preferentially stimulated in a subset of tissues, i.e. soleus, heart and brown adipose tissue. Development of hypoglycemia, which was observed in all of the mouse strains tested, occurred over a period of 3 h to reach values between 2.3 and 3 m*M*.

Transduction of the activated portal sensor signal to target tissues appears to depend in great part on nervous connections. Early studies by Niijima [10–12] showed that portal glucose infusion led to reduction in the firing rates of hepatic vagal afferent nerves. These are connected to the lateral hypothalamus [13, 14] and the nucleus of the tractus solitarius [15]. Activation of the portal

sensor by a portal-peripheral glucose gradient also requires insulin to be present. The absence of a portal-arterial gradient can be replaced by infusion of acetylcholine. In opposite, activation of the sensor can be suppressed by atropine infusion [3, 16]. Furthermore, surgical denervation of the hepatic nerves suppresses portal glucose-induced glycogen storage in the liver [17]. Thus, activation of portal vein afferent vagal nerves are essential to propagate the portal glucose signals, which must be integrated centrally and then transmitted to peripheral organs to regulate glucose homeostasis and probably also to other brain structures involved in the control of food intake.

Study of the Hepatoportal Vein Sensor Using Genetically Engineered Mice

A better understanding of the mechanisms of glucose detection by the hepatoportal sensor and of signal transduction to peripheral tissues was recently gained by the study of genetically modified mice.

Glucose Detection by the Portal Sensor: The β-Cell Model

In pancreatic β-cells, glucokinase catalyzes the enzymatic step with the major control strength on glucose-stimulated insulin secretion. Glucose uptake is not a regulatory step but suppression of GLUT2 expression by gene targeting leads to a severe defect in glucose-induced insulin secretion (GIIS) characterized by a complete loss of first phase and a second phase of much lower amplitude as compared to control islets [18]. The defect in secretory response is specific to glucose since amino acids or glyceraldehyde induce a normal insulin secretion. It is only caused by a restriction in the glucose uptake rate since normal GIIS can be restored by GLUT2 reexpression in isolated GLUT2-null islets using recombinant lentiviruses [19] or by transgenic re-expresion of GLUT2, or GLUT1, in the pancreatic β-cells of GLUT2$^{-/-}$ mice (RIPGLUT2,G2$^{-/-}$ or RIPGLUT1,G2$^{-/-}$ mice) [20]. These rescued mice can live and reproduce normally even though GLUT2 is not present in the other tissues where it is normally expressed (liver, intestine, kidney, and certain glucose-sensing cells).

Stimulation of the Hepatoportal Sensor in RIPGLUT1 × GLUT2$^{-/-}$ Mice

Activation of the hepatoportal sensor by portal vein glucose infusion in RIPGLUT1,G2$^{-/-}$ mice failed to induce hypoglycemia and to stimulate glucose

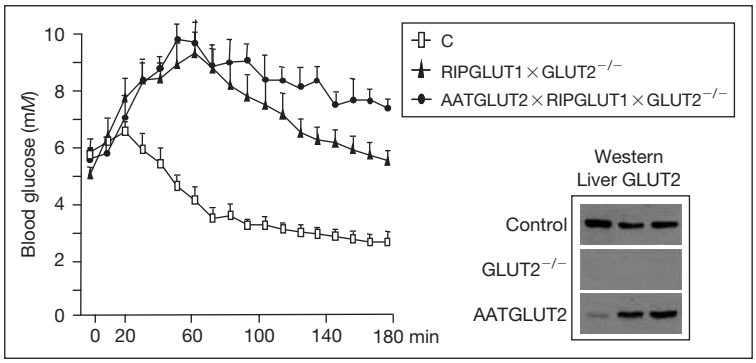

Fig. 2. Absence of hypoglycemic effect in the absence of GLUT2. Control, RIPGLUT1 × GLUT2$^{-/-}$ or RIPGLUT1 × GLUT2$^{-/-}$ mice expressing a GLUT2 transgene in the liver under the control of α_1-antitrypsin (AAT) were infused with glucose into the portal vein. Hypoglycemia developed in the control mice but not in the RIPGLUT1 × GLUT2$^{-/-}$ mice or the same mice reexpressing GLUT2 in the liver. Inset: western blot analysis of GLUT2 reexpression in the liver of the AAT1GLUT2 × RIPGLUT1 × GLUT2$^{-/-}$ mice. These data demonstrate that GLUT2 is required for the function of the portal sensor, which is distinct from the hepatocytes. Reproduced with permission from Burcelin et al. [21].

clearance [21] (fig. 2), suggesting that this sensor may also require GLUT2 to function normally. However, as GLUT2 is the major glucose transporter in liver and GLUT2-null hepatocytes have a >95% reduction in glucose uptake, the absence of hypoglycemia development may be due to the absence of GLUT2 from liver. To formally address this possibility, we generated transgenic mice which re-expressed GLUT2 in liver under the control of the α_1-antitrypsin promoter and transferred the transgene in the RIPGLUT1,G2$^{-/-}$ mice. This completely normalized hepatic glucose uptake and metabolism [22] but failed to restore the hypoglycemic effect of the portal sensor during portal glucose infusion. Thus, GLUT2 is required for the normal function of the portal sensor and this sensor is distinct from hepatocytes. This is in agreement with data published by Bergman and collaborators describing this sensor to be located upstream of the hepatic hilus [23, 24].

These data indicate a similarity between the molecular make-up of the portal sensor and that of pancreatic β-cells. This was further supported by the finding that co-infusion of somatostatin and glucose in the portal vein of control mice suppressed the hypoglycemic effect and the increase in glucose clearance [9] (fig. 1), indicating that somatostatin could inhibit the function of the portal sensor.

Role of GLP-1 on the Hepatoportal Sensor

An interesting question is the potential role of GLP-1 on the activity of the hepatoportal sensor. Indeed, GLP-1 is secreted by L-cells directly into the portal circulation but the stability of its bioactive form, GLP-1-(7-36)amide is extremely short, ~1–2 min, due to its rapid degradation by dipeptydylpeptidase IV, an ubiquitous enzyme also present in endothelial cells at the site of GLP-1 secretion [25]. Therefore, secretion is rapidly followed by inactivation and the portal sensor must be the anatomical site where the highest level of active GLP-1 is found.

Indication that GLP-1 can act on the portal sensor comes from measurement of the firing rate of the hepatic vagal afferent nerves, which was increased by portal infusion of GLP-1 [26]. This effect was, however, not observed with GIP [27]. Balkan and Li [28] also reported that portal vein infusion of GLP-1 increased insulin secretion via a neuronal mechanism distinct from the insulinotropic effect of GLP-1 delivered into a peripheral vein and probably acting directly on the pancreatic β cells.

In our paradigm, the effect of GLP-1 co-infusion with glucose in the portal vein, if it were to further potentiate the action of glucose, would either induce a faster rate of hypoglycemia development and/or a deeper hypoglycemia. In contrast to these expectations, no change in the rate or extent of hypoglycemia development nor in the increase in glucose clearance could be observed by portal co-infusion of GLP-1 with glucose [29]. To evaluate whether this was due to the insensitivity of the sensor to GLP-1 we performed experiments in which we co-infused the antagonist exendin-(9-39) with glucose in the portal vein of control mice. This led to a complete suppression of the hypoglycemic effect, which was replaced by a transient hyperglycemia and a progressive return of the glycemia to basal levels (fig. 3). This response was very similar to that obtained when glucose was infused in the femoral instead of the portal vein, i.e. without activation of the portal sensor. These data thus indicated that the portal sensor was already maximally stimulated by GLP-1 in the normal experimental conditions (6 hours fasted mice) and that exendin-(9-39) displaced GLP-1 from its receptor in the portal sensor, thus suppressing its glucose competence. To further substantiate these observations, similar experiments were performed in GLP-1 receptor-null mice. Portal vein glucose infusion also failed to induce hypoglycemia and to increase glucose clearance. Thus, the hepatoportal vein glucose sensor requires the presence of an active GLP-1 receptor for its glucose competence. The fact that it is already maximally active in the basal state and that exendin-(9-39) can suppress its function suggests that GLP-1 is secreted at sufficient rate in these conditions to maintain the glucose responsiveness of this sensor. Alternatively, the mere presence of the GLP-1 receptor may maintain sufficient basal levels of cAMP for the function of

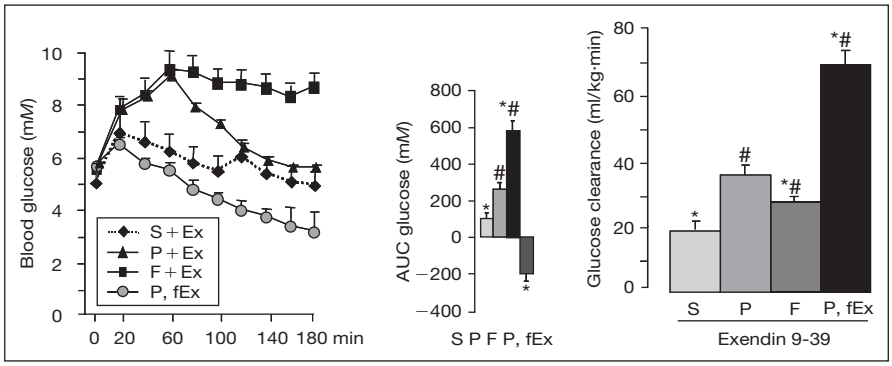

Fig. 3. Inhibition of the hepatoportal sensor by the GLP-1 antagonist exendin-(9-39). Left: Glycemic profiles. Middle: Areas under the glucose curve. Right: Whole body glucose rates. Portal infusion of glucose in the presence of exendin-(9-39) (P + Ex) induces a transient hyperglycemic response and only small increase in whole body glucose clearance. In contrast, infusion of glucose in the portal vein and exendin-(9-39) in the femoral vein (P + fEx) did not prevent development of hypoglycemia nor increase in whole body glucose clearance. Femoral glucose infusion in the presence of exendin-(9-39) (F + Ex) led to an increase in glycemia which did not return to the basal level over the time course of the experiment. S + Ex = Portal saline + exendin-(9-39) infusion. Together, these data indicate that exendin-(9-39) blocks activation of the portal sensor. See text for further discussion of the data. Reproduced with permission from Burcelin et al. [29].

the sensor. In this case, exendin-(9-39) would inactivate the sensor due to its inverse agonist activity at the GLP-1 receptor, as previously reported [30].

In a very recent study, Cherrington and collaborators tested the effect of GLP-1 intraportal infusion on the rate of glucose utilization by peripheral tissues [31]. These experiments, performed in dogs, also indicated that non-hepatic glucose uptake could be increased by portal infusion of GLP-1. These data are very similar but not identical to those obtained in the mouse since exogenous GLP-1 could still stimulate the signal sent by the glucose sensors. This could reflect species-specific differences.

Together the above data indicate that the hepatoportal sensor is composed of glucose sensing units sharing similarity with pancreatic β-cells, i.e. requirement for GLUT2 expression for normal sensing; inhibition by somatostatin; a glucose competence stimulated by the presence of an activated GLP-1 receptor.

Signal Transduction by the Hepatoportal Sensor

The change in firing rate of hepatic afferent nerves by portal vein glucose infusion suggests that the increase in glucose clearance may be due to an

activation of the autonomic nervous system. This may involve a relay through the brainstem and/or the hypothalamus, and the subsequent regulation of tissue glucose uptake by the autonomic nervous system. This would be compatible with recent data describing increased glucose utilization in muscle by activation of the central nervous system, for instance by intracerebroventricular injection of leptin [32].

We attempted to test the importance of these nervous connections by denervating the muscle of one leg of the mice that were prepared for glucose infusion experiments. Measurement of the rate of glucose utilization in the denervated muscle indicated an approximately 50% reduction in glucose uptake as compared to muscle with intact nerve [9]. In the same experimental model, the increase in glucose uptake stimulated during femoral glucose infusion was completely suppressed by denervation. These experiments therefore suggest that the stimulation of glucose uptake by the portal sensor is transmitted in part by an activation of the nervous system but that a part may be independent. Whether soluble factors could participate in this mechanism is not yet known. Such factors have however been postulated to account for the increase in insulin-sensitive muscle glucose uptake observed following portal vein glucose infusion [33, 34].

Role of Muscle Insulin Receptor, GLUT4 and AMP-Kinase in the Stimulation of Glucose Clearance by the Hepatoportal Sensor

The induction of hypoglycemia by the activated portal sensor was accompanied by a slight increase in insulinemia, which was, however, of similar magnitude to that observed during a femoral glucose infusion. Thus, hypoglycemia could not be explained by an exaggerated increase in insulin secretion but rather by an insulin-independent or -sensitizing effect.

To more specifically address the role of insulin in stimulating hypoglycemia and increasing glucose clearance, mice with muscle-specific inactivation of the insulin receptor (MIRKO) [35], were infused with glucose in the portal vein [36]. The same hypoglycemia and increase in glucose clearance rate were observed in MIRKO mice and their control littermates. Similarly, hypoglycemia development and clearance rates were evaluated in mice with muscle-specific inactivation of the GLUT4 gene [37]. Absence of the transporter from muscle prevented development of hypoglycemia and the glycemic profiles obtained over the time-course of the experiments were similar to those obtained when glucose was infused through the femoral vein [36]. Thus, even though insulin action in muscle is not required for increased glucose clearance, glucose uptake still proceeds mainly through GLUT4.

GLUT4 is located in intracellular vesicles which, after insulin stimulation, translocate to, and fuse with the plasma membrane to increase glucose uptake. Other signalling pathways also participate in the control of GLUT4 vesicle translocation. Recently, the metabolic stress sensitive AMP-activated kinase (AMPK) has been demonstrated to be involved in GLUT4 translocation, in response to hypoxia and following activation of autonomic nerve fibers innervating muscles [32, 38].

The possible participation of AMPK in portal vein sensor stimulated glucose uptake was tested using mice expressing a transgenic dominant negative form of AMPK in muscle (AMPK-DN mice) [39]. Portal glucose infusion failed to stimulate development of hypoglycemia and to increase glucose clearance [36].

Together, the above data indicate that the portal sensor induces hypoglycemia by stimulating glucose uptake in muscle by a mechanism that is insulin-independent. This mechanism requires the translocation of GLUT4 to the cell surface and the presence of a dominant-negative form of AMP kinase blocks this response. This suggests the participation of this enzyme in the signal transduction mechanism at the target muscles.

Cellular Basis of the Portal Sensor

The cellular structure of the portal sensor is not yet elucidated. Morphological studies have demonstrated the presence in the portal vein of portal bodies consisting of glomus cells and nerve fibers. Thus, by analogy with the carotid bodies [40], it could be proposed that the glomus cells are involved in glucose sensing and activate vagal afferent. However, at present there is no direct demonstration that the portal bodies identified by Nakabayashi et al. [41, 42] are really the glucose sensing structure of the portal vein. Localization of GLUT2 to these structures would be a strong argument in favor of a direct role in glucose sensing. Different structures may thus be involved in glucose sensing in the portal region. Whether they all control the same physiological function is unknown. Although at present it is difficult to assign specific function to different portal sensing units because they are not firmly identified, functional analysis supports the existence of different type of sensors. Initially, the presence of sensing units connected to the vagal hepatic afferents fibers were described on the basis of their glucose-regulated firing rates [10, 12]. These were decreased by raising portal glucose concentrations but were increased by either GLP-1 [26] or somatostatin [42] infusion. Thus, the effect of these two hormones is opposite to the effect of glucose but similar to each other. In the experiments described above, the activation of hypoglycemia and of glucose

clearance rates required GLP-1 receptor activity [29] but were inhibited by somatostatin [9]. There is therefore no strict correlation between the modulation by glucose, GLP-1 and somatostatin of vagal afferent firing rates and regulated glucose clearance rates.

These data therefore point to the existence of multiple glucose sensing systems in the hepatoportal vein region. Two additional observations further support this proposal. Firstly, in studying the regulation by GLP-1 of the vagal afferent firing rates, Nakabayashi et al., [43] showed that exendin-4, a strong agonist of the β-cell GLP-1 receptor could not mimic GLP-1 action. Furthermore, these firing rates were not altered by the antagonist exendin-(9-39). They therefore concluded that the GLP-1 receptor modulating the activity of these neurons was different from the β-cell receptor. In contrast, our data demonstrated that the exendin-(9-39) suppressed the effect of portal glucose infusion on the increased clearance rate and the absence of the β-cell isoform of the GLP-1 receptor also led to the suppression of the same effect. Secondly, the hypoglycemic effect observed following portal vein glucose infusion is obviously not observed when glucose is delivered intragastrically. As in these conditions, glucose rapidly appears in the portal vein but does not induce hypoglycemia, this suggests that during passage of glucose through the intestinal epithelium or at very early stages of collection into the portal vein, other nerve terminal of the autonomic [44] or enteric nervous system become activated [45]. Together with the sensor activated by direct portal glucose infusion they probably combine to optimize glucose handling to avoid hypoglycemia.

Conclusions

The presence of a glucose sensor in the hepatoportal vein region is now well established. The possibility to test its function in mice with different genetic targeted modifications has provided important new insights in the molecular mechanism of glucose recognition and in the mechanism of signal transduction to target organs. It is now clear that this sensor is GLUT2-dependent and can be modulated by GLP-1 and inhibited by somatostatin. The physiological functions controlled by this sensor include glucose storage in the liver, inhibition of counterregulation and termination of feeding. The recognition that it can also stimulate glucose utilization by a subset of peripheral tissues, by an insulin-independent mechanism, further stresses the role of this sensor in the fine-tuning of glucose homeostasis in the absorptive period. Finally, comparison of different sets of data where the sensor is studied for its role in controlling hepatic vagal afferent nerves firing rates or in controlling glucose clearance, suggests that there is probably not a single mechanism or structure for portal glucose

sensing. A more thorough description of the portal vein innervation and localization of key molecules involved in glucose sensing may shed light on the complexity of this sensing system.

Acknowledgements

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Linear Relationship between Glucokinase Expression, Metabolic Redox State and Insulin Secretion

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Glucose-induced insulin secretion is tightly coupled to the metabolic state of the β-cell. Indicative of the increase in β-cell glucose metabolism is a rise in total NAD(P)H content [1], which is autofluorescent and can thus be measured [2]. The glucose phosphorylating enzyme, glucokinase (GK), has been established as a rate-limiting step for glucose metabolism [3] and the half-maximal glucose dose response for NAD(P)H is similar to the GK EC_{50} [4]. Levels of GK, glucose phosphorylation, NAD(P)H, and insulin secretion can all be measured quantitatively and compared over various amounts of glucose stimulation and genetic manipulations of GK copy number. These results, summarized in the first part of this chapter, show that GK does indeed exercise tight control over downstream metabolic and secretory events. More distal in the pathway, synchronous oscillations of intracellular Ca^{2+} and insulin secretion under glucose stimulation have been observed [5–7] and a linear relationship between the intracellular Ca^{2+} levels and insulin release during the oscillations has been suggested [8]. Although the signaling pathways depend on the β-cell metabolic state, Ca^{2+} events may also regulate the glucose signaling such that it supersedes tight regulation by proximal metabolic events. As shown below, a strikingly linear relationship was found between the redox state and insulin secretion over a broad glucose range and under altered glucose metabolism. Finally, by modulating GK expression levels via changes in the culture media glucose concentrations, we found indications of an allosteric regulation of GK, which further bolsters the evidence for such regulation described in several recent publications [9–13].

