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Editors

Oxidants in Biology

A Question of Balance

Springer

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ISBN 978-1-4020-8398-3 e-ISBN 978-1-4020-8399-0

Library of Congress Control Number: 2008924860

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Preface

This book represents a collection of articles from an international group of authors all of whom were asked to address the question of oxidants from their research viewpoints and to examine them from the perspective of "balance". The rationale for "balance" is because it has become increasingly recognized that the pretense of antioxidants are "good" and oxidants are "bad" are inaccurate. Free radical research was started in the mid-1950's when Gerschman and collaborators (1954) published the free radical theory of oxygen and when Harman (Harman, 1956) published the free radical theory of aging. The subsequent "tsunami" of research results (PubMed gives over 250,000 hits when "antioxidant" and 78,000 hits when "oxidant" were searched), perhaps initially premised on documenting this dichotomy, has increasingly shown that it is a false dichotomy and that any judgement regarding the biological effects of antioxidants and oxidants is inextricably linked to context. By context, this has two aspects. One arises because any oxidant/antioxidant can be made to act as the other depending on circumstances, and in fact is inherent in the terms "oxidant" and "antioxidant" themselves-as these depend on the nature of system utilized to assign their terminologies. Context also has the additional meanings depending not only on the location and the quantity but also the element of time referring not only to the duration of exposure but also at what temporal stage in a particular biological process the production or introduction of either an oxidant or antioxidant takes place.

Based on an assessment of the state of the field, the book has been divided into two sections. One section addresses the issue of oxidants and "balance" from the prospective of well known molecules, with a large corpus of research findings. These chapters have been designated as the "old players", though perhaps with some new tricks. The chapter by Greci et al. reviews the basis of antioxidants with a focus on hydrogen and electron transfer processes and radical scavenging reactions; Forman discusses oxidant molecules (i.e., H_2O_2), Kohen et al. on peroxynitrates, Bocci on O_3; and Goldkorn et al. on the roles of oxidants in lung pathologies. Furthermore, van der Vliet et al. describes the different effects at the cellular levels depending on the level, location and kinds of aldehydes, thus confirming the importance of "balance" in this field. This section also describs the dual roles of antioxidants, e.g., SOD (Domann et al.) and GSH (Cimino et al.), the multiple ways to activate NFkB (Reznick et al.), and the "beneficial/detrimental" effect of iron on erythrocytes (Ciccoli et al.).

The other section addresses the issue of oxidants and "balance" from the prospective of fairly new molecules involved in the oxidative stress pathways with a small corpus of research findings. These chapters have been designated as the "new players" with novel species, mechanisms and effects. Calò et al. focused on the role of p66shc, a potent inducer of oxidation-sensitive mechanism, in the pathophysiology of hypertension and cardiovascular diseases. Of note are the chapters on BARD1 (Irminger-Finger) and Duox (Harper), and of their roles in oxidative stress. In this section new players such as isoprostans (Gardi et al.), oxidation of fatty acids effect on cancer development (Kozubick et al.), release of oxidants by white blood cells (Kubala et al.), and the redox modification as a cause of post-prandial events are discussed.

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Contents

Chapter 1
Hydrogen Peroxide: The Good, The Bad, and The Ugly

Henry Jay Forman

Abstract Hydrogen peroxide (H_2O_2) is a stable intermediate produced from oxygen. Primary sources of H_2O_2 are enzymes that can reduce oxygen with two electrons and the dismutation of superoxide ($O_2^{\bullet-}$). Superoxide, the one-electron reduction product of oxygen is actually kinetically favored as the activation energy for simultaneous two electron reduction is considerably higher. Superoxide production occurs in mitochondria as the result of a leak from the electron transport chain that is pulled forward by dismutation catalyzed by mitochondrial superoxide dismutase; however, mitochondrial production is not clearly regulated by physiological signaling and is likely to be involved in pathologies. A more clearly regulated source of $O_2^{\bullet-}$ and H_2O_2 however, is the NADPH oxidase family (NOX and DuOX) that are found on other cellular membranes. Once thought to be restricted to phagocytes, where the assembly of an active superoxide producing oxidase complex has been well categorized, stimulated H_2O_2 production is now known to occur in almost all cells through NOX and/or DuOX activities.

H_2O_2 participates in pathology through reaction with transition metals that produce hydroxyl radical. H_2O_2 is used to generate hypohalous acids through catalysis by myeloperoxidases and lactoperoxidase. These oxidizing acids kill microorganisms but also damage tissue during inflammation. H_2O_2 also acts as a second messenger in signal transduction through its reaction with key proteins containing critical cysteine residues. These signaling reactions involve reversible oxidation catalyzed by peroxiredoxins and/or other as yet unidentified enzymes that result in intramolecular or mixed glutathione-protein disulfides.

The multiple roles of H_2O_2 in biology are still incompletely understood. The goal here is both to inform the reader about the biological roles of H_2O_2 and the unresolved questions as well as to encourage further investigation of this small but far from simple molecule.

Keywords Hydrogen peroxide, superoxide, superoxide dismutase, catalase, mitochondria, NADPH oxidase, NOX, DuOX, hydroxyl radical, glutathione peroxidase,

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

peroxiredoxin, thioredoxin, protein tyrosine phosphatase, ASK1, signal transduction, myeloperoxidase, lactoperoxidase, hypochlorous acid, hypobromous acid, hypothiocyanic acid

Introduction

About 3.5 billion years ago, a microorganism developed the amazing capacity to produce O_2 from water. O_2, which until then was only about 0.1% of the atmosphere began to increase rapidly. For living organisms, this presented both opportunities and challenges. Some organisms developed the ability to catalyze a concerted four electron reduction of O_2 back to H_2O by the enzyme cytochrome oxidase that was coupled to a much more efficient generation of ATP than had previously been possible. But while many other enzymes also evolved to use O_2 in metabolically important oxidations, those reactions produced the intermediate reduction molecules, superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) and their relative, nitric oxide ($^{\bullet}NO$). These species have been implicated in both physiologically helpful and harmful reactions, with the harmful reactions usually due to non-enzymatic reactions that produce the much more powerful oxidants, hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($OONO^-$). Transition metals in the environment could also react with O_2 to produce the reactive oxygen species necessitating the development of proteins that could bind transition metals in a manner that inhibited $^{\bullet}OH$ formation. This chapter however, primarily concerns H_2O_2 which, like O_2, presents both opportunities and challenges to cells. In the study of the biological roles of H_2O_2, however, the negative aspects of its chemistry and physiological responses have been emphasized and only recently have the positive roles become an increasing focus of biological research. As there are many excellent reviews on the mitochondrial, enzymatic and phagocytic cell sources of H_2O_2, enzymatic elimination of H_2O_2, and the negative consequences of H_2O_2 generation on cell function and viability, this chapter will only briefly describe those areas and will instead focus on the more recent revelations about sources and reactions involved in H_2O_2-dependent redox signaling.

Oxygen Chemistry

The chemistry of O_2 is novel in that its ground state is a triplet; i.e., it has two unpaired electrons that spin in the same direction. H_2O_2 can be formed from O_2 by two electron reduction; however, H_2O_2 is usually formed from O_2 by two sequential one electron reductions (Taube, 1965). This is because the addition of two electrons simultaneously to O_2 requires that one of the spin of one of those electrons has to reverse its spin, which requires that the transition state energy be very high. While formation of H_2O_2 is thermodynamically favorable over the formation of $O_2^{\bullet-}$,

formation of $O_2^{\bullet-}$ has a much lower transition state energy and is therefore kinetically favored. Enzymes that catalyze the formation of H_2O_2 from O_2 likely go through an intermediate formation of $O_2^{\bullet-}$ that is reduced to H_2O_2 before escaping the active site. Regardless, the rapid dismutation of $O_2^{\bullet-}$ at physiological pH causes almost all $O_2^{\bullet-}$ to rapidly become H_2O_2 or O_2 as fast as it is formed (Klug et al., 1972). In the presence of superoxide dismutase (SOD), dismutation is catalyzed at a near diffusion limited rate (Forman and Fridovich, 1973). Inside of the cytosol and mitochondria there are high concentrations of SOD (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973). In some extracellular locations, an extracellular SOD can be found (Marklund, 1984). Therefore, the conditions under which reaction with $O_2^{\bullet-}$ other than dismutation can occur to any significant extent are quite rare. The exceptions are when a reaction with $O_2^{\bullet-}$ has a rate constant competitive with SOD, such as for $^{\bullet}NO$ to form $OONO^-$, in locations where SOD is absent, or when a reactive molecule is within one molecular diameter of where $O_2^{\bullet-}$ is produced. Although H_2O_2 can also be rapidly removed by enzymatic reactions, catalase, which catalyzes the dismutation of H_2O_2 to O_2 and H_2O is sequestered in peroxisomes, and the other enzymes, the glutathione peroxidases and peroxiredoxins have rate constants that are much lower than the rate constants for the SOD family of enzymes (Hofmann et al., 2002; Ursini et al., 1985). This allows the estimated intracellular steady state concentration of H_2O_2 to be in the nanomolar range, which is 100 times the estimated steady state concentration of $O_2^{\bullet-}$ (Boveris and Cadenas, 1997).

Sources of H_2O_2

Mitochondria

In aerobes, the consumption of oxygen is primarily through concerted reduction to water by cytochrome c oxidase that occurs without release of any intermediates under normal conditions. Aerobic organisms have also evolved enzymes that produce superoxide and hydrogen peroxide as products during the oxidation of numerous metabolites (Strobel and Coon, 1971; Massey et al., 1969). Non-enzymatic oxidation of catecholamines and other metabolites can be catalyzed by transition metals (Misra and Fridovich, 1972); however under normal conditions, this is a very minor source. Metabolism of xenobiotics can also cause produce $O_2^{\bullet-}$ either through the direct reduction of oxygen by the enzyme (for example, cytochrome P450) or through the reduction of the xenobiotic to a form that autooxidizes to produce $O_2^{\bullet-}$ (for example, semiquinones and paraquat radical) (Ollinger et al., 1990; Cadenas et al., 1977; Montgomery, 1977). Neutrophils, macrophages and other phagocytes are well-known for their production of $O_2^{\bullet-}$ by a plasma membrane NADPH oxidase that is quiescent until stimulation of the cell (Curnutte and Babior, 1974). Over time evidence accumulated that other cell types also released $O_2^{\bullet-}$ in response to stimuli, and in 1999 it became clear that the phagocyte oxidase was a member of a family of NADPH oxidases that release $O_2^{\bullet-}$ or H_2O_2 and participate

in both cell mediated microbial killing and pathology but also in cell signaling (Suh et al., 1999; Lambeth et al., 2000).

H_2O_2 has long been recognized as being formed in cells even before sources were identified. Indeed, catalase, which dismutes hydrogen peroxide:

$$2H_2O_2 \rightarrow H_2O + O_2$$

was the first enzyme that was given a name. It was not until 1969 however, when McCord and Fridovich (McCord and Fridovich, 1969) isolated superoxide dismutase (SOD) that superoxide production in a biological system began to be considered as a physiologically produced molecule. Superoxide dismutases catalyze the reaction:

$$2O_2^{-} + 2H^{+} \rightarrow H_2O + O_2$$

SODs have been found in the cytosol, mitochondria and extracellular space as distinct proteins (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973; Marklund, 1984). As the rate constraint for the SODs are near the limit of diffusion (Forman and Fridovich, 1973) and the enzymes are abundant in the cytosol and mitochondria, $O_2^{\bullet-}$ is an extremely transient species that, as will be discussed below, will react within approximately one molecular diameter of its site of production.

The production of H_2O_2 by submitochondrial particles was described in 1966 (Jensen, 1966) and in mitochondria in 1972 (Boveris et al., 1972). Soon after, using submitochondrial particles, two laboratories found that $O_2^{\bullet-}$ was produced by the electron transport chain (Forman and Kennedy, 1974; Loschen et al., 1974). It was then shown that all mitochondrial H_2O_2 production results from $O_2^{\bullet-}$ dismutation (Boveris and Cadenas, 1975). Although a debate ensued about the site of $O_2^{\bullet-}$ production, it is now generally agreed is that $O_2^{\bullet-}$ is produced from complexes I and III of the respiratory chain with the actual reaction being the oxidation of ubisemiquinone:

$$Q^{\bullet-} + O_2 \quad Q + O_2^{\bullet-}$$

which, when the ubiquinone is bound to proteins, is thermodynamically unfavorable. When $O_2^{\bullet-}$ is produced in the mitochondria, it is rapidly dismuted by mitochondrial SOD. In fact, the reaction is pulled forward by the presence of mitochondrial SOD (Forman and Kennedy, 1974) and overexpression of the enzyme accelerates H_2O_2 production (Buettner et al., 2006).

While $O_2^{\bullet-}$ could react with another molecule, such as the enzyme aconitase (Hausladen and Fridovich, 1996), any reaction except for that with nitric oxide (Koppenol et al., 1992) is not competitive with SOD. This means that $O_2^{\bullet-}$ can only react with molecules that are within one molecular diameter of its site of its generation. Furthermore, any $O_2^{\bullet-}$ that appears to escape from mitochondria, would have to be produced from a reaction on the outer membrane.

So what happens to H_2O_2 produced inside mitochondria? The glutathione peroxidase and peroxiredoxin found in mitochondria are capable of reducing H_2O_2 very efficiently; however, during exposure of lungs to hyperoxia (Turrens et al., 1982), H_2O_2

produced by the respiratory chain or other mitochondrial sources, may leak into the cytosol. Whether other circumstances can cause the release of H_2O_2 into the cytosol is uncertain. In that regard, whether mitochondrial H_2O_2 production has any relationship to physiological signaling or is involved only in pathology (for example (Poderoso et al., 1996; Brookes and Darley-Usmar, 2002; Jones, 2006)) is under debated.

NADPH Oxidases

Although the pentose phosphate pathway-dependent production of H_2O_2 by phagocytes was demonstrated in 1966 (Selvaraj and Sbarra, 1966) and was later shown to involve primary $O_2^{\bullet-}$ production (Curnutte and Babior, 1974), for more than three decades, this process, the respiratory burst, was considered to be a phagocyte-specific process. Until recently, evidence of physiological generation of H_2O_2 in response to stimulation in other cells (for example (Mohazzab-H et al., 1994; Jones et al., 1994; Irani et al., 1997; Dorey et al., 1989) did not have a well defined source and was not given much attention outside of a small community of participants in the field. Interestingly, over 40 years ago exogenous H_2O_2 was shown to be capable of mimicking the action of the insulin growth factor (Czech, 1976) and insulin and nerve growth factor were shown to stimulate endogenous H_2O_2 production (Mukherjee et al., 1978; Mukherjee and Mukherjee, 1982). Despite these findings and others that described $O_2^{\bullet-}$ and/or H_2O_2 production from non-phagocytic cells (Al-Mehdi et al., 1998; Mohazzab-H et al., 1994), the biological roles of H_2O_2 were considered by most biologists to be only in pathology, toxicology, and the killing of bacteria by phagocytes. Thus, the idea that the generation of $O_2^{\bullet-}$ or H_2O_2 from the respiratory burst could participate in anything other than microbicidal action or inflammatory damage to tissue by phagocytes was not given much attention even when the NADPH oxidase – dependent activation of NF-κB and other signaling pathways was demonstrated to occur under physiological conditions of stimulation of H_2O_2 generation in macrophages (Kaul and Forman, 1996; Torres and Forman, 1999).

This situation began to change dramatically in 1999 with the discovery that the phagocyte oxidase active component, $gp91^{phox}$, was determined to be a member of a class of NADPH oxidases (NOX) that are present in almost cell types (Suh et al., 1999; Lambeth et al., 2000; Griendling et al., 2000). The glycoprotein, $gp91^{phox}$ (also called NOX2) is, like all the NOX proteins, a membrane bound protein that contains flavin and heme cofactors. Upon stimulation $gp91^{phox}$, which is bound to another membrane protein $p22^{phox}$, combines with a cytosolic protein complex ($p47^{phox}$/$p67^{phox}$/$p40^{phox}$) and the small GTPase Rac1/Rac2 to produce the active NADPH oxidase that transfers one electron from NADPH on one side of the membrane to O_2 on the other side to form $O_2^{\bullet-}$ (Shaw et al., 1980). In phagocytes, this happens mostly through the plasma membrane at the site of phagocytosis. In other cells, the Nox proteins can have a different distribution (Hilenski et al., 2004).

The components of the phagocyte NADPH oxidase have all been well characterized and many years of investigation have revealed the complexity of the signaling pathways that activate the assembly of the complex (see (Lambeth et al., 2007) for recent review).

As this information already existed, companion proteins for the other NOX proteins were suspected and identified in a variety of cell types (Griendling et al., 2000); (Tammariello et al., 2000); (Bayraktutan et al., 2000). The search for homologues of $gp91^{phox}$ was in spurred by the observations of stimulated $O_2^{\bullet-}$ production that were associated with mitogenesis in ras-transformed fibroblasts (Irani et al.) and fibroblasts micro-injected with the insert domain of rac1 (Joneson and Bar-Sagi, 1998). When a cDNA for the first of the homologues, NOX1 (originally called Mox1) was transfected into fibroblasts, it too caused increased $O_2^{\bullet-}$ production and cell transformation (Suh et al., 1999; Griendling et al., 2000). At the present time, we now know there are five NOX proteins that produce $O_2^{\bullet-}$ and two closely related DuOX proteins, which produce H_2O_2 rather than $O_2^{\bullet-}$ that are present in a variety of cell types (Lambeth et al., 2007). The expression of the Nox and DuOx protein varies in tissues but so far only $gp91^{phox}$ appears to be expressed in phagocytes. In the past few years much has been learned about the regulation of the other Nox complexes and for some, their activities appear to be turned on or increased in response to stimuli. While much more needs to be determined about their regulation, at this point it appears that Nox proteins are very likely involved in a large variety of redox signaling pathways. For other flavoproteins and mitochondria, which change activity under conditions of substrate availability or energy state, evidence for involvement in signaling is more controversial. Signaling by the Nox proteins has been recently reviewed (Geiszt, 2006; Bedard and Krause, 2007) and the specific involvement of H_2O_2 produced by Nox2 in signaling in macrophages is the final section of this chapter. Regardless, the production of $O_2^{\bullet-}$ and H_2O_2 does have a potential dark side as is well established for the phagocyte oxidase in inflammation and the other Nox proteins may be involved in chronic diseases (Lambeth, 2007; Bedard and Krause, 2007; Geiszt, 2006). Production of H_2O_2 involved in those disease processes may be sufficient to cause significant oxidation of tissue components as in inflammation or may be involved in signaling that produces inflammatory mediators, apoptosis, or unwanted proliferation of cells without directly damaging tissue.

How Is H_2O_2 Eliminated?

As mentioned above, the elimination of H_2O_2 produced in cells is quite efficient. Catalase, which dismutes H_2O_2 to water and O_2, is found in very high concentration within peroxisomes. As such, virtually no H_2O_2 produced by the oxidoreducatases in peroxisomes can escape to the cytosol and any H_2O_2 diffusing into peroxisomes will be very rapidly eliminated. The cytosol and mitochondria contain glutathione peroxidase (GPx) and peroxiredoxin (Prx) isoforms. The GPx use GSH to reduce H_2O_2 to water while five of the six mammalian Prx use thioredoxin (Trx) to reduce H_2O_2 to water.

GSH and Trx have essential roles in a number of other important biological reactions including conjugation of xenobiotic metabolites to GSH, regulation of the cell cycles, and participation in signal transduction (for reviews see (Ketterer, 1982; Meister and Anderson, 1983; Arrigo, 1999)). GSH and Trx react very slowly with hydroperoxides but are rapidly oxidized enzymatically in order to reduce H_2O_2.

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

$$H_2O_2 + Trx(SH)_2 \xrightarrow{Prx} 2H_2O + TrxS_2$$

The oxidized disulfide forms, GSSG and $TrxS_2$, can be rapidly enzymatically reduced to GSH and Trx using NADPH. This makes the removal of H_2O_2 an efficient process that can rapidly restore steady state ratios of GSH/GSSG and Trx/TrxS2. NADPH is restored from its oxidized form, $NADP^+$, by the pentose phosphate shunt, predominantly. Under steady state conditions, GSSG is usually less than 1% of total glutathione and during oxidative stress, elevation of GSSG is generally transient as reduction by GR is relatively rapid. Nonetheless, GSSG can exchange with protein sulfhydryls to produce protein-glutathione mixed disulfides (Brigelius et al., 1983);

$$GSSG + Protein\text{-}SH \rightleftharpoons Protein\text{-}SSG + GSH$$

however, unless the protein thiol is in the ionized (S^-, thiolate) form, this reversible reaction will require catalysis by a protein disulfide isomerase. Thus, mixed disulfides (Protein-SSG) have a longer half-life than GSSG and a significant basal level is found in the cisternae of the endoplasmic reticulum where the GSH/GSSG ratio is lower than in the cytosol and protein folding with disulfide exchange occurs. In the cytosol and nucleus, protein-glutathione disulfide exchange reactions provide an important mechanism for the action of GSH and Trx in cell signaling (see below). Marked decreases in total GSH or sustained large decreases in GSH/GSSG occur under conditions that lead to necrotic cell death. Such conditions can occur with poisoning by agents that conjugate with GSH or with massive production of H_2O_2 and hypochlorous acid (HOCl, see below) during inflammation.

H_2O_2 as a Toxic Molecule

This is where H_2O_2 get really ugly! While H_2O_2 can sit on a shelf in your local pharmacy for months with little decomposition, reaction with the reduced form of a transition metal rapidly produces hydroxyl radical, $^{\bullet}OH$. The most well known of these reactions, the Fenton reaction involves iron:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{g}OH + OH^-$$

Hydroxyl radical reacts with almost any other molecule with a nearly diffusion-limited rate constant to abstract hydrogen and produce another radical:

$$^{\bullet}OH + XH \rightarrow H_2O + X^{g}$$

In this manner, H_2O_2 can indirectly produce free radical reactions that result in damage to cells such as lipid peroxidation, DNA damage, and protein oxidation.

There is no defense against •OH once it is formed. Indeed, as almost everything reacts with •OH at the same extraordinarily fast rate, no molecule can effectively be a hydroxyl radical scavenger. Instead, the best antioxidant strategies that nature has derived to prevent formation of •OH by enzymatically removing H_2O_2 (see above), preventing access to metals by chelation in proteins that do not allow reaction Fenton type reactions, and scavenging of the secondary radicals. For example, the last is achieved for lipid radicals by α-tocopherol.

H_2O_2 also has another dangerous side, the formation of hypochlorous (HOCl), hypobromous (HOBr), and hypothiocyanous (HOSCN) acids (Albrich et al., 1981; Brottman et al., 1996; Wang and Slungaard, 2006) catalyzed by the myeloperoxidases of neutrophils and eosinophils and lactoperoxidase. These potently oxidizing acids are involved in microbicidal action, which helps protect against infection. But, the downside of the production of these oxidants is that they can damage tissues during inflammation. These hypohalous acids oxidize proteins. In addition, these peroxidases also catalyze nitration of proteins using nitrate and H_2O_2 as substrates(Van der Vliet et al., 1997). In chronic inflammation such as in cystic fibrosis, the production of HOCl by neutrophils that cannot successfully attack the biofilm produced by colonizing *Pseudomonas aeriginosa* attacks the epithelial cells of the airway (Van der Vliet et al., 2000). GSH, which is an excellent scavenger of HOCl is usually found in the airway lining fluid; however, in cystic fibrosis patients the airway fluid is low in GSH because of decreased transport (Gao et al., 1999) and scavenging of HOCl therefore is even less effective. The destructive side of H_2O_2's nature certainly draws attention as it should because it is implicated in a very large array of pathologies.

The prevention of oxidative damage is therefore a subject of countless investigations and is a major industry. Sales of antioxidants continue to increase and the search for new ones continues unabated. Nonetheless, while there is a long history of evidence showing that deficiency of antioxidants, such as ascorbic acid and α-tocopherol cause disease, the effectiveness of pharmacological use of antioxidants remains controversial. Even when there is a beneficial effect shown by epidemiological approaches, such as in the use of polyphenols, there is a major question of whether these compounds act as antioxidants or have other effects, such as modulation of signaling enzyme activities (Bain et al., 2003; Stauble et al., 1994)

The Role of H_2O_2 in Signaling

As stated above, during studies of the effects of H_2O_2 on cells, some investigators recognized that rather than causing toxicity, low concentrations of H_2O_2 actually increased cell growth (Burdon et al., 1989, 1990). With research on the biological role of H_2O_2 focusing on pathology, the signaling role of H_2O_2 was not a major focus of investigation until the recent discovery of the NADPH oxidases (see above). In our lab, we had the good fortune of having made the serendipitous discovery

that low concentrations of H_2O_2 enhanced subsequent stimulation of the respiratory burst (Murphy et al., 1995). We also had worked on adaptation to hyperoxia in which we had found that antioxidant enzymes increased in lung epithelial cells. Thus, we were in the right place and right time scientifically to think about H_2O_2 and other products of oxidative stress as signaling molecules (Suzuki et al., 1997; Forman and Cadenas, 1997). Of course, not everyone saw things our way and considered the activation of signaling by oxidants to be either a generalized response to injury, possibly due to increased pathological mitochondrial H_2O_2 generation (see "pink sheets" by unknown reviewers of grant proposals from the 1980's). Even when we demonstrated that endogenous generation by stimulation of the macrophage respiratory burst produced H_2O_2-dependent activation of several signaling pathways (Torres and Forman, 1999; Forman and Torres, 2001), this was considered an oddity of these phagocytes (see turn of the millennium "pink sheets"). As noted in an earlier section, this changed dramatically with the discovery of the NOX family of proteins (Suh et al., 1999; Lassegue et al., 2001) and the demonstration of their involvement in signaling in many cell types.

Mechanism of H_2O_2 Signaling

There are now numerous studies demonstrating that H_2O_2 is involved in signaling. The big question is how. We proposed that H_2O_2 has the characteristics of a second messenger and we and others have been able to demonstrate specific targets, which was the one characteristic that eluded early investigation and is still unresolved for most pathways in which H_2O_2 has been implicated.

Recent advances in understanding how H_2O_2 acts have been the subject of several recent reviews (Forman, 2007; Rhee, 2006; Rahman et al., 2005; Finkel, 2006) Signaling enzymes, such as PTEN, protein tyrosine phosphatases, and the ASK1-Trx complex that are affected by H_2O_2 have been described. Rather than review all of this material again, the remainder of this chapter will focus on the principal unresolved issues regarding how H_2O_2 acts as a second messenger.

Source of H_2O_2 in Signaling

For many pathways for which H_2O_2 (or $O_2^{\bullet-}$) has been suggested to be a second messenger, the source has not been clearly identified. Antibodies for the NOX proteins and their associated proteins are being used to identify where these proteins are in cells but there are debates concerning the precision of these determinations. Unfortunately, no specific inhibitors are available for NOX activity. Apocynin, which inhibits assembly of the NOX2 complex in neutrophils, requires oxidation by myeloperoxidase (Ximenes et al., 2007) and so will not work in all other cells *in vitro*. Diphenyliodonium (DPI) is a general inhibitor of flavoproteins and is

therefore not specific. DPI and apocynin have been useful but more specific inhibitors are needed. Knockout, siRNA and antisense strategies for decreasing these proteins is becoming a useful tool for identifying involvement but so far, with the exception of the phagocyte oxidase components (Price et al., 2002; Dinauer et al., 1997), this has seen limited use (Colston et al., 2005; Modlinger et al., 2006; Mochizuki et al., 2006). Mitochondria as a source of H_2O_2 for signaling outside of the mitochondrion itself is controversial. If mitochondrially generated H_2O_2 is involved in physiological signaling, then much more needs to be resolved concerning its regulation.

Spatial Considerations

As described above, $O_2^{\bullet-}$ will be converted to H_2O_2 within one molecular diameter of its source and H_2O_2 can travel a little bit further before it is destroyed by a Prx or GPx. Thus, the "real estate rule" of location being the most important factor applies to H_2O_2 as a second messenger. With this in mind, the question of where the proteins that interact with H_2O_2 are located in relation to the source needs to be addressed to a greater extent than has thus far been the case. Of course, there is always the possibility that H_2O_2 does not act directly. Some have argued that GSSG produced by the action of GPx or Prx 6 could act on thiol proteins by exchange to inactivate them. The problem with this is that, except under oxidative stress (as apposed to redox signaling conditions) GSSG is a very small fraction of total glutathione in the cytosol or nucleus and so formation of a mixed disulfide is unlikely. Furthermore, unless the target thiol is in the thiolate (S^-) form, the reaction with GSSG will be too slow unless catalyzed by glutaredoxin or other protein disulfide isomerase (PDI) as occurs the cisternae of the endoplasmic reticulum where protein folding occurs (Tu et al., 2000). Thus, this mechanism, while theoretically possible has yet to be demonstrated as physiologically relevant to formation of a mixed disulfide in signal transduction. It would require the presence of both an enzyme that catalyzes the oxidation of GSH to GSSG by H_2O_2 and the PDI to be located very close to the source of H_2O_2.

Kinetics

It seems to be generally agreed that thiol proteins are the site of action for physiological signaling by H_2O_2 (Forman et al., 2004). What needs to be considered however, is how these thiols are modified. Thiols (RSH) react too slowly with H_2O_2 to be part of a physiological mechanism while thiolates (RS^-) can react much faster (Winterbourn and Metodiewa, 1999). Nonetheless, not all thiolates react fast enough with H_2O_2 for the non-enzymatic reaction to account for the H_2O_2-dependent inactivation of the enzyme in which that thiolate participates (Winterbourn and

Metodiewa, 1999). We will describe two cases in which enzymatic oxidation of a thiolate must be involved although assumptions have previously been made that non-enzymatic oxidation of the thiolate occurred. It is important to keep in mind that what happens in a test tube with millimolar H_2O_2 over minutes or even seconds may not actually occur in a cell.

The of Protein Phosphatase 1B (PTP1B)

PTPs, which have a critical thiolate in their active site, play essential roles in many signaling pathways (Raugei et al., 2002; Li and Whorton, 2003; Chiarugi et al., 2001; Lee et al., 1998; Denu and Tanner, 1998; Barrett et al., 1999b; Krejsa et al., 1997). Lee et al. (1998) first demonstrated reversible PTP1B oxidation, consistent with glutathionylation, in cells stimulated by epidermal growth factor. The slow formation of glutathionylated PTP1B was directly demonstrated *in vitro* by incubating the enzyme with a very high concentration of GSSG (Barrett et al., 1999b). More recently, we demonstrated the reversible glutathionylation of PTP1B in macrophages stimulated to produce H_2O_2, which was prevented by the presence of exogenous catalase (Rinna et al., 2006). Thus, glutathionylation of PTP1B can be demonstrated both *in vitro* and in stimulated cells. But, how does this occur? The currently accepted mechanism, which can be clearly demonstrated *in vitro* with the purified enzyme, is that the first reaction involves the formation of a sulfenic acid intermediate:

$$PS^- + H_2O_2 \rightarrow PSOH + OH^-$$

followed by reaction with GSH:

$$PSOH + GSH \rightarrow PSSG + H_2o$$

to form the glutathionylated protein. In actuality, the intermediate is likely to be a sulfenate rather than sulfenic acid as the pKa for sulfenic acid is higher than for the thiol, which was in the thiolate form in that environment. When GSH is not present in the *in vitro* system, the intermediate can go on to form a sulfenyl-amide form of the enzyme that can even be crystallized (Salmeen et al., 2003). While the first reaction can occur *in vitro*, the rate constant is too slow ($9.1\,M^{-1}s^{-1}$ (Denu and Tanner, 1998) to $43\,M^{-1}s^{-1}$ (Sohn and Rudolph, 2003; Barrett et al., 1999a)) to be biologically significant unless the source of H_2O_2 is within one molecular diameter of the PTP1B. For the macrophage, where the production of H_2O_2 occurs outside of the cell, this mechanism seems implausible even though H_2O_2 could diffuse into the cell through an aquaporin (Bienert et al., 2006). Furthermore, increased glutathionylation of PTP1B continued for several minutes after the production of H_2O_2 ceased (Rinna et al., 2006) suggesting that some intermediate was formed. What remains to be determined is the nature of the intermediate. Can H_2O_2 directly oxidize some other PTP thiolates? The rate constants of thiolates with H_2O_2 to form sulfenic acid vary from around 10 to 10^5 $M^{-1}s^{-1}$, which is due to differences in both the

surrounding amino acids and the geometry of the site (Stone, 2004). Thus, in theory, a non-enzymatic reaction is possible but remains to be demonstrated *in vivo*.

Oxidation of the Trx Bound to ASK1

The mitogen activated protein kinase kinase kinase known as apoptosis signal-regulating kinase 1 (ASK1) is an upstream activator of the stress activated protein kinases, p38 MAPK and Jun N-terminal kinase (JNK) (Takeda et al., 2000; Wang et al., 1996). Binding of reduced Trx to ASK1 inhibits its kinase activity while oxidation of Trx causes its dissociation and allows activation of ASK1 and its association with other proteins (Ichijo et al., 1997; Saitoh et al., 1998; Song and Lee, 2003; Salmeen et al., 2003; Nishitoh et al., 1998; Chang et al., 1998; Tobiume et al., 2002; Nishitoh et al., 1998). We recently showed ADP-stimulated H2O2 production in the NR8383 macrophage cell line activated ASK1 through H_2O_2-dependent Trx dissociation, as demonstrated by inhibition with exogenous catalase (Liu et al., 2006). As the non-enzymatic oxidation of Trx by H_2O_2 is relatively slow but Prx's can catalyze this reaction, we assume that one of the Prx is responsible for the physiologic oxidation of Trx associated with ASK1. It has been proposed that the Prx's are inhibitors of redox signaling and that only when H_2O_2 is high enough to overwhelm them does signaling by H_2O_2 occur (Wood et al., 2003). Although this so-called "floodgate" hypothesis is intriguing, it suggests that H_2O_2 signaling results from non-enzymatic oxidation of targets rather than a more regulated, enzyme-catalyzed reaction as we have proposed here in which a Prx is the H_2O_2-sensing mediator of redox signaling. Thus, another challenge to the redox signaling research community is to resolve the true role of Prx's in signaling.

Summary

Hydrogen peroxide is a stable product formed from the two electron reduction of oxygen and the dismutation of superoxide. The one-electron reduction product of oxygen, superoxide, is produced in mitochondria as the result of the reaction of ubisemiquinone with oxygen that is pulled forward by mitochondrial superoxide dismutase. The other major sources of superoxide and hydrogen peroxide are NADPH oxidases, NOX's and DuOX's, that are found on cellular membranes other than the mitochondrial membranes. NOX and DuOX activities are found in a large variety of cells that may express one or more of these enzymes.

H_2O_2 reacts with transition metals to produce hydroxyl radical, which is implicated in a large variety of pathologies. Phagocytes also use H_2O_2 generate hypohalous acids through catalysis by myeloperoxidases. Lactoperoxidase found in some extracellular fluids can also catalyze the production of hypohalous acids, which can kill microorganisms but also damage tissue. More recently, attention has turned to the role of H_2O_2 as a second messenger in signal transduction. This occurs through

reversible oxidation of critical thiols to intramolecular or mixed glutathione-protein disulfides in key signaling proteins catalyzed by peroxiredoxins and/or other as yet unidentified enzymes. Many questions about the signaling role of H_2O_2 remain unresolved

The goal of this chapter was to inform the reader about the biological roles of H_2O_2 and encourage further investigation of this small but far from simple molecule.

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Chapter 2
Peroxynitrite: A Key Molecule in Skin Tissue Response to Different Types of Stress

Meital Portugal and Ron Kohen*

Abstract Skin, the largest tissue exposed to a variety of stresses demonstrates a common cellular biochemical response unrelated to the type of the stress allegedly. In this chapter we will describe a phenomenon in which different insults, unrelated to oxidative events to start with, resulted in an activation of common cellular biochemical pathways. These processes are similar to those evoked following exposure to stressors in which oxidants were shown to be the major metabolites involved in the mechanism of damage.

This chapter strengthen the hypothesis that peroxynitrite ($ONOO^-$) plays a key role in this common mechanism. Peroxynitrite, a product of the interaction of nitric oxide and superoxide radicals, is a potent and versatile oxidant that can attack a wide range of biological molecules. Therefore, tissue oxidative damage can be diminished by prevention of its production or decreasing its level in the different types of stress. The suggested mechanism of its production in the cell and the different metabolites derived from its decomposition are covered.

The literature describing the activation of xanthine oxidase (XO) and nitric oxide synthase (NOS) following exposure of the organism or cells to different types of stress is reviewed. It is suggested that following exposure to the different stressors there is an upregulation of pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6 and INFγ). These cytokines may encourage the activation of XO and NOS leading to an enhanced production of peroxynitrite which in turn cause biological damage.

These biochemical changes can also be reflected in changes in the cellular redox state of which the total antioxidant capacity (TAC) is one of the major components. Therefore, alteration in TAC following exposure to stress is an important factor in the mechanism of damage.

We focus on stressors to skin such as: inflammatory process, exposure to ischemic conditions, diabetic conditions, malignancies, UV irradiation, stretch stress, and effect of drugs.

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

This chapter includes different topics supporting the common mechanism involved in a variety of unrelated stresses. It covers studies of cutaneous injuries in different models (*in vivo, ex-vivo, in-vitro*) that exhibit the markers mentioned above.

Keywords Peroxynitrite ($ONOO^-$), nitric oxide ($NO^\bullet$), superoxide ($O_2^{\bullet-}$) nitric oxide synthase (NOS), xanthine oxidase (XO), pro-inflammatory cytokines, total antioxidant capacity (TAC), glutathione (GSH, GSSG), skin disorders

Introduction

The skin is a highly metabolic tissue, which possesses the largest surface area in the body and serves as the protective layer for internal organs (Regnier et al., 1998). It functions as a biological barrier that defends in physical and biochemical manner against multiple environmental insults. A variety of systemic and internal pathological conditions may be reflected in the skin. These include diabetes mellitus (Ramsey et al., 1999), artherosclerosis (Aliev and Burnstock, 1998; Garasic and Creager, 2001), inflammatory bowel diseases (IBD) (Greenstein et al., 1976), head trauma (Shohami et al., 1999), AIDS (Coopman et al., 1993), mental stress (Picardi and Abeni, 2001), and aging (Richey et al., 1988). Therefore, skin has the ability to serve as an indicator of endogenous disorders. Oxidative stress contributes to adverse effects on the skin, expressed as erythema, edema, wrinkling, photoaging, inflammation, autoimmune manifestation, hypersensitivity, keratinization abnormalities, preneoplastic lesions and skin cancer (Nachbar and Korting, 1995). Air pollution, UV irradiation, micro-organisms, viruses and xenobiotics can serve as source of exogenously-derived ROS, whereas endogenous ROS are generated during normal cellular metabolism, immune reactions and various pathological conditions (Trouba et al., 2002), such as post-ischemic conditions. Among the large variety of oxidants, peroxynitrite ($ONOO^-$) plays a major role in these disorders. Peroxynitrite, which is a product of the interaction between nitric oxide ($NO^\bullet$) and superoxide ($O_2^{\bullet-}$), is a harmful oxidant, that can attack a wide range of biological molecules (Abd-El-Aleem et al., 2000; Beckman, 1996; Ferdinandy, 2006; Halliwell, 1999; Kohen and Nyska, 2002; Richeson, 1998; Szabo, 2003). Recently, there has been a significant increase in scientific information supporting the view that in different organs peroxynitrite is a key molecule in a variety of unrelated pathophysiological processes and insults. However, only limited information is available on the production pathways of peroxynitrite and its role in skin. In this chapter the hypothesis that different types of cutaneous stress have a common mechanism based on peroxynitrite formation will be addressed. According to the suggested mechanism when skin is damaged exogenously or endogenously, there is primary up-regulation of pro-inflammatory cytokines, mainly TNFα and IL-1β (Fuchs et al., 2001; Trouba et al., 2002), leading to enhancement in ROS production, which independently are activators of the

inflammatory cytokines TNFα, IL-1β, IL-6, IL-8, and, hence result in the generation of a positive cytokine loop (Koy, 1996). To date, several agents are known as major mediators of ROS and cytokines activities, e.g. nuclear factor kappa B (NF-κB), NO•, and protein kinases. These agents usually act via cellular pathways such as phosphorylation and gene activation and play a key role in ROS and cytokine cross-talking. The elevation in the oxidants and pro-inflammatory cytokines levels is concomitant with the activation of the enzymes nitric oxide synthase (NOS) and xanthine oxidase (XO) which are the main sources of peroxynirite formation. As peroxynitrite is a very potent and versatile oxidant, the enhancement in its levels may be expressed in changes in the total antioxidant capacity (TAC), which is composed mainly by the low molecular weight antioxidant group (LMWA) (Kohen and Gati, 2000; Kohen and Nyska, 2002). The TAC can also be reflected by the redox balance, which can be measured in biological systems by reduced and oxidized glutathione ratio (GSH/GSSG), (Jones et al., 2000; Schafer and Buettner, 2001).

Peroxynitrite as a Major Mediator of Oxidative Stress in Biological Environments

Peroxynitrite Biochemistry

In 1990, Beckman et al. suggested that *in vivo*, two relatively biologically important free radicals, superoxide ($O_2^{\bullet -}$) and nitric oxide (NO•) combine under physiological conditions to form by a non-enzymatic reaction peroxynitrite ($ONOO^-$) (Beckman et al., 1990; Richeson, 1998). Peroxynitrite is a powerful oxidant, which can attack a wide range of biomolecules (Szabo, 2003). The formation of peroxynitrite is of a second order rate (Eq. 1), and the constant rate of its production is near a diffusion limit rate of (4.3–6.7) × 10^{10} M^{-1} s^{-1} (Czapski and Goldstein, 1995; Goldstein and Czapski, 1995; Huie and Padmaja, 1993; Radi et al., 2001).

$$NO + O_2^{\cdot -} \rightarrow ONOO^- \; v = k[NO][O_2^{\cdot -}] \tag{1}$$

Thus, the formation of peroxynitrite depends on the concentrations of both NO• and $O_2^{\bullet -}$. As the biological half-life of $O_2^{\bullet -}$ (less that 1 ms) is shorter than that of NO• (in the range of seconds) and taking into account the high diffusibility of NO•, peroxynitrite is formed near the sites of $O_2^{\bullet -}$ production. The ratio of NO• and $O_2^{\bullet -}$ is important in peroxynitrite production as both NO• and $O_2^{\bullet -}$ can react with peroxynitrite to form nitrogen dioxide radicals ($NO_2^{\bullet}$), hydroxyl radical (OH•) and other species (Richeson, 1998; Squadrito and Pryor, 1998; Virag et al., 2002). The peoxynitrite biochemical pathways are presented in Fig. 2.1.

Peroxynitrite is a pH-depended anion. At physiological pH it is partially protonated (pKa 6.8) and is in equilibrium with peroxynitrous acid (ONOOH). At pH 7.4 the ratio of $ONOO^-$ to ONOOH is approximately 70% and 30% respectively (Fig. 2.1) (Virag

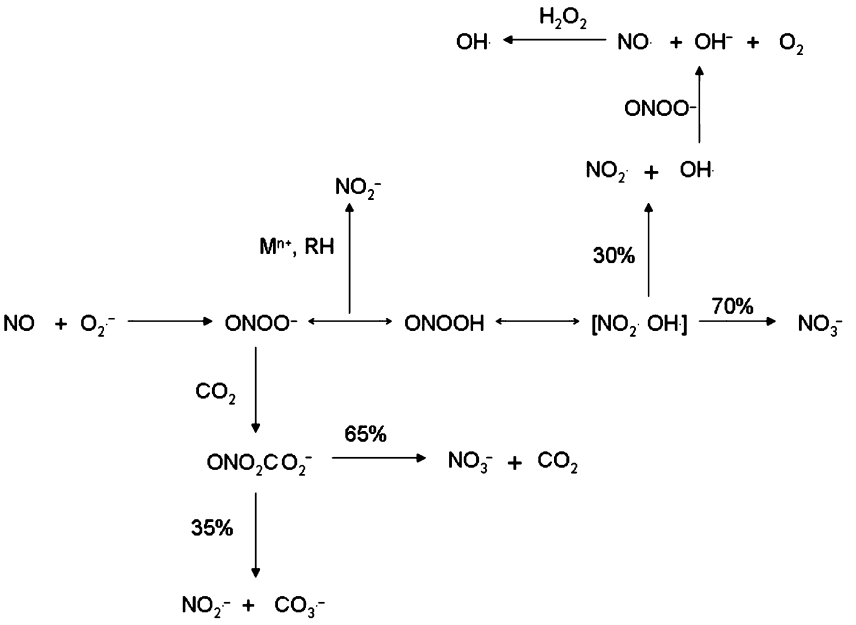

Fig. 2.1 Peroxynitrite reactions in biological systems (Modified from Radi et al., 2001)

et al., 2002). ONOOH, which has a biological half-life near 100 ms, rapidly decomposes forming a highly reactive oxidant species (ROS) (Beckman and Koppenol, 1996; Virag et al., 2002).

Peroxynitrite promotes biological effects via three main reactions: direct redox reactions, reactions with CO_2, and homolytic cleavage (Radi et al., 2001). In the direct reactions peroxynitrite can promote one or two electron oxidation reactions with second order rate constants in the order of 10^3 M^{-1} $s^{-}1$ with thiols and 10^6 M^{-1} s^{-1} with metal centers (Radi, 1998; Radi et al., 2001). In these reactions most of the peroxynitrite yields nitrite radical ($NO_2^{\bullet-}$) (Fig. 2.1). The fate of peroxynitrite in biological environment is largely determined by its reaction with CO_2 (Ferdinandy, 2006; Squadrito and Pryor, 1998). This reaction is very fast ($k = 5.7 \times 10^4$ M^{-1} s^{-1}), and water-soluble LMWA at the concentrations found in tissue can not compete with CO_2 on the peroxynitrite (Ferdinandy, 2006; Squadrito and Pryor, 1998). The reaction with CO_2 yields the short-lived intermediate nitroso-peroxocarboxylate ($ONOOCO_2^-$), which has a half life of less than 1 μs (Alvarez and Radi, 2003; Squadrito and Pryor, 1998). A homolysis of this intermediate accelerates carbonate radical ($CO_3^{\bullet-}$) and $NO_2^{\bullet-}$ formation in ~35% yield (Fig. 2.1) (Radi et al., 2001). These two radicals are highly reactive oxidants that lead to a secondary oxidative stress (Alvarez and Radi, 2003; Radi et al., 2001; Squadrito and Pryor, 1998). At pH 7.4 °C and 37 °C ONOOH can undergo homolysis to $OH^{\bullet}$ and $NO_2^{\bullet}$ with a first order constant of $0.9\,s^{-1}$ in ~30% yield, while the rest of ONOOH isomerizes to nitrate (NO_3^-) (Fig. 2.1) (Beckman et al., 1990; Merenyi and Lind, 1998). $OH^{\bullet}$ is a more powerful oxidant than $CO_3^{\bullet-}$ and $NO_2^{\bullet}$, but it is less selective in target molecule reactions (Radi et al., 2001).

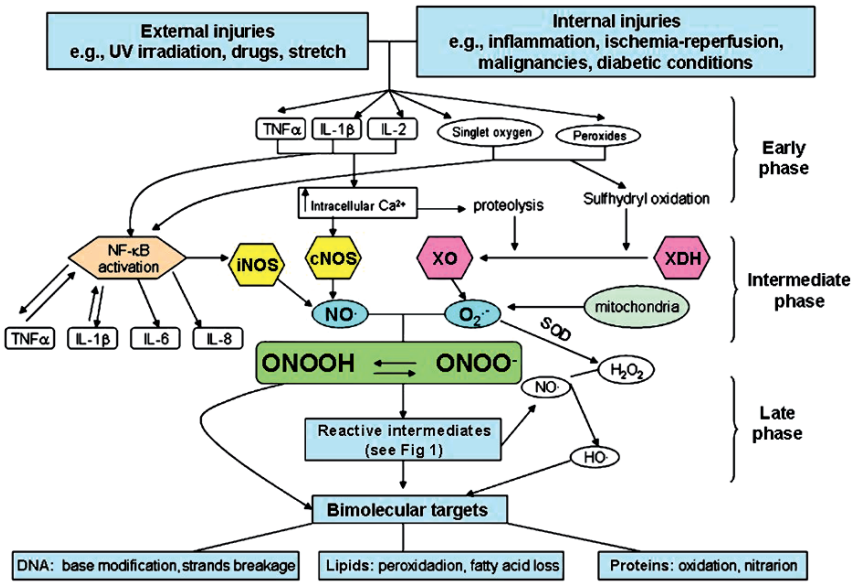

Fig. 2.2 Peroxynitrite as a key molecule in different stresses

Although it is widely accepted that enhanced peroxynitrite formation is cytotoxic, increasing evidence in the literature suggests that physiologic levels of peroxynitrite may serve as regulators of normal cellular functions (Adachi et al., 2004; Ferdinandy, 2006; Herold and Fago, 2005).

Peroxynitrite is more reactive than its parents molecules (Crow and Beckman, 1995; Pryor and Squadrito, 1995; Suarez-Pinzon et al., 2001). It reacts with thiols (Radi et al., 1991b), initiates lipid peroxydation (Radi et al., 1991), causes DNA breakage (Salgo et al., 1995), and induces protein oxidation. In addition, a characteristic reaction of peroxynitrite is the nitration of aromatic residues such as tyrosine and tryptopane residues in proteins (Alvarez et al., 1996; Beckman, 1996). Thus, nitrotyrosine is proposed to serve as an indicator of *in vivo* peroxynitrite formation (Crow and Ischiropoulos, 1996; Virag et al., 2002). Peroxynitrite can also react with enzymes, mainly with those containing a redox active transition metal center (Squadrito and Pryor, 1998), and can trigger some signal transduction pathways through activation of various types of kinases. For example, it triggers and enhances nuclear factor kappa B (NF-κB)-mediated pro-inflammatory signal transduction pathways (Matata and Galinanes, 2002).

Figure 2.2 demonstrates the interplay of peroxynitrite with other molecules following exposure to different types of stress in skin.

As can be seen, the damage mechanism is involved many players. Thus, a diversity of possible agents may have the ability to decrease the oxidative damage in different ways. Table 2.1 demonstrates possible inhibitors for oxidative stress according to the proposed mechanism.

Table 2.1 Possible inhibitors for oxidative stress through the damage mechanism involves peroxynitrite

Inhibitor	Function	Phase (as described in Fig 1)
Anti-inflammatory agents	Anti TNFα, IL-1β, IL-2	Early, intermediate
LMWA e.g., GSH, ascorbic acid, α-tocopherol, etc	Scavenging ROS	Early, late
Oxipurinol, Allopurinol	XO inhibitor	Intermediate
L-NAME, aminoguanidine	NOS inhibitor	Intermediate
Uric acid (UA)	Scavenging peroxynitrite and its reactive intermediates	Late
Superoxide dismutase (SOD)	Dismutation of $O_2^{\cdot}$ to H_2O_2	Intermediate
Nitroxides, e.g., tempol	SOD mimic	Intermediate
Catalase	Degradation of H_2O_2 to H_2O and O_2	Intermediate
Guanidinoethyldisulfide (GED)	Peroxynitrite decomposing agents	Late

Nitric Oxide Synthase (NOS) and Xanthine Oxidase (XO) as Sources for Peroxynitrite Production

As the formation of peroxynitrite depends on the concentrations of both $NO^{\bullet}$ and $O_2^{\bullet-}$, the activities of the enzymes $NO^{\bullet}$ synthase (NOS) and xanthine oxidase (XO) are crucial in this process (Squadrito and Pryor, 1998).

$NO^{\bullet}$ is produced from L-arginine, NADPH, and molecular oxygen by NOS-catalyzed reaction (Marletta, 1994; Nathan and Xie, 1994; Stamler, 1994). The family of NOS consists of three isoforms, the constitutive enzymes cNOS: neural and endothelial NOS (nNON and eNOS respectively) which are Ca^{2+}/calmaduline dependent, and the Ca^{2+}/calmaduline independent inducible NOS (iNOS) (Lee et al., 2000).

Most cell types in skin produce $NO^{\bullet}$ in response to a variety of stimuli. Keratinocytes (Arany et al., 1996), Langerhans cells (Qureshi et al., 1996), dermal fibroblasts (Wang et al., 1996), and melanocytes (Rocha and Guillo, 2001) have reported to express iNOS upon stimulation with inflammatory cytokines (Fig. 2.2) and in a variety of skin disorders including psoriasis, contact dermatitis etc. (Bruch-Gerharz et al., 1996; Trouba et al., 2002).

Xathine oxidase (XO) plays a pivotal role in the catabolism of purines. It converts hypoxanthine to xanthine, and xanthine to uric acid. XO and xanthine dehydrogenase (XDH) are interconvertible forms of the same enzyme, known as xanthine oxidoreductase (XOR) (Harrison, 2002). During the catabolism reaction, XDH reduces NAD^+ to NADH, while XO catalyses the reduction of O_2 leading to a formation of $O_2^{\bullet-}$ and it also causes to H_2O_2 production (Harrison, 2002; Nishino, 1994). Its in agreement that XDH activity is converted to an oxidase that produces $O_2^{\bullet-}$, and H_2O_2. This phenomana may occur reversibily by sulfhydryl oxidation (in oxidative conditions), or irreversibily by limited proteolysis (Fig. 2.2) (Della Corte and Stirpe, 1968; Stirpe and Della Corte, 1970). The latter occurs as a result of a decrease in the intracellular pH followed by Ca^{2+} influx and proteases activation. XO-derived $O_2^{\bullet-}$ formation (Pasquier, 1989) plays a pathogenetic role in a variety of skin disorders, such as ischemia-reperfusion injuries and inflammatory skin diseases (Nishino, 1994; Trenam et al., 1992).

Superoxide Dismutases (SOD)

Superoxide dismutases (SOD) are abundant cellular enzymes catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2, which is neutralized by catalase and glutathione peroxidase (Gpx). The only biomolecule known to well compete with SOD for $O_2^{\bullet-}$ is $NO^{\bullet}$. Hence, under conditions of increased $NO^{\bullet}$ production, $NO^{\bullet}$ can compete with SOD for superoxide, resulting in peroxynitrite formation. On the other hand, SOD or SOD mimic drugs can prevent the formation of peroxynitrite by lowering the concentrations of $O_2^{\bullet-}$ (Squadrito and Pryor, 1998). However, it is important to note that high $NO^{\bullet}$ concentrations can inhibit catalase and GPx, leading to an accumulation of H_2O_2. The latter could interact with transition metals to form $OH^{\bullet}$ (Fig. 2.2) (Nappi and Vass, 1998) and induce DNA fragmentation and cell lysis (Filep et al., 1997).

The Role of Peroxynitrite in Skin Insults

Skin as a Target Organ for a Variety of Injuries

Only limited information is available on the production pathways of peroxynitrite in skin. Therefore, several examples described below cover the important role of peroxynitrite in different and unrelated types of skin injuries: inflammatory processes, ischemic injury, diabetic conditions, malignancies, UV irradiation, stretch stress, and effect of drugs. These injuries demonstrate how up-regulation of pro-inflammatory cytokines may relate to the activation of XO and NOS leading to an enhanced production of peroxynitrite, and how the biological damage of peroxynitrite can be reflected in changes in the cellular redox state of which the LMWA is one of the major components.

Peroxynitrite Is Involved in the Damage Mechanism of Different Types of Cutaneous Stresses

Inflammatory Process

Excessive generation of ROS by the immune system can result in inflammatory responses. This phenomenon can also be induced by skin irritants (Fuchs et al., 2001). Psoriasis is an example of a chronic inflammatory skin disorder that possesses a genetic background. Psoriasis is characterized by a profound epidermal hyperproliferation related to an accelerated and incomplete differentiation, alterations in dermal vasculature, $O_2^{\bullet-}$ and $NO^{\bullet}$ formation, and elevated cellular antioxidant activity (Bruch-Gerharz et al., 1996; Trouba et al., 2002). It has been reported that iNOS is expressed in epidermal keratinocytes of psoriatic skin lesions (Bruch-Gerharz et al.,

1996). This isoform can produce $NO^{\bullet}$ following induction by the pro-inflammatory cytokines INFγ, TNFα, IL-1β and IL-8 (Bruch-Gerharz et al., 1996; Nathan, 1992). Bruch-Gerhats et al. studied the role of iNOS in psoriatic inflammation in psoriatic skin biopsies comparing to healthy biopsis (Bruch-Gerharz et al., 1996). mRNA of iNOS was detected only in skin biopsies from patients with psoriasis, but not in healthy volunteers. Moreover, the expression of iNOS mRNA was localized to the epidermal keratinocytes of psoriatic skin lesions. In addition, they found a complete colocalization of mRNA expression of iNOS with IL-8 mRNA receptors in the basal epidermal compartment and in the focal sites of ongoing neutrophilic inflammation in suprabasal cell layers. Since in psoriatic lesion there is high $NO^{\bullet}$ and $O_2^{\bullet-}$ production, these two molecules might interact immediately to form peroxynitrite (Squadrito and Pryor, 1998; Virag et al., 2002). Therefore, it can be hypothesized that peroxynitrite formation increases in psoriatic lesions.

Lipopolysaccharide (LPS), an outer-membrane component of Gram-negative bacteria can activate inflammatory gene expression through NF-κB. Over the years LPS has commonly been used in experimental models of inflammation (Nakai et al., 2006). It has been demonstrated in mice that the LPS-induced inflammatory response includes activation of iNOS and XO (Nakai et al., 2006). Nakai et al. found that 6hr treatment with LPS increased lipid peroxydation and nitrotyrosine levels in the skin of wild type mice, but not in the skin of mice who are $iNOS^{-/-}$. Moreover, they showed that aminoguanidine and allopurinol, which are iNOS and XO inhibitors respectively, independently suppressed nitrotyrosine formation. The literature also reports that LPS induced accumulation of leukocytes in mice skin at 24 h (Arimoto et al., 2005). IL-8 is known as a potent leukocyte chemokine, which can be mediated by peroxynitrite (Baggiolini et al., 1989). Zouki et al. reported that peroxynitrite can function as an intracellular signaling mechanism mediating IL-1β and TNFα-induced IL-8 expression via activation of Nf-κB. (Zouki et al., 2001).

Inflammatory processes in skin might cause a depletion of the cutaneous GSH. Wheeler et al. demonstrated that in mice exposed to the inflammation inducers psoralen plus UVA irradiation (PUVA), a depletion in both epidermal and dermal GSH levels was demonstrated (Wheeler et al., 1986). Therefore, the decrease in the GSH/GSSG ratio may suggest that the redox balance is interrupted. Such changes are deleterious to cells and tissues.

Ischemia-Reperfusion Conditions

In skin, ischemia appears to contribute frequently to impaired wound healing. Ischemic conditions of wound tissue occur primarily in patients with vascular diseases, diabetes, and in immobilized patients

Following ischemia, $O_2^{\bullet-}$ is produced during the reperfusion phase, and it rapidly reacts with $NO^{\bullet}$ to form peroxynitrite (Cuzzocrea et al., 2001). It is thought that XO has a crucial role in reperfusion injury. One of the mechanisms proposed is the depletion of the cell's ATP under hypoxic conditions, resulting in an elevated concentration of AMP which is catabolized to adenosine, inosine and then to hypoxanthine

(Pasquier, 1989). Concurrently, the conversion of XDH into XO occurs by a protease activated by an elevated cytosilic calcium concentration during ischemia. When reperfusion occurs, the return of oxygen leads to a high production of $O_2^{\bullet-}$ (Pasquier C, 1989; Rees et al., 1994). Rees et al. showed in an animal model that XO activity increased along an ischemic gradient of a skin flap and that the XO enzyme activity correlated with an increase in neutrophils number. Allopurinol significantly reduced the neutrophil number in the distal ends of the skin flaps when compared to untreated animals. Moreover, allopurinol reduced skin necrosis (Rees et al., 1994). The literature also describes that ischemia-reperfusion injury leads to an induction of iNOS expression in rat skin flaps, which results in tissue damage (Kuo et al., 2004). Hence, the elevation of XO and iNOS levels may encourage peroxynitrite formation. Chronic wounds are also known to contain much higher levels of inflammatory cytokines. Work done in our group (unpublished results) using an *in vitro* model of human keratinocytes cell line (HaCaT) showed an increase in IL-6 levels after ischemic conditions for 5 h. We also demonstrated that ischemia-reperfusion injury may affect the redox balance as reflected by intracellular GSH/GSSG ratio and TAC. The ischemic conditions caused an elevation in the GSH/GSSG ratio and in the TAC of LMWA, while reperfusion over-night was demonstrated by decreases of the GSH/GSSG ratio and TAC below the control values. These data indicate that during ischemia a reductive stress rather than oxidative stress occurs due to a lack of the electron acceptor O_2, while reperfusion produces ROS leading to overproduction of $O_2^{\bullet-}$ and $NO^{\bullet}$ radicals. Thus, high levels of peroxynitrite are being formed causing high biological damage.

Diabetic Conditions

Diabetes mellitus is characterized by chronic hyperglycemia, which may cause skin pathologies. Among the severe complications of diabetes, the reoccurrence of skin pathologies such as wounds, gangers plays a major role (Wohlrab et al., 2007). Diabetic state is associated with increase oxidative stress, which plays a pivotal role in the development of diabetic complications (Pacher et al., 2005). This occurs either directly as a result of glucose metabolism through stimulation of advanced glycosylated end-product, enhancement the polyol pathway and mitochomdrial activity, and glucose auto-oxidation (Hunt et al., 1988; Nishikawa et al., 2000; Van den Enden et al., 1995) or indirectly due to inflammatory process and cytokines release (Esposito et al., 2002; Suarez-Pinzon et al., 2001). Oxidative stress has been suggested to be involved in the genesis of both macro- and microvascular problems *via* peroxynitrite (Ellis et al., 2002; Pacher et al., 2005), leading to a restriction of the blood flow and hence to an ischemic insult.

Research in skin biopsies from diabetic patients with chronic venous ulcers showed marked up-regulation of both eNOS and iNOS compared with normal human skin tissue (Abd-El-Aleem et al., 2000). Although this study has demonstrated that both NOS isoforms were up-regulated, analysis of the activity demonstrated that iNOS was the predominant form. Once iNOS was induced, $NO^{\bullet}$ was produced

at a high level (nmoles) over a long period of time. On the other hand, eNOS produced a low level of $NO^{\bullet}$ (pmoles). Therefore, the main source of $NO^{\bullet}$ in chronic venous ulcers is iNOS. Another study on wounds in diabetic mice demonstrated that cutaneous $O_2^{\bullet-}$ levels were markedly increased in these mice. Thus, $NO^{\bullet}$ and $O_2^{\bullet-}$ overexpression in diabetes skin may be involved through the production of peroxynitrite in the pathogenesis of the delay wounds healing.

A recent study done in our laboratory in HaCaT cultures (unpublished results) suggests that cells under hyperglycemia are more vulnerable to ischemic conditions. The effect of hyperglycemia on IL-6 secretion has been examined. The IL-6 levels were significantly higher in hyperglycemic cultures than the untreated ones pointing to inflammatory conditions. It is also known that increased sorbitol production by the polyol pathway in the elevated glucose concentrations results in an intracellular depletion of NADPH, which is crucial for the reduction of GSSG to GSH (Tesfamariam, 1994). The GSH/GSSG ratio and the TAC in hyperglycemic HaCaT cultures were similar to the untreated cultures, however ischemic insult in hyperglycemic cells led to a decrease in the GSH/GSSG ratio and in TAC, which remained constant also after reperfusion. According to our results, contrary to cells under normal glucose levels, which were exposed to ischemia-reperfusion conditions, hyperglycemic cells were not under reductive stress during ischemia, but under oxidative stress. These observations might be due to hyperglycemic conditions preceding to the ischemic insult.

Malignancies

Skin cancer development depends in part on immune surveillance, which is mediated, in part by ROS. Klotz et al. suggested that peroxynitrite activates a pathway of the kinase Akt. The Akt product, the proto-oncogene c-Akt, is a kinase responsible for anti-apoptotic and proliferative response to growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Klotz et al., 2000). The nitration of protein-tyrosine residues impairs phosphorylation of tyrosines crucial for phosphorylation signaling (Klotz et al., 2000). Klotz et al. demonstrated that exposure of human primary fibroblasts to peroxynitrite (either added to the cells as a bolus, or generated *in situ* by SIN-1) resulted in Akt activation as an acute response. They found that phosphorylation of epidermal growth factor receptor (EGFR) plays a primary role in mediating Akt activation. In response to peroxynitrite Akt became phosphorylated, but this appeared not to be important for Akt activation by peroxynitrite, as the inhibitor of EGFR did not alter the response to peroxynitrite. Contrary, the inhibitor of platelet-derived growth factor receptor (PDGFR) abolished Akt activation, indicating an important role for this growth factor in mediating response for Akt activation (Klotz et al., 2000). This reflects selectivity for growth factor receptor tyrosine kinase in transducing signals initiated by oxidative insults.

It is widely known that 12-O-tetradecanoylphorbol-13-acetate (TPA) is a tumor promoter substance. In mice, TPA induced tumor promotion in skin, possibly

through overexpression of $O_2^{\bullet-}$ (Murakami et al., 1997). Another study demonstrated that in basal cell carcinomas and in metastatic tumors of the skin there was an enhancement in iNOS expression (Kagoura et al., 2001). Thus, it could be suggested that in skin malignancies the elevation in iNOS and $O_2^{\bullet-}$ levels leads to peroxynitrite formation.

As oxidative mechanisms have been proposed to play an important role in activating and amplifying the process of tumor promotion, malignancies might also affect GSH/GSSG ratio in skin. A study done by Perchellet et al. examined the impact of TPA, which is one of the essential compounds of mouse skin tumor promotion on isolated epidermal cells from mice (Perchellet et al., 1985). Their results showed a rapid depletion of intracellular GSH and a sharp increase in the GSSG content. The GSH/GSSG ratio dropped from 19 to 4, suggesting that the oxidative stress linked to skin tumor promotion by TPA might rapidly overcome the GSH-dependent antioxidant protective system of the epidermal target cells. Hence, the modifications of the GSH/GSSG ratio might play a key role in skin tumor promotion.

The mechanisms of tumor promotion are known also to involve induction of pro-inflammatory cytokines, mediated through activation on NF-κB (Suganuma et al., 2002). Suganuma et al., demonstrated that TNFα is essential in tumor promotion in mouse skin. TPA produced tumors in 100% of $TNF\alpha^{+/+}$ mice and 78% of $TNF\alpha^{-/-}$ mice and the avarage number of tumors per $TNF\alpha^{+/+}$ mouse was more than five time than in the $TNF\alpha^{-/-}$ group. Moreover, the mRNA expression of IL-1β, IL-6 and iNOS in the skin of $TNF\alpha^{+/+}$ mice was higher that those of $TNF\alpha^{-/-}$ mice. These results suggest a cross-talk between $NO^{\bullet}$ and pro-inflammatory cytokines, probably *via* NF-κB in tumor promotion.

UV Irradiation

Skin exposed to UV irradiation, particularly to UVB (290–320 nm) produces indirectly a large number of ROS, which results in adverse skin changes such as erythema, inflammation, photoaging and skin tumors (Katiyar et al., 1999; Lee et al., 2000).

It has been shown that the cNOS and XO of human keratinocytes can be activated to release $NO^{\bullet}$, $O_2^{\bullet-}$ in a dose-dependent increase, and peroxynitrite release following exposure to UVB irradiation. This was repressed by inhibitors oxypurinol (OP, for XO) and N-Nitro-L-Arginine Methyl Ester (L-NAME, for NOS) (Deliconstantinos et al., 1996). It has been shown that $NO^{\bullet}$ can react with thiol groups to form S-nitrothiols. UVB irradiation caused a 15-fold increase in S-nitrothiols formation (Deliconstantinos et al., 1995, 1996). This may lead to a depletion in the thiol groups and therefore, to a decrease in the GSH/GSSG ratio. Indeed, research in human volunteers demonstrated that UVB exposure of human skin was found to result in a lowering of GSH levels examined at 6–48 h after irradiation (Katiyar et al., 2001).

Human keratinocytes are known to release $NO^{\bullet}$ 24 h after UVB irradiation due to a production of cNOS and iNOS (Chang et al., 2003). $NO^{\bullet}$ is known to enhance

local blood flow and volume in both the superficial and deep vascular of the dermis leading to erythema (Deliconstantinos et al., 1995; Henry et al., 1993). Erythema is also a consequence of various inflammatory stimuli, including pro-inflammatory cytokines. Observations done by our group (unpuplished results), which are based on *ex vivo* skin cultures demonstrated a significant elevation of IL-1β, TNFα, IL-8 and IL-6 levels 24 h after UVB irradiation leading to production of iNOS.

Stretch Stress

As the skin functions as an external barrier, it is commonly vulnerable to mechanical injury. This can be due to routine activities (e.g. sports), weather conditions, burns, trauma, etc. Stretch stress is one example for such a mechanical injury. Studies done in our laboratory focused on an *in vitro* model system for stretch insult in HaCaT cells (unpublished results). We examined the interrelationship between $NO^{\bullet}$ and uric acid (UA), which is the end-product-formed by the XO, but also known as a scavenger of peroxynitrite (Hooper et al., 1998; Scott and Hooper, 2001; Whiteman et al., 2002). Our result showed that nitrites accumulated in the extracellular medium 9–17 h after injury in significantly higher levels than in the untreated culture. These results are indicating the massive release of $NO^{\bullet}$ radicals in the injured cells. At the same time, UA levels, which progressively increased in the untreated culture, remained constant in the injured culture and therefore can be considered as lower than in untreated culture. This data suggest consumption of UA by peroxynitrite and its by-products since it can act as their scavenger.

We also studied the effect of the inhibitors OP for XO, and L-NAME, for NOS, on HaCaT cells exposed to stretch stress. When OP, L-NAME, or both were added to the culture after injury, a significant reduction in $NO^{\bullet}$ and UA levels was observed. Moreover, addition of OP resulted in an increase of $NO^{\bullet}$ levels, probably due to a decrease in the interaction between $O_2^{\bullet-}$ and $NO^{\bullet}$.

Another study observed that extremely high hydrostatic pressure stresses induced a variety of pro-inflammatory cytokines production in normal human dermal fibroblasts (Koyama et al., 2002). Normal human dermal fibroblasts were found to survive and be active in producing IL-6 and IL-8 under extremely high hydrostatic pressure.

Effects of Drugs

Many drugs, systemic or topical, cause side effects which express in oxidative damage to skin. In this section we will focus on the impact of Benzoyl peroxide (BPO) and cocaine in skin.

BPO which is used as a topical antimicrobial agent for acne treatment, can induce an inflammatory reaction mediated by oxidative stress. A study done by Valacchi and colleagues demonstrated that in a human keratinocyte cell line (HaCaT) cytoplasm, BPO interacted with the water soluble antioxidants, resulting

in GSH depletion and a decrease in the intracellular GSH/GSSG ratio which reflects the redox state of the cells (Valacchi et al., 2001). The fluctuation in the cell redox environment induced the gene expression of pro-inflammatory cytokines including IL-1, Il-6, TNFα, and IL-8 (Gosset et al., 1999; Rovin et al., 1997). Topical application of BPO resulted in a depletion of the antioxidant enzyme SOD in mice (Zhao et al., 2000). This depletion may be due to elevation in $O_2^{\bullet-}$. In a study done on HaCaT cell line, treatment with BPO increased the total nitrite concentration, which is an indicator for $NO^{\bullet}$ production (Bellei et al., 2004). Such an elevation in $O_2^{\bullet-}$ and $NO^{\bullet}$ as a consequence of skin exposure to BPO, might lead to peroxynitrite production, which is responsible for oxidative stress.

Cocaine, an alkaloid obtained from the leaves of the coca plant, stimulates the central nervous system (CNS). It can be injected, or smoked, or inhale and is used in medicine as a topical anesthetic (Johanson and Fischman, 1989). Our group studied the acute influence of cocaine in skin using an *in vivo* rat model. Our results showed that 0.5 h after cocaine injection, an elevation in lipid peroxidation was observed in skin, while the UA levels were lower compared to the control group. Since UA can function as a peroxynitrite scavenger, its decrease may be explained due to its consumption as a consequence of the oxidation process. It can be assumed that peroxynitrite formation occurs also in the early phase after cocaine injection by consumption of $NO^{\bullet}$ already existing. This assumption is derived from our results, which showed a decrease of nitrite levels parallel to the increase of lipid peroxidation. These observations are an example for how a CNS stimulant can also affect the skin and lead there to an oxidative damage.

Concluding Remarks

A survey of the current literature indicates that peroxynitrite is a versatile molecule involved in oxidative damage due to different types of cutaneous stresses. This damage is also reflected in the up-regulation of pro-inflammatory cytokines and TAC modifications. From this brief overview on different injuries in skin, it is important to note that although these injuries are apparently unrelated, there is interplay among them, probably as a result of a common mechanism. Ischemic insult can be derived from diabetic conditions and resulting skin ulcers; skin ulcers lead to a mechanical stress and to inflammatory processes; and UV irradiation promotes skin malignancies and inflammation.

It is plausible that the clinical use of NOS or XO inhibitors, peroxynitrite scavengers, as well as inflammatory-cytokines-neutralizing agents might serve as practical and applicable therapeutic regimens against peroxynitrite formation in a variety of skin insults. Therefore, these therapeutical substances have the ability to act indirectly as antioxidants. This ability raises and complicates the question of "what is the definition of antioxidants?" The accepted definition for an antioxidant is based on its ROS scavenging potency, but a novel and wider definition for an antioxidant should include the ability of the substance to inhibit peroxinitrite formation, which is a key player in oxidative stress, and prevent oxidation in general.

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Chapter 3
Cellular and Environmental Electrophiles: Balancing Redox Signaling, Inflammation, and Cell Death Pathways

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Abstract Analogous to reversible phosphorylation of threonine, serine, and tyrosine residues as well established signal transduction mechanisms, the reversible oxidation of protein cysteine residues by reactive oxygen or nitrogen species, e.g. S-glutathionylation or S-nitrosylation, has emerged as an important mechanism of protein function regulation and cell signaling in relation to the activation of NADPH oxidases (NOX/DUOX enzymes) or nitric oxide synthases (NOSs). However, these key protein cysteine residues also present significant targets for a host of electrophilic compounds from either exogenous or endogenous sources. A number of α,β-unsaturated aldehydes and ketones have been identified as important environmental pollutants, bioactive components of ingested foods, or secondary mediators produced endogenously during oxidative processes and the addition of these electrophiles to cysteine thiols, a process known as S-alkylation, may represent an additional mode of "redox" signaling analogous to e.g. S-thiolation. In addition to mediating signals, α,β-unsaturated aldehydes and ketones may compete with or interfere with other pathways that feature thiol/redox dependent signaling. The biological effects of α,β-unsaturated aldehydes and ketones are determined largely by their relative electrophilic character, by acute effects on cellular GSH or redox status and direct S-alkylation of critical protein cysteines, and the activation of phase-2 enzymes involved in GSH synthesis and detoxification and elimination of these electrophiles. The cellular effects are thus determined by a balance between disposal, direct S-alkylation of critical protein targets and more indirect "oxidative stress responses" as a result of increased production of cellular oxidants, all of which are critical in determining apoptotic or necrotic cell death by α,β-unsaturated aldehydes, and their variable pro- and anti-inflammatory properties. The significance of α,β-unsaturated aldehydes as critical mediators of acute and/or chronic disease is gaining increased recognition, fueled by increased identification of critical biological targets for these electrophiles and observations of associations of

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

polymorphisms in important detoxifying enzymes (glutathione S-transferases, GSTs) with chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). This Chapter will address some of the general biochemical and cellular aspects of α,β-unsaturated aldehydes, and discuss mechanisms by which they can regulate cell death pathways and inflammatory responses and the importance of cellular defense mechanisms against these electrophiles.

Keywords Acrolein, cyclopentenone prostaglandins, apoptosis, inflammation, NF-κB, redox signaling

Introduction

The reversible phosphorylation of threonine, serine, and tyrosine residues represent well established mechanisms of signal transduction, controlling a wide range of cellular responses to hormones, growth factors, cytokines, etc. More recently, reversible modifications of protein cysteine residues have emerged as important modes of protein function changes and cell signaling by reactive oxygen or nitrogen species. For example, protein S-nitrosylation or S-glutathionylation of critical susceptible cysteine residues are becoming increasingly implicated in cell signaling pathways in response to reactive oxygen or nitrogen species generated by NADPH oxidases or nitric oxide synthases (Bove and van der Vliet, 2006; Giustarini et al., 2004; Hess et al., 2005). However, these cysteine residues also form susceptible nucleophilic targets for a wide range of reactive electrophilic compounds that react with nucleophiles by Michael addition to form covalent adducts. Many of these electrophiles are xenobiotic, but a host of α,β-unsaturated aldehydes and ketones are also generated within biological systems that can mediate cellular function in large part by direct interaction with susceptible cysteine residues by S-alkylation (Fig. 3.1). Increasing evidence supports the importance of S-alkylation of critical cell targets by endogenously generated α,β-unsaturated aldehydes and ketones as a means of regulating specific cell signaling pathways. For this reason, biological systems have evolved various important defense systems to control unwanted S-alkylation and conversely, evidence is accumulating to suggest that S-alkylation may be another important signaling mechanism, similar to S-nitrosylation or S-glutathionylation (Fig. 3.1).

The paper will focus primarily on the biochemical and cellular properties of α,β-unsaturated aldehydes and ketones and how these electrophils interact with signaling and disposal systems to produce the spectrum of effects associated with these compounds. A major focus will be ACR, structurally the most simple and prototypical α,β-unsaturated aldehyde but occasionally the review will highlight important analogous features of structurally different α,β-unsaturated carbonyl compounds, such as lipid-derived mediators (4-hydroxy-2-nonenal, cyclopentenone prostaglandins, etc.) or dietary electrophiles with noteworthy biological effects (e.g. curcumin) will also be discussed.

