Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life

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SETTING THE SCENE

The field of antioxidants and free radicals is often perceived as focusing around the use of antioxidant supplements to prevent human disease. In fact, antioxidants/free radicals permeate the whole of life, creating the field of redox biology. Free radicals are not all bad, nor antioxidants all good. Life is a balance between the two: Antioxidants serve to keep down the levels of free radicals, permitting them to perform useful biological functions without too much damage (Halliwell and Gutteridge, 2006). This is especially true in plants, as the rest of this issue reveals. Yet some damage is inevitable, requiring repair systems to maintain cell viability. The purpose of this article is to take a broad view of the field, and highlight some of the fascinating differences between plants and other organisms.

HOW DID REDOX BIOLOGY BEGIN?

All animals need O_2 for efficient production of energy in mitochondria. This need for O_2 obscures the fact that it is a toxic mutagenic gas; aerobes survive only because they have evolved antioxidant defenses (Halliwell and Gutteridge, 2006).

Oxygen appeared in significant amounts in the Earth's atmosphere over 2.2 billion years ago, largely due to the evolution of photosynthesis by cyanobacteria. They evolved to use energy from the sun to split water. Thereby, they gained reducing power (hydrogen equivalents) to drive their metabolism, but the by-product, tonnes of O_2, was discarded into the atmosphere (Lane, 2002) as an early case of air pollution. Initially, most of this O_2 was consumed by the formation of the metallic oxide deposits that exist in rocks and ores today. Only when this was largely complete did O_2 build up in the atmosphere. The rise in atmospheric O_2 was advantageous in at least two ways; it led to formation of the ozone (O_3) layer in the stratosphere that protects living organisms from UV-C radiation (that may have helped organisms to leave the sea and colonize land), and it removed ferrous iron (Fe^{2+}) from aqueous environments by forming insoluble ferric complexes. Most Fe^{2+} was precipitated from solution, leaving sea and river waters today containing only trace amounts of soluble iron (Lane, 2002).

What was the advantage of removing Fe^{2+}? This species reacts rapidly with hydrogen peroxide (H_2O_2) to yield highly toxic hydroxyl radical (a superscript $^{\bullet}$ denotes a free radical).

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe(III) + OH^{\bullet} + OH^{-}$$

The above reaction is called the Fenton reaction, after its discoverer in 1876. Fenton chemistry occurs in vivo, but organisms carefully control it by limiting the availability of both Fe^{2+} and H_2O_2 (Halliwell and Gutteridge, 1984, 1990, 2006). It would have been difficult to evolve aerobic life in a world awash with Fe^{2+}.

When living organisms first appeared on Earth, they did so under an atmosphere containing much N_2 and CO_2 but little O_2, i.e. they were anaerobes (Kasting, 1993). Anaerobes still exist today, but usually their growth is inhibited or they are killed by exposure to 21% O_2, the current atmospheric level. As atmospheric O_2 rose, many anaerobes must have died out. Present-day ones are presumably the descendants of organisms that adapted to rising O_2 by restricting themselves to anoxic microenvironments. Other organisms instead began to evolve antioxidant defenses (producing new ones and realigning ancient molecules to new functions) to protect against O_2 toxicity. One of the earliest of these may have been to develop proteins that bind and detoxify iron to protect DNA against Fenton chemistry (Wiedenheft et al., 2005). In retrospect, this development of antioxidants was a fruitful path to follow. Organisms that tolerated O_2 could further evolve to use it for metabolic transformations catalyzed by oxidase, oxygenase, and hydroxylase enzymes, such as the Pro and Lys hydroxylases needed for collagen biosynthesis. Indeed, large multicellular animals need collagen for making their support tissues (bone and cartilage). Best of all, O_2 could facilitate efficient energy production, employing electron-transport chains with O_2 as the terminal electron acceptor. This switch to aerobic metabolism increased the yield of ATP that could be made from food molecules such as Glc by over 15-fold.

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Evolution of efficient energy production allowed the development of complex multicellular organisms, which then also needed systems to ensure that O_2 can be distributed throughout their bodies (mechanisms varying from spiracles in insects to the human vascular system). A further advantage of evolving such systems is that delivery of O_2 can be controlled: for example, most cells in the human body are never exposed to the full force of atmospheric O_2 because blood pO_2 is much lower than that of air. Similarly, insects control the opening of their spiracles to keep internal O_2 low (Hetz and Bradley, 2005).

Photosynthesis: The Ultimate Paradox?

Since O_2 is poisonous and photosynthetic organisms produce it, how could cyanobacteria evolve photosynthesis in a preantioxidant world? Even in present-day plants, which are full of antioxidants, much of the protein synthetic activity of chloroplasts is used to replace oxidatively damaged D1 and other proteins. Were some antioxidants in place already? Perhaps PSII evolved from a manganese-containing form of the enzyme catalase (Lane, 2002; Olson and Blankenship, 2004). Catalases, most of which are haem-containing proteins (Halliwell and Gutteridge, 2006), nowadays (although some bacteria still have manganese-containing ones [Horsburgh et al., 2002]) catalyze the rapid breakdown of H_2O_2.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

If this hypothesis is true, then catalase-like enzymes might have been present prior to a rise in atmospheric O_2. How can this be? Was H_2O_2 present to drive its evolution?

Under an atmosphere mainly composed of N_2 and CO_2 with no O_3 screen, UV radiation must have bombarded the face of the Earth. Even at the low O_2 levels present 3.5 billion years ago (<0.1%), there could have been substantial H_2O_2 levels in rainwater generated by photochemical reactions with traces of O_2 (Lane, 2002). Since Fe^{2+} was freely available, Fenton chemistry was a threat, so H_2O_2 must be eliminated. One suggestion is that the evolutionary precursors of PSII used H_2O_2 as a substrate (Olson and Blankenship, 2004) and only later evolved the increased chemical ferocity needed to split water.

Atmospheric O_2 levels may have been higher at periods in the Earth's history, perhaps reaching 35% by the late Carboniferous era. As plant life flourished, CO_2 levels fell drastically, and huge deposits of coal and oil formed (Graham et al., 1995; Lane, 2002). This increased O_2 concentration may have permitted insects (whose O_2 distribution system depends largely on diffusion) to become larger. For example, the giant Carboniferous dragonfly *Meganeura monyi* was bigger than any dragonfly that exists today (Lane, 2002). The plants and animals existing in Carboniferous times may have had enhanced antioxidant defenses, which would be fascinating to study if they could be resurrected. Even today, some of the plants that evolved at that time can resist damage by elevated O_2 better than plants that evolved more recently (Beerling et al., 1998).

