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Review

A study of free radical chemistry: their role and pathophysiological significance

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Oxygen is one of the most important molecules on Earth mainly because of the biochemical symmetry of oxygenic photosynthesis and aerobic respiration that can maintain homeostasis within our planet's biosphere. Oxygen can also produce toxic molecules, reactive oxygen species (ROS). ROS play a dual role in biological systems, since they can be either harmful or beneficial to living systems. They can be considered a double-edged sword because at moderate concentrations, nitric oxide ($NO^{\bullet}$), superoxide anion, and related reactive oxygen species play an important role as regulatory mediators in signalling processes. Many of the ROS-mediated responses actually protect the cells against oxidative stress and re-establish "redox homeostasis". On the other hand, overproduction of ROS has the potential to cause damage. In the recent decades, ROS has become a focus of interest in most biomedical disciplines and many types of clinical research. Increasing evidence from research on several diseases shows that oxidative stress is associated with the pathogenesis of diabetes mellitus, obesity, cancer, cardiovascular diseases, inflammation, ischaemia/reperfusion injury, obstructive sleep apnea, neurodegenerative disorders, hypertension and ageing.

Key words: reactive oxygen species, reactive nitrogen species, lipid peroxidation, catalytic ions, ascorbic acid

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INTRODUCTION

This is a good time for a broad overview that summarizes the main principles of redox regulation. We are now living in a particularly exciting time of oxidative stress research where information from different fields and independent approaches is falling into place and beginning to reveal an important portrait. In textbook style, this review describes the current knowledge and paradigms but does not discuss future research directions, historical controversies, or experimental models. Moreover, it was not within the scope of this review to deal with all the details. Even the more than 200 references cited here do not cover all relevant publications in the field.

Historic Background

The world of free radicals in biological systems was explored in 1956 by D. Harman who proposed the concept of free radicals playing a role in ageing (Harman, 1956). In 1977, Mittal and Murad provided evidence that the hydroxyl radical ($^{\bullet}OH$) stimulates activation of guanylate cyclase and formation of the "second messenger" cyclic guanosine monophosphate (cGMP). Since then, a large body of evidence has accumulated that living systems have not only adapted to a coexistence with free radicals but have developed various mechanisms for the advantageous use of free radicals in various physiological functions. There are several definitions of the term "free radical", as well as debates about whether the term "free" is unnecessary. "Radical" and "free radical" are frequently used interchangeably. Any reactive molecule with an unpaired electron is traditionally represented by the application of a superscript dot ($^{\bullet}$). Originally, "free" was used by chemists to distinguish between $R^{\bullet}$ and $R^{\bullet}$-$X^{\bullet}$, $R^{\bullet}$ being free "radical" and $R^{\bullet}$ in $R^{\bullet}$-$X^{\bullet}$ being a bound "radical". We accept a simple definition that a free radical is any atom (e.g., oxygen, nitrogen) or group of atoms or molecular species capable of independent existence that contains at least one or more unpaired electrons in the outermost shell configuration (Halliwell & Gutteridge, 1989). Free radicals are also known as reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Halliwell & Gutteridge, 2000). This unpaired electron usually gives a considerable degree of reactivity to the free radical.

MAJOR TYPES OF FREE RADICALS

Reactive Oxygen Species (ROS)

The causes of the poisonous properties of oxygen were obscure prior to the publication of Gershman's free radical theory of oxygen toxicity in 1954, which states that the toxicity of oxygen is due to partially reduced forms of oxygen (Gershman *et al.*, 1954). Oxygen-centred free radicals are those in which an unpaired electron is on an oxygen atom and contain two unpaired electrons in the outer shell. When free radicals "steal" an electron from a surrounding compound or molecule a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction contin-

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Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; *E*', reduction potential; cGMP, cyclic guanosine monophosphate; EPR, Electron paramagnetic resonance spectroscopy; AU, arbitrary units; G; RSNO, nitrosothiols; AH^-, ascorbate anion; DHA, dehydroascorbic acid; SOD, superoxide dismutase; LPO, lipid peroxidation; PUFAs, polyunsaturated fatty acids; CD, conjugated diene; GSH, glutathione; DABCO, 1,4-diazabicyclo[2.2.2]octane; R- alkyl group

ues and can be "thousands of events long" (Valko *et al.*, 2006).

Radicals derived from oxygen represent the most important class of radical species generated by organisms (Miller & Aust, 1988). Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). A major consequence of oxidative stress is damage to nucleic acid bases, lipids, and proteins, which can severely compromise cell functioning and viability or induce a variety of cellular responses through generation of secondary reactive species, ultimately leading to cell death by necrosis or apoptosis (Halliwell, 2001; Klaunig & Kamendulis, 2004; Stocker & Keaney, 2004). However, definitive evidence for this association has often been lacking because of recognised shortcomings with biomarkers and/or methods available to assess oxidative stress status in humans. Emphasis is now being placed on biomarkers of oxidative stress, which are objectively measured and evaluated as indicators of normal biological and pathogenic processes or pharmacologic responses to therapeutic intervention. "Redox" or oxidation-reduction reactions are those reactions that involve exchange of electrons between molecular species.

One of the most common and important oxygen free radicals is the superoxide anion ($O_2^{\bullet-}$), which can be dismutated to form hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($^{\bullet}OH$) in the presence of Fe^{2+} and trace metals (Valko *et al.*, 2005). At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al.*, 2006). Excessive generation of ROS may lead to stimulation of inflammatory process, secretion of chemotactic factors, growth factors, proteolytic enzymes, lipoxygenases, and cyclooxygenases, inactivation of antiproteolytic enzymes and activation of oncogenes and transcription factors (Kehrer, 1993; Jamieson, 1989). It is well established that oxygen free radicals and their metabolites can induce direct cell injury, which may activate a cascade of radical reactions promoting the disease. Permanent modification of genetic material resulting from these oxidative damage incidents represents the first step involved in mutagenesis, carcinogenesis and ageing (Dalle-Donne *et al.*, 2006; Jenner 2003; Sayre *et al.*, 2001; Santos *et al.*, 2005; Wang *et al.*, 1996). In the study of age-related increases in concentrations of oxidised biomolecules, disparities have been observed between intracellular and extracellular proteins. The concentrations of oxidative markers were found to increase with age in extracellular proteins more than in intracellular proteins (Linton *et al.*, 2001). This disparity might be explained by a difference in turnover between extracellular (hours to days) and intracellular proteins (minutes to hours). The difference in homeostatic control between extra- and intracellular proteins might also play a role. ROS-derived radicals operate at low but measurable concentrations in the cells. Their "steady-state" concentrations are determined by the balance between their rates of production and removal by various antioxidants.

The various roles of enzymatic antioxidants (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (vitamins C and E, carotenoids, lipoic acid and others) in the protection against oxidative stress can be found in numerous papers (Catani *et al.*, 2001; Hirota *et al.*, 1999; Miller *et al.*, 2005; Sharoni *et al.*, 2004). Oxidative stress-induced peroxidation of membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, such as the degree of fluidity, and can lead to inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability (Bailey *et al.*, 2003). Products of lipid peroxidation such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein) and isoprostanes are commonly used as biomarkers of oxidative damage (Cracowski *et al.*, 2002; Montuschi *et al.*, 2004). Proteins are major targets for ROS because of their high overall abundance in biological systems. Since proteins are primarily responsible for most of cell activities, their peroxidative damage by ROS is of particular importance. It has been estimated that proteins can scavenge the majority (50%–75%) of reactive species generated (Davies *et al.*, 1999). Exposure of proteins to ROS may alter every level of protein structure from primary to quaternary (if multimeric proteins), causing major physical changes in protein structure. Oxidative damage to proteins is induced either directly by ROS or indirectly by reaction of secondary by-products of oxidative stress and can occur via different mechanisms leading to peptide backbone cleavage, cross-linking and/or modification of the side chain of virtually any amino acid (Dean *et al.*, 1997; Stadtman & Berlett, 1997). Several mechanism *in vivo* produce ROS. $O_2^{\bullet-}$ results from mitochondrial electron transport chain leakage, ischaemia-reperfusion, autooxidation reactions, respiratory burst involving phagocitic cells, and continuous production of $O_2^{\bullet-}$ by the vascular endothelium to neutralise nitric oxide ($NO^{\bullet}$) (Young & Woodside, 2001).

The primary mechanism of $O_2^{\bullet-}$ production during exercise appears to be from mitochondria. H_2O_2 is produced by a variety of intracellular reactions, although the predominant pathway is by dismutation of $O_2^{\bullet-}$ by the enzyme superoxide dismutase (SOD) (McCord & Fridovich, 1988; Halliwell, 1999). By far the most widely known mechanism of formation *in vivo* of the extremely pernicious $^{\bullet}OH$ is the transition metal-catalysed (Fenton chemistry) decomposition of $O_2^{\bullet-}$ and H_2O_2. Within vascular endothelial cells the primary site of ROS generation is the electron transport chain of mitochondria. Although most of molecular oxygen is reduced at complex IV to water, 1–4% of the oxygen is incompletely reduced to $O_2^{\bullet-}$, which can yield other ROS *via* numerous enzymatic or non-enzymatic reactions (Zhang & Gutterman, 2007).