The experiments described in this chapter were all carried out on islets that were isolated from 6- to 12-week-old female mice [14]. These islets were derived from mice of three different backgrounds. One contained an extra copy of the GK gene (gk+/w) [15], another contained a disrupted (null) copy of the GK gene (*gk* d/w) [16] and the C57B (*gk* w/w) were used as wild-type controls. In our lab at Vanderbilt, we have developed several unique, quantitative imaging approaches for the study of pancreatic islet dynamics. All experiments were performed on the microscope stage using a closed chamber of a design similar to that of Bergsten (1995), and samples were kept at 37°C and 5% CO_2 throughout the experiments [2]. First, NAD(P)H was measured non-invasively by two-photon excitation microscopy [2]. Second, GK expression levels were determined by semiquantitative immunofluorescence methods. To maintain even immunoreactivity, islets from different mice or treatments were immobilized in specific locations within the same dish. This minimizes any differences in antibody concentration, time of incubation, or environmental differences during the immunolableing procedure. The results of quantitative confocal imaging performed under identical imaging conditions yields results that can then be directly compared to the NAD(P)H measurements.

In this chapter, we will summarize many of our results regarding the role of GK in regulating NAD(P)H responses to glucose and insulin secretion. These results show the strong control that GK exerts over the entire glucose signaling pathway. In addition, we describe the results of rapid changes of glucose levels in the culture media, which give rise to evidence of allosteric regulation of GK in the β-cell.

Relationship between GK Expression and Metabolism

Under steady-state culture conditions, we have shown that overexpression of GK in transgenic mice [15] results in an increase in the NAD(P)H response and a concomitant rise in insulin secretion, whereas disruption of the GK gene by Cre-mediated recombination [16] decreases the metabolic and secretory response in islets. These results can be summarized as a function of GK gene copy number: 1 copy – heterozygous knock-out, 2 copy – wild-type, 3 copy heterozygous transgenic mouse. In all these cases, GK expression was very uniform cell to cell in whole islets. It is known that culturing islets in high (or low) glucose over a period of days increases (or decreases) GK expression [17]. Table 1 shows our results which demonstrate that culturing in different glucose concentrations can fully compensate for differences in the GK copy number. Based on the traditional view that GK is constitutively expressed [18], our interpretation

Table 1. GK expression levels after 2 days in culture at varying glucose concentrations

[Glucose] GK gene copies	2 m*M*	10 m*M*	20 m*M*
1	0.25 ± 0.11	0.44 ± 0.18	1.25 ± 0.16
2	0.48 ± 0.13	1	1.41 ± 0.05
3	0.61 ± 0.20	1.51 ± 0.11	2.42 ± 0.07

GK was assayed by immunofluorescence microscopy, and normalized to wild-type cultured in 10 m*M* glucose.

Table 2. NAD(P)H response and GK immunofluorescence for different islets cultured for 2 days in 10 m*M* glucose

GK gene copies	NAD(P)H response	GK level
1	0.58 ± 0.06	0.44 ± 0.18
2	1	1
3	1.45 ± 0.09	1.51 ± 0.11

NAD(P)H response is calculated after background subtraction from the images and normalized to the response of the wild-type islets. GK levels are calculated as in table 1.

of this data is that glucose stabilizes GK and thus increases its lifetime. In the presence of high GK turnover (2 m*M* glucose concentration), the reduced transcriptional strength of the single GK gene copy cannot generate enough GK to maintain normal levels. On the other hand, when GK is significantly stabilized in the presence of high glucose concentrations (20 m*M*), the single copy can generate enough transcript to maintain near normal protein levels.

Detailed studies of islets from the different GK gene copy mice cultured in 10 m*M* glucose for two or more days shows a significant correlation between the NAD(P)H response to a change in glucose concentration from 2 to 20 m*M* and GK expression levels (table 2). These data have led us to conclude that culturing islets in different glucose concentrations (over a period of 2–4 days) affects glucose signal transduction almost entirely by changes in GK expression. The metabolic results from heterozygous knock-out mice agree with other published work [19].

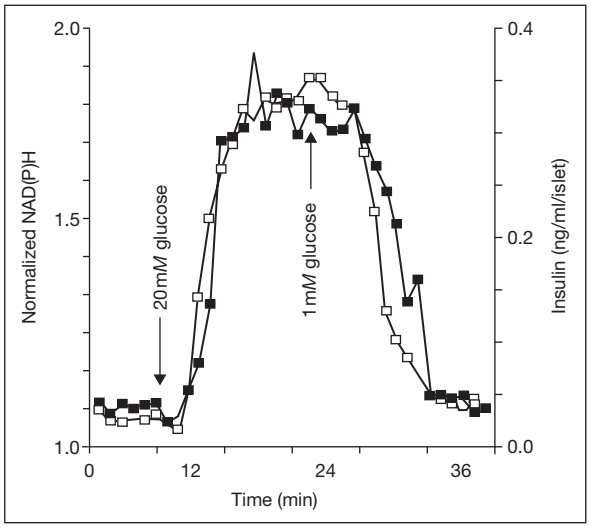

Fig. 1. Simultaneous measurement of NAD(P)H fluorescence and insulin secretion changes in response to glucose. Fifteen islets were perfused with 1 m*M* glucose for a 30-min equilibration period before starting the experiment. The NAD(P)H (white squares) from one of these islets and insulin secretion (black squares) from the entire chamber were monitored for 6 min at 1 m*M* glucose before switching to 20 m*M*. At the 20-min mark, the glucose was decreased back to 1 m*M* glucose. The delays and relatively slow increases seen in here arise from the perfusion system delay times and flow rate (~150 μl/min) used for these experiments (data not shown). Data are from a single experiment.

Relationship between Metabolism and Insulin Secretion

A novel finding from this work was the striking linear relationship between the metabolic and secretory response to glucose. Such a linear response was suggested previously [20], but then abandoned [21]. To investigate this relationship, the NAD(P)H levels and insulin secretion from intact, living pancreatic islets were monitored over time in response to changes in extracellular glucose. To establish the temporal resolution and sensitivity of these assays, the glucose levels were varied from low to high glucose and then back to low glucose as shown in figure 1. The islets were perfused for a 30-min equilibration period with 1 m*M* glucose prior to the start of the experiment and for an additional 6 min at the onset of the experiment. NAD(P)H images and perfusion fractions were collected at 1 min time points for the duration of the experiment. Fractions of the output perfusate were stored at −20°C until the insulin levels were determined by a competitive ELISA method similar to those described

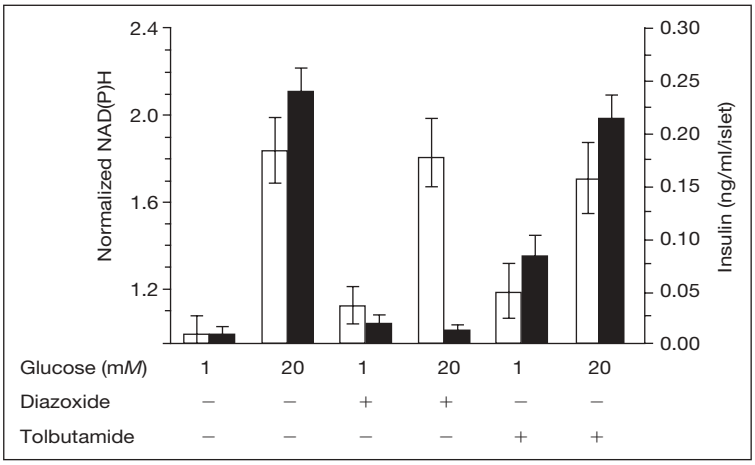

Fig. 2. Uncoupling of metabolism and secretion at the K_{ATP} channel. NAD(P)H autofluorescence (in 1 islet shown white columns) and insulin secretion (from 15 islets shown in black columns) were measured as the islets were perfused with 1 or 20 m*M* glucose in the presence of 0.25 m*M* diazoxide and then 0.1 m*M* tolbutamide. The islets were perfused for 10 min under each condition before collecting an NAD(P)H image and 5 min perfusion buffer fraction. The slight NAD(P)H increases in the presence of the drugs at low glucose are not statistically significant. Data are displayed as mean ± SE (n = 9).

earlier [22–24]. After the glucose level was increased to 20 m*M* at the 6-min point, the maximum NAD(P)H and insulin secretion responses were observed within 10 min. The Y axes for the NAD(P)H and insulin signals have been determined on the fit of NADH and insulin secretion shown in figure 3c.

The metabolic response could be uncoupled from insulin secretion using two pharmacological agents that act on the K_{ATP} channel (fig. 2). Diazoxide acts to keep the channel open, which prevents membrane depolarization and the subsequent events leading to insulin secretion, whereas tolbutamide interacts with the sulfonylurea receptor associated with the K_{ATP} channel and closes the channel which leads to membrane depolarization and eventually insulin secretion. The islets were perfused for 30 min with 1 m*M* glucose for equilibration and then sequentially in 1 and 20 m*M* glucose in the absence of any drug, in the presence of 0.25 m*M* diazoxide, and then in the presence of 0.1 m*M* tolbutamide. In the presence of diazoxide, no increase in insulin output was evident at stimulating glucose levels (20 m*M*), whereas the NAD(P)H levels increased normally in response to glucose. In the presence of tolbutamide, secretion was evident at both low and high glucose concentrations while the metabolic response, as determined by NAD(P)H levels, remained normal at both levels.

Using this approach, we could then assay directly the relationship between the glucose dose responses of both metabolism and insulin secretion. The resulting data, shown in figure 3, are unambiguous, and indicate that under normal conditions insulin secretion is determined by glucose metabolism regardless of downstream molecular events such as membrane depolarization and Ca^{2+} influx.

Glucose metabolism is required for insulin secretion under normal conditions, thus qualitatively similar glucose-stimulated increases in NAD(P)H and insulin secretion are always observed (for review, see [25]). While membrane depolarization and increased intracellular Ca^{2+} activity are necessary for normal insulin secretion, their high capacities for relaying the signal limit their role to a permissive one. Comparison of the NAD(P)H response with insulin secretion suggests that the glucose metabolic state tightly regulates insulin release from pancreatic islets under normal conditions (fig. 3c). Therefore, in a direct metabolic control model, signaling events between metabolism and secretion should also display linear relationships. In fact, comparisons of distal signaling events have found linear relationships between the ATP/ADP ratio and insulin secretion [26, 27], NAD(P)H levels and $^{45}Ca^{2+}$ uptake [20, 28], and $^{45}Ca^{2+}$ uptake and insulin secretion [20, 28].

A key point of interest with the direct control model is that glucose stimulated insulin secretion is a dynamic process characterized by oscillations in membrane depolarization [30], intracellular Ca^{2+} levels [7], and insulin secretion [7]. Similar frequency and amplitude modulations of intracellular Ca^{2+} and insulin release have implied a regulatory role for this distal event in the glucose signaling pathway. If such a regulating role were present, a non-linear effect resembling a 'kink' would be expected in the relationship between NAD(P)H and insulin secretion. The absence of the 'kink' indicates that the events distal to glucose metabolism do not have a regulatory role. Therefore, the effects of Ca^{2+} must be a permissive event leading to insulin secretion rather than a regulatory event. An attractive hypothesis is that the distal oscillations are derived from more proximal metabolic oscillations. However, we and others [29] have not observed oscillations in NAD(P)H fluorescence in islets.

The differences observed between islets from wild-type mice and *gk* d/w islets further confirm the permissive role of intracellular Ca^{2+} signaling. In the *gk* d/w islets, metabolism is significantly reduced, while secretion is reduced much less (0.135 ng/ml/islet for *gk* d/w islets versus 0.195 ng/ml/islet for wild-type, fig. 3c). The further reduction in metabolic response suggests a compensatory mechanism that increases secretion in the presence of a sustained metabolic defect. Since the relationship between metabolism and secretion is still strictly linear over the glucose concentrations where Ca^{2+} oscillations occur, Ca^{2+} signaling is not directly involved in this compensatory mechanism.

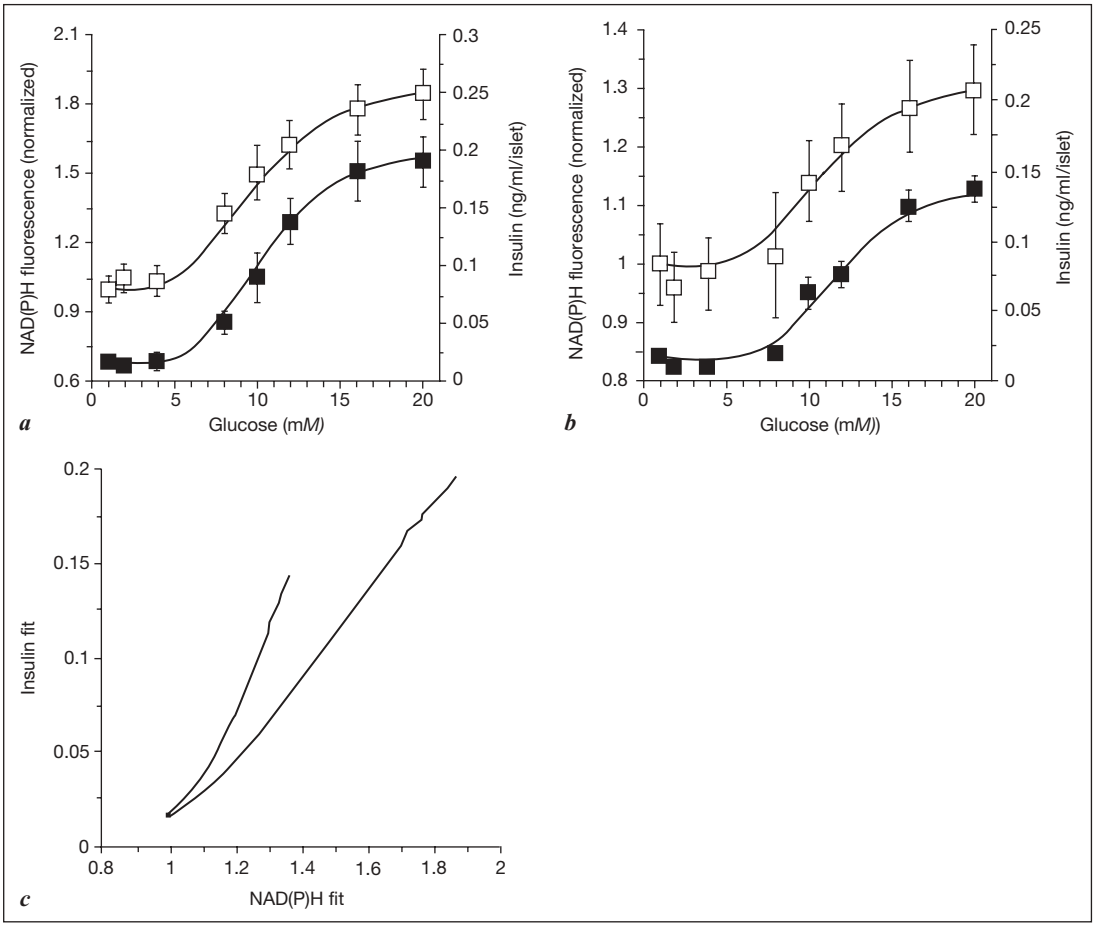

Fig. 3. Glucose dose responses from wild-type and heterozygous GK knock-out islets yield a linear relationship between NAD(P)H and insulin secretion. ***a*** The NAD(P)H response (shown in white squares) and insulin secretion (shown in black squares) from an intact wild-type islet measured as the glucose concentration was increased. Islets were perfused for 10 min under each glucose concentration before collecting an NAD(P)H image and 5-min perfusion buffer fraction. Fits are depicted by the solid black line ($C + (V_{max}/(1+(K/[glucose])^H))$) with values for the NAD(P)H fit: $C = 1$, $V_{max} = 0.92$, $K = 10$, $H = 4$, $\chi^2 = 1.07$ and values for the insulin secretion fit of: $C = 0.015$, $V_{max} = 0.19$, $K = 10.4$, $H = 4.6$, $\chi^2 = 0.91$). ***b*** Similar to ***a***, NAD(P)H response (from a single islet shown in white squares) and insulin secretion (shown in black squares) from a heterozygous GK knock-out islet. Values for the NAD(P)H fit: $C = 1$, $V_{max} = 0.3$, $K = 11$, $H = 4.5$, $\chi^2 = 1.01$; values for the insulin secretion fit: $C = 0.015$, $V_{max} = 0.13$, $K = 12$, $H = 5$, $\chi^2 = 1.36$. ***c*** The two fits from ***a*** or ***b*** were plotted against each other to demonstrate the relationship between the two events. Data are displayed as mean ± SD (n = 19 wt islets, n = 13 $GK^{+/-}$ islets).

To accommodate the linear relationship exhibited between NAD(P)H and insulin secretion with the dynamics of distal events, one of two opposing conclusions can be drawn. First, the metabolic redox state may not be linked with the distal oscillations and one or the other but not both are important in controlling insulin secretion, or secondly, glucose metabolism and oscillatory events are connected by an unknown mechanism. Points against the former conclusion include the requirement of metabolism for glucose to stimulate insulin secretion [1] and the remarkably linear dependence of secretion on the NAD(P)H response shown in figure 3c. In addition, the similar frequency and amplitude modulations of intracellular Ca^{2+} and secretion suggest a modulatory role for the oscillations [31]. Accentuating this point is that the loss of insulin secretory oscillations in vivo may be an early sign of the development of diabetes [32, 33]. Thus, it seems that both the redox state and distal oscillatory events have critical roles in normal insulin secretion. Therefore, since NAD(P)H oscillations are not readily observable in pancreatic islets, it is more likely that the two events are linked in an undetermined manner. Thus, control of insulin secretion by GK is much more linear than might have been anticipated from the number and complexity of downstream processes, such as membrane depolarization and intracellular Ca^{2+} signaling.

Evidence for Allosteric GK Regulation in β-Cells

It has previously been established that long-term reduction (on the order of days) of the media glucose concentration leads to degradation of GK, and thus a reduced metabolic and secretory response. As described above, culturing islets in low glucose over a period of days reduces GK expression, and thus reduces the metabolic and secretory responses [17]. However, rapid changes in the glucose concentration of the culture media can also modulate the NAD(P)H and insulin secretory responses. These rapid changes are easily observed by imaging approaches, which are well-suited to visualization of changes over a wide range of time scales (from milliseconds to hours).