Oxygen Toxicity

All aerobes including plants, aerobic bacteria, and humans, suffer damage when exposed to O_2 concentrations higher than normal (Gilbert, 1981; Balentine, 1982; Halliwell and Gutteridge, 2006), signifying that they have no excess of antioxidant defenses. Indeed, as we learned how to measure oxidative damage (for review, see Halliwell and Whiteman, 2004), it was found to occur in aerobes even at normal O_2 levels. What causes this damage? Many scientists believe that O_2 toxicity is due to excess formation of the superoxide radical $O_2^{\bullet -}$ (Fridovich, 1995), this is the superoxide theory of O_2 toxicity. But let us step back for a moment and examine some basics.

WHAT ARE FREE RADICALS?

A free radical is any species capable of independent existence (hence the term free) that contains one or more unpaired electrons (Halliwell and Gutteridge, 2006). An unpaired electron is one that occupies an atomic or molecular orbital by itself. The simplest free radical is atomic hydrogen. Since a hydrogen atom has only one electron, it must be unpaired. Many free radicals exist in living systems (some bad, some good, and some both), although most molecules in vivo are nonradicals. Radicals can be formed by several mechanisms, such as adding a single electron to a nonradical. They can form when a covalent bond is broken if one electron from the bonding pair remains on each atom (homolytic fission). Some bonds are hard to break, e.g. temperatures of 450°C to 600°C are often required to rupture C–C, C–H, or C–O bonds. Indeed, combustion of organic compounds proceeds by free radical mechanisms. Other covalent bonds fragment more easily: Just trimming your fingernails can cleave disulphide bonds in keratin to generate sulfur radicals (Symons, 1996).

Another example, the O–O bond in H_2O_2, is readily split by exposing it to UV light, generating $OH^{\bullet}$.

$$H-O-O-H \rightarrow OH^{\bullet} + OH^{\bullet}$$

Homolytic fission of one of the O–H covalent bonds in water requires more energy (γ-rays or x-rays) and yields $H^{\bullet}$ and a hydroxyl radical (I write this as $OH^{\bullet}$, but some authors write it as $^{\bullet}OH$, presumably to emphasize the location of the unpaired electron on the oxygen). Formation of $OH^{\bullet}$ accounts for much of the damage done to living organisms by ionizing radiation (von Sonntag, 1987).

The Oxygen Radicals and Related Species

There are many types of free radicals in living systems, but I focus here on the oxygen radicals. In fact,

the O_2 molecule is a free radical (Gilbert, 1981; Halliwell and Gutteridge, 2006); it has two unpaired electrons. It should thus be written as $O_2^{\bullet}$, but nobody ever does so I won't either. The two electrons in O_2 have the same spin quantum number (or, as is often written, they have parallel spins). This is the most stable state, or ground state, of O_2, and is the form that exists in the air around us. Oxygen is, thermodynamically, a potent oxidizing agent. However, if O_2 tries to oxidize a nonradical by accepting a pair of electrons from it, both these electrons must have the same spin to fit into the vacant spaces in the π^* orbitals (Fig. 1). A pair of electrons in an atomic or molecular orbital cannot meet this criterion, since they have opposite spins (+½ and −½). This spin restriction makes O_2 prefer to accept its electrons one at a time, and helps explain why O_2 reacts sluggishly with most nonradicals. By contrast, it often reacts outstandingly fast with other radicals by single electron transfers. Thus, if we heat human bodies or plants to a high enough temperature to cause some homolytic bond fission, O_2 leaps onto the radicals formed and combustion begins (Gilbert, 1981; Halliwell and Gutteridge, 2006). Fortunately, most molecules in living organisms are nonradicals.

Singlet Oxygens

More reactive forms of O_2, the singlet oxygens, can be generated by an input of energy that rearranges the electrons (Foote et al., 1985). In both forms of singlet O_2 the spin restriction is removed (Fig. 1) and the oxidizing ability greatly increased; singlet O_2 can directly oxidize proteins, DNA, and lipids (Foote et al., 1985). The $^1\Sigma g^+$ state rapidly decays to the $^1\Delta g$ state, so only the latter is usually encountered in biological systems. Note that $^1\Delta gO_2$ is not a free radical, it has no unpaired electrons (Fig. 1). Singlet O_2 $^1\Delta g$ (written just as singlet O_2 or $^1O_2$ from now on) is the curse of the illuminated chloroplast; insufficient energy dissipation during photosynthesis can lead to formation of a chlorophyll triplet state that can transfer its excitation energy onto ground-state O_2 to make $^1O_2$ (Holt et al., 2005). This can oxidize chloroplast molecules and can trigger cell death (Wagner et al., 2004). The plant counters this by regulating energy distribution between the light harvesting complexes and by judicious use of certain carotenoids, which can quench both the triplet chlorophyll state and the $^1O_2$ itself (Holt et al., 2005). Singlet O_2 is also sometimes used as a signaling molecule.

Animals are not exempt from singlet O_2 formation; it occurs in the skin and eye (Halliwell and Gutteridge, 2006). Photosensitizers of singlet O_2 can be consumed in dietary plants (e.g. hypericin in St. John's Wort [*Hypericum perforatum*] and psoralens in celery [*Apium graveolens*]) or taken as drugs (e.g. the fluoroquinolone antibiotics), and subsequent sunbathing can cause skin damage (Morison, 2004; Halliwell and Gutteridge, 2006).

Superoxide Radical

If a single electron is supplied to O_2, it enters one of the π^* antibonding orbitals (Fig. 1). The product is superoxide radical, $O_2^{\bullet -}$, full name superoxide radical anion. With only one unpaired electron, superoxide is less radical than O_2, despite its super name (Fridovich, 1995).

Addition of another electron to $O_2^{\bullet -}$ will give O_2^{2-}, the peroxide ion, a nonradical (no unpaired electrons left) with a weaker oxygen-oxygen bond. Addition of two more electrons to O_2^{2-} eliminates the bond entirely, giving two O^{2-} (oxide ions). In biology, the two-electron reduction product of O_2 is H_2O_2, and the four-electron product, water.