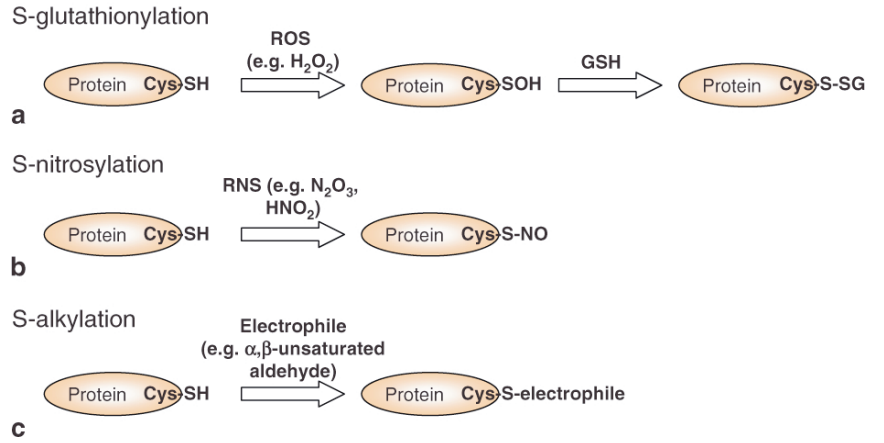
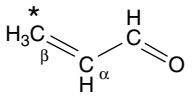


Fig. 3.1 Regulation of protein activity by cysteine modifications. (a) Reactive oxygen species (ROS) can alter protein function by oxidation of critical Cys residues, which result in S-glutathionylation by reaction with highly abundant cellular GSH. (b) Similar protein cysteine modifications by reactive nitrogen species (RNS) lead to S-nitrosylation. (c) Electrophiles can modify protein cysteines by S-alkylation, by Michael addition of α,β-unsaturated carbonyl compounds

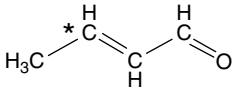
α,β-Unsaturated Aldehydes and Ketones: Environmental Pollutants and Biological Mediators

Biological systems routinely encounter a wide range of electrophilic compounds due to exposure to many diverse xenobiotics as well as various endogenous metabolic products. Among these, the α,β-unsaturated aldehydes and ketones are major components of fuel emissions, wood smoke or tobacco smoke. Acrolein (2,3-propenal; Fig. 3.2), the most abundant and reactive of these is considered to present the greatest non-cancer health risks of all organic air pollutants as ambient levels in urban areas commonly exceeding EPA reference concentrations (Leikauf, 2002; Seaman et al., 2006). For example, environmental acrolein (ACR) has been associated with decreased respiratory function in the United States (Woodruff et al., 2007). ACR is perhaps even more important as an indoor pollutant, being present in cooking areas or various work places (e.g. welding shops). Finally, ACR and several additional α,β-unsaturated aldehydes such as crotonaldehyde are major components of environmental tobacco smoke as mainstream cigarette smoke (CS) contains as much as 90 ppm ACR, and ambient ACR concentrations in smoking areas may reach 0.1–0.5 ppm (Esterbauer et al., 1991; Kehrer and Biswal, 2000; Li and Holian, 1998).

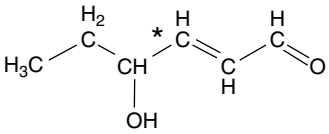
ACR, and other related α,β-unsaturated aldehydes, can also formed endogenously during various oxidative or metabolic processes, perhaps suggesting a biological function for these electrophiles. A number of α,β-unsaturated aldehydes are generated endogenously during conditions of oxidative stress by non-enzymatic oxidation of unsaturated fatty acids, such as linoleic acid and arachidonic acid



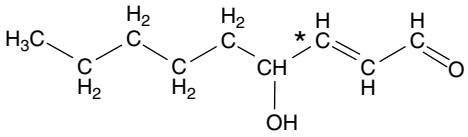
Acrolein (2-propenal)



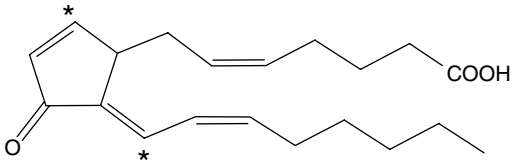
Crotonaldehyde (2-butenal)



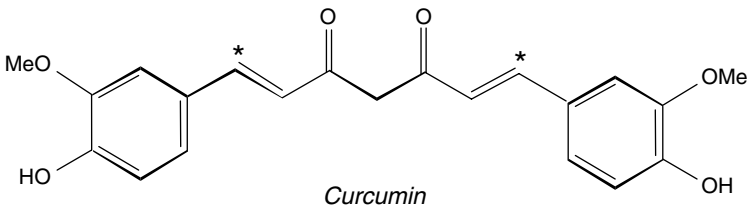
4-hydroxy-2-hexenal



4-hydroxy-2-nonenal



15d-$\Delta^{12,14}$-PGJ$_2$



Curcumin

Fig. 3.2 Structures of the various α,β-unsaturated carbonyls discussed in this review. The double bond between α- and β-carbons adjacent to a carbonyl group forms an electron-deficient center (indicated with an asterisk), due to the electron-drawing properties of the carbonyl group, and forms a common site of attack in each of these electrophiles

(Esterbauer et al., 1991; Petersen and Doorn, 2004; Ichihashi et al., 2001; Uchida et al., 1998; Uchida, 1999). ACR can also be generated during inflammation by oxidation of the amino acid threonine (Anderson et al., 1997) or during oxidative deamination of spermine (Kwak et al., 2003; Tanel and Averill-Bates, 2005). Measurements in airway secretions or aspirates from subjects with chronic airway

inflammation or heavy smokers indicate the presence of ACR and related α,β-unsaturated aldehydes such a 4-hydroxyhexenal and 4-hydroxynonenal (HNE) at concentrations ranging from 10 nM to >10 μM (Andreoli et al., 2003; Annovazzi et al., 2004; Corradi et al., 2004), concentrations that are sufficient to induce major effects on various cellular functional properties, as will be discussed in the following paragraphs.

The α,β-unsaturated aldehydes described above share important characteristic properties with a family of prostaglandin-like compounds the cyclopentenone prostaglandins PGA_2, PGA_1 and PGJ_2, and additional metabolites Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2)(Straus and Glass, 2001). Unlike prostaglandins that act via G-protein coupled prostanoid receptors, cyclopentenone prostaglandins (CyPGs) act via specific interactions with other cellular target proteins. Some activity derives from high-affinity interaction with the nuclear receptor, peroxisome proliferator-activated receptor-γ (PPAR-γ), but increasing evidence suggests that many CyPG's involve the electrophilic α,β-unsaturated carbonyl group (Fig. 3.2). As will be discussed in the following sections, CyPGs share a number of biological effects with α,β-unsaturated aldehydes, such as ACR and HNE, due to their common reactive α,β-unsaturated carbonyl group. Similarly, an important component of the curry spice turmeric, curcumin, contains two adjacent α,β-unsaturated carbonyls (Fig. 3.2), and many of the suggested beneficial health effects of curcumin (Jagetia and Aggarwal, 2007) may be related to similar biological actions involving this functional group.

Cellular Effects of α,β-Unsaturated Aldehydes: Acute Effects and Adaptive Responses

Acute Toxicity by Electrophiles: GSH Depletion and Cellular Redox Changes

Because of the high reactivity of α,β-unsaturated aldehydes with cellular nucleophiles, acute cellular effects of these electrophiles are largely related to depletion of the major cellular nucleophile, GSH, either by direct reaction or in a reaction catalyzed by glutathione S-transferases (GSTs) (Berhane et al., 1994; Tjalkens et al., 1998). High concentrations of ACR, the most reactive of the α,β-unsaturated aldehydes, rapidly depletes cellular GSH, as GST-catalyzed conjugation of α,β-unsaturated aldehydes with GSH prevents covalent modifications of other critical cell targets and serves as an important step in their detoxification, via excretion of GSH-alkyl adducts mediated by multidrug resistance associated proteins (Deeley et al., 2006; Manzano et al., 1996; Paumi et al., 2003; Reddy et al., 2002).

At more moderate concentrations, ACR and other α,β-unsaturated aldehydes induce various stress responses, and (in)activate various signaling pathways that are involved in transcription factor activation and in the regulation gene expression. These effects

then ultimately determine cellular fates, such as the production of cellular (inflammatory) mediators, or cell proliferation, survival or apoptosis (Biswal et al., 2002; Finkelstein et al., 2005; Kern and Kehrer, 2002; Li and Holian, 1998). These are closely related to changes in cellular GSH, suggesting that they are mediated by indirect cellular redox changes due to GSH depletion, or by S-alkylation of critical cell constituent cysteine-containing proteins (Bennaars-Eiden et al., 2002; Hristova et al., 2007; Ji et al., 2001b; Kehrer and Biswal, 2000; Lambert et al., 2007; Li and Holian, 1998). Recent advances in the identification of cellular targets, due to the development of various proteomics strategies, have provided substantial insights into the mechanisms by which ACR and other α,β-unsaturated aldehydes can impact on inflammatory signaling and the regulation of cell apoptosis. The following two sections will focus on these specific aspects of cell signaling by these biologically relevant electrophiles.

Adaptive Responses to Electrophiles: Induction of Antioxidant Defense Systems

The exposure to α,β-unsaturated aldehydes such as ACR induces phase-2 genes as part of a coordinated cellular defense against electrophiles (Dickinson et al., 2004; Kwak et al., 2003; Tirumalai et al., 2002). These include heme oxygenase-1 (HO-1), γ-glutamylcysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, multidrug resistance-associated proteins, GSTs, as well as several antioxidant enzymes. Central to this general induction of phase-2 genes is the activation of the Kelch-like ECH-associated protein (Keap1)-nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf2) pathway. Redox mechanisms that modify the cysteine residues of Keap1 govern the Keap1-Nrf2 interaction and are therefore pivotal for the cellular sensing mechanism for electrophiles (Dinkova-Kostova et al., 2001; Wakabayashi et al., 2004). Electrophiles disrupt the Keap1-Nrf2 complex by modifying two (C273 and C288) of the 25 cysteine residues within Keap1, which results in the liberation and activation of Nrf2. Upon activation, Nrf2, a member of the basic leucine zipper family of transcription factors, translocates to the nucleus and binds to antioxidant (electrophile) responsive elements (ARE/EpRE) within the 5' upstream promoter of these various phase-2 and antioxidant genes (Itoh et al., 2004; Tirumalai et al., 2002; Wakabayashi et al., 2004). Nrf2 activity is normally suppressed due to direct binding to its cysteine-rich protein partner, Keap1, which is anchored to the actin cytoskeleton and sequesters Nrf2 within the cytoplasm and enhances its proteasomal degradation (Fig. 3.3). Because of this Nrf2 response, the acute depletion of cellular GSH by α,β-unsaturated aldehydes is followed by an upregulation of cellular GSH levels, which contributes to increased cellular resistance against these electrophiles (Itoh et al., 2004; Tirumalai et al., 2002). The overall cellular effects of ACR and related α,β-unsaturated aldehydes are therefore balanced by the relative extent of acute responses due to GSH depletion and/or modifications of critical cellular proteins and their relative ability to induce protective phase-2 responses. In turn, this is governed by the effective dose and/or relative

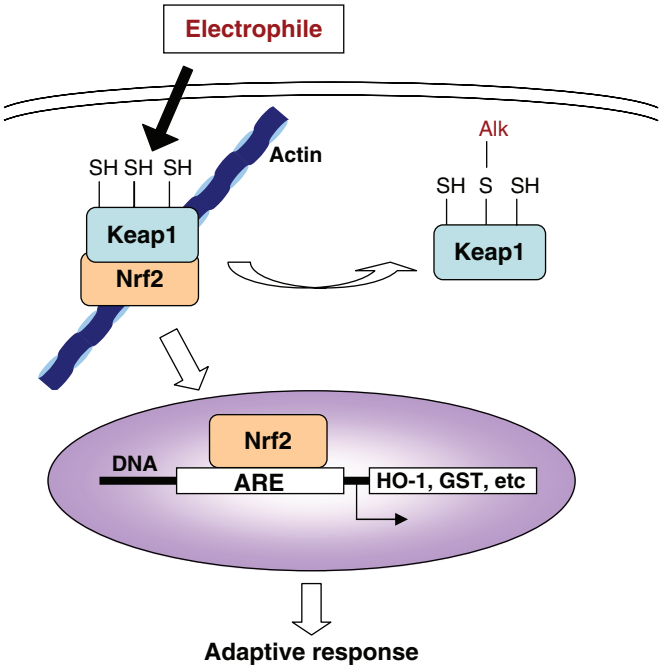


Fig. 3.3 Induction of adaptive responses by electrophiles. Electrophilic compounds can target reactive Cys residues within Keap1, which sequesters the transcription factor Nrf2 within the cytoplasm. Oxidation or alkylation of the Cys within Keap1 dissociates it from Nrf2, allowing translocation of Nrf2 to the nucleus, where it can bind to antioxidant responsive elements (ARE) in the promoter region of various phase-2 or antioxidant genes

reactivity of α,β-unsaturated carbonyl compounds with GSH, their substrate specificity for GSTs, and their relative ability to activate Nrf2 (Biswal et al., 2002; Ceaser et al., 2004; Dinkova-Kostova et al., 2001; Gayarre et al., 2005).

Direct Protein S-alkylation Versus Indirect Redox Signaling

Because of their chemical reactivity, α,β-unsaturated carbonyl compounds such as ACR can directly target nucleophilic sites such as guanine residues in DNA to produce exocyclic DNA adducts, 6-hydroxy-1,N^2-propanodeoxyguanosine and 8-hydroxy-1,N^2-propanodeoxyguanosine adducts, which are mutagenic (Feng et al., 2006; VanderVeen et al., 2001). A recent mapping of distribution of ACR-DNA adducts within the p53 gene of ACR-treated lung cells indicated an ACR-DNA binding pattern similar to the p53 mutational pattern observed in human lung cancers, suggestive of a potential role for ACR in smoking-induced cancer (Feng et al., 2006). Similarly, ACR and other α,β-unsaturated carbonyl compounds can also directly

react with many proteins by Michael addition to highly nucleophilic cysteine residues. However, these residues subject to redox regulation by e.g. S-nitrosylation or S-glutathionylation, being present as a more reactive thiolate ($R\text{-}S^-$) because of their low pK_a (Forman et al., 2004), and these modifications will be further referred to as S-alkylation (Fig. 3.1). Given the presence of directed S-alkylation, the presence of ACR and other compounds capable of s-alkylation is likely to alter those processes and thereby affect protein function and cellular signaling. For the purpose of this chapter, we will limit our discussion largely to effects of α,β-unsaturated carbonyl compounds on protein function through S-alkylation.

Although the cellular effects of ACR (and other α,β-unsaturated aldehydes) may involve direct modifications with critical target proteins, a strong body of evidence also suggests that exposure of cells to non-toxic doses of ACR, HNE, or CyPGs, results in increased cellular production of reactive oxygen species (Hristova et al., 2007; Jaimes et al., 2004; Kondo et al., 2001; Luo et al., 2005; Nardini et al., 2002; Nencioni et al., 2003; Uchida et al., 1999). Therefore, the cellular effects of these electrophiles could also be an indirect result of redox-dependent changes related to protein oxidation or S-thiolation. Although the cellular origin of oxidant production in response to these electrophiles has not been definitively established, most evidence points to increased mitochondrial oxidants production (Kondo et al., 2001; Luo et al., 2005; Nencioni et al., 2003). ACR-induced oxidant production may be due to ACR itself, or to actions of its adduct with GSH, glutathionylpropionaldehyde (Adams and Klaidman, 1993). Alternatively, ACR-induced oxidant production by endothelial cells has been linked to activation of NADPH oxidase (Jaimes et al., 2004), which is paradoxical in light of several studies indicating that ACR can also inhibit NADPH oxidase activity, most likely due to S-alkylation of critical components of this enzyme system (Nguyen et al., 2001; Witz et al., 1987). As illustrated in Fig. 3.4, the overall effects of ACR and other electrophiles on cellular function and/or gene expression are a combined result of direct actions on target proteins and indirect effects due to GSH depletion and oxidant production.

Proteomic Strategies to Detect Protein S-alkylation

To characterize protein modifications by α,β-unsaturated aldehydes, we and several other research groups have designed proteomic screening methods to identify cellular targets for such modifications. Using this approach, we have identified a range of carbonylated proteins in ACR-treated Jurkat T cells, including various stress-related proteins, cytoskeletal proteins, and a number of proteins involved in redox regulation (Table 3.1). Of course, it is important to recognize that global analysis of protein carbonyls is not specific for protein reactions by ACR, as it may include protein modifications by other oxidative mechanisms (Levine et al., 1994). Also, ACR-modified proteins in which the protein-bound aldehydes have engaged in secondary reactions with other biological targets or neighboring amino acids (e.g. by Schiff base formation) (Esterbauer et al., 1991; Furuhata et al., 2002), may not

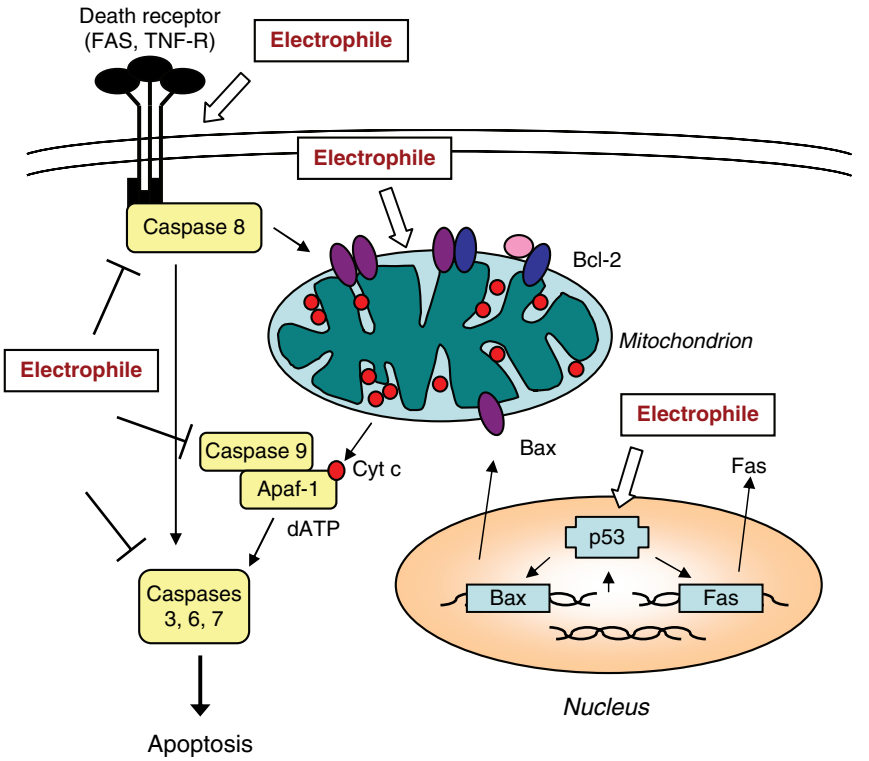


Fig. 3.4 Pro- and anti-apoptotic actions of electrophiles. Apoptosis can be induced by activation of death receptors (e.g. Fas, TNF-R) or by intrinsic mechanisms resulting in loss of mitochondrial membrane potential, activation of Bax, and release of cytochrome c. Both mechanisms result in the activation of caspases with ultimately mediate the apoptotic program. As indicated by white arrows, electrophilic compounds (α,β-unsaturated carbonyls) can initiate apoptotic pathways by promoting loss of mitochondrial membrane potential, activation of death receptors, or activation of the pro-apoptotic transcription factor p53, which upregulates pro-apoptotic genes such as Bax and Fas. Conversely, electrophiles can block apoptosis by direct or indirect inhibition of caspases, by S-alkylation or oxidation of their cysteine residues

be detected. However, as will be illustrated by several examples in the next sections, this general approach has been useful in identifying important cellular target proteins for e.g. ACR.

Controlling Cell Death: Apoptosis Versus Necrosis

Developed and healthy tissues and organs are maintained by a balance between apoptotic cell death, to remove aged or injured cells, and cell proliferation, which serves to replace damaged cells and restore tissue integrity and function. Disruption

Table 3.1 Acrolein inhibits neutrophil apoptosis and their phagocytic uptake

Acrolein (uM)	Macrophages (#PMNs/field)	A549 cells (#PMNs/field)
0	8 ± 2	18 ± 9
10	4 ± 1	11 ± 4
30	1 ± 1	–

Freshly isolated polymorphonuclear neutrophils from human volunteers (Finkelstein et al. 2005) were incubated in the absence or presence of acrolein for 20 h, and 300,000 cells were added to cultured monocyte-derived macrophages (10^4) or alveolar epithelial A549 cells (10^5) in 24 well plates for 1 h. After removal of unattached cells, phagocytosed neutrophils were visualized by peroxidase staining (Savill et al. 1989) and counted (mean ± S.E; n = 3).

of this cellular balance by excessive or dysregulated proliferation or by increased (apoptotic) cell death are therefore believed to result in organ dysfunction and contribute to development of human disease. Many studies have indicated that ACR can inhibit cell proliferation of e.g. epithelial cell or T and B cells lymphocytes, which are critical for repair processes upon epithelial injury or innate or adaptive immune responses (Biswal et al., 2002; Horton et al., 1999; Poirier et al., 2002; Wang et al., 2001). Moreover, while toxic doses of ACR and other α,β-unsaturated aldehydes can induce necrotic cell death, lower doses of ACR and other electrophiles induce a more delayed mode of cell death with many features of apoptosis, (Finkelstein et al., 2005; Li et al., 1997; Nardini et al., 2002; Tanel and Averill-Bates, 2005), HNE (Bruckner et al., 2003; Finkelstein et al., 2005; Ji et al., 2001a; Liu et al., 2000), as well as CyPGs such as 15d-PGJ_2 (Landar et al., 2006; Nencioni et al., 2003). The precise mechanisms by which these electrophiles activate apoptotic signaling are not completely understood, but may include activation of various mitogen-activated protein kinases (MAPK) (Finkelstein et al., 2001; Ranganna et al., 2002; Takeuchi et al., 2001), and more direct activation of death receptor pathways or the pro-apoptotic factor p53 (Tanel and Averill-Bates, 2007a, b), as illustrated in Fig. 3.5.

Although ACR, HNE, and other α,β-unsaturated electrophiles are clearly capable of inducing the apoptotic program, a number of studies have also indicated that some of these α,β-unsaturated aldehydes (specifically ACR and HNE) can also suppress some cardinal apoptotic features, such as activation of executioner caspases, characteristic DNA fragmentation, and expression of typical apoptotic surface markers, such as phosphatidylserine (Finkelstein et al., 2001; Kern and Kehrer, 2002). While the aldehydes can deplete cellular ATP, which is essential for proper execution of the apoptotic program (Finkelstein et al., 2005; Lee and Shacter, 2000), our recent studies suggest that these inhibitory effects on caspase activation are primarily due to cellular redox changes, since they are closely associated with changes in cellular GSH (Finkelstein et al., 2005; Hristova et al., 2007). Indeed, caspases are redox-sensitive proteases that contain an essential cysteine residue that is subject to negative regulation by S-nitrosylation or oxidation (Fadeel et al., 1998; Mannick et al., 1999; Mohr et al., 1997). Similarly, caspases are believed to present direct targets for S-alkylation by α,β-unsaturated aldehydes or other electrophiles (Davis et al., 1997; Nobel et al., 1997). Accordingly, inhibition of Fas-stimulated apoptosis in SKW6.4 cells or Jurkat T cells by non-toxic concentrations of ACR

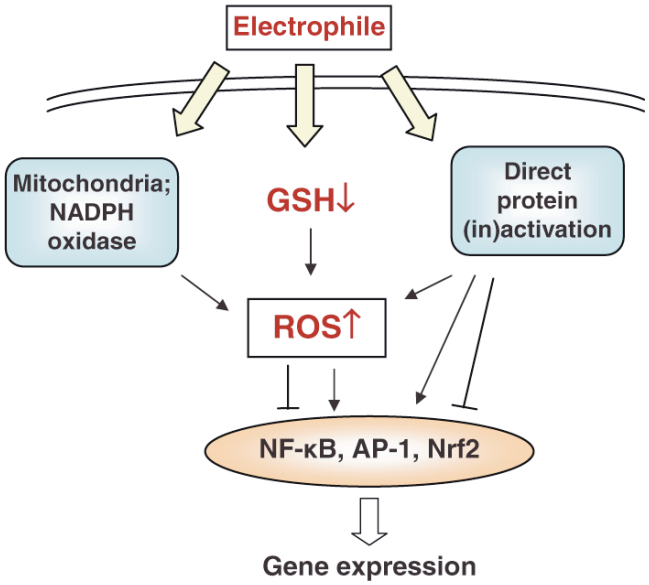


Fig. 3.5 General mechanisms by which electrophiles can impact on cell signaling and gene expression. Electrophiles can deplete GSH and activate oxidant production, by actions on mitochondria or NADPH oxidase. Alternatively, electrophiles can directly target susceptible proteins by S-alkylation. These various actions can have both stimulatory and inhibitory effects on the activation of various redox-sensitive transcription factors, such as NF-κB, AP-1 and Nrf2, and mediate gene expression

were found to be related to direct S-alkylation of the initiator caspase-8 as well as the executioner caspase-3 (Hristova et al., 2007). The overall effects of ACR, or related α,β-unsaturated carbonyl compounds, on cell apoptosis may therefore include both pro-apoptotic signaling, that may be associated with oxidative stress, as well as inhibitory effects, including direct S-alkylation of caspases (Fig. 3.5). The balance between these opposing effects is likely determined by the relative bioactive dose of these electrophiles, their relative reactivity with cellular GSH or other critical target and the status of the redox systems in cells.

The Balancing Effects of Electrophiles on Inflammatory Responses

Pro- and Anti-inflammatory Properties of α,β-Unsaturated Carbonyls

As alluded to in the previous section, ACR may possess pro-inflammatory properties, and may thereby be a major factor involved in chronic airway diseases related to cigarette smoking, such as COPD and asthma (Borchers et al., 1999;

Chalmers et al., 2001; Marwick et al., 2004; Mio et al., 1997; Saetta, 1999). However, studies showing that ACR can directly promote inflammation (Borchers et al., 1999) are countered by a number of studies indicating that ACR also possesses anti-inflammatory properties and reduces airway host defenses against bacterial or viral infection (Jakab, 1977; Li and Holian, 1998). This impairment of innate host defense may result from the fact that non-toxic concentrations of ACR can inhibit the phagocytic properties of alveolar macrophages (Kirkham et al., 2004; Li and Holian, 1998) and suppress the production of pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-12, by alveolar macrophages (Li et al., 1997) or by T lymphocytes (Lambert et al., 2005). These effects of ACR are reflected in epidemiological data showing a strong association between cigarette smoking and the frequency and severity of respiratory tract infections (Haynes et al., 1966; Parnell et al., 1966). It is long recognized that effects of cigarette smoke (CS) on macrophage function or cell toxicity are due largely to volatile thiol-reactive components (Green, 1968; Leuchtenberger et al., 1974), of which ACR is the most reactive and abundant (Esterbauer et al., 1991; Fujioka and Shibamoto, 2006). Similarly, reductions in T helper 1 (Th1) cells within the airways of smokers (Hagiwara et al., 2001), and reduced Th1 cytokine production associated with increased bacterial or viral infection in mice exposed to CS (Drannik et al., 2004; Phaybouth et al., 2006), are consistent with the anti-inflammatory effects of CS, and suppression of innate (Th1) immune responses, in which ACR may play a major role. Similar anti-inflammatory properties are also recognized for other α,β-unsaturated electrophiles such as CyPGs (Straus and Glass, 2001) and α,β-unsaturated carbonyl compounds in food, such as curcumin (Jagetia and Aggarwal, 2007).

Regulation of Th1/Th2 Immune Responses by Electrophiles

Immune responses to inhaled microbes and allergens are initiated by their interaction with various recognition systems and receptors on phagocytic cells, alveolar macrophages, dendritic monocytes, and airway epithelial cells. Adaptive immunity also involves secondary immune responses to inhaled allergens, mediated by presentation of antigen to memory T cells by local antigen presenting cells (APCs), which primarily include alveolar macrophages and dendritic cells (DCs) (Hammad and Lambrecht, 2006; Pynaert et al., 2003; Tang et al., 2001; van Rijt and Lambrecht, 2005). These various immune responses are controlled by production of distinct sets of inflammatory cytokines as IL-4 and IL-10 can negatively regulate Th1 responses and, conversely, IL-12 and IFN-γ are known to suppress Th2 responses (Fig. 3.6). Therefore, the suppressive effects of ACR or inhaled CS on macrophage or DC production of IL-12 and/or IFN-γ (Li and Holian, 1998; Phaybouth et al., 2006;

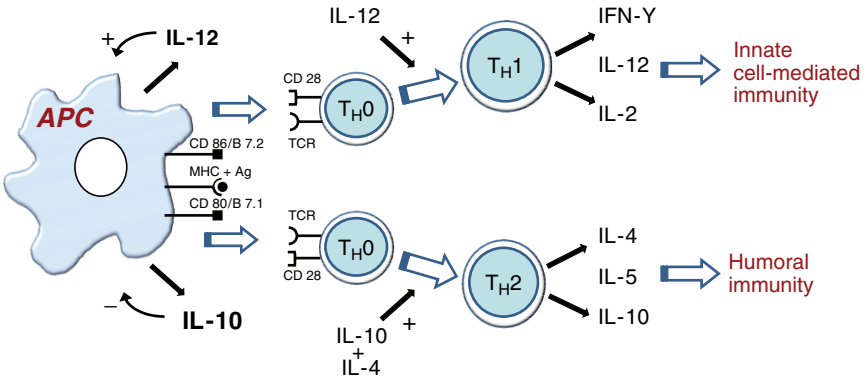


Fig. 3.6 Schematic illustration of regulation of T_H1 and T_H2 immune responses by antigen-presenting cells (APC). Activation of Th0 cells by APCs, through interactions with membrane receptors and T cell receptor (TRC), enhance innate immunity or adaptive (humoral) immunity by inducing either T_H1 or T_H2 responses, which is controlled by the production of specific sets of cytokines. Positive or negative effects of various cytokines on T_H1 or T_H2 responses are indicated

Vassallo et al., 2005) may have important consequences for Th1/Th2 balance and can contribute to development of exaggerated Th2 responses. In support of this notion, several studies using animal models of allergic asthma have shown that exposure to CS can enhance allergic responses in sensitized animals, associated with polarized Th2 immune responses, characterized by enhanced production of Th2 cytokines such as IL-4 and IL-5 and marked suppression of IFN-γ (Moerloose et al., 2005; Rumold et al., 2001; Seymour et al., 1997, 2003). These observations are consistent with clinical and epidemiological data that strongly link direct as well as maternal CS exposure with atopic sensitization (Ronchetti et al., 1990), increased severity of asthma symptoms, increased frequency of medication use, and increased emergency room visits of asthmatic children (Chalmers et al., 2001; DiFranza et al., 2004; Floreani and Rennard, 1999; Moerloose et al., 2005; Nouri-Shirazi and Guinet, 2006; Rumold et al., 2001; Seymour et al., 1997). At this point, it is unclear to what extent ACR or other electrophiles contribute to these CS-related health effects, but given their ability to alter inflammatory-immune processes by S-alkylation of critical proteins or to indirectly affect redox-dependent processes, they are likely to play an important role. Consistent with this suggestion, treatment of patients with multiple sclerosis (a Th1-type cell-mediated autoimmune disease) with cyclophosphamide, which produces ACR during its metabolism (Li and Holian, 1998), was also associated with suppressed IL-12 production and increased production of the Th2 cytokines IL-4 and IL-5 (Comabella et al., 1998). By analogy, a recent study has also implicated 15d-PGJ_2 in controlling T_H1 responses involved in delayed type hypersensitivity (Trivedi et al., 2006).

Regulation of Transcription Factor Activation: Direct and Indirect Mechanisms

A number of studies have shown that the Th1/Th2 cytokine response patterns by APCs are profoundly influenced by alterations in cellular GSH status (Murata et al., 2002; Park et al., 2005). For example, macrophages that contain high GSH were found to produce relatively high levels of IL-12 and reduced levels of IL-6 and IL-10 upon stimulation, whereas reciprocal responses were observed in macrophages that contain less GSH (Murata et al., 2002). Similarly, exposure of APCs to various electrophiles that deplete GSH were found to induce Th2-polarized responses, with reduced production of IL-12 and IFN-γ and enhanced production of IL-10 (Kato et al., 2006; Peterson et al., 1998). The production of inflammatory cytokines in response to allergens or bacterial products involves the activation of various transcription factors that are subject to redox regulation, including nuclear factor (NF)-κB, activator protein-1 (AP-1), hypoxia-inducible factor (HIF)-1. Therefore, it is plausible that ACR and other α,β-unsaturated carbonyls can affect inflammatory cytokine production by interfering with these redox-sensitive transcription factors (Kehrer and Biswal, 2000; Kim and Surh, 2006; Leonarduzzi et al., 2004). For example, although the production of IL-12, IL-10, and other inflammatory cytokines can be regulated at transcriptional, posttranscriptional, and posttranslational levels, activation of NF-κB appears to be a central and critical event in the production of these cytokines in response to various bacterial stimuli or allergens (Trinchieri, 2003), and inhibitors of this critical pathway typically suppress the production of these cytokines (Liu et al., 2006; Trinchieri, 2003). Other transcriptional regulators of these various cytokines include AP-1, which can be enhanced by stimulation of c-Jun-N-terminal kinase (JNK) and/or p38 (Liu et al., 2006; Zhou et al., 2007), and nuclear factor of activated T cells (NFAT) (Lambert et al., 2007). Intriguingly, while transcriptional activation of the anti-inflammatory cytokine IL-10 is mediated by AP-1 and enhanced by stimulation of JNK and/or p38 (Liu et al., 2006; Zhou et al., 2007), JNK activation appears to negatively regulate IL-12 production (la Sala et al., 2005; Utsugi et al., 2003), suggesting that activation of JNK might serve as a critical switch that controls pro- and anti-inflammatory cytokine production and Th2 polarization.

Because ACR and other unsaturated electrophilic carbonyl compounds have been demonstrated to enhance cellular oxidant production (Hristova et al., 2007; Jaimes et al., 2004; Kondo et al., 2001; Luo et al., 2005; Nardini et al., 2002; Uchida et al., 1999), it is possible that their effects on redox-sensitive signaling pathways are mediated by indirect mechanisms due to cellular production of oxidants. Indeed, redox-dependent changes in macrophage-mediated IL-12 production, in response to e.g. H_2O_2, have been associated with reduced activation of NF-κB and increased activation of JNK signaling (Khan et al., 2006; Utsugi et al., 2003). The precise redox-mediated events that affect these signaling pathways have not fully elucidated, but are likely to involve cysteine modifications (S-glutathionylation, S-nitrosation) within critical target proteins (Giustarini et al., 2004; Hess et al., 2005; Pantano et al., 2006). Moreover, several studies have indicated that biological oxidants deregulate the cellular balance between

NF-κB and JNK pathways, which may be critical in their overall effects on inflammatory processes (such as Th1/Th2 polarization) as well as cell survival and apoptosis (Pantano et al., 2006, 2007; Reynaert et al., 2004; Reynaert et al., 2006). It is plausible that electrophiles such as ACR could similarly disrupt this critical signaling balance, by inducing cellular oxidative stress.

Notwithstanding these indirect redox mechanisms, an increasing number of studies demonstrate that ACR and other relevant electrophiles such as HNE and 15d-PGJ_2 can also impact on these redox-sensitive transcription factors by directly targeting critical protein cysteine residues by S-alkylation (Kehrer and Biswal, 2000; Kim and Surh, 2006; Leonarduzzi et al., 2004). In this regard, inhibition of NF-κB activation by ACR (Horton et al., 1999; Lambert et al., 2007; Li et al., 1999; Valacchi et al., 2005), HNE (Ji et al., 2001b; Leonarduzzi et al., 2004), 15d-PGJ_2 (Cernuda-Morollon et al., 2001; Kim and Surh, 2006; Rossi et al., 2000; Straus and Glass, 2001) or curcumin (Jagetia and Aggarwal, 2007) have in each case been associated with direct S-alkylation of specific cysteine residues within either NF-κB p50 (Cys62), p65 (Cys38), or Cys179 within IκB kinase (IKK) a critical upstream kinase involved in NF-κB activation. Recent analysis of ACR modification of p50 revealed alkylation of two amino acids (Cys61 and Arg307) within the DNA-binding domain, whereas crotonaldehyde, a related α,β-unsaturated aldehyde was found to react only with Cys61 of p50 (Lambert et al., 2007). ACR may also inhibit NF-κB activation by more indirect mechanisms such as upregulation of the inhibitor of NF-κB, IκB (Li et al., 1999). Similarly, these α,β-unsaturated carbonyl compounds can also activate JNK and p38 MAPK by enhancing their phosphorylation, or by more direct effects on these MAPKs (Finkelstein et al., 2001; Pugazhenthi et al., 2006; Ranganna et al., 2002; Takeuchi et al., 2001; Tanel and Averill-Bates, 2007b; Wu et al., 2006). For example, HNE was recently found to activate JNK without inducing its phosphorylation, which was associated with direct alkylation of histidine residues (Parola et al., 1998). A more recent study showed that 15d-PGJ_2 can covalently modify c-Jun at Cys269 within the DNA binding domain, and thereby inhibit the DNA binding activity of AP-1 (Perez-Sala et al., 2003). The significance of these modifications for ACR are not known, but it is clear that α,β-unsaturated carbonyls can have variable effects on JNK and AP-1.

Collectively, ACR and other α,β-unsaturated carbonyls can affect inflammatory processes by targeting several cellular pathways by direct modifications of critical proteins within these pathways. The relative importance of these various actions on different cellular signaling pathways for overall cell function or inflammatory cytokine production is, however, not always clear and may depend on the relative reactivity of the α,β-unsaturated carbonyl in question, it cellular location, and the presence of detoxification mechanisms. As an example, a recent study suggests that ACR may exert relatively specific effects on NF-κB-mediated gene expression, based on findings that ACR did not significantly affect gene regulation by AP-1 or NFAT (Lambert et al., 2007). However, these studies do not exclude potential other actions of ACR, and it is unknown whether the same is also true for other biologically relevant α,β-unsaturated carbonyls. Future proteomic strategies may reveal additional relevant cellular targets or changes in cell signaling pathways, and may shed some further light on this.

Electrophiles and Redox Signaling: Interactions with Cellular Redox Systems

As mentioned in previous sections, the cellular effects of ACR and other α,β-unsaturated aldehydes do not only involve direct modifications with critical target proteins, but may also be an indirect consequence of increased cellular production of reactive oxygen species by these electrophiles, by alterations in mitochondrial membrane potential or by activation of oxidant producing enzymes (Hristova et al., 2007; Jaimes et al., 2004; Kondo et al., 2001; Luo et al., 2005; Nardini et al., 2002; Nencioni et al., 2003; Uchida et al., 1999). In fact, the observation that many of the cellular effects of ACR (or related electrophiles) are closely associated with changes in cellular GSH status have led to suggestions that their effects on signaling pathways may be due to alterations in GSH redox status, which would indirectly affect various redox-regulated pathways that are controlled by S-glutathionylation. For example, recent studies have indicated that S-glutathionylation is a means of controlling various signaling pathways, including NF-κB and JNK (Cross and Templeton, 2006; Reynaert et al., 2006) that can be reversed enzymatically by the action of glutaredoxins (Grx) (Reynaert et al., 2006). Analogous redox regulatory systems that operate in cells include proteins of the thioredoxin (Trx) family, which serve to regulate the cysteine/disulfide status of many proteins, to control protein maturation and folding and/or functional activity. In analogy to GSH reductases, that serve to maintain the cellular GSH/GSSG redox balance, the redox status of Trx is controlled by Trx reductases (TrxR), which contain a terminal selenocysteine (SeCys) residue that is critical for their reductase activity (Arner and Holmgren, 2000). Indeed, the Trx/TrxR system is known to be critically involved in the activation of redox-sensitive transcription factors NF-κB and AP-1, and Trx also functions as a redox-sensitive target that controls the activation of JNK (Arner and Holmgren, 2000; Masutani et al., 2005). It has been suggested that the GSH and Trx redox systems may control distinct cellular redox-dependent pathways, based on observations that the redox status of GSH and Trx are regulated independently (Hansen et al., 2006; Watson et al., 2003).

Because Trx and TrxR contain nucleophilic Cys and/or SeCys residues, it should come as no surprise that these proteins can also present direct targets for α,β-unsaturated carbonyls. Indeed, ACR, HNE, as well as 15d-PGJ_2 and curcumin, have been demonstrated to directly interact with Trx and/or TrxR and inhibit their reducing activities (Fang and Holmgren, 2006; Moos et al., 2003; Park et al., 2005; Yang et al., 2004). In fact, TrxR appears to be exquisitely sensitive to a variety of diverse alkylating agents, due to its highly nucleophilic selenocysteine (SeCys) residue (Fang et al., 2005; Fang and Holmgren, 2006; Moos et al., 2003; Nordberg et al., 1998; Park et al., 2005; Wang et al., 2007; Yang et al., 2004). Indeed, changes in TrxR activity by ACR or related aldehydes can be observed in the absence of significant effects on cellular GSH status or inactivation of other redox-related enzymes (Park et al., 2005), suggesting selective and specific modification of this redox system by these electrophiles. Intriguingly, alkylation of TrxR on SeCys not only inhibits its reductase activity, but was in some cases also found to increase its NADPH oxidase activity (Fang et al., 2005; Nordberg et al., 1998), indicating that

alkylation of TrxR may convert an important antioxidant enzyme into an oxidant-producing enzyme. This could therefore present an important mechanism by which electrophiles can shift the cellular balance in favor of pro-oxidant signaling. Indeed, several recent studies demonstrated that alkylation of TrxR (by e.g. 15d-PGJ_2 or curcumin) result in a gain-of-function of TrxR, and actively stimulated cellular pathways involved in e.g. apoptotic cell death (Anestal and Arner, 2003; Cassidy et al., 2006). Alkylation of SeCys in TrxR by environmentally relevant agents such as ACR would also mimic conditions of Se deficiency, which is known to severely compromise TrxR activity (Berggren et al., 1999) and may contribute to altered inflammatory responses and apoptosis in response to e.g. influenza virus infection (Jaspers et al., 2007). The acute effects of electrophiles on TrxR can, however, be compensated for by upregulation of TrxR synthesis in response to activation of Nrf2 (Park et al., 2005), and the overall importance of inactivation of TrxR for cellular redox signaling may be balanced by acute effects on TrxR by direct modifications and more chronic effects due to upregulation of TrxR and other phase-2 genes.

As illustrated in Fig. 3.7, direct actions of electrophiles on Trx/TrxR could have importance consequences for cellular regulation of survival and inflammation, by increased formation of oxidants or by altering the redox balance of Trx. Moreover,

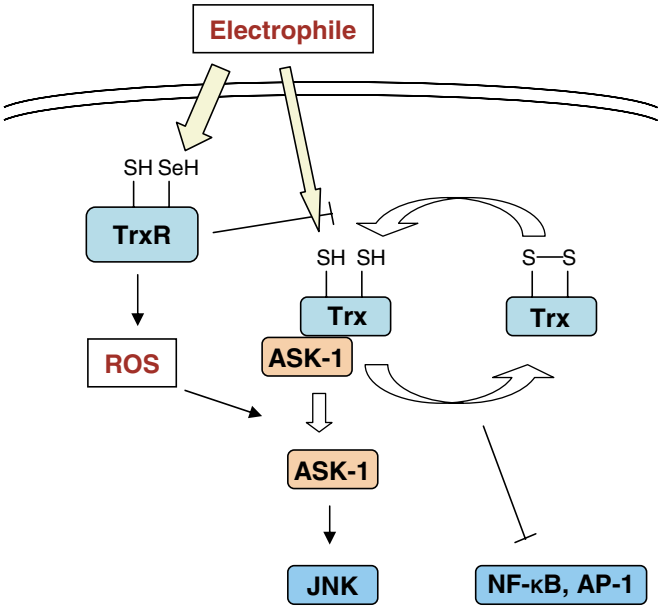


Fig. 3.7 Interactions of electrophiles with the thioredoxin (Trx) system. Electrophiles can induce the alkylation or oxidation of Cys residues on Trx, or inactivate Trx reductase (TrxR) by alkylation of its SeCys residue, which can promote production of ROS. Trx oxidation can result in activation of JNK by initial activation of the apoptosis-regulated kinase (ASK)-1, and inhibit the DNA binding activity of redox-sensitive transcription factors such as NF-κB and AP-1

electrophiles such as HNE have also been demonstrated to directly target other redox proteins, such as members of the peroxiredoxin (Prx) family (Grimsrud et al., 2007). The Prx family include 1-Cys or 2-Cys antioxidant proteins, whose primary function appears to be the metabolism and detoxification of H_2O_2 (Rhee et al., 2005). Indeed, Prx's have recently been implicated as critical regulatory proteins in redox signaling by growth factors and cytokines, and balance redox-dependent regulation of cell proliferation or inflammatory responses (Choi et al., 2005; Yang et al., 2007). Therefore, inactivation of Prx by direct alkylation by ACR or other α,β-unsaturated aldehydes can be expected to further augment oxidative stress responses, by selectively inhibiting this important antioxidant enzyme system.

Defense Systems Against Electrophiles: Implications for Gene-Environment Interactions and Disease Risk

Cellular Defenses Against α,β-Unsaturated Carbonyls

In analogy to the cellular effects of biological oxidants which are controlled by a host of antioxidant and redox regulatory enzymes, the biological properties of biologically relevant electrophiles, including α,β-unsaturated carbonyls, are kept in check by a range of enzymatic systems that serve to metabolize these electrophiles and promote their detoxification and elimination. However, in contrast to various reducing enzyme systems that can reverse protein modifications by S-nitrosylation or S-glutathionylation, protein S-alkylation by most electrophiles is typically thought to be relatively irreversible (Esterbauer et al., 1991), and to date no specific enzyme systems are known to exist that can reverse this modification. Therefore, the biological effects of α,β-unsaturated carbonyl compounds appear to be controlled primarily by the presence of enzyme systems that detoxify and eliminate these compounds to avoid their unwanted interactions with critical cell targets.

As might be expected, mammalian cells are endowed with a broad range of enzymatic systems that serve to detoxify environmental or endogenously produced electrophiles, including biologically important α,β-unsaturated aldehydes. One metabolic route of α,β-unsaturated aldehydes involves either reduction or oxidation of the aldehyde moiety, by aldehyde dehydrogenases (Sladek, 2003), aldo-keto reductases or aldose reductase (Sanli et al., 2003; Srivastava et al., 1999), which reduces their general reactivity and promotes their elimination. However, the most important detoxification mechanism for α,β-unsaturated aldehydes appears to involve their conjugation with GSH, catalyzed by a family of glutathione S-transferases (GST) (Berhane et al., 1994; Choudhary et al., 2005; Petersen and Doorn, 2004; Tjalkens et al., 1998). This conjugation results in saturation of the electron-deficient double bond in these compounds, and avoids their reactivity with other critical cellular nucleophiles, and hence typically reduces toxicity. Moreover, the resulting GSH-alkyl adducts are actively eliminated from cells through multidrug resistance associated proteins (Deeley et al.,

2006), to avoid cytotoxic effects of GSH-aldehyde adducts. The GST enzymes are also critical in the metabolism of other biologically relevant α,β-unsaturated carbonyls, such as CyPGs (Bogaards et al., 1997; Paumi et al., 2004), and can thereby inhibit their biological activity. The GST family consists of five gene classes, that encode soluble GSTs with varying and overlapping specificity for a wide range of endogenous and exogenous electrophilic substrates, and the relative importance for different GST isozymes in the detoxification of specific α,β-unsaturated aldehydes is not precisely known. However, whereas π class GSTs (GSTP1), which are most abundant with the airway epithelium (Strange et al., 2001), are most effective against short chain aldehydes and propenals, such as ACR (Pal et al., 2000), the μ and α classes (GSTM1 and GSTA1 or GSTA4) are more active against hydroxyalkenals and CyPGs (Hubatsch et al., 1998; Pal et al., 2000; Paumi et al., 2004). Furthermore, the different GST isozymes also differ with respect to the formation of stereoselective GSH-conjugates of CyPGs (Bogaards et al., 1997).

In addition to conjugation with GSH, the C = C double bond in α,β-unsaturated aldehydes and ketones can also be metabolized by reduction by alkenal/one oxidoreductase (Aor), an NADPH-dependent reducing enzyme that was initially discovered as a leukotriene B_4–12-hydroxydehydrogenase (Dick et al., 2001; Dick and Kensler, 2004). Overexpression of Aor has been reported to increase the metabolism of α,β-unsaturated carbonyls, such as ACR, 4-hydroxynonena,l and 15d-PGJ_2, and reduce their toxicity or biological effects (Yu et al., 2006). However, the relative importance of Aor, compared to the various GST enzymes, in controlling the biological activities of α,β-unsaturated aldehydes and ketones is not known.

GST Polymorphisms and Their Relation to Disease

The worldwide prevalence of many respiratory diseases is continually increasing, especially in industrialized countries, which is most easily explained by rapidly increasing indoor and outdoor pollution over the past several decades, associated with increased urbanization and a westernized lifestyle. Indeed, ACR is a well-recognized major indoor and outdoor pollutant of considerable health concern, and may be among the main factors contributing to CS-related diseases, including cardiovascular disease, chronic obstructive pulmonary disease (COPD), asthma, and lung cancer (Feng et al., 2006; Leikauf, 2002). However, in spite of these general environmental challenges, not all members of the population are similarly afflicted by these diseases, strongly indicating that genetic factors contribute importantly to disease susceptibility. In this regard, a number of gene polymorphisms have been identified within various GST isozymes that affect their overall activity or substrate specificity, and many studies have attempted to associate these polymorphisms with increased incidence of environmentally relevant diseases, such as lung cancer, allergic asthma, or COPD. Indeed, a number of these studies have demonstrated an association with primarily GSTP1 (the major isoform within the airway), with increased development of atopy or asthma risk (Lee et al., 2004; Mapp et al., 2002; Spiteri et al., 2000; Tamer et al., 2004). Similarly,

several GST polymorphisms have been linked with age-related decline in lung function and COPD (Cheng et al., 2004; Imboden et al., 2007), or with the formation of mutagenic DNA adducts and cancer incidence (Alexandrov et al., 2002; Jourenkova-Mironova et al., 1999; Strange et al., 2001). Although the exact role of these various GSTs and their relevant substrates that may mediate development of these various diseases are not known, many lines of evidence discussed in this chapter suggest that ACR and other α,β-unsaturated aldehydes and ketones may be strong candidates in this regard. Continued studies toward identification of critical modifications in proteins and/or DNA by these electrophiles, and their relationship with disease pathology, will provide more insights into their potential contribution to these various pathologies. Moreover, the abundant presence of a large family of GSTs within cells might suggest that these enzyme systems exist not only to combat environmental xenobiotic electrophiles, but may also be involved in regulation the biological actions of endogenously produced electrophiles. Although these may be produced largely as reactive byproducts associated with cellular (oxidative) metabolism, a growing body of evidence suggest that they may also function as second messengers and perform critical cell functions through S-alkylation of critical target proteins.

Concluding Remarks

The general concept that the biological effects of environmental or biological oxidants are a balance between specific actions on physiological signaling pathways and more harmful toxic responses associated with oxidative stress, clearly also applies to environmental or biological electrophiles, such as α,β-unsaturated aldehydes and ketones. In fact, similar to oxidants, these electrophiles appear to evoke various biological responses by combination of acute effects on critical cell pathways and adaptive responses that serve to mitigate their toxic effects. This balance is determined by the bioactive concentration/dose of these electrophiles, but also by their relative reactivity and specificity by which they may interact with selected cell targets. Analogous to regulation of proteins by S-nitrosylation or S-glutathionylation, electrophiles appear to regulate protein function largely by S-alkylation, and recent proteomic studies over the past several years have identified a growing number of cellular proteins that may be regulated in this fashion.

Much of our knowledge regarding the biological effects of α,β-unsaturated aldehydes and ketones is still based on studies in isolated cell systems, and the significance for overall biology is often unclear. One difficulty in evaluating these *in vitro* studies is that we do not know the relevant bioactive concentrations of these electrophiles. Reported measurements of several α,β-unsaturated aldehydes and ketones in e.g. airway secretions suggest their presence at up to μM levels, but it is unclear how this relates to actual bioactive concentrations within cells. Nevertheless, immunological data using antibodies against protein adducts of ACR or HNE leave no doubt that these aldehydes are capable of modifying proteins under physiological or pathological conditions, and potentially induce functional changes. Curiously,

available antibodies against ACR- or HNE-protein adducts primarily recognize stable adducts with Lys or His residues, even though reactions of these aldehydes with Cys residues might be preferred due to their stronger nucleophilic character. Perhaps this would suggest that Cys-alkyl adducts in biological systems are relatively less stable, and may be degraded by as yet undefined mechanisms. This notion would be consistent with a presumed role of S-alkylation as a reversible signal transduction mechanism, akin to protein phosphorylation or S-thiolation. Future studies that take advantage of new technical approaches to detect S-alkylation within specific proteins are expected to further establish the functional significance of this protein modification, and will increase our appreciation of electrophiles not only as reactive byproducts of cellular metabolism or exogenous toxicants, but also as important biological mediators of inflammatory processes and regulation cell survival and death. Similarly, these advances will also shed additional and potential new light on the importance of GSTs or other enzymes that regulate α,β-unsaturated aldehydes and ketones within cellular systems.

Acknowledgement The authors are grateful to NIH for research support, and to Erik Finkelstein, Jurjen Ruben, Wendy C. Koot, Sjanneke Heuvelmans, and Ziryan Othman for their technical contributions to some of the studies discussed in this review.

List of Abbreviations

ACR	acrolein
Aor	alkenal/one oxidoreductase
AP-1	activator protein-1
APC	antigen-presenting cell
ARE	antioxidant responsive element
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
CyPG	cyclopentenone prostaglandin
DC	dendritic cell
DNPH	dinitrophenylhydrazide
EpRE	electrophile responsive element
GCL	γ-glutamylcysteine ligase
GST	glutathione S-transferase
HIF	hypoxia inducible factor
HO-1	heme oxygenase-1
IFN	interferon
IL	interleukin
JNK	c-Jun-N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
NF-kB	nuclear factor kappa B
NFAT	nuclear factor of activated T cells
Nrf2	nuclear factor-erythriod-2-related factor
Prx	peroxiredoxin
Trx	thioredoxin
TrxR	thioredoxin reductase

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Chapter 4
The Role of Reactive Nitrogen Species (RNS) in the Activation of Nuclear Factor Kappa B (NFkB) and Its Implications for Biological Systems: The Question of Balance

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Abstract The members of redox-sensitive transcription factor nuclear kappa enhancer binding protein (NFkB) contain conserved REL-homology domain (RHD) responsible for DNA binding, dimerization, nuclear translocation as well as interaction with IkB inhibitory proteins. These inhibitory IkBs bind to NfkB and block its nuclear import and transcriptional activity. NFkB pathway plays a central role in the regulation of diverse cellular processes by regulating expression of target genes involved in inflammation, immunity and cell survival. The IKKβ and IKKγ subunits of the IkB kinase (IKK) signalosome are required for the rapid NFkB canonical activation that leads to the IkB phosphorylation and its subsequent release from NFkB heterodimmers RelA/p50 which is then translocated into the nucleus. In contrast, a subset of TNF family members function as biphasic activators of NFkB by activating canonical NFkB pathway as well as noncanoncial route of activation involving protein kinase NIK, phosphorylation of IKKB and the activation of RelB/p52 heterodimers. Nitrogen oxide (NO) and peroxynitrite (ONOO) are highly active reactive nitrogen species (RNS); at low concentrations (5–20 μM), NO has been found to induce rapidly NFkB in the canonical activation pathway in L8 rat myoblasts. At high concentration (above 100 μM), NO has been shown to have inhibitory effect on NFkB activation. The role of peroxynitrite remains contentious: Authentic peroxynitrite and its donors (5–100 μM) are reported to activate NFkB in a noncanonical pathway in skeletal myocytes, for example by tyrosine nitration of IkBα on the expense of its serine phosphorylation and lack of degradation. This finally results in a prolonged nontransient activation of NFkB. Other works observed peroxynitrite induced inhibition of IKKα and concomitant phosphorylation of NFkB-inducing kinase (NIK) and IKKα in cardiac and endothelial cell lines, implying peroxynitrite may induce another noncanonical NFkB activation pathway via IKKα. Thus, this chapter summarizes results of studies concerning the role of RNS and RNS-generating compounds in NFkB signaling. The use of the different experimental models and cell types and the effects of

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

exposure time, RNS concentrations and availability (extracellular vs. intracellular) as well as the significance of the redox state of the cells in the process of NFkB activation, are discussed and the implications for biological systems are also presented.

Keywords NFkB, nitric oxide, peroxynitrite, nitrosylation, nitration, oxidation, disease

Introduction

Reactive Oxygen and Nitrogen Species: General Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are unstable and short-lived chemical entities generated by all aerobic cells. They can be generated in response to specific stimuli as propagators of cell signaling (Suzuki et al., 2000; Droge, 2002), or as by-products of diverse metabolic reactions (Fridovich, 1978; Padgett and Whorton, 1998; Uchida, 2000). The primary cellular sources of ROS and RNS are the mitochondria where oxidative phosphorylation occurs. They can also be generated in the endoplasmic reticulum and nuclear membranes because these organelles contain electron transport chains capable of generating the superoxide radical. ROS and RNS are also generated through the activities of various enzymes that include cytochrome P450 mono-oxygenase, xanthine oxidase and nitric oxide synthases (NOSs), lipoxy genases, cyclo-oxygenase and NADPH oxidase (Halliwell, 1991; Abe and Berk, 1998).

The biological activities of ROS and RNS can be classified as being either regulatory, cytoprotective or cytotoxic. Focussing on regulatory or physiological role of ROS and RNS, substantial evidence exists supporting their role in signal transduction pathways (Palmer and Paulson, 1997; Sauer et al., 2001; Brookes and Darley-Usmar, 2002; Brookes et al., 2002). More specifically, they can activate molecules in specific signaling pathways associated with kinase-linked receptors and kinase cascade phosphorylation mechanisms such as the growth factor (Ras/Raf/mitogen-activated protein (MAP) kinases) or cytokine (Jak/Stat) pathways. ROS and RNS may also function as more direct signaling messengers to: (1) induce gene expression of redox sensitive transcription factors, such as activating protein-1 (AP-1) and nuclear factor-kB (NFkB) (Pinkus et al., 1996), (2) stimulate or down regulate apoptosis (Clement and Pervaiz, 1999), (3) activate cell aging inducers (Lundberg et al., 2000) and (4) modulate cell proliferation (Burdon, 1995).

Reactive Nitrogen Species, Their Chemistry and Availability

The NOS enzymes catalyze the 5 electron oxidation of one N^{ω}-atom of the guanidino L-arginine to produce NO and L-citrulline through cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4)

(Ignarro et al., 2001). The physiological actions of NO include the regulation of vascular tone and blood pressure, prevention of platelet aggregation and inhibition of vascular smooth muscle proliferation. Many of these actions are a result of the activation by NO of the soluble guanylate cyclase and consequent generation of cyclic guanosine monophosphate (cGMP). To act as a messenger nitrogen monoxide needs to bind to guanylate cyclase, which it does at nanomolar concentrations. In contrast, macrophages produce local concentrations of nitrogen monoxide that are two or three orders of magnitude higher than its nM concentrations (Koppenol, 1998). The biological lifetime of nitrogen monoxide is close to 5 s and during this time it can diffuse over several cell diameters, and thereby carry out its function as an intra as well as extra cellular messenger (Lancaster, 1994).

When produced in the presence of appropriate reactive targets, NO can be readily converted into other nitrogen oxide moieties. For example, one of the reactive targets of NO is the cytochrome c oxidase, the terminal enzyme in the electron transport chain, which is inhibited by NO in a manner that is reversible and competitive with oxygen. The consequent reduction of cytochrome c oxidase leads to the release of superoxide anion which under certain circumstances may react with NO to form the powerful oxidant species, peroxynitrite (ONOO) (Moncada and Higgs, 2006).

Superoxide dismutase (SOD) enzyme competes with nitrogen monoxide for superoxide radical. Under normal conditions when physiological nitrogen monoxide concentrations are in the nanomolar range, the amount of the SOD enzyme is sufficient to channel all superoxide towards the dismutation products, dioxygen and hydrogen peroxide. However, in the vicinity of the exposed immunocompetent cells, such as macrophages, the situation is different; local nitrogen monoxide concentrations are in the micromolar range and the rate of peroxonitrate formation is twice the rate of superoxide disappearance through superoxide dismutase (Fielden et al., 1974).

The concept of "reactive exposure" was introduced by Joseph Beckman with his consideration of the formation of peroxynitrite from the reaction of NO with superoxide. According to the proposed principle (Beckman and Koppenol, 1996), the relative amount of a compound (e.g., NO) that reacts with its respective targets is determined by the relative concentrations of those targets and the reaction rates of the compound with each of them. For example, the reaction of NO with superoxide is high (~10^{10} μM/s), but the physiologic concentration is low (~ 0.1–1 nM) (Beckman and Koppenol, 1996). However, because the reaction rate for NO with superoxide is high, even a small increase in the concentration of this reactant will result in a large increase in reactive exposure; a 1-nM increase would lead to a 10-fold increase in reactive exposure (Gow, 2006). Given that the rate constant for reaction of NO with superoxide is higher than that for reaction of superoxide with any of the three SOD isoforms, peroxynitrite will be formed in any cell or tissue where both radicals exist simultaneously (Crow, 2000).

RNS Generation: Exogenous Versus Endogenous Sources of RNS

Endogenous nitric oxide (NO) is synthesized from the L-arginine by a family of NO synthase (NOS) isoenzymes [endothelial NOS (eNOS), neuronal NOS (nNOS)

and inducible NOS (iNOS)] (Ignarro et al., 1987; Nathan, 1992). The NOS isoforms are denoted by descriptive terms, based on the requirement of intracellular calcium transients for full activity. Constitutive NOS enzymes, such as eNOS and nNOS, are activated by a transitory increase generally in cytosolic calcium, which promotes the release of NO over several minutes. A cytokine-inducible NOS isoform is expressed in many cells including macrophages and hepatocytes after the stimulation of immunological or inflammatory reactions. This produces large amounts of NO for several days (Moncada et al., 1991; Kim, 1995). NOS inhibitors, such as N-monomethyl-L-arginine (LNMMA), are commonly used to inhibit NO synthesis, thus allowing the assessment of the input of the effects of NO on the overall response (Palmer et al., 1988).

To study the effects of RNS on the cells, NO-releasing compounds (NO donors) and peroxynitrite donors are valuable tools (Noack and Murphy, 1991). They preserve NO in their molecular structure and exhibit biological activity after decomposition. These chemicals display considerable variation in their structure, stability, and biological activity. Different bioavailability arises from the differences in bioactivation and enzymatic versus nonenzymatic NO release. Examples are organic nitrates, 3-morpholinosydnonimine (SIN-1), sodium nitroprusside (SNP), S-nitrosothiols (e.g. S-nitrosoglutathione) (GSNO), S-nitroso-N-acetylpenicillamine-amine (SNAP), and S-nitrocysteine (CysNO), as well as compounds that contain the N(O)NO-functional group, such as the diethylamine-nitric oxide compound (DEA-NO) and spermine-NO.

Peroxynitrite donor 3-morpholinosydnonimine (SIN-1) is a chemical that decomposes at physiological pH, producing both superoxide and NO, which react immediately to form peroxynitrite (Malan et al., 2003). SIN-1 decomposes slowly over a period of 1–2 h, resulting in the prolonged exposure of the cells to the relatively stable levels of peroxynitrite. In contrast, the half-life of the authentic peroxynitrite under physiological conditions is about 1 s (Radi et al., 2001) and, for that reason, bolus addition of peroxynitrite to the cells results in short-term exposure whereas the addition of SIN-1 most probably mimics the production of peroxynitrite under physiological and pathological conditions (Bar-Shai and Reznick, 2006a).

Macrophages are involved in a number of complex functions in health and disease and their activation is among the first actions to occur in innate immunity to encounter potential pathogens and destroy them primarily by production of ROS and RNS via induction of the NADPH oxidase system and iNOS, respectively (MacMicking et al., 1997).

During stimulation with a wide variety of agents, macrophages activate various cell processes including the respiratory burst, in which an increase in oxygen uptake results in the production of reactive superoxide radicals ($O_2^{\bullet}$) (Leiro et al., 2004). Their production occurs primarily by the activation of a nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase that catalyzes the synthesis of superoxide radicals from oxygen and cellular NADPH, supplied by the hexose monophosphate shunt (Forman and Torres, 2002). The superoxide is also produced through the activity of the enzyme xanthine

oxidase (XO), which catalyzes the oxidative hydroxylation of purine substrates (e.g., xanthine or hypoxanthine) with production of uric acid and generation of $O_2^{\bullet}$ (Cos et al., 1998). The NO molecule is produced in the NADPH-dependent conversion of L-arginine to L-citrulline by the NOS enzymes. Specifically, the NOS2/iNOS isoform is the form of NOS enzymes and it is induced by cytokine and/or microbial stimuli. Superoxide that is produced by the membrane bound NADPH oxidase of macrophages combines with NO to form the peroxynitrite at near the diffusion limit for these two molecules to mediate cytotoxicity by a wide variety of mechanisms (Radi et al., 1991; MacMicking et al., 1997; Hickman-Davis et al., 2001).

In addition to the mediation of cytotoxicity, the RNS serve as important mediators and intracellular signaling molecules (Torres and Forman, 2003; Baran et al., 2004). The average RNS concentration inside randomly distributed target cells increases with increasing macrophage number density and the entry of the extracellular peroxynitrite can rise in a scale relative to its intracellular formation. For example, the surrounding macrophages can double or triple the peroxynitrite concentration in an average target cell. Consequently, it was estimated that a macrophage surrounding a target cell could cause as much as a ten-fold increase in the peroxynitrite concentration inside the target cell (Nalwaya and Deen, 2004). Thus, the RNS production by the macrophages is an important exogenous source of the RNS for the variety of the cells when they face macrophages in their vicinity. The origins of exogenous and endogenous RNS sources are presented in Fig. 4.1.

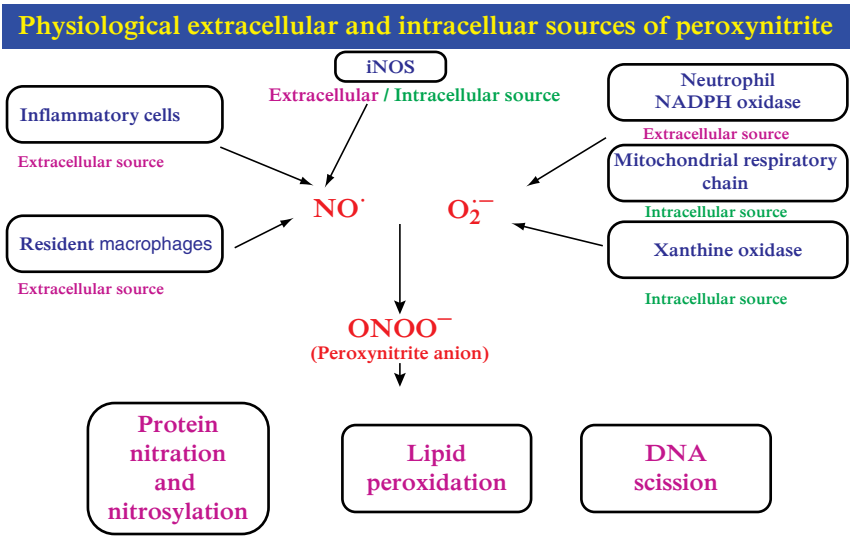

Fig. 4.1 The origins of exogenous and endogenous RNS sources

Cellular Antioxidant Systems

Biological systems are protected from the threat of oxidative attack by a diversity of mechanisms designed to suppress potentially harmful oxidative pathways. An extensive and highly effective range of protective agents and defense antioxidant mechanisms has been developed. The essential function of the cellular antioxidant systems is to lower the steady state intracellular concentrations of ROS and RNS that otherwise might cause excessive damage to cell components. These systems are interrelated and fall into two broad categories – enzymatic and non-enzymatic antioxidant defenses (Freeman and Crapo, 1982). Each tissue, for instance, has an antioxidative potential (AOP), which is determined by those exerting enzymatic and non-enzymatic antioxidants to indicate a need for such protection. These comprise numerous small molecular weight antioxidants to prevent initiation of oxidative damage and/or limit its propagation, enzymes that convert and detoxify ROS/RNS, enzymes to repair oxidative damage when it occurs, and mechanisms to route damaged molecules for destruction and replacement (Sen and Packer, 1996; Hayes and McLellan, 1999).

Pathways mediating the formation of ROS/RNS and their selective dismutation by specific antioxidant enzymes and molecules, indicate that a number of major cellular enzymes that defend against oxidative stress have been conserved through evolution. Superoxide anion is metabolized via the dismutation reaction which is catalyzed by superoxide dismutase (SOD), a cytoplasmic enzyme that is constitutively expressed, and by a mitochondrial enzyme that is induced in response to oxidant stress. The H_2O_2 produced by the dismutation of superoxide is converted by one pathway to H_2O and O_2 by catalase in peroxisomes and by glutathione peroxidase in the cytoplasm, at the expense of reduced glutathione (GSH), leading to the formation of oxidized glutathione disulfide (GSSG) that is recycled back to GSH by glutathione reductase. GSH is derived from glutamate, cysteine and glycine and is the major intracellular non-protein thiol with redox active sulfhydryl moieties with concentrations ranging from 0.5 to 10 millimolar in various organs and cell types (Kosower and Kosower, 1978; Meister and Anderson, 1983). Its principal function is an antioxidant but it also participates and influences other cellular functions (Anderson, 1998; Cotgreave and Gerdes, 1998; Hall, 1999; Halliwell, 1999; Sies, 1999; Filomeni et al., 2002). The unusual γ-peptide bond between glutamate and cysteine prevents its hydrolysis by most peptidases. Another intracellular thiol-containing redox protein with multiple functions possessing active sulfydryl moieties is thioredoxin (TRx) (Tanaka et al., 2000; Powis and Montfort, 2001). TRxs exist in two forms: TRx-1, a cytosolic and nuclear form and TRx-2, a mitochondrial form. Of the two forms, TRx-1 is the more studied type. TRx-1 appears to exert most of its antioxidant properties in cells through TRx peroxidase (TP_x) by removal of H_2O_2 to water and an efficient electron donor to GSH peroxidase (GP_x) (Nishiyama et al., 2001). It performs many biological actions: (1) the supply of reducing equivalents to TRx peroxidases and ribonucleotide reductase; (2) the regulation of transcription factor activity; (3) the regulation of enzyme activity by heterodimer formation; (4) the stimulation of cell growth; and (5) the inhibition of apoptosis (Tanaka et al., 2000; Nishiyama et al., 2001; Powis and Montfort, 2001). The maintenance of adequate yet effective intracellular levels of

GSH and conservation of its cysteinyl moiety, functioning jointly with TRx are crucial for the preservation of cellular reducing environment. These two redox systems are indispensable for neutralizing ROS and RNS and the ensuring of optimal activity of many enzymes and other macromolecules (Droge, 2002).

Oxidative and Nitrosative Stress

The redox state of the cell is the vital balance between the levels of oxidizing and reducing equivalents. Superoxide anion generated by cellular oxidases and NO generated by NOS isoforms, influence the extent of oxidation in this balance. The immediate chemical derivatives of $O_2^{\cdot}$ and NO [(H_2O_2 and peroxynitrite (ONOO)] also contribute to the regulation of this balance. In addition to their harmful actions, these reactive molecules are capable of triggering a multitude of response cascades that influence numerous cellular functions. An upsurge in the generation of ROS and RNS in excess of intrinsic antioxidant buffering capacities results in structural damage to the cell. Oxidative and/or nitrosative stress ensues when pro-oxidative processes overwhelm cellular defense mechanisms (Sies, 1997; Bergendi et al., 1999; Betteridge, 2000). The consequence is cell demise and exacerbation of the disease state (Sevanian and Hochstein, 1985).

Endogenous *S*-nitrosothiols (SNOs) are naturally occurring moieties on proteins in which a sulfur atom from cysteine or homocysteine reacts with nitric oxide (NO) to form an *S*-NO bond. The concept of nitrosative stress has emerged from an understanding that nitrosylation can also reach a hazardous level. In the inflammatory response, many of the same cells that generate reactive oxygen species (ROS) also express NO synthases. Induction of NO synthesis during inflammatory processes represents a defense mechanism, but excessive formation of NO has also been implicated in host tissue injury. In some cases, interactions between nitrosants and oxidants may produce products that are more toxic than either reactant alone. In other instances, nitrosative mechanisms of cellular injury may predominate (Stamler and Hausladen, 1998; Eu et al., 2000). Under such conditions, nitrosylation may directly inhibit critical protein functions (Simon et al., 1996; Eu et al., 2000) and/or promote deleterious oxidative modifications (Stamler and Hausladen, 1998). At the cellular level, nitrosative stress has been linked to inhibition of cell growth and apoptosis, and thus may be widely implicated in NO pathogenesis (Marshall et al., 2000).

Higher concentrations of oxidative species promote the conversion of NO to higher oxide forms, such as nitrogen dioxide and peroxynitrite. One consequence of the production of such species is the formation of nitrotyrosine (Ischiropoulos, 1998). The peroxynitrite is highly reactive molecule that induces many changes in proteins by oxidizing the sulfhydryl groups of cysteine and methionine as well as tryptophan residues and is selectively nitrating tyrosine residues (Pryor and Squadrito, 1995; Radi et al., 2001). The detection of nitrotyrosine (NO_2-Tyr) formation in various inflamed tissues and during the process of aging is recognized as peroxynitrite-triggered mechanism of nitrosative injury (Baian and Murad, 2001; Beal, 2002; Drew and Leeuwenburgh, 2002; Adewuya et al., 2003; Park et al., 2005; Levrand et al., 2005; Bar-Shai and Reznick, 2006a).

RNS and Cell Signaling

NO is a diffusible multifunctional transcellular messenger that has been implicated in numerous physiological and pathological conditions. The biological activities of NO can be divided into cGMP-dependent and cGMP-independent pathways (Schmidt et al., 1993; Schmidt and Walter, 1994) where the activation of guanylate cyclase, formation of cGMP, and concomitant protein phosphorylation are considered the main physiological signaling pathway of NO (Schmidt, 1992). NO is a transducer of the vasodilator message from the endothelium to the vascular smooth muscle. It is also a neurotransmitter in the central and peripheral nervous systems, and participates in non-specific immune responses. Also, NO can affect the cellular functions through post-translational modifications of proteins directly (i.e. nitrosylation and nitration) and indirectly (i.e. methylation and ribosylation). The list of cGMP-independent effects of NO is growing at a rapid rate with the emphasize on the importance and relevance of nitrotyrosine formation (Janssen-Heininger et al., 2002; Bian et al., 2006)

Within mammalian tissues, the concentration of SNOs can vary from nM to μM levels (Kluge et al., 1997; Gaston, 1999), and thiol *S*-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, ranging from regulation of ion channels and G-protein coupled reactions to receptor stimulation and activation of nuclear regulatory proteins.

The altered reduction–oxidation (redox) homeostasis appears to be one of the hallmarks of the processes that regulate gene transcription in physiological and pathophysiological conditions. To accommodate constantly changing microenvironment, cells adjust the pattern of gene expression by adaptive regulation of a host of transcription factors, which bind their respective cognate sites in the regulatory elements of targeted genes. Changes in the pattern of gene expression through ROS/RNS-sensitive regulatory transcription factors are crucial components of the machinery that determines cellular responses to oxidative/redox conditions.

The activities of a variety of nuclear regulatory proteins are affected by proinflammatory signals, reactive nitrogen species, and S-nitrosylation and tyrosine nitration chemistry. One of these RNS-sensitive transcription factors is nuclear factor kappa B (NFkB) (Janssen-Heininger et al., 2000; Reynaert et al., 2004; Bar-Shai and Reznick, 2006a).

NFkb Family: Structural and Functional Characteristics

Although the transcription factor NFkB has been originally recognized in regulating gene expression in B-cell lymphocytes (Sen and Baltimore, 1986), subsequent investigations have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines. NFkB is the functional description for a complex of two variable subunits that come from the Rel/NFkB family. The NFkB/Rel family includes five members: NFkB1 (p50/p105; p50 precursor), NFkB2 (p52/p100; p52 precursor), RelA (p65), RelB (p68) and c-Rel (p75). The members (RelA, RelB, c-Rel,

p52/100 and p50/105) contain Rel homology domain (RDH), a 300 amino acid long sequence that is responsible for the dimer formation, nuclear translocation, sequence-specific consensus DNA recognition and interaction with inhibitory-kB (IkB) proteins, which are the cytosolic inhibitors of NFkB (Ghosh et al., 1998; Chen, 2005).

The best characterized variant of NFkB is the combination of RelA and p50. Inactivated subunits are localized as homo or hetero-dimers in cytoplasm together with IkB, the NFkB inhibitor. The IkB family has four known members (IkBa, IkBb, IkBe and Bcl3). These proteins contain an ankyrin repeat motif, which is important for the maintenance of NFkB in the cytoplasm (Jobin and Sartor, 2000). The carboxyl termini of p105 and p100 also contain IkB-like ankyrin repeats that must be degraded to generate the mature Rel subunits. The translocation and activation of NFkB in response to various stimuli are sequentially organized at the molecular level.