Superoxide anion ($O_2^{\bullet-}$)

Molecular oxygen has a unique electronic configuration and is itself a di-radical with two such unpaired electrons (Miller *et al.*, 1990). If a single electron is added to the ground-state O_2 molecule, it must enter one of the π antibonding orbitals. The product is called superoxide anion ($O_2^{\bullet-}$) and the production of $O_2^{\bullet-}$ occurs mostly within the mitochondria (electron transport chain) of a cell (Halliwell & Gutteridge, 1989; Cadenas & Sies, 1998). Physiological concentrations of $O_2^{\bullet-}$ approach 10 μM (Cuzzocrea *et al.*, 2001) and, compared with other free radicals, $O_2^{\bullet-}$ has a relatively long half-life (Benton *et al.*, 1976) that enables diffusion within the cell thereby increasing the number of potential targets. Beside $O_2^{\bullet-}$, other biologically relevant free radicals derived from oxygen are the perhydroxyl radical (protonated superoxide, $HO_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$), and free radical nitric oxide ($NO^{\bullet}$). With only one unpaired electron, superoxide is less of a radical than is O_2 itself, despite its "super" name. $O_2^{\bullet-}$, arising either through metabolic processes or following oxygen activation by physical irradiation, is considered the *primary* ROS and can further interact with other molecules to generate secondary ROS such

as lipid radicals, either directly or prevalently through enzyme or metal-catalysed processes (Valko *et al.*, 2005). As a redox-active species, $O_2^{\bullet-}$ can reduce some biological materials (e.g., cytochrome *c*) and oxidise others such as ascorbate. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical $O_2^{\bullet-}$ (Valko *et al.*, 2004; Kovacic *et al.*, 2005). Complex I can produce $O_2^{\bullet-}$ as well as hydrogen peroxide (H_2O_2) through at least two different pathways. During forward electron transfer and during reverse electron transfer only very small amounts of $O_2^{\bullet-}$ are produced, less than 0.1% of the overall electron flow (Murphy, 2009; Hansford *et al.*, 1997). Complex I might be the most important site of $O_2^{\bullet-}$ production within mitochondria, with up to 5% of electrons being diverted to superoxide formation (Muller *et al.*, 2008). Measurements on submitochondrial particles suggest an upper limit of 1–3% of all electrons in the transport chain "leaking" to generate $O_2^{\bullet-}$ instead of contributing to the reduction of oxygen to water (Boh *et al.*, 1982). $O_2^{\bullet-}$ is produced from both complexes I and III of the electron transport chain and once in its anionic form it is too strongly charged to readily cross the inner mitochondrial membrane. These two complexes are the main sites of mitochondrial $O_2^{\bullet-}$ production (Barja, 1999; Muller *et al.*, 2004).

Recently it has been demonstrated that complex I-dependent $O_2^{\bullet-}$ is exclusively released into the matrix and no detectable levels escape from intact mitochondria (Muller *et al.*, 2004). This finding fits well with the proposed site of the electron leak at complex I, namely the iron-sulphur clusters of the hydrophilic arm. In addition, experiments on complex III show direct extramitochondrial release of $O_2^{\bullet-}$ but measurements of hydrogen peroxide (H_2O_2) production have revealed that this could only account for less than 50% of the total electron leak even in mitochondria lacking CuZn–SOD. It has been proposed that the remaining 50% of the electron leak must be due to superoxide released to the matrix (Valko *et al.*, 2007). $O_2^{\bullet-}$, in comparison with $^{\bullet}OH$, is far less reactive with non-radical species in aqueous solution. It does react quickly, however, with some other radicals, such as $NO^{\bullet}$ or phenoxyl radicals formed by abstracting hydrogen from the –OH group of the amino acid tyrosine ($k = 1.5 \times 10^9$ $M^{-1}s^{-1}$) (Nagy *et al.*, 2009). The reactivity of $O_2^{\bullet-}$ with non-radicals varies depending on whether studies are carried out in organic solvents or in aqueous solution and pH is also an important determinant (Buettner & Jurkiewicz, 1993).

One of the most popular theories to explain O_2 toxicity has been the Gerschman's free radical theory of oxygen toxicity named the Superoxide Theory of O_2 Toxicity (Gershman *et al.*, 1954), which states that the toxicity of oxygen is due to partially reduced forms of oxygen and due to over-production of $O_2^{\bullet-}$ by components such as enzymes, auto-oxidation, haem proteins, mitochondrial electron transport, endoplasmic reticulum or bacteria (e.g., *Escherichia coli*).

$O_2^{\bullet-}$ in aqueous solution can act as a reducing agent, i.e. a donor of electrons, for example, it reduces the haem protein cytochrome *c* (Harel *et al.*, 1988);

$$\text{cyt } c\ (Fe^{3+}) + O_2^{\bullet-} \rightarrow O_2 + \text{cyt } c\ (Fe^{2+}) \quad (1)$$

$O_2^{\bullet-}$ can also act as an oxidising agent, e.g., it can oxidise ascorbate (Nishikimi, 1975; Fesseden & Verma, 1978);

$$AH_2 + O_2^{\bullet-} \rightarrow A^{\bullet-} + H_2O_2 \quad k = 2.7 \times 10^5\ M^{-1}s^{-1} \text{ at } 25^{o}C \text{ pH} = 7.4 \quad (2)$$

$O_2^{\bullet-}$ does not oxidise NAPH or NADH at measurable rates. However, it can interact with NADH bound to the active site of the enzyme lactate dehydrogenase to form an $NAD^{\bullet}$ radical (Petrat *et al.*, 2005);

$$\text{enzyme–NADH} + O_2^{\bullet-} + H^+ \rightarrow \text{enzyme–NAD}^{\bullet} + H_2O_2 \quad (3)$$

In summary, the tissue toxicity of $O_2^{\bullet-}$ generated extracellularly seems to be based on its direct reactivity with numerous types of biological molecules (lipid, DNA, RNA, catecholamines, steroids, etc.) and from its dismutation to form H_2O_2 and the concomitant reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}); reaction of these two products yields the highly toxic hydroxyl radical that may cleave covalent bonds in proteins and carbohydrates, cause lipid peroxidation, and destroy cell membranes. There are three strategies available to "detoxify" or to prevent formation of locally produced oxygen radicals:

— to deliver SOD (superoxide dismutase) or an SODm (superoxide dismutase mimetic) to the area
— to deliver catalase or a related peroxide scavenger or
— to chelate the trace iron that catalyses the reaction.

Hydroxyl radical ($^{\bullet}OH$)

$^{\bullet}OH$ is the neutral form of the hydroxide ion. It is short-lived (~10^{-9}s) (Pryor, 1966; Pastor *et al.*, 2000) but reacts very rapidly with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA, and organic acids. Indeed, $^{\bullet}OH$ is the most reactive oxygen radical known, with a highly positive reduction potential of +2310 mV (Koppenol & Butler, 1985; Buettner & Jurkiewicz, 1993; Frelon *et al.*, 2003; Jezowska-Bojczuk *et al.*, 2002; Vergely *et al.*, 2003). $^{\bullet}OH$ can be generated in biologically relevant systems by multiple reactions including:

Fenton chemistry (the interaction of copper or iron)

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

Haber-Weiss reaction (free radical formed from $O_2^{\bullet-}$ and H_2O_2)

$$O_2^{\bullet-} + H_2O_2 \rightarrow {}^{\bullet}OH + O_2 + HO^-$$

The Haber–Weiss reaction (Haber & Weiss, 1932) might provide a means to generate more toxic radicals. Although the basic reaction has a second order rate constant of zero in aqueous solution and thus it cannot take place under physiological conditions, the ability of iron salts to serve as catalysts was discussed by those authors. Because transition metal ions, particularly iron, are present at low levels in biological systems, this pathway (commonly referred to as The Iron-Catalysed Haber–Weiss Reaction) has been widely postulated to account for the *in vivo* generation of the highly reactive $^{\bullet}OH$. If $^{\bullet}OH$ radicals meet each other, they can form dimers, thus yielding hydrogen peroxide (Bielski *et al.*, 1984):

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \quad k = 5 \times 10^9\ M^{-1}s^{-1} \quad (4)$$

Although this reaction has a high rate constant very near the diffusion limit (diffusion-controlled), it is unlikely to occur *in vivo* because the steady-state concentration of $^{\bullet}OH$ is effectively zero (Hynes *et al.*, 1988). Thus, when produced *in vivo* $^{\bullet}OH$ reacts close to its site of formation within two molecular diameters (Pryor, 1966). The redox state of the cell is largely linked to the transition metals (iron and copper) and is maintained within strict physiological limits. Preventing metal ions from re-

dox cycling is an alternative mechanism to inhibit •OH formation. Reactions of •OH can be classified into three main types:
— hydrogen abstraction
— addition
— electron transfer.

The reaction of •OH with aromatic compounds often proceeds by addition. For example, •OH adds to the purine base guanine in DNA to form an 8-hydroxyguanine (8-OHdG) radical (Tokiwa *et al.*, 1999). Similarly, •OH can join across a double bond in the pyrimidine base thymine. The thymine radical then undergoes a series of further reactions, e.g. with O_2, to give a thymine peroxyl radical (Cadet *et al.*, 2002).

Peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals

$RO_2^{\bullet}$ and $RO^{\bullet}$ are good oxidising agents, since they have a tendency to accept electrons thereby undergo reduction themselves having highly positive $E^{\circ\prime}$ values (~1000–1600 mV) (Buettner, 1993; Buettner & Jurkiewicz, 1996a), although $RO^{\bullet}$ formed in biological systems often undergoes rapid molecular rearrangement to other radical species. Indeed, $HO_2^{\bullet}$ which is the protonated form (conjugate acid, $pK_a \sim 4.8$) of $O_2^{\bullet-}$ and is usually termed either hydroperoxyl radical or perhydroxyl radical can be regarded as the simplest $RO_2^{\bullet}$. For example, $RO_2^{\bullet}$ radicals oxidise ascorbate and NADH, the latter leading to $O_2^{\bullet-}$ formation in the presence of O_2:

$$RO_2^{\bullet} + NADH \rightarrow RO_2H + NAD^{\bullet} \quad (5)$$

$$NAD^{\bullet} + O_2 \rightarrow NAD^{+} + O_2^{\bullet-}\ k \approx 10^{9}\ M^{-1}s^{-1} \quad (6)$$

Aromatic alkoxyl and peroxyl radicals tend to be less reactive (Casimir, 2006), since electrons can be delocalized into the benzene ring. It has been demonstrated that $RO_2^{\bullet}$ initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent (Aikens & Dix, 1991). These reactions account for much of the stimulation of lipid peroxidation by transition-metal ions in biological systems. The carbon-centred radicals are capable of reacting directly with certain biological molecules including DNA and albumin -SH-groups. $RO_2^{\bullet}$ derived from azo-initiators can induce peroxidation of lipids (Bailey *et al.*, 2004) and can damage proteins, e.g. they inactivate the enzyme lysozyme. The ability of various antioxidants to prevent azo-initiator-induced lipid peroxidation or protein damage is frequently used to assess antioxidant activity, e.g., in the TRAP (Telomere Repeat Amplification Protocol) assay (Falchetti *et al.*, 1998).

Lipid peroxidation (LPO)

Lipid peroxidation (LPO) has been broadly defined by A. L. Tappel as "oxidative deterioration of polyunsaturated fatty acids (PUFAs) ", i.e. fatty acids that contain more than two carbon-carbon double bonds which are the target of ROS (Tappel & Dillard, 1981). Polyunsaturated fatty acids are abundant in cellular membranes and in low-density lipoproteins (LDL). The PUFAs allow for fluidity of cellular membranes. The membranes that surround cells and cell organelles contain large amounts of PUFA side-chains (Dietschy, 1998; Chu & Liu, 2004). Membrane lipids are generally amphipathic molecules, i.e. they contain hydrocarbon regions that tend to cluster together away from water, together with polar parts that like to interact with water. In animal cell membranes the dominant lipids are phospholipids, esters based on the alcohol glycerol (Bartz *et al.*, 2007). Some membranes, particularly plasma membranes, contain significant proportions of sphingolipids and of the hydrophobic molecule cholesterol. The commonest phospholipid in animal cell membranes is lecithin (phosphatidylcholine) (Cherry *et al.*, 2007). Free-radical processes are particularly prone to proceed *via* efficient chain reactions in which the initiating active radical is generated only in very low concentrations (Gutteridge, 1995; Hwang & Kim, 2007; Niki *et al.*, 2005).

A typical example of oxidation is the well-known autoxidation reaction which can occur, for example, in lipids when O_2 concentration is relatively high. The important point is that, when this type of process occurs, numerous product molecules can be formed for each initiating molecule (Sengpiel *et al.*, 1998; Im *et al.*, 2006; Triggaiani *et al.*, 2006). Thus, what might have been a minor generation of radicals becomes an event of real significance. When a chain-breaking antioxidant such as vitamin C is added to the solution, it scavenges $LO^{\bullet}$ and $LO^{\bullet}_2$ radicals and suppresses oxidation. The higher the ascorbic acid concentration, the longer the induction period and the smaller the rate of oxidation during the induction period. The length of induction period is directly proportional to the concentration of vitamin C (Niki, 1991).

Scheme of lipid peroxidation:

$$\begin{aligned}
&\text{Initiation:} && X^{\bullet} + H - CR_3\ (\text{alkyl group}) \rightarrow X - H + {}^{\bullet}CR_3\\
&\text{Chain:} && {}^{\bullet}CR_3 + O_2 \rightarrow R_3C - OO^{\bullet}\\
&&& \downarrow\\
&&& R_3C - OO^{\bullet} + H - CR_3 \rightarrow R_3C - OOH + {}^{\bullet}CR_3\\
&\text{Termination:} && X^{\bullet} + {}^{\bullet}CR_3 \rightarrow R_3C - X\\
&\text{Overall reaction: } R_3C - X + O_2 \rightarrow R_3C - OOH
\end{aligned} \quad (7)$$

Initiation of LPO is caused by an attack upon a lipid of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene (–CH_2–) group (Aruoma *et al.*, 1989). Fatty acids with one or no double bonds are more resistant to such attack than are the PUFAs. An adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom, especially if there is a double bond on both sides of the –CH_2–, yielding bis-allylic hydrogens. The reduction potential of a PUFA•/PUFA couple at pH 7 has been estimated at about $E^{\circ\prime} \approx +600$ mV (Buettner, 1993; Koppenol, 1990). Hence •OH, perhydroxyl radical-$HO_2^{\bullet}$, $RO^{\bullet}$($E^{\circ\prime} \approx +1600$ mV) and $RO_2^{\bullet}$($E^{\circ\prime} \approx +1000$ mV) radicals are thermodynamically capable of oxidising PUFAs and initiating peroxidation (Buettner, 1993).

By contrast, $O_2^{\bullet-}$ is insufficiently reactive to abstract H from lipids; in any case, its charge should preclude it from entering the hydrophobic interior membrane. The most likely fate of carbon radicals under aerobic conditions is to combine with O_2, especially as O_2 is a hydrophobic molecule that concentrates within the interior membranes. The double bond on the carbon atom weakens the carbon-hydrogen bond allowing for easy dissociation of the hydrogen by a free radical. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond. This in turn leaves the carbon with an unpaired electron and hence it becomes a free radical. In an effort to stabilise the carbon-centred free radical a molecular rearrangement occurs. The newly-arranged molecule is called a conjugated diene (CD). The CD then very easily reacts with

oxygen to form a $RO_2^{\bullet}$ (Poirier *et al.*, 2001). Formation of peroxyl radicals has been demonstrated during peroxidation of many membrane systems, using spin-trapping methods (Chamulitrat & Mason, 1989). $RO_2^{\bullet}$ are capable of abstracting H from another lipid molecule, i.e. an adjacent fatty-acid side-chain:

$$ROO^{\bullet} + CH \rightarrow -ROOH + C^{\bullet} \quad (8)$$

This is the propagation stage of lipid peroxidation (Svingen *et al.*, 1979). The carbon radical formed can react with O_2 to form another $RO_2^{\bullet}$ and so the chain reaction of LPO can continue. The $RO_2^{\bullet}$ combines with the hydrogen atom that it abstracted to give a lipid hydroperoxide (LOOH) (Girotti, 1998). This is sometimes shortened to lipid peroxide, although the latter term includes cyclic peroxides as well as LOOH species. A single initiation event can lead to formation of multiple molecules of peroxide as a result of the chain reaction. Another complexity is that the initial H abstraction from PUFA can occur at different points on the carbon chain. Thus peroxidation of linoleic acid gives two hydroperoxides, while that of linolenic acid gives four. Peroxidation of arachidonic acid gives six lipid hydroperoxides, while that of docosahexaenoic acid gives ten (Tallman *et al.*, 2001).

Decomposition of lipid peroxides by heating at high temperatures or by exposure to iron or copper ions generates a hugely complex mixture of products, including epoxides, saturated and unsaturated aldehydes, ketones and hydrocarbons. Thermal homolysis of the O–O bond yields radicals, which can attack other hydroperoxides and PUFAs (Halliwell, 2006):

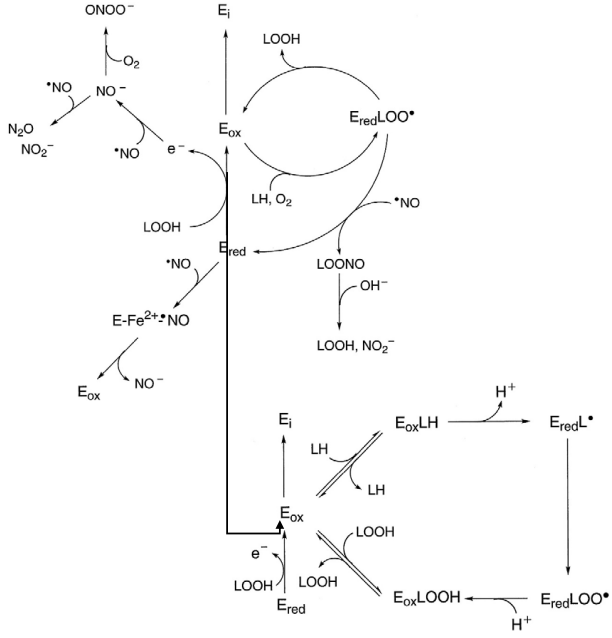
$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH \quad (9)$$

Generation within membranes and lipoproteins of $RO_2^{\bullet}$ and $RO^{\bullet}$, aldehydes and other products of LPO can cause severe damage to the proteins present, e.g., peroxidation of hepatocyte or erythrocyte membranes causes formation of high-molecular-mass protein aggregates within the membrane (Goebel & Schneider, 1981). The surface receptor molecules that allow cells to respond to hormones and cytokines can be inactivated during LPO, as are enzymes such as glucose-6-phosphatase, glycerol-3-phosphate acyl transferase (Thomas & Poznansky, 1990) involved in maintenance of correct balance within cells. Potassium channels can also be damaged as a consequence of lipid peroxidation (Han *et al.*, 2002).