As shown in figure 4, metabolism, glucose usage, and secretion can be reduced in less than 8 h by culturing islets in media containing 0.1 m*M* glucose, but GK immunofluorescence and western blots show no statistically significant change in GK levels. Returning the islets to media containing 10 m*M* glucose restores the full metabolic and secretory responses within less than 4 h, which is too fast for synthesis of new GK protein. This is in contrast to what happens when islets are cultured in 0.1 m*M* glucose for 48 h, where GK expression drops considerably. After returning the islets to 10 m*M* glucose media, it took several days for the responses to return to normal, putatively because new GK had to be

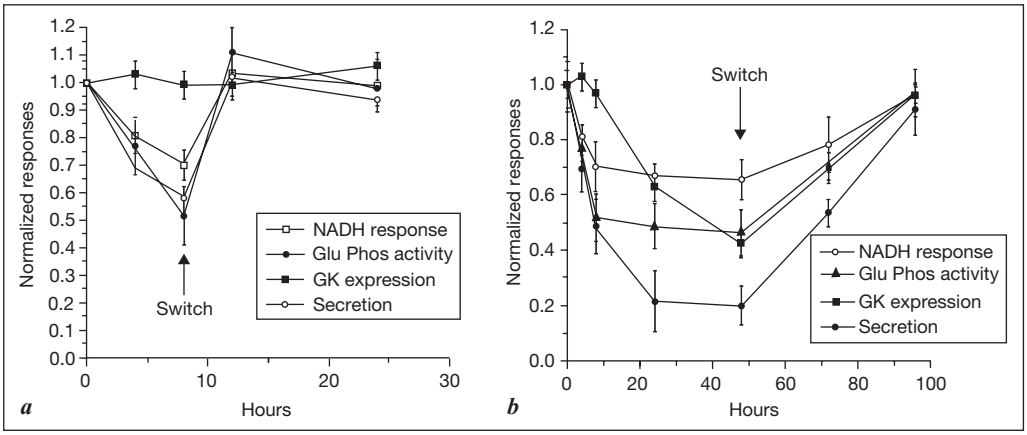

Fig. 4. Effects of low glucose culture over 8 to 48 hours. Islets were cultured in media containing 10 m*M* glucose for 2 days, then switched to 0.1 m*M* glucose at t = 0. At t = 8 h (***a***) or t = 48 h (***b***), the islets were switched back to 10 m*M* glucose (n = 11 for ***a*** and n = 7 for ***b***). GK levels were determined by immunofluorescence, metabolism, glucose utilization, and secretion were determined by the method of Katz and Dunn [34].

synthesized. This data is consistent with a dynamic GK-regulatory mechanism, which may be governed by the GK binding to secretory vesicles. These rapid changes (~4–8 h) can modulate metabolic responses without changes GK expression levels. This is further evidence of allosteric GK regulation, which has recently been shown to correlate with changes in subcellular GK localization [9–13].

Conclusions

The ability to image directly cellular redox changes, and thus intracellular metabolism, in real-time has opened new opportunities for monitoring the details of glucose-stimulated insulin secretion. Because of the tight control exerted by GK over the entire glucose signaling pathway, these redox measurements can often reflect directly on GK activity. Correlation of redox state with insulin secretion provides direct evidence of this tight control, and further defines the role of GK as the 'glucose sensor' of the β-cell. However, many questions remain unanswered at this point. First, how exactly does the allosteric regulation of GK contribute to the overall glucose response in vivo? Second, how are the pulsatile Ca^{2+} and secretion profiles generated in the absence of concomitant oscillation is intracellular metabolism? Finally, how do the various signaling pathways through

the cytoplasm and mitochondria combine to permit non-glucose-stimulated secretion in mice that lack functioning K_{ATP} channels? All of these questions can be addressed in part with the approaches described here, and we are hopeful that important results will continue to be generated by these high-resolution imaging methods.

Acknowledgments

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Glucokinase in β-Cell Insulin-Secretory Granules

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In most biochemistry textbooks, glycolysis has been described to occur in the cytoplasm; for instance, it is stated that 'In eukaryotic cells, glycolysis takes place in the cytosol' [1], and the view is expressed that 'Like the other nine enzymes of glycolysis, hexokinase is a soluble, cytosolic protein' [2]. These descriptions may be defendable at a certain level of understanding; however, it should be kept in mind that the glycolytic enzymes are not localized only in the cytoplasm. Actually, many studies have demonstrated that some of the glycolytic enzymes, including *L*-lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase A, and phosphoglycerate kinase, occur in the cell nuclei, in addition to their being present in the cytosol [3, 4]. *L*-Lactate dehydrogenase was reported to be present in mitochondria as well [5, 6].

By using immunohistochemical and biochemical techniques, Miwa et al. [7] showed for the first time that glucokinase in rat hepatocytes is present in both the cytoplasm and the nucleus. This finding, together with the discovery of glucokinase regulatory protein in rat liver by Van Schaftingen et al. [8, 9], prompted the investigation of the post-translational regulation of liver glucokinase. It is now widely accepted that the glucokinase regulatory protein anchors glucokinase in the nucleus in an inactive state and that the translocation of glucokinase between the cytosol and the nucleus confers a short-term adaptation of glucose metabolism to changes in the glucose concentration in the physiological range [9–15]. A recent study indicated that glucokinase translocation in hepatocytes was impaired in rat models of type 2 diabetes [16].

Pancreatic β-cell glucokinase, acting as a glucose sensor, catalyzes the rate-limiting step for glucose-induced insulin secretion [17–21]. Studies on

β-cell-glucokinase knockout mice [22, 23] and on the metabolic profile of diabetic patients with mutations in their glucokinase gene [24, 25] provided additional support for this concept. Thus, the mechanisms of the post-translational regulation of glucokinase in the β-cell have gained special interest in recent years [26–30], but have not yet been fully clarified in spite of many researchers' efforts. In order to shed light on this matter, investigators have also studied where glucokinase is localized in β-cells and whether glucokinase regulatory protein is present or not in β-cells. Studies on the location of β-cell glucokinase indicated that the enzyme is not detectable in the nucleus but is confined to the cytoplasm [31–33]. On the other hand, reports related to glucokinase regulatory protein in pancreatic islets and insulin-secreting β-cell lines have been contradictory, i.e. two papers reported the presence of the protein or its mRNA in them [34, 35], whereas several others indicated no presence [33, 36–38]. The normal glucose-stimulated insulin secretion in islets isolated from homozygote glucokinase regulatory protein knockout mice suggested that the protein plays no role in the pancreatic β-cells [39]. It is highly possible, therefore, that the level of glucokinase regulatory protein, if any, in β-cells is so low as to be difficult to detect it. These findings and considerations suggest that the situation for the regulation of glucokinase in islets is different from that in liver.

Very recent studies revealed that glucokinase is present in the insulin granules as well as in the cytoplasm of islet β-cells and insulin-secreting β-cell lines [38, 40–42]. In this review, we will show evidence for the existence of glucokinase in insulin granules and outline the possible function of the association of glucokinase with insulin granules.

Evidence for the Association of Glucokinase with Insulin Granules

Double immunostaining of rat pancreatic islets for glucokinase and insulin, glucagon, somatostatin, or pancreatic polypeptide indicated that the enzyme was present in β- and δ-cells, but not in α and PP cells [40]. Glucokinase immunoreactivity was stronger in δ-cells than in β-cells (fig.1).

Jetton and Magnuson [31] reported that in rat islets, glucokinase reactivity was detected only in β-cells with no immunoreactivity detected in α-, δ- or PP cells. They suggested that a small population of δ-cells expressing glucokinase might not have been detected because of the relative scarcity of δ cells. Other studies indicated glucokinase to exist in α-cells [43, 44]. However, the content of the enzyme might be far smaller in α-cells than in β-cells, because raising the glucose concentration from 1 to 10 m*M* did not change the ATP/ADP ratio in cell sorter-purified α cells [45].

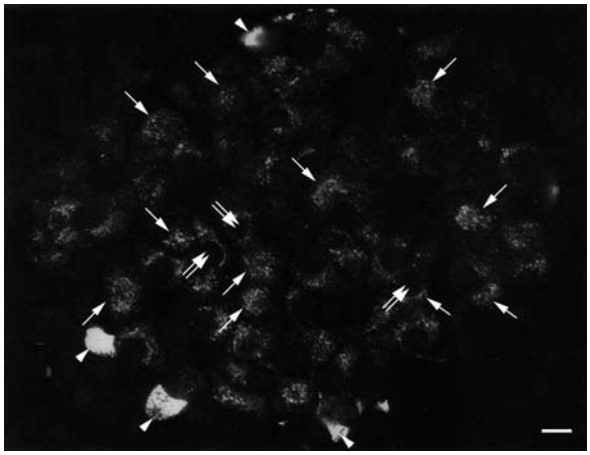

Fig. 1. Immunofluorescence light-microscopic detection of glucokinase in rat pancreas. Frozen sections of the pancreas were permeabilized with 0.5% Triton X-100, blocked with 10% non-fat milk, and incubated with the primary antibody raised in a rabbit against an 11-amino acid C-terminal peptide of glucokinase. After having been washed, the sections were incubated with FITC-labeled donkey anti-rabbit IgG antibody, washed once, and mounted on glass slides with Vectashield mounting medium. Immunofluorescence was analyzed with a confocal imaging system. Glucokinase immunoreactivity is present in β-cells (arrows) and δ-cells (arrowheads). The absence of glucokinase in the nucleus is indicated by the double arrows. Bar = 10 μm. Adapted from Toyoda et al. [40].

As shown in figure 1, glucokinase was not present in the nucleus of β-cells, in agreement with other papers [31–33]. Another notable finding, shown in figure 1, is that glucokinase staining had a punctate appearance in the cytoplasm of β-cells. Such a punctate staining of β-cell glucokinase was repeatedly reported [31–33, 46]. Those papers, however, did not make mention of the possibility of the association of glucokinase with insulin granules.

Colocalization of glucokinase with insulin granules in rat pancreatic β-cells was immunohistochemically shown by Toyoda et al. [40], as shown in figures 2 and 3. Light-microscopic examination demonstrated that a substantial amount of glucokinase was stained in punctate fashion and was costained with insulin (fig. 2). It should be noted, however, that there was also glucokinase immunostaining that was not costained with insulin granules, suggesting that some glucokinase is present in the cytosol and/or is associated with particles, e.g. mitochondria, other than insulin granules. Electron microscopic examination supported the finding obtained by light microscopy that at least part of the glucokinase in β-cells is associated with insulin granules (fig. 3). Another electron microscopic study [41] on the subcellular distribution of glucokinase

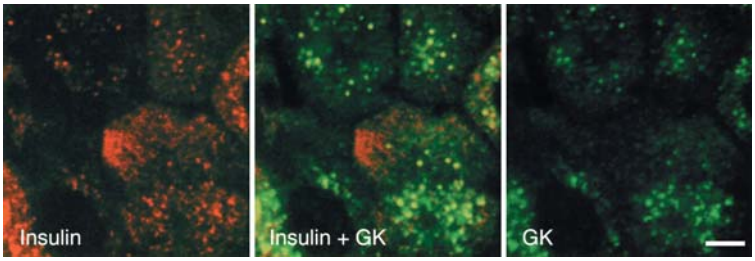

Fig. 2. Colocalization of glucokinase with insulin granules in rat pancreatic β-cells. After glucokinase had been stained as described in figure 1, the sections were incubated with anti-insulin antibody, washed once, incubated with Cy3-labeled donkey anti-mouse IgG antibody, and then analyzed by confocal microscopy. Insulin staining is red; and glucokinase staining, green. The middle panel shows the superimposed images. Yellow color indicates colocalization of glucokinase and insulin. Some of the granules show the colocalization. Bar = 5 μm. Adapted from Toyoda et al. [40].

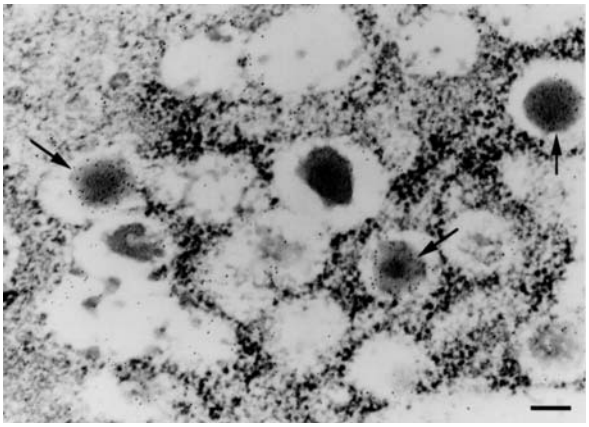

Fig. 3. Demonstration of colocalization of glucokinase with insulin granules in rat pancreatic β-cells by electron microscopic immunocytochemistry. Fixed blocks of the pancreas were dehydrated and embedded in Epon 812. Ultrathin sections were etched with sodium metaperiodate solution and blocked with 5% normal goat serum. The sections were then incubated with anti-glucokinase C-terminal peptide antibody, washed once, and further incubated with anti-rabbit IgG antibody coupled to 5-nm gold particles. They were subsequently stained with uranyl nitrate and examined with a transmission electron microscope under an accelerating voltage of 80 kV. β-Cells were identified by the typical morphological feature of their insulin granules, i.e. the granules have a characteristic dense core surrounded by a clear halo. Arrows indicate typical insulin granules colocalized with glucokinase. Bar = 100 nm. Adapted from Toyoda et al. [40].

in rat pancreatic β-cells also showed the presence of the enzyme associated with insulin granules. In addition, in the report of that study it was stated that glucokinase was associated with only a fraction of the insulin granules.

The subcellular localization of glucokinase in MIN6 cells, a glucose-responsive insulin-secreting β-cell line, was investigated by immunohistochemical and biochemical techniques [38]. In that study immunostaining with an anti-N-19 β-cell glucokinase antibody revealed that the enzyme was present in the cytoplasm and in a granule compartment that was partially colocalized with insulin granules. Light microscopic observation showed the presence of a perinuclear granular staining, and this staining pattern was similar to that observed in rat pancreatic β-cells by Noma et al. [32]. The granular staining of glucokinase was preserved after permeabilization of the cells with digitonin.

Consistent with previous reports [38, 40, 41], Rizzo et al. [42] detected colocalization between glucokinase and insulin granules in βTC3 cells and INS1 cells, both glucose-responsive insulin-secreting β-cells lines. For this detection they used an immunofluorescence staining method employing an antibody against glutathione S-transferase-glucokinase fusion protein. They suggested that the association of glucokinase with insulin granules is likely to be a phenomenon that is not limited to certain cell lines. In addition, they provided evidence that granule association occurs only with glucokinase and is not a general property of all hexokinase isoforms.

The findings of Tiedge et al. [36] that digitonin treatment released 50% of glucokinase from RINm5F cells overexpressing the enzyme and that the residual immunoreactive protein was largely kinetically inactive are compatible with the view that part of the glucokinase associates with insulin granules.

Possible Function of the Association of Glucokinase with Insulin Granules

The association of glucokinase with insulin granules was hypothesized to be a mechanism for protecting the enzyme from degradation [38, 40]. This is analogous to the proposal that glucokinase in hepatocytes is stabilized by association with glucokinase regulatory protein in the nucleus [47]. In this view, glucokinase stored in insulin granules is translocated to the cytoplasm, where the enzyme acts, in response to an increase in the glucose concentration. In one study [38], the distribution of glucokinase between the cytoplasm and the granules in MIN6 cells was reported not to change during incubation of the cells with a high concentration of glucose (25 m*M*). On the contrary, another paper [42] described that glucokinase associated with insulin granules in

βTC3 cells was released into the cytoplasm after glucose stimulation. Noma et al. [32] also reported the translocation of glucokinase in rat pancreatic β-cells, although they made no mention of the association of the enzyme with insulin granules: acute hyperglycemia induced by intravenous injection of glucose changed glucokinase staining from being localized around the perinulear region to become diffusely distributed throughout the cytoplasm. Rizzo et al. [42] suggested that one potential explanation for the lack of consensus in examining glucokinase translocation might arise from the use of such low resolution methods as biochemical fractionation techniques and immunofluorescent microscopy. This is the reason why they used the highly sensitive fluorescence recovery after photobleaching (FRAP) technique to examine the release of glucokinase from insulin granules. Taken together, most available data indicate that it is likely that insulin granules function as a reservoir of glucokinase.

Stubbs et al. [38] proposed a hypothesis that glucokinase associated with insulin granules is involved in direct or indirect coupling between glucose (or glucose-6-phosphate) and ionic events involved in insulin secretion. Glucokinase in the granule may utilize glucose-6-phosphate and ADP in the cytosol to generate ATP, in analogy to the reaction of hexokinase in bound compartments under certain conditions [48], either for the H^+-ATPase, which is involved in acidification of the granule, or for the Ca^{2+}-ATPase, which is involved in the accumulation of Ca^{2+}. Alternatively, glucokinase in the granule may be organized so that some of the enzyme faces the cytoplasmic side and converts glucose and ATP to glucose 6-phosphate and ADP. Glucokinase that is not exposed to the cytoplasmic side utilizes the latter products for the reverse reaction so that the formed ATP is coupled to the H^+-ATPase or Ca^{2+}-ATPase. This hypothesis awaits assessment of its validity.

Rizzo et al. [42] studied the function of glucokinase association with insulin granules in βTC3 cells using FRAP, fluorescence resonance energy transfer (FRET), and biochemical fractionation methods. FRAP measurements and digitonin permeabilization assays indicated that glucokinase translocates from insulin granules to the cytoplasm in response to the increase in glucose concentration. The release of glucokinase from the granule was accompanied by an increase in glucokinase activity and a change in glucokinase conformation, as detected by using FRET. This finding that glucokinase has a lower activity when associated with insulin granules is consistent with the data of Shah et al. [41] that the activity of islet glucokinase was inhibited at a pH range of 5–5.5 [49, 50] presumed to exist inside the granule. Either the inhibition of insulin secretion or of insulin receptor function blocked glucose-stimulated glucokinase translocation and conformational changes [42], suggesting that insulin secretion is required for glucose-stimulated glucokinase regulation.

Thus, changes in glucokinase activity induced by association and dissociation of the enzyme from the granule may be implicated in the regulation of insulin secretion in response to changes in glucose concentration.