The Activation of NFkB Is Essential for the Cells to Survive Diverse Stresses and Diseases

Activation and regulation of the NFkB/Rel transcription family, via nuclear translocation of cytoplasmic components and complexes, play a central role in the evolution of inflammation through the regulation of genes essentially involved in encoding pro-inflammatory cytokines and other inflammatory mediators. Many genes that are induced or upregulated by proinflammatory stimuli contain specific NFkB binding sites within their promotores (Karin and Delhase, 2000; Gosh and Karin, 2002). Rel/NFkB transcription factors are involved in the control of a vast array of processes including its pivotal role in response to stress-like stimuli, initiation and maintenance of immune and inflammatory responses, developmental processes, and cellular growth and programmed cell death (apoptosis). In addition, NFkB is active in a number of disease states, including cancer and metastasis of cancer, arthritis, inflammation, asthma, neurodegenerative diseases and cardiovascular abnormalities (Valen et al., 2001; Celec, 2004; Chen, 2005; Massa et al., 2005; Park et al., 2005). When a cell receives any of a multitude of extracellular signals (inducers), NFkB rapidly enters the nucleus and activates expression of a variety of target genes responsible for the above biological processes.

Activation of NFkB

Canonical, Noncanonical, and Atypical Pathway(s) of NFkB Activation

In unstimulated cells, NFkB is located in the cytoplasm as an inactive NFkB/IkB complex, a mechanism that blocks the recognition of the nuclear localization signal (NLS) by the nuclear import machinery (Haddad, 2002). NFkB activity is tightly

controlled by the inhibitory protein IkBα, complexed to NFkB dimers in the cytosol, thereby preventing the nuclear localization of NFkB and ensuring low basal transcriptional activity (Janssen-Heininger et al., 2002). The heterodimeric NFkB typically presents as the two subunits, p50 (NFkB1) and p65 (RelA) associated with IkB. Upon stimulation, such as with cytokines and lipopolysaccharide-endotoxin (LPS), derived from the cell wall of Gram-negative bacteria, IkBα, the major cytosolic inhibitor of NFkB, undergoes phosphorylation, ubiquitination and subsequent proteolytic degradation, thereby unmasking the NLS on p65 and permitting nuclear translocation of the complex. The IkB phosphorylation creates recognition signal for ubiqitinating enzymes which mark the IkBs for rapid proteosomal degradation (Karin and Ben-Neriah, 2000). After the release, NFkB is transported to the nucleus where it binds specifically to kB enhancer elements of DNA and promotes gene transcription by binding to responsive elements in the DNA, called kB motifs (Janssen-Heininger et al., 2002). The NFkB is able to bind promoter and enhancer regions containing kB sites with the consensus sequence GGGRNNYYCC (N = any base, R = purine, and Y = pyrimidine) (Hayden and Gosh, 2004). Protein kinase A and p300/CBP belong to transcription coactivators that regulate the binding of NFkB in the nucleus. This is an important meeting point of NFkB and cell cycle regulation, as p300 is a substrate for cyclin dependent kinases (CDK) and it affects the RelA subunit of NFkB (Perkins et al., 1997)

In general, the NFkB activation pathways are classified as either the canonical (classical) or the noncanonical (nonclassical). This classification is based on whether activation involves IkB degradation or p100 processing (Pomerantz and Baltimore, 2002). In the classic canonical pathway, which is the main NFkB signaling pathway, stimulating cells with an agonist such as tumor necrosis factor-α (TNFα) or interleukin-1β (IL-1β) activates the IKK complex, which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (also known as IKKγ). The IKKβ, but not IKKα, phosphorylates IkB proteins at two amino-terminal serine residues (ser 32 and ser 36) *in vivo*. This signal-induced phosphorylation targets IkB for ubiquitination and subsequent degradation by the proteasome (Chen et al., 1995).

The involvement of two kinases, NIK and the catalytic IKKα of the IKK complex, is of vital importance in the activation of the alternative, noncanoncial NFkB pathway. The non-canonical pathway of NFkB activation operates mainly in B cells in response to stimulation of a subset of the TNF-receptor (TNFR) superfamily, including receptors for BAFF, lymphotoxin-β (LTβ) and CD40 ligand. Stimulation of these receptors activates the protein kinase NIK, which in turn activates IKKα. IKKα then phosphorylates p100 at two C-terminal serine residues, leading to the selective degradation of its IkB-like domain by the proteasome (Xiao et al., 2001; Senftleben et al., 2001). The mature p52 subunit and its binding partner, RelB, translocate into the nucleus to regulate gene expression. Thus, a hallmark of the unconventional NFkB pathway is inducible p100 processing, leading to liberation of the mature transcription factor p52 in complex with RelB (Hacker and Kärin, 2006). In addition, one of the manifest characteristics of the noncanonical pathway is the slow kinetics of p100 processing, lasting several hours, which stands in apparent contrast to that of the canonical pathway in which the process of IkBα degradation occurs

within minutes. The lower affinity of IKKα for its substrate, p100, in comparison to the affinity of IKK complex to IkBα, has been proposed as the possible cause of the slow kinetics of p100 processing (Kärin and Hacker, 2006).

As stated beforehand, the IkBα phosphorylation at its two serine residues (ser-32 and ser-36) creates recognition signal for ubiqitinating enzymes which mark the IkBs for subsequent rapid proteolytic degradation leading to the unmasking the NLS on p65 and permitting nuclear translocation of the complex. However, it has been shown that the NFkB activation can also be accomplished in the classic activation pathway through the different processes which do not involve the phosphorylation of the IkB proteins at ser-32 and ser-36 amino-terminal residues by the IKK complex. For the instance, tyrosine 42 phosphorylation (P-Tyr-42) of IkBα has been shown to mediate NFkB activation following hypoxia, hypoxia/reoxygenation or tyrosine phosphatase inhibitor (pervanadate) treatment, a mechanism of activation which stands in contrast to the canonical proinflammatory pathways mediating NFkB activation through serine phosphorylation of IkBα (Koong et al., 1994; Fan et al., 2003). The activation of the phosphatidyl inositol-3-kinase (PI3-kinase) pathway has been implicated in NFkB activation induced by tyrosine-42 phosphorylation of IkBα in a way that both the regulatory and the catalytic subunit of PI3-kinase play a role in NFkB activation by the tyrosine phosphorylation-dependent pathway. (Beraud et al., 1999). In contrast to IKK-mediated serine phosphorylation of IkBα, tyrosine phosphorylation of IkBα is capable of activating NFkB in the absence of ubiquitin-dependent degradation of IkBα (Imbert et al., 1996). In their recently published work, Bar-Shai and Reznick (2006a) have shown that exposure of L8 rat myoblasts to peroxynitrite donors resulted in the induction of NFkB activation which was dependent on tyrosine nitration of IkBα, but independent of its serine phosphorylation and degradation. Moreover, prolonged exposure to peroxynitrite resulted in nontransient NFkB activation. Concerning tyrosine phosphorylation as well as tyrosine nitration processes, it has been suggested that the tyrosine phosphorylation and nitration are not mutually exclusive events of the cell signaling pathways and that tyrosine nitration, which extent depends on the concentration of nitrating species, may promote or inhibit tyrosine phosphorylation (Monteiro, 2002). In that way, the peroxynitrite molecule is considered to be a key mediator of the interplay between tyrosine nitration and tyrosine phosphorylation signaling events because it has been shown by numerous investigating teams that peroxynitrite can stimulate tyrosine phosphorylation in different cell systems at its 10 μM – 250 μM concentrations, while at higher μM as well as 1 mM concentration peroxynitrite can lessen or disable tyrosine phosphorylation mechanism resulting in that the tyrosine nitration may become the dominant cell signaling event (Kong et al., 1996; Mondoro et al., 1997; Mallozzi et al., 1997,. 1999; Li et al., 1998; Brito et al., 1999; Di Stasi et al., 1999). While further concerning the NFkB activation pathways, it should be noted that p50 and p52 family members can form homodimers and undergo nuclear translocation as a result of the ubiquitin-proteasome partial processing of their cytoplasmic precursor molecules, p105 (the *Nfkb1* gene) and p100 (the *Nfkb2* gene), respectively, with the IKKα proteolytic processing of p100 leading to noncanonical pathway activation which involves RelB/52 dimers. However, it has been shown

that, similarly to the canonical pathway of NFkB activation, the noncanonical pathway can also display a different mode of NFkB activation such as the binding of p50 homodimer to the Bcl-3, a nuclear IkB family member, as observed to occur in the inactivated skeletal muscle (Hunter et al., 2002; Kandarian and Jackman, 2006). Thus, it seems that both classical and non classical NFkB activation pathways can employ atypical or alternative modes of NFkB activation.

To summarize, stimulation of TNF receptors (TNFRs), the IL-1 receptor (IL-1R) or Toll-like receptors (TLRs) with their cognate ligands activates TNFR-associated factor (TRAF) proteins and subsequently TGFβ-activated kinase 1 (TAK1), which phosphorylates and activates IKKβ. IKKβ then phosphorylates IkB, resulting in its ubiquitination by the SCF–βTrCP ubiquitin-ligase complex and subsequent degradation by the proteasome. The NFkB dimer, which consists of p50 and Rel-A, can then enter the nucleus to regulate the expression of targets genes that are involved in inflammation, immunity and cell survival. In the non-canonical pathway, a subset of receptors belonging to the TNFR superfamily, such as the B-cell receptor for BAFF (BAFF-R), activates the kinase NIK; NIK then phosphorylates IKKα, which in turn phosphorylates the NFkB precursor p100. p100 is subsequently polyubiquitinated and then processed to the mature subunit p52 by the proteasome. Afterward, p52 and its binding partner Rel-B translocate to the nucleus to turn on genes that are important for the maturation of B cells. In addition, atypical or alternative modes of activation exist in both canonical and noncanonical NFkB pathways.

The Upstream IkB Kinases (IKK) Signalosome and Activation of NFkB Dependent Genes

Signals emanating from membrane receptors, such as those for IL-1 and TNF-α, activate members of the MEKK-related family, including NFkB-inducing kinase (NIK) and MEKK1, both being involved in the activation of IkB kinases, IKKα and IKKβ, components of the IKK signalosome (Sen and Packer, 1996; Didonato et al., 1997; Cho et al., 1998; Janssen-Heininger et al., 1999; Shrivastava and Aggarwal, 1999; Hutter and Greene, 2000; Haddad et al., 2001; Chandel et al., 2001). Both kinases are present in cells as part of a high molecular weight complex that also contains a regulatory subunit termed IKKγ or NEMO (NFkB essential modulator) (Chen et al., 1996;Mercurio et al., 1997; Zandi et al., 1997; Rothwarf et al., 1998; Rothwarf and Karin, 1999). This basic trimolecular complex, which may contain an additional substrate-targeting subunit named ELKS (Ducut Sigala et al., 2004), is referred to as the IKK complex.

The IKKs phosphorylate members of the IkB family, including IkBα, at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the proteasome. The inducible degradation of IkB allows release of the NFkB dimers and their nuclear translocation to bind specific kB moieties and set off target gene transcription that encode cytokines such as interleukin-1 (IL-1), IL-12, IL-2, and interferon-β (IFN-β], membrane proteins such

major histocompatibility complex classes I and II, ICAM-1 (intracellular adhesion molecule 1), and E-selectin, TFs like c-Myc and IRF4 (interferon-regulatory factor), and inhibitors of apoptosis, for example c-FLIP (cellular FLICE-like inhibitory protein) and Bcl-XL (Häcker and Karin, 2006).

The ubiquitin-proteasome pathway plays a crucial role in the process of NFkB activation. Ubiquitination is a reversible covalent modification that is catalysed by the enzymatic steps in which ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. (Pickart, 2004). The activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2 or UBC), forming an E2-Ub thioester. Lastly, in the presence of a ubiquitin-protein ligase (E3), ubiquitin is attached to a target protein through an isopeptide bond between the C terminus of ubiquitin and the ε-amino group of a lysine residue in the target protein (Pickart, 2004).

The discovery that TRAF (TNFR-associated factor) proteins are ubiquitin E3 ligases had established missing connection between the process of ubiquitination and the signaling pathways that activate IKK (Deng et al., 2000). The TRAF proteins have a pivotal role in signalling pathways that are involved in the activation of NFkB by many cell-surface receptors, including the TNFR superfamily, the IL-1 receptor (IL-1R) and Toll-like receptors (TLRs) (Chung et al., 2002). Seven members of this family have been identified in the human genome. With the exception of TRAF1, all TRAF proteins contain an N-terminal RING domain, followed by several zinc-finger domains (Chen, 2005).

Among the TRAF family of proteins, TRAFs 2, 5 and 6 are activators of the canonical NFkB pathway (He et al., 2007).

The following model of IKK activation by TRAF6-mediated polyubiquitination has been proposed: Following ligand binding to IL-1R or TLRs, TRAF6 is engaged to the receptor complexes and forms oligomers. TRAF6 oligomerization activates its ligase activity, leading to Lys 63 polyubiquitination of target proteins including TRAF6 itself. Ubiquitinated TRAF6 recruits adaptor protein TAB2 (the protein kinase TAK1 (TGFβ-activated kinase) binding protein) and activates the TAB2-associated TAK1 kinase, which, in turn, phosphorylates IKKβ at two serine residues in the activation loop, thereby activating IKK. Ubiquitin-activated TAK1 also phosphorylates and activates MKK kinases (such as MKK6), which in turn activate the JNK and p38 kinase pathway (Wang et al., 2001).

Some Inducers of NFkB Engage the Canonical Activation Pathway Rapidly While Distinct Class of NFkB Stimuli Use Both Canonical Pathway and Delayed Noncanonical Route of NFkB Activation

The variety of extracellular activating stimuli induces the proteosomal dependent destruction of IkBs and the release of NFkBs to bind DNA and activate the transcription of the target genes. Stress-like inducers of NFkB (including proinflammatory cytokines

such as TNFα1 and IL-1) operate in a classical monophasic capacity to induce the canonical NFkB activation pathway rapidly, largely involving the activation of p65(RelA)/p50 DNA binding activity and transcriptional capacity (Massa et al., 2005).

With the exceptions of UV radiation and the effects of certain DNA-damaging agents, the release of NFkBs from IkBs is mediated by the signalsome complex of IKKα, IKKβ and NEMO/IKKγ (Karin and Ben-Neriah, 2000; Karin et al., 2004; Bonizzi and Karin, 2004). IKKβ/NEMO is indispensable for the phosphorylation of IkBs on a pair of amino-terminal serines (residues 32 and 36 in IkBα). In contrast, IKKα is not required for the phosphorylation of IkBs via the canonical NFkB activation pathway *in vivo*, with the exception of receptor activator of NFkB (RANK) ligand signaling in mammary epithelial cells (Cao et al., 2001). In addition, the potential of IKK and a number of other kinases (casein kinase II, mitogen and stress activated protein kinases, protein kinase C, etc.) to activate NFkB in an IkB independent manner by direct phosphorylation at various sites findings have been shown. However, the meaning of this different activation is not clear by now (Schmitz et al., 2001).

While IKKβ is most important for rapid degradation of NFkB-bound IkBs (IkBα, IkBβ, and IkBε), IKKα directs processing of p100 which leads to the activation of p52:RelB dimmers. This response is considerably slower than the activation of IkB-bound dimers (Bonizzi and Karin, 2004). A distinct class of NFkB stimuli (exemplified by LTβR, BAFF-R, RANK and CD40 ligand) have been shown to function as biphasic NFkB activators, initially acting via the rapid canonical pathway and subsequently traversing into a delayed noncanonical protein synthesis-dependent route (Bonizzi and Karin, 2004; Yamamoto and Gaynor, 2004). In the cytoplasm of resting cells, the kinase NIK is constitutively bound to TRAF3, which results in NIK ubiquitination and rapid turnover. The engagement of BAFF-R stimuli leads to the recruitment and sequestration of TRAF3, which results in NIK stabilization, increased expression, and to its activation by autophosphorylation. NIK forms a complex with p100 and IKKα and directly phosphorylates IKKα at its activation loop, resulting in its activation and IKKα-dependent phosphorylation of p100. Phosphorylated p100 is recognized by the E3 ubiquitin ligase SCFβTrCP, leading to its polyubiquitination and partial proteolytic degradation of its C-terminal ankyrin-repeat region by the 26*S* proteasome. Free p52:RelB dimers translocate to the nucleus and drive gene transcription. However, the information on the molecular events involved in IKKα-dependent processing of p100 is lacking in comparison to the current understanding of IKKβ-dependent IkB phosphorylation, because the knowledge on the alternative pathway is mostly based on genetic and to lesser extent on biochemical analysis.

Dynamic Shuttling of Signaling Molecules of NFkB Pathway Between Cytoplasm and Nucleus

The classical view of the regulation of NFkB is that it is kept inactive in cytoplasm due to binding to IkB proteins which mask the nuclear localization signal (NLS) of

NFkB, thereby preventing the interaction of NFkB with the nuclear import machinery. The NLS of NFkB is unmasked upon degradation of its inhibitor and the transcription factor can be imported into the nucleus, where it binds to specific promoter elements. Besides a number of other genes, NFkB is also inducing the transcription of DNA encoding its own inhibitor (de Martin et al., 1993). After translation in the cytosol, IkB is imported into the nucleus, where it is assumed to dissociate NFkB from promoter regions. The newly formed NFkB-IkB complex is then transported back to the cytosol by the means of a specific nuclear export sequence (NES) of IkB.

The fact that both NFkB and IkB accumulate in the nucleus after inhibition of nuclear export prompted the researchers to test whether upstream signaling molecules of the NFkB pathway shuttle between cytosol and nucleus as well. In effect, a rapid nuclear distribution of the NFkB inducing kinase NIK has been described by Birbach and co-workers (2002). In addition, IKKα has also been shown to translocate to the nucleus. These observations indicate not only that NFkB and its inhibitor are dynamically shuttling between cytosol and nucleus, but also signaling molecules upstream of NFkB (Birbach et al., 2002). Moreover, it has also been found that IKKα is required to activate the transcription of canonical NFkB target genes. That dependence on IKKα is independent of IkBα destruction and the IKKα mechanism of action in the canonical NFkB pathway has been proposed to be nuclear in nature. In this context, IKKα has been shown to migrate into the nucleus and associate with the promoters of NFkB-dependent genes upon TNFα stimulation (Anest et al., 2003; Yamamoto et al., 2003).

NFkB Complex Is Also Found into Mitochondria

The discovery of the dynamic shuttling of NFkB complexes including signaling molecules upstream of NFkB points to the multifaceted aspects of NFkB regulation. The studies indicating that NFkB shuttles into and out of the nucleus in unstimulated cells (Carlotti et al., 2000; Huang et al., 2000a; Tam et al., 2000) have probably raised an idea of exploring the possibility that the members of NFkB family can also be found in the mitochondria. The rationale for that approach may additionally be supported by the comprehension of the vital role of the mitochondria involvement in the execution of many apoptotic pathways in association with the role of NFkB in regulating apoptosis given that NFkB activates several gene products (Bcl-xL, A1/Bfl-2, IAP proteins, TRAF proteins) to inhibit the caspase cascade and, consequently, to block cytochrome *c* release from mitochondria (Baldwin, 2001a, b). Thus, the localization of NFkB to the mitochondria would raise important questions concerning its potential role in regulating apoptosis. In that way, it has been shown that IkBα and p65 are found in the mitochondria of Jurkat T cells and that mitochondrial IkBα interacts with ANT, the adenine nucleotide transporter, which has been assumed to be involved with apoptosis through its ability to regulate the mitochondrial permeability transition (Bottero et al.,

2001). By using electron microscopy and biochemical approaches, Cogswell and co-workers have discovered that the NFkB subunits p50 and p65 along with IkBα (but not IkBβ) are present in the cell mitochondria as well as in the cytoplasm of the growing cells. The stimulation of U937 cells by TNFα treatment lead to the phosphorylation on N-terminal serines and the degradation of the mitochondrially associated IkBα. In contrast to the cytoplasmic IkBα degradation, they observed that mitochondrial degradation of IkBα emerged to be proteasome-independent since the use of the specific proteasome inhibitor (lactacystin) did not block mitochondrial IkBα degradation (Cogswell et al., 2003). Additionally, it is already known that IkB can be degraded by a nonproteasomal mechanism following stimulation of cells with cytokines (Han et al., 1999). Moreover, the key components of the proteasome and ubiquitin ligase associated with IkBα ubiquitination are not reported to be present in the mitochondria. Thus, it is reasonably to assume that the degradation of mitochondrial IkBα might be executed through the proteasome-independent mechanisms.

In connection with the prospective importance of the location of NFkB family members within mitochondria, Guseva and co-authors (2004) have suggested that NFkB-dependent mechanisms operating at the level of the mitochondria contribute to its role in regulating death receptor signaling. They have found NFkB p65 and p50 subunits were expressing DNA binding activity in the mitochondria of prostatic carcinoma cell lines. Since tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) affected DNA binding activity of mitochondria-associated NFkB without a change in the amount of p65 in mitochondria, the authors have suggested that the activation of mitochondrial NFkB is taking place without additional translocation of the NFkB subunits to the mitochondria (Guseva et al., 2004). Furthermore, the glucocorticoid receptor, which has been found in the mitochondria (Scheller et al., 2000), can physically interact with NFkB subunits (Tao et al., 2001). Accordingly, it has been proposed that NFkB/glucocorticoid receptor interactions may function in the mitochondria to regulate key processes involved in cell growth and apoptosis (Cogswell et al., 2003).

RNS and NFkB – The Question of Balance

Nitrogen Monoxide (NO) and NFkB

It is universally accepted that the effects of NO on NFkB activity are concentration-dependent; low concentrations of NO enhance NFkB transactivation and promote its DNA-binding activity by cytokines while high concentrations of NO have inhibitory effect on NFkB activation. Such concentration dependent effects of NO on the state of activity of NFkB originate from the studies in which different NO donors and their different concentrations were used. To test the role of nitrogen monoxide (NO) on the activation of NFkB, Umansky and co-workers (1998) used

endothelial TC10 cells, which express iNOS, to test the NO-generating compound glycerol trinitrate (GTN) (0, 25, 100, 250 μM range) on the DNA-binding activity of NFkB. The authors have shown that GTN at low concentrations enhanced TNF-α or phorbol myristate acetate (PMA) induced NF-kB DNA-binding activity, which was associated with the increased IKKα activity *in vitro* (Umansky et al., 1998). Moreover, NO doses which could inhibit NFkB were not cytotoxic. Since the iNOS is under the control of NFkB binding sites, the authors have concluded this co-stimulatory activity provides a model for self amplifying inflammatory response in the endothelium. It should also be noted that in the cells which do not express iNOS (such as human T cells), NO can induce NFkB via activation of the monomeric $p21^{ras}$ protein through its s-nitrosylation (Lander et al., 1997). Contrary to the effects of low concentrations of NO on NFkB activity, high but still non-toxic NO concentrations, originating from the addition of high concentrations of NO donors or due to increased iNOS activity, have decreased the potential of cytokines to activate NFkB, creating a negative feedback loop in the inflammatory response signal which would limit the extent of inflammation (Shin et al., 1996; Umansky et al., 1998; Peng et al., 1998). The NO effects on NFkB were also investigated on LPS-induced responses in vascular smooth muscle (VSM) cells (Hattori et al., 2004). Various incubations with NO donors (NOR3 and SNAP, 0.01 – 1 mM) and ONOO donor SIN-1 (0.01 – 1 mM) and pure ONOO (0.01 – 1 mM) were done and the results have revealed that NO suppressed LPS-induced IkBα phosphorylation and IKK activity while peroxynitrite sustained NFkB in VSM cells (Hattori et al., 2004).

Various mechanisms by which NO inhibits NFkB were described such as the stabilization of IkB, induction of IkBα mRNA, and blocking of nuclear translocation of NFkB (Peng et al., 1995; Katsuyama et al., 1998; Okamoto et al., 2002) as well as S-nitorsylation of Cys-62 on the NFkB p50 inhibitory subunit (Matthews et al., 1996; dela Torre et al., 1998; Marshall and Stamler, 2001). The reactions of RNS with protein cysteine thiols that result in S-nitrosylation have received an attention because they represent an important post-translational modification, emerging as the prototypic redox-based post-translational modification, often being compared with phosphorylation (Ckless et al., 2004). It may transduce NO-dependent signals such as S-nitrosylation of inhibitory kappa B kinase (IKK) (Reynaert et al., 2004) and the p50 subunit of the transcription factor NFkB (Marshall and Stamler, 2001).

Activated NFkB rapidly translocates into the nucleus where it binds to specific consensus sequences on the promoter-enhancer regions of different genes, stimulating transcription. Its movement from the cytoplasm into the nucleus is rapid, being completed within 20–30 min. Although it is generally accepted that low concentrations of NO have stimulatory effect on NFkB activity while high NO concentrations inhibit it as a part of the negative feedback mechanism that is preventing increased inflammation which, in turn, may lead to tissue damage, it should be noted that in numerous circumstances, the detrimental effects of increased expression of iNOS by prolonged activation of NFkB are attributed to the formation of peroxynitrite from the interaction of NO and superoxide radical. In addition, recent

work of Park et al. (2005) points that tyrosine nitration on p65 subunit may also be the underlying mechanism of NFkB suppression after exposure to NO donor sodium nitroprusside (SNP) (Park et al., 2005). Thus, it seems that presenting the chemistry of RNS donors (their kinetics, transformation, uptake, etc.) may always be warranted when studying RNS-NFkB relation. In that way, it has been recently shown that μM concentrations of NO donors which have shorter half-life, such NOC-9 (t1/2 = 3 min), NOC-7 (t1/2 = 10 min), and NOR-3 (t1/2 = 30 min) were more effective then SNAP (t1/2 = 276 min), NOC-18 (t1/2 = 3,400 min), and SNP (t1/2 = 336 h) in blocking the activation and translocation of p65 subunit of NFkB in vascular endothelial cells, when the donors were added to the cells prior their activation by TNFα (Rogers and Fuseler, 2007). However, in previously activated cells by TNFα, where NFkB had already relocated into cytosol, addition of short half-life NO donors has induced re-translocation of p65 back into the nucleus, suggesting that the activity of NO as an inhibitor or activator of NFkB might be dependent on the state of the cell activation (Rogers and Fuseler, 2007). Also, the fact that TNFα could not provoke NFkB nuclear re-translocation once more in previously activated cells, contrary to the ability of short half-life NO donors, indicates that NO may modulate expression of NFkB-dependent gene products in target cells when cytokines become ineffective, probably due to temporary unavailability of the free TNFα receptor sites and TRADD-TRAF2 transduction pathway (Rothwarf and Karin, 1999). The re-activation of NFkB by NO may perhaps result from the activation of $p21^{ras}$ by NO or by NO-superoxide interaction to produce peroxynitrite which is potent activator of NFkB in different types of cells (Lander et al., 1997; Matata and Galinanes, 2002; Bar-Shai and Reznick, 2006a). By demonstrating relation between the NO donors half-life and the state of cell activity and the NFkB translocation kinetics, Rogers and Fuseler have presented an important dimension to the mechanisms of regulation of NFkB activation and translocation by exogenous nitric oxide donors, in addition to the subjects of the donors bioavailability, their concentrations, and time of exposure.

To protect cells from activation by pro-inflammatory cytokines or other reactive agents, NO released from exogenous donors or inflammatory cells must be delivered rapidly and at high enough concentrations. At sufficiently high concentrations, NO seem capable of reacting with the components of the NFkB signal transduction pathway or directly with specific subunits of the NFkB complex. In that way, exogenous NO was shown to be able to suppress NFkB by preventing proteolytic degradation of IkB, which may be associated with prevention of IkBα phosphorylation and degradation (Peng et al., 1995; Katsuyama et al., 1998). The NO may also interact directly with the NFkB complex to prevent it from binding to DNA by S-nitrosylation of the redox-sensitive C62 residue of the p50 subunit, resulting in its inhibition (Matthews et al., 1996; Schroede et al., 1999).

The inhibitory effect of NO on the NFkB may also occur further upstream in the NFkB activation cascade by interacting with the IKKs, activators of IkB which contain multiple cysteines in their kinase domain, making them potential targets of S-nitrosylation attack by NO (Regnier et al., 1997; DiDonato et al., 1997). Activation of upstream IKKs and IkBs requires phosphorylation at specific sites

and inhibitory effect of NO by S-nitrosylation may result from blocking specific sites of phosphorylation on these kinases, thus preventing the subsequent downstream activation of NFkB. The mechanism of S-nitrosylation of IKK was investigated by Janssen-Heininger group in mouse alveolar type II epithelial C10 cells and Jurkat T cells. The results have shown that different NO-donors such as SNAP or GSNO (100 μM to 1 mM) or SNO (1 mM) were capable of inactivating isolated active IKK by 15 min incubation before the kinase reaction (Reynaert et al., 2004). The authors have shown that both SNAP and GSNO caused a dose-dependent decrease in IKK enzymatic activity *in vitro*. By using biotin derivatization of SNO, the authors revealed that IKKβ, the catalytic subunit required for the canonic NFkB activation, was a direct target for S-nitrosylation with Cys-179 being the main target for attack by SNO. The inhibition of TNFα-induced activation of IKK was detectable at concentrations of SNO as low as 10 μM, which are believed to reflect pathophysiological amounts of extracellular *S*-nitrosothiols (SNO) (Reynaert et al., 2004). Also, TNFα-induced activation of IKKβ was coordinated with enzyme denitrosylation. Moreover, inhibition of NO synthase enhanced the ability of TNFα to activate IKK, illustrating the importance of endogenous NO in regulating the extent of NFkB activation by cytokines (Reynaert et al., 2004). Interestingly, the SNO were not capable of inhibiting the activity of JNK, another serine-directed kinase, although it has been reported that cytokine (IFN-γ)-stimulated iNOS activity inactivated JNK1 in macrophages by S-nitrosylation (Park et al., 2000). This discrepancy may reflect differences in cell type as well as in activating stimulus.

Concerning the inhibition of the IKK activity and blocking of NFkB activity, Levrand and co-workers have found that up to 250 μM peroxynitrite inhibited IKKβ by oxidative chemistry mechanism while simultaneously stimulating the NIK and IKKα. Thus, NO and ONOO seem to have different mode of action on the IKK (Levrand et al., 2005).

The nitration of p65 subunit at two tyrosine sites (Try-66 and -152) by peroxynitrite generated by NO and superoxide may also inhibit NFkB (Park et al., 2005). Park and co-workers (2005) have shown that NFkB activity can be rapidly suppressed by 0.5 mM sodium nitroprusside (SNP), a direct NO donor, and that the constitutively active NFkB target genes m/CAM-1 and cyclin D1 were suppressed by SNP. Immunoprecipitations performed in P19 cell extracts treated with SNP have shown that Tyr nitration was abundant on p65. Moreover, SNP noticeably reduced nuclear level of p65 and simultaneously increased its cytoplasmic level. Although NFkB activity was rapidly suppressed by sodium nitroprusside, a NO donor, the inhibitory effect was actually reversed by deferoxamine(DFO), peroxynitrite scavenger, suggesting a Tyr nitration-mediated mechanism of NFkB inhibition. However, SNP suppression of the *cIAP-1* and h*ICAM-1* genes in HEK293 cells, which were induced by TNFα-activated NFkB, was rescued not only by DFO but also by DTT (an *S*-nitrosylation inhibitor) (Park et al., 2005). Thus, the efficacy of DTT to rescue NFkB-dependent genes suggests that NO may inhibit NFkB by S-nitrosylation of cystine residues or by nitration of tyrosine residues.

Other probable targets for nitrosylation in the NFkB pathway include the epidermal growth factor and src tyrosine kinases which activate NFkB through $p21^{ras}$

(Akhand et al., 1999); tyrosine phosphatases (Li and Whorton, 2003) and NADPH oxidase (Fuji et al., 1997). Thus, S-nitrosylation is the redox-related modification of proteins that has met the criteria of physiological signal which occurs *in vivo*, alters protein function by covalent modification and changes signal amplitude (Marshall et al., 2000) Having in mind the multiple loci of S-nitrosylation in NFkB related pathways, the effects of S-nitrosylation may be indeed considered analogous to those of phosphorylation with a large number of regulatory permutations that combine ultimately to optimize cellular responses (Marshall et al., 2004).

Peroxynitrite Role in NFkB Activation Remains Controversial

Although not a free radical by its chemical nature, peroxynitrite is a powerful oxidant which causes lipid peroxidation, oxidation of protein associated thiol groups, and nitration of amino acids residues. Peroxynitrite-induced protein modifications include protein oxidation on methionine, cysteine, tryptophane or tyrosine residues and nitration of tyrosine or tryptophane residues.

Nitration of free and protein bound tyrosine to yield nitrotyrosine is a well established *in vitro* reaction of peroxynitrite which may affect protein structure and function. Protein tyrosine nitration is a posttranslational modification occurring in a number of diseases and the characteristic presence of tyrosine residues in proteins has been detected in human fluids and tissues in various conditions such as hypertension, atherosclerosis, cardiovascular inflammation and ischemia-reperfusion injury, diabetes, amyotrophic lateral sclerosis, Alzheimer's lesions, or smoking and aging (Leeuwenburgh et al., 1997; Yamakura et al., 1998; Ischiropoulos, 1998; Turko and Murad, 2002). Thus, these findings build a strong association between nitrated proteins found in damaged tissues and numerous diseases and pathological conditions.

The importance of tyrosine nitration as a posttranslational modification in cell signaling is also of interest for the research groups, especially in the light of the pioneering discovery of Kong et al. that peroxynitrite-mediated nitration of a single tyrosine residue in purified cdc2, a cell cycle kinase, prevented its phosphorylation on tyrosine (Kong et al., 1996). Gow and co-workers (1996) have confirmed these observations and also demonstrated that exposing bovine pulmonary artery endothelial cells to authentic peroxynitrite would result in a decrease in the levels of tyrosine-phosphorylated proteins with concomitant increase in nitrotyrosine-containing protein levels, a finding implying that tyrosine nitration interferes with the process of phosphorylation (Gow et al., 1996). On the other hand, it has been shown that nitration of tyrosine residues may simulate phosphorylation and, as a consequence, may result in the constitutively active proteins (MacMillan-Crow et al., 2000; Mallozzi et al., 2001). Thus, peroxynitrite-mediated nitration of tyrosine residues may interfere with signaling processes associated with protein tyrosine phosphorylation. Overall, these observations suggest that protein nitration may be involved in a variety of functions, including the possibility that it may initiate

disease and its progression (Turko and Murad, 2002). Accordingly, the peroxynitrite molecule is considered to be a key mediator of the interplay between tyrosine nitration and tyrosine phosphorylation which can stimulate or disable tyrosine phosphorylation in a concentration-dependent manner, causing tyrosine nitration to become the dominant cell signaling event (Kong et al., 1996; Mondoro et al., 1997; Mallozzi et al., 1997, 1999; Li et al. 1998; Brito et al., 1999; Di Stasi et al., 1999; Monteiro, 2002).

The effect of the peroxynitrite on the state of the NFkB activation is constantly drawing researchers attention after it has been shown that tyrosine nitration impedes with the process of phosphorylation of IkBα molecules and that tyrosine residue at site 42 (Tyr-42) on IkBα is the specific acceptor site for phosphorylation which prevents IkBα degradation, implying that peroxynitrie may activate NFkB by nitrating critical residues in IkBα which otherwise would normally be phosphorylated (Gow et al., 1996; Singh et al., 1996). In addition, the characteristics of tyrosine phosphorylation of being transient and responsive to relatively low concentrations of peroxynitrite, lead to the conclusion that tyrosine phosphorylation is an excellent candidate for mediating signaling events induced by peroxynitrite and that, depending on peroxynitrite local concentrations, nitration and phosphorylation of critical tyrosine residues may be a competitive processes (Brito et al., 1999). In that way, it has been shown that the peroxynitrite affects NFkB activity in a concentration-dependent manner by dephosphorylating IkBα at concentrations of 10–200 μM and that high peroxynitrite concentrations nitrated tyrosine residues in cell lysates which, in turn, have blocked phosphorylation of these amino acids in IkBα molecules, promoting IkBα degradation (Matata and Galinanes, 2002). Thus, it seemed that IkBα molecules containing nitrated tyrosine residues may by themselves be targets for degradation that would result in increased NFkB activity.

However, in contrast to IKK-mediated serine phosphorylation of IkBα, tyrosine phosphorylation of IkBα is capable of activating NFkB even in the absence of ubiquitin-dependent degradation of IkBα (Imbert et al., 1996; Bar-Shai and Reznick, 2006a). We have shown that the induction of NFkB activation in L8 rat myoblasts was dependent on tyrosine nitration of IkBα upon exposure to the authentic peroxynitrite and its donors, taking place independently of its serine phosphorylation and degradation (Bar-Shai and Reznick, 2006a; Bar-Shai et al., 2006). Thus, contrary to the canonic phosphorylation of IkB protein at two serine residues (ser-32 and ser-36), the IkB tyrosine phosphorylation, as well as tyrosine nitration, are two atypical modes of IkB processing which ultimately would result in the NFkB activation. It should also be noted that the tyrosine phosphorylation and nitration are not mutually exclusive events of the cell signaling pathways and that tyrosine nitration may promote or inhibit tyrosine phosphorylation (Monteiro, 2002). The fact that tyrosine nitration can both prevent and reproduce the effects of tyrosine phosphorylation may be one of the reasons for the observation that exposure to peroxynitrite does not block activation of signaling pathways that rely on tyrosine phosphorylation, but rather potently activates some of these (Klotz et al., 2002). In fact, eventual impairment of protein tyrosine phosphorylation by exposure to peroxynitrite is only seen at concentrations of peroxynitrite high enough to lead to tyrosine nitration, which

may become alternative signaling mechanism in transient as well as in nontransient activation of NFkB (Bar-Shai and Reznick, 2006a).

It is interesting that the parent molecules of peroxynitrite, that is NO and superoxide, appear to regulate NFkB activation in an opposing way with superoxide activating and nitric oxide inhibiting NFkB activation (Marshall and Stamler, 2002).

The contrasting roles of superoxide and NO on NFkB activation point toward the importance of understanding circumstances which are regulating peroxynitrite formation and the mechanisms by which peroxynitrite may activate NFkB. In our studies, we have investigated the mechanism of NFkB activation in skeletal myocytes by the RNS. We have seen that both NO and peroxynitrite caused NFkB activation; NO-donor-induced activation of NFkB was transient, dependent on IkBα degradation, and could be decreased by IkBα super-repressor. Conversely, authentic peroxynitrite and its donor SIN-1 induced NFkB activation that was dependent on tyrosine nitration of IkBα, but independent of its serine phosphorylation as well as degradation. Moreover, the presence of IkBα super-repressor did not decrease this activation. In addition, our studies on the IKK activity have revealed that RNS could activate IKK in a different manner; low concentrations of NO donors have activated IKK while higher concentration have shown inhibitory effect on IKK activity *in vitro*. However, peroxynitrite could activate IKK at low as well as high, millimolar concentrations (Bar-Shai and Reznick, 2006a). Moreover, prolonging the time of exposure of skeletal myocytes to peroxynitrite resulted in nontransient NFkB activation which was associated with high iNOS expression. Using proteosome inhibitor MG-132 could not diminish peroxynitrite-induced activation of NFkB while tyrosine nitration inhibitor EGCG has re-established transient NFkB activation in which degradation of IkBα was taking place. Also, the use of EGCG resulted in the decrease of peroxynitrite donor-induced increased iNOS expression. Thus, peroxynitrite was shown to activate NFkB in skeletal myocytes through an alternative mechanism in which IkBα is nitrated on tyrosine and dissociated from NFkB, enabling its nontransient activation and prolonged iNOS expression (Bar-Shai and Reznick, 2006a). Concerning further NFkB activity in skeletal muscle, it should be noted that we have also demonstrated that the activation of proteolytic systems and the degradation of muscle-specific proteins in L6 skeletal muscle cells was mediated by peroxynitrite-induced NFkB activation (Bar-Shai and Reznick, 2006b). This finding may be important having in mind that pathological conditions, such as the muscle dystrophy due to disuse, have been associated with NFkB activation in atrophic muscle which has been achieved via noncanonical but atypical alternate NFkB pathway, that is the binding of p50 homodimer to the Bcl-3, a nuclear IkB family member, (Hunter et al., 2002; Jackman and Kandarian, 2004). Thus, both classical and non-classical NFkB activation pathways can employ atypical or alternative modes of NFkB activation.

Our position on RNS and NFkB is summarized in Fig. 4.2.

It should be noted that peroxynitrite not only nitrates but also potently oxidizes proteins such as oxidizing and inactivating regulating phosphotyrosine phosphatase (PTPase) which, in turn, would lead to enhanced tyrosine phosphorylation

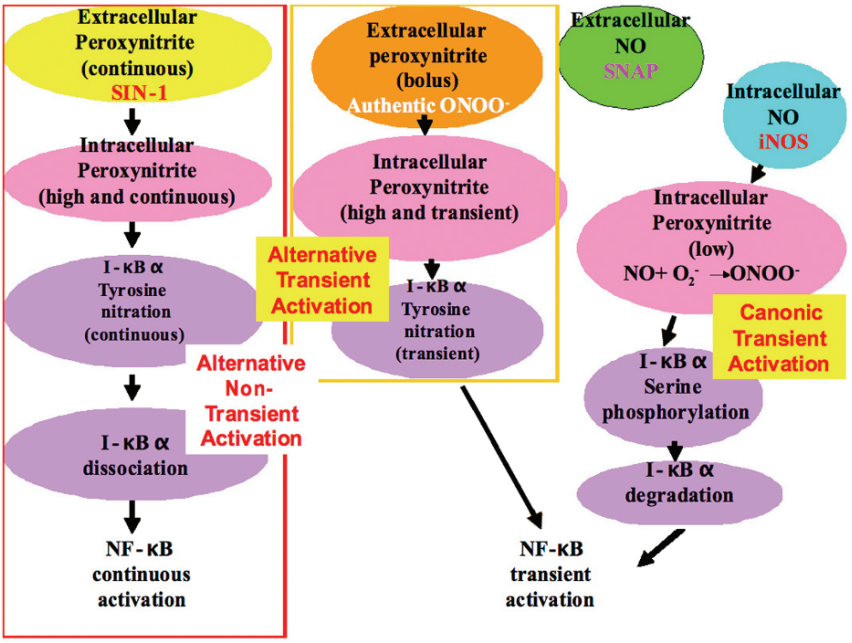

Fig. 4.2 Classic and alternative pathways of NFkB activation by RNS. (SIN-1: 3-morpholinosydnonimine; SNAP: S-nitroso-N-acetylpenicillamine)

(Mallozzi et al., 1997). Recently, Levrand et al. have shown that authentic peroxynitrite has inhibited NFkB activation triggered by inflammatory stimuli (TNFα or lipopolysaccharide) in cardiac and endothelial cell lines. The inhibition of NFkB-DNA binding was completely prevented with the SOD mimetics and antioxidant agent Mn(III)-tetrakis-(4-benzoic acid) porphyrin (MnTBAP), which lead to the conclusion that peroxynitrite-induced NFkB inhibition may be dependent on peroxynitrite oxidative chemistry (Levrand et al., 2005). As mentioned before, recent work of Park et al. (2005) pointed that tyrosine nitration on p65 subunit may also be the underlying mechanism of NFkB suppression after exposure to NO donor sodium nitroprusside (SNP), although SNP-induced suppression of the *cIAP-1* and h*ICAM-1* genes was re-established not only by peroxynitrite scavenger DFO but also by DTT (an *S*-nitrosylation inhibitor), implying that NO, in fact, might be inhibiting NFkB by S-nitrosylation of cysteine residues (Park et al., 2005). On the other hand, Levrand and co-workers have demonstrated that peroxynitrite, while inhibiting a classic NFkB pathway, has strongly activated phosphorylation of NIK and IKKα, the components of the alternative, noncanonical NFkB activation pathway even in the absence of stimulatory signal by LPS or TNFα, suggesting that peroxynitrite molecule alone is involved in the nonclassic pathway of NFkB stimulation (Levrand et al., 2005). As mentioned before, we have also proved that total IKK activity has increased after the exposure to peroxynitrite in vitro, although the separate measurements of IKKα were not performed (Bar-Shai

and Reznick, 2006a). From our experiments, we have concluded that when tyrosine nitration is not blocked, the noncanonic activation of NFkB would take a place. On the other hand, when nitration is blocked with agent such EGCG, canonical NFkB pathway ensue. However, blocking nitration would not exclude the engagement of the oxidatively-induced alternative pathway of NFkB activation by peroxynitrite via NIK and IKKα phosphorylations. Thus, these two peroxynitrite chemistries, oxidative and nitrosive, should not be considered mutually exclusive events. In fact, it may be assumed that the alternative pathways of NFkkB activation and posttranslational modifications may be triggered by peroxynitrite through the nitration and/or oxidation, which may be a matter of redox balance. It will be challenging to assess the relative contributions of either type of reaction of peroxynitrite, tyrosine nitration, or oxidation, to observed signaling effects.

The exposure of the cells to the RNS is associated also with the GSH depletion and, consequently, oxidative stress. We have shown that the cell redox state is an important regulator of NFkB activation because depletion of the cell GSH and the ensuing oxidative stress have caused the augmented NFkB activation, demonstrating that the peak of NFkB activation was inversely associated with cell GSH levels (Sen et al., 1997). It has been further shown that positive correlation exists between the level of NFkB activity and the GSSG/GSH reduction potential (Li et al., 2002). For that reason, it is a bit surprising that there are no too many RNS-NFkB studies in which the cell redox state and subcellular compartmentalization of glutathione were reported in a tandem with the "gel results". This is particularly important having in mind the role of NFkB in apoptosis and the importance of mitochondrial GSH homeostasis in regulating programmed cell death and mitochondria-to nucleus oxidative stress signaling (Storz, 2006). Moreover, the GSH intracellular distribution has been found closely correlated to oxidative stress and genotoxicity, showing negative linear correlations existed between mitochondrial GSH and ROS and between oxidative DNA modifications and nuclear GSH levels (Green et al., 2006). In addition, in the excellent review of Janssen-Heininger (2000), it was emphasized that under the circumstances that lead to iNOS expression, the cells which do not have ability to markedly up-regulate extracellular SOD or MnSOD to maintain the high NO concentrations required for a negative NFkB feedback signaling, would have conditions created for the significant peroxynitrite generation and, ultimately, for different mode of NFkB signaling (Janssen-Heininger, 2000). Thus, the surplus of superoxide anion may be considered an important regulator of the state of NFkB activation by the peroxynitrite, reactive nitrogen species. Also, impaired mitochondrial superoxide homeostasis, expressed by the increase in superoxide radical generation, has been recognized as the inductor of protein kinase D1. This kinase has been identified as a mitochondrial sensor and regulator of a radical-sensing signaling pathway, which relays mitochondrial ROS production to the induction of nuclear genes that mediate cellular detoxification and survival, including the activation of NFkB transcription factor (Storz et al., 2005; Storz, 2007). For all above reasons, it is indeed of particular importance to consider different aspects of the cell redox state when evaluating the link between the RNS and the state of NFkB activation.

Activation of NFkB-Implications to Biological Systems

NFkB and Aging

Denham Harman was the first who has proposed that the damaging effects of reactive oxygen species may play a key role in the mechanism of aging (Harman, 1956). Genetic studies of distantly related species such as *C. elegans, Drosophila melanogaster*, and mice support the free radical hypothesis of aging and the evidence included claims that: (1) variation in species life span is correlated with metabolic rate and protective antioxidant activity; (2) enhanced expression of antioxidative enzymes in experimental animals can produce a significant increase in longevity; (3) cellular levels of free radical damage increase with age; and (4) reduced calorie intake leads to a decline in the production of reactive oxygen species and an increase in life span (Wickens, 2001). In that way, it has been shown that boosting the natural antioxidant systems of *C. elegans* with small synthetic superoxide dismutase/catalase mimetics resulted in an increase of the mean life-span by about of 44% in wild-type worms, while the treatment of prematurely aging worms resulted in 67% life-span increase equaling to their life-span normalization. Thus, oxidative stress appears to be a major determinant of life-span accessible to pharmacological intervention (Melov et al., 2000).

The free radical theory may also be used to explain many of the structural features that develop with ageing including the lipid peroxidation of membranes, formation of age pigments, and cross-linkage of proteins, DNA damage and decline of mitochondrial function. In recent years, several related theories containing the free radical component have also been proposed. They include the mitochondrial theory of aging, which hypothesizes that mitochondria are the critical component in control of aging and that electrons leaking from the electron transport chain (ETC) produce ROS which can damage ETC components and mitochondrial DNA, leading to further increases in intracellular ROS levels and a decline in mitochondrial function (Wallace, 2005). An additional and attractive theory is the molecular inflammatory theory of aging, whereby the activation of redox-sensitive transcriptional factors, such as NFkB, causes the upregulation of proinflammatory gene expression by age-related oxidative stress. Another consideration is the cellular senescence theory of aging, which emphasizes the importance of ROS-induced modulation in various cellular signal responses which regulate expression of a number of genes responding to stress and oxidant damage (Kregl and Zhang, 2007). In many cases, these induced genes are regulated by transcription factors whose structure, subcellular localization, or affinity for DNA is directly or indirectly regulated by the level of oxidative stress (Liu et al., 2005). Examples of aging-related transcription factors known to be redox-regulated include tumor suppressor p53, Forkhead transcription factors, activator protein-1 (AP-1), and NFkB.

There is a good indication that increased life span can be correlated with increased stress resistance and expression of stress response genes such as those encoding for superoxide dismutase (SOD) and HSP (heat shock protein) (Landis

and Tower, 2005). It has been shown that mitochondria-originating oxidative stress regulates the activity of NFkB as well as that of FOXO3a transcription factor of the Forkhead transcription factor family resulting in the induction of the enzyme superoxide dismutase-2 (SOD2) antioxidant gene (Storz, 2006). In addition, an age-associated increase in the DNA binding activities of NFkB and AP-1 has been demonstrated in livers of old animals (Zhang et al., 2004). Thus, versatile NFkB transcriptional activity is associated with the process of aging.

Aging Skeletal Muscle, Exercise, and NFkB

Structural and functional changes in muscle during aging occur in a wide range of species, ranging from *C. elegans* to humans. The structural changes include a reduction in muscle mass and muscle fibers, and a shift of muscle fibers toward type 1 fibers. These structural changes are associated with muscle weakness, reduced endurance capacity, and insulin resistance (Nair, 2005). Aging of skeletal muscle is characterized by increased apoptosis, susceptibility to injury, inflammation, and oxidative stress (Song et al., 2006; Ji, 2007). Performing physical exercise is highly recommended activity for persons of all ages and no damaging habitual exercise may provide some protection against age-related loss of muscle mass and function (Brown et al., 1992; Song et al., 2004). The benefit of exercise has been attributed to the upregulation of the muscle antioxidant adaptation such as an increase in muscle SOD and glutathione peroxidase (GPx) activity (Higuchi et al., 1985; Lawler et al., 1993; Leeuwenburgh et al., 1994). However, increased oxidative stress has been determined as an important mediator of aging and the production of ROS and nitrogen oxide derivatives increases during more demanding exercise resulting in the increased amount of generated peroxynitrite and, with aging, redox modulation of muscle contraction may be altered by the changes in the rates of ROS/RNS production, the levels of endogenous antioxidants, and the sensitivities of regulatory proteins to their action (Reid and Durham, 2002). This may cause a shift in the cross-talk between the cell survival and pro-death signaling pathways. For example, the increased protein nitration with aging was observed both in type II (the semimembranosus) and type I (the soleus) rat muscles (Fugere et al., 2006) and it may affect cross-talk between NFkB and mitogen-activated protein kinases (MAPKs) pathways which are major oxidative stress-sensitive signal transduction pathways in mammalian tissues (Ji et al., 2006). In addition, mitochondrial biogenesis in skeletal muscle, expressed by an increase in mitochondrial volume and structural changes that improve resistance to exhaustion, represent the ROS-induced mitochondria adaptations to exercise (Hood et al., 2006) which embrace the radical-induced activation of NFkB during and following contractile activity (Ho et al., 2005). Ji et al. have found that in rats exercised to exhaustion, there were high levels of NFkB activation in muscles and that treatment with antioxidants such as pyrrolydine dithiocarbamate (PDTC) almost completely abolished the activation of NFkB signaling cascade (Ji et al., 2004). Similarly, recent work by

Ho et al. reported that NFkB activity increased in rat gastrocnemius muscles and that the inhibition of p38 and ERK MAP kinases pathways resulted in as much as 76% inhibition of IKK phosphorylation. This suggested that these kinases may influence the activation of IKK and NFkB during exercise (Ho et al., 2005). Sen (1999) suggested a mechanism for the possible mode of NFkB activation due to exercise. According to this monograph, intense acute exercise may cause the oxidation of glutathione in muscle cells, resulting in increased oxidative stress and NFkB activation (Sen, 1999). Thus, regular exercise would increase glutathione levels in muscle tissue, resulting in decreased oxidative stress which would result in down-regulation of NFkB activity. In addition, it has recently been shown that regular exercise induced increase in NFkB activity in old rats resulted in marked reduction of fiber atrophy and apoptotic signaling in rat skeletal muscle (Song et al., 2006). However, the need for the antioxidants supplementation indicates that free radical-induced muscle damage due to exercise may point to the inability of the aged muscle to respond fully and adequately to the increased oxidative stress stimuli. Data from our animal studies signify that short-term exercise may have damaging effects on the muscle of old animals in comparison to its effects on the skeletal muscle of young animals (Steinhagen-Thiessen et al., 1980; Steinhagen-Thiessen et al., 1981). On the other hand, our data do suggest that the long-term exercise regime and the application of antioxidants may prevent the age-associated oxidant damage and muscle decline (Reznick et al., 1983, 1987, 1989, 1992a, b; Witt et al., 1992).

Muscle Immobilization: The Role of RNS and NFkB

One of the most universal phenomena of aging is the sarcopenia of old age (Carmeli et al., 2002). The loss of muscle mass and function in old age has a grave influence on the quality of life of elderly people. Thus, understanding the biochemical and molecular events leading to this phenomenon is a prerequisite for developing the means to combat the sarcopenia of old age (Carmeli et al., 2002). Skeletal muscle damage under the conditions of disuse-induced atrophy has been demonstrated to be associated with increased activities of both extracellular and intracellular proteolytic systems (Carmeli and Haimovitch, 2006; Carmeli et al., 2006). While the reduction of muscle mass in young animals was associated with increased activities of acid phosphatase (ACP) and matrix metalloproteinases (MMPs) 2 and 9, and with increased ubiquitination of muscle proteins, MMP-2 was continuously active in old muscles in the absence of the expression of MMP-9. In addition, the extent of ubiquitination of myosin heavy chain in muscles of old animals was higher in comparison to young muscles (Zarzhevsky et al., 1999, 2001a, b; Reznick et al., 2003; Bar-Shai et al., 2005a, b). We were able to show that RNS and NFkB have been linked to the activation of different intracellular and extracellular proteolytic systems in skeletal muscle cells by demonstrating that RNS donors caused NFkB activation, and increased activation of proteolytic systems, as well as the degradation of muscle-specific proteins. Antioxidant treatment, tyrosine nitration inhibition

and NFkB molecular inhibition were proven effective in downregulation of NFkB activation and slowing down the degradation of muscle-specific proteins (Bar-Shai and Reznick, 2006b). Moreover, signal transduction through NFkB has been demonstrated in disuse muscle atrophy; NFkB was shown to be biphasically activated in skeletal muscles of old rats showing first the decrease in the activity of the classic p65/p50 dimer which was followed by the increase in its activity with the prolongation of the period of muscle disuse (Bar-Shai et al., 2005b). On the other hand, Kandarian and co-workers have demonstrated the activation of alternative Bcl-3/p50 NFkB pathway in young rats subjected to hindlimb disuse (Hunter et al., 2002; Jackman and Kandarian, 2004). Since NFkB is active both in muscle exercise and disuse atrophy, understanding NFkB pathways and using appropriate inhibitors may prove beneficial in attenuating NFkB -associated muscle damage in disuse atrophy and strenuous exercise (Bar-Shai et al., 2008).

NFkB and Human Diseases

Physiological role of NFkB include its role in the development, proliferation, and effector functions of B and T cells, and expression of many cytokines required for the generation of T cell responses. On the other hand, aberrant activation of the NFkB pathway is involved in the pathogenesis of a variety of human diseases including those related to inflammation, enhanced cellular proliferation, viral infection, and genetic diseases. These include diseases and disease states such as AIDS, atherosclerosis, asthma, arthritis, bone resorption, cancer, diabetes, epilepsy, heart diseases, inflammatory bowel disease, muscular dystrophy, Alzheimer's and Parkinson's diseases, stroke, viral, etc. (Celec, 2004; Kumar et al., 2004; Papa et al., 2006; Mattson and Meffert, 2006).

Recent evidence suggests that the dysfunction of NFkB is also a mediator of some human genetic disorders (Orange et al., 2005; Curtois and Smahi, 2006; Curtois and Gilmore, 2006). Thus, the same machinery that is involved in cell survival, growth, and proliferation can become deregulated in human disease.

NFkB, Oncogenesis, and Tumor Promotion

NFkB participates in regulation of immune and inflammatory responses, proliferation, angiogenesis, and oncogenesis (Lin and Karin, 2003; Aggarwal, 2004; Greten and Karin, 2004). It characteristically induces antiapoptotic gene expression to promote cell survival (May and Ghosh, 1999; Barkett and Gilmore, 1999). On the other hand, the involvement of NFkB both in inflammation and control of the cell growth dos not seem to be coincidental. Pikarsky and co-workers have revealed that NFkB is an important molecular link between inflammation and hepatocellular carcinoma (Pikarsky et al., 2004). The clinical relevance of this link has been supported by findings of high constitutive NFkB activity which was associated with

hepatocellular carcinoma resulting from both hepatitis C virus and hepatitis B virus infections (Tai et al., 2000). Also, the ability of NFkB to suppress apoptosis and to induce expression of proliferation-stimulating proto-oncogenes such as *c-myc* and *cyclin D1* suggest that NFkB participates in many aspects of oncogenesis (Pahl, 1999; Guttridge et al., 1999). In addition, mutations and translocations resulting in constitutive activation of the NFkB pathway are found in tumors derived from many different tissues and its activation correlates with higher grades of malignancy and a poor prognosis (Gilmore et al., 2002; Mattson and Meffert, 2006). Thus, silencing of NFkB can restrain growth and improve the response to antitumor therapy (Tacconelli et al., 2004)

Molecular and Pharmacological Approaches to NFkB Regulation

By using dominant negative phenotype of IkBα mutant which was neither phosphorylated by IKK nor degraded by proteasome, Ghosh and co-workers have provided the first evidence that NFkB pathway could be inhibited; the mutant had sequestered NFkB in cytoplasm and prevented the induction of NFkB specific target genes (Ghosh et al., 1998).

At present, a number of pharmacological agents are known to inhibit NFkB. While the upstream strategies aim to block the activation of NFkB signaling pathway, targeting NFkB would suppress its transactivation. These upstream approaches include the use of selective IKK inhibitors such as deoxyspergualine and AS602868 (Greten and Karin, 2004; Frelin et al., 2005). Targeting the IKKβ kinase with AS602868 to block NFkB activation led to apoptosis of human primary acute myeloid leukaemia cells and increased the apoptotic response induced by the chemotherapeutics doxorubicin, cytarabine or etoposide (VP16) (Frelin et al., 2005). Also, blocking the phosphorylation of IkBs by non-steroidal anti-inflammatory drugs such as aspirin, sodium salicylate, and leflunomide could inhibit NFkB (Yamamoto and Gaynor, 2001). Alternatively, the inhibitory effect on NFkB was achieved by stimulating induction of IkBα synthesis with glucocorticosteroids (Yamamoto and Gaynor, 2001; D'Acquisto et al., 2002). Also, the use of recombinant adenovirus-mediated overexpression of the IkBα gene colud block NFkB activation (Bakker et al., 1999; Oitzinger et al., 2001) and using cell-permeable peptides could block its nuclear transclocation (Lin et al., 1995). Another approach strategy which has been introduced was to block the proteasome degradation of IkB by naturally occurring and synthetic inhibitors of ubiquitin-proteasome. These compounds include lactacystin (a streptomyces metabolite), peptide aldehydes, such as carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG-132), and boronic acid peptides such as PS-341 (Adams et al., 2000). The PS-341 seems suitable for *in vivo* administration. Its use in phase I clinical trials has resulted in a significant antitumor response in chemoresistant multiple myeloma (Mitchell, 2003).

The therapeutic targeting of NFkB by blocking DNA-binding activity by decoy oligonucleotides was demonstrated both in *in vitro* studies and in various animal models of diseases (D'Acquisto et al., 2000; Zingarelli, 2003). Another approach to impede

NFkB activity was to interfere with NFkB mRNA by using NFkB antisense oligonucleotides (D'Acquisto et al., 2002). The redox-regulation-mediated inhibition of NFkB, by using SOD inhibitors, has also been proposed. The rationale for that approach was based on the fact that NFkB up-regulates antioxidant genes and that one of the most important prosurvival factors regulated by NFkB is the antioxidant enzyme MnSOD. Hence, since NFkB activity is increased in different types of cancer, it is reasonable to assume that the expression of MnSOD is higher in malignant cells than in normal cells and, consequently, the former may be more sensitive to SOD inhibitors. In that way, some oestrogen derivates, acting as SOD inhibitors, were shown to selectively kill human leukaemia cells but not normal lymphocytes (Huang et al., 2000a).

Noteworthy are attempts to identify active principles in the natural compounds and traditional preparations which might be blocking NFkB. Green tea polyphenols and red wine polyphenol compound resveratrol inhibit NFkB activation *in vitro* by blocking the activity of IKK (Holmes-McNary and Baldwin, 2000; Yamamoto and Gaynor, 2001). Also, curcumin from turmeric and capsaicin of red pepper, which exhibit considerable anticarcinogenic and antimutagenic activities, are shown potent inhibitors of IKK activity *in vitro* (Pan et al., 2000; Surh et al., 2000). In our laboratory, we have shown that fungal extracts prepared from Marasmius oreades and Cyathus striatus showed significant inhibitory effects on the NFkB activation pathway (Petrova et al., 2006).

The multiple approaches in reducing NFkB activity may be needed because some of the specific targeting strategies may actually not work. For example, the mechanism of NFKB activation by harmful ultraviolet (UV) radiation or by the expression of the Her2/ Neu oncogene involves use of casein kinase II (CK2) instead of IKK to phosphorylate IkBα at a cluster of C-terminal sites that are recognized by CK2 (Kato et al., 2003; Perkins and Gilmore, 2006). Thus, this third pathway of NFkB activation is IKK-independent (Kato et al., 2003; Bing et al., 2005; Perkins and Gilmore, 2006). Also, the comprehensive transcriptional activation of NFkB signaling pathway involves its analogous interaction with redox-sensitive activator protein (AP)-1 and the crosstalk with other signaling pathways such as protein kinase A, phosphatidyl inositol 3 kinase/Akt and MAPKs. Hence, blocking the activity of these pathways may provide alternative approach to control the NFkB activity. However, from a therapeutic perspective, a major problem is that NFkB blockade can cause serious adverse effects such as immunosuppressant effects. In addition, the induction of antiapoptotic factors by NFkB suggests that its complete inhibition would result in unexpected and detrimental side effects. Thus, an important therapeutic goal is to achieve selective modulation of specific functions of NFkB.

Conclusions and Perspectives

The RNS have important effects inside biological systems. While some of these effects, such as protein nitration, were initially recognized as markers of biological damage in different tissues, laboratory works performed by us and other research

groups have established an important role for the RNS in the process of NFkB signaling. The RNS both stimulate and inhibit NFkB via classical and alternative pathways. While the effects of NO are concentration-dependent and related to classical pathway of NFkB activation, dual chemical nature of peroxynitrite (potent oxidant and nitrating species) provides its molecule the ability to concurrently exhibit both inhibitory and stimulatory effects on NFkB. Moreover, depending on the peroxynitrite extra- or intracellular availability, it may cause both transient and nontransient NFkB activation.

Are there still unknown cellular targets for the RNS to induce additional pathways of NFkB activation? NFkB-IkB complex as well as upstream signaling kinases are found to constantly migrate into the nucleus and associate with the promoters of NFkB-dependent genes which raise the possibility those peroxynitrite-activated kinases may be accounted for the prolonged downstream activation of NFkB. Moreover, mitochondria-to nucleus oxidative stress RNS signaling and oxidants derived from mitochondria may target proteins that act further downstream in the NFkB activation cascade. Finally, NO and peroxynitrite present within the mitochondria may also have signaling effect on NFkB localized in mitochondria.

Concerning deregulation of NFkB found in human disease, which is reflected through its continuous activation, mechanisms of NFkB activation by RNS include the induction of alternative NFkB pathways, some of them being characterized by the state of continuous activation of NFkB. Also, the focus in carcinogenesis has been on initiation, and particularly on the direct induction of DNA damage. In that way, pro-carcinogenic mutagenicity induced by the RNS such peroxynitrite, powerful oxidant, cannot be ruled out.

To conclude, the effects of RNS on NFkB include multiple dynamic mechanisms of its activation and inhibition as well. This activation-inhibition dichotomy is related to the concentrations of the RNS present in biological systems, their availability and time of the exposure of the cells, and oxidative and nitrosative chemistry of RNS. Also, the effects are cell-type related and depend on the state of cell activity and the level of the antioxidant regulation. Moreover, NFkB mechanisms of activation include the induction of alternative pathways whose understanding would raise the possibility to induce therapeutic manipulation of signaling pathways to achieve desirable level of NFkB activation by reactive nitrogen species, which are constantly generated both in physiological and pathological conditions. For that reason, further understanding of regulation and control of NFkB activation can be an important therapeutic strategy for inhibiting inflammation and tumor growth.

Appendix

List of Abbreviations

GSH	reduced glutathione
GSSG	oxidized glutathione
IkB	inhibitory kB

IKKs	IkB-kinases
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
MAPKs	mitogen-activated protein kinases
NEMO	NFkB essential modulator
NFkB	nuclear factor kappaB
NIK	NFkB-inducing kinase
NO	nitrogen monoxide
ONOO	peroxynitrite
PI3-kinase	phosphatidyl inositol-3-kinase
RHD	Rel-homology domain
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNOs	S-nitrosothiols
SOD	superoxide dismutase
TNF	tumor necrosis factor-

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Chapter 5
Neutrophil-Derived Oxidants as Modulators of Polyunsaturated Fatty Acid Metabolism

Lukáš Kubala and Antonín Lojek*

Abstract Phagocytes, including polymorphonuclear neutrophilic granulocytes, belong to the first line of defense mechanisms of the body against invading pathogens. Upon their activation under inflammatory conditions neutrophils produce high amounts of reactive oxygen species. These reactive intermediates could react with various molecules in the organism including lipids with polyunsaturated fatty acid as the main susceptible target. This paper is focused on the role of reactive oxygen species produced by neutrophils in the initiation of lipid peroxidation. The oxidation of lipids is considered as a typical hallmark of chronic and acute inflammatory processes. The induction of lipid peroxidation and formation of various oxidized lipid metabolites are mostly distinguished to be deleterious and inflammation inducing processes. In contrary, the oxidation of biologically active pro-inflammatory lipids could have anti-inflammatory effects and over all contribute to termination of inflammatory process. Therefore, lipid peroxidation mediated by reactive intermediates produced by neutrophils could both potentiate and reduce inflammatory reaction. Necessity of fine balance of these processes can be suggested for proper control of physiological inflammatory response.

Keywords Inflammation, phagocytes, reactive oxygen species, myeloperoxidase, lipid peroxidation, polyunsaturated fatty acids

Inflammation and Lipid Peroxidation

The Inflammatory reaction are the physiological response against invading pathogens or harmful physical or chemical effects as part of the process to maintain organismal integrity. It is of necessity a highly controlled and coordinated complex process that includes the concerted and often opposing activities of various cell

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

types and a myriad of mediators. The initial steps of inflammation typically include altered local vascular permeability alongside the mobilization of various inflammatory cells with oxidant producing phagocytes being the most prominent (Nathan, 2006). The inflammatory process with its associated increased production of oxidants then leads to either local or generalized oxidative stress. All types of molecules including lipids are subject to attack from oxidants. Under normal metabolic conditions, it has been estimated that as many as 10,000 oxidative hits per cell per day take place (Sasaki, 2006). However, oxidative hits increase substantially under inflammatory conditions following bursts of cellular metabolism, and or depletion of cellular antioxidants. The induction of lipid peroxidation and concomitant formation of lipid oxidation products with significant biological activities (for reviews see Brennan and Hazen, 2003; Cohen, 2002; Heinecke, 2002; Leitinger, 2003; Nicholls and Hazen, 2005; Rubbo and O'Donnell, 2005; Sasaki, 2006; Shao et al., 2006; Stenke et al., 1994; Sultana et al., 2006) are significant in the pathophysio logy of various chronic and acute inflammatory diseases.

In general, the generation of lipid oxidation products of polyunsaturated fatty acids (PUFAs) are under strict regulatory control as they are potent biologically active compounds involved in intracellular signaling that modulate the inflammatory process with both pro-inflammatory and anti-inflammatory activities. Thus, normally lipid oxidation leads to formation of mediators which are integral to the regulation of physiological functions of organism. However, uncontrolled lipid peroxidation induced by pathological oxidative stress is often considered as a deleterious process when linked to disruption of membranes and cellular dysfunction. Clearly, the control of lipid oxidation as to site, extent and species produced is essential to a normal physiological process while uncontrolled or aberrant lipid oxidation represents a major pathophysiological contributor to acute and chronic inflammatory diseases.

General Mechanism of Lipid Peroxidation

Biological membranes typically consist of a bilayer of phospholipids with fatty acids with hydrophobic hydrocarbon side chains mostly consisting of PUFAs containing two or more double bonds along with other lipid components such as cholesterol. The side chains of the phospholipids are highly susceptible to free radical attack, which can start off lipid peroxidation (for reviews see Halliwell and Gutteridge, 1999; Halliwell and Chirico, 1993; Yin and Porter, 2005). In a peroxide-free lipid system, the initiation of a peroxidation sequence refers to the attack of a reactive oxygen species able to abstract a hydrogen atom from a methylene group ($-CH_2-$) which generates free radicals from polyunsaturated fatty acids. Generally, lipid peroxidation (Fig. 5.1) is characterized by a free radical chain-reaction mechanism consisting of the following basic steps (Halliwell and Gutteridge, 1999; Halliwell and Chirico, 1993; Sultana et al., 2006; Yin and Porter, 2005; Cyberlipid center):

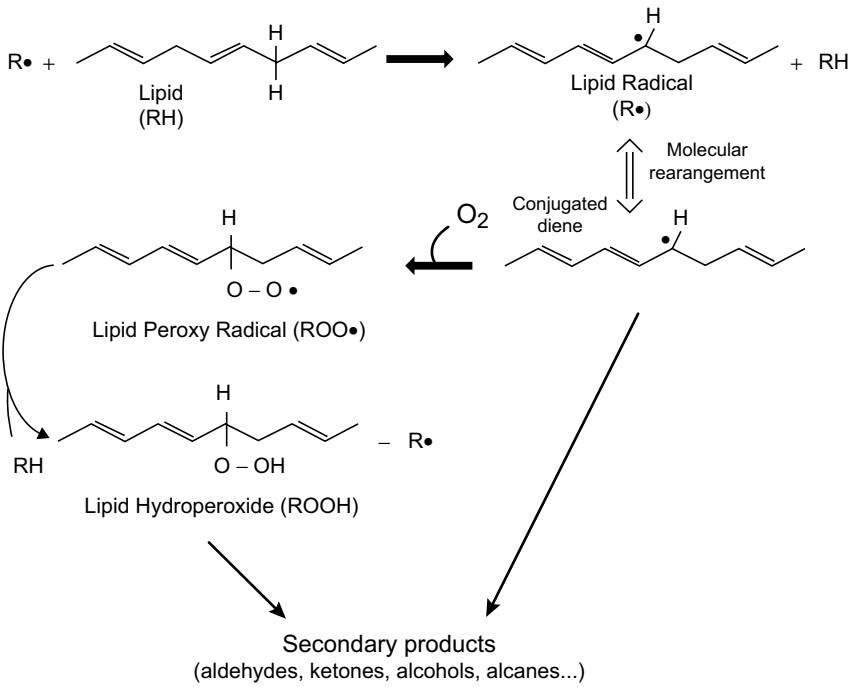

Fig. 5.1 General mechanism of lipid peroxidation. Lipid peroxidation is characterized by a free radical chain-reaction consisting of several basic steps (for details see the main text). ROS attack a methylene group and pull off hydrogen atoms from the PUFA side chain forming free radical. The carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene. In the presence of oxygen, carbon radical combines with oxygen leading to formation of a lipid peroxyl radical. Peroxyl radical is able to abstract hydrogen from another lipid molecule to give a variety of lipid hydroperoxides and cyclic peroxides. Thus, peroxyl radical causes an autocatalytic chain reaction that characterizes the propagation step of lipid peroxidation. Hydroperoxides are further degraded to wide range of products including alcohols, epoxides, and a variety of aldehydes, such as HNE, MDA and acrolein (Figures are modified from Halliwell and Gutteridge, 1999; Sultana et al., 2006; Yin and Porter, 2005; Cyberlipid center)

Step 1 – initiation: The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond. Oxidants attack a methylene group and abstracts or removes hydrogen atoms from the PUFA side chain. The carbon, from which hydrogen atom is abstracted, then has an unpaired electron, making it a free radical. In the absence of oxygen, the carbon radical undergoes a molecular rearrangement to form a conjugated diene. However, in the presence of oxygen, they combine with oxygen to give a peroxyl radical.

Step 2 – propagation: Highly reactive peroxyl radical can oxidize other unsaturated lipids or can attack other cellular molecules e.g. proteins. Peroxyl radical is able to abstract hydrogen from another lipid molecule to give a variety of lipid hydroperoxides and cyclic peroxides especially in the presence of metals such as copper or iron. Peroxyl radical thus causes an autocatalytic chain reaction which

characterizes the propagation stage of lipid peroxidation. Interestingly, hydroperoxides are more hydrophilic than unperoxidized fatty acid side chains. They can migrate to the membrane surface to interact with water, thus disrupting the membrane structure and fluidity, decreasing activity of membrane-bound enzymes (*e.g.*, sodium pumps), altering activity of membrane receptors, and making the membrane leaky. In addition to effects on phospholipids, radicals can also directly attack membrane proteins and induce lipid–lipid, lipid–protein, and protein–protein cross-linking, all of which obviously have effects on membrane function. Hydroperoxides are further degraded to several products including hydrocarbons, alcohols, ether, epoxides, and a variety of aldehydes, such as 4-hydroxy-2,3-trans-nonenal (HNE), malondialdehyde (MDA) and acrolein.

Step 3 – termination: The chain reaction is stopped by interactions between the radicals themselves, or between the radicals and antioxidants, giving rise to nonradical products or unreactive radicals. Generally, any kind of alkyl radicals (lipid free radicals) can react with a peroxyl radical to give non-initiating and non-propagating species such as the relatively stable dimers or two peroxide molecules combining to form hydroxylated derivatives. It is also widely accepted that termination of lipid peroxidation is achieved by reaction of peroxyl radical with alpha-tocopherol that is the main lipophilic "chain-breaking molecule" in the cell membranes. Furthermore, some bonds between lipid peroxides and membrane proteins are also possible to suggest.

Neutrophil-Derived Oxidants

Polymorphonuclear neutrophils and macrophages are often called professional phagocytes because their primary role is to phagocyte and destroy invading microorganisms or damaged cells (for reviews see Klebanoff, 2005; Nathan, 2006; Segal, 2005). Macrophages are often resident in tissues under normal physiological conditions. In contrast, neutrophils are not found in tissues but mainly in the circulation. Neutrophils are highly motile phagocytic cells that can be recruited from the blood across the vascular endothelium into sites of inflammation as a result of production of a number of pro-inflammatory signals.

Activation of neutrophils leads to directed migration, granule mobilization and activation of the NADPH-oxidase, which is associated with a dramatic increase in activity of the hexose monophosphate shunt and the production of large amounts of reactive oxygen species (ROS) (for reviews see Klebanoff, 2005; Segal, 2005; Winterbourn et al., 2000). The generation of ROS is of great importance for microbial killing, and is the means whereby phagocytes provide the first line of defense against bacterial and fungal infections as well as ensuring the proper performance of the immune system.

In human neutrophils, NADPH-oxidase activation takes place at two different cellular sites, in the plasma membrane, resulting in superoxide release to the extracellular milieu (before closure of the vacuole or in the case of soluble stimuli), and in intracellular granules, leading to the production of intracellular oxidants.

Initiation of NADPH-oxidase activity coincides with degranulation, with a lag phase of approximately 20 s (Segal, 2005; Segal et al., 1980).

Superoxide anion radical, generated by NADPH oxidase, is a substrate for the enzyme superoxide dismutase, which catalyzes the formation of hydrogen peroxide from superoxide anion radical. Hydrogen peroxide is relatively stable and capable of diffusing and penetrating cellular membranes, thereby allowing neutrophils to affect processes at a distance.

Most hydrogen peroxide is consumed by myeloperoxidase (MPO), an abundant hemoprotein of azurophilic granules of neutrophils also found in monocytes and some subtypes of tissue macrophages (Klebanoff, 2005; Winterbourn et al., 2000, 2006). MPO has been assumed to primarily mediate host defense reactions. However, MPO has now been suggested to be profoundly involved in the regulation of cellular and tissue homeostasis (Lau and Baldus, 2006; Nauseef, 2001). MPO catalyzes the formation of hypochlorous acid (HOCl) from chloride and hydrogen peroxide. MPO also oxidizes a wide range of reducing substrates to their corresponding radicals. At physiologic pH levels HOCl exists as a mixture of the undissociated acid and the hypochlorite ions, however, when the pH is lowered, as may occur in the phagosome, HOCl predominates, and it can react with excess chloride to form molecular chlorine (Klebanoff, 2005; Winterbourn et al., 2000, 2006).