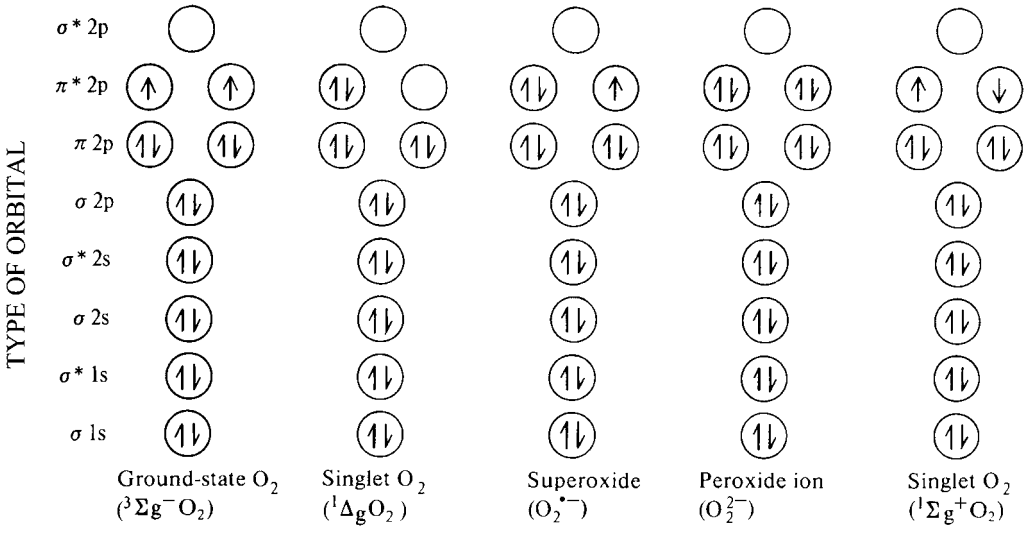


Figure 1. A simplified version of bonding in the diatomic oxygen molecule and its derivatives. The oxygen atom has eight electrons, O_2 has 16 electrons. Adapted from Halliwell and Gutteridge (2006) by courtesy of Oxford University Press.

$$O_2 \xrightarrow[\text{reduction (plus } 2H^+)]{\text{two-electron}} H_2O_2 \text{ (protonated form of } O_2^{2-})$$

$$O_2 \xrightarrow[\text{reduction (plus } 4H^+)]{\text{four-electron}} 2H_2O \text{ (protonated form of } O^{2-})$$

Mitochondria take up O_2 and reduce 95% or more of it to water, a process achieved by cytochrome oxidase. It removes one electron from each of four reduced (Fe^{2+}-haem) cytochrome *c* molecules, oxidizing them to ferric cytochrome *c*, and adds the four electrons on to O_2 as shown above. However, it is impossible to add four (not even two!) electrons to O_2 at once. Cytochrome oxidase is a large and complex multiprotein assembly, both because it catalyzes several reduction steps and also because it must hold onto toxic partially reduced oxygen species until they can be fully reduced to water (Babcock, 1999).

Aspects of Terminology: The Reactive Oxygen Species

Many different terms are used in the literature to describe oxygen radicals and related (nonradical) species such as $^1O_2$ and H_2O_2. I prefer the term reactive oxygen species (ROS), a collective descriptor that includes not only the oxygen radicals but also some nonradical derivatives of O_2 (Table I). Hence, all oxygen radicals are ROS, but not all ROS are oxygen radicals (Halliwell and Gutteridge, 2006). Reactive is a relative term; $O_2^{\bullet -}$ and H_2O_2 are highly selective in their reactions with biological molecules (e.g. H_2O_2 can inactivate chloroplast Fru and sedoheptulose bisphosphatases, but not most other enzymes), whereas $OH^\bullet$ attacks everything around it. The term reactive species has been expanded to include reactive nitrogen, chlorine, and bromine species (Table I). Nitric oxide ($NO^\bullet$), an important signaling molecule in animals and plants, is a free radical, and its radical properties explain many of its biological actions (Halliwell et al., 1999).

WHAT DAMAGE CAN FREE RADICALS AND OTHER ROS DO?

If two free radicals meet, they can join their unpaired electrons to form a covalent bond. Thus, $NO^\bullet$ and $O_2^{\bullet -}$ react fast to form a nonradical product, peroxynitrite (Beckman and Koppenol, 1996).

$$NO^\bullet + O_2^{\bullet -} \rightarrow ONOO^- \text{(peroxynitrite)}$$

At physiological pH, $ONOO^-$ rapidly protonates to peroxynitrous acid, ONOOH. This powerful oxidizing and nitrating agent can directly damage proteins, lipids, and DNA. It can also cause damage by undergoing homolytic fission to give noxious products.

$$ONOOH \rightarrow NO_2^\bullet + OH^\bullet$$

Table I. *Some reactive species*

ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, $^1O_2$, and H_2O_2). All oxygen radicals are ROS, but not all ROS are oxygen radicals. Reactive nitrogen species is a similar collective term that includes $NO^\bullet$ and $NO2^\bullet$, as well as nonradicals such as HNO_2 and N_2O_4. Reactive is not always an appropriate term: H_2O_2, $NO^\bullet$, and $O_2^{\bullet -}$ react fast with few molecules, whereas $OH^\bullet$ reacts fast with almost everything. Species such as $RO_2^\bullet$, $NO_3^\bullet$, $RO^\bullet$, HOCl, HOBr, $CO_3^{\bullet -}$, $CO_2^{\bullet -}$, $NO_2^\bullet$, $ONOO^-$, NO_2^+, and O_3 have intermediate reactivities.