Mechanisms Underlying the Regulation of Glucokinase Association with Insulin Granules

A recent paper reported the mechanism for the regulation of glucokinase association with secretory granules [51]. Using βTC3 cells loaded with a fluorescent indicator for NO, the investigators showed that insulin stimulated NO production in the cells. Inhibition of nitric oxide synthase (NOS) by N^G-nitro-*L*-arginine methyl ester (L-NAME) blocked the stimulatory effect of insulin on the FRAP of YFP-labeled glucokinase to CFP-labeled granules, indicating a requirement for NO production in modulating glucokinase association with secretory granules. Examination of changes in glucokinase conformation by the FRET assay also suggested that NO production is a regulator of this glucokinase association and mediates the effects of insulin treatment. Nitrosylated glucokinase was detected only in immunoprecipitates from insulin-treated cells but not in those from untreated cells or cells treated with L-NAME, suggesting that changes in localization and activity of glucokinase are related to S-nitrosylation of the enzyme. Mutation of cysteine 371 to serine blocked S-nitrosylation of glucokinase and stopped both insulin-stimulated FRAP to CFP-labeled granules and insulin-induced conformational changes of glucokinase. Glucokinase was found to interact stably with neuronal NOS (nNOS) as detected by coimmunoprecipitation and by FRET between CFP-labeled nNOS and YFP-labeled glucokinase. Attachment of a nuclear localization signal sequence to nNOS drove glucokinase to the nucleus in addition to its normal cytoplasmic and granule targeting, suggesting that nNOS may be the primary target for glucokinase on secretory granules. Taken together, the data suggest that NO production is related to the regulation of localization and activity of glucokinase and that the association of glucokinase with insulin granules occurs through its interaction with NOS.

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Discovery and Actions of Glucokinase Activators

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The incidence of type 2 diabetes (T2D) is increasing worldwide at an alarming rate, creating a tremendous challenge for medicine. This challenge is particularly daunting because T2D is a chronic disease of complex (still purely defined) etiology, but almost certainly involves many genes and strong environmental factors. T2D manifests itself by impaired β-cell function and resistance to insulin action. Medical treatment of the disease relies therefore on using drugs that stimulate insulin secretion from β-cells or enhance insulin action in liver, muscle or adipose tissue. However, the choice of drugs is limited to K^+-channel blockers, insulin sensitizers, biguanides (still with an unknown action) and compounds that retard enteric glucose absorption. The pharmaceutical industry clearly appraises this situation as a focus area for the development of new medicines with unique mechanisms of action. One guiding principle in this endeavor is to search for metabolic processes that have a high 'control strength' in the regulation of fuel metabolism or more specifically on glucose homeostasis. The term 'control strength' refers to the impact that physiological, pathological or experimental activation or inhibition of a particular reaction or step of a process (e.g. of an enzyme or a receptor) may exert on a particular parameter or process (e.g. blood glucose or rate of glycolysis).

Glucokinase (GK) which is one of the four known mammalian isozymes that phosphorylate glucose has emerged as a promising drug target using the above criteria. GK serves as the glucose sensor in pancreatic β-cells and plays a critical role in the conversion of glucose to glycogen in the liver. It is now generally accepted that GK has a high 'control strength' in the complex system of glucose homeostasis as compellingly demonstrated by the effect that activating and inactivating mutations of this enzyme have on glucose homeostasis in

humans leading to persistent hyperinsulinemic hypoglycemia of infancy (PHHI) in one case or insulin-deficient hyperglycemia in the other. The syndromes associated with these GK mutations are now collectively described as 'Glucokinase Disease'.

The challenge for the pharmaceutical industry was then to discover molecule(s) that would result in enhanced GK activity, directly or indirectly. When launching a drug discovery effort the most promising approach was to search for fructose-1-phosphate (F1P) mimetics that might block the inhibition of GK by reversing the action of the GK regulatory protein (GKRP) known to exist in liver and possibly playing a role in β-cells as well. A screening assay utilizing recombinant GST-GK inhibited by human GKRP enabled the evaluation of about 120,000 small molecules and resulted in the discovery of a lead compound which not only reversed inhibition of GK by GKRP but also showed direct activation of GK, a hitherto unknown phenomenon in GK kinetics. It was soon demonstrated that GK was allosterically activated by these molecules in a manner best described as nonessential, mixed-type activation.

The development of the GK activators (GKAs) was influenced by the discovery of GK linked PHHI published in 1998. The syndrome was caused by the activating V455M mutation of GK. V455M displayed increased glucose affinity whereas the other kinetic constants were little affected by the mutation. This experiment of nature served as convincing proof of concept that GKAs might be developed into a potential antidiabetic drug. In the meantime, as many as five different activating mutations have been identified in patients with PHHI (T65I, W99R, Y214C, V455M and A456V). These mutations are all located in the region of the enzyme clearly distinct from the substrate binding site. This region of GK is therefore conceptualized and therefore as an allosteric activator site. GKAs have now been found to bind to this very site as shown by the analysis of GK crystallized with one molecule bound to the structure reinforcing the interpretation that GK has indeed such a unique site [1].

The article to follow describes in some detail the biochemical and pharmacological actions of GKAs as illustrated by enzyme assays with recombinant GST-GK, insulin secretion studies with isolated rodent islets and in vivo studies in disease models of T2Ds.

Results and Discussion

Effect of Activators on GK Enzymatic Activity

We have previously shown that activation of GK with small molecules was stereospecific [2]. To expand on this observation we synthesized and tested another pair of stereoisomers. As shown in figure 1, only RO0274375,

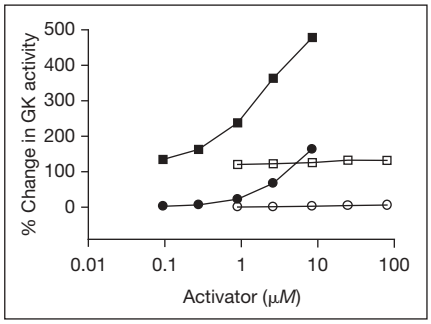

Fig. 1. Enantiomeric activation of GK activity. Effects of RO0274375 (filled symbols) and RO0274374 (open symbols) enantiomers on activation of GK in the absence (squares) and presence (circles) of inhibitor (human GKRP). The effects of test compounds were expressed as a percentage of untreated GK activity. Assay conditions were previously described [2].

the R-enantiomer, increased the enzymatic activity of recombinant human liver GST-tagged GK and reversed the inhibitory actions of the human GKRP in a dose-dependent manner. The concentration of RO0274375 causing a 150% increase in GK activity was 0.20 μ*M* ($SC_{1.5}$) and the concentration required to increase GK activity by 50% in the presence of GKRP was 1.9 μ*M* (IC_{50}). These results are in agreement to the stereoselective activation of GK previously reported for RO0281675 ($SC_{1.5}$, 0.18 μ*M*; IC_{50}, 0.75 μ*M*) [2].

Glucokinase activators RO0274375 and RO0281675 were evaluated in an enzyme kinetic assay to determine the mechanism for GK activation (fig. 2). The results of these experiments were consistent with the rate equation for a nonessential, mixed-type activator and verified by graphical methods and analysis of replots (data not shown). Both compounds increased the enzyme's maximal rate and the half-saturating substrate concentration ($S_{0.5}$) for glucose was lowered (table 1). These effects occurred without affecting the Hill coefficient and K_m for ATP (data not shown). RO0281675 was more effective in increasing the overall catalytic effectiveness of GK as measured by the $V_{max}/S_{0.5}$ ratio. RO0274375 increased the $V_{max}/S_{0.5}$ by 2-fold and RO0281675 by 3-fold relative to untreated GK.

Both compounds displayed similar $SC_{1.5}$ values and effects on V_{max}. However, RO0281675 had a greater impact on the $S_{0.5}$ which suggests that structural differences between these GKAs translate into intrinsic differences on GK kinetics. The lower IC_{50} value observed for RO0281675 (IC_{50}, 0.75 μ*M*) is likely related to its ability to lower the $S_{0.5}$ for glucose more effectively than RO0274375 (IC_{50}, 1.9 μ*M*). Assays using various fixed concentrations of GKRP and GKA showed lines converging to left of the y-axis in a Dixon plot, indicating that GKRP and GKA do not compete with each other for binding to GK (data not shown). Additionally, GKAs do not dissociate the physical interaction between GK and GKRP as determined by pull-down assays (data not shown). We believe that GKAs reverse GKRP inhibition by increasing its Ki

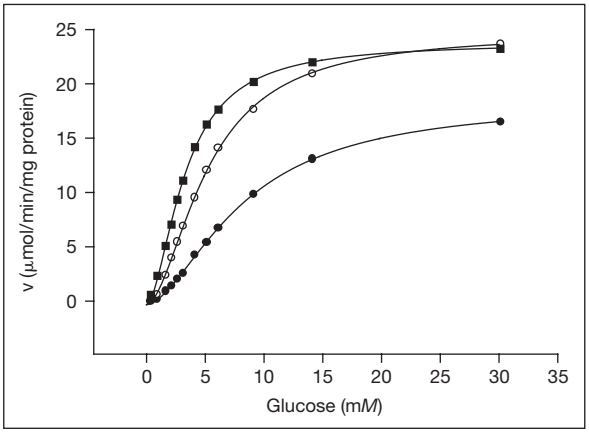

Fig. 2. GK rate versus glucose plot in the absence (filled circles) and presence of 1 μ*M* RO0274375 (open circles) or 1 μ*M* RO0281675 (filled squares). Each compound was evaluated at 7 different concentrations ranging from 0.03 to 30 μ*M* and fit to the velocity equation for a nonessential, mixed-type activator that was modified for an enzyme showing cooperative kinetics. The graph shows actual data points (symbols) and best fit lines. Only one concentration for each GKA is shown for the sake of comparison.

Table 1. Effects of glucokinase activators on GK kinetics

GKA	V_{max} μmol/min/mg protein	$S_{0.5}$ m*M*	$V_{max}/S_{0.5}$
None	18.5	8.2	2.3
1 μM RO0274375	24.7	5.2	4.8
1 μM RO0281675	23.8	3.3	7.2

Kinetic constants derived from the data shown in figure 2.

value rather than causing a physical disruption of the GK/GKRP complex. This may change the equilibrium between cytoplasmic and nuclear bound forms of GK but this needs to proven experimentally. This may have practical implications when evaluating the effects of GKA treatment on fasting and postprandial glucose levels. For example, during post-prandial conditions a greater amount of an active GK pool is accessible to small molecule activation relative to the fasting condition where GK is essentially bound to the regulatory protein in an inactive state.

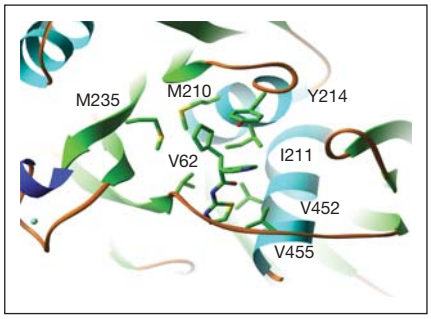

Fig. 3. Close-up of GKA binding site showing amino acid residues making direct interactions with a GKA. Mutations in V62, M210 and M235 cause the MODY-2 phenotype whereas V455M, A456V and Y214A mutations increase the catalytic activity of GK [3–5]. The V455M and A456V are naturally occurring mutations associated with PHHI, whereas the Y214A activating mutant was identified by site-directed mutagenesis.

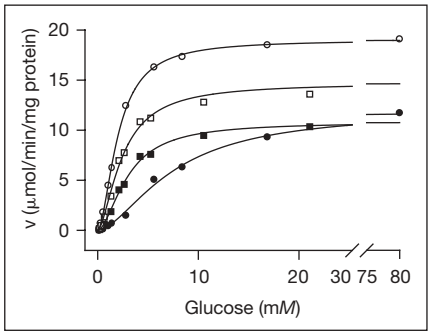

Fig. 4. Rate versus glucose plot for wild type GK (circles) and activation mutant V455M (squares) in the absence (filled) and presence (unfilled) of 20 μ*M* RO0274375. Methods for constructing and purifying site-directed GK mutants was previously described [6].

Many of the known activating GK mutations map to the vicinity of the GKA binding site [1]. In fact, nearly all amino acids that comprise this hinge region of GK, whereby large and small domain movements are believed to pivot, are associated with either inactivating or activating mutations (fig. 3). Therefore, we decided to compare the effects of RO0274375 on wild-type and the gain-of-function, V455M, GK mutation (fig. 4; table 2). Saturating concentrations of RO0274375 decreased the $S_{0.5}$ for glucose (2.4 m*M*) and increased V_{max} by 60% for wild-type GK. These effects were similar to the untreated V455M mutant enzyme ($S_{0.5}$, 3.5 m*M*) except V_{max} was apparently unaltered. However, treatment of the mutant with RO0274375 not only caused a further reduction in the $S_{0.5}$ but also increased V_{max} by 36%. Despite these synergistic effects, wild-type GK was more sensitive to the effects of RO0274375 than V455M as measured by a 6.1- and 1.6-fold change in $V_{max}/S_{0.5}$, respectively. The activating GK mutants that display the greatest impact on the catalytic effectiveness are those that increase both V_{max} and $S_{0.5}$ such as Y214A and V456A, suggesting that an increase in V_{max} is not unique to pharmacological

Table 2. Effect of RO0274375 on wild-type and activating GK mutation

Mutant	RO0274375 μ*M*	V_{max} μmol/min/mg protein	$S_{0.5}$ m*M*	$V_{max}/S_{0.5}$
WT	–	11.9	9.0	1.3
WT	20	19.0 (60%)	2.4 (−73%)	7.9
V455M	–	10.8	3.5	3.1
V455M	20	14.7 (36%)	2.9 (−17%)	5.1

Kinetic constants derived from the data shown in figure 4. Values in parentheses represent the percent change relative to untreated wild-type or V455M mutant GK activity.

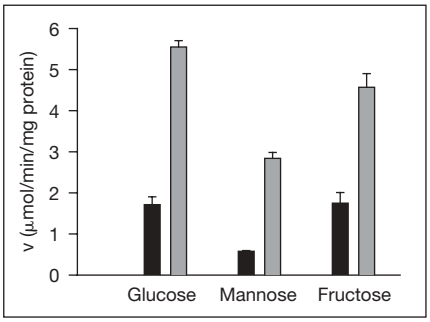

Fig. 5. Activation of GK using alternative substrates. The effects of saturating concentrations of GKA were evaluated using 5 m*M* glucose, 5 m*M* mannose or 200 m*M* fructose as substrates. Assays were conducted as previously described [2] except it was changed to measure ADP production utilizing the pyruvate kinase/lactate dehydrogenase reporter assay.

activation of GK. Augmenting V_{max} is a desirable attribute since this will translate into greater catalysis at blood glucose concentrations typically found in type 2 diabetics.

GK shows broader substrate preference relative to hexokinase I-III. In addition to catalyzing the phosphorylation of glucose, it can also utilize other pyranoses (mannose and deoxyglucose) and furanose (fructose) substrates. These substrates also show kinetic cooperativity like glucose, whereas reports for fructose are conflicting with some showing cooperativity and others showing Michaelis-Menten kinetics. We therefore examined the effects of GK activation using alternative substrates. As shown in figure 5, GKAs increased GK catalysis for glucose, mannose and fructose. The glucose binding site between hexokinase I (high glucose affinity) and GK (low glucose affinity) show no significant differences in the interactions with glucose that would explain these differences in glucose affinity. Furthermore, comparisons of the amino acids

making direct interactions with glucose are nearly identical between the GK and GKA-GK co-crystal. Although activation of GK may have multiple mechanisms the common theme is the stabilization of one GK form, presumably the high affinity form, over others.

Effects of GKAs on Pancreatic β-Cell Metabolism and Secretory Function

Based on the tenets of the GK glucose senor concept GKAs are predicted to have characteristic effects on glucose metabolism and glucose stimulated insulin release closely mimicking the actions of high glucose. A series of experiments was designed to test these predictions and the results were entirely as expected and are described below. GKAs shifted the glucose-stimulated insulin release (GSIR) curve to the left as demonstrated in perifusion studies using isolated pancreatic islets from rat and mouse (fig. 6a, b). In addition to lowering the threshold in a dose-dependent manner, treatment with GKAs also augmented the maximal response to GSIR. The action of RO0281675 was glucose dependent and not duplicated when α-ketoisocaproate was employed as an alternate fuel stimulant at substimulatory levels of 5 m*M* (fig. 6c) and at submaximal concentrations of 25 m*M* (not shown). Additional experiments confirmed that these effects were due to an increase in glucose usage and consistent with changes in the concentration of free intracellular Ca^{2+} (fig. 7). RO0281675 did not alter intracellular Ca^{2+} levels in the absence glucose.

It was demonstrated with isolated rat islets and with a cultured β-cell line derived from hyperplastic mouse islets (βHC9 cells, data not shown) that GKAs augmented respiration in parallel with the enhancement of GSIR (fig. 8). Using P-NMR it was further possible to show that GKAs increased the P-potential of perifused βHC9 cells stimulated with basal glucose (fig. 9). The cellular P-creatine levels rose, inorganic phosphate levels fell while the free ATP levels remained unchanged. It can be extrapolated from these results that the levels of MgADP fell in the presence of the drug, which presumably lead to the closure of K/ATP channels, increased Ca^{++} influx and insulin release.

GKAs proved to be powerful inducers of GK in isolated rat pancreatic islets cultured for several days in low basal (2–6 m*M*) glucose. Under those conditions, β-cell content of GK reached a level comparable to that found in islets cultured in 25–50 m*M* glucose (data not shown). Islets cultured at these basal sugar levels in the presence of 3 μ*M* GKA and then tested in a perifusion system in the absence of the drug, i.e. after the activator had been washed out, showed GSIR comparable to that found in islets cultured in high glucose without drug present (fig. 10).