It is not clear what, if any, ROS other than superoxide anion radical and hydrogen peroxide are produced in significant quantities in the vacuole (Segal, 2005). Theoretically, these two ROS can combine to generate the highly reactive hydroxyl radical via the Haber-Weiss reaction. Hydroxyl radical could also be formed in the presence of hydrogen peroxide and metal ions in the Fenton reaction. However, this reaction does not occur in intact cells under the physiological conditions because lactoferrin inhibits the generation of hydroxyl radical and other free radical reactions by binding free copper and iron. Another suggested pathway for the formation of hydroxyl radical by stimulated neutrophils is through a MPO dependent mechanism. It was proposed that HOCl reacts with superoxide anion radical to form hydrogen peroxide. However, hydroxyl radicals is formed only if no proteins or other biological targets for HOCl are included in the phagosome. If there is ongoing protein release from either the neutrophil or the ingested bacterium, less than 1% of neutrophil superoxide anion radical production could be accounted for by the formation of low levels of hydroxyl radical by this mechanism. Nevertheless, when a new spin trap procedure for the detection of hydroxyl radical was applied to neutrophils by Ramos et al. hydroxyl radical formation was detected (Ramos et al., 1992). Even such low hydroxyl radical production can be of physiological significance, because hydroxyl radical is an extremely reactive radical (Klebanoff, 2005; Lymar and Hurst, 1995; Ramos et al., 1992).

Besides ROS, neutrophils are also able to produce reactive nitrogen species, the production of which is facilitated by inducible nitric oxide synthase, which catalyzes the production of nitric oxide from oxygen, L-arginine, and NADPH. However, the ability of neutrophils to yield nitric oxide is much less than that of macrophages and NO dependent pathways of lipid oxidation are not discussed in this paper.

Neutrophil Dependent Lipid Peroxidation

Different enzymatic and non-enzymatic pathways contribute to the formation of lipid oxidation products by neutrophils. The main enzymatic participants are lipoxygenases, cyclooxygenases, cytochrome P450 mono-oxygenases and others (Cook, 2005). While it is clear that these specific enzymatic pathways and their products such as prostaglandins and leukotriens are crucial in formation of biologically active metabolites of lipid oxidation, other pathways directly connected with respiratory burst of phagocytes my also play key roles. Therefore this review will focus on the induction of lipid peroxidation dependent on ROS produced during respiratory burst of neutrophils and role of enzymes primarily involved in respiratory burst process (Fig. 5.2).

A plethora of studies indicate activated neutrophils participate in lipid and lipoprotein oxidation (Carr and Frei, 2002; Cathcart et al., 1985; Frei et al., 1988; Schmitt et al., 1999; Zhang et al., 2002a, b). Under inflammatory conditions, large amounts of ROS produced by hyperactivated neutrophils can damage all biological molecules including lipids and cholesterol. However, neutrophil-derived oxidants like superoxide anion and hydrogen peroxide are not reactive enough to initiate lipid peroxidation, but this could be induced by neutrophil-derived hydroxyl radical or

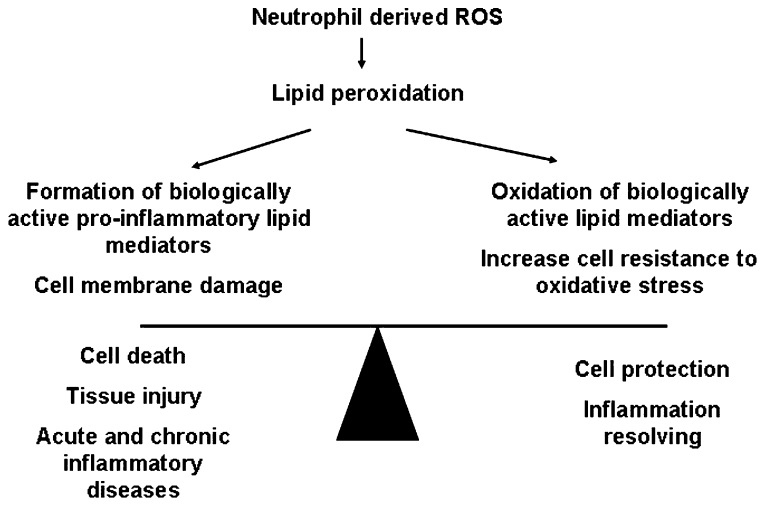

Fig. 5.2 Balance of lipid oxidation. Under inflammatory conditions neutrophil derived ROS significantly contribute to initiation of lipid peroxidation and formation of oxidized lipid metabolites. These processes lead to damage of cellular structures causing tissue injury and to general promotion of acute and chronic inflammatory process. On the other side low levels of biologically active oxidized lipids induce activation of cellular protective mechanisms and increase cell resistance to oxidative damage. Further, neutrophil derived ROS significantly contribute to oxidative catabolism of pro-inflammatory lipid metabolites supporting a termination of inflammatory process. Thus, a well-balanced level of the lipid oxidation is required for the proper functioning of organism

oxidants formed via the reaction of peroxidases with hydrogen peroxide as a substrate. Hydroxyl radical is one of the most reactive oxidants but its physiological importance in the neutrophil-dependent initiation of lipid peroxidation is unresolved due to its very low concentration and extremely short half time (see above). That is why a role for MPO may be suggested given that it generates a wide range of reactive oxidants and diffusible radical species capable of initiating lipid peroxidation. The role of MPO in the mechanism of lipid peroxidation in plasma was demonstrated as the neutrophils isolated from MPO-deficient subjects fail to initiate lipid peroxidation in plasma in contrast to neutrophils isolated from healthy subjects but did so after the addition of isolated MPO (Zhang et al., 2002b). Interestingly, the formation of 9-hydroxy-eicosatrienoic and 9-hydroxy-octadecadienoic acids involved MPO-dependent use of multiple low-molecular–weight substrates (nitrite, tyrosine, and thiocyanate) (Zhang et al., 2002).

Similarly, studies with MPO knockout mice demonstrated that MPO was a major catalyst of lipid peroxidation As peritonitis-triggered formation of F(2)-isoprostanes, a marker of oxidant stress *in vivo*, was reduced by 85% in the MPO deficient mice (Zhang et al., 2002). Moreover, using a *Candida* sepsis model of inflammation significant reduction of plasma isolevuglandins, a family of ketoaldehydes generated by free radical oxidation of arachidonate-containing lipids through the isoprostane pathway, was observed in MPO deficient mice compared to wild types (Poliakov et al., 2003). However, studies questioning the contribution of MPO to neutrophil-dependent formation of lipid signaling mediators exist. For example, Noguchi et al. observed only modest differences in *ex vivo* hydroperoxide formation capacity of leukocytes isolated from wild-type and MPO knockout mice. (Noguchi et al., 2000).

Different mechanisms of initiation of lipid peroxidation, such as Direct catalysis of unsaturated lipid oxidation by MPO are plausible as MPO belongs to the family of heme peroxidases, which are versatile catalysts that perform oxidative reactions of numerous substrates including lipids (Duescher and Elfarra, 1992; Ortiz de Montellano, 1992). Various experimental data support this suggestion. MPO catalyses enantioselective epoxidation of styrene and a number of substituted derivatives, which is believed to be a difficult oxygen transfer process due to the higher redox potential of alkenes (Tuynman et al., 2000). Correspondingly, MPO has been shown to catalyze epoxidation of butadiene (Duescher and Elfarra, 1992). Similarly to MPO, direct epoxidation of styrene and its derivatives has been reported for other heme peroxidases chloroperoxidase from *Caldariomyces tumavo*, cytochrome c peroxidase, and Coprinus cinereus peroxidase (Ortiz de Montellano, 1992; Tuynman et al., 2000). The suggested mechanism of MPO catalyzed reactions, similarly to other peroxidases in general, comprises two consecutive one-electron transfers in the oxidation of organic compounds at the expense of one equivalent of hydrogen peroxide, involving two intermediate forms of the enzyme, compounds I and II, each of which is able to abstract one electron from the substrate to produce a free radical (Klebanoff, 2005; Winterbourn et al., 2000, 2006). The free radical may be subjected to coupling, disproportionation, and reaction with molecular oxygen or with another substrate molecule (Mason and Chignell, 1981). Apart from

this mechanism, a direct oxygen transfer from compound I to substrates will bypass compound II and return the enzyme directly to the native state that is the putative mechanism for chloroperoxidase in the epoxidation of alkenes, the so-called ferryl-oxygen transfer mechanism (Mason and Chignell, 1981; Ortiz de Montellano, 1992). It has also been also suggested that electron transfer from the alkene to compound I may occur first, forming a compound II-like species and an intermediate radical cation that can undergo different fates, leading to the formation of epoxides or aldehydes (Ortiz de Montellano, 1992).

In addition to direct initiation of lipid oxidation, other indirect mechanisms of MPO induced lipid peroxidation may exist. The indirect initiation of lipid peroxidation involve MPO-generated diffusible radical species generated in the presence of low-molecular weight intermediates, such as chloride, nitrite or tyrosine. In the presence of chloride, MPO generates highly reactive HOCl, the major and best characterized oxidant produced by MPO reactions. Reaction of HOCl with unsaturated fatty acids leads to a formation of either chlorhydrins or lipid peroxides (Spickett et al., 2000). Production of chlorhydrins by reaction of HOCl reagent or HOCl generated by the MPO/hydrogen peroxide/chloride system with olefinic double bonds of cholesterol and unsaturated fatty acids was reported by various authors (Arnhold et al., 2001; Carr et al., 1996; Hazell et al., 1994; Heinecke et al., 1994; Panasenko et al., 2002, 2003; Spickett et al., 2000; van den Berg and Winterbourn, 1994; Winterbourn et al., 1992). However, formation of other oxidized forms of unsaturated fatty acids were also observed including epoxides (Hazell et al., 1994; Hazen et al., 1996; Heinecke et al., 1994; Panasenko et al., 2002; van den Berg and Winterbourn, 1994; van den Berg et al., 1993), hydroxyl- and keto-derivatives (Hazell et al., 1994; van den Berg et al., 1993), glycols (Arnhold et al., 2001; Panasenko et al., 2002) and lyso compounds (Panasenko et al., 2003). Further, cholesterol-phosphatidylcholine multilamellar vesicles exposed to a MPO/hydrogen peroxide/chloride system or HOCl reagent produced cholesterol epoxides and chlorohydrins (Heinecke et al., 1994). Hazen et al. found dichlorinated sterol, cholesterol a- and b-chlorohydrins, and a structurally related cholesterol chlorohydrins by examination of LDL cholesterol oxidation by MPO hydrogen peroxide/chloride system under acidic conditions (Hazen et al., 1996). The chlorhydrin formation is a two step reaction involving an electrophilic addition reaction of HOCl (Heinecke et al., 1994; Spickett et al., 2000; van den Berg and Winterbourn, 1994; Winterbourn et al., 1992). Hazen et al. demonstrated LDL cholesterol oxidation to be mediated by molecular chlorine, which may arise either directly from HOCl or indirectly from a chloramines intermediate (Hazen et al., 1996). Conversion of chlorhydrins to epoxides and trans-hydroxy compounds was suggested to require alkaline conditions (van den Berg and Winterbourn, 1994; Winterbourn et al., 1992). Lipid peroxidation is also a possible alternate reaction of HOCl with polyunsaturated fatty acids if pre-formed lipid hydroperoxides, formed by additional radical source, reacts with HOCl, however, the mechanism of the reaction is still unknown (Spickett et al., 2000).

Another low molecular intermediate, converted by MPO/hydrogen peroxide system to an alternative diffusible radical, is nitrite, a decomposition product of

nitric oxide. A one electron oxidation product formed by nitrite/MPO/hydrogen peroxide system is nitrogen dioxide. Nitrogen dioxide acts as a physiological catalyst for the initiation of lipid peroxidation by efficient abstraction of a bis-allylic hydrogen atom (Podrez et al., 1999, 2002; Schmitt et al., 1999). In general, nitrogen dioxide formed in MPO/hydrogen peroxide/nitrite system was shown to nitrite phenolic compounds and proteins confirming that nitrite can serve as a physiological substrate for MPO independent of presence or concentration of chloride (Brennan et al., 2002; Eiserich et al., 1996, 1998). Strong evidence exists that MPO in the presence of nitrite may serve as a physiological mechanism for initiation of lipid peroxidation at the site of inflammation. Podrez et al. described rapid initiation of lipid peroxidation of LDL and phosphatidylcholine molecular species by MPO/hydrogen peroxide/nitrite system leading to formation of NO_2-LDL and multiple lipid peroxidation products including lipid hydroperoxides, suggesting that the oxidant generated by MPO/hydrogen peroxide/nitrite system readily promotes hydrogen atom abstraction in unsaturated lipids (Podrez et al., 1999, 2002). Similarly, exposure of LDL to the MPO/hydrogen peroxide/nitrite system resulted in time-dependent formation of apolipoprotein B-100 adducts which was dependent on the presence of nitrite (Poliakov et al., 2003). Interestingly, the nitrating intermediates formed by neutrophil- and MPO dependent oxidation of nitrite initiated formation of biologically active oxidized lipids including hydroxy-eicosatrienoic acid, hydroperoxy-eicosatrienoic acid, hydroxy-octadecadienoic acid, and hydroperoxy-octadecadienoic acid oxidation products of linoleic and arachidonic acids (Schmitt et al., 1999). The formation of biologically active lipid metabolites by neutrophil- and MPO dependent pathways suggest potential regulatory role of MPO formed lipid peroxides in control of physiological processes including regulation of the course of inflammatory process.

The third type of diffusible radical formed by MPO catalyzed reactions capable of initiating lipid peroxidation is tyrosyl radical. Tyrosyl radical is formed by MPO/hydrogen peroxide system by oxidation of L-tyrosine (Heinecke, 2002; Heinecke et al., 1993; Kapiotis et al., 1997, 2005; Savenkova et al., 1994). Tyrosyl radical is a diffusible resonance-stabilized intermediate which initiates lipid peroxidation by abstracting hydrogen from bis-allylic methylene groups of polyunsaturated fatty acids (Kapiotis et al., 1997, 2005; Savenkova et al., 1994).

Pathological Effects of Lipid Peroxidation

A number of diseases in which lipid peroxidation play a role have been described including atherosclerosis, cancer, diabetes, neurodegenerative and pulmonary disorders among others (Brennan and Hazen, 2003; Cohen, 2002; Leitinger, 2003; Nicholls and Hazen, 2005; Podrez et al., 2000; Rubbo and O'Donnell, 2005; Sasaki, 2006; Sultana et al., 2006). Oxidation of LDL particles and its role in pathogenesis of atherosclerosis is probably one of the most active fields (Leitinger, 2003; Rubbo and O'Donnell, 2005). Lipid peroxidation as a key step in atherogenesis was

first proposed by Chisolm et al. who reported that oxidatively modified LDL could be cytotoxic (Hessler et al., 1983; Morel et al., 1983, 1984). Shortly after that the crucial role of ROS produced by phagocytes in the oxidation of LDL was recognized (Cathcart et al., 1985, 1988). Nowadays, oxidation of LDL is well established as a risk factor for atherosclerosis (Brennan and Hazen, 2003; Nicholls and Hazen, 2005; Podrez et al., 2000; Stocker and Keaney, 2004). However, while it is undisputed that lipid oxidation is a central feature of atherosclerosis, it is still unclear whether this process plays a causative role (Rubbo and O'Donnell, 2005).

Various ROS could be theoretically involved. However looking at ROS produced by neutrophils, LDL constituents are not chemically susceptible to oxidation by superoxide anion since is not reactive enough toward LDL. However, superoxide anion-dependent LDL oxidation can be produced by the near diffusion-limited recombination of superoxide with nitric oxide to yield peroxynitrite, or via formation of hydrogen peroxide which is then used as a substrate for peroxidases (Rubbo and O'Donnell, 2005). Interestingly, MPO has emerged as a potential participant in the promotion and/or propagation of atherosclerosis (for reviews see Brennan and Hazen, 2003; Heinecke, 2002; Lau and Baldus, 2006; Nicholls and Hazen, 2005; Podrez et al., 2000; Shao et al., 2006). Role of MPO in atherosclerosis has been suggested by its presence in an active form in atherosclerotic lesions (Daugherty et al., 1994), detection in lesions of both HOCl-modified epitopes of LDL (Hazell et al., 1994; Marsche et al., 2004) and chlorinated lipid oxidation products α-chloro fatty aldehydes. (Thukkani et al., 2003). Further, reactive intermediates formed by MPO catalyzed reactions lead to the production of biologically active oxidized lipids and to the conversion of low density lipoprotein into an atherogenic form (Podrez et al., 1999; Poliakov et al., 2003).

Thus the effects of oxidised LDL (oxLDL) are suggested to be important in pathogenesis of cardiovascular diseases. (Collot-Teixeira et al., 2007; Stocker and Keaney, 2004). Significant attention has been focused on the oxidation products of cholesterol associated with the peroxidation of PUFA within LDL (Carpenter et al., 2003). Direct radical attack on cholesterol will yield different oxysterols, like 7-hydroxycholesterols, 7-ketocholesterol or the 5,6-epoxides (for review see (Leonarduzzi et al., 2002; van Reyk et al., 2006) and elevated levels of these have been reported in various disease states (multiple sclerosis, senile cataracts, diabetes, atherosclerotic plaques) (for review see van Reyk et al., 2006).

Thus, under various inflammatory conditions a number of lipid peroxidation products with strong biological activities are produced. Among them are lipid hydroperoxides which appear relatively early on in the oxidation and aldehydes which arise from their subsequent breakdown. These products have cellular half-lives upward of 2–3 min and are therefore capable of diffusing to more distant sites within the cell to interact with DNA or proteins (Sasaki, 2006). Aldehydes such as, MDA and HNE have the ability to inactivate phospholipids, proteins, and DNA by binding to or cross-linking between these molecules (Esterbauer and Cheeseman, 1990; Sultana et al., 2006). Considerable attention has been paid to HNE, a major degradation product of n-6 polyunsaturated fatty acids (for review see Dickinson et al., 2003; Leonarduzzi et al., 2005; Petersen and Doorn, 2004; Poli et al., 2004;

Yang et al., 2003). Evidence suggests that high concentrations of HNE are cytotoxic, whereas lower concentrations of HNE stimulate neutrophil chemotaxis and platelet aggregation, inhibit protein synthesis and modulate cell proliferation and gene expression and toxicity (Kumagai et al., 2004; Zarrouki et al., 2007). Similarly, acrolein, one of the most reactive unsaturated aldehyde products of lipid peroxidation, could be rapidly incorporated into proteins, generating a carbonyls or modifying DNA basis with the formation of exocyclic adducts. Acrolein may inactivate the reductase responsible for reducing vitamin E radicals, and, together with depletion of glutathione, this could lead to further lipid peroxidation (Sultana et al., 2006). The pathological role of acrolein has been mostly described in brain tissue where it has been shown to be highly neurotoxic in a time- and concentration-dependent manner.

Other degradation products of hydroperoxides are far less characterized and bear epoxy, alcohol, or ketone group(s), either alone or in combination, even with an aldehyde function (hydroxyaldehydes). Isoprostanes non-enzymatic *in vivo* lipid peroxidation products, formed (for review see (Montuschi et al., 2004, 2007) *specifically* F(2)-isoprostanes exert potent vasoconstrictive activities, thereby suggesting a role in chronic inflammatory diseases such as atherosclerosis. They have also been suggested to be useful *in vivo* biomarkers of lipid peroxidation (Montine et al., 2004; Montuschi et al., 2004, 2007; Sultana et al., 2006). Another arachidonic acid peroxidation product generated by free radical mechanism independent of the cyclooxygenase pathway *in vivo* are the isofurans (for review see Fessel and Jackson Roberts, 2005; Montine et al., 2004). Isofurans are related biosynthetically to the isoprostanes and chemically characterized by a substituted tetrahydrofuran ring structure hence the name. These molecules are of interest as they provide a sensitive index of free-radical peroxidation under conditions of elevated oxygen tension

Oxidative Catabolism of Biologically Active Lipids

Lipid peroxidation reactions as damaging and inflammation promoting processes are based largely on the reasoning that oxidants are directly damaging to the structural makeup of cells, surrounding matrices, and key functional pathways. However, catabolism of pro-inflammatory mediators by their oxidative inactivation could have arguable positive effects. Example of these reactions is the oxidative inactivation of leukotrienes (LTs). LTs, both formed and degraded by leukocytes, are products of lipoxygenase pathway of arachidonic metabolism which contain three conjugated double bonds (Werz and Steinhilber, 2006). LTs are important biologically active lipid metabolites with strong pro-inflammatory effects including stimulation of various leukocyte functions such as chemotactic movement. Cysteinyl LTs consist of LTC4, which contains glutathione in thioesther linkage at C-6, its cysteinylglycine derivative, LTD4, and its cysteinyl derivative, LTE4 (Stenke et al., 1994). Neutrophils degrade LTs by sulphur oxidation of cysteinyl-LTs which was clearly demonstrated to be dependent on MPO (Henderson et al., 1982; Henderson and

Klebanoff, 1983a; Lee et al., 1983; Neill et al., 1985). Another possible site of attack were suggested to be the double bonds of the fatty acid that may be subjected to oxidative cleavage and to halogenation (Henderson et al., 1982). Crucial role of MPO in this process was demonstrated by MPO-dependent LTs inactivation in purified MPO cell-free system and in system containing a supernatant from activated neutrophils (Henderson and Klebanoff, 1983; Lee et al., 1983). Further, MPO deficient neutrophils activated by different stimuli had significantly decreased capacity to degrade LTs, unless MPO was added (Henderson et al., 1982; Henderson and Klebanoff, 1983; Lee et al., 1983). Similarly, MPO deficient monocytes activated by different stimuli did not degrade LTC4, unless MPO was added (Neill et al., 1985). Interestingly, a secondary mechanism of LTs inactivation involving hydroxyl radicals has been recognized in studies with NADPH oxidase-deficient human neutrophils and cell free systems (Henderson and Klebanoff, 1983a, b). However, the significance of this mechanism in normal neutrophils containing active peroxidases is unclear. Chemically synthesized HOCl inactivated such cysteinyl LTs and yields the same chemical products as activated neutrophils or the isolated MPO cell free systems further suggested the role of MPO in this process. The oxidation products of MPO catalyzed degradation of LTs were shown to be biologically inactive (Lee et al., 1983). Similarly to MPO, eosinophil peroxidase (EPO), an abundant peroxidase of eosinophil polymorphonuclear granulocytes, was demonstrated to inactivate LTs biological activity (Henderson et al., 1982). EPO degradation of LTs was observed both in chemical cell-free system with purified EPO and hydrogen peroxide and with isolated eosinophil granulocytes activated with diverse stimuli (Henderson et al., 1982; Weller et al., 1983). EPO inactivated both LTB4 and cysteinyl LTs (LTC4 and LTD4), however, the cysteinyl LTs with faster rate (Henderson et al., 1982). Taken together, the destruction of leukotrienes through MPO and EPO catalyzed oxidative mechanisms could be proposed as an significant regulatory mechanism for down-regulation of inflammatory process.

Interestingly, experimental data with MPO deficient mice revealed that MPO deficiency was connected with increased atherosclerosis in a hypercholesterolemic murine model, higher degree of inflammation induced injury such as liver ischemia reperfusion injury, larger infarct volume after focal cerebral ischemia-reperfusion, and higher susceptibility to neurodegenerative autoimmune encephalomyelitis (Brennan et al., 2001a, b; Ichimori et al., 2003; Takizawa et al., 2002). Thus, it could be speculated that MPO dependent oxidation of various inflammatory mediators including LTs could be responsible for these effects.

Conclusions

Neutrophil derived reactive oxygen species significantly contribute to initiation of lipid peroxidation and formation of oxidized lipid metabolites particularly under inflammatory conditions. The induction of lipid peroxidation and formation of various oxidized lipid metabolites are mostly thought to be deleterious and inflammation inducing processes.

However, the oxidation of biologically active pro-inflammatory lipids by reactive intermediates produced by neutrophils may actually have anti-inflammatory effects and thereby contribute to the termination of inflammatory process. Therefore lipid peroxidation mediated by reactive intermediates produced by neutrophils could both potentiate and reduce inflammatory reaction. Thus, well balanced ratio of lipid oxidation is likely required for the proper functioning of organism.

Acknowledgement The study was supported by grants No. 524/06/1197 and No.525/06/1196 of the Grant Agency of the Czech Republic, grant No. 1QS500040507 of the Grant Agency of Academy of Science of the Czech Republic and research plan AVOZ50040507.

List of Abbreviations

PUFAs	polyunsaturated fatty acids
HNE	4-hydroxy-2,3-trans-nonenal
MDA	malondialdehyde
ROS	reactive oxygen species
NADPH	reduced nicotinamide adenine dinucleotide phosphate
MPO	myeloperoxidase
HOCl	hypochlorous acid
LDL	low-density lipoprotein
oxLDL	oxidised LDL
LTs	leukotrienes
EPO	eosinophil peroxidase

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Chapter 6
Fatty Acids in the Modulation of Reactive Oxygen Species Balance in Cancer

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Abstract Oxidants, in addition to their accepted role as cytotoxicity agents, can mediate specific cellular and molecular responses and expression of genes involved in pathophysiological conditions, such as inflammation and cancer. Their effects are mainly mediated by oxidant-induced cellular oxidation-reduction (redox) imbalance. Dietary fat, especially the content and type of polyunsaturated fatty acids (PUFAs), is the source of oxidants (reactive oxygen and nitrogen species and lipid peroxides) which cause oxidative alteration of biomembranes and modulate inter- and intracellular signalling resulting in changes of cell proliferation, differentiation, and apoptosis. Here, mitochondria (especially oxidation of cardiolipin and activity of cytochrome c oxidase) play an important role. Changes of oxidative/antioxidative balance may thus contribute to deregulation of the above-mentioned processes leading to cancer promotion and progression. On the other hand, the known differences in PUFA content, oxidative metabolism, and antioxidant defence between normal and cancer cells direct the attention to possible application of increased oxidative stress as a novel anticancer strategy, especially by combining PUFAs with other anticancer agents.

Intestinal mucosa is one of the most exposed tissues by diet-derived oxidants. The metabolism and turnover of intestinal epithelial cells are regulated by various endogenous factors. However, they can also be modulated by dietary components. In addition to PUFAs, short-chain fatty acids like butyrate represent important regulators of epithelial cell kinetics and may also affect oxidative status in the tissue.

Our own results are based on the suggestion that in the colon many various agents (exogenous, endogenous) may operate together. Thus, signals from nutritional compounds and endogenous factors regulating cell growth, differentiation, and apoptosis are integrated within the cell and have a substantial impact determining the final phenotype, metabolism, and kinetics of colon epithelial cell population.

We investigated the effects of selected types of n-3 and n-6 PUFAs, sodium butyrate (NaBt), their mutual interaction, as well as their interaction with endogenous

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

regulators from the TNF family on *in vitro* proliferation, differentiation, and apoptosis of normal and/or transformed colon epithelial cells and the association of cytokinetic changes with modifications of cellular lipids, oxidative metabolism, and intracellular signalling.

Keywords Dietary lipids, polyunsaturated fatty acids, butyrate, cytokinetics, oxidative/antioxidative balance, signal transduction, tumour promotion, colon cancer

Introduction

A number of experimental and epidemiological studies support the idea that lipid nutritional components play an important role in the aetiology of certain cancer types such as colon, prostate, and breast (Bartsch et al., 1999; Giardina et al., 1999). Essential polyunsaturated fatty acids (PUFAs) cannot be synthesized by mammals and are required in the diet for the maintenance of health. As components of cell membrane phospholipids (PLs), they play a key role in the metabolism of both normal and malignant cells (Lunn and Theobald, 2006). PUFAs are divided into two main types: the n-6 series, derived from linoleic acid (LA, 18:2, n-6), e.g. γ-linolenic acid (GLA, 20:3) or arachidonic acid (AA, 20:4), and the n-3 series, derived from α-linolenic acid (ALA, 18:3, n-3), e.g. eicosapentaenoic acid (EPA, 20:5) or docosahexaenoic acid (DHA, 22:6). Their availability depends on external supply in the diet and thus, the changes of cell membrane composition become independent of the genome. It has been shown that the quantitative and qualitative content of PUFAs, particularly the ratio of n-3 and n-6 type in the diet, is highly important (Simopoulos, 2002). These PUFAs may assert themselves in the initiation stage of carcinogenesis, especially through enzymatic systems activating or metabolizing procarcinogens such as prostaglandin G/H synthase and cytochrome P-450, and play the main role in the promotion stage of carcinogenesis as well as in the metastatic process, affecting directly or indirectly various cellular and molecular events. In spite of much contradiction in the literature, it is generally thought that high calorie and fat intake is a risk factor in cancer development and that n-6 PUFAs (from plant oils rich in linoleic acid) can be positively correlated with this process. On the other hand, PUFAs of the n-3 series, ALA, and especially EPA and DHA acids (rich in fish oil), were shown to be protective against cancer development (for review see (Diggle, 2002; Moyad, 2005)). However, the precise role of n-6 and n-3 PUFAs in carcinogenesis and the mechanisms of their effects on various cell types remain yet to be elucidated.

Luminal nutrients and the products of their metabolism (oxidants, mutagens, carcinogens) directly or indirectly influence intestinal metabolic homeostasis, induction of inflammation, and the balance between cell growth and death. Among them, the types of dietary fat and fibre are thought to have the most significant impact on colon carcinogenesis (Chapkin et al., 2002). Epidemiological and experimental studies

confirm a protective role of dietary fibre, whose effects are partially associated with short-chain fatty acids, especially butyrate, produced in the gastrointestinal tract by fibre anaerobic fermentation (Sengupta et al., 2006). Moreover, mutual interaction of butyrate and PUFAs (particularly of the n-3 type present in fish oil) and their beneficial effects in colon inflammation and carcinogenesis is supposed. In addition to the many mechanisms and pathways modulated by these components in various cellular systems *in vitro* and *in vivo*, the changes of oxidative metabolism and redox balance remain one of the most important topics (Ng et al., 2005; Sanders et al., 2004a).

Effects of Redox Balance on Cytokinetics and Its Role in Carcinogenesis

Reactive Species in Regulation of Cytokinetics

Balance in signals regulating proliferation, differentiation, and apoptosis (cytokinetics) is a key factor for the maintenance of cell and tissue homeostasis. This is controlled by both extrinsic and intrinsic signals and their discrepancy may lead to the development of pathogenesis of various diseases. Reactive oxygen species (ROS), such as superoxide anion ($O^{\cdot}_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$) along with reactive nitrogen species (RNS), e.g. nitric oxide (NO), are important for several normal biological processes. However, they play an important role in initiation and progression of pathological states such as diabetes, atherosclerosis, arthritis, ischemic reperfusion injury, neurodegenerative disorders, and malignant diseases (for review see (Valko et al., 2007)). Under normal conditions, the intracellular level of ROS is tightly balanced by antioxidants and scavenging systems. Disruption of such a balance as a result of the presence of xenobiotics, carcinogens, chronic inflammation, metabolic changes, and other events may significantly contribute to the development of cancer (for review see (Storz, 2005; Valko et al., 2006; Waris and Ahsan, 2006; Wu, 2006)).

Probably the best known mechanism how cellular redox imbalance can lead to cancer is induction of oxidative DNA lesions (Valko et al., 2004). ROS-induced DNA breaks, cross-links, and base modifications result in genomic instability and mutation, which can directly result in the development of malignancy. However, the variety of ROS effects indicates that their contribution to cancer development can mechanistically occur also at other levels. It has been clearly shown that ROS can directly interact with various signalling pathways and modulate expression of numerous genes involved in the regulation of cytokinetics and malignant transformation (Boonstra and Post, 2004; Gius and Spitz, 2006; Havens et al., 2006; Valko et al., 2006). Moreover, the role of ROS themselves in cancer is complicated through their "two-faced" effect. On the one hand, oxidative stress is clearly associated with higher cancer risk, but, on the other hand, ROS can play an antitumorigenic role through the induction of senescence and apoptosis (Ryter et al., 2007).

These effects of ROS are crucial for the action of anticancer therapies such as photodynamic therapy (Triesscheijn et al., 2006) and some types of chemotherapy (Chou and Dang, 2005).

Lipid Peroxidation

Unsaturated phospholipids (PLs) and cholesterol make biomembranes and other lipid-containing structures prominent targets for oxidation by ROS and also non-radical species such as ozone, oxygen or peroxynitrite. This can lead to lipid peroxidation and processes which affect the structure and function of the target system (Girotti, 1998). A cascade of reactions leads to final products of lipid peroxidation, which is malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal (HNE) (Cheng and Li, 2007). MDA is a mutagenic and carcinogenic aldehyde which can directly react with DNA bases and form adducts. HNE is a less mutagenic but more toxic compound which is able to modify proteins and their function (West and Marnett, 2005). Basically, there are two fates for HNE-modified proteins, either degradation by proteasome or cross-linking and their accumulation (Grune and Davies, 2003). This can be reflected in the modulation of signal transduction, gene expression (West and Marnett, 2005), regulation of cytokinetics, and development of pathological states (for review see (Dwivedi et al., 2007; Petersen and Doorn, 2004)). Lipid peroxidation is implicated in various diseases including atherosclerosis, arthritis, or neurodegenerative disorders. However, its role in cancer development and progression is not fully understood. Oxidative stress has been demonstrated as an important factor in metastasis, as it is involved in the loss of cell adhesion. ROS-induced DNA damage has also been linked to metastatic progression (Malins et al., 1996). Thus, finding practical ways for modulation of lipid peroxidation and its negative effects could have an extensive impact in the chemoprevention and therapy of various diseases. From this point of view, the inhibition of oxidative stress and induction of lipid peroxidation by nutritional antioxidants (Aviram et al., 2005; Singh et al., 2005) and modulation of intracellular detoxification and antioxidative systems (Caro and Cederbaum, 2006; Niki et al., 2005) seem to be the most prospective approaches.

A detailed description of the complex role of ROS, RNS, and lipid peroxidation in the multi-step process of cancer development would be helpful for approaching specific procedures for the modulation of their diverse effects uniquely in chemoprevention (decrease of DNA damage), cancer progression (inhibition of migration and metastasis), and therapy (boosting of efficiency).

Fatty Acids in Cell Physiology and Cancer

The main physiological role of PUFAs is concerned with the structure and function of cell membranes, and with the synthesis of intermediate compounds that regulate cellular metabolism and function. Changes of the fatty acid spectrum influence

membrane fluidity, potential, ligand-receptor interactions, and other membrane-mediated processes (Los and Murata, 2004). PUFAs play an important role in transduction of signals from extracellular space and function as inter- and intracellular mediators and modulators of the cellular signalling network (Di Marzo, 1995). They have been shown to control the events in the nucleus, mainly by the interaction with intracellular receptors and transcription factors, thus directly affecting gene transcription (Deckelbaum et al., 2006).

For a considerable time, the research of PUFA effects has been focused on the changes of oxidative metabolism, i.e. ROS and RNS production, and lipid peroxidation and modulation of the production of biologically active regulatory intermediates formed from PUFAs, which include prostaglandins (PGs), leukotrienes (LTs), hydroxy acids, and other products, collectively termed eicosanoids. They are derived predominantly from AA and have many functions including regulation of intracellular calcium and second messenger molecules, and modulation of inflammation and host defence (Soberman and Christmas, 2003). The effects of PUFAs and their metabolites on various levels of cell organization and their interaction with other endogenous or exogenous factors can finally significantly influence cell proliferation, differentiation, and apoptosis of various cell types (Rudolph et al., 2001). Undoubtedly, the action of PUFAs is complex and involves a number of integrated signalling pathways that are necessary to be elucidated. Fig. 6.1 schematically shows the most important levels of cell organization where PUFAs exert their effects through modulation of redox balance.

Studies on the effects of PUFAs on cell proliferation and their role in carcinogenesis have had a long history. More than 20 years ago, Cornwell and Morisaki summarized the knowledge about the PUFA effects on the cells and defined the "fatty acid and prostaglandin paradox" (Cornwell and Morisaki, 1984). Considering many experimental data they reported that low concentrations of n-6 PUFAs as well as PGs stimulate cell growth, while higher concentrations of these compounds inhibit proliferation. Moreover, they recognized the important role of lipid peroxidation and antioxidants in these effects. Since then, a lot of further *in vitro* and *in vivo* experimental studies and some clinical and epidemiological data have supported the idea that lipid nutritional components play an important role in the regulation of cytokinetics and in the aetiology of certain types of cancer, particularly in colon, breast, and prostate (Bartsch et al., 1999; Griffiths et al., 1997; McEntee and Whelan, 2002).

Recently, it has been suggested that a complex view on lipid interactions, which defines the resulting "lipidome" – the individual lipid profile, is necessary. Rather than individual fatty acids, a complex indicator combining the increased content of monosaturated fatty acids and the low n-6/n-3 ratio could be associated with protection against certain types of cancer (Kang, 2005). Thus, the lipidome could become a template for the detection of cancer risk in relation to diet and for dietary recommendations aimed at cancer prevention (Bougnoux et al., 2006). Recently, the experiments using introduction of the newly revealed fat-1 gene (encoding an n-3 fatty acid desaturase) in cells and laboratory animals have confirmed anti-inflammatory and anticancer effects of n-3 PUFAs (Hudert et al., 2006; Xia et al., 2006).

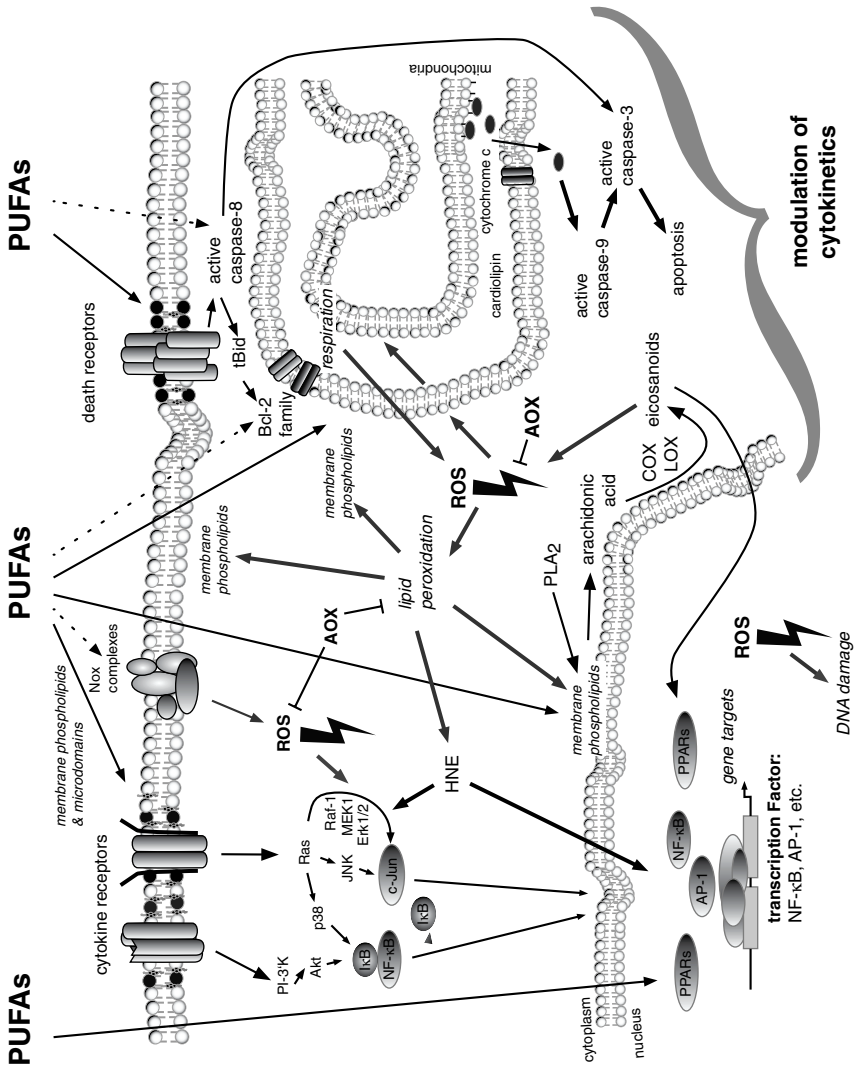

Fig. 6.1 Mechanisms of PUFA effects involving oxidative metabolism at various levels of cell organisation (straight line ~ direct effects, dotted line ~ indirect effects)

Because of inconsistence of experimental, clinical and epidemiological data, further studies elucidating and verifying the cellular and molecular mechanisms of n-6 and n-3 PUFA action are necessary for a better understanding of their role in human cancer risk (Larsson et al., 2004; Siddiqui et al., 2004).

Impact of PUFAs on Anticancer Therapy

Experimental as well as several preclinical studies have shown that certain PUFAs, especially of the n-3 type, may actually depress tumour growth and enhance tumour responsiveness to radiation and anticancer drugs (Conklin, 2002). Supplementation of tumour cells with PUFAs changes membrane properties (increased fluidity, an elevated unsaturation index, enhanced transport capabilities) and may shift the uptake and intracellular distribution of chemotherapeutic drugs. This results in an increased sensitivity to chemotherapy, especially in resistant tumour cell lines, and causes selective enhancement of cytotoxicity to tumour cells and protection of normal cells. Modulation of redox balance is supposed to be an important mechanism of increasing the efficacy of many types of anticancer therapies by PUFAs. These include radiation, hyperthermia, photodynamic therapy, and some chemotherapeutic drugs such as doxorubicin, epirubicin, CPT-11, 5-fluorouracil, and tamoxifen (Kokura et al., 2002; Norman et al., 2003; Sagar and Das, 1993). The anticancer activity of cisplatin has been shown to be effectively supported by fish oil diet under a conditioned balance of oxidation and antioxidation adjusted by vitamins E and C (Yam et al., 2001). Therefore, it appears that the administration of n-3 PUFAs might be a relatively non-toxic form of supportive therapy improving cancer treatment outcomes and slowing or preventing the recurrence of cancer. In addition, certain PUFAs may also prevent or reduce some of the side effects of the therapies and tumour cachexia (Jho et al., 2004).

PUFAs and Redox Balance

As mentioned above, long time ago it was known that both cancer promoting and antiproliferative and apoptotic effects of certain PUFAs reported in various systems in *vitro* and *in vivo* were associated with the modulation of oxidative metabolism and the induction of oxidative stress. Susceptibility of double bonds of PUFAs to oxidation is the reason for the production of various types of biologically active metabolites such as eicosanoids, ROS and RNS, and lipid peroxides, which may significantly alter the redox balance in cells and tissues (Maziere et al., 1999).

Superoxide and hydrogen peroxide at low concentrations are effective stimulators of transformed cell growth. In contrast, high concentrations of the same oxidative species have been shown to be highly cytotoxic. NO and its reactive products are mutagenic and have the potential to produce nitration, nitrosation, and deamination

of DNA bases. Tumour-derived NO promotes tumour growth and metastasis. This is associated with activation of COX-2 and an increased production of PGE_2 (Chen et al., 2006). The oxidation of unsaturated lipids in complex biological tissues and thus the kind and quantity of oxidative species produced *in vivo* depend on the amount and type of fat used as a dietary source, cell and tissue fatty acid composition, the amount and type of antioxidants present, the activity of antioxidative enzymes like glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), the quantity and nature of metal ions, divalent cations, pH, etc. If lipid peroxidation overwhelms the normally efficient cellular protective mechanisms, it causes genotoxicity or cytotoxicity and subsequently cell death (Salganik, 2001). Thus, the final response depends on the environment where these reactions take place, and on the sensitivity of the cells to the particular level/type of oxidative species. During the last twenty years, several studies have shown that the addition of some PUFAs to a range of malignant cells *in vitro* or *in vivo* results in cell death, while normal cells are unaffected (Begin, 1987). This is associated with the fact that PUFAs augmented free radical generation and formation of lipid peroxides selectively in the tumour cells compared to normal cells despite the fact that the uptake of fatty acids in the tumour cells was lower (Das et al., 1987). Many types of tumour cells have a low concentration of PUFAs that can be attributed to the loss or decreased activity of desaturases and thus a decreased metabolism of LA and ALA to longer chain fatty acids such as GLA and AA or EPA and DHA. Tumour cells have a low level of cytochrome P450 enzymes which can initiate and propagate LP (Gonzales et al., 1995). Moreover, malignant cells have markedly enhanced levels of antioxidants (e.g. vitamin E and oleic acid) and low levels of SOD, GPx, and CAT enzymes. The decreased susceptibility to LP and the relatively increased antioxidant capacity might contribute to a decrease in the cytotoxic and cytostatic effects of ROS and aldehydes. The resultant lowering in the production of the substrates for LP is believed to lead to a failure of regulation of cell division. It is suggested that ROS, RNS, and LP are powerful inducers of p53 activity, influence the expression and activity of members of the Bcl-2 family, activate caspases and shorten telomeres, thus inducing apoptosis of cancer cells. They were also shown to play a role in the metastatic process and angiogenesis (Das, 2002).

Oxidative Changes of Cell Membranes

The cell membrane responds to various exogenous stimuli by activating membrane-associated enzymes. Phospholipases A_2 cleave PLs in an sn-2 position of the glycerol backbone liberating PUFAs which serve as a substrate for various types of oxygenases, especially cyclooxygenase (COX) and lipoxygenases (LOX). Lipid hydroperoxides (LOOHs) formed by LOX are short-lived intermediates which are quickly reduced to hydroxy acids and transformed to carbonyl and epoxy derivatives like leukotrienes. It has been reported that LA, the most abundant PUFA in the diet, is even a better substrate for LOX than AA and many other LA-derived oxidation

products like unsaturated aldehydes that induce apoptosis have been detected in oxidized LDL (Spiteller, 2002). The important role of a specific type of 15-LOX for dioxygenation of the most abundant PUFAs (LA, ALA, AA, EPA, DHA) in both free and esterified forms and its connection with the activity of PLA_2 has been reported (Nigam and Schewe, 2000).

Changes of the cell membrane associated with cell stimulation, aging, wounding, cell proliferation, etc. may cause lipid peroxidation. Varying exposures to prooxidants modulate proliferation, apoptosis or necrosis depending on the intensity of the stimulus. It is hypothesized that proliferation is connected with enzymatic LP, while a localized switch to non-enzymatic LP induces apoptosis or necrosis. All these processes are also dependent on membrane fatty acid composition. Supplementation of EPA or DHA in the diet causes their rapid incorporation into the membrane PLs instead of AA, thus altering the lipid composition, lipid raft formation, and membrane subdomain distribution of signal transducing molecules (Seo et al., 2006). PUFAs are thought to be essential components of lipid rafts which represent small platforms of proteins and lipids rich in cholesterol and sphingolipids, and their phospholipid acyl chain contents saturated fatty acids (Stillwell et al., 2005). These structures provide a particular biophysical property of the cell membrane called liquid-ordered state and are functionally implicated in the compartmentalization, modulation, and integration of cell signalling. They are also involved in modulating important processes including cell growth, survival, and adhesion (Pohl et al. 2004). Many proteins involved in signal transduction are modified by acyl moieties to be attached to the plasma membrane and/or concentrated in lipid rafts (Stillwell and Wassall, 2003).

PUFA Metabolic Pathways

Cancer development often associates with activation of PLA_2, elevated AA liberation, and changes in the expression of enzymes controlling the synthesis of eicosanoids – COX and LOX (for review see (Furstenberger et al., 2006)). LOX and COX activity and eicosanoids synthesis can be modified by various diet compounds including PUFAs (Larsson et al., 2004; Wada et al., 2007). Most of the beneficial effects of n-3 PUFAs, particularly their antithrombotic, anti-inflammatory and probably also anticarcinogenic effects, are attributed to the inhibition of the AA metabolism. These PUFAs, namely EPA, compete for the same enzyme to form less bioactive 3-series PGs and thromboxanes, and the 5-series LTs. Recently, using lipidomics approach, anti-inflammatory mediators derived from EPA and DHA called resolvins have been recognized. DHA metabolites comprise several resolvins (RvD1 to RvD6) and docosatrienes. The resolvin formed from EPA, RvE1, inhibited the activation of NF-kB by TNF-α (Serhan, 2005).

There is strong evidence that hydrolysis of phospholipids by PLA_2 and generation of eicosanoids lead to the production of reactive intermediates and lipid peroxidation, which can mediate cytotoxic effects of some PUFAs (Dommels et al., 2003;

Rouzer and Marnett, 2003). COX- and/or LOX-mediated lipid peroxidation can also lead to formation of protein adducts and modulation of transcription (West and Marnett, 2005) and/or to DNA damage and mutations through generation of DNA adducts (Marnett, 2002). Moreover, both COX and LOX enzymes are peroxidases which are able to oxidize and activate various procarcinogens (xenobiotics) and initiate the development of cancer disease (Lim et al., 1999; Vogel, 2000; Wiese et al., 2001). From these observations we can presume that the modulation of the COX and LOX metabolism by PUFAs might be another mechanism how PUFAs affect intracellular redox balance and modulate various signalling pathways. The complexity and importance of the lipid-mediator system in physiology and pathophysiology highlight the potential effect of diet and nutrition on human health. Diet is the source of precursors for oxidants, antioxidants and various lipid mediators, and determines to a great extent the concentration and availability of each mediator (Weylandt and Kang, 2005).

Modulation of Signal Transduction by PUFAs Through ROS

It has been proved by several studies that intracellular redox balance plays an important role in the modulation of signal transduction by various mechanisms. Besides direct effects of PUFAs on signal transduction pathways, several indirect mechanisms of their action may also exist. They may involve the action of their oxidative metabolites or modulation of intracellular redox balance. A detailed description of the positions where PUFAs and lipid metabolism can interact with signal flow in the cells is behind the scope of this review. However, in the following paragraphs we would like to emphasize briefly at least some of the most important mechanisms how PUFAs can modulate signal transduction through ROS and oxidative metabolism.

Mitogen-activated protein kinases (MAPK) are generally well known molecules involved in various cascades of signal transduction. It is generally accepted that MAPK activity can be modulated by ROS to the intent of activation and/or suppression (for review see (McCubrey et al., 2006)). Interestingly, PUFAs and their metabolites can also participate in the regulation of MAPK pathway activity through ROS. It has been shown that AA released by oxidative stress is involved in both ERK and JNK activation and regulates cell cycle progression (Tournier et al., 1997).

ROS are produced not only as by-products of cellular metabolism but also specifically through unique enzymatic systems – NADPH oxidase (NOX) and dual oxidase (DUOX). These enzymes generate ROS as a response to different signals such as cytokines and calcium. It plays a significant role in immunity, mitogenic signalling, modification of extracellular matrix, and possibly also in the development of various diseases including cancer (for review see (Bedard and Krause, 2007; Lambeth, 2004; Ushio-Fukai, 2007)). Recently, it has been shown by Rossary A. et al. that PUFAs can specifically regulate the ROS level through NOX activity, without the participation of intracellular SOD. The authors demonstrated

that DHA can induce ROS production through NOX activation (Rossary et al., 2007), which triggers antioxidant response via the HO- 1 enzyme (Arab et al., 2006a b). These observations indicate a novel mechanism of the antioxidant effect of DHA and its role in the regulation of cellular and tissue redox balance.

Cholesterol, fatty acids, and other bioactive lipids may serve as sources for precursors of nuclear receptor ligands (for review see (Chawla et al., 2001)). Peroxisome proliferator-activated receptors (PPARs) are transcription factors which play an important role in cellular signalling (Ehrmann et al., 2002). PPARs are essential in regulating the normal physiological function such as lipid metabolism and also in the development of several diseases, e.g. atherosclerosis and diabetes. In cancer, PPARs can regulate both proliferation and differentiation of malignant cells. Oxidative metabolites of AA and LA are natural ligands of PPARs (Chawla et al., 2001). However, details about signal transduction and interactions between PPAR family members are not known. It has been shown that 13-*S*-hydroxyoctadecadienoic acid, a primary metabolite of 15-LOX oxidation of LA, inhibits PPAR-β/δ suppressive functions, activates PPAR-γ, and induces apoptosis in colorectal cancer cells (Shureiqi et al., 2003; Zuo et al., 2006).

Events on Mitochondria

Mitochondria are the main source of intracellular ROS production, and also the main target for their damaging effects (Orrenius et al., 2007). The interaction of diverse macromolecules including phospholipids (containing PUFAs) with ROS may impair the function of these organelles and significantly contribute to the induction of cell death. Mitochondria-generated ROS have been shown to play an important role in the release of cytochrome c (cyt c) and other proapoptotic proteins, which can trigger caspase activation and subsequent apoptosis (Ott et al., 2007a).

Cyt c is bound to the inner mitochondrial membrane by an association with cardiolipin (CL), a unique anionic phospholipid containing four fatty acid residues. During apoptotic signalling, the interaction between cyt c and CL limits the amount of cyt c released from mitochondria. Binding of CL with cyt c yields the cyt c/CL complex that, in the presence of ROS, acts as a potent CL-specific peroxidase and generates CL hydroperoxides (Kagan et al., 2005). This modulation of CL leads to a decreased binding affinity for cyt c and represents an important and early step in inducing the mitochondrial apoptotic pathway(Ott et al., 2007b). It has been reported that NO inhibits the peroxidase activity of the cyt c/CL complex and blocks CL oxidation, acting as an antioxidant protecting the mitochondrial membrane from oxidative damage and subsequent cell death (Vlasova et al., 2006). The oxidized CL does not bind cyt c effectively, which facilitates cyt c mobilization from the inner mitochondrial membrane (IMM). CL susceptibility to oxidation correlates well with the unsaturation index of the CL acyl chains. Unlike its monounsaturated molecular species, polyunsaturated CLs are readily oxidized by cyt c (Kagan et al., 2005).

It is also important to emphasize that cyt c release during apoptosis is a two-step process that involves (1) the above-mentioned detachment of cyt c from the IMM, which is followed by (2) permeabilization of the outer mitochondrial membrane (OMM) and the release of cyt c into the cytosol (Ott et al., 2002). The proapoptotic members of the Bcl-2 family such as Bid, Bax or Bak are known to induce the loss of OMM integrity followed by a release of proapoptotic mediators including cytochrome c into the cytosol (Green and Reed, 1998). Bid is a crucial proapoptotic protein integrating the action of other Bcl-2 proteins on the mitochondrial membrane. Its cleavage fragment, tBid, is known to transfer the apoptotic signal to mitochondria. The ability of PUFAs to stimulate Bid cleavage has also been demonstrated, with more pronounced effects in the case of EPA and DHA compared to the effects of AA (Arita et al., 2001). In addition to binding cyt c at the level of IMM, interactions of CL with tBid may also be very important. The significant role of CL in targetting tBid to mitochondria has been reported in some cells (Esposti et al., 2003; Lutter et al., 2000), while it was less pronounced in others (Choi et al., 2007). The possible impact of modifications of CL molecule on the suggested interactions therefore deserves further attention.

Importantly, CL may act as an important factor regulating the functions of cyt c at the level of mitochondria. As CL is a very dynamic molecule, the effective manipulations of its structure are of great importance as they could have a high impact on the overall modulation of the mitochondrial apoptotic pathway (Kagan et al., 2006). Interestingly, CL has been shown to have the most diet-responsive and changeable fatty acid composition among phospholipids. Incorporation of DHA into CL was associated with the induction of oxidative stress and apoptosis in colon cancer cells *in vitro* (Watkins et al., 1998) and *in vivo* (Hong et al., 2002; Chapkin et al., 2002). DHA containing six double bonds provides an excellent substrate for lipid peroxidation, which in turn decreases CL binding affinity for cyt c and facilitates cyt c release. Therefore, it has been suggested that manipulations of CL susceptibility to oxidation via incorporation of PUFAs could be an important factor modulating the cell sensitivity/resistance to apoptotic signals (Bayir et al., 2006). Thus, the nutritional manipulation using e.g. fish oil that is capable of stimulating the incorporation of highly unsaturated n-3 PUFAs into the mitochondrial CL may be extremely beneficial in overcoming cancer cell resistance to drug-induced apoptosis.

Redox Balance in Colon Carcinogenesis

Proliferation, differentiation, apoptosis, and extrusion of epithelial cells into the colonic lumen are regulated by genetic programmes and endogenous regulatory factors and additionally modulated by exogenous compounds. Lipid peroxides and ROS generated especially from high-fat diet rich in PUFAs, higher production of endogenous ROS, and higher susceptibility to DNA oxidative damage in the colon compared with the small intestine could explain the higher cancer susceptibility of this tissue (Sanders et al., 2004a). The challenge of oxidants initiates intestinal

oxidative stress and, depending on the degree of thiol redox imbalance, enterocytes will either proliferate or die by apoptosis. These cellular responses do not exhibit a linear but rather a "bell-shaped" function that is directly dependent on the cellular redox status. A fundamental difference in the control of intestinal cell apoptosis was also indicated in response to acute or persistent peroxide challenge. In the molecular responses of enterocytes, an oxidant- and redox-sensitive nuclear transcription factor NF-κB, which is linked to the signalling pathways governing the expression and suppression of candidate proliferative as well as apoptotic genes, has been shown to play a role (Aw, 1999). Our understanding of oxidative stress and redox imbalance, redox effects on specific cell cycle responses and redox regulation of NF-κB activation and signalling influencing gene expression is very important with regard to the risk of colon inflammation, epithelial cell transformation, and colon carcinogenesis.

It was demonstrated that excessive production of ROS is a direct or indirect cause of mucosal damage in inflammatory bowel disease (IBD). Although the intestinal mucosa contains a wide variety of endogenous antioxidant defence mechanisms, their levels are relatively low (Buffinton and Doe, 1995). Thus, the IBD mucosa is under constant oxidative stress with a significant impact on intestinal tissue homeostasis which can then facilitate the formation of colon adenomas and carcinomas. However, the role of the individual components of oxidative metabolism is not quite clear. Examining the potential role of iNOS, NO, and peroxinitrite in intestinal inflammation revealed their dichotomous function as both beneficial and detrimental molecules (Kubes and McCafferty, 2000).

Significant changes in the antioxidant capacity of the colorectal cancer tissue, which lead to enhanced action of ROS and lipid peroxidation, were indicated. The study on patients operated on for colorectal cancer showed increased activities of SOD, GPx and glutathione reductase, and a decreased activity of CAT in the cancer tissue. The levels of the non-enzymatic antioxidant (glutathione, vitamin C and E) were also decreased (Skrzydlewska et al., 2001).

Activated phospholipases release membrane-bound arachidonate, thus making it available for conversion to bioactive eicosanoids. In the intestine, they are produced by immune cells as well as by colon epithelial cells, and mediate colonic secretory effects of many different compounds such as interleukins, TNF-α, and ROS. COX and LOX products play a significant role in IBD and colon carcinogenesis (Krause and DuBois, 2000). Many reports on increased activities of COX-2 and 5- and 12-LOX in colon cancer cells and tissue were published. From this knowledge, the chemopreventive and therapeutic use of non-steroidal anti-inflammatory drugs (NSAIDs) was derived (Abir et al., 2005; Goodman et al., 2004; Sinicrope and Gill, 2004). A significant correlation between iNOS activity, COX-2 expression, and PGE_2 production was found to be associated with the promotion of colon tumour angiogenesis (Cianchi et al., 2004). Recently, a co-operation of p53 and NF-κB in the upregulation of COX-2 expression in colon cancer cell lines has been reported (Benoit et al., 2006).

Based on the knowledge described above, an important part of the therapeutic strategy against colon cancer development is aimed at preventing or attenuating

oxidative stress. In addition to inhibitors of COX and LOX expression and many other agents with antioxidative capacity, the most widely investigated dietary components with respect to colon cancer prevention are butyrate (from dietary fibre) and n-3 PUFAs (especially those found in fish oil) (Lupton, 2000). Based on experimental studies *in vitro* and *in vivo* and some clinical and epidemiological data it is supposed that the diet rich in n-3 PUFAs and fibre protects against IBD (Belluzzi, 2004; Seidner et al., 2005; Teitelbaum and Allan Walker, 2001) and colon cancer development (Andoh et al., 2003; Boudreau et al., 2001; Jho et al., 2004; Roynette et al., 2004). However, the reported data are often contradictory and the mechanisms by which these dietary components function have not been clearly elucidated yet. In addition to many other mechanisms, it is suggested that dietary lipids and fibre work co-ordinately to alter antioxidant activity and balance in a manner that may create a pro-oxidant environment in colonocytes.

Fibre and Short-Chain Fatty Acids

The effects of highly fermentable fibre are partially associated with short-chain fatty acids (SCFA), especially butyrate, produced in the gastrointestinal tract by anaerobic bacterial fermentation (Andoh et al., 2003). It is generally thought that it serves as the main oxidative fuel for the normal colonic epithelium, increases normal cell proliferation, and its withdrawal results in apoptosis. On the other hand, sodium butyrate (NaBt) can decrease the proliferation of neoplastic colonocytes *in vitro* and *in vivo*, and induces their differentiation as well as apoptosis (Velazquez et al., 1996). The data on butyrate effects on colon cancer are rather confusing leading to what is called "butyrate paradox". The reasons for these discrepancies include differences between the *in vivo* and *in vitro* systems, concentration and timing of butyrate administration, physiological conditions during the study, dependence on cell type, stage of differentiation, and the presence of other factors. The precise molecular mechanisms of butyrate effects are still far from being fully clarified. A gene array and proteome analysis of human colon cancer cell lines revealed that the genes (mostly transcription factors) and proteins linked to the cell growth, apoptosis, and oxidative metabolism are most significantly affected (Iacomino et al., 2001). The most commonly reported mechanism by which butyrate modulates gene expression involves alteration of chromatin structure subsequent to increased histone acetylation (Mei et al., 2004).

In patients with ulcerative colitis, utilization, particularly of butyrate, seems to be impaired and treatment with butyrate was shown to be beneficial. It reduced the apoptosis of colonocytes and the production of inflammatory cytokines like IL-8, increased mucin production, and adhesion molecule expression (Kruidenier and Verspaget, 1998). Butyrate was also shown to alter dramatically the cellular response to inflammatory stimuli. NF-κB activation by TNF-α is down-regulated in cells that undergo butyrate-induced growth arrest and differentiation. On the other hand, NaBt- and TNF-α-combined treatment of human colon adenocarcinoma

cell lines modulates their differentiation and potentiates apoptosis (Giardina et al., 1999; Kovarikova et al., 2000). Several studies have shown that NaBt chemopreventive effects are associated with its ability to induce detoxifying and antioxidative genes and enzymes. Apoptosis induced by NaBt was shown to be associated with depletion of intracellular glutathione (Louis et al., 2004) and increased gene expression and activity of GST, glutathione reductase, peroxidase, and CAT (Beyer-Sehlmeyer et al., 2003; Ebert et al., 2003; Sauer et al., 2007). These changes could affect the biotransformation ability of colon cells and reduce damaging effects of dietary carcinogens as well as oxidants like 4-HNE, thus protecting cells from cancer initiation (Pool-Zobel et al., 2005). Butyrate was shown to reduce COX-2 expression and activation in adenoma and colon cancer cell lines (Tong et al., 2005). Preincubation of colon carcinoma cells with NaBt reduced their metastatic ability *in vivo*. NaBt-treated cells were also more susceptible to oxidative stress induced by oxidants such as menadione (Li et al., 2004).

Interaction of PUFAs with Butyrate and Endogenous Regulators

It has been proposed by ourselves and others that PUFAs and butyrate may operate together in the colonic lumen and their interactions have a substantial impact on the metabolism and kinetics of colon epithelial cell population. Moreover, they can modulate the effects of endogenous regulators such as cytokines and apoptotic inducers. In a number of studies published by researchers from the University of Texas, protective effects of fish oil compared to corn oil using rat model colon carcinogenesis were documented (Lupton, 2004). The apoptotic removal of cells with azoxymethane-induced DNA damage was more efficient in the colon of rats receiving fish-oil diet compared to corn-oil diet (Chang et al. 1998). In addition to this initiation stage, similar effects of fish oil were detected throughout the promotion stage. In these studies fish oil results in a higher degree of colonocyte differentiation and apoptosis. DNA microarrays revealed that the chemopreventive effects of fish oil are due to a direct action of n-3 PUFAs (Davidson et al., 2004). Dietary fish oil as well as pure n-3 PUFAs are incorporated into colon cell mitochondrial PLs increasing the unsaturation index, ROS production, cyt c release, and activation of caspase-3, which results in increased apoptosis (Hong et al., 2002). DHA was shown to decrease the expression of iNOS, COX-2 and the related proinflammatory genes and down-regulated NF-κB isoforms in human colon cancer cells (Narayanan et al., 2003).

A combination of fish oil with pectin (a significant butyrate source) was still more efficient in the upregulation of colon cell apoptosis (Sanders et al., 2004b). During the initiation phase of tumour induction fish oil/pectin stimulate apoptosis in the upper portion of the crypt and down-regulate DNA damage. The suggested mechanisms involve inactivation of COX-2 and upregulation of apoptosis via ROS mechanisms. Considering the degree of unsaturation of n-3 PUFAs in combination with rapid colonocyte oxidation of butyrate, they may alter cellular ROS production

and antioxidant capacity. Pre-treatment of immortalized mouse colonocytes with DHA but not LA primed the cells for butyrate-induced apoptosis by enhancing the unsaturation of mitochondrial PL, especially CL, resulting in an increase of ROS. Moreover, expression of the antiapoptotic Bcl-2 protein, which possesses antioxidant properties suppressing membrane lipid oxidation, was decreased by DHA and butyrate (Ng et al., 2005).

Data from experiments following main determinants of antioxidative capacity (SOD, CAT, GPx) showed that the activity of these enzymes, especially CAT, is influenced by dietary fibre. Furthermore, the combination of fish oil with pectin resulted in an even lower and imbalanced activity of CAT and SOD. Dietary pectin and, to a lesser extent, dietary fish oil were shown to elevate the SOD/CAT ratio compared with cellulose and corn oil, respectively. The greatest enhancement was seen after the combination of fish oil and pectin in the diet.

The effects of model PUFAs of the n-6 (AA) and n-3 (DHA) types and of butyrate on colon epithelial cells have also been investigated in our laboratory. Previously we demonstrated that colon cancer HT-29 cells pre-treated with AA or DHA became more sensitive to apoptotic effects of NaBt (Hofmanova et al., 2005a) as well as to death receptor-mediated apoptosis induced by TNF-α or anti-Fas monoclonal antibody (CH-11) (Hofmanova et al., 2005a). Apoptosis was also increased after simultaneous treatment of HT-29 colon adenocarcinoma cells with TRAIL and DHA (Vaculova et al., 2005). It was suggested that ROS may play a role in these effects because AA and especially DHA induced dose-dependent ROS production and lipid peroxidation in these colon cancer cells, which can be enhanced by NaBt.

Based on differences reported in the fatty acid composition of tumour and normal tissue (Das, 2002; Martin et al., 1996), we have supposed different activities of fatty acid metabolism enzymes and anti-oxidative defence and thus a distinct response of normal and cancer cell lines to fatty acid treatment. Comparing the effects of AA and DHA in normal FHC and cancer colon cell lines, a higher dose-dependent sensitivity of FHC cells was observed. Here, antiproliferative and apoptotic effects of PUFAs correlated with the more intensive oxidative response of FHC cells in comparison with cancer cells (our unpublished results). These results were in accord with those directed to practical application. We reported that FHC cells are more sensitive to supplementation of various clinically used parenteral lipid emulsions to cultivation media than are HT-29 cells, mainly due to their stronger oxidative response, which can be reversed by the addition of α-tocopherol (Hofmanova et al., 2005b).

Recently, our experiments have been aimed at investigating cell lipid and oxidative metabolism changes and their correlation with the modulation of cytokinetic parameters, particularly apoptosis, in normal FHC and cancer HCT-116 colon epithelial cell lines. Suggesting mutual interaction of dietary lipid components in the colonic lumen we investigated the effects of AA, DHA or NaBt as single agents or in combination. The panel of the data in Fig. 6.2 represents an example of our results which showed parameters reflecting cell uptake of fatty acids and accumulation of triacylglycerols in the cytoplasm, changes in mitochondria, production of ROS, and apoptosis. From these data it is apparent that accumulation of cytoplasmic lipid

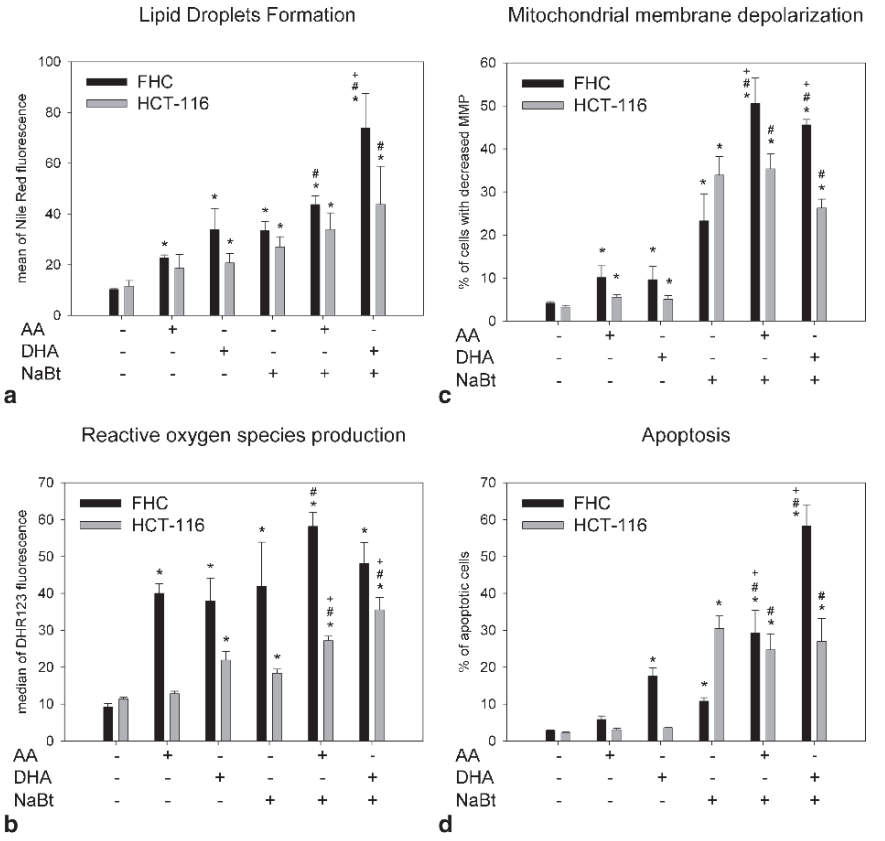

Fig. 6.2 (A), accumulation of triacylglycerides in cytoplasmic lipid droplets (Nile red fluorescence); (B), production of reactive oxygen species (dihydrorhodamine-123, DHR-123 fluorescence); (C), changes of mitochondrial membrane potential (MMP, TMRE fluorescence) detected by flow cytometry; and (D), percentage of cells with apoptotic nuclear morphology (DAPI staining) using fluorescence microscopy. All parameters were evaluated after 48 h of FHC or HCT-116 cell treatment with AA (50 μM), DHA (50 μM), NaBt (3 mM), or their combination. The symbols denote significant ($P < 0.05$) changes compared to untreated cells (*) or PUFAs (#) and/or NaBt (+) treated cells

droplets and ROS production after both PUFA and NaBt were higher in FHC than in HCT-116 cells and that they were additionally increased after combined treatment. This was finally reflected in a higher percentage of apoptotic FHC cells. In spite of the fact that in HCT-116 cells NaBt induced a more intensive apoptotic response than in FHC cells, it was not modulated by combination with PUFAs. Our results imply mutual interaction of dietary fatty acids and a distinct response of normal and cancer colonic cell populations to these compounds. Moreover, we showed that there is a relationship between the changes in cell lipids, mitochondria, oxidative metabolism, and the apoptotic response. Detailed mechanisms of these effects are under study. Our research can help to clarify the role of dietary lipid components

in colon carcinogenesis and to design appropriate approaches in cancer prevention and therapeutic application using these compounds.

Conclusions

Lipid dietary components, particularly the ratio of various types of fatty acids, play an important role in the development of certain types of cancer, such as colon, breast, and prostate. Now it is clear that the modulation of oxidative/antioxidative balance through various mechanisms and pathways is included in these effects. PUFAs and their metabolism are sources of various types of ROS, RNS, and products of lipid peroxidation, simultaneously modulating also some components of the cell antioxidative system. The effects of PUFAs are presented at various levels of cell organization from changes of membrane structure and function, modulation of intracellular signalling and mitochondrial function to changes of gene transcription. In addition to DNA damage and xenobiotic metabolism, the changes of redox balance have a substantial impact on the intracellular signalling pathways and expression of genes regulating cell proliferation, differentiation, and apoptosis. Imbalance of cell and tissue homeostasis can then promote cancer development and malignant transformation.

Dietary PUFAs and their metabolites play a significant role especially in the self-renewing tissue of the colon, where they are in direct contact with epithelial cells and where the strict control of the balance between proliferation, differentiation, and apoptosis in the crypts is highly important. Moreover, their interaction with other compounds such as the product of dietary fibre – butyrate and endogenous regulatory molecules affects colon cytokinetics. Based on ample experimental evidence about the beneficial effects of n-3 PUFAs (from fish oil), butyrate (from fibre), and especially their combination, these compounds are seriously considered for the prevention as well as therapy not only of the colon but also of other types of cancers. For this purpose, further research aimed to specify the mechanisms of their action in various cell types including the specific role of individual components of the oxidative/antioxidative systems is necessary.

Acknowledgements The authors would like to thank Zuzana Koubková and Lenka Stixová for technical assistance. This work was supported by the Grant Agency of the Czech Republic, projects No. 524/07/1178, No. 204/07/0834 and No. 301/07/1557, and the Grant Agency of the Academy of Sciences of the Czech Republic, project No. 1QS500040507.

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Chapter 7
The Question of Balance: The Interaction Between Blood and Ozone

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Abstract That an oxidant can act either as a useful physiological messenger or a death signal is exemplified by the behaviour of blood treated with different ozone doses. Blood is an ideal tissue because is composed of plasma, which has a wealth of reductants, which can be easily measured before and after ozonation as well as cells able to cooperate to modulate the oxidant properties of ozone. Blood cells contain not only GSH and thioredoxin but enzymes able to promptly reduce oxidized compounds, thus restoring the initial antioxidant status. Our work over the last 16 years has identified important compounds generated *ex vivo* during the initial reaction of ozone with some plasma components and how these activate biochemical pathways with therapeutic effects after transfusion of the ozonated blood in the donor. We have shown that appropriate ozone doses are not toxic and elucidated the subtle interaction between the generated compounds and the antioxidant defences.

Introduction

ROS Produced in Physiological Conditions Stimulate the Maintenance of a Normal Redox State and Are Critical for Cell Survival

During the last 2.5 billions year, oxygen (O_2) handling has become essential for the aerobic life. It is an unusual molecule because, in spite of having two unpaired electrons in its outer orbital, making it a free radical, it is unusually stable (Green and Hill, 1984). However about 2–3% of oxygen used by mitochondria, via the complex I and III, during the process of oxidative phosphorylation will leak from the respiratory chain to form anion superoxide, $O^{\bullet-}_2$, (Buetler et al., 2004). NAD(P)H

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

oxidases, present in cell membranes of fibroblasts, endothelial and vascular smooth muscle cells and particularly phagocytes, directly produce superoxide. Other enzymes such as nitric oxide synthase (NOS), xanthine oxidase, cytochrome P450, lipoxygenases and even heme oxygenases, during abnormal situations, e.g. ischemia–reperfusion or initial inflammation, may be involved in superoxide production. The reduction of superoxide, discovered by McCord and Fridovich in 1968, is performed by mitochondrial (Mn), cytosolic (Cu/Zn) and extracellular (ec) superoxide dismutases(SODs), that catalyze its dismutation to hydrogen peroxide as follows:

$$2O^{\bullet-}_2+2H^+ \rightarrow H_2O_2+O_2$$

Hydrogen peroxide is not a radical molecule because it has paired electrons but it has been included among the reactive oxygen species (ROS) because it is an oxidant on its own right. Its relative stability allows measuring it in plasma: in normotensive subjects its concentration is of 2.5 μM (Lacy et al., 1998). Remarkably, it has a half-life of about 1–2″ in plasma but less than 1″ when generated in blood (Bocci et al., 1998a). In this case the intracellular concentration of hydrogen peroxide will be at the most of 0.25 μM while the maximal intracellular concentration that can be generated for signalling purposes may reach 0.5–0.7 μM (Stone and Yang, 2006). As it is a unionized molecule, in the presence of an extracellular-cytosolic gradient, it passes through the cell membrane but the intracellular concentration is only about 1/10 of the extracellular one (Antunes and Cadenas, 2000; Stone and Yang, 2006). It appears ubiquitous as it has been detected in urine and in exhaled air (Halliwell et al., 2000a, b). Depending upon its local concentration and cell-type, hydrogen peroxide can either induce proliferation or cell death (Urschel, 1967; Stone and Collins, 2002; Ardanaz and Pagano, 2006). It can regulate vascular tone by causing constrictions of vascular beds or vasodilation although it remains uncertain if it acts as an endothelium-derived hyperpolarizing factor (Matoba et al., 2002). When ozone induces a sudden production of hydrogen peroxide in plasma, its intracellular presence is always transitory because, as we shall describe, reductants and enzymes promptly reduce it to water. During blood ozonation, hydrogen peroxide, suddenly generated in plasma, permeates lymphocytes and, when it reaches the cytosol it activates a tyrosine-kinase, that phosphorylates NF-kB and the release and translocation into the nucleus of the heterodimer p50 - p65 which able to regulate the expression of over 50 genes. Importantly this process is checked by a phosphatase or inhibited by intracytoplasmic antioxidants, thus very transitory.

Anion superoxide can free and reduce Fe^{3+} from ferritin:

$$O_2^{\bullet-}+Fe^{3+}+ \rightarrow Fe^{++}+O_2$$

Obviously an excess of hydrogen peroxide in the presence of Fe^{++}, can give rise to the very reactive hydroxyl radical by way of the Fenton-Jackson reaction:

$$H_2O_2+Fe^{++}+ \rightarrow OH^{\bullet}+OH^{-}+Fe^{3+}$$

Moreover hydrogen peroxide in the presence of anion superoxide can generate another hydroxyl radical via the iron catalyzed Haber and Weiss's reaction. Hydroxyl radicals, are highly reactive with a nanosecond half-life that can cause covalent cross-linking of enzymes or propagate deleterious free radical reactions in a variety of molecules such as DNA, proteins and lipids. Obviously these types of dangerous reactions should and can be avoided by precisely calibrating the ozone dose against the antioxidant capacity of blood. Similarly, in the presence of hydrogen peroxide, one should avoid the activation of the enzyme myeloperoxidase, which, by catalyzing the oxidation of halide ions, forms hypochloric acid (OCl^-). Notably, ozonation of physiological saline, not only generates H_2O_2 but also NaOCl as it has been shown by Ueno et al. (1998).