Free Radicals	Nonradicals
ROS	ROS
Superoxide, $O_2^{\bullet -}$	H_2O_2
Hydroxyl, $OH^\bullet$	Hypobromous acid, HOBr[a]
Hydroperoxyl, $HO_2^\bullet$ (protonated superoxide)	Hypochlorous acid, HOCl[b]
Carbonate, $CO_3^{\bullet -}$	Ozone, O_3[c]
Peroxyl, $RO_2^\bullet$	Singlet oxygen ($O_2{}^1\Delta g$)
Alkoxyl, $RO^\bullet$	Organic peroxides, ROOH
Carbon dioxide radical, $CO_2^{\bullet -}$	Peroxynitrite, $ONOO^-$[d]
Singlet $O_2{}^1\Sigma g^+$	Peroxynitrate, O_2NOO^-[d]
	Peroxynitrous acid, ONOOH[d]
	Peroxomonocarbonate, $HOOCO_2^-$
Reactive chlorine species	Reactive chlorine species
Atomic chlorine, $Cl^\bullet$	Hypochlorous acid, HOCl[b]
	Nitryl chloride, NO_2Cl[e]
	Chloramines
	Chlorine gas (Cl_2)
	Bromine chloride (BrCl)[a]
	Chlorine dioxide (ClO_2)
Reactive bromine species	Reactive bromine species
Atomic bromine, $Br^\bullet$	Hypobromous acid (HOBr)
	Bromine gas (Br_2)
	Bromine chloride (BrCl)[a]
Reactive nitrogen species	Reactive nitrogen species
Nitric oxide, $NO^\bullet$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^\bullet$[c]	Nitrosyl cation, NO^+
Nitrate radical, $NO_3^\bullet$[c,f]	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$[d]
	Peroxynitrate, O_2NOO^-[d]
	Peroxynitrous acid, ONOOH[d]
	Nitronium cation, NO_2^+
	Alkyl peroxynitrites, ROONO
	Alkyl peroxynitrates, RO_2ONO
	Nitryl chloride, NO_2Cl
	Peroxyacetyl nitrate, $CH_3C(O)OONO_2$[c]

[a]HOBr and BrCl could also be regarded as reactive bromine species. [b]HOCl and HOBr are often included as ROS. [c]Oxidizing species formed in polluted air that are toxic to plants and animals. [d]$ONOO^-$, ONOOH, and O_2NOO^- are often included as ROS. [e]NO_2Cl can also be regarded as a reactive nitrogen species. [f]This species may cause formation of allergenic nitrated proteins in pollens.

Peroxynitrite also reacts with CO_2:

$$ONOO^- + CO_2 \rightarrow ONOOCOO^-$$

$$ONOOCOO^- \rightarrow NO_2^{\bullet} + CO_3^{\bullet -}.$$

Both $NO_2^{\bullet}$ (nitrogen dioxide) and $CO_3^{\bullet -}$ (carbonate radical) are powerful oxidizing agents: not as bad as $OH^{\bullet}$ but not nice either. Thus, any system producing $NO^{\bullet}$ and $O_2^{\bullet -}$ can cause biological damage, and this occurs in many human diseases (Beckman and Koppenol, 1996; Halliwell et al., 1999; Greenacre and Ischiropoulos, 2001; Alvarez and Radi, 2003). It may happen in plants as well, since they can make both $O_2^{\bullet -}$ and $NO^{\bullet}$ (Vanin et al., 2004), but few data are available.

However, most biological molecules are nonradicals. When a free radical reacts with a nonradical, a new radical results, and chain reactions may occur. There are several types of reactions (Halliwell and Gutteridge, 2006).

(1) A radical may add to another molecule; the adduct still has an unpaired electron. For example, $OH^{\bullet}$ adds to position 8 in the ring structure of guanine in DNA; the initial product is an 8-hydroxy-2′-deoxyguanosine radical. Among other fates, this can undergo oxidation to the mutagenic lesion 8-hydroxy-2′-deoxyguanosine, 8OHdG (Evans et al., 2004). Hydroxyl radical also attacks other bases and the deoxy-Rib sugar in DNA, producing massive damage (Evans et al., 2004).

(2) A radical may be a reducing agent, donating a single electron. Thus the toxicity of paraquat (PQ^{2+}) to plants, animals, and bacteria involves its reduction to paraquat radical cation ($PQ^{\bullet +}$) by cellular enzymes.

$$\underset{\text{paraquat}}{PQ^{2+}} \xrightarrow{1e^-} PQ^{\bullet +},$$

followed by $PQ^{\bullet +}$ reducing O_2 to $O_2^{\bullet -}$:

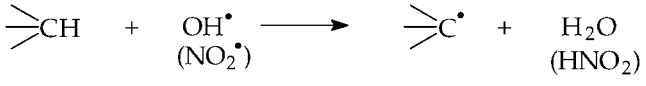
$$PQ^{\bullet +} + O_2 \rightarrow PQ + O_2^{\bullet -}.$$

Thus, paraquat catalyzes $O_2^{\bullet -}$ formation (Halliwell and Gutteridge, 2006).

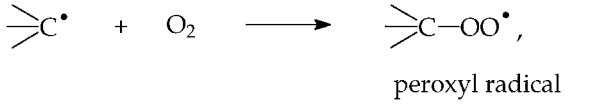
(3) A radical may be an oxidizing agent, taking a single electron from a nonradical to leave an unpaired electron behind. For example, $OH^{\bullet}$ oxidizes carbonate to carbonate radical.

$$CO_3^{2-} + OH^{\bullet} \rightarrow OH^- + CO_3^{\bullet -}$$

(4) A reactive radical (e.g. $OH^{\bullet}$ or $NO_2^{\bullet}$) may abstract a hydrogen atom from a C–H bond, e.g. from a hydrocarbon side chain of a polyunsaturated fatty acid (PUFA) residue in a membrane. As $H^{\bullet}$ has only one electron, an unpaired electron remains on the carbon.

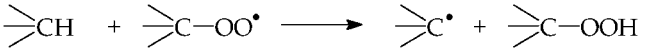


Carbon-centered radicals react fast with O_2 (like many radicals do) to generate peroxyl radicals



peroxyl radical

which are reactive enough to both oxidize membrane proteins and attack adjacent PUFA side chains, propagating a chain reaction.



A new $C^{\bullet}$ radical is formed to continue the chain, and a lipid hydroperoxide forms. If the PUFA chain is long enough, the peroxyl radicals can whip around and abstract $H^{\bullet}$ from the same PUFA, giving cyclic peroxides (Fam and Morrow, 2003).

A single initiation event thus has the potential to generate multiple peroxide molecules by a chain reaction. The initial $H^{\bullet}$ abstraction from a PUFA can occur at different points on the carbon chain, giving complex mixtures of peroxides. Singlet O_2 can react directly with PUFAs to give peroxides, e.g. it converts linoleic acid to 9-, 10-, 12-, and 13-hydroperoxides (Foote et al., 1985). The lifetime of singlet O_2 in the hydrophobic interior of membranes is greater than in aqueous solution. Hence, illumination of lipids in the presence of sensitizers of $^1O_2$ formation induces rapid peroxide formation. Such reactions are important in the eye and in plants.