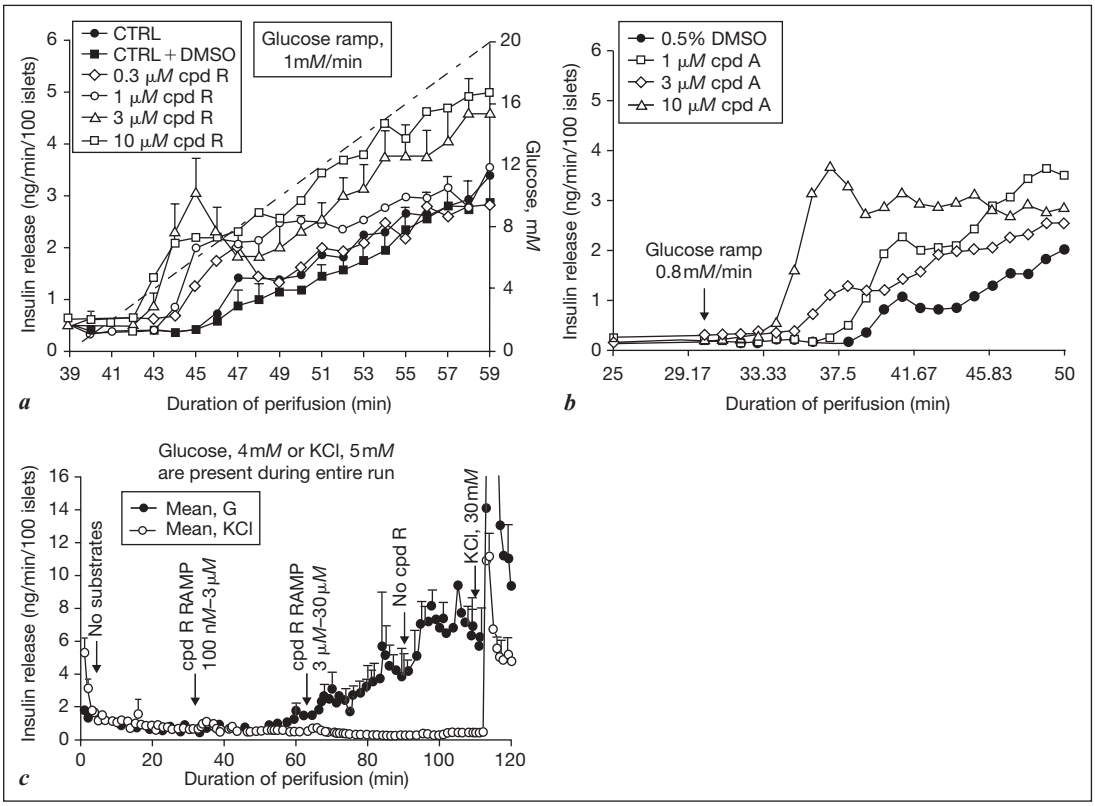

Fig. 6. GKAs lower the threshold for GSIR. Effects of RO0281675 (cpd R) on GSIR in freshly isolated rat islets (***a***) and RO0274375 (cpd A) in isolated mouse islets, cultured for 4–5 days in 10 m*M* glucose (***b***). Following a 39-min pre-perifusion phase with a balanced salt solution in the absence of glucose [2], a glucose ramp was applied from 0 to 20 m*M* (1 m*M*/min) in the presence of the test agent. Studies with mouse islets were performed similarly (***b***). ***c*** Dose effect of RO0281675 and glucose specificity for GSIR in freshly isolated rat islets. Following a 32-min perifusion phase with 4 m*M* glucose (filled circles) or 5 m*M* alpha-ketoisocaproate (open circles), RO0281675 was applied at a slow rate 0.1 to 3 μ*M* (0.1 μ*M*/min) and proceeded by a faster rate (1 μ*M*/min) before drug wash-out and high K^+ stimulus. There were 2–4 replicates for each condition.

The results of these comprehensive studies clearly distinguish GKAs from widely used antidiabetic agents that directly inhibit the SUR-1/Kir-6.2 potassium channel complex. Glyburide for example, serves as glucose-independent insulin releasing drug which may lower the P-potential of the β-cell [7] but does not function as an inducer of GK in pancreatic islets cultured in 3 m*M* glucose in contrast to what is described above for GKAs.

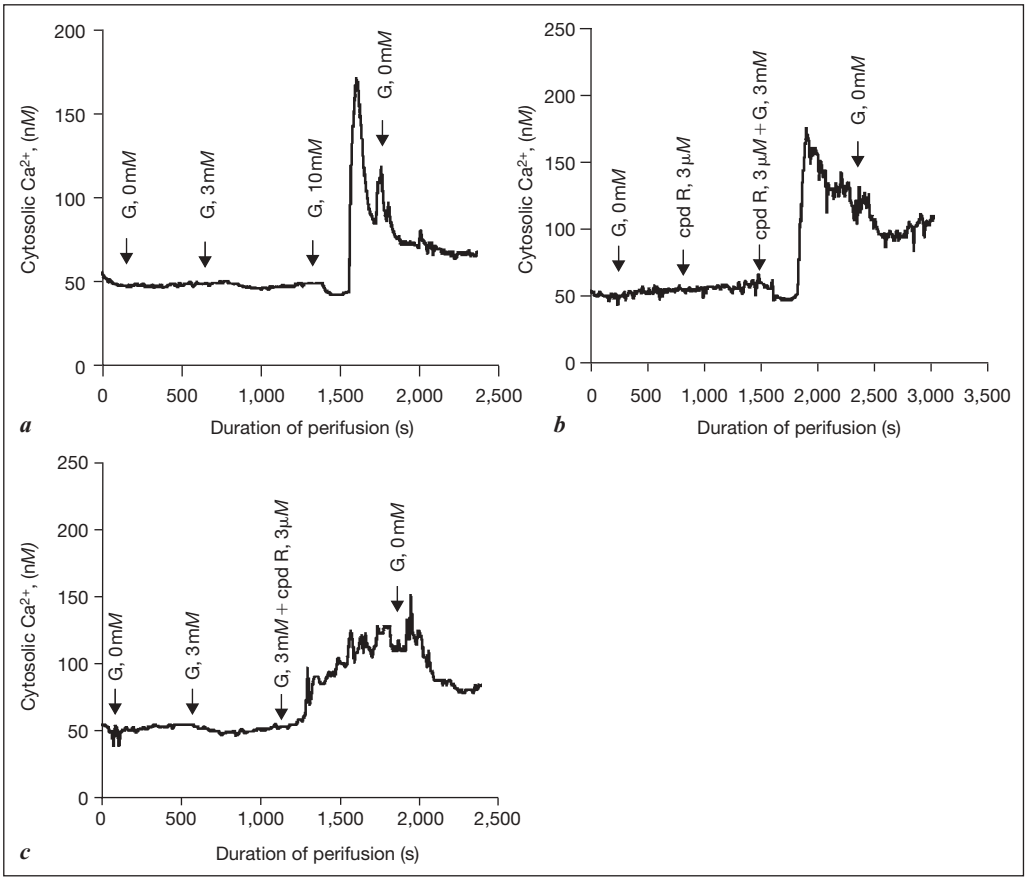

Fig. 7. Response of β-cell cytosolic Ca^{2+} to low glucose in the presence of RO0281675. Isolated mouse pancreatic islets were cultured in 10 m*M* glucose for 3–4 days and loaded with Fura-2 prior to the sequential treatment with: (***a***) 3 m*M* glucose followed by 10 m*M* glucose; (***b***) 3 μ*M* RO0281675 followed by 3 μ*M* RO0281675 plus 3 m*M* glucose, and ***c*** same as ***b*** except glucose was added before treatment with the combination of glucose and GKA. ***a–c*** It can be seen that RO0281675 (cpd R) sensitize islets to glucose but are ineffective in the absence of sugar.

It is anticipated that GKAs will augment all known actions of glucose in pancreatic β-cells. For example, cAMP levels and insulin biosynthesis should be elevated. From these studies with isolated pancreatic rodent islets and β-cell lines it is reasonable to extrapolate that GKAs will sensitize the β-cell to glucose and augment GSIR. The efficacy of GKAs will obviously depend on the β-cell mass, the β-cell GK content, the availability of insulin stores, effective

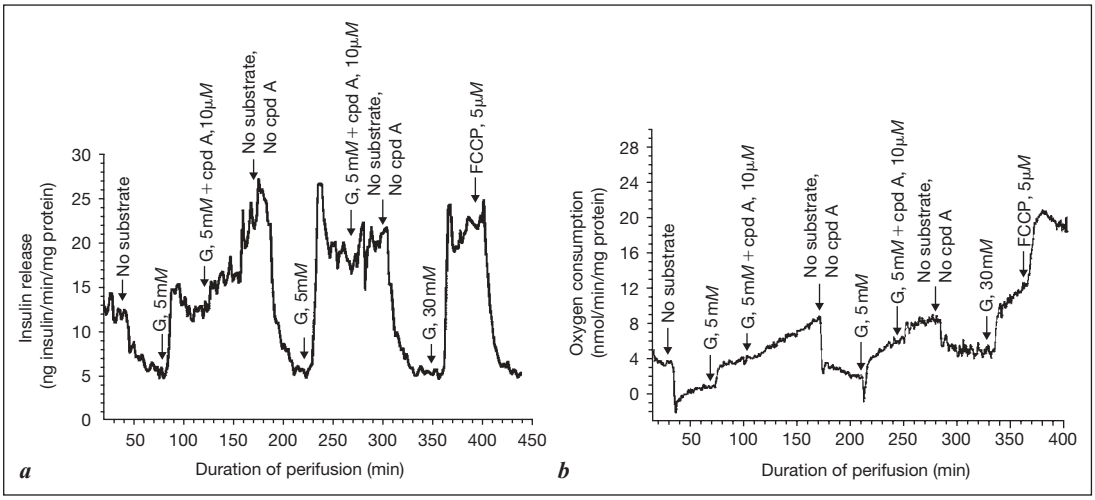

Fig. 8. GKAs stimulate respiration of isolated rat islets in parallel to enhancing GSIR. Approximately 5,000 isolated rat islets were cultured at 10 m*M* glucose for 4–5 days prior to perifusion. The islets were layered between macrodex beads to facilitate a uniform flow of the perifusion medium. Oxygen electrodes were placed in the inflow and outflow lines and the A–V difference of O_2 concentrations and usage were determined. DMSO at 0.5% was present throughout. The temporal insulin release profile (***a***) and corresponding O_2 consumption pattern (***b***) during the various experimental interventions are indicated. One out of three representative experiments is shown.

coupling of stimulus-secretion and unimpaired operation of insulin granule exocytosis. In vivo studies with normal and diabetic rodents as discussed below demonstrate that these requirements are met and that the GKAs stimulate the β-cells to secrete more insulin into the bloodstream resulting in lower blood glucose.

In vivo Effects of GKAs

Administration of a single oral dose of RO0281675 reduced blood glucose levels in wild-type C57 mice in a dose-dependent manner (fig. 11a). The nadir in blood glucose levels and peak insulin levels (fig. 11b, c) occurred between 45 and 60 min following administration and is coincident with the drug's t_{max}. Similarly, a single oral dose of RO0281675 given to *ob/ob* mice, KK/Upj-Ay/J mice, Wistar rats and Goto-Kakizaki rats effectively lowered basal blood glucose levels (fig. 12a–c). It can be seen from these results that pharmacologic activation of GK results in a hypoglycemic state in normal rodents and near normalization of blood glucose levels in rodent models of type 2 diabetes. Based

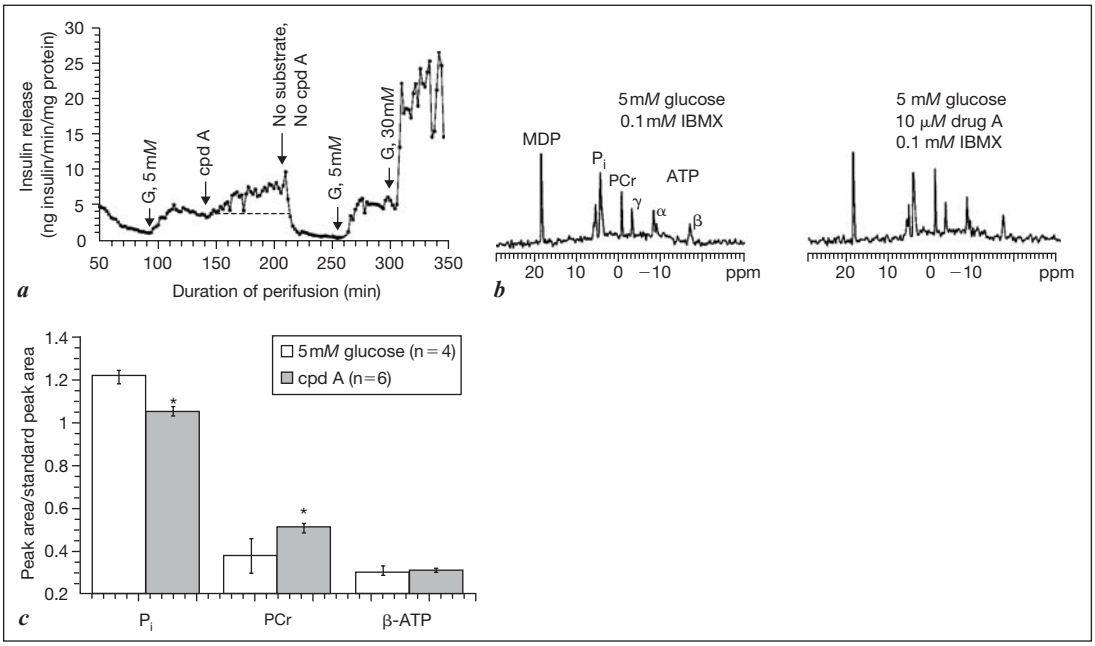

Fig. 9. GKAs increase the P-potential of βHC9 cells perifused with low glucose. βHC9 cells incorporated in microscopic agarose beads were perifused in the presence of 0.5% DMSO and treated as indicated in the graph. The chamber containing beaded cells was inserted into the NMR spectrometer for online determination of the phosphorus spectra. Insulin release (***a***) and P-NMR spectra analysis (***b***) were measured in the presence and absence of 10 μ*M* RO0274375 (cpd A). ***c*** P-NMR summary of five experiments. The spectra peaks are denoted: MDP = standard; P_i = inorganic phosphate; PCr = P-creatine; ATP = α, β and γ peaks of the nucleotide are labeled.

on the glucose sensor paradigm for glucose homeostasis and the mechanism by which the kinetic constants are altered by pharmacological activation, these results can be anticipated and are further substantiated based on biochemical and clinical insights into activating GK mutations in humans. Although mathematical modeling can accurately predict glucose thresholds in these patients, it is difficult to apply it to small molecule activation due to the uncertainty of the drug concentration at its site of action. Furthermore, it would be difficult to use it to explain the differences in glucose thresholds in drug treated Wistar and Goto-Kakizaki rats primarily due to differences in GK expression levels, distribution, disease status, etc. between the 2 models. In summary, we speculate that the reason why the same dose and plasma drug exposure levels of RO0281675 in a normal rodent cause's hypoglycemia, but near normoglycemic levels in

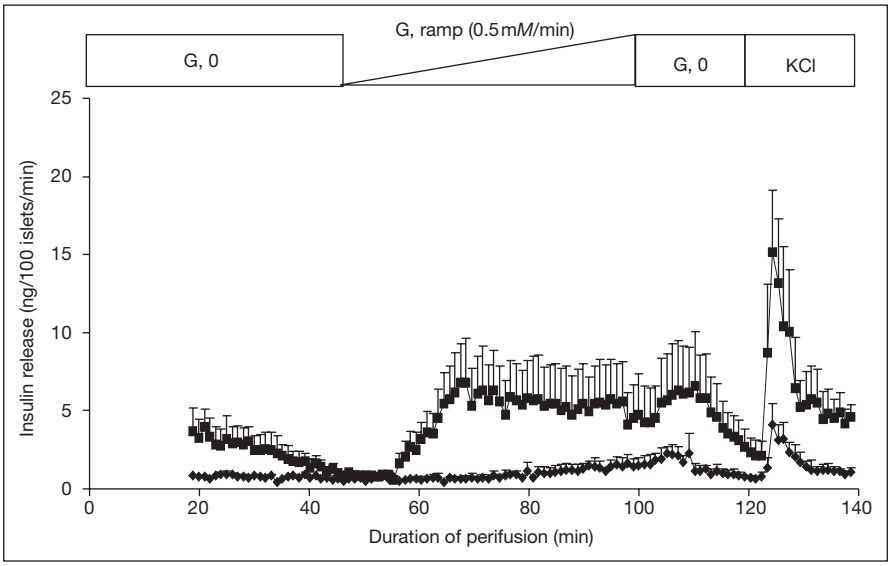

Fig. 10. Preservation of glucose responsiveness by prolonged exposure of isolated islets to GKAs cultured at low glucose. Rat islets were cultured at 6 m*M* glucose for 4–5 days in the absence and presence of 3 μ*M* cpd *M*. Islets were perifused in the absence of GKA and a glucose ramp applied (0.5 m*M* increment/min) after complete washout of the drug. It was also shown in parallel studies that the GK content in drug treated islets was increased 2.5-fold relative the untreated islets (not shown here).

diabetic rodents, is due to the underlying defects in glucose homeostasis associated with the disease models.

A single oral dose of RO0281675 administered 120 min. prior to an oral glucose tolerance test (OGTT) improved the glucose excursion in *ob/ob* mice and DIO-C57Bl/6J mice as well as in C57Bl/6J mice, Wistar rats and Goto-Kakizaki rats (fig. 13a–d). GKAs controlled the glucose excursions without augmenting insulin levels at the time when the glucose challenge was initiated (peak insulin levels occur at 45–60 min and the OGTT was given 120 min following administration of GKA). We have found GKAs to be similarly efficacious regardless of when the OGTT was given in relation to GKA treatment (i.e. OGTT initiated at 30 or 120 min after drug administration). In contrast, sulfonylureas were more effective when the OGTT was given during drug induced peak insulin levels rather than administering the OGTT when insulin levels return to their baseline values (~120 min following drug treatment). Taken together, these results suggest that the liver is an important secondary site of RO0281675 action, as expected due to the presence of GK in hepatocytes.

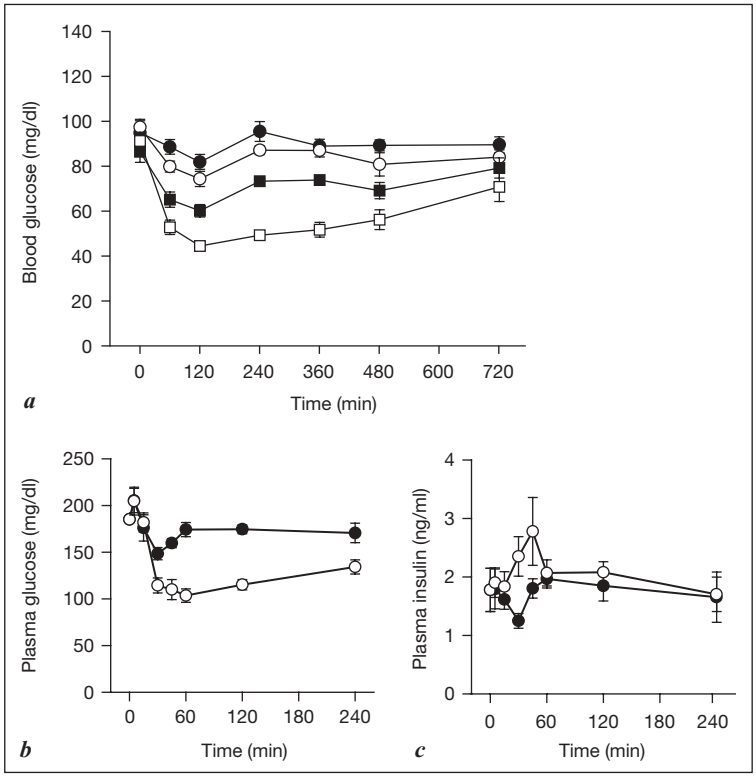

Fig. 11. Glucose lowering and insulin releasing effects in mice. ***a*** Blood glucose levels in male C57Bl/6J mice (Jackson Labs) treated with a single oral dose of vehicle (filled circles), 3 mg/kg (open circles), 10 mg/kg (filled squares) or 30 mg/kg (open squares) RO0281675. Simultaneous plasma glucose levels (***b***) and insulin (***c***) levels measured for each time point in vehicle (filled circles) or 15 mg/kg RO0281675 (open circles) treated C57Bl/6J mice. All mice were fasted for 2 h prior to oral administration of test compound. Food was withheld until the end of the study. Mice had free access to water. Glucose and insulin concentrations determined as previously described [2]. All animal procedures were approved by the Institutional Animal Care and Use Committee. All results are reported as the mean ± SEM.