$NO^\bullet$ is a relatively unreactive free radical with a half life of 1–2″ formed by NO synthase. We have shown that, during blood ozonation depending upon the ozone concentration, from pico to nanomolar concentrations of nitric oxide are generated. This compound mediates relevant processes as vasodilation, platelet stability and host-defense. NO binds partly to cysteine 67 in hemoglobin (Stamler, 2004; Foresti et al., 2006) and to GSH. The formation of more stable nitrosothiols thus enables NO to have pharmacological actions far distant from its synthesis site. During pathological situations, or using an excessive ozone dosage, micromolar concentrations of nitric oxide can be generated and can either aggravate an inflammatory state or, by reacting with anion superoxide, peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS) are formed. Peroxynitrite reacts with an array of biomolecules inducing lipid peroxidation, cross-linking and carbonyls (Stadtman and Levine, 2000). Furthermore either protonation or oxidation of peroxynitrite can generate an oxydryl molecule and nitrogen dioxide (NO_2). These molecules are able to form nitro-adducts and carcinogenic nitrosamines.

Another series of compounds formed in different amounts in both physiological or pathological situations are the lipid oxidation products (LOPs). As an example (Pryor, 1994), a hydroxyl radical, reacting with an unsaturated fatty acid (PUFA) as arachidonic acid (LH), bound to albumin or present in membrane phospholipids, produces a lipid molecule radical ($L^\bullet$):

$$OH^\bullet + LH^\bullet + \rightarrow L^\bullet + H_2O$$

The lipid molecule radical, by reacting with oxygen, forms a peroxyl radical, LOO•, which can be either reduced to a hydroperoxide, LOOH or to a final aldehyde such as malonyldialdehyde (MDA) or the typical 4-hydroxy-2,3-trans-nonenal (4-HNE). Needless to say given the myriad of PUFA's in biological systems, ozonation of plasma lipids leads to the generation of a bewildering mixture of aldehydes. These compounds are intrinsically toxic because they can inactivate enzymes, other lipids and nucleic acids. Unlike ROS, they are fairly stable in vitro at 37 °C in ozonated blood (Bocci et al., 1998a). Once again, their toxicity depends upon their final concentration and location because *in vivo*, after the slow reinfusion of carefully ozonated blood, they undergo a marked dilution in the blood and extravascular fluids, detoxification via aldehyde-dehydrogenases and GSH-transferases, and

excretion via the bile and urine (Bocci, 2005, 2006a), with the very few remaining molecules eventually binding to cells. Interestingly, in line with the concept of a dynamic balance, the physiological plasma level of 4-HNE ranges between 0.3 and 0.7 μM (Siems and Grune, 2003; Forman and Dickinson, 2004) where its stimulates the synthesis of GSH-transferases and aldehyde dehydrogenases (Dianzani, 1998; Awasthi et al., 2005). The problem of detoxification of aldehydes has been extensively discussed elsewhere (Bocci, 2002, 2005).

Due to the presence of oxygen, evolution has been forced to develop interacting mechanisms for protecting living beings against the threat of ROS. Of these systems, the critical role of hydrophilic (~50 μM ascorbic acid, ~300 μM uric acid, GSH, thioredoxin and other electron donors) lipophilic (vitamin E, bilirubin) compounds, proteins like albumin acting either as oxidant scavenger and/or Fe^{++}, Cu^{+} chelator (transferrin, ferritin, ceruloplasmin) are noteworthy. In addition, a large series of antioxidant enzymes like SOD, catalase, GSH-peroxidases, GSH-reductases, peroxiredoxins, as well as glucose-6-phosphate dehydrogenase as one of the key enzyme of the pentose phosphate pathway supplying the constantly required NADPH as a reductant. The maintenance of an optimal balance of GSH/GSSG, of NAD + /NADH and NADP + /NADPH is critical for the cell

The interaction of the various components of the antioxidant system (Mendiratta et al., 1988a, b), is made quite effective by the recycling of its components and is sufficient to deal with the stresses due to ROS, LOPs and RNS for long periods of the life of any organism (Soberman, 2003). However aging and particularly chronic inflammatory diseases cause an often irreversible disruption of the control of the redox state that progressively aggravates the pathology. In contrast to these inherent systems, ozonation of blood provides a precisely measurable and small perturbation of the oxidant-antioxidant balance that, within a few minute is re-equilibrated (Bocci et al., 1998b) and activates a number of biochemical pathways in different cells to produce biological and therapeutic effects

Why Study the Reactions Between Blood and Ozone?

Ozone therapy has been a poorly known and empirical complementary approach treated with scepticism by orthodox medicine was sceptical about it. In fact, a distinguished ozone chemist has declared that “ozone is toxic, no matter how you deal with it and it should not be used in medicine”. In 1989, while studying the induction of interferon-gamma (IFNγ) by oxidizing agent, I was asked for an explanation of the apparently beneficial effect of ozonated blood re-transfused in donor patients affected by chronic hepatitis C. Ozone was known as a potent oxidant and periodate and galactose-oxidase had been shown to induce the synthesis of IFN-gamma in blood mononuclear cells (BMC) (Dianzani F, personal communication 1987) We then decided to evaluate whether human BMC, briefly exposed to small ozone doses, would produce this cytokine. Because this labile gas must be produced *extempore* and represents about 2–4% of the gas mixture made up with medical oxygen

this experiment required careful technique. We were able to demonstrate (Bocci and Paulesu, 1990) the ozone dose-dependent production of IFNγ. Our observation, extended to other cytokines, was confirmed by other (Cho et al., 2001), evaluating the ozone as an inducer of proinflammatory cytokines in the lung. In the 1990s, there was a general consensus that ROS and LOPs were involved in many human pathological conditions and, at the very least, they could perpetuate a chronic oxidative stress. We soon realized that ozone was an excellent generator of free radicals. Thus the idea of using ozone in medicine appeared misguided but it was unclear whether ozone can really be always toxic given the principle ascribed to Paracelsus (1493–1541) that the dosage makes it either "a poison or a remedy". In 2005, John Timbrell entitled his book "The poison paradox; chemicals as friends and foes" reminding us two essential facts: firstly, it is the dose that makes a chemical toxic and secondly and more important, toxicity results from the interaction between chemicals and biological defences. Recently a more objective view has been taken by considering that hydrogen peroxide and two gases such as NO and CO, produced in normal conditions, have an essential role in physiology and yet they can become toxic when produced in excessive amounts overwhelming the antioxidant defences. Moreover, ROS and LOPs are produced continuously and participate in a variety of crucial physiological functions but can evoke negative effects when location, time of exposure and concentration are inappropriate. What follows is a brief review of our results showing how judicious ozone doses alters the balance between positive and negative effects of oxidants and trigger a number of biological activities.

An Objective Evaluation of the Action of Ozone on Whole Human Blood

The use of modest i.e. therapeutic dosages ranging from 0.21 μmol/mL (10 μg/ml of gas per ml of blood) up to 1.68 μmol/mL (80 μg/ml of gas) results in the blood's antioxidant system neutralizing ozone while not preventing the helpful biologic activities.

This then raises the issue of the behaviour and fate of ozone upon contact with body fluids. Analyzing these interactions, essential concepts are that:

1. As any other gas, ozone dissolves physically in pure water according Henry's law in relation to the temperature, pressure and ozone concentration. Only in this situation ozone does not react and, in a tightly closed glass bottle, the ozonated water (useful as a disinfectant) remains active for several days.
2. On the other hand, ozone reacts immediately upon biological fluids (physiological saline, plasma, lymph, urine). This means that contrary to the belief that ozone penetrates through the skin and mucosae or enters into the cells, after the above mentioned reaction, ozone as ozone no longer exists.

In order of preference, ozone reacts with abundant polyunsaturated fatty acids (PUFA), bound to albumin, antioxidants such as ascorbic and uric acids, thiol

compounds with –SH groups such as cysteine, reduced glutathione (GSH) and albumin. If the ozone dose is increased further, carbohydrates, enzymes, DNA and RNA also become targets as all of these compounds act as electron donors such that, they undergo oxidation and thus are "damaged".

3. The main reaction for PUFA and ozone:

$$\text{R-CH = CH-R'} + O_3 + H_2O \rightarrow \text{R-CH = O} + \text{R'-CH = O} + H_2O_2$$

shows the simultaneous formation of one mole of hydrogen peroxide (included among reactive oxygen species, ROS) and of two moles of LOPs (Pryor, 1994; Uppu et al., 1995).

The resulting hydrogen peroxide, which is a non radical oxidant then acts as an ozone messenger responsible for eliciting several biological and therapeutic effects (Bocci, 2002, 2006a, 2007; Bocci and Aldinucci, 2006; Stone and Yang, 2006). A gain as noted earlier ROS are not always harmful but rather in physiological amounts, they act as regulators of signal transduction and represent important mediators of host defense and immune responses (Baeuerle and Henkel, 1994; Bocci et al., 1993, 1998b; Los et al., 1995; Grisham, 2004). Under normal conditions, the formation of hydroxyl radicals in blood is practically impossible because all the iron is chelated and none is released free. Ozone causes the generation of hydrogen peroxide and chemiluminescence in both physiological saline and plasma (Allen and Loose, 1976; Bocci et al., 1998a). In saline there is a consistent and prolonged increase while plasma both chemiluminescence and hydrogen peroxide increase immediately but decay very rapidly with a half-life of less than 2 min. This suggests that both antioxidants and traces of enzymes rapidly quench hydrogen peroxide (Bocci et al., 1998b) and its reduction is so fast in ozonated blood that it is currently experimentally impossible to measure. The transitory gradient of hydrogen peroxide in plasma likely produces as best a submicromolar gradient in the cytosol of RBC, which nonetheless is indispensable for the activation of biochemical pathways. Interestingly, the formation of nitrogen monoxide ($NO^{\bullet}$) in human endothelial cells exposed to ozonated serum has been shown (Valacchi and Bocci, 2000). However the use of ozone within the therapeutic range likely produces neither peroxynitrite, nor other RNS, nor hypochlorite anion.

As noted repeatedly, ROS generation must be precisely calibrated to achieve a biological effect without any damage. With respect to ozone, this can be achieved by regulating the ozone dose (ozone concentration as μg/ml of gas per ml of blood in 1:1 ratio) against the antioxidant capacity of blood that can be measured (Rice-Evans and Miller, 1994) and, if necessary, strengthened by oral administration of antioxidants (Polidori et al., 2004) before and throughout ozone therapy. A very enlightening finding was achieved by evaluating the variation of the total antioxidant status (TAS) in plasma after ozonation and 1 min mixing of the liquid-gas-phases of either fresh blood or the respective plasma withdrawn from the same five donors. We have shown (Bocci et al., 1998b) that, after ozonation of plasma with either a medium, or a high (40 μg/ml or 80 μg/ml of gas per ml of plasma, respectively)

ozone concentration, TAS levels progressively decrease at first and then remain stable after 20 min. The literature clearly shows that the therapeutic ozonation modifies only temporarily and reversibly the cellular redox homeostasis.

4. LOPs production follows peroxidation of PUFA present in the plasma: they are heterogenous and can be classified as lipoperoxides (LOO•), alkoxyl radicals (LO•), lipohydroperoxides (LOOH), isoprostanes and alkenals, among which HNE and MDA have been extensively studied (Pompella et al., 1987). The lipid radicals and aldehydes are intrinsically toxic and are far more stable than ROS (Bocci et al., 1998a) in vitro. However they have a brief half-life in body fluids owing to a marked dilution,, excretion (via urine and bile), and metabolism by GSH-transferase) and aldehyde dehydrogenases (Bocci, 2005). If the state of the oxidative stress is not too far advanced, small amounts of ROS and LOPs can elicit the upregulation of antioxidant enzymes on the basis of the phenomenon described under the term of "hormesis". The oxidative preconditioning or the adaptation to the chronic oxidative stress has been now demonstrated experimentally (Bocci, 1996; Leon et al., 1998; Larini et al., 2003, 2004). The increased synthesis of enzymes such as superoxide-dismutase (SOD), GSH-peroxidases (GSH-Px), GSH-reductase (GSH-Rd) and catalase (CAT) has been repeatedly determined in experimental animals and in patients (Bocci, 2007). Interestingly, Iles and Liu (2005) have just demonstrated that HNE, by inducing the expression of glutamate cysteine ligase, causes an intracellular increase of GSH, which plays a key role in antioxidant defense. Furthermore LOPs induce oxidative stress proteins, one of which is heme-oxygenase I (HO-1 or HSP-32) which, after breaking down the heme molecule, delivers very useful compounds such as CO and bilirubin (Maines, 1988; Foresti et al., 2006). Bilirubin is a significant lipophilic antioxidant and CO cooperates with NO in regulating vasodilation by increasing cGMP Moreover, Fe^2 + is promptly chelated by upregulated ferritin. The induction of HO-1 after an oxidative stress has been described in thousands of papers as one of the most important antioxidant defence and protective enzyme (extensively reviewed by Bocci, 2005, pp. 85–96).

Submicromolar LOPs levels can be stimulatory and beneficial, while high levels can be toxic (Bocci, 2005, 2007). Too low concentrations are practically useless (at best elicit a placebo effect), too high may elicit a negative effect (malaise, fatigue) so that they must be just above the threshold level to yield an acute, absolutely transitory oxidative stress capable of triggering biological effects without toxicity.

Which Are the Biological Effects Elicited by ROS and LOPs?

The ozonation process is therefore characterized by the formation of ROS and LOPs acting in two phases. This process happens either *ex vivo* (as a typical example in the blood collected in a glass bottle) or *in vivo* (after an intramuscular

injection of ozone) but, while ROS are acting immediately and disappear (early and short-acting messengers), LOPs, via the circulation, distribute throughout the tissues and eventually only a few molecules bind to cell receptors. Their complex pharmacodynamic facilitates their roles as late and long-lasting messengers.

The erythrocytes mop up the hydrogen peroxide also produced by ozone: GSH is promptly oxidized to GSSG and the cell, extremely sensitive to the reduction of the GSH/GSSG ratio, immediately corrects the unbalance by either extruding GSSG, or reducing it with GSH-Rd at the expenses of ascorbate or of the reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as a crucial electron donor. Next, the oxidized NADP is reduced after the activation of the pentose phosphate pathway, of which glucose-6-phosphate dehydrogenase (G-6PD) is the key enzyme. We have determined a small but significant increase of ATP formation (Bocci, 2002) but, whether this is due to the activation of the pentose cycle or to phosphofructokinase or to both remains to be clarified. Moreover the reinfused erythrocytes, for a brief period, enhance the delivery of oxygen into ischemic tissues because of a shift to the right of the oxygen-hemoglobin dissociation curve, due either to a slight decrease of intracellular pH (Bohr effect) or/and an increase of 2,3-diphosphoglycerate (2,3-DPG) levels.

Ozone acts as a mild enhancer of the immune system, by activating neutrophils and stimulating the synthesis of some cytokines (Bocci et al., 1993, 1998b) and once again the crucial messenger is hydrogen peroxide. Hydrogen peroxide, after entering into the cytoplasm of blood mononuclear cells (BMC), by oxidizing selected cysteines, activates a tyrosine kinase, which then phosphorylates the transcription factor Nuclear Factor kB (Baeuerle and Henkel, 1994; Los et al., 1995) allowing the release of an heterodimer (p50 + p65) eventually responsible for causing the synthesis of several proteins, among which, the acute-phase reactants and numerous interleukins. In the past, we have measured the release of several cytokines from ozonated blood upon *in vitro* incubation. Once the ozonated leukocytes return into the circulation, they home in lymphoid microenvironments and successively release cytokines acting in a paracrine fashion on neighbouring cells with a possible reactivation of a depressed immune system (Bocci, 2005). This process, described as the physiological cytokine response, is a part of the innate immune system and helps us to survive in a hostile environment.

During ozonation of blood, particularly if it is anticoagulated with heparin, we have noted an ozone-dose dependent increase of activation of platelets (Bocci et al., 1999; Valacchi and Bocci, 1999) with a consequent release of typical growth factors, which will enhance the healing of chronic ulcers in ischemic patients. Whenever possible, the use of heparin as an anticoagulant is preferable to sodium citrate because, by not chelating plasmatic Ca^{++}, reinforces biochemical and electric events.

During the reinfusion of the ozonated blood into the donor, endothelial cells will be activated by LOPs resulting in an increased production of NO, plasma S-nitrosothiols and S-nitrosohemoglobin (Stamler, 2004; Foresti et al., 2006)). While NO has a half-life of less than one second, protein-bound-NO can exert vasodilation also at distant ischemic vascular sites with relevant therapeutic effect.

The paradoxical concept is that ozone eventually induces an antioxidant response capable of reversing a chronic oxidative stress is common in the animal and vegetal kingdom and there is good experimental evidence that this phenomenon is a characteristic of all living beings. Moreover it is already supported by our findings of an increased level of antioxidant enzymes and HO-1 during ozone therapy. It also suggests that a judicious use of ozone, in spite of acting as an oxidant, enhances the antioxidant capacity, which represents the critical factor for overcoming chronic viral infections, ischemia and cell degeneration.

Conclusion

This article evaluates blood ozonation and uses this system to emphasize how different amounts of ROS and LOPs produced in physiological, or in pathological conditions, or using ozone overdoses, can be either useful as signalling agents or detrimental. It is discouraging to note that many scientists and clinicians, without knowing anything about the chemistry and biochemistry of ozone, continue to express scepticism against the appropriate use of ozone in medicine, thus depriving patients of a valid therapeutic support. Indeed it is not easy to find another example able to emphasize so well how the either positive or negative interaction of ozone and blood is really a question of balance.

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Chapter 8
Iron and Erythrocytes: Physiological and Pathophysiological Aspects

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Abstract It is generally accepted that iron when released from the macromolecular complexes (ferritin, transferrin, heme proteins, etc.) normally sequestering it, represents a source of oxidative stress. The reductive release of iron from ferritin has received extensive consideration, while the release of iron in an oxidative manner from heme proteins has been relatively little studied. We have shown that iron is released from hemoglobin or heme in a non protein-bound desferrioxamine-chelatable form (DCI) in a number of conditions in which the erythrocytes are subjected to oxidative stress. Such conditions can be related to toxicological events (haemolytic drugs) or to physiological situations (erythrocyte ageing, reproduced in a model of prolonged aerobic incubation) or pathological conditions (thalassemia, diabetes mellitus), but can also result from more subtle circumstances in which a state of ischemia-reperfusion is imposed on erythrocytes (e.g. childbirth). The released iron could play a central role in oxidation of membrane proteins, in particular band 3 protein, and in autologous IgG binding, one of the major pathways for erythrocyte removal. Iron chelators able to enter cells (such as ferrozine, quercetin, and fluorbenzoil-pyridoxalhydrazone) prevent both membrane protein oxidation and IgG binding. The increased release of iron observed in β-thalassemia patients and newborns (particularly premature babies) suggests that fetal hemoglobin is more prone to release iron than adult hemoglobin. In newborns the release of iron in erythrocytes is correlated with plasma non-protein-bound iron and may contribute to its appearance. Since disulfide-cross-linked band 3 dimers are the minimal aggregates with enhanced affinity for autologous IgG, we have observed that such oxidatively modified band 3 is present in a much higher percentage of newborns (especially premature) than of adults and that a correlation between free iron level and IgG binding is evident in erythrocytes. The IgG binding is present also in a much higher percentage of diabetic subjects than of controls. Iron release is higher in diabetic erythrocytes showing IgG binding and a significant correlation between iron release

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

and IgG binding is also evident. Therefore a large part of newborn (and possibly thalassemic) and diabetic erythrocytes are ready to be eliminated. Thus the iron appears an element of conflicting effect, it can be either beneficial or detrimental to the red cells, depending on whether it serves as a micronutrient or as a catalyst of free radical reactions, with consequent decrease of their life-span.

Keywords Oxidative stress, iron release, erythrocytes, Band 3 protein, newborns, β-thalassemia, diabetes mellitus

Introduction

The extremely large amount of iron in all the living cells cannot be only explained by the fact that this metal is one of the more abundant elements in the earth's crust, but rather by the unique abilities of iron, due to its physico-chemical properties, to vary oxidation state, redox potential and electronic spin configuration in response to different liganding environments. Such properties enable iron to play an essential role in an astonishing array of biological reactions. Iron exerts its functions either in the form of nonheme containing proteins (e.g., iron-sulphur proteins) or in the form of hemoproteins. A number of Fe-containing proteins catalyze key reactions in energy metabolism, respiration, and oxygen delivery to tissues, DNA synthesis, regulation of citric acid cycle, etc.; several other reactions, e.g., with oxygen (monooxygenases and dioxygenases), with peroxides (peroxidase, catalase, ferrioxidase), etc., are also catalyzed by iron (Miller et al., 1990; Ryan and Aust, 1992).

The widespread use of iron in living organisms gave rise to a paradox, as also noticed for oxygen. On the one hand, by serving its multifunctional role in biological functions, iron represents an enormous advantage for the complex mechanisms of life; on the other hand, if not appropriately shielded, iron can readily participate in one-electron transfer reactions that can lead to the production of extremely toxic free radicals. To overcome these problems, multicellular organisms have developed Fe-binding proteins known as transferrins, which complex iron, transport it in the circulation (serum transferrin) or in other media (ovotransferrin, lactoferrin) and are taken up by the cell mainly (serum transferrin) via a receptor mediated mechanism. Once transferrin is taken up and iron is released within the cell, nature has faced the most critical problem in iron handling. The problem was to discover mechanisms whereby iron can be utilized in a form that is soluble under physiological conditions, bioavailable and non-toxic. "Nontoxic" means that it is not able to elicit the Haber Weiss reaction, which describes the sum of reduction of Fe^{3+} by superoxide and the well known Fenton reaction and gives rise to very toxic oxygen species ($OH^{\bullet}$ in particular).Thus, the complexation of iron within a cell necessitates that the iron storage protein, primarily ferritin, must guard its iron in a form that will be assimilated into the very many molecules whose function depend on its physico-chemical properties, but cannot be released indiscriminately to elicit pathological consequences.

Throughout past decades, major acquisitions have been obtained by the discovery of a post-translational regulatory system (the iron-responsive elements [IREs]-iron-regulatory proteins [IRPs] system) controlling the expression of ferritin, transferrin receptor, and erythroid δ aminolevulinic acid synthase. IRPs have presumably evolved in such way that they simultaneously "sense" and "control" cell iron level, with the latter function being aimed at keeping the intermediate pool of transit or chelatable iron at critical level to ensure an adequate iron supply for important cell functions, while avoiding the toxic events such as oxidative stress, associated with an expansion of this pool (Cairo and Pietrangelo, 2000). A great deal of studies have shown that iron can be released from iron containing proteins under a number of conditions. The release of iron from ferritin, the most concentrated source of iron (up to 4,500 Fe^{3+} atoms per molecule), has been extensively studied (Minotti, 1993). Any compound capable of reducing Fe^{3+} within the iron core of ferritin in the presence of a suitable chelator is capable of releasing ferritin iron (particularly $O_2^{\bullet-}$, $NO^{\bullet}$, and the "redox cycling" class of xenobiotics). In addition to this reductive release from ferritin, iron can also be released in an oxidative manner from heme-proteins or heme, as shown by a number studies from our laboratory (Ferrali et al., 1992) (with erythrocytes) or other laboratories (Gutteridge, 1986; Puppo and Halliwell, 1988) (with hemoglobin). It is thus conceivable that "free" iron is immediately conveyed into an intermediate, labile iron pool, and that this pool represents a steady state exchangeable and readily chelatable iron compartment.

This pool has recently appeared as a substantial reality. Cabantchik and coworkers (Breuer et al., 1996; Konijn et al., 1999) have recently detected a chelatable intracellular iron pool by introducing a method based upon quenching of the fluorescent chelator calcein by metal ions. Used in conjunction with permeant and impermeant Fe^{2+} and Fe^{3+} chelators, these studies, carried out in human K 562 cell line, have suggested that the concentration of Fe in this pool (LIP) is 0.2–0.5 μM and that it is composed primarily of Fe^{2+}.

Iron Release in Erythrocytes Exposed to Oxidative Stress

As mentioned above, it is generally accepted that iron, released from macromolecules normally sequestering it, represents the source of iron-catalyzed oxidative stress, such as lipid, protein and DNA oxidation. Iron redox cycling in fact promotes the Fenton reaction in which the potent oxidant hydroxyl radical is produced (Halliwell and Gutteridge, 1985). Normally iron is transported, utilized and stored in specific proteins (transferrin, ferritin, haem proteins, etc.) which prevent or minimize its reactions with reduced oxygen species. Thus, to be redox cycling active, iron has to be released from its macromolecular complexes. The release of iron from its transport or store proteins has been extensively investigated (Thomas and Aust, 1985; Monteiro and Winterbourn, 1989; Reif, 1992; Minotti, 1993). In this review we discuss our and other studies showing that iron is released in a non-protein-bound desferrioxamine (DFO) chelatable form (DCI) (sometimes as referred to free iron) when erythrocytes

are exposed to oxidative stress. The iron is released in a DFO-chelatable form when mouse erythrocytes are exposed to oxidative stress, i.e., are incubated with a number of oxidizing agents such as phenylhydrazine, divicine, isouramil, acrolein, phenylhydroxylamine, and others (Ferrali et al., 1989, 1990, 1992; Ciccoli et al., 1999a). Iron is released from hemoglobin or its derivatives (Ferrali et al., 1990) and the release is accompanied by methemoglobin (Met-Hb) formation. If the erythrocytes are depleted of glutathione (GSH) (which can be easily obtained by a short preincubation with diethylmaleate), the release of iron is accompanied by lipid peroxidation and hemolysis (Ferrali et al., 1992). Iron acts from the inside of the cell. In fact, in experiments (Ferrali et al., 1992) in which mouse erythrocytes were preloaded with DFO (preincubation with a large excess of DFO), washed, depleted of GSH and then incubated with phenylhydrazine, the released iron was chelated at intracellular level (iron was extracted from the erythrocytes as ferrioxamine, that is, as DFO plus iron complex) and lipid peroxidation and hemolysis were prevented. This did not occur when the amount of intracellular DFO was not sufficient to chelate all the released iron. Extracellularly added DFO (erythrocytes not preloaded with DFO) did not prevent lipid peroxidation and hemolysis.

As known, DFO penetrates cells in minimal amounts only (Lloyd et al., 1991). Therefore, further studies were carried out with substances capable of penetrating cells and of chelating iron. One of these substances is the flavonoid quercetin (Afanas'ev et al., 1989) which, contrary to its rutinoside rutin, can cross the cell membrane (Sorata et al., 1984; Ben-Hur et al., 1993). As mentioned above, incubation of GSH-depleted mouse erythrocytes with phenylhydrazine resulted in release of iron as well as in lipid peroxidation and hemolysis. The addition of quercetin resulted (Ferrali et al., 1997) in a marked protection against lipid peroxidation and hemolysis, in spite of a similar release of iron. Similar results of protection were seen when the erythrocytes were challenged with acrolein (Ferrali et al., 1997), which induces GSH depletion by directly binding GSH, and promotes iron release as well as lipid peroxidation and hemolysis (Ferrali et al., 1989, 1990). The protection afforded by quercetin seems to be due to intracellular chelation of iron, since a semistechiometric ratio between the amount of released iron and the amount of quercetin necessary to prevent lipid peroxidation and hemolysis was observed (Ferrali et al., 1997).

Iron Release, Membrane Protein Oxidation and Erythrocyte Ageing

As known, the removal of old erythrocytes from the blood is accomplished by the mononuclear phagocytes that can distinguish senescent from mature cells. It is generally accepted (Kay et al., 1983; Kay, 1984; Low et al., 1985; Beppu et al., 1996) that the recognition of senescent cells depends on the appearance of a neo-antigen on the surface of senescent cells (senescent cell antigen (SCA)), to which autologous IgG antibodies bind. The latter ones attach to macrophages, initiating in such way the elimination of old cells (Lutz et al., 1987). It has been suggested (Kay, 1984; Beppu et al., 1996) that oxidative alterations of membrane proteins, particularly band 3, underlie SCA formation. It has been shown (Beppu et al., 1990), for instance,

that the incubation of human erythrocytes with oxidizing systems, such as ADP/Fe^{3+} or xanthine/xanthine oxidase/Fe^{3+}, promotes the binding of autologous IgG and that the antibody involved in the binding is an antiband 3 antibody. In human or calf erythrocytes, phenylhydrazine induced iron release, but not lipid peroxidation and hemolysis, probably because these cells could not be completely depleted of GSH (Signorini et al., 1995). Nevertheless, in human erythrocytes, phenylhydrazine induces the clustering of band 3 and the binding of autologous IgG (Low et al., 1985). In view of the possibility that the release of iron in a reactive form is a relevant factor in the generation of senescent antigen (SCA), we studied (Signorini et al., 1995), in a model of rapid in vitro ageing of erythrocytes, the relationships among iron release, oxidation of membrane proteins and binding of autologous IgG, that is, the formation of SCA. Since it has been reported that the aerobic incubation of erythrocytes in buffer markedly accelerates the erythrocyte ageing as measured by vesciculation (Bocci et al., 1980), we incubated calf erythrocytes in saline phosphate buffer for 24, 48 and 60 h under aerobic conditions and compared the results with those obtained under anaerobic conditions. The aerobic incubation induced a marked and progressive iron release (36 ± 2 nmol/ml erythrocyte suspension 50%, at 60 h), which was accompanied by an extensive Met-Hb formation. No substantial iron release or intensive Met-Hb formation occurred under anaerobic conditions, thus suggesting again the involvement of an oxidative stress in the release of iron. The release of iron was also accompanied by oxidative alterations of membrane proteins that were detected (Signorini et al., 1995) by both polyacrylamide gel electrophoresis (PAGE) and infrared spectroscopy (IR). PAGE electrophoresis showed the appearance of new bands in the range of 66–45 kDal and other alterations. These bands, which were not seen after the anaerobic incubation, are considered as an index of erythrocyte ageing and are thought to originate from the oxidative degradation of protein band 3 (Kay et al., 1983; Kay, 1984). IR showed in the aerobically incubated sample a consistent increase of carbonyl groups (absorption increase in the 1685–1650 cm^{-1} range) and other alterations of the spectrum all related to protein oxidation. In order to investigate whether the protein alterations seen during the aerobic incubation were related to the appearance of SCA, the membranes of erythrocytes incubated under aerobic or anaerobic conditions were challenged with autologous IgG (Signorini et al., 1995). The membranes from aerobically incubated cells were found to bind an extremely higher amount of autologous IgG than those from the anaerobically incubated ones, which indicates that the aerobic incubation gives rise to the appearance of SCA. Further studies were carried out to investigate whether the release of iron seen during the aerobic incubation could be related to the appearance of SCA. It was confirmed (Signorini et al., 1995) that the incubation of erythrocytes with phenylhydrazine, which, as mentioned above, induces a marked release of iron, also promotes a considerable binding of autologous IgG to the erythrocyte membrane. Also divicine and isouramil, which derive from the potentially toxic glucosides contained in fava beans (Vicia Faba) and which, like phenylhydrazine, induce iron release, promoted autologous IgG binding (Ferrali et al., 1997). Therefore in three conditions in which a marked release of iron occurs - namely prolonged aerobic incubation (*in vitro* ageing), incubation with phenylhydrazine and

incubation with divicine and isouramil – formation of SCA also occurs. Additional support for the hypothesis that the release of iron is indeed causally related to the formation of senescent antigen was found in experiments in which iron chelators able to enter cells were used in the attempt to chelate at the intracellular level the iron released during the aerobic incubation. It was found (Signorini et al., 1995) that the addition of the iron chelator ferrozine during the aerobic incubation completely prevents the formation of SCA. The addition of ferrozine also prevents all the membrane protein oxidative alterations seen with infrared spectroscopy. These results strongly suggest that ferrozine, which freely enters the cells, is capable of chelating at intracellular level all the released iron and to prevent in such way the alterations of membrane protein, probably produced by the redox cycling of the released iron and related to the formation of SCA. Also, the addition of quercetin during the aerobic incubation significantly prevented SCA formation. Quercetin was also able to prevent SCA formation and membrane protein alterations in human erythrocytes incubated with divicine and isouramil (Ferrali et al., 1997).

Moreover, similar results have been obtained (Ferrali et al., 2000) by using, as protective agent, a synthetic acyl hydrazone, fluor-benzoil-pyridoxal-hydrazone (FBPH), which is an iron chelator and enters the cells. This compound completely prevented lipid peroxidation and hemolysis in GSH depleted mouse erythrocytes incubated with phenylhydrazine and the formation of SCA in human erythrocytes incubated aerobically for 48–60 h. The IgG binding was detected, in these experiments, by using an anti-IgG antibody labeled with fluorescein and by examining the cells for fluorescence with confocal microscopy. The non incubated cells (0 time) showed no fluorescence, the aerobically incubated cells showed extensive fluorescence that is extensive binding, and the cells incubated aerobically in the presence of the FBPH showed much less fluorescence. The concentrations of FBPH tested (100 and 200 μM) showed proportional responses.

Finally, a progressive iron release was found (Signorini et al., 1995) to occur in human erythrocytes incubated aerobically for 40 and 60 h in saline phosphate buffer or stored in their own plasma at 4 °C for 15 and 35 days. Therefore an iron release seems to be really related to the ageing of red blood cells, and it is likely to occur even under physiological conditions. Chelation of iron at intracellular level could represent a means of prolonging the storage time of red cells in blood stores.

These results suggest the possibility that a metal-catalyzed oxidation of membrane proteins underlies erythrocyte ageing. A divalent cation capable of redox-cycling, such as iron released from its complexes, would bind to a divalent cation binding site on the protein (it is known that proteins possess such sites). Reaction with O_2 or H_2O_2 will generate active oxygen species which will oxidize amino acid residues near that cation binding site. The overall scheme would be the following: an oxidative stress in the erythrocytes will promote iron release, which in turn will promote oxidation of membrane proteins and the consequent formation of SCA, that is, the ageing of erythrocytes.

Consistent with the above observations, a number of studies by Hebbel and coworkers (Repka et al., 1993; Browne et al., 1998) have shown that iron decompartmentalization occurs in thalassemic and sickle red cells and that iron can be

found associated with the cytoplasmic surface of the membrane in which several discrete iron compartments (denatured hemoglobin, free heme, molecular iron, etc.) can be demonstrated. In particular, molecular iron (Browne et al., 1998) would be able to cycle between ferric and ferrous states and thereby participate in several redox reactions. Lutz et al. (1987) and Turrini et al. (1991) have shown that one of pathways leading to the erythrocyte ageing is the clustering of band 3 to which autologous IgG bind and that such clustering can be caused by protein oxidative cross-linking and similar processes.

Action at a Distance of Free Iron

Additional studies showed (Ferrali et al., 1993) that iron released from an erythrocyte lysate under conditions of oxidative stress is able to induce oxidative reactions and membrane damage even at a distance. It was, in fact, found that iron, released in a ghost-free erythrocyte lysate by the oxidant phenylhydrazine, is capable of crossing a dialysis membrane still in a redox active form, as shown by the fact that when the dialysate is added to membranes (erythrocyte ghosts, liver or brain microsomes) oxidative reactions are readily activated in the latter ones, such as lipid peroxidation and protein aggregation. Thus iron, once released from hemoglobin, is capable of activating oxidative reactions at a distance. In an in vivo situation, this may mean that iron, released from hemoglobin oxidation (for instance, after hemorrhagic events), can induce oxidative alterations in nearby cells, thus amplifying the hemorrhagic damage to a great extent.

Iron Release in Thalassemic Erythrocytes

As known, oxidative alterations somewhat similar to those occurring in aged erythrocytes have been found (Kahane, 1978; Flynn et al., 1983) in erythrocytes with hereditary abnormal hemoglobins, which are removed from the blood stream with an accelerated rate. In β-thalassemia, owing to the reduced synthesis of β chains, imbalance between α and β chains with a marked excess of α chains and persistence of HbF ($\alpha_2 \gamma_2$) occurs. It has been suggested (Scott et al., 1993; Brunori et al., 1975) that such an excess of α chains is the source of oxidative stress.

We studied (Ciccoli et al., 1999) erythrocytes from patients with β-thalassemia major (all continuously transfused), with β-thalassemia intermedia (mostly nontransfused) and with β-thalassemia minor (all nontransfused). Erythrocytes with thalassemia intermedia had an extremely higher content (0 time value) of DCI (16 ± 5 nmol/ml erythrocyte suspension 50%) as compared to controls (1 ± 0.3 nmol/ml) while no statistically significant increase was seen in erythrocytes with thalassemia major and with thalassemia minor. Upon aerobic incubation for 24 h, the release of iron in β-thalassemic erythrocytes (24 ± 5 and 13 ± 2 nmol/ml in thalassemia intermedia

and major, respectively) was far greater than in controls (6 ± 1 nmol/ml), with the exception of thalassemia minor. Met-Hb content (0 time) and formation (after 24 h incubation) were markedly increased in erythrocytes with thalassemia intermedia.

The percentage of HbF was highest in erythrocytes from subjects with thalassemia intermedia (43 ± 17%). It was increased, compared to controls, in erythrocytes with thalassemia major (11 ± 4%), and to a much lesser extent (at least when detectable) in thalassemia minor. When the individual values for DCI content (0 time) in thalassemia major and intermedia were plotted against the corresponding values for HbF, a positive correlation was seen. Also, a positive correlation was seen between free iron release (after 24 h incubation) and HbF values. Since, as stated above, in β-thalassemia the persistence of HbF is related to the lack or deficiency of β chains and therefore to the excess of α chains, the observed correlation between free iron and HbF is in agreement with the hypothesis that an excess of α chains represents a pro-oxidant factor. This is probably due to the fact that α chains are more prone to release iron in reactive form. In α-hemoglobin chains loaded-erythrocytes (an interesting model of β-thalassemic cells) membrane bound heme and iron were markedly elevated and the cells were more prone to oxidative stress (Scott et al., 1993). In inside-out membranes from β-thalassemic and sickle cells, elevated amounts of heme iron and, especially, free iron were observed (Repka et al., 1993). This form of iron may thus represent the trigger for the oxidative damage seen in β-thalassemic cells. It may also represent the mechanism of formation of SCA, or any other membrane event responsible for the early removal of erythrocytes from the blood stream.

Iron Release in Erythrocytes from Newborn Infants

Since, as above reported, iron release seems to be related to the erythrocyte ageing, and since in newborn infants an accelerated removal of erythrocytes occurs, we investigated whether an increased DCI content and an increased susceptibility to release iron could be observed in erythrocytes from term and preterm newborns. The erythrocyte DCI content was significantly increased in preterm but not in term newborns as compared to adults while the release of iron after aerobic incubation (24 h) was increased in both preterm and term erythrocytes (Ciccoli et al., 2003). Met-Hb formation was also increased in both groups of babies. Interestingly, the level of plasma non-protein-bound iron (NPBI)[1] which was not detectable in adults,

[1] As known, in several newborns NPBI is detectable in plasma (Evans et al. 1992, Berger et al. 1995, Dorrepaal et al. 1996). This iron was initially looked for and detected with ≥100% transferrin saturation, but subsequently, as techniques for its detection became more sophisticated, non-transferrin-bound iron (NTBI), subsequently referred to as NPBI, was also found in conditions where transferrin was not fully saturated. The origin of this form (or forms) of iron, which is also detectable in plasma of many patients with iron overload, such as hereditary and secondary hemocromatosis (Hershko et al. 1978, Aruoma et al. 1988, Porter et al. 1996, Breuer et al. 2000) or other metabolic disease (Lee et al. 2006), is not clear. It is likely that NPBI is one of the several contributing factors to the observed overall oxidative stress in iron overload conditions and in severe diseases of newborns.

was relatively high (3.6 ± 0.5 nmol/ml) in preterm and, although to a lower extent (1.2 ± 0.3 nmol/ml), in term newborns (Ciccoli et al., 2003). When term plus preterm newborns were divided into two groups, normoxic and hypoxic, according to previously published (Buonocore et al., 1999) criteria (cord blood pH ≤ 7.21, APGAR score[2] ≤ 6 at 5 min, and fraction of inspired oxygen needed for resuscitation immediately after delivery ≥0.4), it was found that both iron release (after aerobic incubation) and plasma NPBI level were markedly higher in the hypoxic newborns compared to normoxic ones. Similar results were also obtained when the preterm and term infants were considered separately on the base of cord blood pH. Therefore, iron release and plasma NPBI are higher when conditions of hypoxia occur. In fact, when the values for iron release and plasma NPBI were separately plotted against cord blood pH values, significant negative correlations were seen (Ciccoli et al., 2003) in both cases. NPBI was correlated with iron release seen in all the newborns.

The different results seen with different cord blood pH values suggest that "hypoxic environment" and subsequent reoxygenation promote iron release. To test this possibility a model of hypoxia or hypoxia-reoxygenation was accomplished (Ciccoli et al., 2004) by incubating erythrocytes from newborns and adults for 24 h under hypoxic condition (exposure to a mixture of 4% O_2, 6% CO_2 and 90% N_2) at 37 °C or for 16 h under hypoxic condition and subsequently for 8 h in aerobic condition (normoxia, room air). Control erythrocytes from both newborns and adults were incubated for 24 h or 16 + 8 h in aerobic conditions. The incubation in hypoxic conditions induces greater iron release than incubation under aerobic conditions. The iron release observed after 16 h and 24 h of hypoxia was greater in newborns (25.5 ± 4.3 nmol/ml; 43.9 ± 3.1 nmol/ml) than in adult erythrocytes (10.9 ± 1.7 nmol/ml; 14.8 ± 1.8 nmol/ml), and it increased in newborn erythrocytes with the duration of hypoxia. Reoxygenation of hypoxic (16 h) erythrocytes induced a further increase in iron release. The highest levels of iron release and Met-Hb formation occurred in newborn erythrocytes after 24 h of hypoxia. Since iron release is related to oxidative stress, it appears, paradoxically, that hypoxia represents a condition of oxidative stress, especially in newborn erythrocytes. Thus, these in vitro studies confirms our previous results (Ciccoli et al., 2003) in which much higher iron release was observed in hypoxic than in non-hypoxic newborns, again suggesting that a hypoxic environment is a factor promoting iron release.

IgG Binding to Band 3 Dimers in Newborn Erythrocytes

As mentioned above in two conditions in which an accelerated removal of erythrocytes occurs, namely β-thalassemic (major and intermedia) and perinatal period, the erythrocyte DCI (content and release after incubation) is greatly

[2] The APGAR score is an index of clinical evaluation of the newborn at birth.

increased as compared to normal adult erythrocytes. β-Thalassemic and newborn erythrocytes show a high content in fetal hemoglobin (HbF) (even higher in preterm infants). HbF, at least under many experimental conditions, has greater oxygen affinity than adult hemoglobin and is more subjected to denaturation and oxidation (MacDonald et al., 1983; Lubin et al., 1993; Robson and Brittain, 1996; Rossi et al., 2006a). Birth is an oxidative challenge for the newborn, due to the sharp postnatal transition from the relatively low oxygen intrauterine pressure (pO_2 20–25 Torr) to the significantly higher oxygen extrauterine environment (pO_2 100 Torr). Such oxidative challenge is exacerbated by the low efficiency of natural antioxidant systems in the newborns, particularly in preterm babies (Saugstad, 1996; Bracci and Buonocore, 1998). In addition in most newborns (Evans et al., 1992; Berger et al., 1995; Dorrepaal et al., 1996) NPBI is detectable in plasma. Furthermore, F_2-isoprostanes, prostaglandin F_2-like compounds formed by free radical-catalyzed lipid peroxidation of phospholipid-bound arachidonic acid and considered the most reliable marker of oxidative stress (Morrow et al., 1996; Delanty et al., 1996; Montuschi et al., 2004), are significantly higher in plasma of newborns than of healthy adults and are higher in preterm than in term newborns (Comporti et al., 2004). Lutz et al. (1987) and Turrini et al. (1991) have shown that the clustering of band 3, to which autologous IgG bind with consequent erythrocyte removal, can be produced by oxidative cross-linking of membrane proteins. Turrini et al. (1994) have demonstrated that disulfide-cross-linked band 3 dimers (clusters) are the minimal band 3 aggregates with enhanced affinity for anti-band 3 antibodies.

We have shown (Rossi et al., 2006) that such oxidatively modified band 3 (the 170 kDa band which marks the erythrocytes for removal) is present in as much as in 74% of preterm newborns, in 21% of term newborns, and in 10% of adults. During erythrocyte ageing *in vitro* (0, 24 and 48 h aerobic incubation), the appearance of the band occurred much faster with erythrocytes from newborns (particularly preterm) that with those from adults. When the blots for the 170 kDa band were quantified by scanning densitometry it was seen that the 0 time values were significantly higher in preterm compared to term and adult values. After aerobic incubation a progressive increase in the optical density was observed in each group and the densities were higher in preterm than in other groups. The course of iron release during the various incubations was analogous to that of the 170 kDa band blots, and significant correlations were found at 0 and 48 h. Methemoglobin formation roughly paralleled iron release. Esterified F_2-isoprostanes (markers of oxidative stress) and $O_2^{\bullet-}$ production in the nonincubated (0 time) erythrocytes were much higher in newborn (preterm and term) than in adult erythrocytes. Thus dimers of band 3 with autologous IgG are found under conditions in which oxidative stress can be detected in erythrocytes or in plasma: namely in newborns or in aged erythrocytes. Moreover the binding of autologous IgG to band 3 dimers was (Ciccoli et al., 2004) much greater in newborn than in adult erythrocytes exposed to hypoxia, (in which also iron release is more elevated), again suggesting that the level of iron release is related to the extent of band 3 clustering and that hypoxia accelerates removal of erythrocytes from blood stream.

Iron Release and IgG Binding to Band 3 Dimers in Diabetic Erythrocytes

Oxidative stress is involved in the pathogenesis of diabetes and particularly of its long term complications (Jay et al., 2006) and exacerbated by hyperglycemia, can damage red blood cells with consequent possible decrease of their life-span (Sailaja et al., 2003). Recently we have observed that the binding of autologous IgG to band 3 dimers is present in 47.8% of diabetic subjects and in 15% of controls, and that no difference in percentages between type 1 and type 2 diabetics is evident (Rossi et al., 2006). Iron release is higher in diabetic erythrocytes showing IgG binding and a significant correlation between iron release and IgG binding is found. This suggests that in diabetic subjects a large part of the erythrocytes is ready to be eliminated by the phagocytic system and reflects an accelerated turnover of red cells. So intraerythrocyte free iron and band 3 dimers appear to be strictly related in diabetic erythrocytes too, which are likely exposed to an increased oxidative stress, as shown by increased plasma levels of esterified F_2-isoprostanes (Gopaul et al., 1995; Rossi et al., 2006) and NPBI (Lee et al., 2006), markers of oxidative stress. The increased erythrophagocytosis and the subsequent possible exocytosis of iron (Yuan et al., 1996a, b) can play an important role in LDL oxidation, mechanism underlying atherosclerotic injuries and long term diabetic complications (Li et al., 2004).

Conclusion

As previously mentioned the reductive release of iron from ferritin has received extensive consideration, while the release of iron in an oxidative manner from heme-proteins has been relatively little studied. It has been calculated (Ladner et al., 1977) that in the planar structure of heme the distance Fe^{2+} -porphyrin nitrogen is about 2.1 Å. If Fe^{2+} is oxidized to Fe^{3+} as in methemoglobin, the structure looses its planar symmetry and iron is moved away from the plan center of about 0.1 and 0.2 Å in α and β chains, respectively. This implies a shift from a low to a high spin condition; in the latter the bond of the metal to nitrogen is less stable. Such a decrease in bond stability could account for the release of iron in concomitance with methemoglobin formation. Obviously the amount of released iron is by far lower (on a molar base) than that of methemoglobin formed and could represent the part that exceeds the reducing capacity of erythrocyte methemoglobin reductase.

The studies reviewed in this article show that iron is released from hemoglobin in a number of conditions in which the erythrocyte is subjected to oxidative stress. Such conditions can be clearly linked to pathological or toxicological events (hemolytic drugs), but can also result from more subtle situations in which a state of ischemia-reperfusion is imposed on erythrocytes (e.g., childbirth, especially when newborns are severely premature). The released iron could play a central role

in oxidation of membrane proteins and band 3 alterations, with consequent erythrocyte removal from the blood stream. This could be also one of the mechanisms of the hyperbilirubinemia observed in hemolytic anemias and in neonates.

Finally, since the released iron has a tendency to diffuse from the erythrocytes into the surrounding medium (Ciccoli et al., 2004), such a diffusion, together with the higher susceptibility to release iron found in newborn erythrocytes as compared to adult erythrocytes, could explain, at least in part, the appearance of plasma free iron, which is assumed to play an important role in the so called "free radical disease" of the neonate

In conclusion, we have briefly reviewed the role of iron with regard to oxidative damage to lipid and protein membranes particularly of erythrocytes, employing both *in vitro* and *in vivo* studies. Iron metabolism is very complex. Much progress still needs to be made in order to understand the contribution of iron to oxidative stress and disease, and the various possible chemotherapeutic strategies, since iron is an element of conflicting effects, that is it can be either beneficial or detrimental to the cell, depending on whether it serves as a micronutrient or as a catalyst of free radical reactions.

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Chapter 9
The Adventures of Superoxide Dismutase in Health and Disease: Superoxide in the Balance

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Abstract This chapter focuses on superoxide dismutases (SOD), a group of enzymes important for removing biologically generated superoxide anion radical ($O_2^{\bullet -}$). These enzymes function by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and oxygen, and their action helps to protect cells from oxidation of lipids, proteins and DNA. These enzymes are crucial in maintaining a proper balance of superoxide within specific cellular compartments that is essential for normal cellular signaling and stress responses. Three forms of SOD exist in humans: copper and zinc containing SOD (CuZnSOD), manganese containing SOD (MnSOD) and extracellular SOD (ecSOD). Each of the three SOD proteins plays a unique physiological role based in part on its tissue distribution and sub cellular localization. For example CuZnSOD is generally found in the cytosol, but can also be localized in the nucleus under certain conditions. CuZnSOD protects proteins, lipids and nuclear DNA from oxidation. In contrast, MnSOD, an enzyme required for life in an oxygen atmosphere, is located specifically in the mitochondrial matrix and protects the respiratory machinery and mitochondrial DNA from oxidative damage. ecSOD is found both extracellularly and on the plasma membrane. Its expression is greatest in endothelial cells of blood vessels where its key role appears to be regulating superoxide interactions with nitric oxide. Superoxide and nitric oxide react to form peroxynitrite, a toxic species. This reaction decreases the bioavailability of nitric oxide so it can no longer function to maintain vascular tone and health. The balance of superoxide and nitric oxide is therefore vital for proper vasculature function. In disease processes, dysregulation of SODs results in a pathogenic imbalance of superoxide, leading to profound effects on cells and tissue. For example, aberrant structure or activity of CuZnSOD has been linked to the neurological disease amyotrophic lateral sclerosis (ALS) and developmental abnormalities seen in Down's syndrome. Similarly, decreases in the function of MnSOD

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

have been shown to play a role in carcinogenesis. Finally, cardiovascular disease is associated with poor vascular tone following lowered nitric oxide bioavailability. Decreased ecSOD function results in increased peroxynitrite formation that causes damage to the vasculature, thus accelerating cardiovascular disease. In summary, SODs are an essential group of enzymes that must be present at appropriate levels, structure, and locations to maintain a healthy balance of superoxide, necessary for development and disease prevention.

Keywords Free radicals, antioxidants, cancer, amyotrophic lateral sclerosis, atherosclerosis, Down's syndrome

Introduction

Life in an oxygen rich atmosphere provides living organisms with the luxury to utilize oxidative phosphorylation as an efficient source of energy production, but it also comes with a price, which is the production of biologically damaging oxygen radicals and other reactive oxygen species. Approximately 2% of all of the oxygen we consume is converted to superoxide via normal physiological processes such as respiration and innate immune responses. During the generation of cellular energy in the form of ATP, the electron transport chain produces superoxide when single electrons slip off the electron transport chain during transfer from complex I (NADH dehydrogenase) (Turrens and Boveris, 1980), complex II (succinate dehydrogenase) (Zhang et al., 1998), and complex III (cytochrome c reductase) (Cadenas et al., 1977; Han et al., 2001). The other major source of superoxide generation in the body is the plasma membrane bound NADPH oxidases. A variety of cells, particularly neutrophils, employ these enzymes to produce superoxide for conversion to hydrogen peroxide used as fuel for peroxidases that generate additional oxidizing species to fight bacteria and other infections. In addition, vascular cells produce both superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) to regulate vascular tone (review by (Griendling et al., 2000). Superoxide has various specialties within the body, explaining the need for multiple superoxide dismutase enzymes (SOD) to regulate concentration and availability for each specialized location. When superoxide levels are not properly regulated by their enzyme catalyzed dismutation, cellular pro-oxidants are increased and the risk of human disease increases. The focus of this chapter will be the balancing act that SODs perform with respect to controlling superoxide levels in various cellular compartments in particular tissues of the body.

Types of SOD in Humans, Localization and Function

To survive in the oxygen atmosphere of earth, aerobic organisms have evolved a group of enzymes capable of dismuting the superoxide radical (McCord et al., 1971). Discovered in 1969 by McCord and Fridovich the enzymes are now known

as superoxide dismutase (SOD) enzymes (McCord and Fridovich, 1969). SOD enzymes function to dismute superoxide to hydrogen peroxide and molecular oxygen by the following reaction.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Superoxide by itself is a relatively unreactive free radical; most of its deleterious effects are through iron catalyzed Haber Weiss interactions that produce hydroxyl radical as follows.

$$O_2^{\bullet-} + Fe^{+3} \rightarrow O_2 + Fe^{+2} \quad (2)$$

$$Fe^{+2} + H_2O_2 \rightarrow HO^{\bullet} + OH^- + Fe^{+3} \quad (3)$$

Humans have developed three types of superoxide dismutases (SODs); each is encoded from a separate gene and serves a different purpose depending on subcellular localization. The first SOD discovered contains both copper and zinc; hence, its name copper and zinc containing superoxide dismutase (CuZnSOD). This is a homodimeric protein, composed of two active sites each containing one copper and one zinc atom. The metals are essential for proper enzyme function. Copper provides the catalytic function of the enzyme by facilitating a single electron transfer from superoxide, while zinc stabilizes the protein structure and prevents the copper from leaving the active site.

$$E\text{-}Cu^{2+} + O_2^{\bullet-} \rightarrow E\text{-}Cu^{1+} + O_2 \quad (4)$$

$$E\text{-}Cu^{1+} + O_2^{\bullet-} + 2H^+ \rightarrow E\text{-}Cu^{2+} + O_2 \quad (5)$$

CuZnSOD protein is encoded by the gene *SOD1* located on human chromosome 21. This enzyme is predominantly located in the cytoplasm, but has also been found in the nucleus and lysosomes under certain conditions (Chang et al., 1988; Crapo et al., 1992). Its localization allows it to protect proteins, lipids and DNA from oxidation and it is an important regulator of intracellular superoxide signaling.

The second SOD we will discuss is the manganese containing SOD (MnSOD). It is a homotetramer with four active sites each containing a manganese atom that constitutes the catalytic center of the protein. The MnSOD tetrameric protein forms a channel for superoxide to enter and react with manganese (Leveque et al., 2001). Equations 6 and 7 outline the overall reaction of the enzyme to dismute superoxide (Hsu et al., 1996).

$$Mn^{3+} + O_2^{\bullet-} \rightarrow [Mn^{3+} \text{ -- } O_2^{\bullet-}] + 2H^+ \rightarrow Mn^{2+} + O_2 \quad (6)$$

$$Mn^{2+} + O_2^{\bullet-} \rightarrow [Mn^{2+} \text{ -- } O_2^{\bullet-}] + 2H^+ \rightarrow Mn^{3+} + H_2O_2 \quad (7)$$

MnSOD is encoded by the gene *SOD2* on human chromosome six. During translation the protein is translocated into the mitochondrial matrix where it is found

primarily between cristae (Slot et al., 1986). Superoxide is generated in the mitochondria from the one-electron reduction of molecular oxygen. MnSOD reduces oxidative stress in the mitochondria by catalyzing the rapid dismutation of superoxide formed by the electron transport chain during respiration. This reaction proceeds with an impressively rapid rate constant ($k = 1.5 \times 10^9\ M^{-1}\ s^{-1}$) (Pick et al., 1974). Overall, the action of MnSOD protects the respiratory machinery and mitochondrial DNA, thus ensuring maintenance of mitochondrial integrity.

Finally we will discuss extracellular superoxide dismutase (ecSOD), the most recently discovered form of SOD. The ecSOD gene, *SOD3*, is located on human chromosome 4. The protein, which exists in cells as a homotetramer, is similar to CuZnSOD in that it uses copper for its catalytic function and zinc to maintain protein structure. Unlike the previously described SODs, ecSOD is predominantly located in the extracellular space (Marklund, 1982). It has recently been demonstrated that ecSOD is an important determinant of the total superoxide dismutase in vasculature, comprising one-third to one-half of the total vascular SOD activity, where its key role is regulating the concentration of superoxide in the vessel wall available to interact with nitric oxide ($NO^{\bullet}$) (Stralin et al., 1995). Superoxide in the vasculature limits $NO^{\bullet}$ bioavailability by reacting with it to form peroxynitrite ($ONOO^-$), a highly reactive and oxidizing species. The reaction between superoxide and $NO^{\bullet}$ is fast (rate constant = $6.7 \pm 0.9 \times 10^9\ M^{-1}\ s^{-1}$) (Huie and Padmaja, 1993), and thus ecSOD competes with $NO^{\bullet}$ for superoxide to preserve $NO^{\bullet}$ availability for maintaining proper vascular tone and health. The reaction of superoxide with $NO^{\bullet}$ not only destroys $NO^{\bullet}$, but also generates the highly damaging $ONOO^-$ molecule. Thus ecSOD is vital to maintain vascular health.

Low Levels of SOD Leads to Increased Levels of Superoxide

The importance of each of the three forms of SOD in mammals is perhaps best demonstrated by observing the phenotypes produced upon their inactivation in mouse models. For example, *SOD1* (CuZnSOD) knockout mice develop normally and grow to maturity, but more than half develop hepatocarcinomas, presumably from increased hepatic oxidative stress (Elchuri et al., 2005). The *SOD2* (MnSOD) knockout mice encounter severe crisis and die within the first ten days to three weeks depending on their genetic background. These mice die with dilated cardiomyopathy, accumulation of lipids in liver and skeletal muscle, and metabolic acidosis (Li et al., 1995). While the *SOD2* heterozygotes grow into adulthood they are also severely afflicted by reduced MnSOD activity (50% of normal). They have elevated levels of oxidative damage as evident by accumulation of 8-oxo-2-deoxyguanosine (8-oxo-dG) in their nuclear and mitochondrial DNA and display an increased incidence of cancer (Van Remmen et al., 2003). In contrast, *SOD3* (ecSOD) deficient mice develop normally and the effects of the SOD deficiency are only seen when the mice are under stress. For example, in response to induced

hypertension these mice display an exaggerated hypertensive response compared to their wild type counterparts. These *SOD3*-/- mice had increased vascular levels of superoxide that prevented them from responding appropriately to their secretion of nitric oxide in order to reduce blood pressure (Jung et al., 2003).

Taken together, the findings highlighted above show that mice without SOD have severe biological consequences; however, this does not mean that low levels of SOD enzymes are negative in all cases. Individual cell types or specific cells within populations can be characterized by their minimal levels of SOD expression. For example, immunohistochemistry studies have shown that stem cells do not strongly express the antioxidant enzymes CuZnSOD, MnSOD or catalase. Conversely, when these cells differentiate, the differentiated cells stain strongly for these same antioxidant enzymes (Oberley et al., 1990). The cellular levels of both CuZnSOD and MnSOD have been determined to increase during development of mammals (Pittschieler et al., 1991). This phenomenon has been shown in other eukaryotes such as the slime mold, where it has been shown that SOD increases during differentiation and over-expression of CuZnSOD is able to cause non-dividing cells to re-enter the cell cycle (Allen et al., 1988). The increase in SOD is most likely directly a result of increases in ROS that is observed at the beginning of differentiation events (Hansberg et al., 1993). In support of this, increased MnSOD expression has been shown to promote cellular differentiation. One hypothesis states that this is due to the protection against ROS damage conferred by high MnSOD; by protecting mitochondria from oxidative damage the cells are ensuring an adequate energy source is available for differentiation. An alternate hypothesis states that MnSOD may be modulating the intracellular redox state and the expression of second messengers and genes required for continued differentiation (St Clair et al., 1994).

High Levels of SOD Lead to Decreased Superoxide

Superoxide levels are important messengers in intracellular signaling, particularly in response to hypoxic stress. Hypoxia inducible factor-1 (HIF-1) is a transcription factor that responds to the oxygen tension inside the cell. Under conditions of hypoxia the transcription factor induces transcription of genes that activate glyco lysis and signal for angiogenesis to increase oxygen and nutrients to the site of hypoxia. HIF-1 not only promotes glycolysis, but also apparently inhibits oxidative phosphorylation. HIF-1 activates pyruvate dehydrogenase kinase PDK1, an enzyme that phosphorylates pyruvate dehydrogenase and inhibits it from using pyruvate to fuel the mitochondrial TCA cycle, thus decreasing oxidative phosphorylation. The effect of this activity, coupled with the induction of most of the enzymes that mediate glycolysis, causes a shift from ATP production by oxidative phosphorylation and towards glycolysis (Papandreou et al., 2006). To increase oxygen at a hypoxic site the number and density of blood vessels can be increased to promote greater blood flow to the area. HIF-1 signals this response by inducing transcription of

vascular endothelial growth factor (VEGF). VEGF triggers a pro-angiogenic cascade in order to increase blood flow and nutrients to the hypoxic regions (Liu et al., 1995). The activity of the HIF-1 transcription factor is indirectly controlled by the cellular oxygen content, which regulates the stability of one of the subunits, HIF-1α (Wang and Semenza, 1993). In the presence of oxygen, the HIF-1α subunit is rapidly degraded. Degradation is initiated by HIF-prolyl hydroxylase (PHD). Hydroxylated prolines in the oxygen dependent degradation domain of the HIF-1α protein provide a signal for the tumor suppressor protein von Hippel-Lindau (VHL) to ubiquitinate the protein so that is can be degraded by the 26S proteasome. The absence of oxygen removes the cofactor necessary for hydroxylation and changes the state of the iron in the enzyme's active site allowing HIF-1α to encounter HIF-1β and activate transcription of downstream targets. In spite of the presence of oxygen, high concentrations of superoxide reduce the iron cluster of PHD to change the protein conformation and prevent hydroxylation of HIF-1α, thus activating the HIF-1 signaling pathway. MnSOD is able to decrease superoxide levels, and at physiologically relevant concentrations can inhibit the hypoxic induction of the HIF-1α transcription factor (Wang et al., 2005).

von Hippel-Lindau disease is a disease where both alleles of the *VHL* gene are inactivated. This inhibits the degradation of HIF-1α, leading to increased transcription of its downstream targets, such as VEGF. Victims of this disease commonly face hemangioblastomas, highly vascular tumors of the blood vessels, in the central nervous system and retina (reviewed by (Richard et al., 1998)). In addition, it has been shown that HIF-1α expression is increased in various human tumors types when compared to normal tissue (Talks et al., 2000; Zhong et al., 1999). This evidence suggests that not only can the over-expression of HIF-1α lead directly to tumor formation, but it can also confer an advantage to tumor cells because HIF-1 transcription targets promote growth and survival in response to stress.

Aberrant Regulation of SOD Leads to Human Disease

As described previously, the consequences of imbalanced SOD can have dramatic cellular and organismal consequences that correspond to the development of human diseases. Indeed, all three SODs are associated with human diseases. The overexpression of CuZnSOD during development is associated with Down's syndrome. Toxic gain of function mutations in the *SOD1* gene can cause neuronal death and result in amyotrophic lateral sclerosis (ALS). Decreases in MnSOD activity are frequently seen in many forms of human cancer. Similarly, decreased ecSOD aids in the progression of atherosclerosis. The variety of diseases related to aberrant expression of SOD demonstrates the breadth of activities for enzymes with the same catalytic function but in different cell types and intracellular locations. We will discuss the pathogenesis and treatment options currently understood for each of these diseases.

Down's Syndrome

It has long been known that Down's syndrome is caused by a trisomy of chromosome 21. As cytogenetic analysis advanced, researchers pinpointed a partial duplication that result in Down's phenotypes. They have been able to identify the specific region necessary to cause the development of Down's phenotype, that is 21q21–21q22, this region contains many genes including *SOD1* (Delabar et al., 1987; Huret et al., 1987). One case in particular was instrumental in confirming the significance of over-expression of CuZnSOD in Down's syndrome. In 1987 a young boy was identified as having Down's syndrome, but had a normal cyto genetic analysis. Further investigation found that he had a small duplication 2,000–3,000 kb on chromosome 21; Southern analysis found three copies of *SOD1* and more importantly 3/2 the SOD activity of his normal parents. The boy presented with poor speech development and psychomotor and mental retardation; the only Down's phenotype absent was hypotonia (Huret et al., 1987). Thus, gene dosage for CuZnSOD activity and a proper balance between superoxide and SOD activity appears to be an important determinant of this disease.

Amyotrophic Lateral Sclerosis

The next disease we will discuss is also caused by alterations of CuZnSOD activity, but in this example it is mutations rather than aberrant expression that lead to the disease phenotype. Amyotrophic lateral sclerosis (ALS) is an adult onset progressive neurodegenerative disease that affects the brain cortex, brainstem and spinal cord. The disease causes motor neuron death leading to muscular atrophy. The disease was first described in 1869; nevertheless little new knowledge about the mechanisms underlying the disease process was obtained for the next 100 years, and an effective treatment is still a mystery. One of the known causes of the inherited form of the disease is a dominant missense mutation of the *SOD1* gene (Rosen et al., 1993). This is the case for approximately 20% of familial (heritable) cases of ALS and 4% of sporadic cases of ALS (Gamez et al., 2006). There are many mutations of *SOD1* described that cause the familial form of the disease, and we know that all these mutations do not cause a loss of SOD function (Reaume et al., 1996). Indeed, SOD activity of the mutant proteins is typically normal in these individuals. Thus, rather than loss of SOD activity, it appears that these mutations confer a toxic gain of function to the protein, the precise nature of which is still debated. There are over 100 known mutations in the *SOD1* gene that have been shown to result in ALS. The strongest evidence of a role for *SOD1* mutations is the variety of transgenic mouse models that have been created [G93A (Gurney et al., 1994)], [G37R (Wong et al., 1995)], [G85R (Bruijn et al., 1997)]. The mechanisms underlying the etiology of the disease process at the molecular level and its resulting pathogenesis are poorly understood. Insights into *SOD1* mutations gives hope to understand all forms of ALS with the eventual promise of an interventional therapeutic.

There are currently three leading hypotheses proposed to explain the mechanisms leading to the degeneration of motor neurons in ALS (Fig. 9.1). One model suggests that mutant CuZnSOD associates with chromogranins in secretory vessels within neurons and glial cells to facilitate secretion into the extracellular space. It is proposed that this elicits an immune response by activating microglia to produce proapoptotic signals that ultimately kill both upper and lower motor neurons (Urushitani et al., 2006). Another model of disease pathogenesis follows findings from other neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease. All of these have been shown to be caused by accumulation of toxic aggregates within neurons causing neuronal death. Indeed such aggregates containing the mutant CuZnSOD protein have been found in both familial and sporadic ALS including all mouse models examined. Specifically, protein aggregation has been shown when there is decreased protein structural stability and enhanced aggregate formation (Stathopulos et al., 2003). These aggregates are detectable by the onset of clinical disease. In some cases (e.g., $SOD1^{G85R}$ mice) they represent the first pathologic sign of disease, and later during disease progression they increase markedly in abundance (Bruijn et al., 1997). Several possible toxicities of the protein aggregates have been proposed, including aberrant cellular biochemistry; loss of protein function through CuZnSOD co-aggregation within the aggregates; depletion of protein folding chaperones; dysfunction of the proteasome overwhelmed with undigestable misfolded protein; and inhibition of specific organelle function including mitochondria and peroxisomes through mutant aggregation onto or within such organelles. However, insoluble aggregates are not found in all cases of ALS, thus diminishing the argument that this is the universal mechanism causing pathogenesis. Lastly we will discuss a model that involves mitochondrial dysfunction as the putative cause of ALS. This model was developed following the observation that there are morphological abnormalities of mitochondria prior to neuronal degeneration in ALS transgenic mice (Dal Canto and Gurney, 1994). Not only are physical features of mitochondria altered, but also function of electron transport chain leading to impaired ATP production and increased oxidative stress (Jung et al., 2002). It has been shown that expression of mutant *SOD1* increases superoxide levels in neuronal cells. Interestingly, forced expression of MnSOD is able to protect from neuronal death, further supporting the role of oxidative stress in the neuronal death that causes ALS symptoms (Zimmerman et al., 2007). The damaged mitochondria have been shown to initiate apoptosis that involves translocation of Bax from the cytosol to the mitochondria, and cytochrome c translocation from the mitochondria to cytosol in mice and humans (Guegan et al., 2001). Other studies have shown that mutant *SOD1* in mitochondria triggers release of cytochrome c, thus activating caspase cascade and neuronal death in the absence of aggregate formation (Takeuchi et al., 2002). Taken together these studies provided strong evidence for aberrant mitochondrial metabolism in ALS and highlight the role of a balance between superoxide and SOD in maintaining neuronal health.

Since ALS can be caused by many types of mutations in a single gene, it seems reasonable that preventing expression of the mutant protein will stop disease progression.

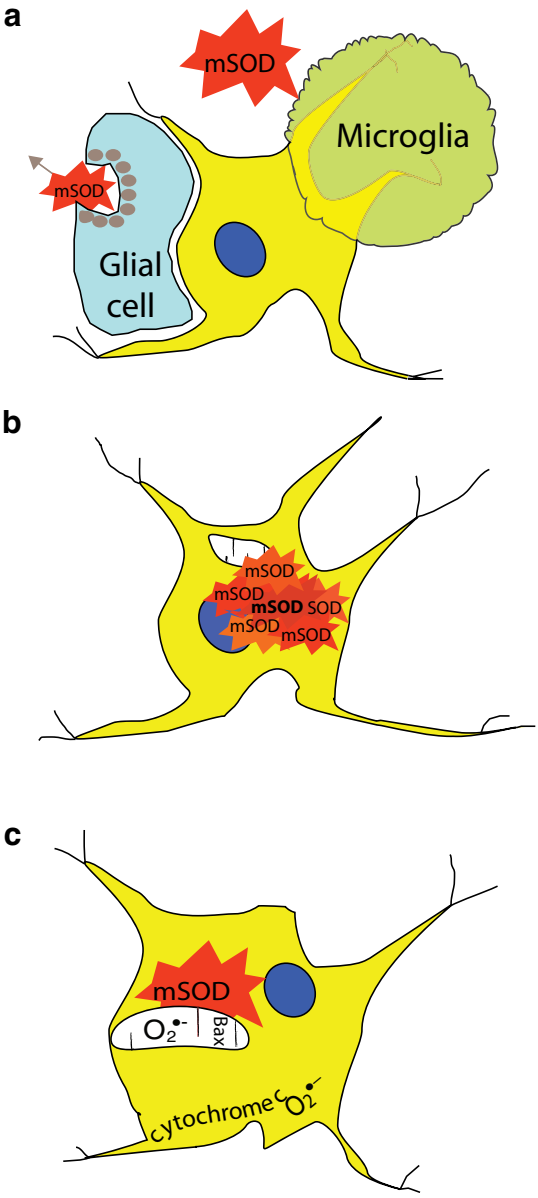

Fig. 9.1 Three proposed mechanisms of neuronal toxicity in motor neurons of ALS patients. (A) The first hypothesis suggests that mutant CuZnSOD (mSOD) is associated with chromogranins in secretory vessels within glial cells to facilitate secretion of the mutant protein into the extracellular space. This elicits an immune response whereby microglial cells are activated to initiate neuronal cell killing. (B) The second hypothesis states that mutant CuZnSOD accumulates and causes protein aggregation leading to neuronal toxicity. (C) The final model suggests that expression of mutant CuZnSOD leads to increased intracellular superoxide levels. This can initiate apoptosis as indicated by the translocation of Bax to the mitochondria and the mitochondrial release of cytochrome c, both responsible for initiation of a caspase cascade.

Degrading the RNA before it is translated using RNA interference (RNAi) is currently being tried with successful outcomes in mouse models (Ralph et al., 2005; Raoul et al., 2005). RNAi is particularly ideal for this disease because it is able to specifically target *SOD1*, ideally degrading mutant forms without interfering with wild type protein. One challenge is effective delivery of siRNA or shRNA. The other will be to identify cases early enough to maximize effectiveness. Nevertheless, some RNAi studies in mice show promise for human treatment. Using lentiviral vectors based on equine infectious anemia virus (EIAV), investigators can target motor neurons with intramuscular injections. The virus expresses shRNA for the *SOD1* gene, providing 95% gene transfer efficiency. This treatment significantly delays onset of disease, and reduces life long hind limb dysfunction. This type of treatment is beginning clinical studies (Ralph et al., 2005).

Cancer

As previously noted, mice deficient in SOD expression are at a greater risk of developing cancer. The most profound effects are seen in cases of MnSOD deficiency. Expression patterns have also been examined in human cancer; it has been shown that MnSOD expression is decreased in many human cancers (Soini et al., 2001). There are three well described explanations of how MnSOD activity is lowered in tumor cells. The first is mutations in the gene, either in the coding region (Hernandez-Saavedra and McCord, 2003) or within important regulatory sites (Xu et al., 2002) The second mechanism is loss of heterozygosity of the portion of chromosome 6q where the *SOD2* gene resides (Bravard et al., 1998; Liang et al., 1994; Walker et al., 1994). A third mechanism of *SOD2* gene silencing is abnormal DNA methylation of the gene leading to decreased transcription (Hitchler et al., 2006; Hodge et al., 2005; Huang et al., 1997, 1999). Taken together, these findings have led to the suggestion that MnSOD is a tumor suppressor gene (Bravard et al., 1992). In support of this concept it has been shown that over-expression of MnSOD decreases cell growth and malignant characteristics in human cancer cells (Church et al., 1993; Cullen et al., 2003; Lam et al., 1997; Ough et al., 2004; Weydert et al., 2003; Zhang et al., 1999; Zhong et al., 1997). With this knowledge it can be hypothesized that increasing SOD activity in the context of cancer will have benefits to human health by slowing the growth of cancer or inhibiting its progression. Other than gene transfer studies to over-express MnSOD, another approach is the use of SOD mimetics: small, stable molecules with intracellular SOD activity (Salvemini et al., 1999). Another possibility would be to induce epigenetic modifications to increase *SOD2* mRNA expression. This has proven successful in cell culture using the DNA methyltransferase inhibitors (Hitchler et al., 2006; Hodge et al., 2005) (Fig. 9.2).

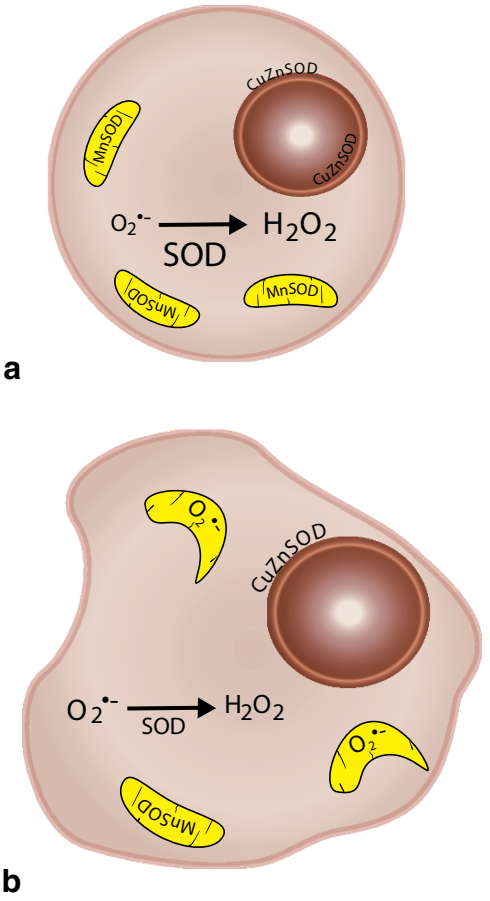

Fig. 9.2 Intracellular changes in oxidative stress during malignant transformation. (A) Schematic diagram of a normal cell, showing high levels of SOD enzymes and concomitantly low level of superoxide, due to the dismutation of superoxide to hydrogen peroxide. The resulting hydrogen peroxide is involved in many cellular processes and is broken down by a variety of peroxidase enzymes within the cell. (B) Transformed cells have decreased levels of MnSOD, which leads to an increase in superoxide levels and oxidative stress within the cell, thus contributing to its malignant phenotype.

Cardiovascular Disease

Cardiovascular disease is another significant cause of death worldwide. Although multiple factors contribute to the development of cardiovascular disease, perhaps none are more important than the balance of superoxide and nitric oxide in the vasculature. Nitric oxide is essential in regulating vascular tone and thrombogenicity in blood vessels. Not surprisingly an imbalance of this molecule

results in a disease state known as atherosclerosis, a process where cholesterol, calcium, and other substances build up in vessel walls leading to the obstruction of blood vessels. This process involves many free radical reactions, including lipid oxidations, inflammation, thrombosis, and apoptosis of vascular smooth muscle cells.