In general, the overall effects of LPO are to decrease membrane fluidity, make it easier for phospholipids to exchange between the two monolayers, increase the leakiness of the membrane bilayer to substances that do not normally cross it other than through specific channels, and inactivate membrane-bound enzymes. Cross-linking of membrane proteins decreases their lateral and rotational mobility. Continued oxidation of fatty-acid side-chains and their fragmentation to produce aldehydes and hydrocarbons such as pentane will eventually lead to loss of membrane integrity. Peroxidation of erythrocyte membranes causes them to lose their ability to change shape and squeeze through the smallest capillaries (Goebel & Schneider, 1981; Thomas & Poznansky, 1990).

Oral administration of large doses of peroxidised fatty acids or lipids to animals leads to disease processes (Armstrong *et al.*, 1984), e.g., heart damage, fatty liver or damage to lymphoid tissues. Not only enzymes but also receptors and transport proteins can be important early targets of oxidative damage. Damage can occur to proteins involved in maintenance of essential ion gradients between cells and extracellular fluids, such as the Ca(II)-ATPase and Ca(II)/Na(I) exchange systems that keep intracellular Ca(II) levels much lower than extracellular levels. The Na(I), K(I)-ATPase system in the plasma membranes keeps intracellular K(I) high and Na(I) low when compared with levels in extracellular fluids.

In summary, LPO is a free radical-related process that in biological systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or non-enzymatically. This latter form is associated mostly with cellular damage as a result of oxidative stress, which also involves cellular antioxidants. It is an important process in oxygen toxicity. Free radicals are generated in a number of metabolic reactions, and lipids containing polyunsaturated fatty acids in cell membranes and lipoproteins are targets of free radical-mediated oxidation (Halliwell & Gutteridge, 2000). This process of LPO consists of three components: initiation in which free radicals are formed, propagation of the radical chain reactions, and termination (Miller & Aust, 1988; Porter, 1984; Buettner, 1993). Iron or other catalytic metals usually are required to initiate LPO and the free radicals generated, such as a lipid-derived carbon-centered radical, lipid peroxyl radical ($LOO^{\bullet}$), and lipid alkoxyl radical ($LO^{\bullet}$), propagate the chain reactions. Termination of LPO occurs when the free radicals in the chain propagation step react with other free radicals or antioxidants to form nonradical short-chain hydrocarbon compounds (Scheme 1). Free radicals inflict damage by attacking polyunsaturated fatty acids, thus setting off a deleterious chain reaction that ultimately results in their disintegra-



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Scheme 1. Potential sites of nitric oxide reaction during 15-LOX oxidation of lipid (adapted from O'Donnell *et al.*, 1999).
Three sites of potential NO· reaction are shown. (i) During peroxide (LOOH) activation of LOX, 2 mol of NO· are consumed *via* reaction with an electron (e^-) released from the ferrous enzyme (E_{red}) to form nitroxyl anion (NO^-). Secondary reactions of NO^- will consume further NO· molecules, for example, reaction of NO^- with O_2 or with further NO· molecules, as shown. (ii) During dioxygenase turnover, NO· is consumed through reaction with $E_{red}LOO^{\bullet}$ to form reduced inactive enzyme (E_{red}) and an organic peroxynitrite (LONOO). This hydrolyses to the hydroperoxide (LOOH) and nitrite (NO_2^-). (iii) At higher NO· concentrations a ferrous nitrosyl complex is formed, which slowly decomposes, yielding active enzyme. NO is consumed by enzymatic turnover of LOX.

tion into malondialdehye, 4 hydroxy-2-nonenal and other harmful by-products. Polyunsaturated fatty acids (PUFA) are believed to be one of the keys for understanding the damage that can be done to cells by free radicals.

Oxidative stress enhances LPO, implicated in the promotion and progression stages of carcinogenesis and development of atherosclerosis, in particular under conditions of chronic inflammation and infections, and in worsening the initial tissue injury caused by ischaemic or traumatic brain damage. Common pathways involve biologically relevant ROS and RNS, which can be generated by biochemical redox reactions, phagocytes, and upregulation of stress-response enzymes like cyclooxygenase-2, lipoxygenases and inducible nitric oxide synthase (Bartsch & Nair, 2006). The resulting oxidative stress is currently implicated in over 100 human and animal diseases, including cancer, inflammatory, infectious, cardiovascular and neurological diseases. Exocyclic etheno- and propano-DNA adducts, which are formed by LPO endproducts such as 4-hydroxy-2-nonenal and malondialdehyde, are strong pro-mutagenic DNA lesions causing point mutations (Barbati *et al*., 2010; Valko *et al*., 2005).

Ascorbate radical ($A^{\bullet-}$)

Human and animal tissues contains many antioxidants, water-soluble compounds such as ascorbic acid and glutathione and lipid-soluble antioxidants such as α-tocopherol and ubiquinones. Ascorbic acid or vitamin C is especially significant. The most important reaction in the inhibition of oxidation by ascorbic acid must be scavenging of oxygen radicals such as hydroxyl, hydroperoxyl, lipid peroxyl and lipid alkoxyl radicals. It is reported that ascorbic acid reacts with $^{\bullet}OH$ at a rate constant of 7.2×10^{9}–1.3×10^{10} $M^{-1}s^{-1}$ depending on the pH (Bielski, 1982; Cabelli & Bielski, 1983), which shows that the reaction is very fast and diffusion-controlled. However, this does not mean that vitamin C is a specific $^{\bullet}OH$ scavenger, because $^{\bullet}OH$ is so reactive that it can react with many other compounds at about similar rate. Vitamin C reacts with $O_2^{\bullet-}$ at a rate constant of 10^4–10^5 $M^{-1}s^{-1}$ (Bielski *et al*., 1975) and scavenges $O_2^{\bullet-}$ at a rate constant of 1.6×10^4 $M^{-1}s^{-1}$ (Cabelli & Bielski, 1983). Singlet oxygen reacts with vitamin C at a rate constant of 8.30×10^6 $M^{-1}s^{-1}$ (Chou & Khan, 1983).

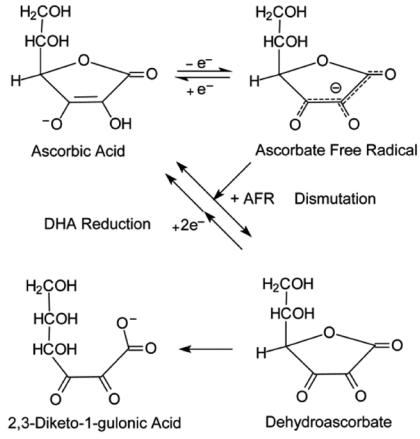
Oxidative damage to biomolecules is inhibited by antioxidants. Frei and co-workers (Frei *et al*., 1988; 1989, Frei & Gazianot, 1993) have shown that vitamin C is a powerful antioxidant preventing LPO in plasma exposed to various types of oxidative stress. It is known that ascorbate can switch from anti- to pro-oxidant activity *in vitro*, depending on its concentration and the presence of redox-active metal ions, and thus contribute to the formation of $^{\bullet}OH$, which in turn may cause lipid, DNA, or protein oxidation (Samuni *et al*., 1983; Bendich *et al*., 1986). No pro-oxidant effect of ascorbate was observed up to a concentration of 5 mM. This confirms that in blood plasma transition metal ions are bound tightly and are not available for free radical reactions. In human plasma ascorbate is the main water-soluble antioxidant (Frei *et al*., 1989). Compared to average concentrations of ascorbate in human blood plasma (27–51 μM) (Lentner, 1984), its levels in human tissues are generally far higher. Its concentration is particularly high in the cornea, lens, and aqueous humor of the eye (up to 1.5 mM) and in adrenal and pituitary glands (up to 2.5 mM). Brain, heart, liver, spleen, kidneys and pancreas also contain high concentrations of ascorbate (up to 0.8 mM) (Lentner, 1984).

Vitamin C effectively scavenges superoxide and other ROS (Bendich *et al*., 1986) and plays an important role in the regulation of intracellular redox state through its interaction with glutathione (Meister, 1994; Winkler *et al*., 1994). Ascorbate is an antioxidant because of the shared ability of the hydroxyl groups on carbons-2 and -3 to donate a hydrogen atom (both an electron and a proton) to a variety of oxidants, including oxygen- and nitrogen-based free radicals, peroxides and superoxide (Buettner, 1993). Ascorbate oxidation is reversible, which allows for recycling from its oxidised forms. Ascorbate can be one-electron oxidised by radicals and oxidants in two successive steps. The first one yields $A^{\bullet-}$, which owing to electron delocalisation over a conjugated tri-carbonyl system is surprisingly stable and can be detected at 10 nM concentrations in biological fluids by EPR (Buettner & Jurkiewicz, 1993; Coassin *et al*., 1991; Mehlhorn, 1991), avoiding the detection of artefactual signals arising from freeze/thaw processes (Pietri *et al*., 1990), and can subsequently be oxidised to dehydroascorbic acid (DHA), unstable and degraded to potentially toxic compounds. In order to prevent the accumulation of toxic ascorbate metabolites, cells are equipped with efficient regenerating systems. One way to achieve this is by transporting extracellular DHA to the cell interior after which it can be reduced to ascorbate. Due to the low reduction potential of the $A^{\bullet-}/AH-$ ($E^{\circ\prime}=+282$ mV), AH^- is able to give up one single electron to any free radical that can arise in biological systems or to regenerate oxidised biological radical scavengers such as vitamin E (Sharma & Buettner, 1993; Pietri *et al*., 1990; Vergely *et al*., 1998). Instead of undergoing further oxidation, two molecules of $A^{\bullet-}$ are thought to react and dismutate to form ascorbate and dehydroascorbate (Bielski *et al*., 1975). DHA is unstable at physiologic pH, with a half-life of about 6 minutes (Drake *et al*., 1942; Winkler, 1987). With hydrolysis of the lactone ring it is irreversibly converted to 2,3-diketo-1-gulonic acid (Bode *et al*., 1990; Chatterjee, 1970). Ascorbate loss due to ring-opening of DHA is wasteful of the vitamin, and cells have developed redundant mechanisms to recycle DHA back to ascorbate (Scheme 2). $A^{\bullet-}$ can be generated by an equilibrium reaction of ascorbate with dehydroascorbic acid, by transition metal-dependent (Me) oxidation of ascorbate or by autoxidation of the ascorbate di-anion (Eqn 10).