To determine whether RO0281675 has a direct effect on the liver, in addition to its action on β-cells, we studied 18-hour-fasted conscious Sprague-Dawley rats maintained on a pancreatic clamp (fig. 14a, b). During the hyperglycemic phase of the study, in which blood glucose and plasma insulin levels were clamped at the same levels in both the vehicle and RO0281675 treatment groups, RO0281675 increased glucose disposal rates. As expected, hyperglycemia per se decreased net endogenous glucose production (EGP) in the vehicle group and treatment with RO0281675 appeared to reverse EGP in

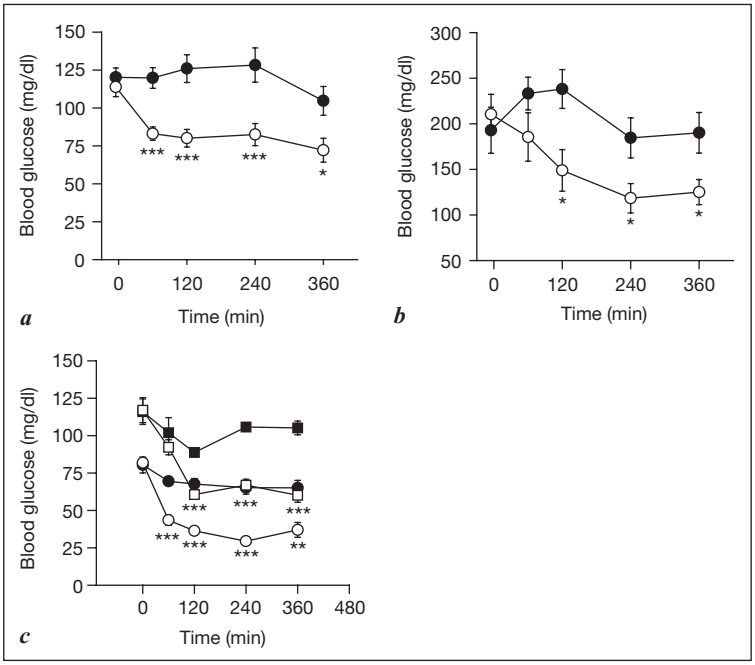

Fig. 12. Acute effect of RO0281675 on basal blood glucose levels in rodents. ***a*** Blood glucose levels in 5-week-old female *ob/ob* mice (Jackson Labs) treated with a single oral dose of vehicle (n = 11/time point) (filled circles) or 15 mg/kg RO0281675 (n = 11/time point) (open circles). ***b*** Glucose levels in 7-week-old male KK/Upj-Ay/J mice (Jackson Labs) treated with a single oral dose of vehicle (n = 6/time point) (filled circles) or 50 mg/kg RO0281675 (n = 6/time point) (open circles). ***c*** Glucose lowering effects in 7-week-old male Wistar rats (Charles River Laboratories) (circles) and Goto-Kakizaki rats (Charles River Laboratories) (squares) orally administered vehicle (n = 5–6/time point) (filled symbols) or RO0281675 (n = 5–6/time point) (open symbols). Experimental details are described in figure 11. A Student's t test was used to test for statistical significance (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.005$).

favor of hepatic glucose uptake. Similar experiments in male ZDF-Gmi rats, where hyperglycemia did not suppress EGP, GKAs significantly reduced EGP (fig. 14c). We attribute the effects on EGP to the stimulatory effect of RO0281675 on hepatic GK. Increases in hepatic glucose-6-phosphate, fructose-6-phosphate, lactate and glycogen levels were also observed in the RO0281675 group compared to the vehicle group.

Chronic treatment with RO0281675 was evaluated in a 40-week food admixture study in DIO-C57Bl/6J mice. Both DIO and RO0281675-treated DIO groups developed obesity during the study (fig. 15a). Mice in the DIO group gained slightly more weight than those in the RO0281675-treated DIO

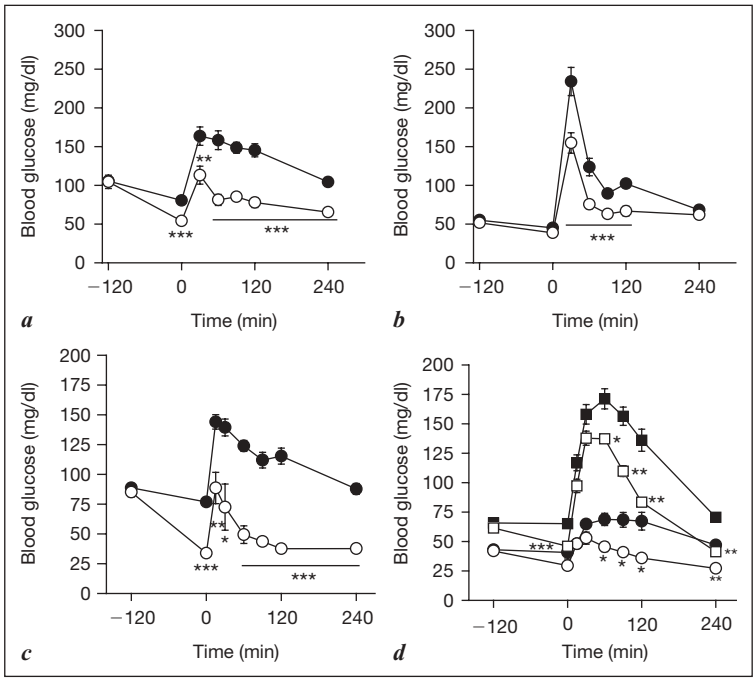

Fig. 13. Acute effects of RO0281675 on an oral glucose tolerance test in rodents. A single oral dose of vehicle or RO0281675 was administered 120 min prior to glucose challenge (2 g/kg) in: (***a***) 19-week-old male DIO C57Bl/6J mice (vehicle, filled circles; 15 mg/kg RO0281675, open circles); (***b***) 9-week old female *ob/ob* mice (Jackson Labs) (vehicle, filled circles; 10 mg/kg RO0281675, open circles; (***c***) 8-week-old male C57Bl/6J (vehicle, filled circles; 50 mg/kg RO0281675 open circles), and (***d***) 8-week-old male Wistar rats (Charles River Laboratories) (circles) and Goto-Kakizaki rats (Charles River Laboratories) (squares) (vehicle, filled symbols; 50 mg/kg RO0281675, open symbols). Rodents were fasted overnight prior to administration of test compounds. Glucose determined as described above.

group. Body weight increases were accompanied by increases in blood glucose levels in the DIO group that were not seen in the RO0281675-treated DIO group (fig. 15b). Transgenic mice with an extra copy of the full GK gene were also resistant to obesity induced hyperglycemia [8]. On study day 199, an OGTT was conducted in mice that were fasted for 2 hours prior to glucose challenge. Mice in the DIO group were glucose intolerant compared to mice in the control group. In contrast, mice in the RO0281675-treated DIO group showed a marked improvement in the glucose excursion (data not shown). Food intake per day was similar within the DIO and RO0281675-treated DIO groups and for both groups, was lower than that seen for the control group. This was expected since mice fed a high-fat diet adjust food consumption due to the high caloric

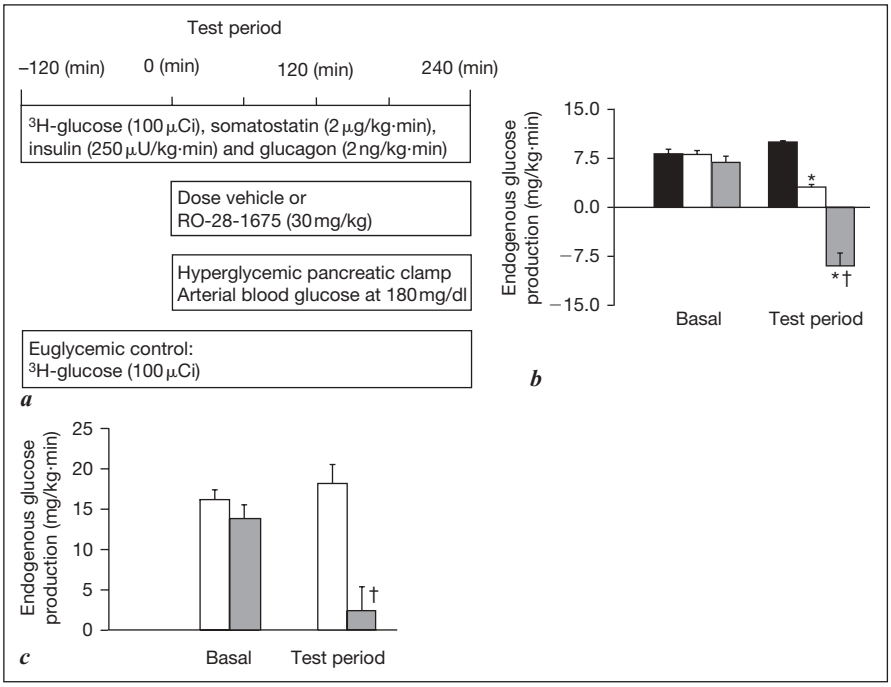

Fig. 14. Effect of RO0281675 on hepatic glucose production during a pancreatic clamp experiment. ***a*** Schematic diagram of the pancreatic clamp experiment described previously [2]. ***b*** Effect of RO0281675 on endogenous glucose production in Sprague-Dawley rats. ***c*** Effect of GKA in 9- to 12-week-old male ZDF-Gmi rats (Charles River). Filled bars = Euglycemic control; empty bars = vehicle; stippled bars = 30 mg/kg RO0281675. A Student's t test was used to test for statistical significance (*$p < 0.05$ compared to euglycemic control; †$p < 0.05$ compared to vehicle).

content of the food. The daily dose of RO0281675 in the RO0281675-treated DIO group ranged from 55 mg/kg on study day 15 to 40 mg/kg on study day 169 and decreased primarily due to an increase in body weight from study days 15–169. Both the DIO and RO0281675-treated DIO groups were hyperinsulinemic relative to the control group with the DIO group having higher insulin levels than the RO0281675-treated DIO group (data not shown). However, this difference between treatment groups was not statistically significant.

Perspective

Biochemical and genetic studies had identified GK as a promising drug target for developing a new class of antidiabetic agents. A drug discovery

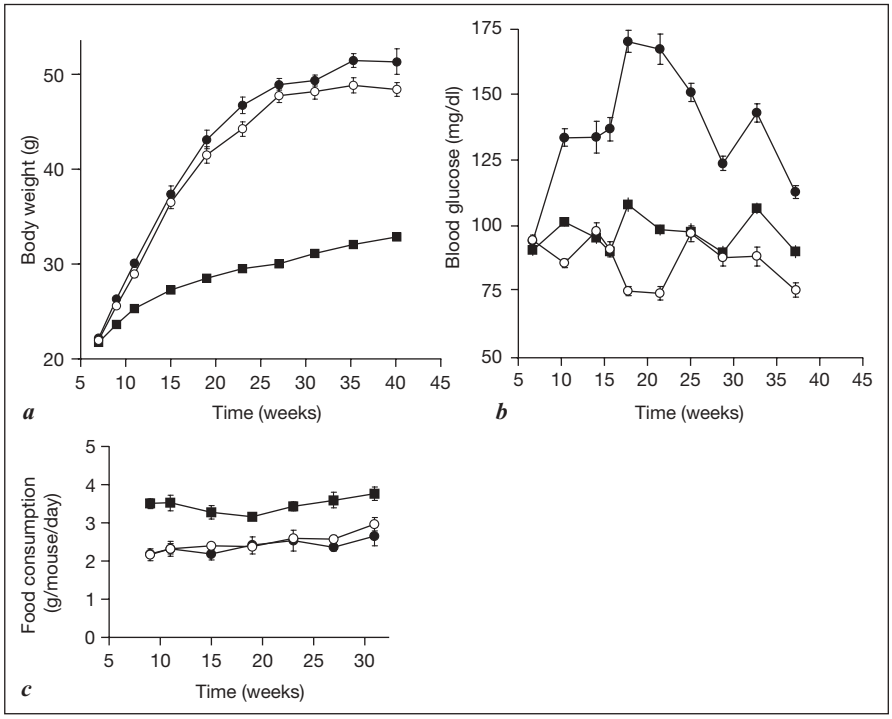

Fig. 15. GKAs prevent the development of hyperglycemia in diet induced obese mice. Three groups (n = 24/group) of C57Bl/6J mice (age, 6 weeks) were placed on a regular chow (control group, squares), a high-fat diet (BioServe, F1850) (DIO group, filled circles) or a high-fat diet supplemented with RO0281675 (RO0281675-treated DIO group, open circles) for 28 weeks. Body weight (***a***), blood glucose (***b***) and food consumption (***c***) were evaluated periodically.

project launched to find molecules that might activate GK directly or indirectly was successful and resulted in the discovery of a new class of compounds which enhance GK activity by binding to a hitherto unknown allosteric activator site on the enzyme. These compounds, called GKAs, enhance GSIR from the pancreatic β-cell and augment hepatic glucose metabolism. They lower blood sugar in normal and diabetic animals as a result. It remains to be seen in extensive clinical trials whether GKAs, alone or in combination with other antidiabetic drugs, do indeed improve glucose homeostasis in patients with type 2 diabetes. The outcome of all laboratory experiments and the PHHI phenotype in humans bode well for the outcome of such trials. Availability of GKAs will undoubtedly also facilitate the ongoing studies and deepen our understanding of the role of GK in the pancreatic β-cells, other GK containing neuroendocrine cells and in the liver cells.

The discovery of GKAs and their proposed use for the treatment of T2D in man illustrates fundamental aspects of modern medicine which faces the difficult task of finding effective long-term therapies for chronic diseases of an aging population, often diseases with multifactorial and largely unknown etiologies. Essential hypertension and T2D are typical examples. High blood pressure is now treated aggressively and successfully with a wide variety of drugs: e.g. β-blockers, calcium channel blockers, calcium channel inhibitors and diuretics. Each one of these has a different mechanism of action and it is probably safe to say that none of these compounds normalizes the primary defect or the root cause of the disease. Nevertheless, the pathogenetically important manifestation of the disease, namely high blood pressure, can be normalized by these drugs, one by one or in combinations. Since there is ample evidence that high blood glucose per se is indeed the direct cause of the long-term complications of any form of diabetes mellitus including retinopathy, kidney disease, neuropathy and large vessel disease leading to heart disease and stroke, lowering of blood glucose remains the primary goal of antidiabetic therapies. Lowering of blood glucose and achieving normalization of glucose homeostasis may then be accomplished by a variety of drugs with very different mechanisms of action, alone or in combinations. The strategy is very realistic in view of the fact that T2D is due to a failure of the insulin producing β-cells and decreased efficiency of insulin action on liver, muscle and fat cells (usually referred to by the term 'insulin resistance'), whatever the underlying molecular genetic basis may be, but clearly requiring different means of attack in therapy. GKAs described here offer a new weapon in this battle to complement or even replace available ones (e.g. SUR drugs, insulin, insulin sensitizers etc). They promise to normalize glucose homeostasis by enhancing insulin secretion and hepatic action of the hormone even though there is little evidence to suggest that GK defects are a primary cause of the disease (at least not for the vast majority of cases). Treatment of the predominant, pathogenetically relevant manifestation, that is hyperglycemia, turns out to be effective in this situation very similar to drug treatment of essential hypertension. In fact, these two examples illustrate that the common place saying 'to treat the symptoms is of little long-term benefit' (or something to that effect) may just not be true.

Summary

Pacreatic GK is the molecular sensor for GSIR in β-cells and in the liver GK plays an important role in hepatic glucose metabolism. We describe the action of a novel class of small-molecular-weight GKAs that bind to an allosteric site on GK and increase its maximal rate and glucose affinity. GKAs lowered the threshold for GSIR in freshly isolated rat islets and potentiated insulin release following oral administration to rodents. GKAs lowered blood

glucose levels in numerous diabetic rodent models and showed no signs of tolerance as assessed by a 40-week study in DIO mice. Pancreatic clamp experiments in normal and disease rodent models demonstrated the ability of GKAs to increase hepatic glucose utilization and suppress endogenous glucose production. These results suggest that GKAs have a dual mechanism of action distinct from currently known insulin secretagogues. These findings may lead to the development of a new medicine for the treatment of type 2 diabetes. In addition, the discovery of GKAs represents one of the first de novo designed allosteric enzyme activators which may initiate the search for enzyme activators for other drug targets.

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Balancing Hepatic Glucose Disposal and Production

Key Regulatory Genes and Therapeutic Opportunities

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Type 2 diabetes occurs as a consequence of the failure of three regulatory systems that control fuel homeostasis. First, the normal capacity of insulin to stimulate glucose uptake and inhibit lipolysis in peripheral tissues such as muscle and fat is impaired, a condition referred to as insulin resistance. Second, insulin secretion becomes dysregulated, with higher than normal amounts of insulin secreted under basal (fasted) conditions, and lesser secretory responses to glucose and its potentiators under anabolic (fed) conditions. Finally, the normal balance between glucose disposal and storage on the one hand, and glucose production on the other, is perturbed in liver, such that net hepatic glucose output is increased. The purpose of this chapter is to review recent work relevant to the last of these metabolic lesions, with particular focus on identification of genes that play important roles in controlling hepatic glucose balance, and the potential utility of a sub-set of these genes as targets for lowering of blood glucose levels in diabetes.

Derangements of Hepatic Glucose Metabolism in Diabetes

In normal animals, hepatic glucose production occurs at a high rate in the fasted state. This high rate of glucose production from the liver is curtailed in the fasted to fed transition in response to the rise in blood glucose and the consequent stimulation of insulin secretion from islet β-cells. The net rate of hepatic glucose production represents the balance between pathways that contribute to formation of glucose (gluconeogenesis and glycogenolysis), and

pathways that consume or store glucose (glycogen synthesis, glycolysis, and the pentose monophosphate shunt). The relative activities of opposing pathways such as glycolysis/gluconeogenesis and glycogen synthesis/glycogenolysis are controlled in large measure by the ratio of the pancreatic hormones insulin and glucagon. A low insulin:glucagon ratio in the fasted state favors glycogenolysis and gluconeogenesis, while a high insulin:glucagon ratio in the fed state favors glycogen synthesis, glycolysis, and other pathways of glucose disposal.