One important risk factor for developing atherosclerosis is high levels of circulating low density lipoproteins (LDL). Large amount of LDLs circulating in the bloodstream increases the amount available to adhere to the wall of a blood vessel. It has been proposed that endothelial cell dysfunction is sufficient to initiate permeability to lipoproteins, leading to an accumulation of LDLs in the wall of the blood vessels (Ross, 1999). Besides cholesterol, LDLs contain predominantly polyunsaturated fatty acids (PUFAs) including linoleic acid and arachidonic acid (Esterbauer et al., 1987). The long lipid chains and *bis*-allylic methylenes of these two PUFAs make them highly oxidizable by reactive oxygen species (ROS) or reactive nitrogen species (RNS), particularly peroxynitrite, in the vascular system (Cosgrove et al., 1987; Wagner et al., 1994). Another piece of evidence to support the role of radical induced lipid peroxidation as the key step to initiating plaque formation is that the lipid soluble anti-oxidant α-tocopherol is able to partially inhibit LDL oxidation (Dieber-Rotheneder et al., 1991). Oxidized LDLs (oxLDLs) elicit an immune response (Graham et al., 1993; Parums, 1990) and form immune complexes that facilitate macrophage phagocytosis of the oxLDL (Griffith et al., 1988; Klimov et al., 1985). Macrophages that have engulfed lipids are known as foam cells. The accumulation of lipids and foam cells is known as a "fatty streak". This pathology can stimulate additional changes in the surrounding vascular cells to form a bona fide atherosclerotic plaque. The plaque is characterized by the accumulation of a lipid core with foam cells and necrotic debris under a fibrous cap of extracellular matrix, smooth muscle cells, and collagen (Keaney, 2000).

Increased inflammation in the plaque can lead to increased apoptosis of vascular smooth muscle cells, thus weakening the plaque and making it more vulnerable to rupture by shear forces and mechanical stress in the vessels (Keaney, 2000). Studies have demonstrated that atherosclerosis patients have increased oxidative stress and increases in mitochondrial damage of the vascular smooth muscle cells and macrophages that causes the cells to undergo apoptosis and therefore weaken plaques (Ballinger et al., 2002; Madamanchi and Runge, 2007) making them more susceptible to rupture. Plaque rupture is detrimental because the ruptured caps move to smaller vessels, reducing blood flow to the organs being fed by the vessels which causes necrosis in the tissue and organ malfunction. Blood flow can also be reduced when the exposed vessel wall that is damaged is recognized by platelets and induces thrombosis. The reduced blood flow can cause tissue death in the heart (heart attack) or brain (stroke).

Atherosclerotic lesions increase production of ROS, especially superoxide which oxidizes lipids, participates in inflammation and reduces nitric oxide bioavailability. In response to the increased superoxide radicals, the body increases the expression of extracellular superoxide dismutase (ecSOD) to remove superoxide,

thus preserving nitric oxide and helping to protect against plaque formation and thrombosis (Fig. 9.3). Atherosclerosis is a disease of age caused by dysfunction of endothelium, hypothesized to be due in part to increased levels of superoxide and decreased activity of ecSOD as one ages. Therefore it is reasonable to propose that treatments that increase SOD activity in the vasculature will prove to be effective therapies for atherosclerosis. Recently adenoviral gene transfer has been examined as a mechanism to upregulate ecSOD expression in vascular cells. Brown et al. administered adenoviral ecSOD to old and young rats. They found that old rats had elevated levels of superoxide and when ecSOD was given their superoxide levels were decreased to the levels of young rats, a finding that was associated with improved vascular function. In contrast, ecSOD therapy had no effect on young rats (Brown et al., 2006). Another study using the same treatment demonstrated improved $NO^{\bullet}$ availability, improved endothelial function in response to over-expression of ecSOD (Fennell et al., 2002). Taken together these studies support a role for therapeutic strategies aimed at increasing vascular targeted SOD activity in the prevention and treatment of cardiovascular disease.

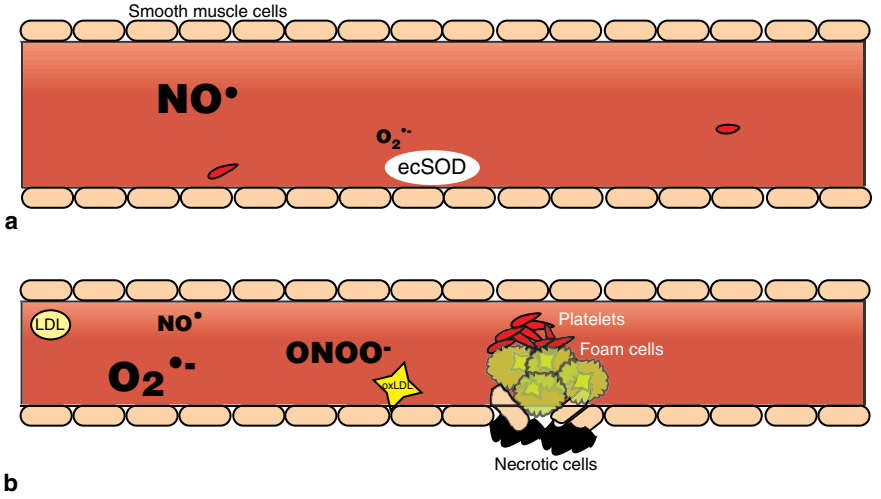

Fig. 9.3 ecSOD prevents atherosclerotic lesions in blood vessels. (A) Healthy vasculature expresses abundant ecSOD to keep the concentration of superoxide low, in turn preventing interactions with nitric oxide that decrease its bioavailability. (B) Atherosclerotic vessels are commonly deficient in ecSOD activity. This causes an increase in superoxide that reacts with nitric oxide, reducing $NO^{\bullet}$ bioavailability and producing peroxynitrite. Oxidants including peroxynitrite are responsible for the oxidation of low density lipoproteins that embed in the vessel walls. Macrophages engulf the oxidized lipids and become foam cells. The accumulation of oxidized lipids and foam cells initiates the formation of an atherosclerotic plaque on the vessel wall. This eventually leads to apoptosis of smooth muscle cells and weakening of the vessel wall. Thrombosis surrounding the plaques is the final event leading to decreased blood flow and tissue death in downstream organs.

Conclusions

Superoxide has various functions within the body, explaining the need for specialized superoxide dismutase enzymes (SOD) to regulate concentration and availability for each precise location. Aberrant SOD expression alters the body's oxidant state and increases the risk of human disease processes. Mutations in CuZnSOD leads to neuronal cell death and muscle atrophy culminating in ALS. Decreases in MnSOD expression allows for increased superoxide accumulation and, in turn, increased cellular damage found in most human cancers. Decreased ecSOD activity in the vasculature causes a decrease in nitric oxide availability and function as well to cause accumulation of the powerful pro-oxidant peroxynitrite, both of which are implicated in the progression of cardiovascular disease. The role of the SOD enzymes is essential in the development and progression of each of the diseases discussed, providing direction for therapeutic intervention towards the development and administration of SOD targeted therapy. Modern advances in the treatment of these diseases includes using molecular biology approaches to decrease or increase specific SOD enzymes within an affected group of cells. This has been shown with virus directed administered shRNA to degrade toxic *SOD1* mutants specifically to motor neurons to treat ALS. In contrast, amplifying SOD activity could be effective treatment for diseases exacerbated by oxidative stress, such as cancer and atherosclerosis. Administration of *SOD2* to cancer cells has overwhelmingly shown prevention or reduction of malignancy. At the same time SOD can reduce lipid oxidation and improve vasculature function in atherosclerotic tissue. In conclusion, restoring the balance of SOD using therapeutic approaches holds promises for the treatment of a variety of life threatening diseases.

Acknowledgments This work was supported National Institutes of Health grants CA73612 to FED and CA115438 to LWO.

List of Abbreviations

SOD	superoxide dismutase
CuZnSOD	copper and zinc containing superoxide dismutase
MnSOD	manganese containing superoxide dismutase
ecSOD	extracellular superoxide dismutase
8-oxo-dG	8-oxo-deoxyguanosine
HIF-1	hypoxia inducible factor-1
PHD	prolyl hydroxylase domain containing protein
VHL	von Hippel Lindau
VEGF	vascular endothelial growth factor
PDK1	pyruvate decarboxylase kinase 1
ALS	amyotrophic lateral sclerosis
LDL	low density lipoprotein

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Chapter 10
Glutathione Metabolism: Favorable Versus Unfavorable Effects

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Abstract The aim of this paper is to review current knowledge concerning the different perspectives that are actually investigated to better clarify the role of GSH in organism health.

Glutathione (GSH) is widely found in all forms of life and plays an essential role especially in aerobic organisms. In animals, including humans, and in plants, GSH is the predominant non-protein thiol and acts as a redox buffer, in particular keeping with its own SH groups those of proteins in a reduced condition. Because of the cysteine residue, GSH is readily oxidized non-enzymatically to glutathione disulfide (GSSG) by electrophilic substances; GSSG is in turn reduced to GSH by the NADPH-dependent glutathione reductase. Cellular GSH concentrations are markedly reduced in response to oxidative stress and the GSH/GSSG ratio is often used as an indicator of the cellular redox state. Furthermore, GSH acts as an endogenous trapping for reactive intermediates derived from exogenous and endogenous chemicals preventing unwanted reactions of chemically reactive molecules with important cell constituents. In fact, GSH is able to form adducts with a wide range of reactive intermediates, such as quinoneimines, nitrenium ions, arene oxides, quinones, imine methides, Michael acceptors and 4-hydroxy-2-nonenal.

Although GSH conjugation has been identified as an important detoxification reaction, other biological and/or toxic effects of some GSH-adducts have been described. For example, oxyeicosanoid GSH-adducts do not represent just inactivation products, but they can both retain or show novel biological activities. However, several papers describe the biotransformation and bioactivation of a range of nephrotoxic compounds, evidencing that this bioactivation is dependent on GSH S-conjugate formation and activation of cysteine S-conjugates by cysteine conjugate β-lyase. Furthermore, recent evidences show that GSH may conjugate to nitric oxide (NO) to form an S-nitroso-glutathione (GSNO) adduct. GSNO represents one of the major transport forms of NO in biological systems, may give rise to transnitrosation

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

and S-thiolation reactions. GSNO has been shown to have several pharmacological activities, including the inhibition of platelet aggregation and a protective effect during the exposure of cells to oxidants. Finally, recent studies have also highlighted the ability of GSH and of its catabolites to promote oxidative processes, by participating in metal-ion-mediated reactions leading to formation of reactive oxygen species and free radicals.

Keywords GSH, GSSG, glutathione adducts, GSNO, protein S-glutathiolation, GSH-dependent bioactivation, GSH-dependent oxidative processes

Introduction

Glutathione is widely found in all forms of life and plays an essential role in the health of organisms and particularly in aerobic organisms. Glutathione (GSH; γ-glutamylcysteinylglycine) is the main low-molecular-weight non-protein thiol (molecular weight 307.4 g mol^{-1}) present in animal organisms, both at cellular (mainly in the cytosol, with a small amount in mitochondria, nuclear matrix and peroxisomes) and extracellular levels (Wu et al., 2004; Smith et al., 1996; Lu, 2000), and acts as a redox buffer (Wu et al., 2004). In fact GSH contains a cysteine residue, so that it is readily oxidized non-enzymatically to glutathione disulfide (GSSG) by electrophilic substances, such as free radicals and reactive oxygen/nitrogen species (ROS/RNS). The GSH + 2GSSG concentration is usually denoted as total glutathione in cells (Sies, 1999). Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (Lu, 2000; Griffith, 1999); a loss of intracellular GSH may be also related to GSSG efflux from cells. One has to point out that the understanding of GSH metabolism is complicated by the compartmentalization of substrates and their metabolism at both the organ and subcellular levels, so that changes in plasma GSH levels may also reflect changes in GSH synthesis in specific cell types.

The [GSH]:[GSSG] ratio is an efficacious marker of the cellular redox state (Griffith, 1999), since GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells; however it may be influenced by other redox couples, e.g. $NADPH/NADP^+$ and $thioredoxin^{red}/thioredoxin^{ox}$ (Jones, 2002; Schafer and Buettner, 2001). Adequate GSH concentrations are necessary for several physiological processes, such as cell proliferation of cells (Aw, 2003), spermatogenesis and sperm maturation (Sies, 1999), activation of T-lymphocytes and polymorphonuclear leukocytes, cytokine production, immune responses, response to influenza virus infection (Cai et al., 2003). On the other hand, shifting the [GSH]:[GSSG] redox toward the oxidizing state activates several signaling pathways, which reduce cell proliferation and increase apoptosis (Sen, 2000).

GSH plays essential functions in the health of organisms. In fact, GSH is a major defence pathway involved in: (1) deactivation of ROS, either via direct GSH-ROS

interaction or via the activity of the selenium-containing enzyme GSH peroxidase (Dickinson and Forman, 2002a; Fang et al., 2002; Lei, 2002); (2) detoxification of xenobiotics, by the formation of less-toxic GSH-xenobiotic conjugates often requiring the catalytic activity of GSH S-transferases, that is a family of Phase II detoxification enzymes (Fang et al., 2002; Hayes et al., 2005). Furthermore, GSH is involved in the removal of formaldehyde, a carcinogen produced from the metabolism of methionine, choline, methanol and several xenobiotics, by formaldehyde dehydrogenase, which converts formaldehyde and GSH to S-formyl-glutathione (Townsend et al., 2003).

Besides these fundamental antioxidant and antitoxic roles, glutathione modulates many other vital functions in animals. Many electrophilic physiological metabolites (e.g., estrogen, prostaglandins, and leukotrienes) are substrates for GST-mediated addition of GSH (Fang et al., 2002), although biological significance of such GSH-adducts is generally unclear. Furthermore the GSH conjugation pathway may have an important role in bioactivation of drugs and xenobiotics, giving cytotoxic and nephrotoxic metabolites.

GSH is able to conjugate also with NO to form an S-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (Fang et al., 2002), which have a critical role in several physiological processes (e.g., the regulation of lipid, glucose, and amino acid utilization) (Guarino et al., 2003); André and Felley-Bosco (2003) have recently demonstrated that the targeting of endogenous NO is mediated by intracellular GSH. GSH has been shown to glutathiolate several proteins; besides to represent an important cellular response to oxidative stress, protein S-glutathiolation plays a vital role in various physiological and pathological processes (Shackelford et al., 2005; Hill and Bhatnagar, 2007; Townsend et al., 2003).

Finally, recent studies have also highlighted the ability of GSH and of its catabolites to promote oxidative processes, by participating in metal-ion-mediated reactions leading to formation of ROS ad free radicals; the membrane-bound γ-glutamyl-transferase (GGT) plays a fundamental role in these processes.

The aim of this chapter is to give an overview of how glutathione can have different cellular effect depending on the different interaction with proteins or metabolites.

Glutathione Metabolism

GSH is synthesized in all cell types and especially in liver cells. Liver GSH synthesis occurs predominantly in perivenous hepatocytes and, to a lesser extent, in periportal cells (Bella et al., 2002). Among extra hepatic cells, erythrocytes have a relatively high turnover rate for GSH and may contribute up to 10% of whole body GSH synthesis in humans (Reid et al., 2000; Lyons et al., 2000).

GSH is synthesized from glutamate, cysteine, and glycine in two adenosine triphosphate-dependent steps by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (GS) (Fig. 10.1): (1) γ-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme γ-glutamylcysteine synthetase (glutamate

cysteine ligase); (2) glycine is added to the C-terminal of γ-glutamylcysteine via the enzyme GS.

GSH is an unusual tripeptide because it has an amide bond between the γ-carboxyl group of the glutamate residue and the γ-amino group of cysteine (Stark et al., 2003; Dalton et al., 2004) (Fig. 10.1). The peptidic γ-linkage between the γ-carboxyl group of glutamate and the amino group of cysteine protects GSH from intracellular peptidase-catalysed hydrolysis. GSH can be transported out of cells via a carrier-dependent facilitated mechanism (Townsend et al., 2003) and leaves the liver either intact or as γ-Glu-$(Cys)_2$. The transport of extracellular GSH or GSSG into cells is thermodynamically unfavourable, due to the concentration gradient across the plasma membrane, but γ-Glu-$(Cys)_2$ is readily taken up by extrahepatic cells for GSH synthesis.

The rate-controlling enzyme of GSH synthesis is GCS (Lu, 2000); furthermore γ-glutamylcysteine may be a substrate also for γ-glutamylcyclotransferase, but GSH synthesis is favored in animal cells due to the higher affinity and activity of GS (Griffith, 1999). GCS transcription/activity is affected by different conditions and agents. In fact it may be increased by oxidative and nitrosative stress, inflammation, GSH depletion and conjugation, heavy metals and antioxidants (Townsend et al., 2003; Lu, 2000); conversely it may be decreased by dietary protein deficiency, dexamethasone, erythropoietin, tumor growth factor, hyperglycemia and GCS

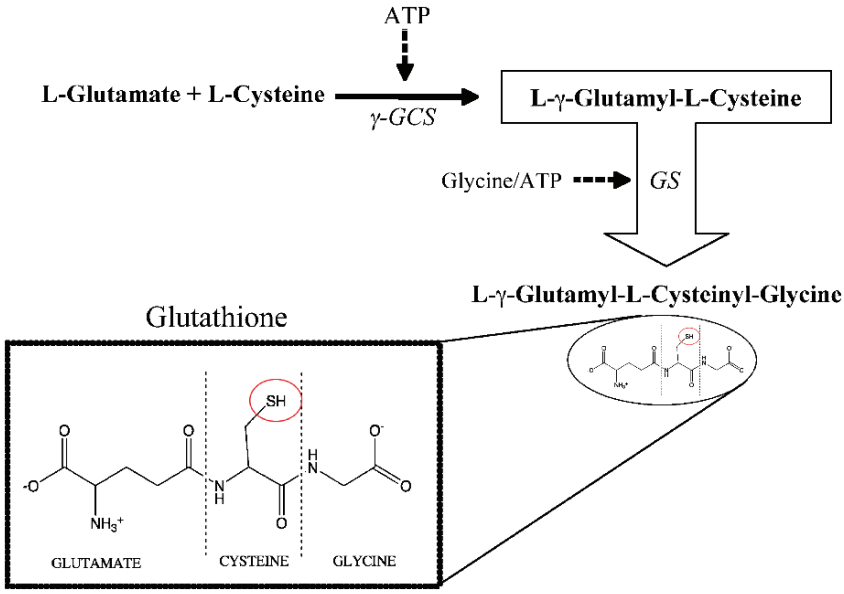

Fig. 10.1 *De novo* synthesis of glutathione (GSH). GSH (L-γ-glutamyl-L-cysteinyl-glycine) is synthesized from amino acids (L-glutamate and L-cysteine) by the action of γ-glutamylcysteine synthetase (γ-GCS), and glutamyl synthase (GS); this reaction requires energy and is ATP-limited. The reaction center of GSH is the -SH group present on the Cys residue

phosphorylation. At cellular levels, the upregulation of GCS expression in response to oxidant stress, inflammatory cytokines, and buthionine sulfoximine–induced GSH depletion is mediated by nuclear factor-κ B, and GCS S-nitrosation by NO donors reduces enzyme activity (Townsend et al., 2003; Lu, 2000).

There is evidence that the dietary amino acid balance and in particular the adequate provision of sulfur-containing amino acids have an important effect on protein nutrition and therefore on GSH homeostasis (Lu, 2000; Yu, 2002; Persaud et al., 1996). Cysteine (an essential amino acid in premature and newborn infants and in subjects stressed by disease and contained in relatively small amounts at intracellular level) (Jahoor et al., 1999) is generally considered the limiting amino acid for GSH synthesis in humans and in other animal species (Lyons et al., 2000; Jahoor et al., 1999; Chung et al., 1990). Thus, factors stimulating cell cysteine uptake generally increase intracellular GSH concentrations (Lu, 2000), and oral or intravenous administration of cysteine or its precursors (such as cystine and N-acetylcysteine) enhances GSH synthesis and prevents GSH deficiency due to several nutritional and pathological conditions (Townsend et al., 2003). Furthermore, since cysteine may be generated from methionine catabolism (primarily in hepatocytes), also dietary methionine can support GSH synthesis in vivo. Glutamate used for GSH synthesis is formed both at extracellular and intracellular level (Reeds et al., 1997). Glutamine is an effective precursor of the glutamate for GSH synthesis in many cell types (Johnson et al., 2003); in fact parenteral nutrition supplementation with glutamine maintains tissue GSH levels and improves survival in pathological conditions such as reperfusion injury, ischemia, acetaminophen toxicity, chemotherapy, inflammatory stress and bone marrow transplantation (Oehler and Roth, 2003). Glutamate plays a regulatory role in GSH synthesis through two mechanisms: (1) the uptake of cystine (since glutamate and cystine share the system X_c^- amino acid transporter) (Lu, 2000), and (2) the prevention of GSH inhibition of GCS (since GSH is a nonallosteric feedback inhibitor of GCS, and the binding of GSH to the enzyme competes with glutamate) (Griffith, 1999). Finally due to changes in its availability or in its hepatic oxidation, also glycine may become a limiting factor for GSH synthesis (Yu et al., 2002; Persaud et al., 1996; Grimble et al., 1992).

The important reaction center of GSH is the -SH group present on the Cys residue (Fig. 10.1). This allows GSH to act as a sulfhydryl buffer in the cell, which maintains the Cys residues of proteins in the reduced state. In this process, GSH is oxidized to glutathione disulfide according to:

$$2GSH \rightarrow GSSG + 2e^- + 2H^+$$

However, the oxidation of GSH and subsequent reduction of GSSG, form a cyclic process (Fig. 10.2) because the NADPH-dependent enzyme glutathione disulfide reductase (GRx), reverts it from GSSG, so that glutathione is found almost exclusively in its reduced form. In healthy tissue, more than 90% of the total glutathione pool is in the reduced form and less than 10% exists in the disulfide form.

When it is transported to the extracellular milieu, GSH is rapidly metabolized by GGTs (Dalton et al., 2004; Lieberman et al., 1995), which catalyzes ATP-dependent

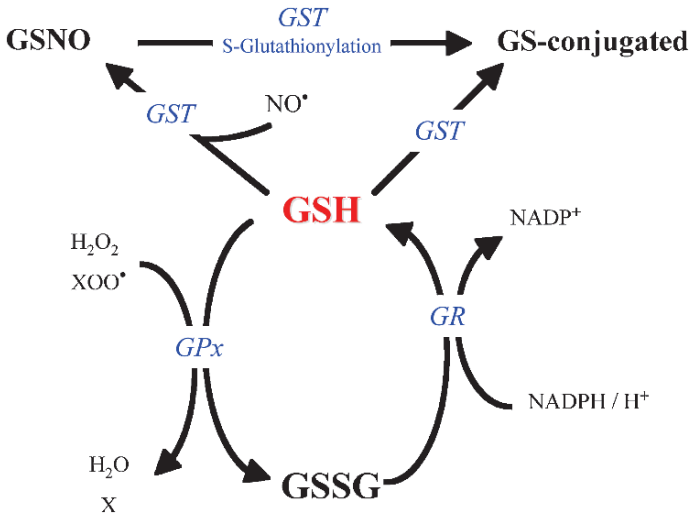

Fig. 10.2 Glutathione cycling. GSH undergoes to the glutathione-peroxidase (GPx) coupled reaction, thereby detoxifying reactive oxygen species (such as hydrogen peroxide (H_2O_2) or hydroperoxide radicals) and being oxidized to glutathione disulfide (GSSG), which is recycled back to GSH by the action of glutathione reductase (GR) at the expense of reduced nicotinamide ($NADPH/H^+$), thus forming the redox cycle. Through glutathione-S-transferase (GST) GSH serves also as a natural trapping agent for chemically reactive compounds, can conjugate the free radical nitric oxide ($NO^•$), to form S-nitrosoglutathione (GSNO), and, as well as GSNO, can glutathiolate proteins

cleavage of the γ-glutamyl amide bond to cysteine, forming cysteinylglycine, and transfers the glutamyl residue to cystine or other amino acids (Dalton et al., 2004). External dipeptidases then cleave cysteinylglycine to cysteine and glycine, which may be re-uptaken into the cells and employed as substrates for GSH biosynthesis (Dickinson and Forman, 2002a, b). Also γ-glutamylcystine can be uptaken into the cytosol and reduced to γ-glutamylcysteine to be employed for GSH synthesis by GS (Dalton et al., 2004). Thus, also under conditions rapidly consuming GSH, intracellular concentration of GSH may be efficiently restored through these biochemical pathways (Dalton et al., 2004). In fact inhibition of GGT or of amino acid membrane transporters can significantly affect intracellular GSH concentrations (Carter et al., 1997; Jian et al., 2005).

Biological Effects of S-Nitrosoglutathione

S-Nitrosothiols (compounds with the structure R-SNO) are endogenous metabolites of nitric oxide (NO) that have been detected in extra- and intracellular spaces (Hogg, 2000, 2002; Zhang and Hogg, 2005). It has been suggested that they represent a more stable metabolite of NO that can either be stored or transported.

However, today it is evident that the biological properties of S-nitrosothiols concern three main reactions, NO release, transnitrosation and S-thiolation, and that RSNO can also have direct effects, so evidencing that S-nitrosothiols are more than simply NO donors.

Since transnitrosation is a reaction allowing NO transfer from species to species inside the cells, it represents a potential mechanism affecting protein activity. Several studies suggested that nitrosation of a protein thiol by GSNO can lead to inactivation/activation of enzymes and influence significantly cell regulatory control. However, numerous studies employ GSNO as a NO donor molecule, but only few of them are addressed to how cells actually metabolize this compound.

As well as S-nitrosothiols generally, GSNO has a potent inhibitory effect on platelet aggregation (Radomski et al., 1992); besides the NO scavenger oxyhemoglobin, the copper chelator bathocuproine sulfonate, the thiol blocking agent dithionitrobenzine sulfonic acid and the vicinal dithiol modifier phenyl arsine oxide are potent inhibitors of GSNO-induced platelet aggregation; this means that a copper(I)-containing protein and a protein disulfide isomerase on the platelet surface are involved in this effect (Gordge et al., 1995, 1996; Shah et al., 2003; Root et al., 2004). Interestingly, GSNO can inhibit platelet aggregation in human circulation without affecting vascular tone. Conversely, GSNO is able to elicit bronchodilator effects; the GSNO-induced relaxation on canine tracheal smooth muscle is partially independent on cGMP formation (Perkins et al., 1998).

S-Nitrosothiols have been shown to protect against cellular toxicity associated with oxidative stress (Rauhala et al., 1996); this protective effect is due to NO release, followed by a radical-radical termination reaction of NO with the propagating free radicals. GSNO has been shown to be protective against polyunsaturated fatty acid oxidation, against increased monolayer permeability induced by oxidative stress in lung epithelium and against damage induced by oxidized low density lipoproteins in endothelial cells (Chamulitrat, 1998; Gutierrez et al., 1996; Struck et al., 1995). Also the protective effect of GSNO against reperfusion injury, appears to require NO release (Brunner, 1997; Konorev et al., 1995), although the exact involved mechanisms are yet unclear.

GSNO has also been demonstrated to have a dual role in apoptosis, being both protective and toxic depending on concentration and cell type (Zeng et al. 2001).

Finally, GSNO can act as a substrate/inhibitor for several GSH-utilizing enzymes, such as γ-glutamyltranspeptidase and glutathione peroxidase.

Protein S-Glutathiolation

Protein S-glutathiolation plays a vital role in both normal and pathophysiological processes and represents an important cellular response to oxidative stress. For example, protein S-glutathiolation increases in certain diseases (such as sickle cell anemia and diabetes mellitus) and in aging (Al-Abed et al., 2001; Goodman, 2004; Lim et al., 2003; Mallis et al., 2002; Thomas and Mallis, 2001).

Cysteine thiol groups are among the cellular constituents most readily damaged by oxidant exposure. Modifications of cysteine side chains of proteins can lead to loss of protein activity or folding, which require intramolecular disulfides. In addition, such modifications may result from specific addition reactions (e.g., protein nitrosylation, sulfenic acid formation, addition products of protein thiols with electrophilic metabolites or xenobiotics) (Hess et al., 2005; Poole et al., 2004; Esterbauer et al., 1991), or can result in the formation of sulfinic and sulfonic acids.

To avoid potentially irreversible cysteine thiol oxidation, cells have developed multiple antioxidant defences, including protein S-glutathiolation. However, although protein glutathiolation is considered primarily to be a protective mechanism for preventing irreversible oxidation of protein thiols, recent evidence suggests that controlled glutathiolation reactions have multiple functions, including regulation of cell metabolism and division, modulation of transcription factor activity and gene expression, regulation of protein processing and ubiquitination, modulation of specific protein activity following oxidative stress, modulation of ROS/RNS-initiated signal transduction cascades, inhibition of the tumor-promoting effects of oxidants (Shackelford et al., 2005; Hill and Bhatnagar, 2007).

The mechanism suggested for the generation of glutathiolated proteins has been a direct thiol-disulfide exchange; the products of this reaction are a glutathiolated protein (PSSG) and GSH (Gilbert, 1982, 1984; Saurin et al., 2004). However, since the intracellular concentration of GSSG in most cells is very low and since several proteins become glutathiolated without a measurable increase in GSSG levels (DeLucia et al., 1975), direct uncatalyzed thiol disulfide exchange is unlikely to explain protein glutathiolation under basal conditions and even under oxidative conditions. Hence, GSH is now hypothesized to directly glutathiolate proteins with activated thiols during oxidative stress. However some specific subcellular domains (e.g., endoplasmic reticulum and mitochondria) can contain higher concentrations GSSG (Fewell et al., 2001; Ravindranath and Reed, 1990) that thus could mediate generation of glutathiolated proteins by direct thioldisulfide exchange.

The generation and the abundance of glutathiolated proteins could also be regulated by NO, since S-nitroglutathione (GSNO) is a potent glutathiolating agent. A mechanism of protein glutathiolation may be GSH nitrosylation to form GSNO (Gaston et al., 1993), which in turn could glutathiolate proteins (Gaston et al., 1993; West et al., 2006); an additional mechanism of protein glutathiolation may be a direct group transfer reaction involving a N-hydroxysulfenamide-like intermediate generated from the protein nucleophilic attack on the nitrogen of GSNO (Chandra et al., 1997; Srivastava et al., 2001). Besides this direct GSNO-induced protein glutathiolation, GSNO could glutathiolate proteins through more reactive intermediates (Tao and English, 2004).

Protein glutathiolation appears to be a regulatable process, because several enzymes such as glutaredoxins and protein-disulfide isomerase (Gravina and Mieyal, 1993; Peltoniemi et al., 2006) catalyze protein deglutathiolation. The existence of mechanisms controlling deglutathiolation confirms that glutathiolation is a protective and reparative strategy, as demonstrated by the detection of glutathiolated proteins in cells and tissues exposed to oxidants or oxidative stress (Fratelli

et al., 2002; Clavreul et al., 2006; Cross and Templeton, 2004; Adachi et al., 2004a; Eaton et al., 2002). Glutathiolation could also be a restorative strategy for thiols modified by NO or oxidized to sulfenic acid. Finally addition of glutathione to proteins may have a cell signaling role that could trigger alarm, elicit adaptive responses, inhibit cell survival or facilitate cell apoptosis (Ghezzi, 2005).

Protein glutathiolation has been shown to have an important role also under physiological conditions and changes in glutathiolation status of specific proteins are required for physiological responses. For example, arterial relaxation in response to acetylcholine increases NO-mediated glutathiolation of several proteins including actin (West et al., 2006), and changes in actin glutathiolation during ischemia (Chen and Ogut, 2006) could mediate changes in cell shape and contractility, while inhibition of actin glutathiolation has been shown to prevent stress fiber formation and disassembly of actin-myosin (Fiaschi et al., 2006). Furthermore glutathiolation also appears to be an important regulator of calcium homeostasis, because stimulation of calcium uptake by NO was shown to be produced by peroxynitrite-mediated glutathiolation of sarco/endoplasmic reticulum calcium ATPase (Adachi et al., 2004), and faster calcium release during tachycardia appears related to NADPH-oxidase mediated glutathiolation of cardiac ryanodine receptors (Sanchez et al., 2005). Finally activation of NADPH oxidase can lead to increased glutathiolation of Ras and thus to an increase in Ras hypertrophic activity (Adachi et al., 2004).

The number of known proteins that can undergo S-glutathiolation is relatively large; among the better studied S-glutathiolate proteins there are carbonic anhydrase III, glyceraldehyde-3-phosphate dehydrogenase, H-ras, microsomal glutathione S-transferase and HIV-1 protease (Shackelford et al., 2005). Interestingly, a significant amount of glutathione is bound covalently to proteins, being, in healthy adults, GSH bound to proteins for 5–25% of the total content in red cells, for 30% in the liver (Isaacs and Binkley, 1977) and for 50% in endoplasmic reticulum (Bass et al., 2004). Being about 3% of all proteins bound to GSH under normal conditions (Klatt and Lamas, 2000; Thomas et al., 1995; Ziegler, 1985) it can be noticed that almost any protein in solution can undergo S-glutathiolation following incubation with GSH and diamide or S-nitrosoglutathione (Klatt et al., 2000).

Currently, a significant number of clinical disorders or pathophysiological entities have been related to redox post-translational modifications. These comprise cardiovascular disease, diabetes (Adachi et al., 2005) and neural protein conformational disorders (Dinoto et al., 2005) among others. Some proteins, including those mentioned below, modified by S-glutathionylation have been demonstrated to have a pathophysiological relevance also in relationship to NO production (Martinez-Ruiz and Lamas, 2007).

Glutathionyl hemoglobin has been widely documented in disease states such as uremia or diabetes (Niwa, 2006); since it is currently believed to be a consequence of the oxidative stress underlying these diseases, it might be a very useful marker of oxidative stress in such pathological conditions. Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is a protein regulating the intracellular storage of Ca^{2+}, as it enhances refilling of Ca^{2+} sarcoplasmic and endoplasmic reticula. S-glutathionylation

by peroxynitrite activates SERCA during arterial relaxation by NO (Adachi et al., 2004). S-thiolation of SERCA occurs in vivo, so suggesting a potential pathophysiological role for S-thiolated SERCA in vascular disease. The oncogenic protein Ras may be S-glutathionylated; this modified protein may be a mediator of signaling in vascular smooth muscle cells and in endothelial cells, and may mediate cardiac myocyte hypertrophy in a strain-stimulated model (Adachi et al., 2004; Pimentel et al., 2006). Finally glutathionyl caspase-3 plays an important role in the regulation of cell death promoted by TNF-α (Pan and Berk, 2007).

GSH Adducts

Extensive production of reactive intermediates may occur in the metabolism of several endogenous and exogenous chemicals; these reactive intermediates can form protein-adducts, RNA-adducts or in certain cases DNA-adducts; furthermore they may have a sufficient long life lived to be transported across the plasma membrane and to form adducts with plasma proteins (Lee et al., 2005; Swenberg et al., 2001; Yocum et al., 2005).

GSH serves as a natural trapping agent for chemically reactive intermediates derived from drugs, xenobiotics and endogenous compounds (Evans et al., 2004; Blair, 2006), resulting in formation of GSH adducts (Fig. 10.3) and sometimes causing depletion of intracellular GSH.

GSH-adducts of a wide range of reactive intermediates have been studied intensively in vitro, including: quinoneimines (acetaminophen), nitrenium ions (clozapine), arene oxides (carbamazepine), quinones (estrogens), imine methides (3-methylindole), Michael acceptors (valproic acid metabolites) and HNE (from lipid hydroperoxides)

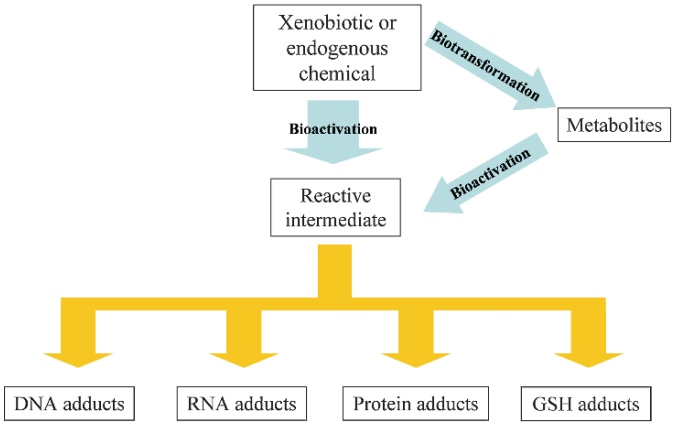

Fig. 10.3 Formation of reactive intermediates, GSH-adducts, RNA-adducts, protein-adducts and DNA adducts in vivo

(Evans et al., 2004). A large number of studies have been conducted on the metabolism of acetaminophen-GSH-adducts and have provided an excellent model to explain how endogenous GSH-adducts are metabolized.

An overdose of the analgesic acetaminophen can cause fatal, hepatic centrilobular necrosis (James et al., 2003), since acetaminophen is metabolically activated by cytochromes P450 (CYPs) to N-acetyl-*para*-benzoquinoneimine, a reactive metabolite depleting GSH and interacting with proteins (Gillette, 1982; Hinson et al., 1981; Baillie and Slatter 1991; Bessems and Vermeulen, 2001); N-acetylcysteine administration can restore GSH levels and thus is the currently used antidote for acetaminophen toxicity (Dargan and Jones, 2003).

On the other hand, the acetaminophen-GSH-adduct is transported from the cytosol to the extracellular space and then metabolized by GGTs to form an acetaminophencysteinylglycine adduct and glutamylcystine. The cysteinylglycine adduct is then converted by a dipeptidase into free glycine and a cysteine adduct, which is transported back into cells and acetylated by N-acetyltransferases (NATs) to a mercapturic acid derivative and excreted. Furthermore, the cysteine adduct derived from acetaminophen can also induce nephrotoxic effects, very likely due to its conversion to a free, unstable thiol by β-lyase (Baillie and Slatter 1991; Bessems and Vermeulen, 2001). As more extensively described below in this chapter, a similar metabolic pathway has been described for GSH-adducts derived from anesthetic reagents and environmental chemicals and also results in toxic metabolites formation (Anders, 2004, 2005). However, same authors have hypothesized that the nephrotoxicity of the acetaminophen-cysteine-adduct is related to an interaction with the γ-glutamyl cycle and to the consequent depletion of renal GSH stores (Stern et al., 2005).

Endogenous GSH Adducts

Endogenous Eicosanoid GSH-Adducts

Leukotrienes (LTs): LTC_4 is generated by GST-mediated conjugation of the epoxide intermediate LTA_4 with GSH, then cleaved by GGTs to form the cysteinylglycine-adduct LTD_4, which is converted to LTE_4 by dipeptidases (Lam and Austen, 2002; Penrose et al., 1992; Carter et al., 1997; Hammarstrom et al., 1985; Huber and Keppler, 1987). LTE_4 is acetylated by NATs (Sala et al., 1990; Huber et al., 1990) to give N-acetyl-LTE_4, a mercapturic acid derivative similar to that formed in the metabolism of the acetaminophen-GSH-adduct.

In asthma, LTD_4 (the GGT metabolite of LTC_4) and other cysteinyl LTs induce and aggravate bronchoconstriction, vascular permeability, edema, and inflammation (Habib et al., 1998); furthermore LTD_4 is more potent than the parent LT in the induction of mucus formation and in eosinophil accumulation (Habib et al., 1998). Cleavage of LTD_4 to LTE_4 drastically decreases its biological activity (Dahlen et al., 1980;

O'Byrne, 1994). Two classes of LT receptors ($CysLT_1$ and $CysLT_2$) have been identified but only $CysLT_1$ receptor antagonists have been described to date and are currently available for the treatment of asthma (Krawiec and Jarjour, 2002). Finally LTD_4 possesses a number of other biological activities including exacerbation of ventricular arrhythmias and impairment of ventricular function and coronary flow (Carry et al., 1991; Lee et al., 1993).

Oxyeicosanoids: Many electrophilic eicosanoids containing α,β-unsaturated ketones and biosynthesized during oxidative metabolism of arachidonic acid are substrates for GST-mediated addition of GSH (Wang and Ballatori, 1998; Murphy and Zarini, 2002). Some of these eicosanoids are LTB_4 and 5-oxo-eicosatetraenoic acid (5-oxo-ETE), hepoxilin A_3, 13-oxo-octadecadienoic, PGA_1, PGA_2, PGD_2, PGE_2, and PGJ_2.

While the biological significance of GSH-adducts of endogenous oxyeicosanoids is unclear, it is evident that some of them are not inactive products, but retain activity of the parent compound (e.g., the GSH-adduct of hepoxilin A_3 causes membrane hypopolarization in hippocampal CA1 neurons) or possess novel biological activity (e.g., 5-oxo-ETE-derived FOG_7 has chemotactic properties) (Murphy and Zarini, 2002; Powell and Rokach, 2005).

Endogenous Estrogen GSH-Adducts

Estrogens undergo CYP-mediated metabolism to catechol estrogens that can be further oxidized to semiquinones and then to quinones (Raftogianis et al., 2000; Rogan and Cavalieri, 2004), which can then undergo to a NAPDH-dependent 2-electron reduction back to the catechol. This sets up a redox cycle in which quinone and ROS are constantly produced. GST-mediated formation of GSH-adducts represents a termination of this cycle because the adducts are rapidly secreted into the extracellular milieu. Thus estrogen GSH-adduct formation is very likely a protective pathway against the formation of oxidative damage arising from catechol estrogens (Hachey et al., 2003; Dawling et al., 2004; Todorovic et al., 2001; Markushin et al., 2005). Interestingly, estrogens and catechol estrogens have a role in the development of breast cancer, and CYP1A1, CYP1B1 and GSTP1 have been shown to metabolize estradiol and to be all expressed in breast tissue (Hachey et al., 2003; Dawling et al., 2004).

Endogenous Catecholamine GSH-Adducts

5-S-cysteinyl derivatives of dopamine (DA), 3,4-Dihydroxy-L-phenylalanine (DOPA) and dihydroxyphenylacetic acid were detected in the brain of humans and other mammalian species (Rosengren et al., 1985; Fornstedt et al., 1986) with a regional distribution similar to that of DA. Thus may be hypothesized that autoxidation of catechols to quinones and subsequent GST-mediated DA-GSH-adduct

formation occur; 5-S-cysteinyl-adducts are then formed by GGT- and dipeptidase-mediated metabolism. One has to note that cysteine- and N-acetylcysteine-DA-adducts have been suggested to be involved in cell death and neuronal damage occurring in Parkinson disease (Spencer et al., 1998).

GSH-Dependent Bioactivation of Xenobiotics and Nephrotoxicity

The kidneys act as filters to remove toxic waste products from the blood via glomerular filtration. Furthermore, the kidneys are active metabolically because they are capable of carrying out extensive oxidation, reduction, hydrolysis, and conjugation reactions. Enzyme systems involved in renal drug metabolism are similar to those present in liver and other extrarenal tissues, but, unlike other organs and tissues, are differentially distributed among the nephron cell populations (Lash, 1994); for example, CYP450 is found exclusively in renal cortex, and PG synthetase in the medulla.

Thus renal drug metabolism pathways and in particular the GSH conjugation pathway may have an important role in bioactivation of drugs and xenobiotics (Fig. 10.4) (Lash, 1994).

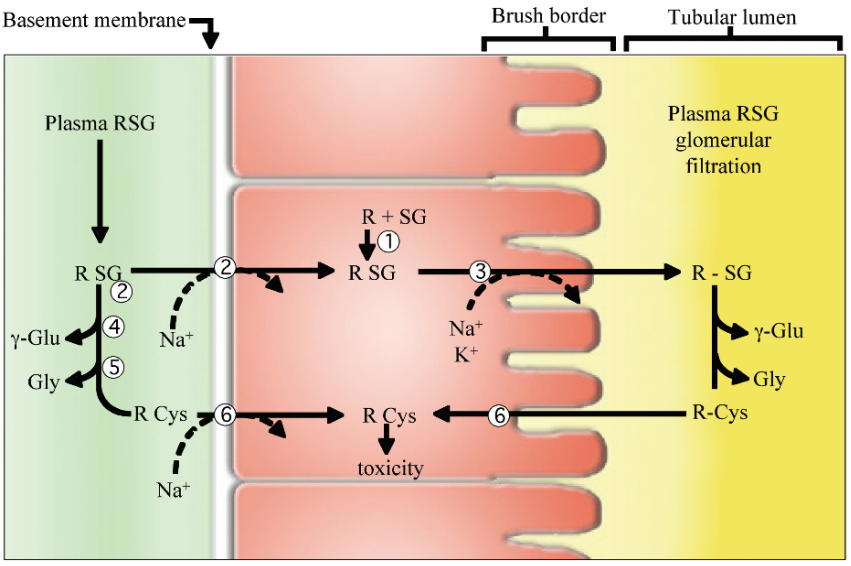

Fig. 10.4 Uptake of glutathione conjugates (RSG) and cysteine conjugates (R-Cys) by the kidney cell. RSG can be produced in the cell itself (1) or enter the cell from the blood via the organic ion transport system (2), after which it is transported to the tubular lumen (3). In the lumen, and probably also in the capillary on the basolateral side of the cell, glutamine (γ-Glu) and glycine (Gly) are separated (4, 5), leaving R-Cys, which is subsequently transported into the cell (6)

The biotransformation and bioactivation of a range of nephrotoxic and cytotoxic halogenated hydrocarbons has been described (Anders, 2005; Dekant et al., 1994; Anders and Dekant, 1998) evidencing that it is dependent on GSH S-conjugate formation and activation of cysteine S-conjugates by cysteine conjugate β-lyase. Briefly, this pathway includes: glutathione transferase-catalyzed glutathione S-conjugate formation, hydrolysis of the conjugates by GGT and dipeptidases to give the corresponding cysteine S-conjugates, active uptake of the cysteine S-conjugates by the kidney, and bioactivation by cytosolic and mitochondrial β-lyases.

The liver is quantitatively the most important site of GSH conjugation (Hinchman and Ballatori, 1990), but the general point of view is that the ultimate toxic metabolites derived from GSH S-conjugates are generated in the kidneys. However nephrotoxicity of hexafluoropropene appeared to be directly associated with intrarenal formation of the GSH S-conjugate (Koob and Dekant, 1990); thus interorgan processing of GSH S-conjugate depends, very likely, on the chemical properties of the particular compound.

Metabolism of GSH and of GSH S-conjugates is a complex process involving the sequential action of several enzymes localized in various tissues. Liver cells actively extrude GSH and GSH S-conjugates into plasma and bile for translocation to kidney and small intestine, respectively. The GSH S-conjugates secreted into bile are degraded to cysteine S-conjugates by GGT and dipeptidase (Lash, 1994), while metabolites from small intestinal epithelium are returned to the liver via the portal vein.

Finally, cysteine or N-acetylcysteine S-conjugates are delivered via the circulation to the kidneys, where, due to the presence of glomerular filtration and active transport systems on both brush-border and basal-lateral plasma membranes, high intracellular concentrations of these conjugates are produced (Lash, 1994). Once inside the renal epithelial cell, the ultimate toxic metabolite is generated.

The cysteine S-conjugate may be N-acetylated to form the mercapturic acid. Although the highly polar, chemically stable mercapturates generally are excreted, kidneys as well as other organs and tissues have deacetylase activities able to regenerate the cysteine S-conjugate. For many compounds, the cysteine S-conjugate is bioactivated so generating reactive, toxic metabolites. However, some GSH or cysteine S-conjugates do not need bioactivation to induce toxicity, because they are chemically unstable and form reactive episulfonium ions which that can bind covalently to cellular macromolecules (Elfarra et al., 1985). The localization of transport systems delivering the conjugates to kidney cells and the presence of enzymes bioactivating these conjugates within those cells, determine the target site specificity of injury.

Several haloalkyl or haloalkenyl cysteine S-conjugates are bioactivated by β-lyase. β-lyase is a pyridoxal phosphate-dependent enzyme found in renal cytosol and mitochondria. This enzyme cleaves the carbon-sulfur bond, thereby generating a reactive, sulfur-containing compound (Lash, 1994), and is named β-lyase because all the conjugates that are substrates have the common chemical property of possessing a good leaving group on the 13-carbon atom.

Besides that in the kidney, β-lyase activity is also present in liver and in the intestinal microflora (Larsen, 1985). This evidences that tissue and cell type specificity of cysteine S-conjugate toxicity is due not only to the presence of β-lyase

in kidney cells, but also to other factors such as membrane transport processes and detoxification pathways.

Examples of this bioactivation pathway are dichloroacetylene and 2-bromo-2-chloro-1,1-difluoroethylene (degradation products formed from the general anesthetics trichloroethylene and halothane respectively), which have been shown to undergo bioactivation by the β-lyase pathway (Chiu et al., 2006; Caldwell and Keshava, 2006; Anders, 2005).

Bioactivation of Dichloroacetylene

Trichloroethylene is a general anesthetic and analgesic that was widely used into the 1970s due to its advantages (in particular nonflammability, maintenance of cardiovascular stability, and very low incidence of postoperative side effects) in comparison with other inhaled anesthetics, such as diethyl ether and cyclopropane. Its use was early demonstrated to be occasionally associated with cranial nerve neuropathies (Hewer, 1943; McAuley, 1943). The toxicity of trichloroethylene appeared to be due to its metabolite dichloroacetylene (Anders, 2005). Despite the apparent safety of modern carbon dioxide absorbents, they have been implicated in the production of toxic degradation products of some anesthetics. In fact the most likely cause to explain the toxicity of trichloroethylene is dichloroacetylene formed by a chemical reaction of trichloroethylene with soda lime; for this reason to avoid soda lime during anesthesia with trichloroethylene has been recommended.

Dichloroacetylene is highly unstable, but is stabilized by high concentrations of trichloroethylene, which during anesthesia may limit its decomposition and the formation of possible toxic degradation products; anyway, the most abundant degradation product of dichloroacetylene is phosgene, together with small amounts of other compounds, but these compounds have not been implicated in trichloroethylene-induced neurotoxicity (Reichert et al., 1980).

Data obtained in experimental animals indicate that the mercapturate S-(1,2-dichlorovinyl)-N-acetyl-L-cysteine (Fig. 10.5A) is the major metabolite of dichloroacetylene (Kanhai et al., 1991), so demonstrating that glutathione-dependent metabolism is the major pathway of dichloroacetylene metabolism.

The reaction of dichloroacetylene with glutathione is catalyzed by rat hepatic and renal glutathione S-transferases to give S-(1,2-dichlorovinyl)glutathione (Kanhai et al., 1989), which is hydrolyzed by γ-glutamyltransferase and dipeptidases to give S-(1,2-dichlorovinyl)-L-cysteine (Fig. 10.5A). Besides to be detoxificated by N-acetylation to give S-(1,2-dichlorovinyl)-N-acetyl-L-cysteine, S-(1,2-Dichlorovinyl)-L-cysteine undergoes a β-lyase catalyzed β-elimination to give 1,2-dichloroethenethiolate, pyruvate, and ammonia. Thiolate may lose chloride to give chlorothioketene or may tautomerize to give chlorothionoacetyl chloride. Thioketene is highly unstable in aqueous environments, thus the toxicity of S-(1,2-dichlorovinyl)-L-cysteine is very likely due to thionoacyl chloride (Völkel and Dekant, 1998).

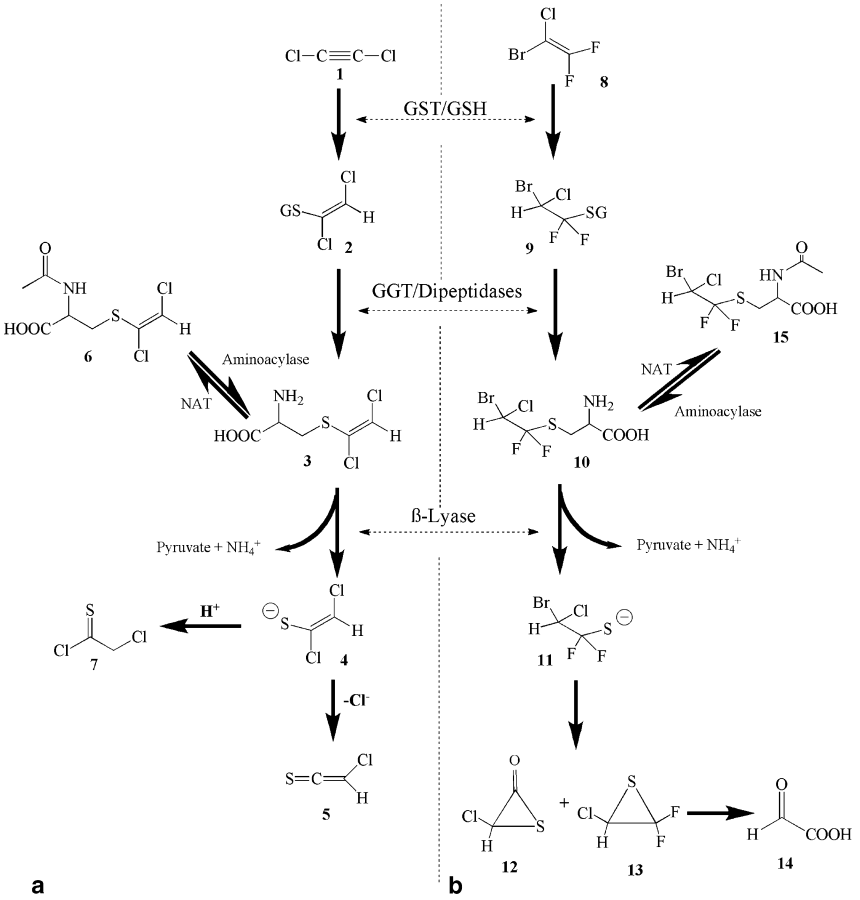

Fig. 10.5 Glutathione S-transferase- and cysteine conjugate β-lyase-dependent bioactivation of (A) dichloroacetylene [**1**] and (B) 2-bromo-2-chloro-1,1-difluoroethylene [**8**]. [**2**], S-(1,2-dichlorovinyl)gl utathione; [**3**], S-(1,2-dichlorovinyl)-L-cysteine; [**4**], 1,2-dichloroethenethiolate; [**5**], chlorothioketene; [**6**], S-(1,2-dichlorovinyl)-N-acetyl-L-cysteine; [**7**], chlorothionoacetyl chloride; [**9**], S-(2-bromo-2-chloro-1,1-difluoroethyl)glutathione; [**10**], S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine; [**11**], 2-bromo-2-chloro-1,1-difluoroethanethiolate; [**12**], 2-chloro-α-thiolactone; [**13**], 2,2-difluoro-3-chlorothiirane; [**14**], glyoxylic acid; [**15**], S-(2-bromo-2-chloro-1,1-difluoroethyl)-N-acetyl-L-cysteine; NAT, N-acetyltransferase

Dichloroacetylene is neurotoxic, hepatotoxic, and especially nephrotoxic and nephrocarcinogenic in experimental animals (Anders, 2005). The nephrotoxicity of dichloroacetylene is associated with β-lyase-dependent bioactivation (Kanhai et al., 1989). Interestingly, nephrotoxicity, which is a prominent feature of dichloroacetylene-induced toxicity in rodents, was apparently not observed in human subjects anesthetized with trichloroethylene. Although the evidence strongly indicates that dichloroacetylene undergoes β-lyase-dependent bioactivation in rodents, the failure to observe nephrotoxicity in human subjects may be attributed to the low β-lyase activities present

in human kidney tissue (Iyer and Anders, 1996; McCarthy et al., 1994). A possible role of β-lyase-dependent bioactivation in the observed neurotoxicity of dichloroacetylene needs further investigation.

Bioactivation of 2-Bromo-2-Chloro-1,1-Difluoroethylene

Halothane is a volatile anesthetic characterized by lack of flammability and very desirable anesthetic properties. By few years after its introduction into clinical anesthesia practice in 1956, there have been some case reports of halothane-associated fulminant hepatitis. Halothane-associated hepatitis is today recognized as due to its CYP450-dependent metabolism to trifluoroacetyl chloride, which interacts with lysine residues in liver proteins to give neoantigens and thus to induce allergic hepatitis (Pohl, 1990).

2-Bromo-2-chloro-1,1-difluoroethylene, which has not been implicated in the pathogenesis of halothane-associated hepatitis, is a minor impurity in halothane, formed in the presence of soda lime and present in the breath of human subjects during halothane administration (Anders, 2005). 2-Bromo-2-chloro-1,1-difluoroethylene reacts readily and nonenzymatically with sulfur nucleophiles, such as GSH and cysteine, forming S-(2-bromo-2-chloro-1,1-difluoroethyl)-N-acetyl-L-cysteine, that is present in the urine of halothane-anesthetized humans (Wark et al., 1990).

In fact, the addition of GSH to 2-bromo-2-chloro-1,1-difluoroethylene gives S-(2-bromo-2-chloro-1,1-difluoroethyl)glutathione, which may undergo GGT- and dipeptidase-catalyzed hydrolysis to give (2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine (Fig. 10.5B). The cysteine S-conjugate can undergo N-acetylation, giving the mercapturate S-(2-bromo-2-chloro-1,1-difluoroethyl)-N-acetyl-L-cysteine, or undergo β-lyase-dependent bioactivation, forming 2-chloro-α-thiolactone, 3-chloro-2,2-difluorothiirane and finally glyoxylic acid as a terminal metabolite (Finkelstein et al., 1995, 1996).

2-Bromo-2-chloro-1,1-difluoroethylene and its glutathione and cysteine conjugates S-(2-bromo-2-chloro-1,1-difluoroethyl)glutathione and S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine are nephrotoxic in rodents (Finkelstein et al., 1992). The nephrotoxicity of the S-conjugates is characterized by diuresis and increases in urine glucose and protein concentrations, in blood urea nitrogen concentrations, in kidney/body weight percentages, and in serum glutamate-pyruvate transaminase.

Furthermore S-(2-bromo-2-chloro-1,1-difluoroethyl)-glutathione and S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine, but not S-(2-bromo-2-chloro-1,1-difluoroethyl)-DL-α-methylcysteine (which cannot undergo β-lyase-catalyzed bioactivation), are cytotoxic in cultured LLC-PK1 cells (Finkelstein et al., 1992). These data demonstrate that the observed nephrotoxicity of 2-bromo-2-chloro-1,1-difluoroethylene is attributable to the formation of glutathione and cysteines S-conjugates by the β-lyase pathway.

However, there are no data about possible nephrotoxicity of 2-bromo-2-chloro-1,1-difluoroethylene and of its glutathione and cysteines S-conjugates in humans during halothane anaesthesia.

Prooxidant Effects of GSH

GSH is able to bind metal cations (Ciriolo et al., 1990); this interaction with metal ions can have important bearings on the cellular redox environment because thiol compounds, especially if in the dissociated thiolate anion form, can reduce metal cations, such as iron and copper. Electrons can be then transferred in turn from metal ions to molecular oxygen, thus generating superoxide anions that will easily dismutate to the strong prooxidant hydrogen peroxide (Fig. 10.6). In this way a true "redox cycling" of metals is induced and can proceed with even minimal concentrations of metal ions as long as electron donors (thiols) and acceptor (molecular oxygen) are available in the system (Paolicchi et al., 1999; Pompella et al., 2003).

GSH itself is rather less efficient than other thiols but the removal of glutamic acid by membrane-bound GGT from extracellular GSH produces a marked increase in the metal-reducing ability, resulting in a promotion of oxidative processes (Paolicchi et al., 1999, 2002; Spear and Aust, 1994; Pompella et al., 2003). Thus sites of GGT activity might act as sites of promotion of metal ion reduction and redox cycling, with consequent stimulation of oxidative processes; this hypothesis has been confirmed by findings obtained in several physiopathological conditions (e.g., cancer, human atherosclerosis, kidney ischemia, cell proliferative and apoptotic signals) (Dominici et al., 2003; Paolicchi et al., 2002).

However, GGT-derived cysteinyl-glycine is a strong nucleophile and thus can conjugate electrophilic agents, so contributing to extracellular detoxification of electrophilic compounds drugs, as demonstrated in normal and cancer cells (Daubeuf et al., 2002; Paolicchi et al., 2003).

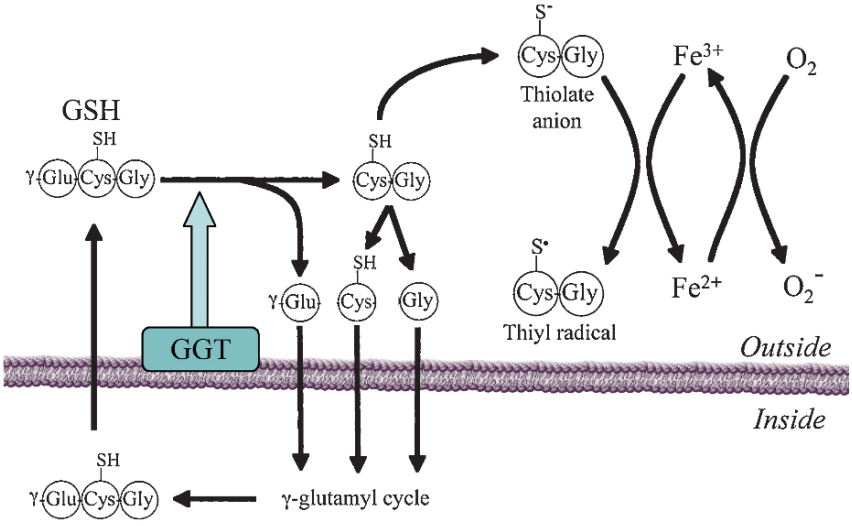

Fig. 10.6 Prooxidant reactions originating from GGT-dependent catabolism of extracellular GSH

Finally, the prooxidant properties of GSH are also due to its capability to bind NO, so forming GSNO, that, as previously discussed in this review, can serve as a transport form for this potent prooxidant species involved in several pathophysiological conditions.

Conclusions

This chapter gives an overview of the different roles that GSH appears to play in living organisms. GSH is a major defence pathway involved in deactivation of ROS and in detoxification of xenobiotics. Besides these fundamental antioxidant and antitoxic roles, glutathione modulates many other vital functions in animals. Many electrophilic physiological metabolites are substrates for GST-mediated addition of GSH. Furthermore the GSH conjugation pathway may have an important role in bioactivation of drugs and xenobiotics, giving cytotoxic and nephtotoxic metabolites. GSH is able to conjugate also with NO to form an S-nitrosoglutathione adduct, which may have a critical role in several physiological processes. GSH has been shown to glutathiolate several proteins; besides to represent an important cellular response to oxidative stress, protein S-glutathiolation plays a vital role in various physiological and pathological processes. Finally, recent studies have also highlighted the ability of GSH and of its catabolites to promote oxidative processes. Determining how GSH metabolism-related pathways are related with physiological processes may be critical for understanding the mechanisms of, and eventually developing therapies for, several physiopathological conditions in which these pathways surely play a fundamental role.

List of Abbreviations

cGMP	cyclic guanosine monophosphate
CYP	cytochrome P-450
CYS	cysteine
DAdopamine	
DOPA	3,4-dihydroxy-L-phenylalanine
DOPAC	dihydroxyphenylacetic acid
DP	dipeptidase
EET	epoxyeicosatrienoic acid
ETE	eicosatetraenoic acid
FOG_7	5-Oxo-7-glutathionyl-8,11,14-eicosatrienoic acid
GCL	glutamate cysteine ligase
GGT	γ-glutamyltransferase
GPx	glutathione peroxidase
GR	glutathione reductase
GS	GSH synthase
GSH	glutathione
GSNO	S-nitrosoglutathione
GSSG	glutathione disulfide

GST	glutathione-S-transferase
HIV	human immunodeficiency virus
HNE	4-Hydroxy-2-nonenal
LT	leukotriene
NADPH	nicotinamide adenine dinucleotide phosphate reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate oxidized form
NAT	N-acetyltransferase
NO	nitric oxide
ODE	octadecadienoic acid
PG	prostaglandin
PSSG	glutathiolated protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSNO	S-nitrosothiols
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SNAP	S-nitroso-N-acetylpenicillamine
TNF-α	tumor necrosis factor-α
XOO•	peroxide radical
γ-GCS	γ-glutamylcysteine synthetase

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Chapter 11
Dual Roles of Oxidative Stress in the Lungs

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Introduction

The redox state of the cell plays an important part in processes such as tumor progression, aging, atherosclerosis, chronic inflammatory processes, lung injuries and neurodegenerative diseases. Increased levels of intracellular reactive oxygen species (ROS) above normal basal levels are defined as cellular oxidative stress. ROS include superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals and hydrogen peroxide (H_2O_2), which can be further changed into highly reactive hydroxyl radicals via iron-catalyzed Fenton reactions under pathological conditions. $O_2^{\bullet-}$ can also rapidly react with nitric oxide (NO) to generate a stable free radical, peroxynitrite ($OONO^{\bullet-}$), which is a strong cytotoxic oxidant. Typically, oxidative stress is generated by increased production of ROS and/or by damage to the antioxidant defense system during cellular processes (Sies, 1997; Hoidal, 2001; Sen and Packer, 1996). Oxidative stress can also be introduced by exogenous sources such as air pollutants and cigarette smoke. Since the lungs contain the largest surface area of epithelial and endothelial cells of any organ, they are at high risk for injury from inhalation of high concentrations of ROS.

Reactive oxidants are associated with the pathogenesis of pulmonary diseases and affect various cell functions, from proliferation to apoptosis. While it is desirable to prevent cell death and tissue injury induced by oxidants in diseases such as asthma or acute respiratory distress syndrome, the opposite is sought in cancer. Oxidants have been shown to exert growth control on airway epithelial cells by modulating upstream growth factor receptor function (Ravid et al., 2002 2004; Goldkorn et al., 2005; Khan et al., 2006). Conversely, H_2O_2-mediated oxidative stress modulates ceramide levels to induce apoptosis in lung epithelium (Goldkorn et al., 1991, 2003; Chan and Goldkorn, 2000; Goldkorn and Ding, 1997; Barak et al., 2001, 2005; Goldkorn, 1994, 2001; Lavrentiadou et al., 2001; Ravid et al., 2003; Castillo et al., 2007; Honn, 1995). Additionally, depletion of the antioxidant glutathione (GSH) in lung epithelial cells results in ceramide accumulation,

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

suggesting that ceramide elevation, coupled to oxidative stress, initiates apoptosis (Lavrentiadou et al., 2001).

Given that ROS are short-lived and diffusible, localization of ROS signals in particular subcellular compartments suggests sequential and spatially organized redox signaling pathways in mammalian cells, which control various cellular roles. A variety of signaling pathways are initiated in response to oxidative stress, through which the cell's fate is determined. Oxidative stress and ROS can elicit and regulate both physiological and pathological processes. But, many of the molecular mechanisms modulating ROS-induced cellular events still require characterization. Many of these signaling pathways are generated at the cell surface and therefore involve the cell membrane. First, the activation of the ceramide/sphingomyelin pathway, which plays a key role in the induction of cellular death in response to ROS, will be reviewed. Then, the involvement of cholesterol-enriched cell membrane domains known as lipid rafts is presented. These lipid rafts are platforms for the initiation or transduction of signals and may modulate protein activity through a direct change in local membrane structure or by allowing protein–protein interactions to occur with higher affinity/specificity or both. Lastly, examples related to the induction of membrane-associated tyrosine kinase activation are discussed.

Oxidative Stress and Pulmonary Disease

ROS are involved in multiple biologic processes ranging from normal tissue homeostasis to various human diseases. Initially, the involvement of ROS in disease was explained with simplistic chemistry in which critical cell proteins and lipids were randomly oxidized and became inactive for their roles in normal cell function (Halliwell and Gutteridge, 1990). Accordingly, antioxidants were viewed simply as free radical scavengers. More recently, ROS have been recognized to function as signaling molecules, and several studies describe such roles for ROS in the pathogenesis of pulmonary diseases such as acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary diseases (COPD), asthma, and interstitial pulmonary fibrosis (Hoidal, 2001, Halliwell and Gutteridge, 1990; Rahman, 2005).

Pulmonary emphysema, which together with chronic bronchitis comprises COPD, is a very widespread lung disease with no effective treatments. Such lung injury diseases adversely affect more than 16 million people in the United States, with another 50 million estimated to be at risk (American Thoracic Society, 1996; Murphy, 2000). Eighty five percent of COPD cases are directly attributed to cigarette smoking and smokers are ten times more likely to develop COPD than nonsmokers. These diseases have no effective treatments because they involve destructive and lasting enlargement of distal airspaces and alveolar walls (Petrache et al., 2005; Elias and Lee, 2005; National Heart, Lung, and Blood Institute, 1985), which eventually leads to impaired oxygenation.

Traditionally, the pathogenesis of a lung disease such as emphysema has been linked to chronic lung inflammation causing a protease-antiprotease imbalance

(National Heart, Lung, and Blood Institute, 1985; Shapiro, 2000). However, this hypothesis does not explain why alveolar cells and alveolar wall structures are lost irreversibly. Another recent hypothesis presents the loss of alveolar wall structures through epithelial apoptosis (Petrache et al., 2005; Elias and Lee, 2005; Shapiro, 2000; Kasahara et al., 2000). Indeed, clinical studies have shown elevated levels of apoptosis in alveolar walls of emphysematous lungs (Kasahara et al., 2000; Segura-Valdez et al., 2000). Subsequently, direct evidence was provided linking apoptosis and emphysema (Petrache et al., 2005). When mice were given a single intracellular injection of active caspase-3 (executioner of apoptosis), the treatment resulted in alveolar epithelial apoptosis followed by alveolar wall damage and airspace enlargement (Aoshiba et al., 2003; Tuder et al., 2003).

Therefore, it has recently been suggested that alveolar cell apoptosis is a vital step in COPD (Petrache et al., 2005; Elias and Lee, 2005; Kasahara et al., 2000, Tuder et al., 2003; Rangasamy et al., 2004). This could explain the unique nature of alveolar septal damage in emphysema when compared with other lung diseases that are also characterized by inflammation and elevated matrix proteolysis. The irreversible pattern of the disease even when the patient stops smoking (Tuder et al., 2003) could well be accredited to the frequent interface between apoptosis, inflammation, oxidative stress and matrix proteolysis. Because ceramide is a second messenger molecule that modulates cell apoptosis (Goldkorn et al., 1998, 2005; Chan and Goldkorn, 2000; Lavrentiadou et al., 2001; Ravid et al., 2003; Hannun and Obeid, 2002), oxidative stress (Lavrentiadou et al., 2001), and proteolysis (Reunanen et al., 1998), we propose that deregulated ceramide generation (up-regulation) is critical for the induction of alveolar destruction as presented in the pathogenesis of lung injury diseases such as COPD. Therefore, the elucidation of the molecular mechanism(s) that modulate the sphingomyelin/ceramide pathway, which is critically involved in the pathogenesis of these diseases, is essential and may lead to the development of improved mechanism-specific drugs for their treatment or prevention.

ROS Modulate Ceramide Generation and Apoptosis

Agonists of the ceramide pathway (Strum et al., 1994; Jaffrezou et al., 1996, 1998; Obeid et al., 1993; Dbaibo et al., 1993; Andrieu et al., 1995; Kim et al., 1991; Cifone et al., 1994; Tepper et al., 1995; Martin et al., 1995; Boucher et al., 1995) include stress-inducing agents (Hannun, 1996), such as UV (Verheij et al., 1996), ionizing radiation (Haimovitz-Friedman et al., 1994; Santana et al., 1996), and reactive oxidants (Goldkorn et al., 1998, 2003; Chan and Goldkorn, 2000; Barak et al., 2001; Lavrentiadou et al., 2001; Ravid et al., 2003; Castillo et al., 2006). Emerging data suggest that oxidative stress and activation of the sphingomyelin catabolic pathway are closely coupled in apoptotic signaling (Heinrich et al., 2000; Chalfant et al., 2002; Gulbins and Grassme, 2002; Siskind et al., 2002; Futerman and Hannun, 2004). Recent studies have defined ceramide as a second messenger

and assigned sphingomyelin signal transduction an active role in biological processes (Goldkorn et al., 1991; Goldkorn and Ding, 1997; Honn, 1995).

It was previously observed that either administration of exogenous H_2O_2 or enhancement of endogenously generated H_2O_2 (by administration of aminotriazole) effectively depleted cellular GSH and initiated ceramide-induced apoptosis (Lavrentiadou et al., 2001). Both scenarios are relevant to lung epithelium. H_2O_2 is a ubiquitous molecule and able to cross cell membranes freely. It is present in several air pollutants, including the vapor phase of cigarette smoke. It is also detected in exhaled air of humans (Williams and Chance, 1983), and the amount of exhaled H_2O_2 appears greater in subjects with pulmonary disease (Sznajder et al., 1989) and in cigarette smokers (Nowak et al., 1996). However, the key intracellular sites of ROS generation that contribute to apoptosis in lung epithelium are still unknown. It is possible that an early burst of ROS generation also participates in initiating the apoptotic cascade in various models. This was demonstrated by using aminotriazole, a specific inhibitor of catalase, which augments endogenously generated H_2O_2. Administration of aminotriazole led to rapid build-up of ceramide, a proximal event in the apoptosis model (Lavrentiadou et al., 2001). Furthermore, H_2O_2 was found to act directly on cell membrane preparations devoid of nuclei, stimulating sphingomyelin hydrolysis to ceramide through a Mg^{+2}-dependent neutral sphingomyelinase (nSMase) (Chan and Goldkorn, 2000; Goldkorn et al., 1998). This was the first evidence that Mg^{+2}-dependent nSMase activity resides in the membrane of human airway epithelial (HAE) cells.

Several Types of SMases Have Been Identified

Ceramide is synthesized through either a de novo pathway involving serine palmitoyl-CoA transferase and ceramide synthase, or from breakdown of membrane sphingomyelin (N-acylsphingosin-1-phosphocholine) (Fig. 11.1), a phospholipid preferentially concentrated in the plasma membrane of mammalian cells (Merrill and Jones, 1990). Sphingomyelin catabolism occurs via the action of sphingomyelin-

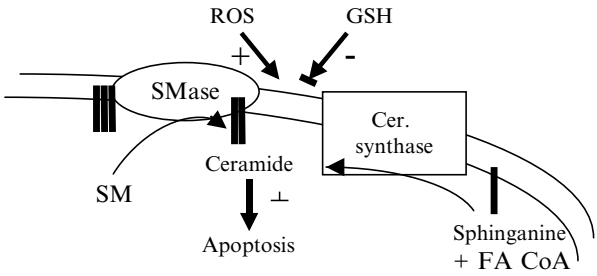

Fig. 11.1 Coupling between reactive oxygen species (ROS) and ceramide pathway induces apoptosis in airway epithelial cells via a SMase. SM, shingomyelin; GSH, glutathione; FA, fatty acid

specific forms of phospholipase C termed sphingomyelinases (SMases), which hydrolyze the phosphodiester bond of sphingomyelin, yielding ceramide and phosphorylcholine. Ceramide then serves as a second messenger, leading to apoptotic DNA degradation.

The main forms of SMases are distinguished by their pH optima (Cifone et al., 1994; Castillo et al., 2006,Schutze et al., 1992; Lawler et al., 1998; Jayadev et al., 1995; Wiegmann et al., 1994; Okazaki et al., 1994). Human and murine acid sphingomyelinase (aSMase; pH optimum 4.5–5.0) have been cloned and determined to be the products of a conserved gene, while Mg^{2+} -dependent or -independent neutral SMases (nSMase; pH optimum 7.4) have yet to be molecularly characterized. Interestingly, membrane nSMase does not gain access to the signaling events activated by the lysosomal aSMase and vice versa, indicating that ceramide action may be determined by the subcellular site of its production. The aSMase, recently implicated in the development of acute lung edema (Goggel et al., 2004), has lysosomal and secretory isoforms that contribute to extracellular increases in ceramide (Kolesnick and Fuks, 2003).

Very little is presently known about the regulatory mechanisms of SMases. Recently, it was shown that H_2O_2-generated oxidative stress modulates (up-regulates) only nSMase and not aSMase (Chan and Goldkorn, 2000). Moreover, aSMase plays a dual role in reactive nitrogen species-induced apoptosis in airway epithelial cells (Castillo et al., 2006). In one instance, $ONOO^-$ generated ceramide through the activation of aSMase, resulting in apoptosis. In another instance, NO generated ceramide through the induction of the de novo biosynthetic pathway involving ceramide synthase, but no apoptosis was observed. The absence of apoptosis was explained by the observation that NO exposure increased the protein-protein interaction between aSMase and caspase-3, with aSMase sequestering caspase-3 and thereby blocking an apoptotic response (Castillo et al., 2006).

GSH Modulates Ceramide Generation and Apoptosis

Both lung epithelial cells and the epithelial lining fluid (ELF) contain relatively high concentrations of GSH, the main antioxidant in the lung epithelium. Low GSH levels have been shown to be essential for ceramide generation, whereas high GSH levels inhibit the production of ceramide (Lavrentiadou et al., 2001). Low levels of GSH and elevated levels of ceramide correlate with the induction of apoptosis in lung epithelial cells. Furthermore, GSH and N-acetylcysteine (NAC), but not other thiol-containing antioxidants or oxidized GSH (GSSG), inhibit H_2O_2-mediated induction of ceramide and apoptosis. Therefore, GSH plays a critical role in preventing lung epithelial cell apoptosis. In this model, inhibitory effects on ceramide production were observed with both extracellular and intracellular GSH. The effects of extracellular GSH are primarily applicable to lung epithelium. It is interesting that even a short exposure of cells to 250 μM H_2O_2 for 1 h, followed by growth in regular medium, was sufficient to induce apoptosis (Lavrentiadou et al.,

2001). This demonstrates that the events that control the fate of the cells occur within this hour, during which GSH is depleted and ceramide is generated. Therefore, it was proposed that in lung epithelial cells, a membrane-linked nSMase may exist in an inactive form inhibited by high levels of both intra- and extracellular GSH present in ELF, thus maintaining low levels of ceramide (Fig. 11.1). The inhibition of such a SMase may render lung cells less sensitive and less susceptible to oxidants, to which they are ordinarily exposed. This would increase the threshold for ceramide elevation required for the induction of apoptosis. However, once oxidant levels increase, they decrease GSH levels, thereby overcoming its inhibitory effect on nSMase. Therefore, ceramide is elevated and the apoptotic pathway is initiated.

Ceramide Accumulation, Caspase Activation, and Bcl2 Inhibition of Apoptosis

The literature presents conflicting studies with respect to the placement of ceramide generation relative to caspases in the apoptotic cascade (Hannun and Luberto, 2000; Hofmann et al., 2000; Dbaibo et al., 1997; Takeda et al., 1999). Different stimuli acting at diverse sites to activate ceramide accumulation have been shown to trigger apoptosis (Ravid et al., 2003). This supports the hypothesis that an increase in ceramide levels, per se, is sufficient to initiate the apoptotic cascade in lung epithelial cells (Fig. 11.2) and that ceramide accumulation is the causative signal for apoptosis induction and not just an outcome of epithelial cell death.