$$\text{Ascorbate}^- + \text{Dehydroascorbate} \leftrightarrow 2A^{\bullet-} + H^+$$

$$\text{Ascorbate} + Me^{n+} \rightarrow A^{\bullet-} + Me^{(n-1)+}$$

$$\text{Ascorbate}^{2-} + O_2 \rightarrow A^{\bullet-} + O_2^{\bullet-} \qquad (10)$$



Scheme 2. Ascorbate oxidation and recycling

The ascorbate anion (AH^-) can be considered the major endogenous water-soluble antioxidant in biological systems. Hence measurement of $A^{\bullet-}$ has been used as a non-invasive biomarker of oxidative stress in humans, e.g., in body fluids and reperfused organs. $A^{\bullet-}$ that is generated by donation of a single electron to a radical species is reduced back to ascorbate by NADH-dependent reductases present in microsomal membranes (Lumper *et al.*, 1967; Schulze *et al.*, 1970), as well as by cytosolic thioredoxin reductase (May *et al.*, 1998). $A^{\bullet-}$ reduction occurs with high affinity, with apparent K_m values for $A^{\bullet-}$ of 2 μM or less. Since ascorbate is primarily a one-electron donor, these processes likely account for the bulk of ascorbate recycling in the cell. If there is $A^{\bullet-}$ generated in excess of what the enzyme systems can handle, $A^{\bullet-}$ dismutation both regenerates ascorbate and forms DHA. The latter is reduced by redundant high capacity but low affinity systems in all mammalian cells. For example, endothelial cells and macrophages possess both GSH and NADPH-dependent mechanisms for recycling ascorbate (May *et al.*, 2001, 2003), although GSH-dependent ascorbate recycling has not been observed in HL-60 leukemic cells (Guaiquil *et al.*, 1997) or human skin keratinocytes (Savini *et al.*, 2000). GSH and other cellular thiols can also directly reduce DHA to ascorbate (Winkler *et al.*, 1994), although this process is not as efficient as enzyme-mediated reduction. Since no vascular cells can synthesise ascorbate directly, their intracellular ascorbate concentrations are determined by the combined actions of ascorbate transport into the cell and recycling within the cell.

Singlet oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) was first observed in 1924 and then defined as a more reactive form of oxygen (Halliwell & Gutteridge, 2000). It is the most important in biological systems with ample higher energy state molecular oxygen species but is not a radical since it contains no unpaired electrons. Although not a free radial, it can be formed in some radical reactions and also can lead to others since it is one of the most active intermediates involved in chemical and biochemical reactions. We need to remember that its lifetime strongly depends on which solvent it is generated in, e.g. in water it is about 3.8 μs, in hexane is 31 μs and in C_6F_6 (hexafluorobenzene) is about 3900 μs. (Foote *et al.*, 1995). It has been demonstrated that $^1O_2$ can react with many kinds of biological molecules such as DNA, proteins and lipids (Briviba *et al.*, 1997). Since oxygen is ubiquitous and efficiently quenches electronically excited states, $^1O_2$ is likely to be formed following irradiation in countless situations and involved in various chemical and biological process as well as in several disease processes which can lead to excessive singlet oxygen formation, especially many different porphyrias (these diseases are caused by defects in the biosynthesis of haem).

Both physical and chemical methods can generate singlet oxygen. Singlet oxygen is most often generated in the laboratory by so called photosensitization reactions (a certain molecule is illuminated with light, absorbs it and the energy raises the molecule into an excited state). Popular sensitizers include the dyes rose Bengal and toluidine blue, but also many biological compounds such as riboflavin, bilirubin or retinal. One well-established reaction used in the laboratory to generate singlet oxygen is the reaction of H_2O_2 and the hypochlorite ion OCl^-, the ionized form of hypochlorous acid (Kanofsky, 1989);

$$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2 \text{ (singlet)}$$

Singlet oxygen can react with other molecules either chemically or can transfer upon them its excitation energy, returning to the ground state, while the other molecule (the reaction partner) enters the excited state. The latter phenomenon is known as quenching of singlet oxygen. In the laboratory practice, several compounds are used as singlet oxygen scavengers. They include; azide, histidine, DABCO and 2-phenylisobenzofuran (Foote *et al.*, 1995). It is important to note that tocopherols quench and react with singlet oxygen and might protect membranes against this species (Traber, 1994). $^1O_2$ can react directly with carbon-carbon double bonds to give hydroperoxides and cause rapid peroxidation, however, the overall contribution of $^1O_2$ to lipid peroxidation is still uncertain.

Singlet oxygen is difficult to detect unambiguously but it can be detected by three different approaches:

— by scavengers: scavengers can inhibit reactions dependent on $^1O_2$; for example, azide, carotene, ascorbate, DABCO, thiols and histindine (Wilkinson & Brummer, 1981; Foote *et al.*, 1995);

— D_2O (deuterium oxide): D_2O can be used to detect $^1O_2$ since the lifetime of singlet oxygen is 10-fold longer in D_2O than in H_2O (Parker & Stanbro, 1984). So, if a reaction in aqueous solution is dependent on singlet oxygen, it becomes greatly potentiated when it occurs in D_2O instead of in H_2O.

— luminescence: as $^1O_2$ decays back to the ground state, some of the energy is emitted as light in the infrared region at 1268 nm (Krinsky, 1979).

Other detection methods include ESR, calorimetry, photo ionization and mass spectroscopy.

Ozone (O_3)

Ozone is a form of elemental oxygen and is an irritating, acrid-smelling, colourless gas. It is not a free radical but a triatomic oxygen molecule and is much more unstable than O_2. It is also a much more powerful oxidizing agent than ground-state oxygen. It is a very reactive gas and even at low concentrations is irritating and toxic. Ozone can be formed when a mixture of O_2 and NO_2 is exposed to bright light. The concentration of NO_2 in air is usually very low, because N_2 and O_2 do not react at normal temperatures. However, in the hot mixture of gases inside the cylinders of internal combustion engines, nitrogen and oxygen can react:

$$N_2 + O_2 \xrightarrow{\text{Temp}} 2NO$$

NO formed inside car engines reacts spontaneously with O_2 in air to form NO_2:

$$2NO + O_2 \rightarrow 2NO_2$$

Nitrogen dioxide is a red-brown gas that dissociates when irradiated with bright light:

$$NO_2\ NO + \xrightarrow{\text{light}} O$$

The oxygen atom formed in this process is extremely reactive and readily attaches to a molecule of O_2, forming ozone.

($O_2 + O \rightarrow O_3$) (Wellburn, 1994).

Ozone levels as low as 0.5 p.p.m can cause lung damage in a few hours and also induce inflammation, activating pulmonary macrophages and recruiting neutrophils to the lung. Damage to macrophages can also decrease resistance to infections. Ozone irritates the eyes and can oxidize proteins and lipids (Schmut *et al.*, 1994; Berlett *et al.*, 1996). Most of the inhaled O_3 probably reacts in

the human body with ascorbate, GSH or urate. These compounds seem especially effective as O_3 scavengers. Unlike $NO_2^{\bullet}$, O_3 does not appear to directly induce lipid peroxidation, although free radical products resulting from its reactions with PUFAs might be able to do so (Halliwell & Gutteridge, 2000).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is liquid and is toxic to most cells in the 10–100 μM range but is not a free radical, although it can be formed from two $^{\bullet}OH$ radicals:

$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2$ $k = 5 \times 10^9$ $M^{-1}s^{-1}$ (Bielski *et al.*, 1984)

Although this reaction has a high rate constant, it is unlikely to occur *in vivo* since the steady-state concentration of $^{\bullet}OH$ is effectively zero. Several enzymes can generate H_2O_2 *in vivo* e.g., xanthine, urate, and D-amino acid oxidases. In addition, any biological system that generates $O_2^{\bullet-}$ will also produce H_2O_2 by $O_2^{\bullet-}$ dismutation. However, H_2O_2 is only a weak oxidizing and reducing agent and is generally poorly reactive at physiological levels (Brodie & Reed, 1987) but is capable of inactivating several enzymes and oxidize keto-acids such as pyruvate and 2-oxoglutarate. It can react with iron and possibly copper to form much more damaging species such as $^{\bullet}OH$. It is important to know that H_2O_2 can degrade haem proteins including myoglobin, haemoglobin and cytochrome *c* (Gutteridge, 1986).

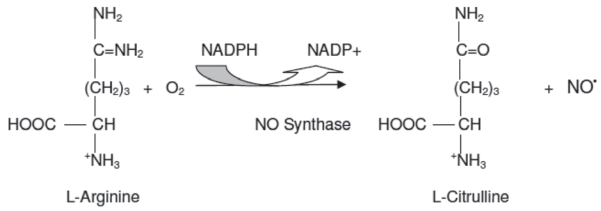
H_2O_2 has also been reported to stimulate synthesis of the chemokine MIP-1α by macrophages (Shi *et al.*, 1996). It can affect the proliferation of cells and facilitate phagocyte adherence to endothelium by up-regulating expression of such adhesion molecules as E-selectin, ICAM-1 and VCAM-1 (Lo *et al.*, 1993). It also leads to oxidative DNA damage by oxo-copper complexes (Zastawny *et al.*, 1995).