In diabetes, insulin insufficiency and/or insulin resistance are the prevalent conditions. Under these circumstances, the liver functions in a manner analogous to the fasted state, with a preferential activation of gluconeogeneis and glycogenolysis. Subjects with poorly controlled, insulin-deficient type 1 diabetes exhibit a gross defect in suppression of hepatic glucose production following glucose ingestion, in concert with a failure to take up glucose and a severe defect in liver glycogen storage. These patients also have increased rates of gluconeogenesis [1, 2]. In type 2 diabetes, where insulin levels and action are partially retained, defects in suppression of hepatic glucose production and liver glycogen storage are also observed, but to a lesser extent than in poorly controlled type 1 diabetes. Nevertheless, the defect in suppression of hepatic glucose production in type 2 diabetes is thought to account for approximately one-third of the defect in total body glucose homeostasis [1]. Thus, in both major forms of diabetes, an imbalance in hepatic glucose production and disposal makes a major contribution to development of hyperglycemia and other perturbations in fuel homeostasis. In the remaining sections of this chapter, we will review recent studies in which genetic engineering techniques have been used to identify proteins that are capable of influencing the overall balance between hepatic glucose production and disposal. We will also discuss the potential utility of a subset of these proteins as therapeutic targets for treatment of both major forms of diabetes.

Manipulation of the Balance between Glucose Phosphorylation and Glucose-6-Phosphate Hydrolysis

The balance between hepatic glucose production on the one hand and glucose disposal and storage on the other is ultimately determined by the relative rates of glucose phosphorylation and glucose-6-phosphate hydrolysis (fig. 1). The terminal step of gluconeogenesis is the hydrolysis of glucose-6-phosphate (G6P) to free glucose, catalyzed by the glucose-6-phosphatase (G6Pase) enzyme complex. The complex is comprised of a catalytic subunit sequestered within the endoplasmic reticulum (ER), a glucose-6-phosphate translocase known as T1 that delivers glucose-6-phosphate to the catalytic subunit, and putative ER glucose and inorganic phosphate transporters (T2, T3) that move

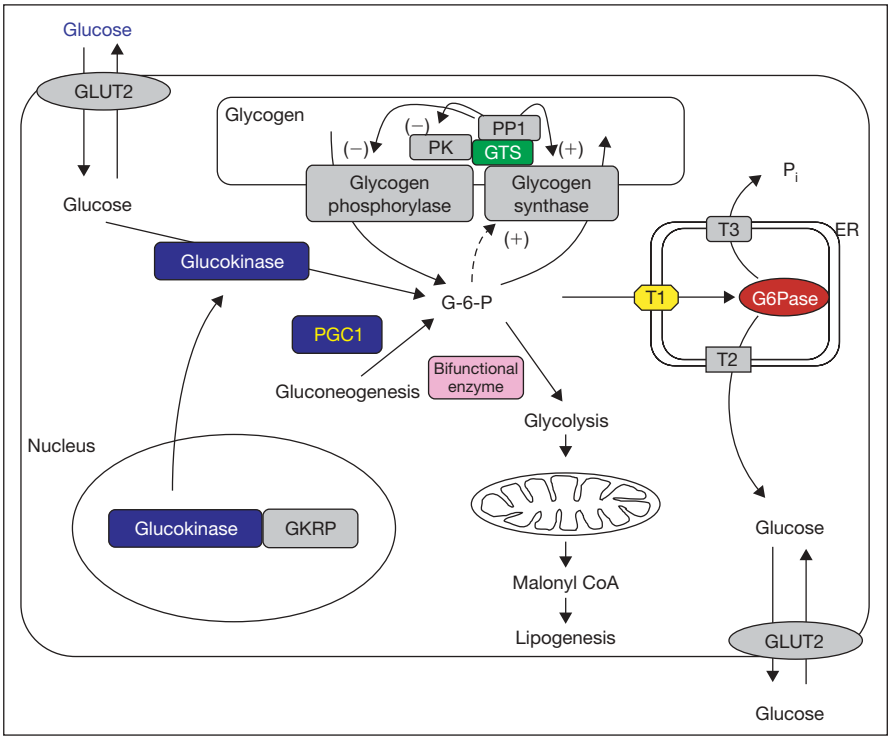

Fig. 1. Regulatory steps in control of the balance between glucose disposal and glucose production in liver. The figure shows some of the key regulatory steps controlling hepatic glucose disposal and production, and emphasizes how these steps are compartmentalized. Examples of this include translocation of glucokinase from the nucleus to the cytoplasm in response to nutritional stimulation, localization and regulation of enzymes of glycogen metabolism via the glycogen targeting subunits (GTS) of protein phosphatase-1, and sequestration of the glucose-6-phosphatase catalytic subunit (G6Pase) in the endoplasmic reticulum (ER). The figure shows that glucokinase overexpression can affect lipid homeostasis by increasing malonyl CoA levels, which diverts fatty acids from oxidative to esterification pathways. In contrast, overexpression of glycogen targeting subunits is predicted to enhance glucose disposal by activation of glycogenesis, possibly without affecting lipid metabolism. PP1 = Protein phosphatase-1; PK = phosphorylase kinase; T1, T2, T3 = components of the glucose-6-phosphatase enzyme complex serving as translocases for glucose-6-phosphate, glucose, and inorganic phosphate, respectively; GKRP = glucokinase regulatory protein; G-6-P = glucose-6-phosphate.

the reaction products back into the cytosol [3, 4]. Glucose phosphorylation in liver is primarily catalyzed by glucokinase (hexokinase IV). This enzyme has a lower affinity for glucose and a higher catalytic capacity than other members of its gene family, and is limited in terms of its tissue distribution to liver, the islets

of Langerhans, and certain specialized neuroendocrine cells in the pituitary and gastrointestinal tract [5, 6].

Studies on Components of the Glucose-6-Phosphatase System

One mechanism for impairing normal hepatic glucose disposal might be to increase the activity of the G6Pase enzyme complex. Indeed, expression of the gene encoding the catalytic subunit of G6Pase is increased in animal models of type 1 and type 2 diabetes [7–9]. In liver of the Zucker diabetic fatty (ZDF) rat, the concentration of the G6Pase catalytic subunit and the overall enzymatic activity of the G6Pase complex are increased by approximately 2.5-fold relative to lean ZDF Wistar controls [8]. Moreover, variables associated with the diabetic state such as hyperglycemia and hyperlipidemia cause increased expression of the G6Pase catalytic subunit both in vitro and in whole animal studies [10–12].

The foregoing studies do not prove that increased G6Pase activity can contribute directly to impaired metabolic regulation. In addressing this point, overexpression of the G6Pase catalytic subunit in rat hepatocytes was found to cause a large (8-fold) increase in G6P hydrolysis and an attendant 25% reduction in intracellular G6P levels [13]. This led to substantial decreases in glycolytic flux and glycogen deposition, and a parallel increase in gluconeogenesis. Later experiments showed that overexpression of the T1 translocase in rat hepatocytes caused a 58% increase in glucose-6-phosphate hydrolysis, leading to a similar (50%) inhibition of glycogen formation as observed with overexpression of the catalytic subunit, but a much smaller impairment of glycolytic flux [14]. The more pronounced effect on glycogen deposition may be explained in part by the observation that overexpression of the T1 translocase enhanced glucose-1-phosphate, but not fructose-6-phosphate hydrolysis, given that the former is an intermediate in glycogen synthesis [14]. These studies showed that increased expression of the T1 translocase or the G6Pase catalytic subunit are capable of altering the overall rate of G6P hydrolysis by the G6Pase enzyme complex, but that the catalytic subunit has the greater control strength in this system.

A recombinant adenovirus containing the cDNA for the G6Pase catalytic subunit was used to cause a 2.5-fold increase in enzyme activity in liver of normal rats [8]. These animals exhibited several of the abnormalities associated with early-stage type 2 diabetes, including glucose intolerance, hyperinsulinemia, a marked decrease in hepatic glycogen content, and increased peripheral (muscle) triglyceride stores [8]. These findings are consistent with the idea that increased activity of the G6Pase complex in liver can make a significant contribution to the development of type 2 diabetes, and clearly establish the importance of tight control of the balance between glucose phosphorylation and G6P hydrolysis in regulation of hepatic glucose balance.

Alteration of Glucose Phosphorylating Capacity in Liver

As summarized above, an increase in the rate of hepatic glucose production causes a condition resembling early stage type 2 diabetes. The question then arises, will the converse maneuver of enhancement of hepatic glucose disposal improve metabolic status in diabetes? Three distinct approaches to increasing hepatic glucose disposal can be envisioned: (1) an increase in the rate of glycogen storage by engineering of enzymes or regulatory proteins that regulate this process; (2) an increase in the rate of glycolytic flux by engineering of steps distal to glucose phosphorylation, and (3) an increase in the rate of glucose phosphorylation, which will presumably increase both glycolysis and glycogenesis. All of these approaches have been attempted, and the results obtained are summarized below.

The metabolic impact of increased hepatic glucose phosphorylation has been studied via recombinant adenoviruses encoding hexokinase I or glucokinase [15, 16] and by transgenic animals with additional copies of a glucokinase transgene [17]. In vitro studies demonstrated a very limited impact of hexokinase I overexpression on glycolytic rate and glycogen deposition in hepatocytes, whereas glucokinase overexpression caused profound increases in both variables [15]. The difference in efficacy of the two proteins is likely explained by the fact that hexokinase I activity is strongly inhibited by the product of the reaction, glucose-6-phosphate, while glucokinase is not subject to such regulation [16].

These studies provided the impetus for testing of the role of glucokinase in control of blood glucose levels in vivo. A recombinant adenovirus containing the glucokinase cDNA was infused into normal rats at rates designed to cause moderate (3-fold) or large (7-fold) increases in hepatic glucokinase enzymatic activity [18]. Moderate overexpression of glucokinase in liver had no significant impact on circulating glucose, free fatty acids or triglyceride levels. In contrast, animals with the higher degree of overexpression exhibited a 38% decrease in blood glucose levels and a 67% decline in circulating insulin levels, as well as increased liver glycogen stores [18]. Similar findings were reported in transgenic mice with additional copies of the glucokinase gene [17]. However, the decrease in glucose levels engendered by the adenovirus-mediated expression of glucokinase came at the expense of a 190% increase in circulating triglycerides and a 310% increase in circulating free fatty acids [18]. Thus, in normal animals, levels of glucokinase overexpression associated with a decline in blood glucose are accompanied by equally dramatic increases in circulating lipids, raising concerns about manipulation of glucokinase activity as a viable strategy for treatment of diabetes.

These findings differ in some respects from other work in the context of rodent models of diabetes. In one study, near-normalization of blood glucose levels was achieved in streptozotocin-diabetic mice transgenic for glucokinase

expression under control of the PEPCK promoter [19]. Surprisingly, the high levels of fatty acids, triglycerides, and ketone bodies found in streptozotocin diabetes were lowered in the transgenic mice. One possible explanation for this finding is that the glucose lowering effect of glucokinase resulted in a higher rate of utilization of lipids for energy production throughout the body. Moreover, it should also be noted that these animals are completely insulin-deficient, making it difficult to predict the metabolic impact of increased glucokinase expression in the presence of the hormone, as would be the case in patients with type 1 diabetes receiving insulin therapy or in patients with type 2 diabetes. Two studies have reported the effects of glucokinase overexpression in mouse models of type 2 diabetes induced by high fat feeding [20, 21]. In one of these, FFA did not rise in response to feeding of the high fat diet in these mice, although liver triglyceride content was increased [20]. This increase in hepatic triglyceride was found in both the glucokinase transgenic and non-transgenic mice. In the second study, circulating FFA and TG were not raised by the high fat diet, and were also not affected by adenovirus-mediated overexpression of glucokinase in liver, although insulin and glucose levels were substantially reduced [21]. However, animals in these two studies exhibited a 40% [20] or a 2.2-fold [21] increase in hepatic glucokinase activity, respectively, clearly less than the 6-fold increase achieved in the adenovirus studies in normal rats [18].

In another recent study, a small molecule activator of glucokinase enzyme activity was shown to improve glucose homeostasis in an animal model of type 2 diabetes, but no information about circulating lipid levels was provided [22]. One would actually anticipate that the improvement in glucose disposal in response to the allosteric activator of glucokinase would not be accompanied by hyperlipidemia, given that the drug stimulates glucokinase activity both in liver and pancreatic islets. The increase in islet glucokinase activity results in increased insulin secretion, which would be predicted to suppress lipolysis and favor lipid storage. In contrast, in studies of adenovirus-mediated delivery of glucokinase to liver of normal rats, glucokinase was overexpressed only in liver, and insulin levels were decreased secondary to the fall in blood glucose concentrations [18]. With the small molecule activator of glucokinase, the long-term effects of chronic elevations in insulin levels due to sustained activation of islet glucokinase activity is a potential concern. Chronic hyperinsulinemia represents a sustained anabolic state, possibly leading to overstorage of lipids in tissues such as muscle and liver. Tissue lipid overstorage has been linked to the development of insulin resistance [1, 2]. A small molecule activator of glucokinase may also have the potential for causing hypoglycemia, as both hepatic glucose clearance and insulin secretion are stimulated by these molecules. Further investigation of these issues will be required.

Finally, another interesting approach for manipulation of glucose phosphorylation in liver is to alter the expression of the glucokinase regulatory protein (GKRP). GKRP binds to glucokinase and sequesters the enzyme in the nucleus and inhibits its activity under fasted conditions. In the fasted/fed transition, GKRP dissociates from glucokinase and the active enzyme is translocated to the cytosol. Remarkably, adenovirus-mediated overexpression of GKRP in liver of mice fed on a high fat diet caused a dramatic lowering of their elevated blood glucose levels and restored insulin levels to near-normal, suggesting that insulin resistance was ameliorated [23]. Curiously, glucokinase enzymatic activity in liver was actually reduced in the GKRP expressing mice, but this is probably explained by the fall in insulin levels and the consequent decrease in glucokinase gene transcription. In support of this idea, adenovirus-mediated co-expression of glucokinase and GKRP in isolated hepatocytes resulted in higher levels of glucokinase protein and enzyme activity when GKRP and glucokinase were co-expressed compared with overexpression of glucokinase alone, consistent with a potential role for GKRP in stabilization of the glucokinase protein and maintenance of a pool of recruitable enzyme. Administration of GKRP virus to mice fed on the high fat diet did not affect their circulating TG or FFA levels, but all of the foregoing concerns about the potential for hypoglycemia and long-term perturbations of lipid homeostasis discussed in the context of manipulation of glucokinase activity also pertain to this approach.

Alteration of Glucose Phosphorylating Capacity in Muscle

In a parallel set of studies, several laboratories have investigated the metabolic impact of overexpression of glucokinase in skeletal muscle [24–29]. Impetus for this work came from a study demonstrating that adenovirus-mediated overexpression of glucokinase in isolated human myocytes enhanced glucose uptake and glycogen synthesis in these cultures [24, 27]. More importantly, these effects occurred in an insulin-independent manner, and the expression of glucokinase altered the glucose dose-response relationship for glucose uptake to reflect the kinetic features of glucokinase, e.g., glucose uptake was regulated in response to changes in glucose concentration in the range from 1 to 30 m*M* in muscle cultures with overexpressed glucokinase, as opposed to control cultures, where maximal glucose uptake was observed at <5 m*M* glucose [24]. Moreover, differentiated myoblast cells engineered for glucokinase overexpression were able to lower blood glucose levels in mice rendered diabetic by streptozotocin injection [27]. Finally, adenovirus-mediated expression of glucokinase in hind limb skeletal muscle of normal [25] or ZDF [28] rats enhanced glucose tolerance and improved insulin sensitivity, respectively. A similar protective effect against diet-induced insulin resistance and hyperglycemia was

reported in transgenic mice with muscle glucokinase expression [29]. While studies in this area have clearly been of interest, issues such as the potential for hypoglycemia and changes in lipid homeostasis remain to be explored, not to mention the development of vectors that can safely be used for human gene therapy.

Manipulation of Steps Distal to Glucose Phosphorylation

Alterations in the Pool of Fructose-2,6-Bisphosphate, an Allosteric Regulator of Phosphofructokinase

Another approach to lowering of blood glucose levels in diabetes is to enhance hepatic glycolysis by engineering of steps distal to glucose phosphorylation. This has been achieved by construction of recombinant adenoviruses containing either wild-type or doubly mutated 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, also known as the bifunctional enzyme [30–32]. This enzyme catalyzes both the synthesis and degradation of fructose-2,6-bisphosphate, the potent allosteric activator of phosphofructokinase in liver. The doubly mutated version of the bifunctional enzyme (Ser32Ala, His258Ala) can not be phosphorylated by protein kinase A, an event which normally suppresses its kinase function, and also has diminished fructose-2,6-bisphosphatase activity, favoring formation of fructose-2,6-bisphosphate. As expected, overexpression of the wild-type or doubly mutated enzymes increases fructose-2,6-bisphosphate levels in isolated cells [30] or in liver of whole animals [31, 32], with larger increases engendered by expression of the mutant form.

The metabolic impact of overexpression of the bifunctional enzyme is similar in many respects to that observed with glucokinase overexpression. Thus, overexpression of this enzyme in normal mice resulted in lowering of blood glucose levels, but also resulted in a significant increase in circulating FFA and TG levels [31]. These animals differed from animals with overexpressed glucokinase in that liver glycogen content was decreased rather than increased. In mice with streptozotocin-induced type 1 diabetes, blood glucose levels were partially normalized, and more effectively by the doubly mutated bifunctional enzyme construct [31]. Similar to findings with glucokinase overexpression in STZ-induced diabetes, the doubly mutated form of bifunctional enzyme partially normalized circulating FFA and TG levels. Finally, overexpression of the doubly mutated form of bifunctional enzyme in liver of two mouse strains that develop syndromes resembling type 2 diabetes (KK/HIJ and KK.Cg-Ay/J strains) also resulted in partial normalization of blood glucose levels, lowering (but not normalization of) circulating insulin levels, and a modest reduction (but not normalization of) the elevated circulating FFA and TG

levels [32]. As suggested for animals with glucokinase overexpression, the modest decrease in circulating lipids caused by overexpression of the bifunctional enzyme in the type 1 and type 2 diabetes models may have occurred because the fall in circulating glucose made the animals more dependent upon lipid oxidation for energy production. As is the case for glucokinase, more work is required to fully understand the utility of bifunctional enzyme as a safe and efficacious target for diabetes therapy, particularly with regard to the potential for causing hypoglycemia and a complete understanding of effects of lipid metabolism.