The antiapoptotic activity of Bcl2 has been thought to be due to its ability to prevent Bax/Bak from initiating mitochondrial cytochrome-C release followed by inhibition of caspase-3 activity (Green and Kroemer, 2004). The role of Bcl2 overexpression

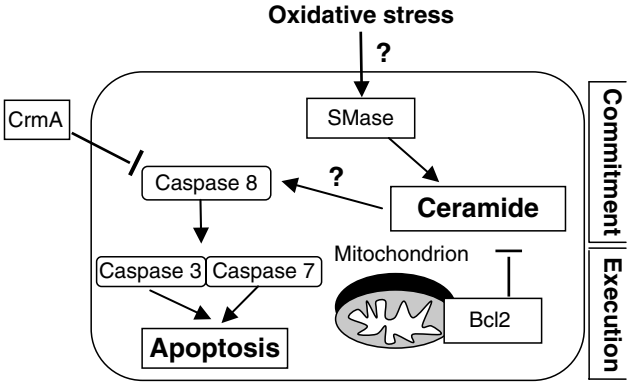

Fig. 11.2 Mapping the cellular sites of interaction between the ceramide-generating machinery and members of the apoptotic signaling cascade, such as the caspases and Bcl2

in modulating the ceramide pathway, however, is controversial (Jaffrezou et al., 1998; Tepper et al., 1999). Only a few reports demonstrate that Bcl2 regulates ceramide formation (Sawada et al., 2000), whereas most studies indicate that Bcl2 blocks apoptosis but does not affect ceramide generation (Dbaibo et al., 1997; Allouche et al., 1997). For instance, one study demonstrated that Bcl2 protects against H_2O_2- and C_6-ceramide-induced caspase-3 activation and cell death (Ravid et al., 2003). In addition, Bcl2 inhibited ceramide generation in response to inducers of apoptosis and also reduced the basal cellular levels of ceramide production, which suggests that the modulation of the ceramide pathway may be a target for the antiapoptotic effects of Bcl2 (Fig. 11.2).

Lung Cells Express High Levels of Sphingolipid Enzymes and Derivatives

Several recent studies implicate sphingomyelin hydrolysis in acute lung injury (Petrache et al., 2005) and pulmonary edema (Goggel et al., 2004). However multiple questions still emerge from these observations (Zimmerman and McIntyre, 2004), and additional characterization of the ceramide pathway and lung injury are still needed. Reports regarding septic patients and their increase in ceramide levels suggest that the ceramide pathway has a pivotal role in contributing through its proapoptotic activity to multiple organ dysfunction syndrome (MODS) (Delogu et al., 1999; Claus et al., 2005). However, the exact cellular and molecular mechanism(s) underlying ceramide-mediated apoptosis are still unknown, and thus, the role of ceramide in the pathobiochemistry of disease still needs to be addressed.

Recently, Petrache et al. (2005) reported that blockade of the vascular endothelial growth factor receptor resulted in de novo ceramide synthesis, pulmonary cell apoptosis and emphysema-like disease in mice. Additionally, patients with smoking-induced emphysema were shown to have increased lung ceramides, suggesting that ceramide upregulation may have a pivotal role in the pathogenesis of this disease (Petrache et al., 2005). Other studies have shown that accumulation of ceramide, triggered by hydrogen peroxide (H_2O_2), induces apoptosis of HAE cells (Chan and Goldkorn, 2000; Lavrentiadou et al., 2001; Ravid et al., 2003). Under oxidant exposure, a lung SMase is activated and displays continued ceramide generation and pro-apoptotic signaling, thus leading to the pathological apoptosis that causes lung injury. In searching for a specific SMase that is modulated by oxidative stress, nSMase2 was cloned from monkey lung tissue and HAE cells, and was shown to be up-regulated by an oxidant (H_2O_2) and is inhibited by an antioxidant (GSH). Furthermore, mice and rats exposed to cigarette smoke demonstrated increased apoptosis in the bronchial epithelium as well as significant levels of nSMase2 and increased ceramide (Goldkorn unpublished data, 2007). Human emphysematous lung biopsy sections also displayed significantly higher levels of nSMase2 staining compared to normal lung tissue (Goldkorn unpublished data, 2007). The correlation

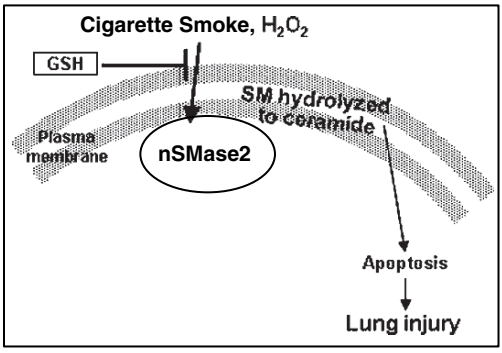

Fig. 11.3 Schematic model of nSMase2 modulation by cigarette smoke (CS)–oxidative stress. CS-induced oxidative stress up-regulates nSMase2 activity and leads to elevation in ceramide generation (hydrolysis of sphingomyelin (SM)) and thus exacerbated apoptosis in airway epithelial cells which causes lung injury. GSH blocks this effect of CS

between cigarette smoke, nSMase2, ceramide, and apoptosis suggests that there is a link between cigarette smoke oxidants such as H_2O_2 and nSMase2-generated ceramide in lung cell apoptosis (Fig. 11.3).

Other Oxidants – Other Sphingomyelinases

In addition to ROS, reactive nitrogen species (RNS) are also components of cigarette smoke and environmental pollutants that affect lung epithelial cell function by modulating cell proliferation or apoptosis (Castillo et al., 2007). RNS have been implicated in the pathophysiology of inflammatory lung diseases such as asthma and COPD. But the molecular mechanisms and signaling events involved in lung cell injury by RNS are still poorly understood. RNS, like ROS, may also damage lung tissue via the generation of ceramide, an inducer of apoptosis (Pautz et al., 2002). Indeed, RNS exposure in different cell types has generated ceramide and promoted apoptosis (Pautz et al., 2002; Pilane and LaBelle, 2004).

RNS, such as NO, have often been viewed as being "double-edged sword" molecules. In several model systems, NO has been shown to promote apoptosis, while in others it has not. Ceramide may be generated in the cell through several different pathways such as through the hydrolytic action of a SMase on sphingomyelin or through the de novo synthesis pathway by the condensation of palmitoyl-CoA and serine. A third way ceramide may be generated is through the impairment of the degradative process of ceramide via ceramidases. Previous studies using retinal photoreceptor cells or renal mesangial cells have found that NO exposure via NO donors (sodium nitroprusside or spermine NO) may generate ceramide via the activation of acidic sphingomyelinase or through the degradation of neutral ceramidase, respectively, triggering an apoptotic response (Sanvicens and Cotter, 2006;

Franzen et al., 2002). Similarly, Rabkin found that the NO donor sodium nitroprusside (SNP) promoted ceramide generation via ceramide synthase in cardiomyocytes, resulting in cell death (Rabkin, 2002).

Recently, a novel anti-apoptotic response to NO exposure (via the NO donors 3-morpholine-syndnonimine (SIN1) or papa-NONOate) was observed in HAE cells. NO exposure via the NO donors increased cellular ceramide levels via ceramide synthase but did not trigger an apoptotic response. Rather, exposure to the NO donors promoted an increase in the protein–protein interaction between acidic sphingomyelinase (aSMase) and caspase-3, with aSMase sequestering caspase-3 and preventing its cleavage (Fig. 11.4). In contrast, when aSMase was silenced in HAE cells or was knocked out in mice, an increase in cleaved caspase-3 was observed (Fig. 11.5). This elevated caspase-3 cleavage was further augmented upon NO exposure (via SIN1 or papa-NONOate) of HAE cells and could be prevented by an inhibitor to ceramide synthase. These results demonstrated a novel mechanism of NO modulation of apoptosis, in which HAE cells exposed to NO induced ceramide generation via ceramide synthase. However, this ceramide induction did not lead to apoptosis unless aSMase was knocked down, allowing the release of caspase-3, its activation and execution of apoptosis. These findings were the first to show a novel way NO prevents apoptosis by increasing the interaction between aSMase and caspase-3 in lung epithelial cells.

Following the observation that NO exposure failed to induce apoptosis in HAE cells, the NO donor papa-NONOate was combined with a superoxide anion donor (DMNQ) to generate $ONOO^-$, after which an increase in cellular ceramide levels and apoptosis were observed. Pretreatment with the antioxidant GSH did not prevent $ONOO^-$-induced apoptosis, but did prevent H_2O_2-induced apoptosis. Analysis of the ceramide-generating enzymes revealed a differential response triggered by the oxidants. $ONOO^-$ exposure did not affect nSMase activity but instead specifically activated aSMase. The specificity of each enzyme was confirmed using siRNA to knock down both nSMase2 and aSMase. Silencing nSMase2 prevented H_2O_2-induced

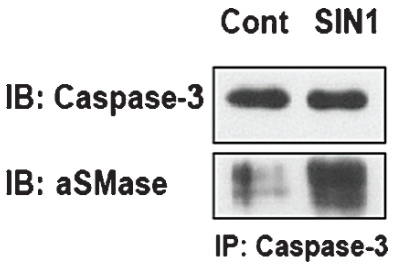

Fig. 11.4 NO exposure enhances the interaction between aSMase and caspase-3 in HAE cells. HAE cells were treated with 100 μM SIN1 or vehicle control for 15 min. Cells were harvested and lysates were subjected to immunoprecipitation with a caspase-3 antibody. Immunoprecipitates were separated by SDS-PAGE and immunoblotted for aSMase and caspase-3 (From Castillo et al., *Exp. Cell Res*. 313: 816–823)

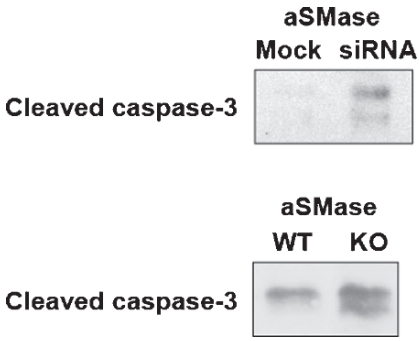

Fig. 11.5 The absence of aSMase allows for caspase-3 cleavage both in vitro and in vivo. HAE cells were mock or aSMase siRNA transfected. 48 h post-transfection, cell lysates were analyzed by immunoblot analysis for cleaved caspase-3 (*upper*). Whole lung homogenate from wild type (C57BL6) or aSMase$^{-/-}$ mice was analyzed by immunoblot analysis for cleaved caspase-3 (*lower*) (From Castillo et al., *Exp. Cell Res*. 313: 816–823)

apoptosis, but had no effect on $ONOO^-$-induced apoptosis. On the other hand, silencing of aSMase markedly impaired $ONOO^-$-induced apoptosis, but did not affect H_2O_2-induced apoptosis. These findings support the hypothesis that ROS and RNS modulate ceramide levels to induce apoptosis in HAE cells. However, it appears that different oxidants modulate different enzymes of the ceramide-generating machinery to induce apoptosis in airway epithelial cells, thereby adding to the complexity of how oxidative stress promotes lung cell injury (Fig. 11.6).

Lipid Rafts, Ceramide, and ROS-Induced Cell Death

The cell membrane can form lipid-enriched microdomains called lipid rafts, which are capable of forming platforms that reportedly play a role in membrane protein sorting and the construction of signaling complexes (Li and Gulbins, 2007). Because ROS can react with various molecular targets such as proteins and lipids, ROS may interact (directly or indirectly) with raft components and regulate the signaling roles of lipid rafts. On the other hand, membrane platforms may regulate ROS-generating enzymes such as NADPH oxidase, thereby augmenting ROS production.

Traditionally, lipid rafts have been described as relatively small structures enriched in cholesterol and sphingolipids, within which associated proteins are likely to be concentrated. Sphingomyelin, the most prevalent component of the sphingolipid fraction of the cell membrane, is composed of a highly hydrophobic ceramide moiety and a hydrophilic phosphorylcholine head group. Microdomain formation is promoted by the tight interaction between the cholesterol sterol ring system and the ceramide moiety of sphingomyelin. Recently, a novel membrane

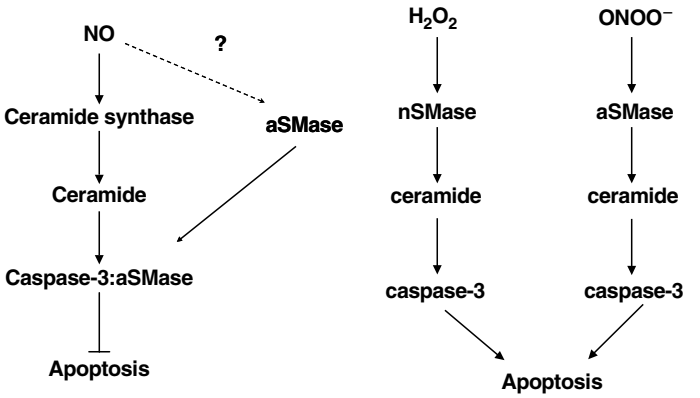

Fig. 11.6 ROS and RNS modulate the activity of different SMases in HAE cells. Different oxidant species modulate components of the ceramide-generating machinery with varying effects. NO exposure activates ceramide synthase and generates ceramide. Apoptosis is prevented due to the up-regulation of the caspase-3:aSMase interaction, which prevents caspase-3 cleavage and apoptosis. H_2O_2, on the other hand, activates nSMase to generate ceramide and induce apoptosis. $ONOO^-$ induces apoptosis via the activation of aSMase, which also generates ceramide (From Castillo et al., *Exp. Cell Res*. 313: 816–823 and 2680–2686)

domain type, i.e., ceramide-enriched membrane domains, has been described. The tight interaction of ceramide molecules with each other results in the formation of stable and tightly packed ceramide-enriched membrane macrodomains or platforms (Li and Gulbins, 2007).

The formation of ceramide-enriched platforms has been implicated in many signaling pathways, including signaling via CD95, CD40, CD20, interleukin-1 receptor, the PAF receptor, CD5, LFA-1, FcγRII, DR5, CD28, and TNF and many stress stimuli, including certain bacterial and viral infections (Li and Gulbins, 2007). Many studies have shown that in response to oxidative stress, lipid rafts or platforms may participate in cellular apoptotic signaling during the activation of various death receptors. For example, the death receptors Fas and tumor necrosis factor receptor 1 (TNFR1) are localized in lipid rafts and the receptors in lipid rafts can interact to stabilize the raft further and allow raft aggregation, or clustering, and the recruitment of raftophilic molecules to the complex, thereby producing massive signaling action (Li and Gulbins, 2007). Furthermore, ROS have also been shown to act downstream of lipid raft formation in apoptosis signaling. For example, Fas and TNFR1 activation generates ROS in response to stimulation and this is attributed to the production of superoxide ($O_2^{\cdot -}$) due to the formation of lipid raft-derived NADPH oxidase platforms (Li and Gulbins, 2007; Morgan et al., 2007). ROS could then enhance various SMase activities, thereby generating more ceramide and promoting further lipid raft platform formation. The resulting feed forward action creates a redox signaling network via lipid raft or ceramide-enriched membrane platforms, which can amplify or fine-tune redox-regulated transmembrane signaling (Li and Gulbins, 2007).

ROS Interact with Src, Caveolin-1, and EGF Receptor

Inasmuch as oxidative stress can trigger an apoptotic response in cells, it can also trigger a pro-survival or proliferative response. Receptor tyrosine kinases (RTKs) and other tyrosine kinases, such as members of the Src family, have been shown to be activated by oxidative stress via two possible mechanisms. First, ROS can inactivate the catalytic cysteines of protein tyrosine phosphatases that are usually recruited to the complexes during activation, thereby enhancing tyrosine kinase activity (Morgan et al., 2007; den Hertog et al., 2005; Nakashima et al., 2002, 2005). Second, ROS appear to mediate a direct activation of RTKs via direct oxidation of specific cysteine residues that result in structural changes that favor an active conformation (Giannoni et al., 2005) or by the formation of specific intermolecular disulfide bonds with other molecules within the complex (Morgan et al., 2007; Nakashima et al., 2002; Rhee et al., 2005). Lipid rafts are thought to play a role in the activation process by causing the aggregation of the kinases and allowing their crosslinking (Morgan et al., 2007; Nakashima et al., 2002; Rhee et al., 2005), thus promoting activity. There are many examples of ROS activation of tyrosine kinases and their downstream events presented in the literature. For instance, c-Src kinases phosphorylate Tyr-14 of caveolin-1, which regulates the trafficking/localization of caveolae in such places as focal adhesions (Volonte et al., 2001; del Pozo et al., 2005), is required for caveolar internalization, and may thus regulate the composition/localization of certain lipid rafts (del Pozo et al., 2005). Additionally, c-Src is reportedly required for the activation of JNK by H_2O_2 (Morgan et al., 2007) and Src family kinases are critically involved in the control of cytoskeletal organization and in the generation of integrin-dependent signaling responses in fibroblasts, inducing tyrosine phosphorylation of many signaling and cytoskeletal proteins (Tatosyan and Mizenina, 2000). Furthermore, H_2O_2 has been reported to activate pro-survival pathways including PI3 kinase/Akt and ERK1/2 in various cell lines (Ravid et al., 2002; Khan et al., 2006; Yang et al., 2006).

The epidermal growth factor (EGF) receptor (EGFR) is implicated in a number of cancers and its oncogenic potential is linked to its inability to undergo clathrin-mediated endocytosis and lysosomal degradation (Franklin et al., 2002; Eccles et al., 1994). The process of EGFR down-regulation is highly dependent upon the ability of the E3 ubiquitin ligase, c-Cbl, to bind the receptor, thereby facilitating its entry into the clathrin-coated pits and lysosomal sorting (Mosesson et al., 2003; Levkowitz et al., 1999; Huang et al., 2006; Jiang and Sorkin, 2003). H_2O_2-induced oxidative stress has been shown to activate and aberrantly phosphorylate the EGFR in human airway epithelial cells, which impedes the clathrin-mediated endocytosis and subsequent lysosomal degradation of the receptor (Ravid et al., 2002; Goldkorn et al., 1998; Gamou and Shimizu, 1995). Concomitantly, H_2O_2 activates Src, which in turn hyperphosphorylates caveolin-1 (a proposed trigger for caveolar endocytosis) and dynamin-2, which is thought to localize at the neck of caveolae such that activation of its GTPase activity leads to vesicle fission

(Shajahan et al., 2004; Oh et al., 1998). Through this route of caveolar endocytosis, H_2O_2-activated EGFR is trafficked to a perinuclear region, where the receptor remains active (Fig. 11.7). This results in prolonged downstream activation of proliferative molecules such as Akt and ERK1/2 (Ravid et al., 2004). This may have significant ramifications in tumorigenesis, since Wang et al. (2002) have reported that EGFR signaling in the endosome is both sufficient to activate major signaling cascades involved in cell proliferation and to suppress apoptosis induced by serum withdrawal and the lack of receptor turnover has been shown to mediate tumor promotion in non-neoplastic rat liver epithelial cells (Huang et al., 2001).

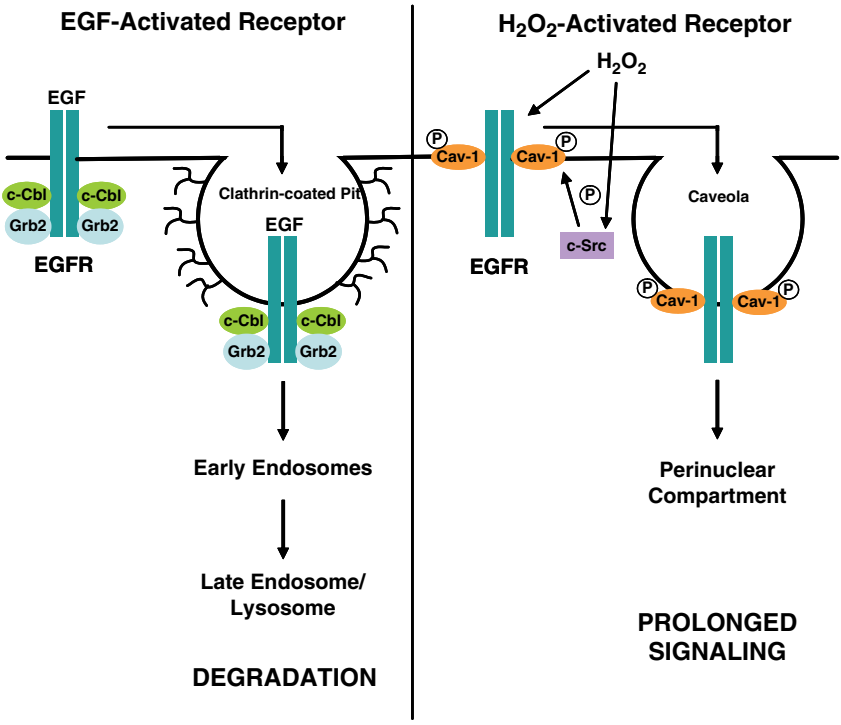

Fig. 11.7 Schematic model for the role of caveolin-1 in EGFR trafficking. When exposed to EGF, EGFR recruits both c-Cbl and Grb2, which are required for the receptor to enter clathrin-coated pits, where it is sorted for lysosomal degradation. Under oxidative stress, EGFR is precluded from entering clathrin-coated pits by its failure to recruit both c-Cbl and Grb2. However, the activated EGFR does accumulate in a perinuclear compartment via caveolae-mediated endocytosis that is dependent on Src phosphorylation of caveolin-1 (From Khan et al., J. *Biol. Chem.* 281: 14486–14493)

Conclusions

Oxidative stress can elicit myriad effects in cells, ranging from cell death to growth and proliferation. What tips the scales between life and death still remains a mystery. Having touched on the contributions of sphingomyelinases, ceramide, lipid rafts, and tyrosine kinases, it is quite evident that many pieces of the puzzle are still missing. The extent of ROS production, the site and source of their generation, the cell type, and the antioxidant status of the cell are all likely to affect the final outcome. Further delineating and understanding the mechanisms of sphingomyelinase activation and ceramide signaling will be helpful in identifying therapeutic targets and points of intervention in the pathogenesis of degenerative pulmonary diseases.

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Chapter 12
Antioxidants: How They Work

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Abstract In this overview, peroxidation processes which lead to the degradation of organic material and the mechanisms by which antioxidants work are discussed. Special attention is focused on hydrogen and electron transfer processes and on radical scavenging reactions. Most natural and synthetic antioxidants during their action generate species which could exert a pro-oxidant effect, therefore the balance between antioxidant/pro-oxidant aspects is also discussed.

Keywords Antioxidants, pro-oxidants, free radicals, electron transfer, hydrogen transfer, radical scavenging, nitroxides radicals, vitamins

Introduction

Peroxidative processes caused by free radicals are directly or indirectly involved in various human disease states (e.g. inflammation, carcinogenesis, ischemia/reperfusion injury) (Halliwell and Gutteridge, 1999) and ageing. Oxygen, which is vital to aerobic organisms, may become toxic through its metabolites such as superoxide anion, hydroperoxyl and hydrogen peroxide (McCord, 2000), which are reactive oxygen species (ROS) all involved in the so called "oxidative stress". A peroxidative process takes place every time carbon-centred radicals are formed. Figure 12.1A shows that this process occurs in a cyclic fashion through the formation of carbon-centred radicals (R•) and peroxyl radicals (ROO•) while Fig. 12.1B shows how this process may be inhibited by using either preventive antioxidants which inhibit the initiation process (initiated by transition metal ions, ROS, ionizing radiation or UV radiation) or chain-breaking antioxidants which inhibit the formation of either peroxyl radicals and/or new carbon-centred radicals.

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

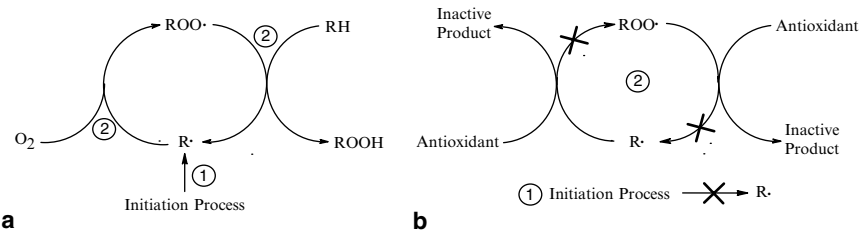


Fig. 12.1 The peroxidative process (A) and it's inhibition (B) by preventive antioxidants ①, and chain-breaking antioxidants ②

How Antioxidants Work

All biological systems exposed to oxygen undergo peroxidative degradation in different ways (Halliwell and Gutteridge, 1999) and the same degradation may also occur in polymers (Scott, 1990). All these organic systems need to be protected by antioxidants in order to maintain their physical, chemical and functional properties. An antioxidant may therefore be defined as a compound which inhibits oxidation by either reacting with ROS to yield harmless products and/or by disrupting free radical chain reactions. In broader terms, Halliwell and Gutteridge define an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation" (Halliwell and Gutteridge, 1999). However, each antioxidant during its activity generates or can generate species that could promote oxidation, hence all antioxidants possess either directly or indirectly pro-oxidant properties. In fact a good antioxidant is one that generates compounds having a low propensity to promote peroxidative processes. Indeed, the pro-oxidant effect of antioxidants has been the object of studies which have shown antioxidants to be harmful when administered in high doses in an ordinary diet (Halliwell, 2000). Therefore it should be stressed that it is possible for a compound to show both pro- and anti-oxidant properties depending on concentrations, experimental conditions, stage of oxidation and the presence of a reaction partner (co-antioxidant).

Following is a treatise on some of the different mechanisms by which antioxidants work and the possible pro-oxidant effects that can be generated during their activity. It is important to underline here that not all antioxidants exert their inhibitory action through the same mechanism and that the same antioxidant may inhibit free radical processes by more than one mechanism.

To explain the different mechanisms, the antioxidants reported in Fig. 12.2 have been chosen as examples.

Inhibition by Hydrogen Transfer

Hydrogen atom transfer very often represents the key reaction through which a chain-breaking antioxidant exerts its action. This is the typical mechanism for phenolic

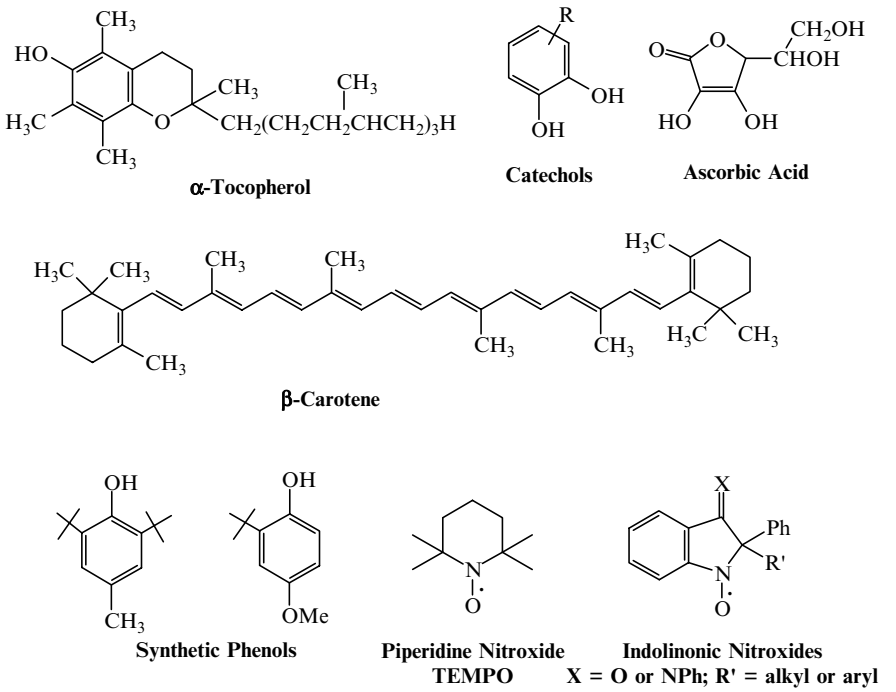


Fig. 12.2 Selected natural and synthetic antioxidants

antioxidants which will be mainly discussed in the present section, as well as for other antioxidants such as carotenoids (Mortensen and Skibsted, 1998).

Most synthetic and naturally occurring antioxidants are phenolic compounds for which the generic term ArOH will be used since, by definition, they contain at least one hydroxyl group attached to a benzene ring. They owe their activity to the donation of the phenolic hydrogen atom to the chain carrying peroxyl radicals by reaction 1, which is a much faster reaction than the attack of the peroxyl radicals on the organic substrate, reaction 2, the propagating step of the peroxidative process.

$$\mathrm{ROO^{\bullet} + ArOH \xrightarrow{K_{inh}} ROOH + ArO^{\bullet}} \quad (1)$$

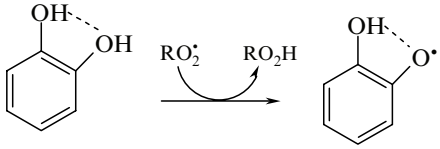
$$\mathrm{ROO^{\bullet} + RH \xrightarrow{K_2} ROOH + R^{\bullet}\left(\xrightarrow{O_2} ROO^{\bullet}\right)} \quad (2)$$

For example, α-tocopherol (Vit E), the major lipid-soluble, peroxyl radical-trapping antioxidant in human blood plasma, reacts with peroxyl radicals with a rate constant of about 10^6 $M^{-1}s^{-1}$ (Burton et al., 1983) a much higher value than those reported in the literature for reaction 2, typically 10^1 $M^{-1}s^{-1}$ (Ingold, 1969).

Theoretically, ArOH could donate its phenolic hydrogen atom to carbon-centred radicals formed in the initiation step of peroxidative processes (Fig. 12.1A) and hence acting as an antioxidant also in this case but, practically, these radicals, once

formed, immediately react with molecular oxygen leading to peroxyl radicals, reaction close to be diffusion controlled ($k \sim 10^9\ M^{-1}s^{-1}$) (Ingold, 1969).

The antioxidant properties of Vit E and of all the other phenolic antioxidants is a consequence of the relatively weak bond dissociation enthalpy (BDE) of the O-H bond. The phenolic hydrogen atom of Vit E (BDE = 77.93 kcal/mol) (Borges dos Santos and Martinho Simoes, 1998) is rapidly abstracted in an exothermic reaction by ROO• to give an alkyl hydroperoxide ROOH having a BDE (O-H) of ca. 88 kcal/mol (Berkowitz et al., 1994). The lower BDE value of the phenolic hydrogen in Vit E compared to that of an unsubstituted phenol (BDE ~ 88 kcal/mol) (Denisov and Denisova, 1999) derives from its particular structure, i.e. alkyl and alkoxyl moieties on the aromatic ring which stabilize the phenoxyl radical ArO• thus favouring its formation. (Burton and Ingold, 1986; Wright et al., 1997). In fact, it is well known that electron releasing groups, especially if in conjugated positions, reduce the BDE O-H value in a substituted phenol (Brigati et al., 2002). Moreover, the influence of a substituent (hydroxyl or alkoxyl) is also due to the possible formation of intramolecular hydrogen bonds with the phenolic O-H thus stabilizing the starting phenol and/or the corresponding phenoxyl radical.



(3)

If, on the one hand, phenolic hydrogens involved in intramolecular H-bonding, such as in Ubiquinol-10 ($Q_{10}H_2$) and in catechols, are less reactive toward peroxyl radicals than free hydroxyl groups, on the other hand, their reactivity is less affected by the nature of the solvent since H-bonding between the phenolic O-H and hydrogen-bond-accepting (HBA) solvents is prevented (de Heer et al., 1999, 2000; Wright et al., 2001). This means that, in HBA solvents, their hydrogen donating ability towards peroxyl radicals is increased if compared to ArOHs lacking intramolecular H-bonding (phenolic hydrogen atoms involved in H-bonds with the solvent are not abstracted) (MacFaul et al., 1996). In addition, the presence of an intramolecular H-bond in the phenoxyl radical (reaction 3), as in catechol derivatives, decreases further the O-H BDE value (Lucarini et al., 2002) rationalizing the very good antioxidant properties of natural products containing catechol moieties (Jovanovic et al., 1996; Rice-Evans et al., 1996) (polyphenolic compounds).

The chemical structure of an antioxidant is therefore important in determining its activity. However, the microenvironment in which the antioxidant works is equally important: in relevant biological systems such as heterogeneous aqueous/lipid phases H-bonding, differential solvatation and diffusion phenomena have also to be taken into account when the kinetics of peroxyl radical trapping by antioxidants are considered (Barclay et al., 1990; Castle and Perkins, 1986; Pryor et al., 1988).

The efficiency of Vit E as antioxidant is also due to the fact that tocopheryl radical, formed in the inhibition process, is a relatively persistent free radical which does not, in general (see later), react with molecular oxygen or with substrate RH to

continue the peroxidative chain, but is eventually destroyed by reacting with a second peroxyl radical in a fast coupling reaction (k= 1–8 × 10^8 $M^{-1}s^{-1}$) (Jonsson et al., 1993), thus terminating two potential radical chains (Bowry and Ingold, 1995).

Moreover, Vit E may be easily regenerated by several co-antioxidants, namely ascorbic acid (Vit C), $Q_{10}H_2$, etc. Vit C, which at physiological pH is largely ionized in the ascorbate form, may easily reduce tocopheryl radical back to tocopherol (k= 8.3 × 10^6 $M^{-1}s^{-1}$) (Davies et al., 1988) *via* either a hydrogen atom transfer or a concerted electron and proton transfer mechanism (Bisby and Parker, 1995).

In this way, the two vitamins, E and C, show a synergistic behaviour (Doba et al., 1985; Niki et al., 1984, 1985) and their mixtures display much better antioxidant properties than expected on the basis of the activity of the single components, even though the two vitamins have different locations, being Vit E lipid soluble and Vit C water soluble. Synergism with Vit E has been described also for $Q_{10}H_2$ (Frei et al., 1990) and for flavonoids (Jia et al., 1998; Pedrielli and Skibsted, 2002) and in both cases recycling of Vit E proceeds through the transfer of two hydrogen atoms (transferred sequentially) from the co-antioxant to two tocopheryl radicals.

These are only some of the aspects of the complex situation which occurs each time an antioxidant exerts its action. In fact, as already stated in the introduction, each antioxidant may also have a pro-oxidant activity as observed for Vit E in an aqueous dispersion of human low density lipoprotein (LDL) when no co-antioxidants, such as Vit C or $Q_{10}H_2$, are present. In fact, tocopheryl radical ArO• formed in a LDL particle, too small to hold more than one radical at a time (Bowry and Ingold, 1999), is too water-insoluble to escape from that particle into the aqueous surrounding medium, thus it could abstract an active bisallilyc hydrogen atom from polyunsaturated fatty acid molecules [BDE ~ 75 kcal/mol (McMillen and Golden, 1982) vs. 77.93 kcal/mol for Vit E] starting a new radical-chain process, the so-called Tocopherol-Mediated-Peroxidation (Bowry and Stocker, 1993).

$$ArO^{\bullet} + -CH{=}CH - CH_2 - CH{=}CH - \longrightarrow ArOH + -CH{=}CH - \dot{C}H - CH{=}CH - \quad (4)$$

Although reaction 4 is rather slow (k ~ 0.1 $M^{-1}s^{-1}$) (Ingold et al., 1993), it is faster than the coupling of ArO• with a peroxyl radical diffusing from the surrounding medium into the LDL particle. Therefore, increasing levels of Vit E, in oxidative conditions, without a concomitant increase in co-antioxidants, may result in increased levels of α-tocopherol radicals which can no longer be efficiently detoxified by co-antioxidants.

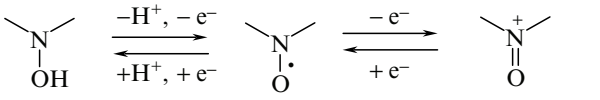
Similarly, polyphenols, flavonoids, ubiquinols as well as ascorbic acid may all also have a pro-oxidant activity through different mechanisms according to the specific antioxidant considered. For example, ascorbic acid can switch from antioxidant to pro-oxidant activity depending on its concentration and on the presence of free transition metal ions by reducing Fe(III) into Fe(II), triggering metal-catalyzed free radical reactions (Halliwell et al., 1987; Higson et al., 1988; Samuni et al., 1983).

Inhibition by Electron Transfer

Antioxidants may also inhibit peroxidative processes by the transfer of electrons to or from radical or non-radical species involved in oxidative stress, according to the redox potentials of the species involved.

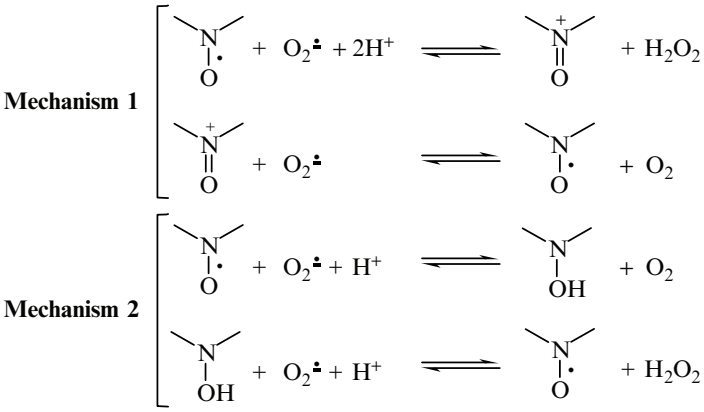
Nitroxide radicals (see Fig. 12.2) can act as preventive antioxidants through this mechanism. These compounds are among the most stable free radicals known that have been widely exploited as biophysical probes, spin labels and contrast agents for many biophysical/medical studies (Kocherginsky and Swartz, 1995). Because of their versatile ability to deactivate free radicals and of their low toxicity (Damiani et al., 2000a), nitroxides have also been thoroughly investigated as antioxidants for all those systems subject to oxidation (Mitchell et al., 1997).

Via single electron transfer reactions, nitroxides can be reduced to hydroxylamines or oxidized to oxoammonium cations, thus they can afford protection by acting both as oxidazing and as reducing agents, and in some cases they can be continuously recycled (Scheme 1).



Scheme 1

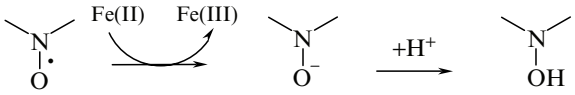
For example, piperidine nitroxides have been proposed to dismutate superoxide radical (Goldstein et al., 2003; Krishna et al., 1992) *via* Mechanism 1 reported in Scheme 2:



Scheme 2

A detailed study of the electrochemical properties of a series of aliphatic and aromatic nitroxides and of oxygen, in the same experimental conditions (DMF/H_2O 6:4)suggests that nitroxide radicals can detoxify superoxide radical *via* two different mechanisms. Mechanism 1 takes place when the oxidation potential of the nitroxide is less positive than the reduction potential of the protonated superoxide radical anion; instead, Mechanism 2 takes place when the reduction potential of the nitroxide is less negative than the oxidation potential of superoxide. Hence different types of nitroxides may destroy superoxide radical through the intermediate formation of the oxoammonium cation or the hydroxylamine, depending on their redox potentials (Carloni et al., 1996). Although dismutation of superoxide radical by nitroxides is significantly slower than dismutation by superoxide dismutase (SOD), detoxification of superoxide radical by nitroxides can nevertheless take place *in vivo*.

Nitroxides can inhibit transition metal-mediated damage by oxidizing the reduced form of transition metals such as Cu (I) or Fe (II) while they themselves are reduced to hydroxylamines (Scheme 3) (Bar-On et al., 1999; Damiani et al., 1999a; Krishna et al., 1996), which can in turn act as antioxidants *via* hydrogen donation (see below).



Scheme 3

Recent reports suggest that nitroxides may reduce peroxyl radicals (Goldstein and Samuni, 2007): in this case, nitroxides are oxidized to their corresponding oxoammonium cations. The formation of oxoammonium cations is not a harmless process since these species appear to be responsible for the pro-oxidant activity of nitroxides when they are used at relatively high concentrations (Aronovitch et al., 2007; Dragutan and Mehlhorn, 2007; Offer et al., 2000). Indeed, oxoammonium cations can oxidize many organic as well as many inorganic molecules found in biological systems (de Nooy et al., 1995; Schnatbaum and Schäfer, 1999).

Possible inhibition of peroxidative processes by an electron transfer mechanism has also been postulated for antioxidants such as carotenoids (CarH). These compounds in fact have low oxidation potentials (E_{ox} from 0.55 to 0.797 V vs. SCE) (Liu et al., 2000) and could therefore be oxidized by oxygen-centred radicals such as peroxyls, alkoxyls (RO•) and hydroxyl that have sufficiently positive reduction potentials (Jonsson, 1996; Merényi et al., 1994): the electron transfer process between β-carotene and a peroxyl radical, is in fact exoergonic and can give rise to the formation of the β-carotene radical cation (β-CarH $^{+\bullet}$) (reaction 5).

$$ROO^{\bullet} + \beta - CarH \rightarrow ROO^{-} + \beta - CarH^{+\bullet} \quad (5)$$

Pulse radiolysis studies on the interaction of carotenoids (Hill et al., 1995) and retinoids (Rozanowska et al., 2005) with trichloromethylperoxyl radicals have confirmed the formation of the radical cation in some cases and hence the feasibility of an electron transfer process. However, with some of the studied compounds and depending on the solvent used, the radical cation was not detected or another species that has not been well identified was formed prior to the carotenoid radical cation ($CarH^{+\bullet}$). The interaction of CarH with radicals by an electron transfer mechanism has nevertheless been proposed for the reaction with nitrogen dioxide, which has been recognized as a potential initiator of the lipid peroxidation chain reaction. (Everett et al., 1996; Koppenol, 1996)

Carotenoids may however deactivate radicals (X•) by allylic hydrogen donation forming a neutral, resonance stabilized carotenoid radical (Car•) (reaction 6) or by addition to double bonds (reaction 7, see below).

$$X^{\bullet} + CarH \longrightarrow XH + Car^{\bullet} \quad (6)$$

$$X^{\bullet} + CarH \longrightarrow X - CarH^{\bullet} \quad (7)$$

The antioxidant properties of carotenoids will of course not only depend on their reactivity towards free radicals, but also on the reactivity of the radicals deriving from their antioxidant activity ($CarH^{+\bullet}$, Car• or X-CarH•). In fact it has been claimed that β-carotene can have pro-oxidative effects: for example it has been shown that dietary supplementation with β-carotene may lead to deleterious health effects in some subpopulations such as smokers (Omenn et al., 1996). For this reason it is important to determine the stability of the carotenoid radical cation that can be formed by the electron transfer interaction of the carotenoid with radical species: electrochemical studies have shown that the stability of $CarH^{+\bullet}$ depends on the medium and on the oxidation potentials of the radical itself. In general, in aqueous solution and in the absence of other oxidizable species, $CarH^{+\bullet}$ seem to be rather long lived. The possible life of $CarH^{+\bullet}$ is of potential importance because the value of its reported one-electron reduction potential (Edge et al., 2000) suggests that it is capable of oxidizing protein components such as tyrosine (Mortensen et al., 2001).

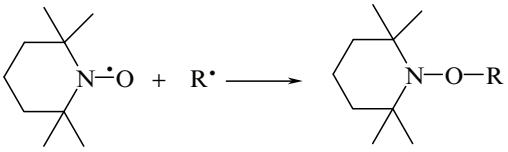
Nevertheless, it has been shown that $CarH^{+\bullet}$ is efficiently reconverted to the parent carotenoid by species such as Vit C (Burke et al., 2001) and Vit E (Mortensen and Skibsted, 1997). For example in liposomes, β-$CarH^{+\bullet}$ is able to interact with water-soluble Vit C even though the parent hydrocarbon carotenoid probably resides entirely in the non-polar region of the liposome: this surprising behaviour is due to the different properties of radical cations compared to their parent species (reorientation to the aqueous phase of cell membranes/liposomes). These observations have suggested that the deleterious effects of

β-carotene in heavy smokers can be related to the generation of β-$CarH^{+\bullet}$ by for example $NO_2\bullet$, and to the low levels of Vit C found in the serum of heavy smokers, which is therefore unable to deactivate the relatively long-lived $CarH^{+\bullet}$.

Inhibition by Radical Scavenging

All radicals taking part in a peroxidative process may be inactivated by coupling reactions with other radical species. These reactions have very low activation energies therefore their rates are almost diffusion controlled (Ingold, 1973).

Nitroxide radicals, mentioned earlier, can deactivate radicals also through this mechanism thereby acting as chain-breaking antioxidants. These compounds, similarly to phenoxy radicals, are very unreactive towards non-radical molecules, but they react with carbon-centred radicals at an almost diffusion controlled rate (Chateauneuf et al., 1988) leading to the formation of alkoxyamines as shown in reaction 8 for TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), one of the most widely studied aliphatic nitroxides.



(8)

Nitroxides with an aromatic structure such as indolinonic nitroxides (Fig. 12.2), besides reacting with carbon-centred radicals (Brownlie and Ingold, 1967) are able to couple at high rates with oxygen-centred radicals such as •OH, RO• and ROO• (Berti et al., 1977a, b; Cardellini et al., 1989; Damiani et al., 1999a, 2001; Greci, 1982), to

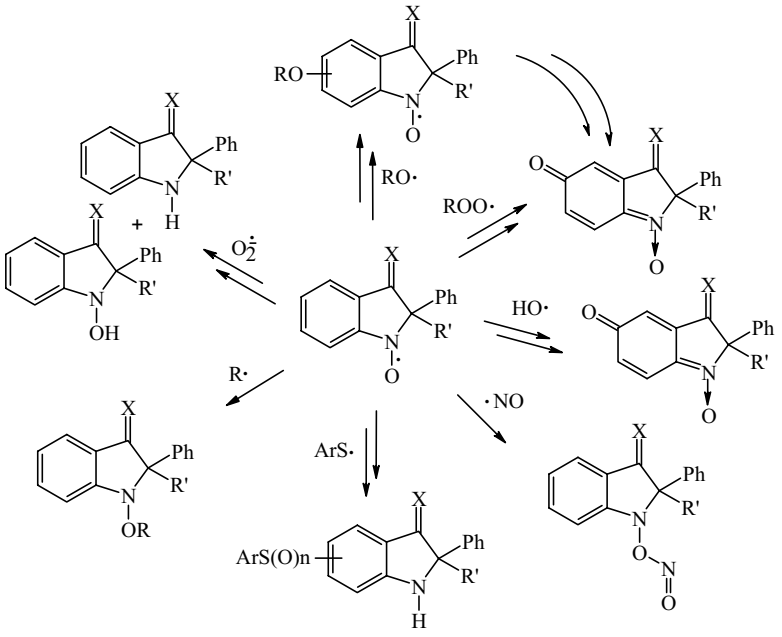
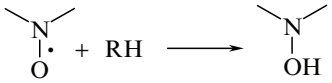


Fig. 12.3 Reactivity of indolinonic nitroxides with different radicals

form non-paramagnetic compounds. The reactions through which this class of aromatic nitroxides exert their antioxidant activity are summarized in Fig. 12.3.

On the basis of their variegated reactivity, indolinonic nitroxides have been tested as antioxidants towards several important biological targets (proteins, lipids, DNA, LDL) submitted to oxidative stress (Antosiewicz et al., 1995, 1993, 1997; Damiani et al., 1994, 1999b, 2000b, c, 2002; Tanfani et al., 1994): in all cases these compounds exhibited excellent antioxidant activity.

However, besides the well documented antioxidant activity of nitroxide radicals, a certain pro-oxidant effect exerted by these compounds cannot be excluded. Nitroxides may in fact abstract labile hydrogens leading to the formation of new radicals (reaction 9), and therefore capable of initiating a peroxidative process, which becomes significant only at high concentrations of nitroxides.



(9)

Nevertheless, if hydrogen abstraction were to take place in viable biological systems, the hydroxylamine formed may in turn work as a hydrogen donor antioxidant (Noguchi et al., 1999); in fact, the O-H bond of nitroxides such as TEMPO and indolinonic nitroxides have a BDE (Amorati et al., 2003) which is even lower than that of Vit E.

Consequently, with regards to nitroxides, the antioxidant and pro-oxidant effects are a question of balance. The antioxidant effects outbalance the pro-oxidant ones when nitroxides are used at low concentrations, when they have a low propensity to oxidation and when the oxoammonium cation is relatively unstable and short-lived.

Inhibition of peroxidative processes can also be performed by antioxidants that possess conjugated double bonds, by scavenging radicals *via* addition reactions: for example radical addition to carotenoid type antioxidants (Burton and Ingold, 1984) yields a resonance stabilized radical adduct (X-CarH•) (reaction 7) which can couple with a second radical to form an addition product (X-CarH-X) (reaction 10).

$$X^{\bullet} + CarH \longrightarrow X - CarH^{\bullet} \tag{7}$$

$$X^{\bullet} + X - CarH^{\bullet} \longrightarrow X - CarH - X \tag{10}$$

β-Carotene can add directly two alky (X• = R•) and/or alkoxyl (X• = RO•) radicals giving rise to a non radical adduct. In contrast, peroxyl radical (X• = ROO•) addition yields an unstable intermediate radical adduct (ROO-CarH•) that collapses to an epoxide

(CarH-epoxide) releasing an alkoxyl radical (reaction 11).

$$ROO - CarH^{\bullet} \longrightarrow CarH - epoxide + RO^{\bullet} \tag{11}$$

These results suggest that peroxyl radical scavenging by initial radical addition is inherently inefficient: the reaction in fact captures a peroxyl radical but releases an epoxide and an alkoxyl radical. Thus, peroxyl addition contributes to an antioxidant effect only if the released alkoxyl radicals are subsequently scavenged. ROO-CarH•

radical may also then reversibly trap oxygen to form a reactive peroxyl radical (ROO-CarH-OO•) (reaction 12). This reaction is shifted to the right and promotes β-carotene autoxidation at high oxygen tensions (Liebler and McClure, 1996).

$$ROO - CarH^{\bullet} + O_2 \longrightarrow ROO - CarH + OO^{\bullet} \quad (12)$$

Conclusions

Peroxidative processes are inhibited essentially through three mechanisms: hydrogen transfer, electron transfer and radical scavenging reactions. The antioxidant activity of all the compounds described in this chapter, regardless of the mechanism through which they work, is explicated by deactivating and/or scavenging ROS. However, their antioxidant activity is almost always accompanied by a pro-oxidant one which becomes significant only under particular conditions as discussed in this chapter. In fact recent results seem to point out that excessive supplementation for prevention of several diseases with β-carotene, Vit A and Vit E and other antioxidants, seem not to be as beneficial as expected (Bjelakovic et al., 2007; Halliwell, 2000). Therefore it might be possible that a significant pro-oxidant activity due to heavy supplementation with antioxidants could have negative consequences on ones health.

List of Abbreviations

ArO• = phenoxyl radical
ArOH = phenolic compound
BDE = bond dissociation enthalpy
Car• = carotenoid radical
CarH = carotenoids
$CarH^{+\bullet}$ = carotenoid radical cation
HBA = hydrogen-bond-accepting
LDL = low density lipoprotein
$Q_{10}H_2$ = ubiquinol-10
R• = carbon-centred radical
RO• = alkoxyl radical
ROO• = peroxyl radical
ROO-CarH-OO• = carotenoid peroxyl radical
ROOH = alkyl hydroperoxide
ROS = reactive oxygen species
SOD = superoxide dismutase
TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl
Vit C = ascorbic acid
Vit E = α-tocopherol
X• = generic radical
X-CarH• = carotenoid radical adduct
X-CarHX = carotenoid addition product
β-CarH = β-carotene

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Chapter 13
The Role of DUOX Isozymes in the Respiratory Tract Epithelium

Richard Harper

Abstract Increasingly, reactive oxygen species such as superoxide and hydrogen peroxide are recognized to be intentionally generated intracellularly to serve important cellular functions. A key protein family responsible for the regulated generation of reactive oxygen species in multiple cell types is the NOX/DUOX enzyme family. Two family members, DUOX1 and DUOX2, appear to be highly expressed in tissues of endodermal origin including the thyroid, respiratory tract, and gastrointestinal tract. In this chapter, we will focus our review on DUOX proteins in the respiratory tract. We will discuss a brief history of the discovery of the DUOX isoforms, the estimated hydrogen peroxide-generating capacity of DUOX in respiratory tract epithelium, putative functions of the DUOX enzymes, and some regulatory factors responsible for DUOX gene expression and oxidase activity.

Keywords DUOX1; DUOX2; respiratory tract; oxidase; peroxidase; host defense

Introduction

Recent evidence suggests that reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are not mere byproducts of cellular respiration, but are intentionally generated to serve important cellular functions (Terada, 2006; Finkel, 2003). A key protein family responsible for the regulated generation of ROS in multiple cell types is the NOX/DUOX enzyme family (Lambeth, 2002). The prototypical member of this family is gp91*phox* (NOX2), which is responsible for the respiratory burst in neutrophils (Gabig and Babior, 1979; Dusi et al., 1995; Hohn and Lehrer, 1975). Suh et al. were the first to characterize NOX1 (initially named Mox1; for mitogenic oxidase) as a non-mitochondrial, non-phagocytic enzyme capable of regulated ROS production (Suh et al., 1999). Since this discovery,

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

there has been an exponentially growing body of literature characterizing the function of the non-phagocytic NOX/DUOX family members (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) in various aspects of human biology and disease (Lambeth, 2004; Leto and Geiszt, 2006). In this chapter, we will focus our review on the expression and function of DUOX proteins in the respiratory tract.

Discovery and Localization of DUOX Family Members

Initial identification of the DUOX2 was made by Corinne Dupuy and colleagues using biochemical techniques to isolate the known membrane-bound NADPH oxidase in thyroid tissues (Dupuy et al., 1999). In this report, they described the isolation of a 1,210 amino acid protein, $p138^{Tox}$, which contained a gp91phox-like region and two EF-hand domains. EF-hand domains contain calcium binding sites and serve as regulatory sub units for enzymatic activity. The presence of EF-hand domains suggested that $p138^{Tox}$ enzymatic activity may be regulated by calcium. Additionally, expression of $p138^{Tox}$ was upregulated by forskolin and the gene localized to Chromosome 15q15. Shortly after this discovery, Xavier De Deken described the isolation of two human thyroid NADPH oxidases (De Deken et al., 2000). These genes, thyroid oxidase 1 (ThOX1) and thyroid oxidase 2 (ThOX2), were identified by low stringency hybridization screening of thyroid cDNA libraries using a 1.3-kilobase fragment of gp91phox. Similar to Dupuy's findings, both ThOX1 and ThOX2 contained a gp91phox-like region and two EF-hand domains, and both genes were upregulated by forskolin. In addition, increased sequence information identified novel N-terminal extensions for both proteins that contained a peroxidase-like domain. The 1,210 amino acid C-terminal portion of ThOX2 was identical to $p138^{Tox}$, which strongly suggested that these two proteins were the same protein. Because both proteins express dual oxidases, a heme peroxidase domain and an NADPH oxidase domain, ThOX1 and ThOX2/$p138^{Tox}$ have been renamed DUOX1 and DUOX2, respectively.

Further characterization of DUOX enzyme expression in human tissues demonstrated *DUOX* mRNA transcripts in multiple non-thyroid human tissues including heart, kidney liver, pancreas, placenta, GI tract, prostate, testis, and the respiratory tract (Caillou et al., 2001; Edens et al., 2001; Geiszt et al., 2003; El Hassani et al., 2005). Further characterization of DUOX in the lung demonstrated that DUOX expression is likely limited to the respiratory tract epithelium (Geiszt et al., 2003). Although PCR evidence exists that several NOX family members are potentially expressed in the respiratory tract, expression levels of the other Nox family members are a thousand-fold less abundant compared to DUOX (Schwarzer et al., 2004). Based on in situ hybridization experiments, it appears that DUOX1 is predominantly located in proximal respiratory tract epithelial cells whereas DUOX2 is localized to submandibular or salivary glands during basal conditions (Schwarzer et al., 2004). However, protein confirmation of these findings has been limited due to the absence of DUOX isoform-specific antibodies.

Functional Role(s) of DUOX

The capacity for respiratory tract epithelium to generate H_2O_2 appears to be dependent upon the specific cell type utilized. For example, in unstimulated human primary tracheobronchial epithelial cells, the rate of H_2O_2 production was 0.5–2.5 nmol/10 min/10^6 cells and this production increases to 2–5 nmol/10 min/10^6 cells after stimulation with ATP (Moskwa et al., 2007). In HBE1 cells, a papilloma-transformed human tracheobronchial epithelial cell line (Yankaskas et al., 1993). H_2O_2 production was 0.03–0.17 nmol/10 min/10^6 cells and this production increases to 0.26–0.45 nmol/10 min/10^6 cells after stimulation with ATP (Wesley et al., 2007). In a human pulmonary mucoepidermoid carcinoma cell line, H_2O_2 production increased from 0.002–0.003 nmol/10 min/10^6 cells to 0.012–0.014 nmol/10 min/10^6 cells after PMA stimulation (Nakanaga et al., 2007). Although laboratory to laboratory variation may account for these differences, it appears that primary respiratory tract epithelial cells have the highest capacity for DUOX-mediated H_2O_2 generation. In parallel, we have observed that *DUOX1* or *DUOX2* mRNA expression and functional activity is higher in primary tracheobronchial epithelial cells compared to HBE1 cells (Xu and Haper, 2007).

It is well-accepted that DUOX2-mediated H_2O_2 production is critical for iodide oxidation during thyroid hormone synthesis (Igo et al., 1964; Degroot et al., 1972; Clark et al., 1975; Lissitzky, 1976; Rigutto et al., 2007; Song et al., 2007). Observations that mutations in *DUOX2* gene result in congenital hypothyroidism (Moreno et al., 2002; Vigone et al., 2005) confirm the available biochemical and expression data. However, similarly convincing evidence for the function of DUOX isoforms in the respiratory tract are lacking. Several compelling models to explain the expression and function of DUOX isoforms in the respiratory tract have been proposed. The location of DUOX isoforms in the respiratory tract epithelium and their homology to gp91phox strongly implicated the role of DUOX proteins in host defense.

Several mechanisms exist to provide innate host defense in the respiratory tract epithelium (Antunes and Cohen, 2007; Thacker, 2006; Grubor et al., 2006; Beisswenger and Bals, 2005; Ng et al., 2004; Zhang et al., 2000). The upper respiratory tract epithelium and conducting airways are structured to provide multiple layers of protection against invading organisms. This includes a tight epithelial barrier where the apical surface of the respiratory tract is in close apposition to prevent invasion. This apical surface is bathed in an airway surface liquid that is rich in antimicrobial peptides hostile to bacterial and viral products. Resting on top of this airway surface liquid is a mucous layer that provides a physical barrier against infection. Mucus that has ensnared bacteria and virus particles is cleared from the airway by ciliary action provided by the respiratory tract epithelial cells. The generation of hypothiocyanate in the airway surface liquid appears to be a critical part of the host defense for those bacteria and viral particles that are able to penetrate the initial mucociliary barrier (Goldman and Smith, 1973; Ratner and Prince, 2000).

Conner and others previously described the importance of lactoperoxidase (LPO) in maintaining sterility in the respiratory tract (Gerson et al., 2000; Conner

et al., 2002; Salathe et al., 1997; Wijkstrom-Frei et al., 2003). In this model, LPO catalyzes a reaction between H_2O_2 and thiocyanate (SCN^-) to generate the bactericidal product hypothiocyanate ($OSCN^-$) (Gerson et al., 2000; Conner et al., 2002; Salathe et al., 1997; Wijkstrom-Frei et al., 2003; Conner et al., 2007).

$$H_2O_2 + LPO \rightarrow LPO - O + H_2O$$

$$LPO - O + SCN^- \rightarrow OSCN^- + LPO$$

Absence of this abundant protein, nearly 1% of the total protein in airway surface liquid, results in significant colonization and infection of respiratory tract epithelium with bacteria. Although the source of H_2O_2 in the respiratory tract was not initially known, DUOX isoforms have since been verified to be the predominant enzymes responsible for H_2O_2 generation in the respiratory tract. In addition, Geiszt et al. demonstrated evidence to suggest that DUOX localization next to LPO production is consistent with this model (Geiszt et al., 2003).

Recently, Conner and Banfi independently, characterized the transport of SCN^- from the basolateral surface to the apical surface of the respiratory tract where enzymatic activation of SCN^- to $OSCN^-$ will occur (Moskwa et al., 2007; Conner et al., 2007). This transport appears to be the rate-limiting step in the respiratory tract and is dependent upon the presence of functional CFTR chloride channels. The rate of SCN- transport (1.5–1.8 nmol/10 min/cm^2) (Conner et al., 2007) parallels the rate of OSCN- formation (1.4–1.7 nmol/10 min/cm^2) (Moskwa et al., 2007) in respiratory tract epithelium. This is consistent with the notion that SCN^- is immediately converted to $OSCN^-$ once SCN^- is transported to the apical surface. However, two different model systems, rat versus human, were used in these reports, which limits our ability to directly compare the two studies. Of importance, LPO, DUOX-mediated H_2O_2, and apical SCN^- are all required for bacterial killing of S. aureus or P. aeruginosa (Moskwa et al., 2007). The capacity of DUOX isoforms to generate H_2O_2 in respiratory tract epithelium (5 nmol/10 min/cm^2) is more than adequate to support this model.

In parallel to the putative antibacterial role for DUOX enzymes, our recent data suggest DUOX isoforms are important for innate antiviral host defense. The observation that DUOX2 expression is substantially increased by IFN-γ and rhinovirus provides circumstantial evidence for this notion (Harper et al., 2005). In addition, respiratory tract epithelial cells are known to generate H_2O_2 in less than 1h after RV infection (Kaul et al., 2000; Biagioli et al., 1999). RV-induced H_2O_2 production is inhibited by diphenyleneiodonium (DPI), which indicates the involvement of a flavoprotein, likely an NADPH oxidase, in this process. Therefore, DUOX appears to be the best candidate responsible for RV-induced H_2O_2 production in respiratory tract epithelium. The presence of EF-hand domains on DUOX isoforms suggests a mechanism by which RV infection can immediately induce DUOX-mediated H_2O_2 production (e.g. membrane-associated calcium flux (Geiszt et al., 2003; Forteza et al., 2005). The specific contribution of DUOX isoforms in respiratory tract epithelial innate host defense against viral infection remains to be determined.

Alternative functions for DUOX isozymes in the respiratory tract have recently been suggested. Hydrogen ion concentration at the apical surface of respiratory tract epithelium serves important host defense functions. Not only does lower pH increase the efficiency of known respiratory tract peroxidases such as LPO, but an acidic environment is known to decrease viability of some respiratory viruses (Greenberg, 2007; Whitton et al., 2005; Hayden, 2004). Therefore, an attractive model for DUOX is the ability of this protein to simultaneously acidify the airway surface liquid to optimize LPO enzymatic activity and provide the H_2O_2 needed for LPO to convert SCN^- to $OSCN^-$. Recent data from Horst Fischer and colleagues support the role of DUOX in H^+ production and H^+ secretion. By using chemical inhibitors of DUOX (Schwarzer et al., 2004) and DUOX1-specific RNAi transcripts, they were able to demonstrate an approximately 50% reduction in intracellular acid production. These data suggest that under basal conditions there is DUOX-mediated turnover of NADPH to NADP + that is responsible for approximately 50% of H + production. Based on this model, it is presumed that activation of NADPH oxidase activity in the respiratory tract, and specifically activation of DUOX1, will result in a parallel increase in H + secretion. However, this presumption has not been formally tested. Similarly, it is not known if increased activation of DUOX2 will result in a similar increase in intracellular H + production or H + secretion.

DUOX1 appears to perform a specific role in signaling events related to mucin secretion and wound healing. Wesley et al. recently demonstrated that ATP-mediated P2 purinergic receptor activation was necessary for cell migration and wound closure in primary and virus-immortalized tracheobronchial epithelial cell cultures (Wesley et al., 2007). This functional activity correlated with increased DUOX1-dependent H_2O_2 production. Wound closure was significantly decreased in cells expressing siRNA against DUOX1, but not in cells expressing non-target siRNA transcripts. Together, these data strongly suggested that DUOX1-generated H_2O_2 is a necessary signal for adequate wound closure in human tracheobronchial epithelium. Although not explicitly tested, it is likely that release of intracellular ATP during injury induces purinergic receptor-mediated calcium flux, which directly triggers DUOX1 oxidase activity via EF-hand domain activation. Similarly, Shao et al. demonstrated that DUOX1 appears to be a critical protein necessary for phorbol 12-myristate 13-acetate- or human neutrophil elastase-mediated MUC5AC production in human tracheobronchial epithelial cell cultures (Shao and Nadel, 2005).

Regulation of DUOX Expression and Enzymatic Activity

As mentioned, the initial identification of DUOX isoforms demonstrated that *DUOX* mRNA is upregulated by forskolin in thyroid tissues (Dupuy et al., 1999; De Deken et al., 2000). Thyroid stimulating hormone also increased *DUOX1* and *DUOX2* mRNA expression in thyroid tissues after 24 h of treatment (Pachucki et al., 2004). However, DUOX promoter activity does not appear to depend upon thyroid-specific regulators consistent with the expression of DUOX in multiple tis-

sue types (Christophe-Hobertus and Christophe, 2007). Data for the regulation of DUOX expression in the respiratory tract is similarly limited.

We recently demonstrated differential expression of *DUOX1* and *DUOX2* after treatment of human tracheobronchial epithelial cells with inflammatory cytokines. *DUOX1* increased in response to the Th2 cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13). DUOX2 mRNA expression increased significantly in response to the Th1 cytokine interferon-γ (IFN-γ), and increased modestly to interleukin-1a or interleukin-1b. Of potential importance, the nature of gene expression was significantly different between the two genes in response to their respective cytokines. DUOX1, for example, increased maximally three- to sixfold in response to IL-4 at varying doses of the cytokine. In comparison, DUOX2 increased linearly with increasing doses of IFN-γ more than 15- to 20-fold. Although the fold-induction was substantially higher for DUOX2 compared to DUOX1, DUOX2 expression levels began to wane after 48 h. However, DUOX1 levels continued to increase 72 h after cytokine treatment. These qualitative differences in regulation and the differential regulation of DUOX1 and DUOX2 by cytokines suggest both enzymes perform distinct functions in the respiratory tract epithelium. Localization of DUOX1 and DUOX2 protein in the respiratory tract epithelium, and identifying proteins that co-localized with the DUOX isoforms will be instrumental in determining the functions of each protein.

Current evidence suggests that constitutive DUOX-mediated oxidase activity is directly proportional to the total DUOX protein available in respiratory tract epithelial cells. Based on mRNA data, DUOX1 is the predominant isoform expressed under basal conditions in airway cell cultures. Therefore, it is likely that DUOX1 provides the majority of DUOX-mediated H_2O_2 under basal conditions. Cytokine treatments that increase *DUOX* mRNA levels, and presumably DUOX protein levels, induce a parallel increase in H_2O_2 production (Harper et al., 2005). This suggests that one method to regulate DUOX oxidase activity is to regulate the absolute level of protein in respiratory tract epithelium. Based on our previous data, it appears that maximal inducible expression of *DUOX1* or *DUOX2* result in similar levels of H_2O_2 generation in respiratory tract epithelium (Harper et al., 2005). Although DUOX2 is more inducible by cytokine stimulation compared to DUOX1, it is possible that the protein levels of DUOX2 or DUOX1 are similar when maximally induced by their respective cytokines. Antibodies that differentiate between DUOX1 and DUOX2 will provide insights into the absolute protein levels for each isoform.

DUOX-mediated H_2O_2 generation can also be rapidly and robustly induced above constitutive levels in respiratory tract epithelium (Geiszt et al., 2003; Wesley et al., 2007; Forteza et al., 2005). Prior to the identification of DUOX2 in thyroid tissue, it was established that thyroid cells required calcium for H_2O_2 generation (Ameziane-El-Hassani et al., 2005; Leseney et al., 1999; Nakamura et al., 1987). Similar to Nox5, DUOX family members contain two EF-hand domains suggesting that DUOX oxidase activity will increase in response to calcium (De Deken et al., 2000; Lambeth et al., 2007; Dupuy et al., 1988). Further evidence in thyroid and respiratory tract epithelium has confirmed that Ca^{++} influx is likely the predominant

factor regulating inducible DUOX-mediated NADPH oxidase activity. Treatment of respiratory tract epithelial cells with agonists for calcium mobilization, including ionomycin, thapsigargin, or ATP, has resulted in the rapid increase in H_2O_2 flux. This activity was blocked by nonspecific inhibitors of DUOX enzymes or siRNA against DUOX1.

There is some evidence that additional regulatory proteins are required for DUOX-mediated enzymatic activity. Schwarzer et al. demonstrated that known NOX2 regulatory proteins, p40phox, p47phox, and p67phox, are co-expressed with the DUOX isozymes in respiratory tract epithelium (Schwarzer et al., 2004). Shao et al. demonstrated that p47phox and p67phox proteins were identified in the membrane fraction of respiratory tract epithelial cells after PMA treatment. This localization corresponded to PMA-induced, DUOX1-mediated H_2O_2 production (Shao and Nadel, 2005). However, family members that are more closely related to NOX2 do not use these phox regulatory units for enzymatic activity (Banfi et al., 2003). There is direct evidence in thyroid tissues that cytosolic phox regulators, including Rac, do not provide regulatory functions for DUOX. And, patients with autosomal recessive chronic granulomatous disease, in which there are defects in cytosolic phox regulators, do not exhibit hypothyroidism. Together, these latter data suggest that the DUOX isozymes do not rely on these cytosolic phox regulatory units. However, differences in the predominant isoform found in thyroid tissue (DUOX2) versus respiratory tract epithelium (DUOX1) may be responsible for these contradictory data. Further studies with knock down of various phox regulatory units in respiratory tract epithelium will be able to definitively settle this issue.

The model that DUOX isoforms are simply H_2O_2 generators in the respiratory tract epithelium is consistent with current data, but presents potential teleological inconsistencies. For example, if the predominant function of the DUOX proteins is to generate H_2O_2, why are *two* bulky DUOX proteins maintained in the airway rather than a single, and smaller, isoform of the NOX/DUOX family? This question can be partially addressed by the observation that DUOX1 and DUOX2 are differentially regulated in respiratory tract epithelium. Although H_2O_2 is produced by both DUOX proteins, it is possible that the functional signalasomes that assemble around DUOX1 are substantially different from the proteins that cluster around DUOX2. The presence of two different oxidases in the same cell type, allow efficient utilization of H_2O_2 as a single signal that produces two very distinct effects.

Similarly, it is unclear why two peroxidase-containing DUOX proteins are present in airway epithelium if several other heme peroxidases (e.g. LPO, MPO, and others) are abundantly present in airway surface liquid (Gerson et al., 2000; Salathe et al., 1995). Several investigators have suggested that the peroxidase domains within DUOX1 or DUOX2 are not functional (Lambeth et al., 2007; Dupuy et al., 1988; Banfi et al., 2003; Salathe et al., 1995; Geiszt and Leto, 2004). It is possible that the peroxidase domains represent the vestigial remains of an ancestoral protein that has not been selected against. Or, the peroxidase domains may provide regulatory functions for the DUOX isozymes independent of heme peroxidase activity.

However, biochemical analysis of purified DUOX protein suggests that DUOX has functional heme peroxidase activity (Edens et al., 2001; Ha et al., 2005). DUOX peroxidase domains isolated from humans, C. elegans, and Drosophila demonstrated peroxidase activity. In addition, we have recently shown that *DUOX2* is required for functional peroxidase activity in respiratory tract epithelium (Harper et al., 2006). Therefore, based on physical proximity, it is likely that DUOX2-generated H_2O_2 is utilized by its own peroxidase rather than diffusing to LPO some distance away. Together, these data provide strong evidence that DUOX is not solely an H_2O_2 generator to assist the antibacterial functions of LPO but is performing distinct functions apart from other respiratory tract peroxidases. The demonstration of heme incorporation into the DUOX peroxidase domain has not been confirmed to date, and therefore, the functional capacity of the DUOX peroxidase domain remains controversial. Therefore not only are there questions related to the levels of oxidants generated via the DUOX pathway – i.e. oxidative balance but in addition there are questions related to the balance of oxidant generation between the DUOX isoforms and what these may signal.

Summary

There is growing interest in understanding the function and importance of DUOX proteins in the respiratory tract. Based on the current body of literature, it is clear that DUOX is important for antibacterial host defense functions in the respiratory tract as part of the DUOX/LPO/SCN^- system. Evidence that DUOX isozymes are important for mucin secretion, proton secretion, and wound healing strongly supports the notion that these family members are critical for multiple functions in the respiratory tract. Regulation of DUOX enzymatic activity by calcium flux and regulation of protein expression by immune-modulating cytokines provide insights into additional potential functions for these family members. Protein isolation techniques that allow specific identification of each isoform are still required to fully characterize the role of DUOX1 and DUOX2 in the respiratory tract epithelium.

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Chapter 14
Hypertension, Diabetes, Oxidative Stress, and Cardiovascular Remodeling: Making the Connection with $p66^{shc}$

Elisa Pagnin, Angelo Avogaro, and Lorenzo A. Calò*

Abstract An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of cardiovascular disease, including hypertension and diabetes.

In the last few years, new factors playing a major role in oxidative stress signaling and oxidative stress-related responses leading to cardiovascular remodeling, atherogenesis and organ damage have been identified. One of these factors is $p66^{shc}$ which is a splice variant of $p52^{shc}$/$p46^{shc}$, adaptor proteins controlling mitogenic signaling from tyrosine kinases to Ras. Although the domain organization is identical in the three isoforms, $p66^{shc}$ shows substantial differences. It does not activate Ras and competitively inhibits signaling by the other isoforms. Furthermore, $p66^{shc}$ is implicated in signaling pathways linking oxidative stress to apoptosis.

The major role played by $p66^{shc}$ in oxidative stress-related response has been extensively demonstrated in $p66^{shc}$ knock-out mice which, compared to the wild type, show resistance to oxidative stress, prolonged lifespan, reduction of systemic oxidative stress and oxidative stress induced apoptosis, reduction of plasma LDL oxidation and early atherogenic lesions which are restored by $p66^{shc}$ over-expression, therefore confirming $p66^{shc}$ as a potent inducer of oxidation-sensitive mechanisms. In addition, a link between angiotensin II (Ang II) and $p66^{shc}$ has been demonstrated in an animal model through the protective effect of $p66^{shc}$ genetic deletion on the Ang II-induced myocardial damage, therefore linking this protein with the pathophysiology of hypertension and cardiovascular disease.

Studies from our laboratory in humans have been the first to provide information at molecular level of the role of $p66^{shc}$ in the pathophysiology of type 2 diabetes and its complications as well as insulin signaling showing that $p66^{shc}$ is overexpressed in type 2 diabetic patients. Conversely, in a human model of vascular hyporeactivity and increased insulin sensitivity Bartter's/Gitelman's syndrome, which presents a mirror image of hypertension, we have shown that $p66^{shc}$ expression is downregulated.

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

This chapter examines the biology of $p66^{shc}$, details its involvement in oxidative stress, reports the findings provided by our ongoing studies in humans and, on the basis of the available data, outlines the linkages between $p66^{shc}$, hypertension, diabetes and cardiovascular remodeling in humans and how these could open possible therapeutic approaches to oxidative stress induced hypertensive and diabetic complications via the pharmacologic targeting of $p66^{shc}$.

Keywords $p66^{shc}$, oxidative stress, hypertension, diabetes mellitus

Introduction

Vascular diseases are chronic inflammatory disorders with an underlying abnormality in redox-mediated signals in the vasculature. A change from low to high levels of oxidative stress results in a shift from an atheroprotective to a proatherogenic pattern of gene expression favoring inflammation and vascular dysfunction (Kunsch and Medford, 1999). Thus, oxidative stress occurring in response to hyperglycemia, dyslipidemia, hypertension is now considered as the molecular mechanism linking these risk factors with micro and macrovascular disease (Cai and Harrison, 2000).

In particular, increased oxidative stress is widely accepted as a factor in the development and progression of diabetes and its complications (Ceriello, 2000; Baynes and Thorpe, 1999; Baynes 1991).

Diabetes mellitus is usually accompanied by increased production of free radicals (Baynes and Thorpe, 1999; Baynes, 1991; Chang et al., 1993; Young et al., 1995) or impaired antioxidant defenses (Halliwell and Gutteridge, 1990; Saxena et al., 1993; McLennan et al., 1991). Oxidative stress, in addition, has been shown to be involved in the induction of vascular smooth muscle cell growth and lipid peroxidation (Harrison et al., 2003) and Brownlee has suggested oxidative stress as the unifying mechanism in the pathobiology of diabetic complications given that it is the common feature of the pathways mediating hyperglycemia-induced cell damage such as increased polyol pathway flux, increased advanced glycation end (AGE) product formation, activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux (Brownlee, 2005).

The ultimate effects of vascular smooth muscle cell growth and lipid peroxidation are hypertension and its complications such as vascular remodeling, and atherosclerosis (Dhalla et al., 2000; Luft, 2001). Increased oxidative stress is, in fact, involved in a number of harmful events for the vascular wall such as the inactivation of nitric oxide, induction of endothelial dysfunction, and cell proliferation.

Patients with hypertension show increased levels of oxidative stress related products together with decreased activity of endogenous antioxidant enzymes in blood and mononuclear cells. Vascular reactive oxygen species (ROS) production is also elevated in a range of different experimental models of hypertension, including

mineralocoricoid (Beswick et al., 2001), renovascular hypertension (Heitzer et al., 1999; Jung et al., 2004) and angiotensin II-induced (Laursen et al., 1997; Rajagolapan et al., 1996). Angiotensin II (Ang II) is a well known promoter of oxidative stress via upregulation of NAD(P)H oxidase (Griendling et al., 1994; Ushio-Fukai et al., 1996; Zafari et al., 1998) and the effects of its long term signaling pathways in the vasculature, which ultimately lead to vascular remodeling and atherosclerosis, are determined mostly through production of ROS and activation of redox sensitive genes (Harrison et al., 2003; Dhalla et al., 2000; Luft, 2001). This is true also for aldosterone; a number of reports from different laboratories included our own have shown that aldosterone is able to generate ROS, to increase the expression of oxidative stress related proteins such as $p22^{phox}$, PAI-1 and TGFβ (Keidar et al., 2003; Gerling et al., 2003; Calò et al., 2003), thereby directly contributing to endothelial dysfunction and cardiovascular remodelling in its non classic target tissues such as vascular wall and heart.