The overall effects of lipid peroxidation are to decrease membrane fluidity, make it easier for phospholipids to exchange between the two halves of the bilayer, increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels (e.g. K^+ and Ca^{2+}), and damage membrane proteins, inactivating receptors, enzymes, and ion channels. Iron and copper irons accelerate lipid peroxidation by two mechanisms (Halliwell and Gutteridge, 1984). First, they convert H_2O_2 to $OH^{\bullet}$ by splitting the O–O bond (the Fenton reaction, in the case of Fe^{2+}). In an analogous reaction, they can split lipid hydroperoxides,

$$LOOH + \underset{(Cu^+)}{Fe^{2+}} \rightarrow \underset{(Cu^{2+})}{Fe(III)} + LO^{\bullet} + OH^-$$

$$LOOH + \underset{(Cu^{2+})}{Fe(III)} \rightarrow \underset{(Cu^{2+})}{Fe^{2+}} + LOO^{\bullet} + H^+,$$

giving alkoxyl ($LO^{\bullet}$) and more peroxyl ($LOO^{\bullet}$) radicals to keep the chain reaction going.

Continued oxidation of fatty acid side chains and their fragmentation to produce aldehydes and hydrocarbons (see below) will eventually lead to loss of membrane integrity, e.g. rupture of lysosomal or central vacuolar membranes. In addition, some end products of lipid peroxidation have direct damaging effects. These include the isoprostanes (IPs), cyclic peroxides formed from PUFAs with at least three double bonds, including linolenic acid, arachidonic acid (F_2 IPs), eicosapentaenoic acid (F_3 IPs), and docosahexaenoic acid (F_4 IPs, sometimes called neuroprostanes). Increased formation of IPs is observed in many human diseases and after exposure of animals to a range of toxins (Fam and Morrow, 2003). Some act as

vasoconstrictors and can damage tissues. Linoleate-derived IPs (phytoprostanes) are abundant in plants but their roles have not been studied as much as the animal IPs (Mueller, 2004).

Decomposition of lipid peroxides accelerated by iron and copper ions or by heating (e.g. the use of oxidized cooking oils) generates a complex mixture of toxic products including epoxides, saturated aldehydes, unsaturated aldehydes, ketones, and hydrocarbons such as ethane and pentane (Esterbauer et al., 1991). Particularly toxic aldehydes include malondialdehyde (formed from peroxidation of linolenic, arachidonic, or docosahexaenoic acids), and 4-hydroxynonenal, formed from linolenic and arachidonic acid peroxides. Both bind avidly to membrane proteins, inactivating enzymes and receptors. Both can attack DNA, forming mutagenic lesions (Esterbauer et al., 1991).

ANTIOXIDANT DEFENSES

Making Less ROS

How do organisms deal with O_2 toxicity? One strategy is to minimize the levels of O_2, or deter its conversion to ROS (Halliwell and Gutteridge, 2006). Some mobile organisms avoid O_2 toxicity by swimming away from high O_2 regions, and *Caenorhabditis elegans* can cluster together to regulate the group O_2 level to the value they like (Gray et al., 2004). The most important source of $O_2^{\bullet -}$ in vivo in many (perhaps all) aerobic animal cells is probably the mitochondrial electron-transport chain (in plants, chloroplasts make a lot of $O_2^{\bullet -}$ as well, of course). Whereas cytochrome oxidase releases no ROS (a beautifully evolved enzyme complex), some earlier components of the mitochondrial electron-transport chain can leak electrons directly to O_2, although passing the bulk of them onto the next component in the chain (Turrens, 2003). This leakage generates $O_2^{\bullet -}$. Whereas mammalian cytochrome oxidase is saturated at low O_2 tensions, the rate of electron leakage (and hence $O_2^{\bullet -}$ production) by mitochondria is, in general, increased by raising O_2 levels (although it is not quite so simple; leakage is also favored by high levels of reduced carriers, which can drop when O_2 is high).

Aerobes may have evolved to pack the redox constituents of the mitochondrial and other electron-transport chains together in a way that makes escape of electrons to O_2 to form $O_2^{\bullet -}$ less likely. Plants may additionally use alternative oxidase systems to minimize mitochondrial $O_2^{\bullet -}$ formation (Moller, 2001), and both plants and animals have uncoupling proteins in their inner mitochondrial membranes. These may act as antioxidants, e.g. by allowing more proton leak and preventing a back up of electrons in the chain to escape to O_2. Formation of 4-hydroxynonenal or $O_2^{\bullet -}$ within mitochondria seems to activate uncoupling proteins, which should then decrease the membrane potential and limit $O_2^{\bullet -}$ formation (Brand et al., 2004). Proteins that bind metal ions (such as transferrin, ferritins, and metallothioneins) and so hinder both Fenton chemistry and the acceleration of lipid peroxide ion by iron and copper, are other mechanisms deterring ROS formation (Halliwell and Gutteridge, 1990).

Scavenging ROS

The superoxide dismutase enzymes (SODs) remove $O_2^{\bullet -}$ by catalyzing its dismutation, one $O_2^{\bullet -}$ being reduced to H_2O_2 and another oxidized to O_2.

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

Animals have SODs containing active-site manganese (MnSOD) in the mitochondrial matrix, plus SODs with copper and zinc (CuZnSOD) in the mitochondrial intermembrane space and in the rest of the cell (Fridovich, 1995). Plants have more or less the same, but some have iron-containing SODs (FeSOD) in the chloroplast (in addition to CuZnSOD; Alscher et al., 2002). Bacteria often have CuZnSOD plus MnSOD and/or FeSOD; a few even have nickel-containing SOD (Halliwell and Gutteridge, 2006). Whatever the metal, all SODs catalyze the above reaction (Fridovich, 1995; Halliwell and Gutteridge, 2006). Some anaerobic bacteria cope with $O_2^{\bullet -}$ (generated if they are transiently exposed to O_2) in a different way: they use superoxide reductase (Niviere and Fontecave, 2004) proteins, which catalyze the overall reaction.

$$O_2^{\bullet -} + \text{cellular reductant} + 2H^+ \rightarrow H_2O_2$$

Unlike SOD, no O_2 is produced, an obvious advantage for an anaerobe, although the H_2O_2 has to be dealt with.