H_2O_2 production rates by cells and organelles are often in the range of a few nmoles per minute. Therefore, methods for measuring H_2O_2 in the biological material should be sufficiently sensitive. These methods include reaction with dichlorofluorescin diacetate, polarographic detection (O_2 electrode) and histochemical staining methods.

REACTIVE NITROGEN SPECIES (RNS)

Nitric oxide ($NO^{\bullet}$)

Nitric oxide ($NO^{\bullet}$) contains an unpaired electron in a π*2p antibonding orbital, thus it is a paramagnetic molecule and a free radical. $NO^{\bullet}$ is generated by specific nitric oxide synthases (NOSs), which metabolise arginine to citrulline with the formation of $NO^{\bullet}$ *via* a five electron oxidative reaction (Ghafourifar & Cadenas, 2005) (Scheme 3). It is moderately soluble in water (7.4 ml/dl at 0°C) and is more soluble in organic solvents, therefore it can readily diffuse through the cytoplasm and plasma membranes (Chiueth, 1999). In human tissue $NO^{\bullet}$ has a half-life of only a few seconds (~3–5 s) (Czapski & Goldstein, 1995; Ignarro *et al.*, 1993), because it binds avidly with haemoglobin (Hb). It has a greater stability in an environment with a lower oxygen concentration (half-life ~ 15s). $NO^{\bullet}$ reacts with molecular oxygen to produce nitrite (NO_2^-) (Ignarro *et al.*, 1993; Czapski & Goldstein, 1995). The kinetics of this reaction was studied by several research groups (Lewis & Deen, 1994; Kharitonov *et al.*, 1994) and has been found to follow



Scheme 3. Oxidation of L-arginine to L-citrulline plus $NO^{\bullet}$ (adapted from Ghafourifar & Cadenas, 2005)

second order kinetics with respect to $NO^{\bullet}$ and first order with respect to O_2. Inactivation of $NO^{\bullet}$ in aerobic solutions is therefore governed by a third-order law with an overall rate constant in the range of 6.3 to 11.5×10^6 $M^{-1}s^{-1}$ (Kharitonov *et al.*, 1994; Czapski & Goldstein, 1995). Consequently, $NO^{\bullet}$ is relatively stable at concentrations in the nanomolar range (half-life of ~80 min at 100 nM) but is rapidly inactivated at higher concentrations (half-life of ~50 s at 10 μM). Therefore, when $NO^{\bullet}$ is generated by a donor compound the autoxidation reaction will be negligible in the initial phase but will become progressively faster with increasing $NO^{\bullet}$ concentration. The maximum rate constant of $NO^{\bullet}$ disappearance was found $k = 2 \times 10^5$ $M^{-1}s^{-1}$ due to its binding with Hb (Hakim *et al.*, 1996). The 4:1 binding ratio between NO and Hb may be used as a tool to quantitate $NO^{\bullet}$ release in some biological assays. If the unpaired electron is removed by one-electron oxidation, nitrosonium cation (NO^+) is produced. One-electron reduction would give nitroxyl anion (NO^-). There are three isoforms of NOS:

neuronal NOS (nNOS) — Type I

inducible NOS (iNOS) — Type II

endothelial NOS (eNOS) — Type III

$NO^{\bullet}$ possesses other unique and important chemical properties that are also critical with respect to its biology. $NO^{\bullet}$ is almost exclusively a monomeric radical species at room temperature and pressure, so its reactions with other radicals, such as $O_2^{\bullet-}$ or alkyl radicals, are extremely facile. The inherent radical nature of $NO^{\bullet}$ and its reactions with other free radicals present in biological systems are important to some of its possible biological actions. For example, it has been proposed that $NO^{\bullet}$ can be toxic through reaction with superoxide ($O_2^{\bullet-}$) to generate peroxynitrite ($ONOO^-$), an oxidising agent capable of modifying a variety of biological molecules (Pryor *et al.*, 1994). The reaction of $NO^{\bullet}$ with O_2 generates species such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) that may have biological significance. Dinitrogen trioxide is a potent nitrosating agent that can alter protein function *via* nitrosation of critical nucleophilic residues (Eqn 11) (Kissner *et al.*, 1997).

$$
\begin{array}{l}
NO^{\bullet} + O_2 \rightarrow {}^{\bullet}OONO \qquad k = 1.9 \times 10^{10} M^{-1}s^{-1} \\
\quad \downarrow \\
{}^{\bullet}OONO + NO^{\bullet} \rightarrow ONOONO \\
\quad \downarrow \\
ONOONO \rightarrow NO_2 \rightarrow \text{Lipid peroxidation, vasodilatation at low } pO_2 \\
\quad \downarrow \\
NO_2 + NO^{\bullet} \rightarrow N_2O_3 \\
\quad \downarrow \\
N_2O_3 + \text{protein nucleophile} \rightarrow \text{Nuc-NO} + NO_2^-
\end{array}
\qquad (11)
$$

NO_2 is also a free radical species that, unlike NO•, is a fairly potent oxidant, $E^{\circ'}$ for the NO_2/NO_2^- couple = 1040 mV. There are a variety of potential reaction pathways by which NO_2 can cause oxidation of biological molecules: hydrogen atom abstraction, addition to unsaturated bonds, and electron transfer reactions (Huie, 1994). However, it has been postulated that the lipophilicity of NO• and O_2 allows their concentration to be high enough for this reaction to occur within cell membranes (Liu *et al.*, 1998). The chemistry of reactions of NO• and derived species with thiols appears to be an important aspect of NO• biology (Stamler, 1995). Modification of biological molecules by NO• may occur *via* reaction with a thiol function; for example, nitrosothiols (RSNO) can be formed by reaction with NO• or, more likely, NO•-derived species. RSNO formation can be accomplished by the mechanism shown in Eqn 13 with a nucleophilic thiol. Also, RSNO formation has been postulated to occur *via* direct reaction of NO• with a thiol followed by reaction of the thiol–NO• intermediate with O_2 (Eqns 12, 13) (Gow *et al.*, 1997).

$$NO^{\bullet} + RSH \rightarrow RSN^{\bullet}\text{-}OH \quad (12)$$
$$RSN^{\bullet}\text{–}OH + O_2 \rightarrow RSNO + O_2^{\bullet-} + H^+ \quad (13)$$

Finally, RSNO formation can occur *via* metal-mediated processes whereby the metal binds NO• and acts as an electron acceptor when reacted with a thiol (Wayland & Olson, 1974; Liu *et al.*, 1998; Wade & Castro, 1990; Ford 2004). This chemistry can be accomplished by, for example, ferric haem proteins, ultimately resulting in the generation of a ferrous nitrosyl adduct (M^n= Fe^{3+}-haem, M^{n-1}=Fe^{2+}-haem) in a process referred to as "reductive nitrosylation." As indicated above, NO• can react with $O_2^{\bullet-}$ to generate potentially deleterious oxidants such as peroxynitrite ($ONOO^-$) and NO_2 (Hsiai *et al.*, 2007; Patcher *et al.*, 2007). Indeed, it has been hypothesised that much of the toxicity associated with high levels of NO• is a result of formation of these oxidants. However, the ability of NO• to react with radicals also predicts that it can have antioxidant properties. That is, NO• can combine with another radical leading to termination of radical chain reactions. Probably the best example of the antioxidant properties of NO• is the effect it can have on lipid peroxidation (Wink *et al.*, 1993; Hogg *et al.*, 1993; Rubbo *et al.*, 1994, 1995; Struck *et al.*, 1995). Free radical chain processes occur in membranes because the membrane PUFA are susceptible to radical initiation processes and undergo the well-known PUFA radical chain autoxidaton (Pryor, 1966, 1976). Lipid alkoxyl (LO•) and peroxyl (LOO•) radicals are important intermediates in these lipid autoxidation processes. Nitric oxide can behave as an antioxidant or as a pro-oxidant in lipid autoxidations, depending on the experimental conditions (O'Donnell *et al.*, 1997; 1999; O'Donnell & Freeman, 2001; Hiramoto *et al.*, 2003). The antioxidant action of NO• occurs by chain-breaking termination reactions of NO• with LO• and LOO• radicals, as in Eqns 14,15.

$$LO^{\bullet} + NO^{\bullet} \rightarrow LONO \quad (14)$$
$$LOO^{\bullet} + NO^{\bullet} \rightarrow LOONO \quad (15)$$

The reaction of nitric oxide with LOO• results in the formation of an alkyl peroxynitrite (LOONO) which can homolyse to generate a geminate radical pair, NO_2 and an alkoxyl radical (LO•). Both of these radicals can initiate further radical reactions (Goldstein *et al.*, 2004; Zhao *et al.*, 2004). About 86% of these radical pairs from LOONO rapidly recombine to give unreactive alkyl nitrates ($LONO_2$) (Goldstein *et al.*, 2004), indicating that NO• can be an effective antioxidant. However, the remaining 14% of the radical pairs formed in the homolysis of LOONO become free NO_2 and LO• radicals (Eqs 16, 17) (Abuja *et al.*, 1997).

$$LOONO \rightarrow LO^{\bullet} + NO_2 \text{ (14\% yield)} \quad (16)$$
$$LOONO \rightarrow LONO_2 \text{ (86\% yield)} \quad (17)$$

Thus, 14% of the NO• and LOO• radicals that react to form LOONO get effectively converted into NO_2 and LO•, a much more reactive pair. For instance, in abstracting a hydrogen atom from a doubly allylic position, the rate constants for LOO• and LO• are 31 M^{-1} s^{-1} and ~ 10^7 M^{-1} s^{-1}, respectively (Abuja *et al.*, 1997), and although NO• cannot abstract a hydrogen atom from a doubly allylic position in a PUFA, the rate constant for the reaction of NO_2 with linoleic acid is 2×10^5 M^{-1} s^{-1} (Prutz *et al.*, 1985). In summary, through the sum of reactions 15–17, 86% of the LOO• and NO• radicals go on to form the stable product $LONO_2$, and 14% form the more reactive radicals LO• and NO_2.