In addition to the identification of oxidative stress as a factor contributing to cell damage, new factors involved in oxidative stress signaling have been identified. Some of them are important for the oxidative stress-mediated signaling that leads to cardiovascular remodeling, atherogenesis and target organ damage. A pivotal role of $p66^{shc}$ has been recently established using $p66^{shc}$ knock-out mice. These mice are resistant to oxidative stress and compared to their wild type mice also show reduction of systemic oxidative stress (Migliaccio et al., 1999), as well as plasma LDL oxidation and early atherogenic lesions (Napoli et al., 2003). In fact in $p66^{shc}$ knock-out mice a prolonged lifespan compared to wild type mice has been shown (Migliaccio et al., 1999). Conversely, induction of $p66^{shc}$ increases the intracellular ROS availability, which, in turn, affects the rate of oxidative damage. $p66^{shc}$ knock out cells show a reduction of the apoptosis induced by oxidative stress, which is restored by $p66^{shc}$ over-expression.

$p66^{shc}$: Description, Protein Sequence and Mechanism of Action

ShcA proteins are adaptor proteins that exist in three different isoforms with relative molecular masses of 46, 52 and 66 kDa. $p52^{shc}$ and $p46^{shc}$ share an carboxy-terminal SH2 domain, a proline-rich region (CH1) containing critical tyrosine phosphorylation sites implicated in Ras activation and a carboxy-terminal phosphotyrosine binding domain (PTB) (Pelicci et al., 1992; Luzi et al., 2000). $p66^{shc}$ is composed of the entire $p52^{shc}/46^{shc}$ sequence and an additional amino-terminal proline-rich region (CH2) which contains a phosphorylation site at Ser36. Recently it has been established the presence of a new functional region named CB (cytochrome C binding) which is partially shared by $p52^{shc}$ (Giorgio et al., 2005) (Fig. 14.1).

An alternative translation initiation site of the same mRNA causes the production of $p52^{shc}$ and $p46^{shc}$ (Pelicci et al., 1992), whereas $p66^{shc}$ transcription occurs via an alternative promoter found in the first intron of the Shc locus (Ventura et al., 2002). $p52^{shc}$ and $p46^{shc}$ are ubiquitously co-expressed while $p66^{shc}$ expression is

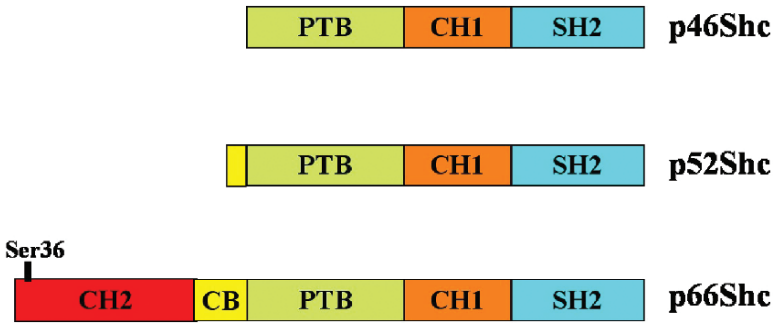

Fig. 14.1 Schematic representation of the molecular structure of Shc proteins

absent or at very low levels in hemopoietic cell lines, normal peripheral blood lymphocytes and brain (Pelicci et al., 1992). Silencing of $p66^{shc}$ gene expression is controlled at least in part by epigenetic modifications of its promoter. Analysis of the $p66^{shc}$ gene promoter in cells expressing different levels of the protein has revealed an inverse correlation between promoter methylation and $p66^{shc}$ expression. This mechanism was confirmed when Ventura and coworkers showed that treatment with demethylating agents results in $p66^{shc}$ expression in cells where it is normally undetectable (Ventura et al., 2002).

It is well established that the stimulation of Shc proteins by different growth factors and cytokines causes phosphorylation on tyrosine residues (Pelicci et al., 1992; Rozakis-Adcock et al., 1992; Pronk et al., 1993; Ruff-Jamison et al., 1993; Cutler et al., 1993), which, in turn, induces the formation of a stable complex with the adaptor protein Grb2 and the Ras guanine nucleotide exchange factor SOS (Son of Sevenless) (Skolnik et al., 1993; Egan et al., 1993; Aronheim et al., 1994; Kavanaugh and Williams, 1994; Ohmichi et al., 1994). These events lead to the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway when $p46^{shc}$ and $p52^{shc}$ are involved. In contrast, activated $p66^{shc}$ is unable to affect MAPK activity, whereas it inhibits c-Fos promoter activation (Migliaccio et al., 1997).

The mechanism through which $p66^{shc}$ acts as oxidative stress effector has been investigated by multiple groups. Nemoto and Finkel (2002) have demonstrated that $p66^{shc}$ activation induces the inactivation of FOXO3a, a transcriptional factor that increases the expression of genes encoding antioxidant enzymes (mitochondrial superoxide dismutase (MnSOD) and catalase). In wild-type fibroblasts, stimulation with insulin or hydrogen peroxide led to a rapid and significant increase in FOXO3a phosphorylation, which is not observed in $p66^{shc-/-}$ fibroblasts. That $p66^{shc}$ induces reduced transcription of antioxidant enzymes strongly links it to the progression of the damage induced by reactive oxygen species (ROS).

More recently, Giorgio et al. (Giorgio et al., 2005) have demonstrated that $p66^{shc}$ proapoptotic activities were due to its acting as a redox enzyme to generate mitochondrial hydrogen peroxide, which in turn induces opening of the permeability transition pore (PTP) eventually leading to cellular apoptosis. In particular,

proapoptotic signals induce release of $p66^{shc}$ from the putative inhibitory TIM-TOM-mtHsp70 complex. Active $p66^{shc}$ oxidizes the reduced cyt c and catalyzes the reduction of molecular oxygen to hydrogen peroxide. The PTP opening by hydrogen peroxide leads then to swelling and apoptosis.

The role for $p66^{shc}$ in relation to oxidative stress remains incompletely defined as experiments conducted in mouse embryo fibroblasts suggest that p66shc is a stress-response protein. That is because $p66^{shc}$ was activated upon exposure to agents that induce oxidative stress, such as UV light or H_2O_2. Conversely, Trinei et al. (2002) have shown that p66shc is an oxidative stress inducer, downstream of p53, increasing ROS production in MEF. For example, when p66shc was reintroduced in both $p66^{shc-/-}$ and $p53^{-/-}$ cells, it induced an increase in ROS production.

Mitotic arrest can be induced by increased ROS, which then elevates oxidative stress levels and subsequent cellular damage (Campisi, 2001; Balin et al., 2002; Droge, 2002; Hutter et al., 2002; Favetta et al., 2004).

The tumor suppressor protein p53 and the stress adaptor protein $p66^{shc}$ play a vital role in triggering this process in somatic cells. Although no correlation was observed between p53 expression levels under varying oxidative stress conditions and early embryo arrest, a correlation between the induction of oxidative stress and $p66^{shc}$ expression was observed (Favetta et al., 2007). Favetta et al. (2004) were able to show that $p66^{shc}$ was significantly elevated in arrested 2- to 4-cell embryos alongside with an increased level of ROS. Thus $p66^{shc}$ appears as a link in oxidative stress induced cellular events. Wu et al. have reported that suppression of $p66^{shc}$ results in upregulation of some antioxidant enzymes under basal conditions and others in the presence of oxidative stress (Wu et al., 2006). Interestingly, only catalase mRNA was reduced in both control and $p66^{shc}$-deficient cells after H_2O_2 exposure which was also shown to be the case in AML-2/DX100 cells where exposure to H_2O_2 caused downregulation of catalase and upregulation of other antioxidant enzymes (Oh et al., 2004). Of note, $p66^{shc}$ has been reported to inhibit members of the forkhead transcription factor family, which induce transcription of various antioxidant enzymes (Purdom and Chen, 2003). Thus elevated levels of $p66^{shc}$ might diminish a cell's ability to deal with oxidative stress, thereby promoting irreparable cellular damage and subsequent cell death. The converse also appears in another study where it is reported a decreased $p66^{shc}$ levels in mice fed with low advanced glycation/lipoxidation product diet that was associated with a reduction in systemic AGE accumulation and amelioration of insulin resistance, albuminuria, and glomerulosclerosis as well as extension of their lifespan (Cai et al., 2007).

It is interesting to note that, recently, Khanday et al. have demonstrated that $p66^{shc}$ is crucial for the rac1 activation mediated by Sos (Khanday et al., 2006). Rac1 is necessary for the activation of the multi-component enzyme NAD(P)H oxidase that catalyzes the production of superoxide by the reduction of molecular oxygen using as electron donor NAD(P)H. These findings fit with the results of studies from our laboratory demonstrating increased mononuclear cell gene expression of both $p22^{phox}$, a subunit of NAD(P)H oxidase (Avogaro et al., 2003) and $p66^{shc}$ (Pagnin et al., 2005) in type 2 diabetic patients. NAD(P)H oxidase is also involved in long term signaling mechanisms mediated by Ang II which cause

the cardiovascular remodeling, common to hypertension, atherosclerosis and heart failure, by modulating the cell oxidative state (Griendling et al., 1997; Dzau, 2001).

The latest proposed mechanism of action of $p66^{shc}$ in response to oxidative stress and as cause of ROS production, has been put forward by Pinton et al. (2007). They demonstrated that $p66^{shc}$ is required for early mitochondrial responses to an oxidative challenge (H_2O_2). These responses include mitochondrial fragmentation and suppression of Ca^{2+} signal propagation to the mitochondria, followed by execution of apoptosis in murine fibroblasts. $p66^{shc}$ undergoes phosphorylation mainly at Ser36, unique for the longest Shc isoform, after exposure to oxidative stress such as UV light or H_2O_2 (Kao et al., 1997; Migliaccio et al., 1999; Yang and Horowitz, 2000; Le et al., 2001; Nemoto and Finkel, 2002). Phosphorylation at Ser 36 is required to confer increased susceptibility to oxidative stress and it is critical for the cell death response elicited by oxidative damage (Skulachev, 2000). Pinton et al. have demonstrated that protein kinase C β (PKC β) is responsible for the phosphorylation of $p66^{shc}$ on Ser36 in response to H_2O_2. This consequently causes a binding to Pin 1, a peptidyl-prolyl isomerase, which helps in the translocate $p66^{shc}$ from the cytosol to the mitochondria (Pinton et al., 2007). The activation of $p66^{shc}$ at mitochondrial level, then, requires its dephosphorylation and dissociation from mtHsp70 which is followed by the interaction with cytochrome C to produce H_2O_2, which can promote opening of the mitochondrial permeability transition pore as described by Giorgio et al. (Giorgio et al., 2005). The different mechanisms of action of $p66^{shc}$ are represented in Fig. 14.2.

$p66^{shc}$, Diabetes, Hypertension and Cardiovascular Diseases

Increased oxidative stress plays an important role in vascular dysfunction and atherogenesis. Enhanced production of ROS affects vascular reactivity and atherogenesis by modulating multiple signaling pathways and transcriptional events. Several studies have demonstrated a direct involvement of $p66^{shc}$ in oxidative stress-induced cardiovascular damage and diseases. In dogs, ventricular pacing-induced cardiomyopathy which is characterized by increased ROS and mitochondrial dysfunction; it has been shown that ventricular pacing induces a progressive, time of pacing-dependent, $p66^{shc}$ overexpression that correlates both with parameters of ventricular dysfunction and with cytochrome C release and procaspases activation, compared to the heart of normal dogs which contains a very little $p66^{shc}$ (Cesselli et al., 2001). $p66^{shc-/-}$ mice showed blood pressure, heart rate and left ventricular wall thickness similar to that of wild type littermates while chronic infusion of non pressor doses of Ang II induced myocardial hypertrophy and apoptosis only in $p66^{shc\,+\,/\,+}$ but not $p66^{shc-/-}$ animals. These results suggest that $p66^{shc-/-}$ mice are resistant to the proapoptotic/hypertrophic action of Ang II (Graiani et al., 2005). Additional evidence comes from the findings of Napoli et al. who reported that deletion of $p66^{shc}$ was associated with reduced vascular cell apoptosis (Napoli et al.,

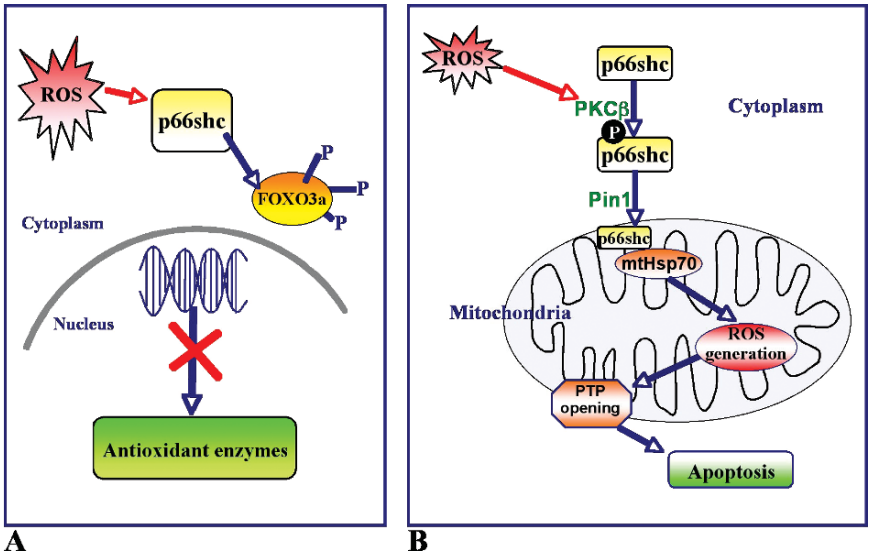

Fig. 14.2 Mechanisms of action of p66^{shc}. Panel A. p66^{shc} activation by ROS induces FOXO3A phosphorylation and consequent translocation from nucleus to cytoplasm. Panel B. ROS induce Ser36 p66^{shc} phosphorylation through PKCβ. This consequently causes a binding to Pin which helps in p66shc translocation to the mitochondria. The subsequent dissociation from mtHsp70 is followed by the interaction with cytC, production of ROS and opening of the permeability transition pore (PTP)

2003), and tissue damage (Zaccagnini et al., 2004). In addition, Francia et al. have demonstrated that the inactivation of the p66^{shc} gene protects against age-dependent, ROS-mediated endothelial dysfunction (Francia et al., 2004).

These findings suggest that the p66^{shc} is part of a signaling pathway linked to to endothelial integrity. Menini et al., in fact, has recently shown that p66$^{shc-/-}$ mice do not develop diabetic glomerulopathy after blocking hyperglycemia-induced ROS overproduction and oxidant-dependent renal tissue injury (Menini et al., 2006). On the other hand, Camici et al. have demonstrated in streptozotocin-treated p66$^{shc-/-}$ mice the absence of any impairment of endothelium dependent relaxation, peroxynitrite generation, nitrotyrosine expression, and lipid peroxidation in contrast to streptozotocin-treated wild type animals (Camici et al., 2007).

These results suggest that p66^{shc} is involved in the cellular and molecular mechanisms underlying the oxidative stress mediated cardiovascular complications in type 2 diabetes. This association has been further strengthened by our study where we have analyzed the involvement of p66^{shc} directly in type 2 diabetic patients (Pagnin et al., 2005). We have, in fact, shown that mononuclear cell p66^{shc} gene expression is increased in type 2 diabetic patients compared to healthy controls. Moreover, this increased p66^{shc} mRNA levels shown in diabetic patients correlates with a higher plasma concentration of 8-isoprostanes, a classic marker of oxidative stress.

Studies from our laboratory have extensively demonstrated a blunted long-term Ang II signaling and reduced redox state in Bartter's/Gitelman's syndrome patients who are characterized by increased level of Ang II and aldosterone yet reduced vascular reactivity, normo-hypotension (Calò, 2006; Calò and Pessina, 2007) and increased insulin sensitivity (Davis et al., 2006). Moreover, these patients show a reduced $p22^{phox}$ and PAI-1 expression (Calò et al., 2003; Pagnin et al., 2004), reduced susceptibility of LDL to oxidation (Calò et al., 1998) and activation of NO system with increased production of NO (Calò et al. 1995, 1996, 1999), a known antioxidant and antiapoptotic factor. With respect to $p66^{shc}$ and BS/GS, we have found an absence of upregulation of $p66^{shc}$ i.e. no differences in BS/GS mononuclear cell $p66^{shc}$ gene expression compared to healthy controls (Calò et al., 2007) despite the presence of high levels of Ang II and aldosterone and activation of the renin-angiotensin-aldosterone system present in BS/GS patients. The fact that Bartter's/Gitelman's syndrome patients do not develop hypertension and cardiovascular remodeling despite high levels of Ang II and activation of the renin-angiotensin-aldosterone system, has led to the concept that they represent the mirror (opposite) image of hypertension and atherosclerosis. Therefore BS/GS patients are a good human model to delineate the mechanisms involved in vascular tone regulation, atherogenesis and remodeling and our findings in these patients therefore strengthen the potential linkage between NAD(P)H oxidase and $p66^{shc}$.

Summary

There is an increasing recognition that oxidative stress represents a major factor in the damage associated with a variety of human diseases, such as cardiovascular diseases. The term "oxidative stress" describes a condition characterized by chronically elevated level of ROS.

In this context, we have demonstrated an increased $p66^{shc}$ gene expression in type 2 diabetic patients (Pagnin et al., 2005) and a downregulation in Bartter's/Gitelman's syndrome patients (Calò et al., 2007 submitted), a human model of altered vascular tone regulation which is characterized by a clinical picture mirror (opposite) image of hypertension, therefore supporting a role for increased $p66^{shc}$ in the pathophysiology of diabetes and hypertension and their long term complication such as cardiovascular remodeling and atherogenesis.

However, apparent dual nature of p66shc activity as demonstrated by its association with the production of oxidative stressors as well as responding to oxidative stress, suggests that it plays a role in determining the "balance" between those activities. The demonstration of $p66^{shc}$ involvement in the pathophysiology of type 2 diabetes and hypertension in humans, suggest that affecting $p66^{shc}$ and its roles in that balancing may be prove to be an important therapeutic target for the prevention of these diseases and of the development and progression of diabetic and hypertensive complications.

List of Abbreviations

NOS	nitric oxide synthase
ROS	reactive oxygen species
Ang II	Angiotensin II
PMA	phorbol myristate acetate
NAD(P)H oxidase	nicotinamide adenine dinucleotide (phosphate) oxidase
RAAS	renin-angiotensin-aldosterone system
PKC	protein kinase C
AGE	advanced glycation end product
MAPK	mitogen-activated protein kinase
PKC β	protein kinase C β
BS/GS	Bartter's/Gitelman's syndromes

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Chapter 15
A Role of BRCA1-Associated Protein BARD1 in Oxidative Stress Response and Signaling and Proliferation Control

Irmgard Irminger-Finger[1]* and Shazib Pervais[2]

Abstract More than a decade ago, the *BRCA1* and *BRCA2* genes, responsible for familial breast cancers, were discovered. About 50 percent of women diagnosed with breast cancer have inherited mutations in BRCA1 or BRCA2 that predispose them to breast and ovarian cancer. Although there are several thousand publications concerning analysis of structure, expression, and function of these genes, no treatment methods for breast cancer have been developed that based on the accumulated knowledge. BRCA1 and BRCA2 are large proteins that interact with many other proteins of diverse functions. One particular protein, BARD1, a binding partner of BRCA1, might crucially regulate the tumor suppressor function of BRCA1 and act as a tumor suppressor in its own right. The functions attributed to BARD1 might make it indispensable for cell viability. This might explain why BARD1 mutations are rarely found in cancer, but aberrant truncated forms are overexpressed. Disappointingly, while screening for mutations in the predisposition genes BRCA1 and BRCA2 is now routinely carried out, no treatment methods have been developed that are based on our knowledge of BRCA1 and BRCA2 functions, which leaves mutation carriers without hope for future treatment. It will be interesting how dissection of the functions of BARD1 will open new avenues for cancer treatment. Here we discuss that BARD1 expression can be regulated in a cell cycle dependent way, in a hormone dependent was, and by hypoxia and oxidative stress. Understanding the way how BARD1 is activated will be important the understanding of its role in tumorigenesis and in the search for treatment targets.

Keywords BARD1, BRCA1, BRCA2, breast cancer, ovarian cancer, apoptosis, cancer therapy

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

Introduction

The BRCA1-associated RING domain 1 (BARD1) was discovered in a yeast two-hybrid screen as a binding partner of BRCA1 (Wu et al., 1996). BARD1 and BRCA1 have several features in common, namely, similar protein structure, embryonic lethality of knockouts in mouse models (McCarthy et al., 2003), and induction of genetic instability when depleted from cells (Irminger-Finger et al., 1998; Joukov et al., 2001; McCarthy et al., 2003). The prevalent opinion in the field is that BARD1 only acts as an accessory protein for BRCA1. However, several reports have demonstrated the BRCA1-independent functions of BARD1, primarily in apoptosis (Fig. 15.1). Additionally, data have accumulated in the last three years that suggest a role of BARD1, as well as BRCA1 and BRCA2, in mitosis, which might link their functions and explain their role in maintaining genetic stability. The expression of BARD1 is upregulated in most proliferating tissues and it is low in quiescent cells. This is consistent with the activation of BARD1 transcription by the transcription factor E2F (Ren et al., 2002). The diverse functions of BARD1, in regulation of cell proliferation and apoptosis and its role as tumor suppressor in normal cells and as oncogene when expressed as truncated isoform in tumors, suggest that it is of importance to understand the regulators of BARD1 expression, such as hypoxia, and hormones.

BARD1: A Stress-Response Factor?

The function of BARD1 as inducer of apoptosis was discovered in cells treated with doxorubicin, a chemotherapeutic drug. Doxorubicin induces DNA damage, therefore BARD1 transcriptional activation was thought to be induced by a DNA damage pathway (Irminger-Finger et al., 2001). However, doxorubicin also generates

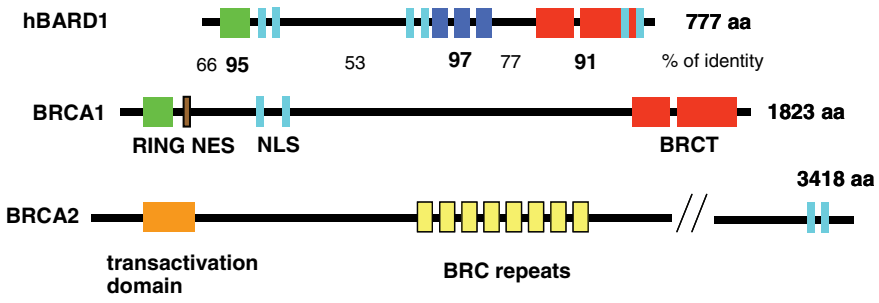

Fig. 15.1 The BARD1 protein. (A) BARD1 domain structure compared to BRCA1. RING (green), ANK (blue), and BRCT (red) domains are indicated and location of potential NLS (light blue). Evolutionary conservation is indicated as percentage of identical amino acids between mouse and human sequence within distinct regions

reactive oxygen species (ROS) in the cell, which then generate DNA damage. Although a role of BARD1 in DNA damage is well established, a direct activation of BARD1 by ROS cannot be excluded. Indeed, UV exposure leads to BARD1 transcriptional activation (Irminger-Finger et al., 2001), supporting the notion that BARD1 can be upregulated by ROS.

Further support that BARD1 is a cellular stress sensor was obtained from in vivo experiments. Neither BRCA1 nor BARD1 expression is found in the normal brain, but upregulation of BARD1 associated with apoptotic markers is seen after hypoxia induced by artery ablation (Irminger-Finger et al., 2001). In a mouse model of brain stroke, artery ablation was performed in one hemisphere. It was shown that BARD1 was expressed in the region surrounding the necrotic center of the stroke, the penumbra (Irminger-Finger et al., 2001), where apoptosis and regeneration is observed. Another example of hypoxia effects on BARD1 is presented in the developing placenta. A role for BARD1 in CTB invasion was suggested, based on its expression in invasive CTB (Li et al., 2007). When CTB invade into the maternal duodenum they transgress a region of hypoxia before reaching the maternal blood supply (Bischof and Irminger-Finger, 2005). Indeed, in this tissue hypoxia is a factor that presumably triggers CTB invasion. Culture of CTB under hypoxic and anoxic conditions indeed showed upregulation of BARD1 when cells were grown in hypoxia (Li et al., 2007). Thus, hypoxia acts as transcriptional activator of BARD1, but the intermediates in this signaling pathway remain to be determined.

Indications are that BARD1 might be expressed specifically in tissues undergoing stress and resulting apoptosis. The pathway of BARD1-dependent apoptosis appears to be induced by genotoxic stress wherein *BARD1* is transcriptionally upregulated (Irminger-Finger et al., 2001). Furthermore, over-expression of exogenous *BARD1* leads to apoptosis associated with p53 stabilization and activation of caspase 3, in various cell lines (Irminger-Finger et al., 2001). The apoptotic function of BARD1 is dependent on functional p53, but is independent of and even inhibited by BRCA1, as demonstrated in p53 or BRCA1 deficient cell lines, respectively (Irminger-Finger et al., 2001). BARD1 co-immunoprecipitates with p53. The BARD1-p53 interaction leads to p53 stabilization, as BARD1 up-regulation, as a result of genotoxic stress, or overexpression of exogenous BARD1 is accompanied by an increase in p53 protein levels but not mRNA levels (Irminger-Finger et al., 2001). The activation and stabilization of p53 is thought to be dependent on its phosphorylation at multiple sites by a number of kinases, including phosphorylation on serine 15 (Meek, 1994; Milczarek et al., 1997). In the absence of BARD1, phosphorylation of serine 15 is lost, whereas overexpression of exogenous BARD1 can catalyze the phosphorylation of serine 15 (Fabbro et al., 2004; Feki et al., 2005). BARD1 directs phosphorylation of p53 at serine 15 by interacting with a DNA-damage response kinase. Indeed, BARD1 binds to Ku-70, a subunit of DNA-dependent protein kinase (DNA-PK) (Feki et al., 2005). DNA-PK plays an important role in the repair of double-stranded DNA breaks by a nonhomologous end-joining pathway and in maintaining genomic stability. The region of BARD1 that interacts with p53 does not involve the RING finger domain as deletion mutants lacking this domain co-immunoprecipitate with p53 (Feki et al., 2005).

The minimal region required for p53 binding spans residues 510–604 between ANK and BRCT domains and it is this region that is required for BARD1-dependent apoptosis (Feki et al., 2005; Jefford et al., 2004) (Fig. 15.2). The same region of BARD1 also harbors two known cancer predisposing mutations (Fig. 15.4) C557S and Q564H (Ghimenti et al., 2002; Jefford et al., 2004; Karppinen et al., 2004; Thai et al., 1998). The Q564H missense mutation of BARD1 cannot induce apoptosis when transfected in cultured cells (Irminger-Finger et al., 2001), implying that the region of BARD1 around Q564H is necessary for its pro-apoptotic tumor suppressor function. Thus BARD1 acts as a mediator between pro-apoptotic stress and p53-dependent apoptosis (Fig. 15.3).

Upregulation of Aberrant BARD1 Isoforms in Cancer: A Marker for Hormone-Dependent Cancers and Others?

In hormone-dependent tissues, BARD1 expression seems to be regulated in a hormone-responsive way, since the expression of BARD1 mRNA was correlated with hormonal changes in various mouse tissues (Irminger-Finger et al., 1998). Hormonal regulation was also observed in the germinal cells of rat testis, cultured in vitro with or without addition of hormones (Feki et al., 2004). Indeed, testosterone seems to induce BARD1 expression to counterbalance follicle stimulating hormone (FSH)-dependent repression of BARD1. Hormone-dependent expression of BARD1 was also followed in human cytotrophoblasts (CTB) where a maximum of BARD1 expression is seen at 10 weeks of pregnancy, and correlates with human chorionic gonadotropin (hCG)expression. Treatment of cultured CTB with hCG

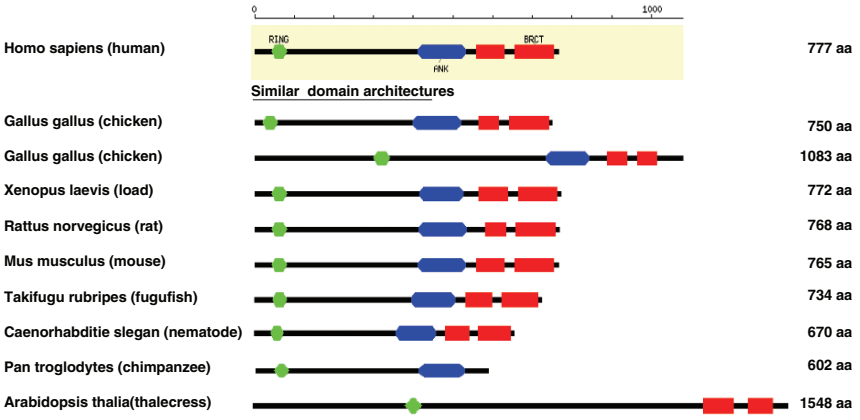

Fig. 15.2 Structural conservation of BARD1 proteins. An alignment of BARD1 protein sequences from different species shows complete conservation of structural organization of RING (green), ANK (blue) and BRCT (red) domains

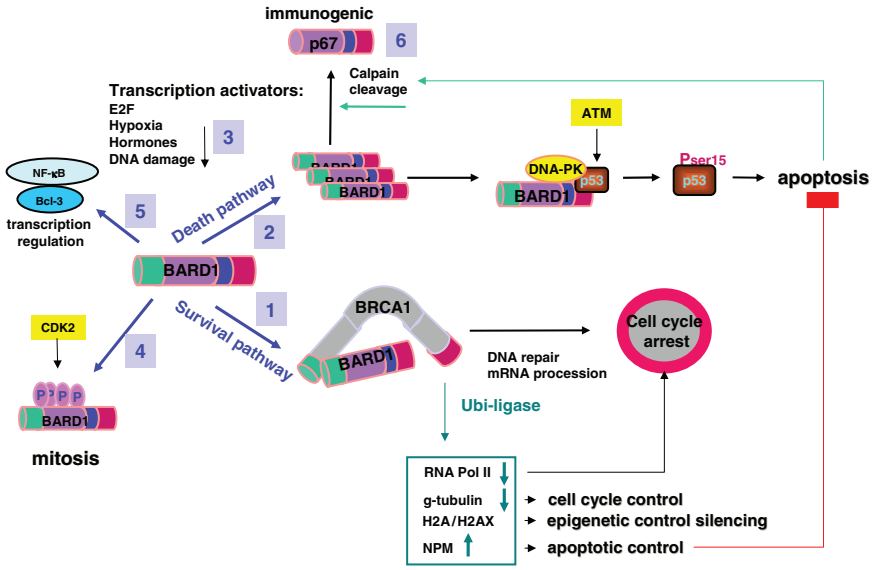

Fig. 15.3 BARD1 and BRCA1BARD1 pathways and functions. BARD1 participates in two pathways: (1) survival pathway as BRCA1BARD1 heterdimer and (2) death pathway in a BRCA1-independent function in apoptosis. BRCA1BARD1 acts in survival pathway. BRCA1BARD1 ubiqitin ligase activity leads RNAPolII degradation and cell cycle arrest, to γ-tubulin degradation and regulation of centromere duplication, to H2A/H2AX ubiquitylation and epigentic control, and to NPB ubiquitylation. Upregulated NPB is a known inhibitor of apoptosis, it causes centromere amplification and genetic instability; hence NPM antagonizes BARD1 functions.
In the death pathway (2) BARD1 is upregulated by DNA damage, UV, and hormones (3). Increased BARD1 stabilizes p53 and facilitates its phosphorylation by DNA-PK. The role of BARD1 in p53 phosphorylation on ser 15 by ATM is unknown. Posttranslational modification through phosphorylation by CDK2/cyclins might regulate BARD1 interaction with BRCA1 and trigger its mitotic activity (4). BARD1 has transcriptional activity by modulating NF-kB functions (5). The proteolytic cleavage product p67 is immunogenic and has anti-tumorigenic properties (6)

increased BARD1 expression in vitro (Li et al., 2007). Finally, estrogen response elements were described in intron seven of the BARD1 gene and BARD1 expression is regulated by estrogen receptor alpha (Creekmore et al., 2007). The BRCA1-BARD1 heterodimer, on the other hand, targets the estrogen receptor alpha for degradation (Eakin et al., 2007), thus providing a regulatory feedback loop.

With respect to hormone dependent cancers, immunostaining for BARD1 expression in tumors revealed that high expression of BARD1 in tumors with *BARD1* mutations, as well as in tumors with *BRCA1* mutations. High expression was also found in a series of spontaneous ovarian, breast, and lung cancers. The upregulated protein localized exclusively to the cytoplasm, while low level of expression was found in the nucleus of adjacent healthy tissue (Wu et al., 2006). Expression was maximal in ovarian clear cell carcinoma, an ovarian cancer with worst prognosis, with close to 100 percent positive cells. The extent of upregulated

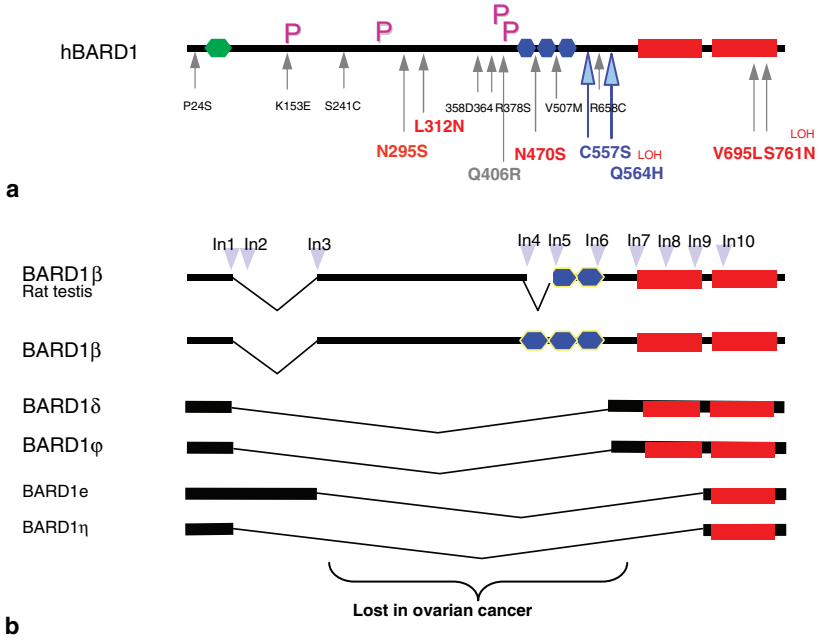

Fig. 15.4 BARD1 mutations and splice variants. **(A)** Schematic drawing of BARD1 protein structure with RING (green), ANK (blue), BRCT (red) motifs indicated. Phosphorylation sites are marked with P. Mutations are marked in red and polymorphisms in black underneath. The Q406R mutation was found recently in ovarian cancer (Wu et al., 2005). **(B)** Splice variants found for *BARD1*: BARD1β in preleptotene spermatocytes (Feki et al., 2004), BARD1δ in ovarian cancer cell line (Feki et al., 2005) and in HeLa cells (Tsuzuki et al., 2005). The N-terminal exons were frequently lost in ovarian cancer (Wu et al., 2005) and in human CTB (Li et al., 2007)

BARD1 expression was correlated with other indicators of poor prognosis, such as tumor type in ovarian cancers and tumor size and stage for breast cancer, which was not the case for non-small-cell lung cancer.

In most cases of ovarian cancer, but not in clear cell carcinomas, the N-terminal epitopes were deleted, suggesting that tumors recruit different isoforms of BARD1, which might have lost tumor suppressor functions and possibly acquired oncogenic potential. In support of this view, a rat ovarian cancer cell line, which is resistant to apoptosis, did not express full length *BARD1*, but *BARD1*$\ddot{\delta}$ a splice variant lacking exons 2 through 6, was identified (Fig. 15.4) (Feki et al., 2005). The *BARD1*$\dot{\delta}$ isoform was also found in HeLa cells in addition to full length *BARD1* (Tsuzuki et al., 2005).

Consistent with the notion of oncogenic *BARD1* isoforms in cancers, upregulated *BARD1* expression was described as one of the markers for treatment failure in embryonic central nervous system tumors (Pomeroy et al., 2002). Another example of oncogenic activity is the interaction of BARD1 with the Ewing's sarcoma product EWS and oncogenic fusion protein EWS/FLI1 (Spahn et al., 2002).

BARD1 isoforms were also found in CTB (Fig. 15.4) (Li et al., 2007), and since CTB are invasive cells, reminiscent of cancer cells, it can be expected the upregulated truncated forms observed in cancer correspond to the isoforms described in CTB.

Since isoforms are lacking regions required for tumor suppressor functions, e.g. interaction with BRCA1, it is important to investigate which of the activators of BARD1, such as hormones and oxidative stress, affect the expression pattern of isoforms.

The tissue specific expression of *BARD1* suggested a role in tissue homeostasis. BARD1, as well as BRCA1 are expressed in most proliferative tissues, and expression of murine *Bard1* is highest in testis and in spleen (Ayi et al., 1998) (Irminger-Finger et al., 1998). Specifically, *Bard1* expression in the testis was found in spermatogonia and in spermatocytes, cell types that undergo proliferation and apoptosis (Feki et al., 2004).

Although *Bard1* and *Brca1* showed correlated expression in most proliferative tissues of the mouse, in breast, ovary, and uterus, the expressions of *Bard1* and *Brca1* were differentially regulated in accordance with the ovulatory cycle. Specifically, *Bard1* levels were increased from diestrus to postestrus while *Brca1* expression was decreased from estrus to postestrus (Irminger-Finger et al., 1998). These data imply that BARD1 might have a role in the endometrium during the postestrus period. This hypothesis is consistent with the finding that an inherited mutation of *BARD1* is associated not only with breast and ovarian, but also endometrial cancer (Thai et al., 1998), a cancer that has not been associated with *BRCA1* mutations. Similarly, in the mouse mammary gland, BARD1 is expressed in the lining of the glands, where it could be important for the control of proliferation (Wu and Irminger-Finger, 2007). BRCA1 acts as a competitor of the apoptosis pathway. The importance of a BARD1 dependent apoptosis pathway is corroborated by resistance of cells expressing *BARD1*-antisense RNA (Irminger-Finger et al., 2001) and ovarian cancer cells that lack full length *BARD1* (Feki et al., 2005) to doxorubicin-induced apoptosis.

These findings are consistent with a model where binding of BARD1 to BRCA1 induces survival and repair functions, but an excess of BARD1 over BRCA1 leads to apoptosis induction (Fig. 15.3). In cells deficient of BRCA1 due to inactivating mutations, an excess of BARD1 over BRCA1 could induce p53-dependent apoptosis. This could explain why p53 mutations or deletions are frequent in cancers with BRCA1 mutations (Reedy et al., 2001). However, the status of *BARD1* has not been investigated in this context.

Conclusions

Of the multiple functions of BARD1, two major pathways emerge: BARD1 as an essential component of the BRCA1BARD1 ubiquitin ligase and BARD1, required for p53-mediated apoptosis. The BRCA1-dependent function resides at the N-terminus of BARD1, the apoptosis function within the C-terminus. The C-terminus also

harbors the rare cancer associated and germ line mutations. A function of BARD1 in mitosis seems required for maintaining of genetic stability. Since depletion of BARD1 rapidly causes genomic instability leading to non-viable cells, it will be important to investigate whether BARD1 repression is found in premalignant tissue in response to oxidative stress. It is possible that BARD1 repression and/or expression of isoforms devoid of tumor suppressor functions occurs at onset of tumorigenesis and causes genomic instability. In summary, there is clear evidence that BARD1 expression is governed by stress and hormones, but further research is needed to clarify the pathways involved and the nature of the balances that govern the end results.

Acknowledgements This work was supported by grant from the SNSF 3100-68222 to I I-F.

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Chapter 16
Postprandial Events as a Trigger for Redox Unbalance: Role of Dietary Lipid Hydroperoxides and Antioxidants

Fausta Natella*, Arianna Ramberti, and Cristina Scaccini

Abstract In the late '70s Zilversmit hypothesized that the dramatic postprandial modification of the plasma lipid pattern could have a significant effect on atherogenesis. Since then, several evidence has been collected, and it has been demonstrated that many events happening during the postprandial phase, such as oxidative stress and inflammation, are strongly linked to atherosclerosis.

At the moment, there is clear scientific evidence that during the postprandial phase there is an impairment of the redox status (postprandial oxidative stress) that could be determined by (1) the aspecific intake of macronutrients, whose catabolism can lead to the production of oxygen-radical species, or (2) the intake of oxidized/prooxidant species, which, when absorbed, can directly modify the redox balance.

Thus eating regular meals throughout the day can result in a constant oxidative condition that depends on the relative intake of oxidizable or oxidized nutrients and that of antioxidants. This chapter will focus on the role of dietary lipid hydroper oxides and antioxidants in determining the extent of postprandial oxidative stress.

Keywords Atherosclerosis, oxidative stress, antioxidant, postprandial phase

Introduction

Multiple biological pathways can mediate the effect of diet on atherosclerosis. In the past, the diet-heart relation was founded on the "lipid hypothesis of atherosclerosis", postulating a primary role of diet in modulating serum lipid level, particularly total cholesterol and LDL/HDL ratio. At the present, all the schemes for the pathogenesis of atherosclerosis include oxidative stress, sub clinical inflammation,

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

endothelial dysfunction and insulin sensitivity, conditions likely to be affected during the postprandial phase.

This chapter will review the studies on the effect of meal consumption on redox status in humans, describing mechanisms and possible physio-pathological consequences.

Text

Postprandial Phase and Atherosclerosis

The postprandial phase is a complex and highly specialized physiological process, whose aim is to deliver nutrients to tissues. The postprandial lipemia is the period of time going from the ingestion of food to the post-absorptive phase, when all the components of the lipid transport system return to equilibrium (Ebenbichler et al., 1995). The length of the postprandial phase depends on both meal composition and inter-individual genetic differences. It is noteworthy that, in the western society, an individual spends the majority of the day in postprandial state.

Since the '50s, when postprandial triglyceridemia was reported to be higher in cardiovascular disease (CVD) patients than in control (Albrink and Man, 1959), the postprandial hyperlipemia has been considered a significant risk factor for cardiovascular disease. At the end of the '70s, Zilversmit proposed chi omicron remnants as having a significant role in atherogenesis (Zilversmit, 1979). Only in the '90s, postprandial levels of triacylglycerides started to be considered an independent risk factor for CVD.

At the present it can be stated that:

1. Postprandial levels of triacylglycerols and triacylglycerol-rich lipoproteins correlate with the risk for coronary hearth disease stronger than the concentrations of triacylglycerols in the post absorptive state (Zilversmit, 1979; Patsch et al., 1992; Cohn, 1998) and independently from other risk factors (including fasting LDL and HDL cholesterol) (Ginsberg et al., 1995).
2. Postprandial levels of chylomicron remnants (Karpe et al., 1994; Kugiyama et al., 1999) and VLDL remnants (Phillips et al., 1993) are related to the rate of progression of coronary lesions in patients with premature coronary artery disease.
3. Postprandial hyperlipemia induces alterations in metabolism and composition of all major lipoproteins (Ebenbichler et al., 1995; Cohn, 1998).
4. Chylomicron remnants and VLDL remnants induce cholesterol accumulation into cultured macrophages and smooth muscle cells (Floren and Chait, 1981; Van Lenten et al., 1985; Huff, 2003).
5. LDL isolated during postprandial lipemia is more susceptible to oxidation *in vitro* (Lechleitner et al., 1994; Natella et al., 2001) and induces a greater cholesterol accumulation into cultured macrophages than fasting LDL (Lechleitner et al., 1994).

Postprandial Oxidative Stress

Aerobic life is based on the oxidative metabolism. Nutrients oxidation provide energy, but such oxidation can induce the formation of reactive oxygen species (ROS), which, in turn, can attack and damage lipids, proteins and nucleic acids. When the organism is not able to cope with all the ROS produced and to avoid oxidative damages an "oxidative stress" is induced; the "oxidative stress", in fact, is a metabolic condition common to cells, organs and organisms that is characterized by an oxidative surcharge (Sies et al., 2005).

The consumption of a high-fat or high-carbohydrate meal has been demonstrated able to induce a condition of oxidative stress (Ursini and Sevanian, 2002a; Sies et al., 2005).

Several *in vivo* human studies demonstrated that a high-fat meal:

- Induces a decrease of total plasma antioxidant capacity and/or the oxidation of plasma antioxidants, such as ascorbic acid, uric acid, GSH and protein thiol groups (Ceriello et al., 1998; Natella et al., 2002)
- Reduces the activity of some antioxidant enzymes, such as plasma glutathione peroxidase (Natella et al., 2007) and paraoxonase (an enzyme associated to HDL and endowed with esterase activity, which hydrolyzes oxidized phospholipids on lipoproteins and inhibits the oxidation of LDL) (Sutherland et al., 1999)
- Induces the increase of lipid hydroperoxides (LPO) or other by-products of lipid peroxidation in plasma and chylomicrons (Ursini et al., 1998; Natella et al., 2002)
- Induces an increase of human LDL susceptibility to oxidative modification *ex vivo* and the appearance of LDL(-) *in vivo* (Lechleitner et al., 1994; Natella et al., 2001, 2007). LDL (-) is an electronegative LDL subfraction that is enriched with oxidized lipids (Sevanian et al., 1996), accounts in vitro for several biological events known as pro-atherogenic (Demuth et al., 1996; Sanchez-Quesada et al., 2003) and correlates positively with common atherosclerotic risk (Moro et al., 1998; Ziouzenkova et al., 1999)

Moreover, *in vivo* human studies report that the consumption of a single meal can also determine many other events that are key elements in the atherosclerotic process, such as:

(a) A transient endothelial dysfunction (Plotnick et al., 1997; Vogel et al., 1997; Williams et al., 1999; Esposito et al., 2003)
(b) An increased propensity to blood coagulation (Mutanen and Freese, 2001) and platelet aggregation (Fuhrman et al., 1986)
(c) A transient inflammatory response, as evidenced by:

- The increase of neutrophil count (van Oostrom et al., 2003)
- The activation of monocytes and neutrophils (van Oostrom et al., 2004)
- The increase of proinflammatory cytochines, such as IL-8 (van Oostrom et al., 2003), IL-10 (Twickler et al., 2003), IL-6 and TNF-α (Nappo et al., 2002)
- The increase of plasma concentration of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Nappo et al., 2002)

- The activation of NF-κB in peripheral blood mononuclear cells (Blanco-Colio et al., 2000)

It is noteworthy that some, if not all, these events are prevented by the presence of antioxidants in the meal (Plotnick et al., 1997; Nappo et al., 2002). Thus, the impairment of the redox status could represent the "primary" effect of postprandial state, while transient endothelial dysfunction, inflammation and the other observed events could be "secondary" effects.

Which Is The Cause of Postprandial Oxidative Stress?

The postprandial oxidative stress can be caused by the increase of reactive oxygen species produced during the catabolism of lipids and carbohydrates (Marfella et al., 2001).

Besides this aspecific mechanism, the postprandial oxidative stress could be directly caused by the ingestion of oxidized molecules (for ex. lipid peroxidation by-products). In this case, the typology of the meal (nutrient and fat pattern, cooking method and time, etc) could represent the main determinant of the extent of postprandial oxidative stress and, as a consequence, could increase or decrease the risk for atherosclerosis.

Staprans et al. demonstrated, in fact, that lipid peroxides-rich diets accelerate the development of atherosclerosis in animal models (Staprans et al., 1996; Staprans et al., 1998; Staprans et al., 2000). The mechanism of this event could be based on the fact that oxidized fats from the meal escape the gastrointestinal reduction and pass into the blood stream, where they could attack lipoproteins (LDL), promoting atherogenesis.

Oxidized Lipids in Food

A number of scientific evidences indicate that a western-style diet contains a significant amount of oxidized fats. PUFAs, particularly susceptible to oxidation, during cooking easily undergo auto oxidation processes. The extent of lipid peroxidation is enhanced by the presence of catalysts, as heme iron containing proteins (as in the case of meat and fish) (Sesink et al., 1999). The lipid peroxides in food can reach the concentration of 600 nmol/g of food (Yagi et al., 1986) and Wolff et al. (Wolff and Nourooz-Zadeh, 1996) estimate a typical dietary intake of lipid hydroperoxides of 1.5 mmol/day. The extent of lipid peroxidation is higher in cooked and fried foods (Frankel, 1984; Kanner and Lapidot, 2001).

Oxidized cholesterols are present in processed foods (dehydrated, irradiated, or exposed to high temperature) and in all cholesterol-containing food that are preserved in the presence of oxygen (Leonarduzzi et al., 2002). An estimate of the

mean intake of oxysterols with diet is of some mg/day per person (van de Bovenkamp et al., 1988).

Formation of Oxidized Lipids in the Gastrointestinal Tract

Products of lipid oxidation can also be generated in the gastrointestinal tract during digestion. The alkaline pH in the intestine and the presence of already oxidized lipids and catalytic transition metals can create conditions promoting free radical formation (Terao et al., 1995). Further, Kanner and Lapidot, mimicking *in vitro* what happens in the gastric environment, demonstrated that the low pH of gastric fluid promoted lipid peroxidation catalyzed by metmyoglobin or iron ions. Incubating heated meat (where both metmyoglobin and lipid hydroperoxides are present) in simulated gastric fluid resulted in a great increase of lipid hydroperoxides and malondialdehyde concentration (Kanner and Lapidot, 2001). This event was prevented by the addition of dietary antioxidants (Gorelik et al., 2005).

Gastrointestinal Tract Defense System

The gastrointestinal tract represents the interface between the organism and the external environment and, for this reason, is particularly exposed to xenobiotics (i.e. drugs, toxins, oxidant agents) (Halliwell et al., 2000). Thanks to its complex physic and biochemical defense system, the gastrointestinal tract is the main site of detoxification after the liver (Liska, 1998). Within the defense system, it is important to mention the activity of phase I and phase II enzymes (the family of cytochrom P450, glutathione S-transferase, glucuronidases) (Bjorkhem and Diczfalusy, 2002; Kaminsky and Zhang, 2003), antioxidant enzymes (superoxide dismutase, catalases, glutathione peroxidases, etc), metal-chelating proteins (metallothioneine, ferritine, etc) and molecules endowed with antioxidant action (glutathione, thioredoxine, etc.).

Dietary Oxidized Lipids Absorption

Despite the efficient gastrointestinal defense system, a minimum amount of dietary oxidized lipids can escape reduction, penetrate into plasma and be incorporated into lipoproteins. Several *in vivo* studies, both on animals and humans, demonstrate that lipid hydroperoxides, cholesterol oxides, and other by-products of lipid peroxidation from lipid hydroperoxide-enriched food escape the reduction in gastrointestinal tract and are found in blood in the postprandial phase (Naruszewicz et al., 1987; Staprans et al., 1993; Wolff and Nourooz-Zadeh, 1996; Vine et al., 1997; Grootveld

et al., 1998). It may be interesting to mention that plasma lipid hydroperoxides increase even after a meal not enriched of lipid peroxides (Ursini et al., 1998; Natella et al., 2002).

A recent human study demonstrates that oxidized cholesterol, when ingested, is incorporated into chylomicrons and chylomicron remnants, and then transferred within the plasma compartment from exogenous to endogenous lipoproteins (VLDL, LDL and HDL), LDL displaying the highest levels of incorporation (Staprans et al., 2003). According with these results, a postprandial increase of both LDL susceptibility to oxidation (Lechleitner et al., 1994; Natella et al., 2001) and LDL(-) concentration was observed after the ingestion of a "high-peroxides" meal (Natella et al., 2007).

Can Dietary Antioxidants Affect Postprandial Increase of Lipid Hydroperoxides?

Dietary antioxidants play a key role in reducing the postprandial increase in lipid hydroperoxides (Fig. 16.1). Ursini et al., in an *in vivo* study, demonstrated that the consumption of red wine can effectively reduce postprandial increase of plasma lipid peroxides (Ursini and Sevanian, 2002). It was also observed that the addition of wine to a "high-peroxides" meal preserves plasma antioxidants and inhibits the increase of LDL susceptibility to oxidative modification (Natella et al., 2001). Similar results were obtained in a study where the test meal was supplemented with proanthocyanidins (Natella et al., 2002). In this study, it was observed that a "high-peroxides" meal induces an increase of lipid peroxides in plasma and in chylomicrons, which was not observed when the same meal is supplemented with proanthocyanidins.

Antioxidants could minimize the postprandial increase of lipid hydroperoxides (in plasma and lipoproteins):

1. Preventing the formation of new peroxides or reducing dietary lipid hydroperoxides in the digestive tract, independently on their bioavailability. In fact, in the presence of catalytic metals, the decomposition of dietary lipid hydroperoxides in the stomach leads to an increase of their concentration. In the presence of phenolic antioxidants not only peroxidation is prevented, but also original hydroperoxides disappear since they are decomposed in a peroxidatic reaction where polyphenols act as hydrogen donors (Kanner and Lapidot, 2001).
2. After absorption, contributing to the plasma antioxidant capacity by reducing lipid hydroperoxides and/or sparing endogenous antioxidants at plasma level and in LDL.
3. Inducing the activity of detoxifying enzymes, thus reducing dietary lipid hydroperoxides in gut or in liver. Several *in vitro* (carried out on isolated enzymes or on cultured enterocytes) and *in vivo* studies (carried out on rats, mice and humans), demonstrate that dietary antioxidants, such as phenolic compounds, can modulate the activity of many detoxifying enzymes, e.g. phase I and phase II enzymes and antioxidants enzymes (Moon et al., 2006). In animal models the supplementation of diet with phenolic compounds induces the increase of

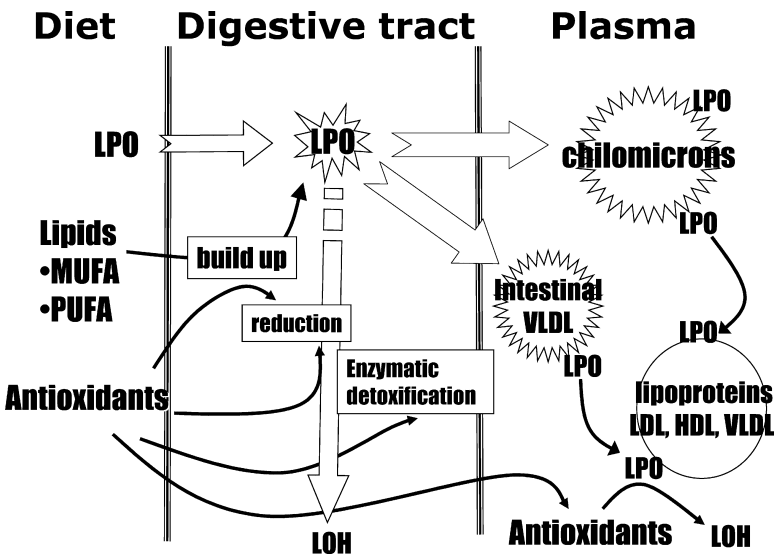

Fig. 16.1 Role of dietary lipids and antioxidants: from food to lipoproteins. Schematic representation of the postprandial events bringing to LPO incorporation in lipoproteins. Possible mechanisms by which dietary antioxidants reduce or prevent the LPO postprandial appearance in plasma. (LPO: lipid hydroperoxides; LOH: lipid alcohol; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; VLDL: very low density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein)

the activity of glucuronidase (van der Logt et al., 2003), glutathione S-transferase (Hurst et al., 1998), glutathione peroxidases, glutathione reductase, super oxide dismutase and catalase (Cai and Wei, 1996; Appelt and Reicks, 1999) in the gastrointestinal tract.

With this regards, we studied if an "enhancement" of the gastrointestinal antioxidant system could help in preventing the postprandial increase in LDL susceptibility to oxidation and LDL(-) formation. We found that a 10-day selenium supplementation significantly inhibits the meal-induced increase in LDL susceptibility to oxidation, LDL(-) concentration and plasma MDA (Natella et al., 2007). Our suggestion was that selenium optimized the function of the gastrointestinal system for the removal of food-derived hydroperoxides preventing their absorption, increasing the activity of selenium-dependent gastrointestinal glutathione peroxidases.

Conclusion

The antioxidants/prooxidants balance in the postprandial phase can represent a novel approach to reinterpret the oxidative hypothesis of atherosclerosis.

In this view, antioxidants may attenuate the risk of CVD by reducing the potential prooxidant effect of lipid hydroperoxides rich foods, and relevance emerges on the modality of antioxidants consumption, trivially just "with or without food".

Biography of the corresponding author

Dr. Fausta Natella, born in Italy in 1971, Degree in Biology, PhD on "Cellular and Molecular Physiology", researcher at the National Institute for Research on Food and Nutrition, in Rome. Research interest: dietary modulation of the oxidative stress in humans. Particular interest is given to the study of biological and pathological effects of dietary elements that are preventive (antioxidants) and inductive (oxidizable and/or oxidized lipids) on the oxidative stress.

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Chapter 17
F_2-Isoprostanes: Markers and Mediators of an Imbalanced Redox Status in the Liver

Concetta Gardi*, Beatrice Arezzini, Cinzia Signorini, Daniela Vecchio, Barbara Monaco, and Mario Comporti

Abstract Previous studies suggested a relation between oxidative stress and collagen hyperproduction. Carbon tetrachloride-induced hepatic fibrosis has been considered to be mediated by aldehydic lipid peroxidation products. In the present study we investigated whether collagen synthesis is induced by F_2-isoprostanes, the most proximal products of lipid peroxidation and known mediators of important biological effects. By contrast with aldehydes, F_2-isoprostanes act through receptors able to elicit definite signal transduction pathways. In a rat model of carbon tetrachloride-induced hepatic fibrosis, plasma F_2-isoprostanes were markedly elevated for the entire experimental period; hepatic collagen content also increased. When hepatic stellate cells from normal liver were cultured up to activation (expression of α-SMA) with F_2-isoprostanes in the concentration range found in the *in vivo* studies (10^{-9} to 10^{-8}M), a striking increase in DNA synthesis, in cell proliferation and in collagen synthesis was observed. Total collagen content was similarly increased. All these stimulatory effects were reversed by the specific antagonist of thromboxane A_2 receptor, SQ29548. Moreover, F_2-isoprostanes markedly increased the production of transforming growth factor-β1 by U937 cells, considered a model of liver macrophages. The data provide evidence for the possibility that F_2-isoprostanes generated by lipid peroxidation in hepatocytes mediate hepatic stellate cell proliferation and collagen hyperproduction seen in hepatic fibrosis.

Keywords 8-epi-$PGF_{2\alpha}$, hepatic stellate cells, isoprostane receptor, liver fibrosis, thromboxane A_2 receptor, SQ29548

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G. Valacchi, P.A. Davis (eds.) *Oxidants in Biology*,

Introduction

A great deal of information has been obtained during the last decades on oxidative stress in animal models of experimental pathology. A transfer of the animal studies to human pathology could be therefore anticipated, since oxidative and peroxidative processes could be rightly implicated in the explanation of diseases and syndromes. In the bulk of these studies, evidence has been searched for the occurrence of oxidation products of lipid, proteins and nucleic acids and/or for the decrease of the antioxidant level.

However, in human pathology, the available biological samples are generally restricted to blood, urine or expired air small bioptical samples. Therefore, the great number of studies on oxidative stress, although bringing forth very interesting results, had not, however, allowed the evaluation of oxidative stress in human pathology, at least on a large scale. This was due to the fact that a reliable and non invasive method to monitor lipid peroxidation *in vivo* with the only use of blood and urine was lacking. In fact, all the methods used (detection of conjugated dienes, lipoperoxides, aldehydes) are poorly reproducible and unreliable when carried out in plasma due to the extreme reactivity and instability of the species to be dosed; or they imply the use of tissues and are therefore hardly feasible in humans. Some years ago, however, the group of Morrow and Roberts (Morrow et al., 1990a, b) demonstrated the production of a series of prostaglandin F_2-like compounds named F_2-isoprostanes, which are formed *in vivo* and *in vitro* by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid (Morrow et al., 1992a), a pathway which is independent of the cyclooxygenase pathway. Because isoprostanes, initially formed *in situ* on phospholipids (Morrow et al., 1992a) are released into the circulation and because these prostanoids are less reactive than other lipid peroxidation products such as lipid hydroperoxides and aldehydes, they can be found more easily in plasma and urine. Therefore, F_2-isoprostanes can nowadays be considered as the markers of oxidative stress (lipid peroxidation) and can be used to evaluate oxidative status in a number of human pathologies (Morrow and Roberts, 1996; Roberts and Morrow, 2000).

Isoprostanes as Agonists of Biological Effects

Besides being markers of oxidative stress, F_2-isoprostanes appeared to be mediators of important biological effects. Some of these effects are summarized in Table 17.1.

The first one to be revealed (Takahashi et al., 1992; Fukunaga et al., 1993, 1997) was the vasoconstriction of renal glomerular arterioles, as demonstrated by the direct infusion of 8-epi-$PGF_{2\alpha}$ (the most represented isomer of the series) into the renal artery. It appears to act through the activation of receptors analogous to those for thromboxane A_2 (TxA_2) (Takahashi et al., 1992). This effect is believed to be

Table 17.1 Effect of F_2-isoprostane in various pathological conditions

Disease	Target cells	Effects	References
Hepatorenal syndrome 1993; 1997;	Renal glomerular arterioles	Vasoconstriction	Takahashi et al., 1992; Fukunaga et al., Fukunaga et al., Holt et al., 1999
Hepatorenal syndrome	Muscle vascular cells and endothelial cells	Increased DNA synthesis and cell proliferation	Takahashi et al., 1992; Yura et al., 1999
Retinopathy of prematurity	Retinal endothelial cells	Endothelin-1 release; increased Ca^{2+} transients; increased thromboxane production and vasoconstriction	Lahaie et al., 1998
Streptozotocin-induced diabetes	Glomerular cells	Increased production of TGF-β1	Montero et al., 2000
Hepatic fibrosis	Hepatic stellate cells	DNA and collagen synthesis	Comporti et al., 2005

very important in the explanation of the Hepato-Renal Syndrome (Holt et al., 1999), in which the initial production of F_2-isoprostanes would occur in the liver; the latter would induce vasoconstrictory effects in the kidney resulting in the full feature of renal failure.

Furthermore, 8-epi-$PGF_{2\alpha}$ has shown to increase DNA synthesis and cell proliferation in muscle vascular cells and endothelial cells (Takahashi et al., 1992; Yura et al., 1999). Also these effects are probably due to activation of receptors related to those of TxA_2 (TxA_2r). Eight-epi-$PGF_{2\alpha}$ potently contracts retinal vessels, elicits endothelin-1 release from retinal preparation, increases thromboxane production in the retina and cultured endothelial cells and also increases Ca^{2+} transients in retinal endothelial cells (Lahaie et al., 1998). All these effects may play a role in the retinopathy of prematurity, since it has been suggested that oxidative stress such as reoxygenation after an asphyxic episode is frequently encountered in premature newborns (Reynaud and Dorey, 1994; Phelps and Rosenbaum, 1984) and the isoprostane-induced generation of thromboxane (Lahaie et al., 1998) may produce vasoconstriction with ischemia of the retina. Because ischemia and tissue hypoxia precede angiogenesis (Reynaud and Dorey, 1994; Phelps and Rosenbaum, 1984), the overall pathway may be relevant in the revascularization of the retinopathy of prematurity and, with the obvious changes, in the revascularization of the retinopathy of diabetes; in prematurity (Flynn et al., 1992; Comporti et al., 2004) and in diabetes (Davi et al., 1999; Gopaul et al., 1995) increased levels of plasma isoprostanes have been reported. Finally, 8-epi-$PGF_{2\alpha}$ seems to mediate the increased production of TGF-β1 in kidney mesangial and glomerular cells exposed to high ambient glucose such as that produced by streptozotocin-induced diabetes (Montero et al., 2000).

Isoprostanes as Possible Mediators of Hepatic Fibrosis

Increased deposition of collagen and other extracellular matrix-proteins is a feature of many chronic diseases affecting the liver, lung, arteries and nervous systems. In CCl_4 experimentally-induced hepatotoxicity, besides the classical steatonecrosis, fibrosis also develops and evolves into cirrhosis in the chronic intoxication.

The relation between oxidative stress and collagen hyperproduction was first proposed by Chojker et al. (1989) who observed that the addition of ascorbic acid and iron to cultured fibroblasts strongly stimulates lipid peroxidation and, at the same time, the production of collagen and procollagen alpha 1 (I) mRNA; the effects are reproduced by the addition to the same fibroblasts of malonaldehyde, which is one of the end products of lipid peroxidation.

One effective fibrogenic mediator is TGF-β which strongly stimulates the production of matrix proteins (particularly collagen) in various cellular types (Sporn and Roberts, 1985; De Bleser et al., 1997; Knittel et al., 1996). In chronic CCl_4 intoxication (Armendariz-Borunda et al., 1990) an increase in TGF-β mRNA occurs in nonparenchymal cells. Among liver nonparenchymal cells, hepatic stellate cells (HSC) (lipocytes or Ito cells) represent a very important source of production of matrix proteins. The activation of HSC, which occurs quickly even in culture, is accompanied by an increased production of matrix proteins, by cellular proliferation and by the typical change from the resting to the myofibroblast-like phenotype (expression of smooth muscle-alpha actin) (α-SMA).

It has been reported (Parola et al., 1993) that lipid peroxidation induced *in vitro* in human HSC or the treatment of the latter with 4-hydroxynonenal (the most cytotoxic aldehyde originating from lipid peroxidation (Benedetti et al., 1980), stimulates the expression of procollagen α1 (I) gene. Also, the treatment of various lineages of macrophages with 4-hydroxynonenal induces mRNA production and synthesis of TGF-β1 (Leonarduzzi et al., 1997). Finally, 4-hydroxynonenal added to cultured HSC up regulates the synthesis of procollagen α1 (I) (Zamara et al., 2004).

Since aldehydic lipid peroxidation products, have been reported, as mentioned above, to induce collagen expression and synthesis, it has been investigated (Comporti et al., 2005). whether analogous effects can be obtained with F_2-isoprostanes, the most proximal derivatives of peroxidizing arachidonic acid. One potential advantage of isoprostanes over aldehydes is that while aldehydes can interact with cellular macromolecules by addition processes only, isoprostanes could possess receptors able to induce specific signal transduction pathways.

An elevated level of plasma F_2-isoprostanes has been reported (Morrow et al., 1992b; Comporti et al., 2004) to be associated with acute CCl_4 intoxication. Furthermore, in a model of hepatic fibrosis induced by chronic CCl_4 intoxication, it has been observed (Comporti et al., 2005) that the levels of plasma F_2-isoprostanes (even if lower than that found in the acute intoxication) were maintained elevated for the entire experimental period, with a sharp peak during the early stages of the intoxication and a decrease to ~500 pg/ml later. This level was maintained up to the final period of treatment, when a new peak at the seventh and last week was seen. Such an increase in plasma F_2-isoprostanes level was accompanied by an

increase in hepatic collagen content (measured as hydroxyproline) and a progressive fibrosis that finally results in a clear cirrhosis (Comporti et al., 2005).

Effect of F_2-Isoprostanes on HSC

In parallel studies (Comporti et al., 2005), HSC were isolated from normal liver, cultured and treated with F_2-isoprostanes in the range of concentrations found in the *in vivo* studies in order to evaluate the effects of these prostanoids on cell proliferation and collagen synthesis. Addition of F_2-isoprostanes (10^{-10}–10^{-8} M) to cultured activated HSC induced a 2–4 fold increase in DNA synthesis (measured by tritiated thymidine incorporation) and an increase in cell number (231 ± 26% and 197 ± 42% with 10^{-9} M and 10^{-8} M isoprostanes, respectively, as compared to the vehicle control).

A similar 2.5 fold increase was also seen in collagen synthesis (Fig.17.1A) and total collagen content (Fig.17.1B). The relative collagen production, i.e. the percentage of collagen production over the total protein production was increased by 3.0–3.5 fold. The most active concentrations were between 10^{-8} and 10^{-9} M (= 10nM and 1nM), exactly as those found in the *in vivo* intoxication (3,000–500pg/ml = 9.0–1.5pmol/ml = 9.0–1.5nM).

On the other hand, 4-hydroxynonenal similarly added to cultured HSC at much higher (0.1–5 μM) concentrations stimulated DNA synthesis to a much lower extent (nearly 1.6 fold at 1 μM concentration) as compared to isoprostanes. No effect was seen on collagen synthesis (Comporti et al., 2005).

Effect of F_2-Isoprostanes on TGF-β1 Production by U937 Cells

It is generally believed (De Bleser et al., 1997; Knittel et al., 1996; Gressner, 1996; Pietrangelo, 1996) that activation of HSC follows the release of soluble factors (cytokines, mainly TGF-β) by cells of macrophage lineages such as Kupffer cells or liver macrophages. The effects of F_2-isoprostanes on TGF-β release by the human promonocyte cell line U937, assumed as a model of Kupffer cells or liver machrophages, was also studied (Comporti et al., 2005). A concentration dependent increase in the production of TGF-β1 by U937 cells stimulated with F_2-isoprostanes has been reported (Comporti et al., 2005). No effect was seen on TGF-β production by HSC.

Search for 8-epi-$PGF_{2\alpha}$ Receptors in HSC and for Subsequent Signal Transduction Pathways

In view of the results obtained with the addition of isoprostanes to cultured HSC, in further studies the binding of 8-epi-$PGF_{2\alpha}$ to HSC was investigated to get information on the receptor(s) involved in the binding. Since, as previously stated, most of

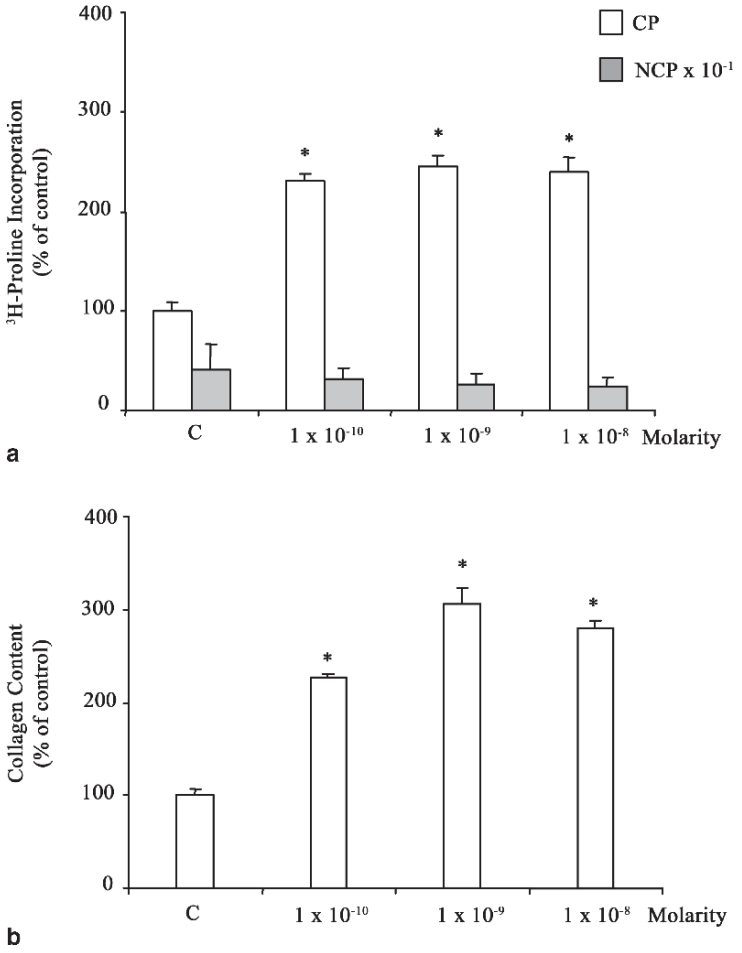

Fig. 17.1 Effect of F_2-isoprostanes on synthesis and total content of collagen in HSC treated with 8-epi-$PGF_{2\alpha}$. **(A)** Collagen synthesis was evaluated as ^{3}H-proline incorporation (dpm) per microgram of DNA; **(B)** Collagen content was evaluated in the media of HSC using the Kit Sircol Collagen Assay.All data are expressed as percentage, assuming the control as 100%. Data are the means ± s.e.m. of three experiments.$^*P < 0.05$ v*s* control. C, control; CP, collagen protein; NCP, noncollagen protein

the known effects of isoprostanes appear to be mediated through receptors identical or analogous to those of thromboxaneA_2, activated HSC were incubated in the presence of both 8-epi-$PGF_{2\alpha}$ and the specific TxA_2r antagonist SQ29548, in a molar ratio of 1:10. The stimulatory effect of 8-epi-$PGF_{2\alpha}$ on DNA synthesis was almost completely abolished by TxA_2r antagonist (Fig. 17.2). This could suggest that much of the effect of 8-epi-$PGF_{2\alpha}$ is mediated by TxA_2 receptors. Binding studies are carried out at the present to characterize the population of TxA_2 receptors on HSC.

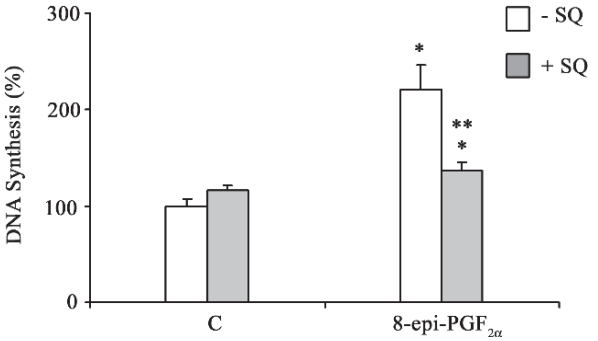

Fig. 17.2 Effect of F_2-isoprostanes and SQ29548 on DNA synthesis of HSC. Cell were treated with 10^{-9} M 8-epi-$PGF_{2\alpha}$ in the presence or absence of SQ29548 in a molar ratio 1:10. Results are expressed as percentage, assuming the control as 100%. Each sample was run in quadruplicate. Date are the mean ± s.e.m. of three experiments. $^{*}P < 0.05$ *vs* Control; $^{**}P < 0.05$ *vs* 8-epi-$PGF_{2\alpha}$ C, control; SQ, SQ29548

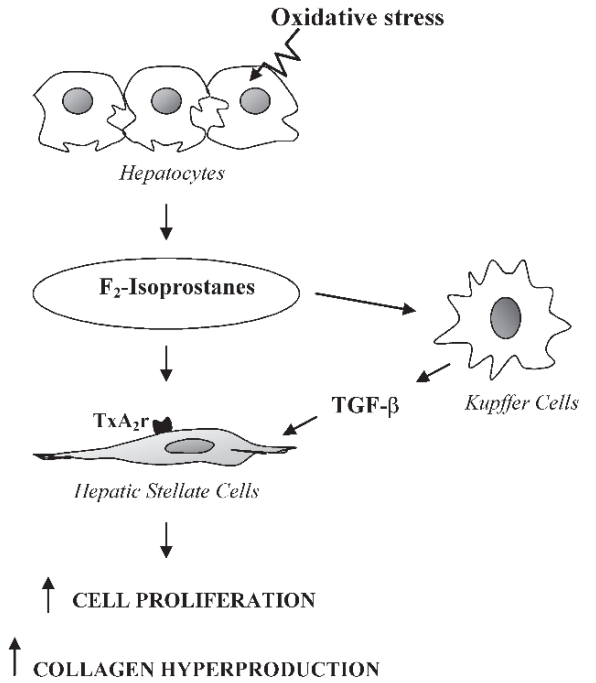

Fig. 17.3 Possible pathways of activation of hepatic stellate cells by F_2-isoprostanes. Oxidative stress-induced F_2-isoprostanes can directly stimulate hepatic stellate cells through the TxA_2r or indirectly stimulate hepatic stellate cells by increasing TGF-β release by hepatic macrophages

Conclusions

An imbalance of the oxidative status in the liver may lead to the release of F_2-isoprostanes by hepatocytes. The assumption that F_2-isoprostanes are generated in hepatocytes is supported by a 40 year long lasting literature (Comporti et al., 1965, 1985; Recknagel and Ghoshal, 1966; Recknagel and Glende, 1973; Slater, 1972) demonstrating (*in vivo* and *in vitro* studies) that CCl_4 induces lipid peroxidation in the liver and in particular in the endoplasmic reticulum of hepatocytes. Lipid peroxidation is induced in both acute and chronic (Comporti et al., 1971) CCl_4 intoxication and there is no indication that the process occurs in mesenchymal (non parenchymal) cells. Moreover, recently has been shown (Morrow et al., 1992b; Comporti et al., 2004) that both free and total (sum of free plus esterified) isoprostanes are dramatically increased in the liver after CCl_4 intoxication.

F_2-isoprostanes generated in hepatocytes by lipid peroxidation can stimulate HSC through receptors related to TXA_2r, inducing an hyperproduction of collagen and cell proliferation. Alternatively (or in addition), F_2-isoprostanes can indirectly stimulate HSC by increasing the production of TGF-β by Kupffer cells or macrophages (Fig. 17.3).

In conclusion, F_2-isoprostanes seems to play an important role in the stimulation of HSC and development of hepatic fibrosis. Future studies are needed to clarify the signaling pathways set into motion by these lipid peroxidation products.

List of Abbreviations

α-SMA	α-smooth muscle actin
HSC	hepatic stellate cells
SQ29548	[[1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(Phenylamino)carbonyl] hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid]]
TxA_2	thromboxane A_2
TxA_2r	thromboxane A_2 receptor

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