Superoxide dismutases and reductases must work together with enzymes that remove H_2O_2. Catalases are not the most important in this context since there is little or no catalase in mitochondria and chloroplasts, where much $O_2^{\bullet -}$ is generated (Halliwell and Gutteridge, 2006). Most or all catalase in plants and animals is in peroxisomes, to deal with H_2O_2 produced by oxidase enzymes acting on such substrates as glycollate, urate, and D amino acids (Schrader and Fahimi, 2004). Plants are rich in peroxidases, enzymes that remove H_2O_2 by using it to oxidize a cosubstrate. Many plant peroxidases are nonspecific, using multiple cosubstrates (horseradish [*Armoracia lapathifolia*] peroxidase is perhaps the most-studied example); ascorbate peroxidases in plant chloroplast and cytosol can remove H_2O_2 by using vitamin C as a cosubstrate, oxidizing it to a (poorly reactive) ascorbyl free radical (Mano et al., 2001).

Until recently, it was thought that the most important H_2O_2-removing enzymes in animals are glutathione peroxidases, a family (Brigelius-Flohe, 1999) of selenium-containing enzymes that remove H_2O_2 by coupling its reduction to water with oxidation of reduced glutathione (GSH), a thiol-containing tripeptide (glu-cys-gly).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

The product, oxidized glutathione (GSSG), consists of two GSH linked by a disulphide bridge, and can be converted back to GSH by glutathione reductase enzymes.

At least four types of GPx exist. One is the classical enzyme, often now called GPx1. Mammalian body fluids contain low levels of a different form, GPx3. Another type, GPx2, is found in the cells lining the gastrointestinal tract and may help to metabolize peroxides in ingested food lipids. The fourth member of the family is phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx4), with the ability to reduce not only H_2O_2 but also fatty acid hydroperoxides (to alcohols) that are still esterified in lipids of membranes or lipoproteins. In all four types of GPx, the selenium is essential for catalysis (Brigelius-Flohe, 1999). In marked contrast, selenium plays little role in plants. The chloroplast appears to lack selenoprotein GPx activity (indeed selenoprotein GPx enzymes are rare in plants) and ascorbate peroxidase takes over some or all of the job of H_2O_2 removal (Mano et al., 2001). GPx-like activity has been identified in chloroplasts and cytoplasm in some plant species, and genes similar to those encoding GPxs in animals (most commonly resembling PHGPx genes) have been identified in several plant genomes. Plant GPxs have Cys rather than seleno Cys at the active sites, which decreases their catalytic activity as compared with selenoprotein GPx enzymes. Indeed, at least some of the plant enzymes may prefer thioredoxin to GSH as a substrate (Herbette et al., 2002; Rodriguez Milla et al., 2003).

It has now been realized that peroxiredoxins may be the most important H_2O_2-removal systems in animals, bacteria, and possibly plants (Rhee et al., 2005). They are homodimers and contain no prosthetic groups: The redox reactions are dependent on Cys at the active sites. There are at least three classes: the typical 2-cys (the most common), the atypical 2-cys, and the 1-cys peroxiredoxins. In all cases, H_2O_2 oxidizes an -SH group on the peroxiredoxin to a sulfenic acid, cys-SOH. In the 2-cys enzymes, this reacts with another -SH on the protein to give a disulphide that is then reduced by thioredoxin. In 1-cys peroxiredoxins, another cellular reductant (not yet identified) regenerates the -SH group.

Peroxiredoxins are slower at catalyzing H_2O_2 removal than GPx, although the large amounts present (up to 0.8% of total soluble protein in some animal cells) and their low K_m for H_2O_2 (<20 μM) can compensate for this, as can their presence in all subcellular organelles and in the cytosol. Peroxiredoxins are readily inactivated by H_2O_2, the eukaryotic ones being more susceptible to this than bacterial ones (Georgiou and Masip, 2003). Cells have various mechanisms for reactivating oxidized peroxiredoxins (Rhee et al., 2005). In animals, selenium is essential for the activity of thioredoxin reductase, the enzyme that keeps thioredoxins in the reduced state for peroxiredoxins (and for the many other metabolic activities of thioredoxins). Thus lack of selenium in animals impairs the peroxiredoxin system as well as GPx (Su et al., 2005).

Finally, there are sacrificial antioxidants, agents that are preferentially oxidized by reactive species to preserve more important biomolecules. For example, ascorbate can scavenge most ROS, including $O_2^{\bullet -}$, $OH^{\bullet}$, $RO_2^{\bullet}$, and ONOOH, as can GSH. Tocopherols (Tocs) are good scavengers of peroxyl radicals and help to protect membranes against lipid peroxidation by interrupting the chain reaction. Reaction is with the phenolic -OH group of the Toc structure (Halliwell and Gutteridge, 2006).

The essence of the antioxidant actions of ascorbate and Toc is that the radicals they form are poorly reactive (Smirnoff, 2001; Halliwell and Gutteridge, 2006). Plants can make all the ascorbate and Tocs they want, we poor humans need to eat the plants to get them! Most animals can still make ascorbate, but the terminal step in its synthesis generates H_2O_2 (Puskas et al., 1998).

$$L - \text{gulono} - \gamma - \text{lactone} + O_2 \xrightarrow[\text{oxidase}]{\text{Gulonolactone}} L - \text{ascorbate} + H_2O_2$$

Is this why humans, evolving on a plant-rich diet, lost this enzyme? It is an interesting speculation. Also interesting, ascorbate synthesis in plants uses a different pathway that does not make H_2O_2 (Smirnoff, 2001).

Other examples of sacrificial antioxidants include carotenoids, urate, plasma albumin, and GSH.

WHAT'S SO BAD ABOUT SUPEROXIDE?

Transgenic animal experiments show that SODs are important enzymes in animals. Even bacteria and yeasts (*Saccharomyces cerevisiae*) lacking SODs are pretty sick (for review, see Halliwell and Gutteridge, 2006) and plants have problems as well (e.g. Rizhsky et al., 2003). In one study (Lebovitz et al., 1996), most MnSOD-knockout mice died within 10 d after birth, with cardiac abnormalities, fat accumulation in liver and skeletal muscle, metabolic acidosis, and severe mitochondrial damage in heart and, to a lesser extent, in other tissues. Those mice that manage to survive longer than 10 d soon succumb to a variety of pathologies, including anemia, retinal defects, and neurodegeneration (Melov et al., 1998). Even MnSOD (+/−) heterozygous mice, which at first seem normal, show increased mitochondrial oxidative damage as they age, as well as increased nuclear oxidative DNA damage and elevated cancer rates (Van Remmen et al., 2003).

Mice lacking CuZnSOD have also been obtained. When young, they appear normal (although they are more sensitive to ROS-generating toxins) but as they age, neurological damage, hearing loss, and cancers (especially liver cancer) can develop at an accelerated

rate. They have reproductive problems and show impaired vascular function (Elchuri et al., 2005).