Although NO_2 is a more reactive and a more powerful oxidant than is NO•, reactions of NO_2 with closed-shell molecules are relatively slow compared with those of •OH. However, NO_2 reacts rapidly with other radicals. This is one reason nitrotyrosine is formed from the reaction of NO_2 with protein tyrosyl radicals (Squadrito & Pryor, 2002).

$$NO_2 + O_2^{\bullet-} \rightarrow O_2NOO^- \quad (18)$$

Just as the rate of reaction of NO• with $O_2^{\bullet-}$ (Eqn 18) to form $ONOO^-$ is fast and close to the diffusion limit ($k = 3.8\text{–}6.7\times10^9$ $M^{-1}s^{-1}$) (Huie & Padmaja, 1993; Goldstein *et al.*, 1995; Kobayashi *et al.*, 1995), so is the reaction of NO_2 with $O_2^{\bullet-}$ to form peroxynitrate fast ($k = 4.5\times10^9$ $M^{-1}s^{-1}$) (Løgager & Sehested, 1993). This rate is approximately three times greater than that of the superoxide dismutase-catalysed dismutation of $O_2^{\bullet-}$. Therefore NO• is capable of outcompeting SOD for $O_2^{\bullet-}$ in conditions such as atherosclerosis, I-R, increased shear stress and exercise, in which production of NO• and $O_2^{\bullet-}$ is concomitantly increased. This implies that when both $O_2^{\bullet-}$ and NO_2 are present in the same environment, they will most likely react to form peroxynitrate. Because of the higher reactivity of NO_2 relative to NO•, the formation of peroxynitrate might be somewhat less likely than the formation of $ONOO^-$. Nevertheless, $ONOO^-$ appears to be formed under a variety of experimental conditions. Goldstein and collaborators (Goldstein *et al.*, 1998; Hodges & Ingold, 1999; Alvarez & Radi, 2001; Kirsch *et al.*, 2001), Uppu and collaborators (Uppu *et al.*, 2000) and others have implicated peroxynitrate during the *in vitro* decomposition of $ONOO^-$ in the presence of certain substrates that lead to the formation of $O_2^{\bullet-}$ and NO_2. Since $O_2^{\bullet-}$ is ubiquitous in aerobic organisms and $NO_2^{\bullet}$ can be formed endogenously by several pathways, the formation of peroxynitrate could be more widespread than presently recognised, and possible roles for $ONOO^-$ in oxidative biology should be studied further. The biochemistry of peroxynitrate is very different from that of $ONOO^-$. For example, in contrast to $ONOO^-$, for peroxynitrate it is the conjugate base (O_2NOO^-) that is kinetically unstable. Biological oxidations by peroxynitrate would then result from reactive intermediates that are formed during its decomposition and/or from direct oxidations by peroxynitrate. For example, theoretical and experimental data suggest that singlet oxygen (Eqn 19) may be produced during the unassisted decomposition of peroxynitrate (Goldstein *et al.*, 1998; Khan *et al.*, 2000; Martinez *et al.*, 2000; Merenyi

et al., 2000; Olson *et al.*, 2003). Peroxynitrate (O_2NOO^-) is a more powerful two-electron oxidant than peroxynitrite; their reduction potentials are $E^{\circ\prime}$(pH 7) = 1.59 V *vs* $E^{\circ\prime}$(pH 7) = 1.37 V, respectively (Goldstein *et al.*, 1998). Knowledge of the reaction kinetics of peroxynitrate with biological molecules is very limited. The kinetics of the reaction of peroxynitrate with methionine were studied recently, affording $k = 34\ M^{-1}s^{-1}$ at pH 7.4 and 25°C (Squadrito & Pryor, 2002), which compares with 181 $M^{-1}s^{-1}$ for the reaction of methionine with $ONOO^-$ under similar conditions (Pryor *et al.*, 1994). We are only beginning to understand the delicate interplay of the radical reactions and the generation of secondary reactive species downstream from the formation of NO• and how these reactions can integrate with biochemical processes.

$$O_2NOO^- \rightarrow {}^1O_2\ (\text{singlet}) + NO_2^- \qquad (19)$$

NO• is an abundant reactive radical that acts as an important oxidative biological signalling molecule in physiological processes including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation or immune regulation (Bergendi *et al.*, 1999). Interest in NO• arose because of the discovery of its multiple important physiological roles (Gow *et al.*, 2000; Stratford *et al.*, 1997). NO• normally functions not only to reduce platelet aggregation and leukocyte adhesion to the endothelium, but to promote vascular smooth muscle relaxation and reduce endothelial cell cytokine production. NO• concentrates in lipophilic cellular regions with a partition coefficient of 8:1, and can inhibit LPO e.g., by 15-lipoxygenase (15-LOX), a thousand times more potently than α-tocopherol (O'Donnell *et al.*, 1999). NO• has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu nitric oxide reacts with oxygen and water to form nitrate (NO_3^-) and nitrite (NO_2^-) anions (Bryan, 2006). Since NO• relaxes smooth muscle in blood vessel walls resulting in lower blood pressure, $O_2^{\bullet-}$ can be a vasoconstrictor by removing NO•. Thus, excessive vascular $O_2^{\bullet-}$ production could contribute to hypertension and vasospasm (Darsley-Usmar *et al.*, 1995; Bailey *et al.*, 2010).

A role for NO• has also been demonstrated in such human diseases as malaria where NO• appears to be partly involved in resistance to malarial infection (Riley *et al.*, 2006), in cardiovascular disease (Levy *et al.*, 2009; Strijdom *et al.*, 2009; Rudolph & Freeman, 2009), acute inflammation (Tilg & Moschen, 2008), cancer (Nanni *et al.*, 2009; Parkins *et al.*, 1995), neurodegenerative diseases (Knott & Bossy-Wetzel, 2009; Zhu *et al.*, 2007), and diabetes (Szabo, 2009; Dawson *et al.*, 2009; Sobrevia & Mann, 1997). In inflammatory conditions, such as occurs in reperfusion, inducible nitric oxide synthetase can increase nitric oxide concentration to thousands of times normal levels. During reperfusion, abnormally high amounts of $O_2^{\bullet-}$ convert almost all available NO• to $ONOO^-$ regarded as the agent causing most of the damage to brain capillary endothelial cells (Schaller & Graf, 2004). Damage to the endothelium not only increases oedema (tissue swelling due to "leakiness"), but causes endothelial protrusions "blebbs" which can block capillaries (Ono *et al.*, 1993). Moreover, NO• has been implicated in adult respiratory distress syndrome, septic shock, hypertension, thrombosis, renal failure, AIDS encephalopathy, bronchospasm, stroke and male impotence (Bailey *et al.*, 2009; Canning *et al.*, 2001; Adamson *et al.*, 1996; Maree *et al.*, 1994). Nitric oxide readily binds certain transition metal ions, and many of its physiological effects are exerted as a result of its initial binding to Fe^{2+} haem groups in the enzyme guanylate cyclase (Archer *et al.*, 1993). For example, NO• synthesised by the vascular endothelial cells that line the interior of blood vessels presumably diffuses in all directions, but some of it will reach the underlying smooth muscle, bind to guanylate cyclase and active it. As a result more cyclic GMP is made, which lowers intracellular free Ca^{2+} and relaxes the muscle, dilating the vessel and lowering blood pressure (Torfgard & Adler, 1994).

TRANSITION METAL IONS

All metals in the first row of the d-block in the periodic table, except zinc, contain unpaired electrons and can thus qualify as free radicals. The ability of transition metal ions to undergo facile one-electron oxidation or reduction makes them obvious potential chemical partners for reactions involving biological free radicals with advantageous or deleterious biological effects. Iron is by far the most abundant transition metal in the human body where it plays a role in oxygen binding (haemoglobin) and electron transport. It is therefore a potential mediator of •OH generation under normal physiological conditions in the iron/copper-catalysed Haber–Weiss reaction. Because of the central and essential roles of iron in the metabolism of all aerobic organisms, humans have evolved some peculiar ways of dealing with it. These peculiarities provide opportunities to cause diseases related to iron absorption, transport, and metabolism, as well as for the exacerbation of general mechanisms of disease involving free radical injury. Iron-catalysed generation of ROS has been implicated in the pathogenesis of many disorders including atherosclerosis (Salonen *et al.*, 1992) cancer (Loeb *et al.*, 1988), ischaemia reperfusion injury (White *et al.*, 1985; Katoh *et al.*, 1992) and conditions of iron overload (Burkitt & Mason, 1991), such as haemochromatosis, which is one of the most prevalent genetic disorders in Western countries. The excess iron induces cellular injury and functional abnormalities in hepatocytes by lipid peroxidation in lysosomal, mitochondrial and microsomal membranes (Britton *et al.*, 1987). Lipid peroxidation is a likely outcome of oxidative stress in biological systems, and its measurement is often used as a method of assessing the degree of oxidative damage.