Enhanced Glycogen Synthesis and Glucose Disposal Achieved via Altered Expression of Glycogen Targeting Subunits of Protein Phosphatase-1

Another approach to glycemic control might be to enhance hepatic glucose disposal via changes in glycogen metabolism. For example, inhibition of hepatic glycogen phosphorylase activity via a pharmaceutical approach results in improved glucose homeostasis in an animal model of diabetes, diabetic *ob/ob* mice [33, 34]. Our own studies have focused on a family of proteins known collectively as glycogen targeting subunits of proteins phosphatase-1 (hereafter referred to generally as glycogen targeting subunits). The glycogen targeting subunits are part of a larger family of more than 50 protein phosphatase-1 (PP1)-binding proteins that deliver the enzyme to a wide array of substrates and cellular addresses, allowing PP-1 to participate in diverse cellular processes such as glycogen metabolism, cell division, vesicle fusion, and ion channel function [35–45].

Four members of the glycogen targeting subunit gene family have been well described [35]. The targeting subunit that is preferentially expressed in striated skeletal muscle, termed G_M/R_{Gl}, was the first to be cloned [46, 47]. G_L is a 35-kD protein that is preferentially expressed in liver [48]. Protein targeting to glycogen (PTG) [49], also known as PPP1R5 [50], and a fourth form, PPP1R6 [51], are similar in size to G_L but differ from G_L and G_M/R_{Gl} in that they are expressed in a wide variety of tissues based on Northern blot analysis. The sequence homology among family members is centered around the PP1- and putative glycogen-binding regions. All four family members contain the consensus PP1 binding motif (R/K) (V/I) XF. The COOH-terminal two-thirds of G_M/R_{Gl} shares no homology with other family members, but contains a hydrophobic sarcoplasmic reticulum-binding domain [46]. G_M/R_{Gl} also contains two potential phosphorylation sites that are absent from the other targeting subunits.

Overexpression of PTG in 3T3 L1 cells or rat hepatocytes results in potent activation of glycogen synthesis [49, 52], and in hepatocytes, glycogen

synthesis is stimulated even in the complete absence of carbohydrates or insulin in the culture medium [52]. These data suggest that PTG overexpression activates the conversion of gluconeogenic precursors into glycogen. PTG overexpression also prevented the normal glycogenolytic action of agents such as forskolin and glucagon [52]. This led to studies in which the recombinant adenovirus was used to overexpress PTG in liver of normal rats [53]. Overexpression of PTG in liver improved whole-body glucose tolerance, but in contrast to glucokinase overexpression, this was achieved without perturbation of lipid homeostasis. ^{13}C-glucose NMR was used to study the pathways of glycogen synthesis in these animals, with the finding that PTG overexpression activated both direct (from glucose) and indirect (from gluconeogenic precursors) routes of glycogen synthesis. Finally, overexpression of PTG in liver of normal rats prevented the normal activation of glycogenolysis in response to fasting, consistent with the finding of impaired glycogenolysis in response to forskolin and glucagon in PTG-overexpressing hepatocytes. Thus PTG expression resulted in improved glucose tolerance, but also appeared to cause a form of glycogen storage disease when overexpressed in liver of normal animals.

These findings led to investigation of the possibility that other members of the family of glycogen-targeting subunits, such as G_M/R_{Gl} or G_L, might have regulatory features that would allow them to stimulate glucose disposal while still allowing substrate- and hormone-mediated regulation of glycogen turnover. To this end, recombinant adenoviruses containing G_L, G_M/R_{Gl}, and a truncated version of G_M/R_{Gl} that lacked its C-terminal 700 amino acids, termed $G_M\,\Delta C$ were prepared. When controlled for extent of overexpression, the targeting subunits were found to exhibit a rank order of glycogenic potency in hepatocytes of $G_L > PTG > G_M\,\Delta C > G_M/R_{Gl}$ [54, 55]. Furthermore, cells with overexpressed $G_M\,\Delta C$ were unique in that glycogen was efficiently degraded in response to lowering of media glucose concentrations, stimulation with forskolin, or the combination of both maneuvers [55].

The effects of two types of genes that stimulate glucose disposal, glucokinase and glycogen targeting subunits, were compared in more detail in isolated hepatocytes [55]. Overexpression of either glycogen targeting subunits or glucokinase stimulated glycogen synthesis in response to glucose and insulin. Glycogen synthesis in GK overexpressing cells was more dependent on the extracellular glucose concentration than in cells with overexpressed glycogen targeting subunits. Overexpression of GK also caused a much larger stimulation of glucose transport than did overexpression of glycogen targeting subunits. Glucokinase overexpression, but not glycogen targeting subunit overexpression, significantly increased lactate production. Interestingly, co-overexpression of a single glycogen targeting subunit and glucokinase resulted in an additive effect on glycogen synthesis. This suggests that targeting

subunits and glucokinase activate glycogen synthesis through different pathways. To investigate this further, NMR methods were used to measure the labeling pattern of carbons in glycogen-glucose in hepatocytes incubated with ^2H_2O [55]. This allows an estimate of the relative contributions of carbon emanating from the level of TCA cycle intermediates, triose phosphates, or hexose phosphates to glycogen synthesis, as reflected by the relative deuterium labeling of carbons 6, 5, and 2, respectively [56, 57]. This analysis demonstrated that cells with overexpressed glycogen targeting subunits synthesize a larger portion of their glycogen from the level of TCA cycle intermediates than cells with overexpressed glucokinase. These results suggest that overexpression of glucokinase increases the pool of glucose-6-phosphate [16], consequently activating glycolysis, glucose uptake, and glycogen synthesis through a 'push' mechanism. Overexpression of glycogen targeting subunits has little effect on glucose-6-phosphate levels [52], and therefore seems to activate glycogen synthesis more through a 'pull' mechanism that increases the contribution made by gluconeogenesis to glycogen synthesis. The increased glycogen synthesis from gluconeogenic precursors in cells with glycogen targeting subunit overexpression suggests that the effect of these molecules to enhance glucose tolerance may occur in part via diversion of gluconeogenic flux away from hepatic glucose production and into glycogen deposition.

These in vitro findings led to a comparison of the metabolic effects of hepatic overexpression of $G_M\,\Delta C$, native GM, and the most glycogenic of all the isoforms, G_L in vivo. At similar levels of overexpression in liver, $G_M\,\Delta C$ but not G_M or G_L lowered blood glucose levels towards normal during an oral glucose tolerance test (OGTT) in insulin-resistant, glucose intolerant, rats fed on a high-fat diet [58]. Liver glycogen levels after OGTT were increased by more than 100% in both G_L and $G_M\,\Delta C$ overexpressing rats compared with animals that received a β-galactosidase control adenovirus. However, further analysis showed that rats with overexpression of G_L had markedly elevated liver glycogen levels in both the fed and fasted state compared with control animals. In contrast, rats with overexpression of $G_M\,\Delta C$ had normal liver glycogen content in the fed state and efficiently degraded liver glycogen in the fasted state to near-normal fasting levels. Thus, overexpression of the highly glycogenic targeting subunit G_L caused glycogen stores to be replete in the fasted state, allowing little further synthesis of glycogen to occur during glucose challenge. In contrast, animals with overexpressed $G_M\,\Delta C$ were able to respond to glycogenolytic signals, thereby allowing them to maintain low fasting glycogen levels, in turn providing storage capacity during the glucose challenge.

In light of these encouraging findings in a model of diet-induced glucose intolerance, we investigated whether $G_M\,\Delta C$ could also reverse frank hyperglycemia associated with STZ-induced diabetes [59]. Three new findings

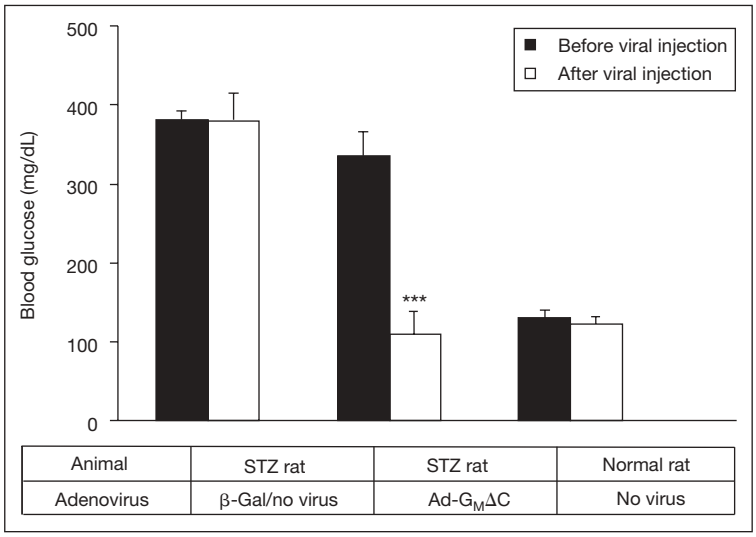

Fig. 2. Lowering of blood glucose levels in STZ-treated animals by hepatic expression of G_M ΔC. Normal rats received a single dose of streptozotocin (STZ-Rat) or no streptozotocin (Normal rat). Control STZ-injected rats received either no adenovirus or a virus expressing the β-galactosidase gene (β-GAL/no virus). Other STZ-injected rats received the AdCMV-G_M ΔC virus. The asterisk (*) indicates that blood glucose was lower in animals after treatment with AdCMV-G_M ΔC virus than before treatment, with $p < 0.001$. Data taken from Yang and Newgard [59], with permission.

emerged from these studies. First, G_M ΔC expression in liver was sufficient to fully normalize blood glucose levels (fig. 2) and glycogen storage (fig. 3) in this model of insulin-deficient diabetes. Importantly, and in contrast to what was observed with hepatic overexpression of GK, other circulating metabolites, including free fatty acids and triglycerides, were not perturbed in these experiments. Second, this normalization occurred despite very low levels of liver glucokinase expression (fig. 3), and was also remarkable in that it occurred in the face of a 68% reduction in circulating insulin levels [59]. This is consistent with our observation that glycogen synthesis in hepatocytes with overexpressed glycogen targeting subunits is glucose-dependent, but largely insulin-independent [54, 55]. Finally, G_M ΔC expression in liver curtailed food intake in STZ-injected rats, which were otherwise hyperphagic (fig. 4). This reduction in food intake occurred despite dramatic lowering of circulating insulin and leptin levels in the STZ-injected animals.

It was quite surprising that G_M ΔC expression in liver was able to lower blood glucose levels and normalize liver glycogen content in STZ-injected rats in the face of a dramatic reduction in glucokinase expression. The mechanism

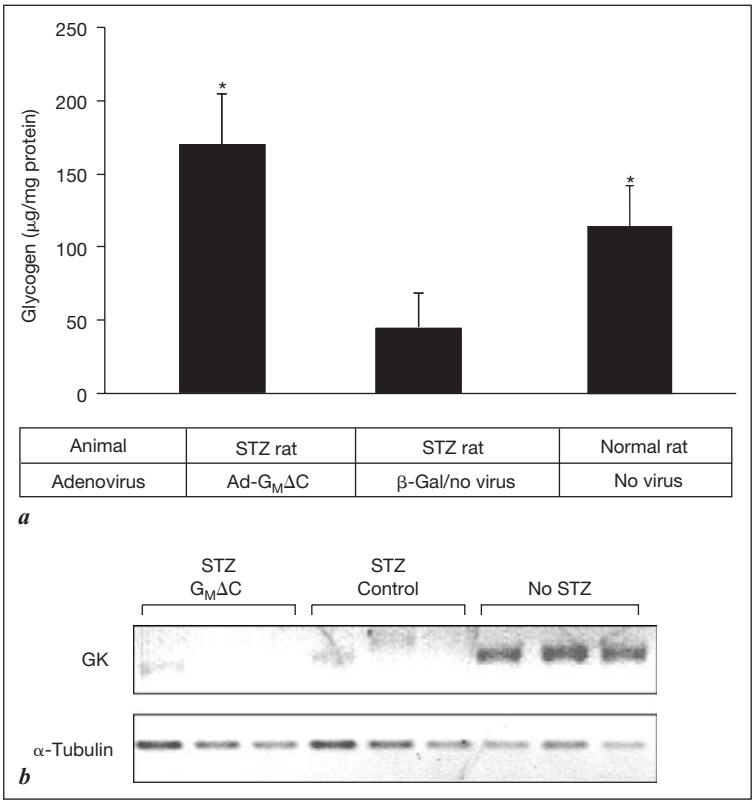

Fig. 3. Normalization of liver glycogen metabolism in STZ-induced diabetes by hepatic expression of G_M ΔC despite low levels of liver glucokinase. ***a*** Liver glycogen content in normal fed rats (Normal rat), in control STZ-injected rats that received either a β-galactosidase adenovirus or no adenovirus (β-GAL/No virus), or STZ-injected rats that received AdCMV-G_M ΔC. ***b*** Glucokinase mRNA in liver of the same groups of rats used to study liver glycogen content. Data taken from Yang and Newgard [59], with permission.

by which G_M ΔC overcomes the decrease in glucokinase expression in insulin-deficient animals remains to be defined. One possibility is that G_M ΔC, by activating glycogen synthesis, exerts a 'pull' on the glucose-6-phosphate pool, preventing a rise in glucose-6-phosphate levels that would cause product inhibition of an alternative glucose phosphorylating enzyme that is expressed in liver, hexokinase I. Alternatively, G_M ΔC expression may divert gluconeogenic precursors away from the glucose-6-phosphatase reaction and into the glycogen storage pathway, thereby contributing to lowering of glucose levels via a decrease in hepatic glucose production as suggested earlier and in agreement

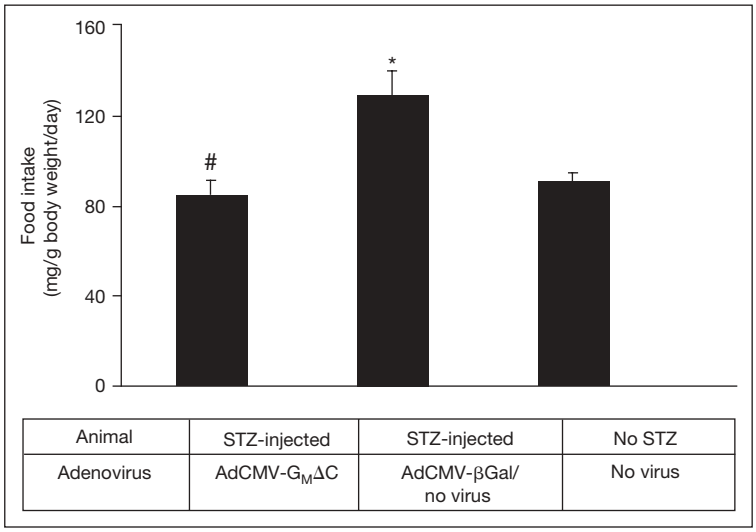

Fig. 4. Expression of $G_M\Delta C$ in liver of STZ-injected rats normalizes food intake. Normal rats received either a single bolus of 60 mg/kg streptozotocin (STZ-injected) or no streptozotocin injection (No STZ). One group of STZ-injected rats was treated with AdCMV-βGAL adenovirus or received no viral treatment (βGAL/no virus). A separate group of STZ-injected rats received the AdCMV-G_M ΔC virus. Food intake was measured during 3 successive 24-hour periods, beginning 3 days day after viral treatment. Data represent the mean intake/24 h ± SEM for 8 animals in each STZ-treated group and 6 in the No STZ group. The asterisk (*) indicates that STZ-injected rats had increased food intake relative to non-injected controls, with $p < 0.001$. The symbol # indicates that AdCMV-GMΔC-treated, STZ-injected rats had reduced food intake relative to STZ-injected control rats, with $p < 0.001$. Data taken from reference Yang and Newgard [59], with permission.

with our ^2H_2O NMR studies [55]. While these ideas will require further investigation, it is now clear that normalization of blood glucose can be achieved by manipulation of steps distal to glucokinase, even when this important enzyme is present at very low levels.

Another possible mechanism contributing to lowering of blood glucose in animals with hepatic G_M ΔC expression is their decrease in food intake relative to controls. This is clearly not the only mechanism at work, however, based on a study by others of the effects of leptin infusion in STZ-induced diabetic rats [60]. In these studies, infusion of leptin at a rate that allowed circulating levels of the hormone to be restored to those found in non-STZ-injected controls resulted in complete normalization of food intake. Hyperglycemia was partially ameliorated in these animals, but clearly not normalized (glucose levels fell from 24.3 to 17.2 m*M*). Further studies will be

required to fully understand the link between hepatic $G_M < C$ expression and regulation of food intake.

Modulation of Glucose Disposal in Liver: A Real Therapeutic Opportunity?

In this chapter, we have reviewed several methods for enhancing hepatic glucose disposal that have emerged via recent genetic engineering studies in experimental animals. We conclude by discussing the validity of each of these steps as potential drug target opportunities for treatment of human diabetes. The pros and cons of targeting glucokinase or glycolytic steps distal to glucose phosphorylation have already been discussed. In brief, these approaches show considerable promise, assuming that potential concerns about long-term impact on lipid homeostasis and hypoglycemic episodes can be addressed. With regard to the glycogen targeting subunits, we have now established that expression of G_M ΔC in liver not only reverses glucose intolerance in insulin resistant rats but also normalizes blood glucose in STZ-induced diabetic animals. However, we recognize that while G_M ΔC is an effective reagent for treatment of diabetes in rodents, its application to human diabetes is hampered by the lack of safe and efficacious vectors for delivery of foreign genes to liver of diabetic patients. Small molecules that affect glycogen targeting subunit in useful ways in liver may be difficult to design, as liver expresses a mixture of G_L and PTG, which are targeting subunit isoforms that when overexpressed seem to confer a lack of response to normal glycogenolytic signals. Nevertheless, the surprising demonstration of a glucose lowering effect of G_M ΔC in the background of depressed hepatic glucokinase expression and insulin insufficiency may place new focus on drugs that activate liver glycogen storage as a means of controlling blood glucose.

Other groups have focused on other targets in glycogen metabolism, including glycogen phosphorylase (GP) and glycogen synthase kinase 3 (GSK-3). Several compounds have been described that directly inhibit glycogen phosphorylase and glycogenolysis in primary cell cultures and in animals, and some of these inhibitors also inhibit gluconeogenesis in vitro [33, 34].

In addition, GSK-3 inhibitors have been used to mimic the effect of insulin to activate glycogen synthesis in vitro [61]. Again, some of these compounds also repress the expression of gluconeogenic genes in vitro, and improve oral glucose tolerance in Zucker diabetic fatty rats, mostly by enhancing liver glycogen synthesis [62, 63]. However, since GSK-3 plays a key role in the regulation of many cellular functions [64], the potential side effects of this class of compounds must be carefully evaluated.

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