It follows that $O_2^{\bullet -}$ can cause severe damage. But why? Superoxide in aqueous solution does not react with many biomolecules. However, those few that it does attack are very important. First, its reaction with $NO^{\bullet}$ to give $ONOO^-$ is fast. A second clue came from studies with bacteria (Imlay, 2003). Superoxide inactivates several enzymes important in energy production and amino acid metabolism. These enzymes have iron-sulfur clusters; inactivation is caused by oxidation of the cluster, leading to release of iron, followed by Fenton chemistry. The oxidized enzymes can be repaired in vivo by reassembling the iron clusters. Indeed, even the low basal levels of $O_2^{\bullet -}$ production in *Escherichia coli* damage these enzymes, and activity is maintained by constantly repairing them (Imlay, 2003). If $O_2^{\bullet -}$ levels rise (e.g. at elevated O_2), inactivation rates accelerate, repair cannot keep up, and metabolic pathways are inhibited. Similar damage to Fe-S cluster enzymes occurs in yeast and animals. Superoxide can release additional iron from ferritins, and degradation of haem proteins by H_2O_2 can also release iron. Peroxynitrite also displaces iron from Fe-S proteins (Halliwell and Gutteridge, 2006).

What about H_2O_2? Knockout of catalase or GPx1 in animals does not cause many problems, but removal of both GPx1 and GPx2 predisposed mice to inflammation and cancer of the intestines, and knockout of GPx4 is embryonic lethal (Chu et al., 2004; Halliwell and Gutteridge, 2006). Knockouts of peroxiredoxins in mice also cause problems (anemia, cancer, etc.) later in life (Lee et al., 2003).

ROS: NOT AS BAD AS THEY LOOK

Antioxidant defenses are not 100% effective, since oxidative damage to DNA, proteins, and lipids is demonstrable in all aerobes under ambient O_2. Indeed, this damage may contribute to the age-related development of cancer in animals (Ames, 1983; Halliwell, 2002) and perhaps even to ageing itself. Hence, even 21% O_2 is toxic. So why are all the ROS not eliminated? Probably because ROS perform important roles, so the challenge was to evolve antioxidant defenses that allow such roles while minimizing damage. Mechanisms to cope with oxidative damage are required, e.g. to repair oxidized DNA, reassemble [Fe-S] clusters in enzymes, repair oxidized Met residues on proteins (using Met sulfoxide reductases, some of which are selenium requiring in animals, but not in plants; Moskovitz and Stadtman, 2003), and destroy oxidized lipids and proteins.

In what ways could ROS be beneficial? Plants provide lots of examples. ROS production in animals by phagocytes and by other cells in the gastrointestinal and respiratory tracts is a defense against microorganisms (Fang, 2004; Donko et al., 2005). Everyone is familiar with regulation of cellular processes by phosphorylation and dephosphorylation of enzymes and transcription factors, but we have realized in the past decade that regulation by oxidation and reduction (redox regulation) is equally important. Not only that, the two systems cross talk, i.e. the redox state of the cell influences phosphorylation, and vice versa. For example, binding of ligands to growth factor receptors on animal cells activates protein kinases that then phosphorylate and activate subsequent proteins in the signal cascade. Often at the same time, cellular ROS levels increase and facilitate the signaling. Although some kinases can be directly affected by ROS (e.g. some isoforms of protein kinase C), in general ROS tend not to stimulate phosphorylation directly. Instead, they increase net phosphorylation by inhibiting protein dephosphorylation (Rhee et al., 2005). Protein phosphatase enzymes are constantly active in cells, but can be attacked and inactivated by ROS.

Thus, the ligand binding increases kinase activity, and the ROS assist by transiently inactivating phosphatases. Where do the ROS come from? Sometimes the ligand increases $O_2^{\bullet -}$ production, e.g. by activating $O_2^{\bullet -}$-producing NADPH oxidase enzymes. These were originally described in phagocytes (Fang, 2004), but are now known to be widespread in animal (and plant, see the rest of this issue) cells. In addition, when cells are exposed to extra H_2O_2 (e.g. at a site of injury or inflammation or when NADPH oxidase enzymes are activated), the peroxiredoxins are partially inactivated to allow signaling, neatly explaining why the animal enzymes are more sensitive to inactivation. The cell then rapidly makes more peroxiredoxin, and reactivates the inactive form, so that the extra H_2O_2 can be removed after it has done its job (Georgiou and Masip, 2003; Rhee et al., 2005).

Mitochondrial ROS production is often thought of as a nuisance, an unavoidable consequence of electron leakage under O_2, a view consistent with the severe phenotype of MnSOD-knockout mice. However, another view is that variations in mitochondrial H_2O_2 production are a signal that advises the cytoplasm and nucleus what the mitochondria are doing, leading to changes in nuclear gene transcription via redox regulation and phosphorylation of transcription factors. Mitochondrially targeted antioxidants are proving useful in attempts to study the physiological roles of mitochondrial ROS (Sheu et al., 2006). Chloroplast ROS may perform similar roles.

OXIDATIVE STRESS AND DAMAGE

What happens if the balance between ROS and antioxidants is upset? Having too many ROS in relation to the available antioxidants is said to be a state of oxidative stress. Sies (1991) defined this term as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage. Such damage is often called oxidative damage, which has been defined as the biomolecular damage caused by

attack of reactive species upon the constituents of living organisms (Halliwell and Whiteman, 2004). Increased oxidative damage can result not only from more oxidative stress, but also from failure to repair or replace damaged biomolecules. Oxidative stress can result from decreases in antioxidant levels, e.g. mutations decreasing the levels of MnSOD. Depletions of dietary antioxidants and other essential dietary constituents (e.g. copper, iron, zinc, and magnesium) can also cause it. For example, children with the protein deficiency disease kwashiorkor suffer oxidative stress, involving low GSH levels (lack of sulfur-containing amino acids in the diet) and iron overload (inability to make enough transferrin; Fuchs, 2005). Oxidative stress can also be due to increased ROS production, e.g. by exposure to elevated O_2, the presence of toxins that produce ROS (e.g. paraquat), or excessive activation of natural systems producing ROS, e.g. inappropriate activation of phagocytes (Halliwell and Gutteridge, 2006).