The Fenton chemistry

Fenton chemistry is a prime example of damaging free radical reactions catalysed by transition metals. A mixture of H_2O_2 with a Fe^{2+} salt oxidises many different organic molecules and can provoke a whole series of radical reaction. *In vivo*, •OH are most likely generated from superoxide anions *via* an iron-catalysed Fenton reaction (Halliwell, 1982).

EPR (electron paramagnetic resonance spectroscopy) studies demonstrated the ability of iron bound to transferrin to catalyse •OH formation in the presence of $O_2^{\bullet-}$ (Bannister *et al.*, 1982). However, these results have subsequently been questioned (Baldwin *et al.*, 1984). Another source of iron is the intracellular pool, where iron is principally bound to ferritin. It has been recently shown *in vitro* that $O_2^{\bullet-}$ is capable of releasing iron from ferritin, thereby allowing the formation of •OH (Thomas *et al.*, 1985). Alternatively, the presence of low molecu-

lar weight iron chelates has been hypothesised (Jacobs, 1977).

The mechanism of Fe^{2+}-initiated Fenton reaction is as follows:

rate constant ($M^{-1}s^{-1}$) at pH = 5
(Walling, 1975; Kwan & Voelker, 2002)

a. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \; k = 5.7 \times 10^2 \; M^{-1}s^{-1}$
b. $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2/O_2^- + H^+ \; k = 2.6 \times 10^{-3} M^{-1}s^{-1}$
c. $H_2O_2 + {}^{\bullet}OH \rightarrow HO_2/O_2^- + H_2O \; k = 3.3 \times 10^7 M^{-1}s^{-1}$
d. $Fe^{3+} + HO_2/O_2^- \rightarrow Fe^{2+} + O_2 + H^+ \; k = 3.1 \times 10^5 M^{-1}s^{-1}$
e. $Fe^{2+} + {}^{\bullet}OH \rightarrow Fe^{3+} + OH^- \; k = 3.2 \times 10^8 M^{-1}s^{-1}$
f. $Fe^{2+} + HO_2/O_2^- \rightarrow Fe^{3+} + H_2O_2 \; k = 6.6 \times 10^6 M^{-1}s^{-1}$
g. $HO_2/O_2^- + HO_2/O_2^- \rightarrow H_2O_2 \; k = 2.3 \times 10^6 M^{-1}s^{-1}$
h. $^{\bullet}OH + HO_2/O_2^- \rightarrow H_2O + O_2 \; k = 8.9 \times 10^9 M^{-1}s^{-1}$
i. $^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \; k = 5.2 \times 10^9 M^{-1}s^{-1}$ (20)

The generally accepted mechanism for the Fenton process identifies $^{\bullet}OH$ as the active oxidising intermediate in the system (Haber & Weiss, 1932, 1934; Barb *et al.*, 1951; Walling, 1975). According to this mechanism (Eqn 20), the combination of ferrous iron and hydrogen peroxide induces a series of chain reactions initiated by the degradation of peroxide to $^{\bullet}OH$ and the hydroxide ion (reaction 20a). The hydroxyl radical serves as a chain carrier that may react with Fe^{2+}, H_2O_2, or any organic species present. These reactions may either propagate the chain cycle through the production of additional radicals (superoxide and its conjugate acid, reaction 20c) that can reduce Fe^{3+} back to Fe^{2+} (reaction 20d), or terminate the chain by oxidising Fe^{2+} (reactions 20e, f). When reaction 20b is taken into account, Fe^{3+} may also be considered a chain carrier, producing Fe^{2+} and superoxide, although this cycling occurs at a much slower rate ($k_2 \ll k_3, k_4$). Additional chain termination reactions include the minor radical-radical recombination pathways (reactions 20f, g, h). Depending on the type of organic species present, reactions with $^{\bullet}OH$ may either propagate the chain by producing $HO_2^{\bullet}/O_2^{\bullet-}$ or organic radicals capable of reducing Fe^{3+} directly, or terminate the cycle by scavenging $^{\bullet}OH$ (Walling, 1975). Extrapolated to biological systems, this information suggests that tissues exposed to an increased concentration of iron and/or copper (e.g., liberated from internal stores) may be prone to oxidative damage related to the metal ion-O_2-mediated free radical production. This might indeed be so, because transition metals, when liberated from intracellular stores, are probably present in reduced forms (Keyer & Imlay, 1996; Qian & Buettner, 1999). If it is taken for granted that an increased pool of low molecular mass iron and copper is present in ischaemic tissues (Nayni *et al.*, 1985; Gower *et al.*, 1989; Voogd *et al.*, 1992), it becomes apparent that reperfusion, which induces tissue injury in a mechanism involving $^{\bullet}OH$ (Bolli, 1991), creates particularly favourable conditions for the metal ion-O_2 reaction to occur. This is because:

— this reaction is fast enough to account for the reperfusion-induced production of free radicals;
— O_2, catalytic metals, and their reductants (including enzymatically produced $O_2^{\bullet-}$) are abundant;
— intracellular pH rapidly increases (Bauza *et al.*, 1995) in the reperfused tissue (Harris & Aisen, 1973) and facilitates metal ion-O_2 chemistry.

Oxygen-derived active species, including free radicals, have been implicated in tissue injury following ischaemia and reperfusion of the heart (McCord, 1985; Garlick *et al.*, 1987; Arroyo *et al.*, 1987; Zweier *et al.*, 1987) and brain (Krause *et al.*, 1988; Cao *et al.*, 1988; Watson & Ginsberg, 1989) as well as in various other pathologies (Halliwell & Gutteridge, 1984). Their production from relatively low-reactive species has been proposed to be mediated by redox-active metal ions (Aust *et al.*, 1985; Chevion, 1988). Indeed, circumstantial evidence has been presented to support the causative role of newly mobilised redox-active iron in tissue injury (Nayini *et al.*, 1985; Holt *et al.*, 1986). Iron chelation provides protection against tissue injury following ischaemia (Myers *et al.*, 1985, 1986), whereas the addition of Fe^{3+} and Fe^{2+} to the perfusate increased the rate of injury in hearts subjected to ischaemia and reperfusion (Bernier *et al.*, 1986; Karwatowska-Prokopczuk *et al.*, 1992). The recovery of myocardial high-energy phosphate metabolism and left ventricular contractility after a period of global ischaemia suggest that iron-catalysed $^{\bullet}OH$ formation plays a key role in the pathogenesis of reperfusion injury. Despite these theoretical considerations, relatively little is known about the role of iron in the pathogenesis of postischaemic damage.

CONCLUSIONS

The radicals NO ($NO^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) play an important role in biological regulation. Superoxide gives rise to other forms of ROS that serve as mediators in many regulatory processes. Most redox-responsive regulatory mechanisms in bacteria and mammalian cells serve to protect the cells against oxidative stress and to reestablish redox homeostasis. The oxidative induction of protective enzymes by proteins or the inhibition of NOS by NO are prominent examples. Redox regulation of other physiological responses in higher organisms is embedded in these basic mechanisms of redox homeostasis. The relatively large number of isoforms of NAD(P)H oxidase and NOS indicates that nature has "learned" to use free radicals to her advantage in processes not directly related to protection against oxidative stress. The production of superoxide and NO, respectively, by these enzymes is strictly regulated by hormones, cytokines, or other inducing mechanisms. The resulting oxidative species, in turn, act as secondary messengers to control a variety of physiological responses. The regulation of vascular smooth muscle relaxation, the monitoring of the oxygen concentration in the regulation of respiratory ventilation and erythropoietin production, and the enhancement of signaling cascades from various membrane receptors are prominent examples. The enhancement of signal transduction from a given receptor by stimulation of ROS production through this or other receptors may serve two physiological purposes.

First, it provides a basis for cooperativity, and second, the membrane receptor may function simultaneously as a sensor for the extracellular ligand and as a sensor for the inner metabolic state of the individual cell. The cooperativity between angiotensin II receptor and EGF receptor is a well-studied example, but other examples will likely be found. Because hydrogen peroxide has a relatively long half-life and crosses membranes, the cooperativity principle may even extend to other cells in the vicinity. By enhancing the intracellular signaling pathways of lymphocytes, ROS from activated macrophages and neutrophils may contribute decisively to the activation of the antigen-specific immune response and may allow the immune system to respond to minute amounts of invading pathogens. Signaling pathways involving JNK, p38 MAPK, and the transcription factors AP-1 and NF-κB are particularly responsive to redox regulation. The capacity of ROS to damage proteins and to hasten their proteolytic degradation has been employed as a regula-

tory mechanism in several cases, e.g., in the degradation of the transcription factor subunit HIF-1α and the NF-κB inhibitor IκB. The inhibition of protein tyrosine phosphatases is well-defined on a molecular basis and provides an example of redox regulation by loss of function. In other cases, NO or ROS induce a gain of function in a signaling protein. This mechanism is involved in the regulation of vascular tone and the functional activation of bacterial proteins. The oxidative enhancement of membrane receptor signaling and the corresponding downstream signaling pathways are not well-characterized at the molecular level but are likely to involve a simultaneous induction of several different redox-sensitive signaling proteins. This redundancy does not preclude selective effects. The *in vivo* relevance of redox-sensitive signaling cascades is strongly suggested by the mere existence of the many NAD(P)H oxidase isoforms and by the apparent dysregulation of physiological responses in various disease-related oxidative stress conditions. However, the relative contributions of individual redox-sensitive signaling proteins to redox-regulated processes *in vivo* are presently obscure.

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