What do cells do when under oxidative stress? It depends on the cell and the level of stress applied (Fig. 2). Usually intracellular free Ca^{2+} levels rise; so do levels of iron catalytic for free radical reactions (Fig. 2). Several cell types respond to mild oxidative stress by proliferating, which can be good in wound healing but bad if it leads to tissue fibrosis (Cave et al., 2005). Cells may adapt to the stress by up-regulation of defense and/or repair systems. This may completely protect against damage, protect against damage to some extent but not completely, or sometimes overprotect; the cells are then resistant to higher levels of oxidative stress imposed subsequently. Adaptation need not always involve increases in antioxidants:

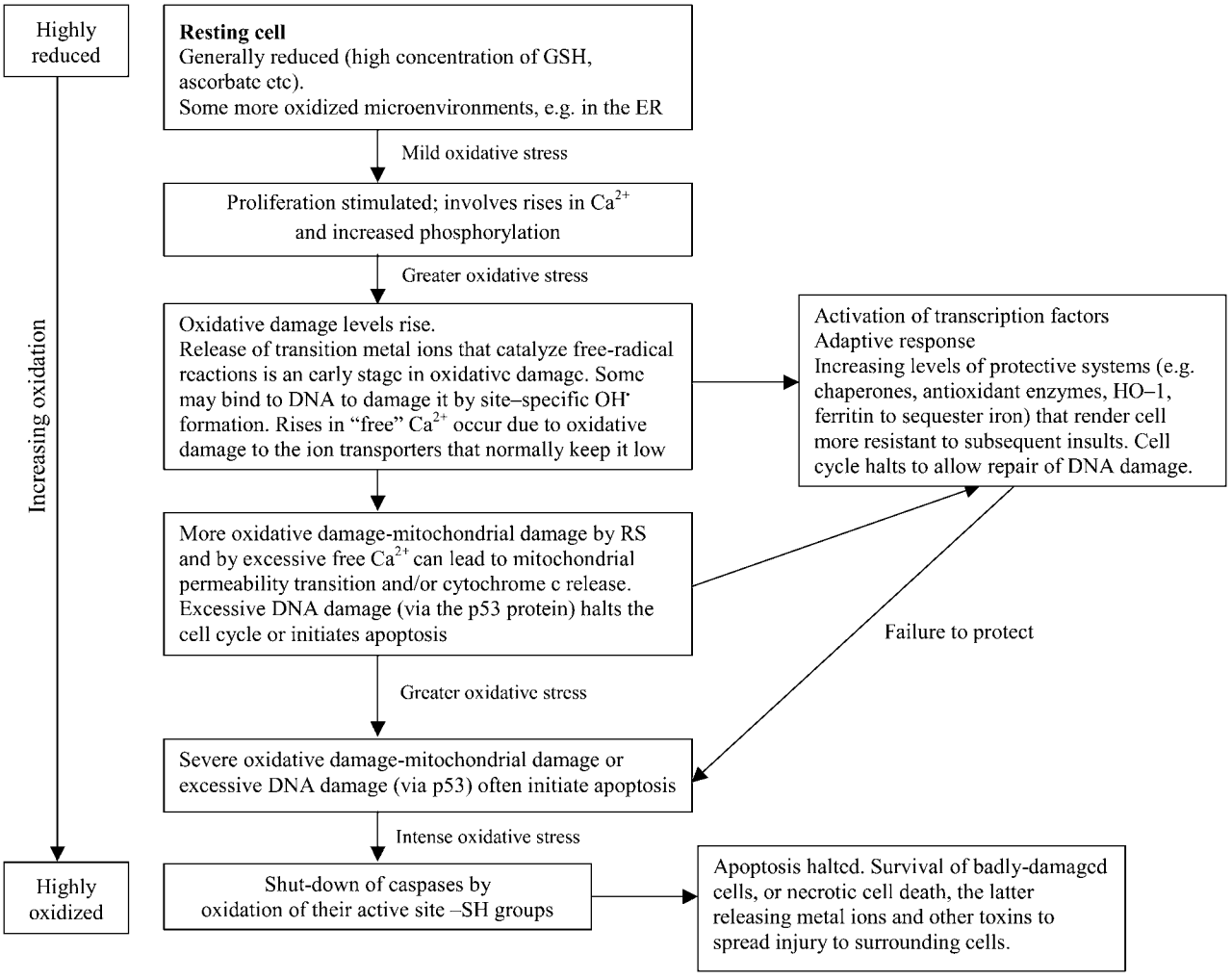


Figure 2. How cells respond to oxidative stress. Adapted from Halliwell and Gutteridge (2006) by courtesy of Oxford University Press. Stimulation of proliferation by low levels of reactive species is associated with increased net phosphorylation of multiple proteins. The cell is generally a reducing environment, especially the mitochondria (GSH/GSSG > 100) and cytosol (GSH/GSSG > 100), but less so in the endoplasmic reticulum (ER) lumen (GSH/GSSG = approximately 3), since a more-oxidizing environment is required for optimal protein folding and disulphide bridge formation. HO-1, Haem oxygenase 1; RS, reactive species.

there can be decreases in ROS-producing systems, increases in other protective mechanisms (such as chaperones), or changes in oxidative damage targets (e.g. *E. coli* under oxidative stress can replace a fumarase enzyme sensitive to inactivation by $O_2^{\bullet -}$ with one that resists $O_2^{\bullet -}$ [Liochev and Fridovich, 1993]). Moderate oxidative stress usually halts the cell cycle, or can drive cells into senescence; the cell survives but can no longer divide. Severe oxidative damage, especially to DNA, may trigger death by apoptosis, necrosis, or mechanisms with features of both. Indeed, ROS act as triggers of apoptosis, and as participants in apoptosis induced by other mechanisms, in both plants and animals.

SUMMARY

ROS are all over the place in plants, animals, and aerobic bacteria. We cannot live without them or we will probably die from infections. Yet, they often kill us in the end; over the long human lifespan, the continual damage by ROS, if not properly repaired (and repair efficiency tends to drop in the aged) can contribute to the age-related development of cancer, neurodegenerative diseases, and many other disorders (Ames, 1983; Halliwell and Gutteridge, 2006). This may be a by product of evolution; ROS are essential for defense against infection and signaling, keeping you alive until your reproduction has finished and children have grown up. Who cares if they kill you in the later post-reproductive years? Evolution doesn't. So why does taking antioxidant supplements not make us live healthily for ever? Simply because the human body regulates the ROS/antioxidant balance so carefully that feeding antioxidants does not disturb it much, and oxidative damage does not decrease (Halliwell, 1999, 2000).

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