Free Radicals and other reactive species in Disease

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Introduction

Free radicals can be generated in a wide variety of chemical and biological systems, including the formation of plastics, the ageing of paints, the combustion of fuels and in the human body. In living organisms, the levels of free radicals and other 'reactive species' are controlled by a complex web of antioxidant defences, which minimize (but do not completely prevent) oxidative damage to biomolecules. In human disease, this 'oxidant–antioxidant' balance is tilted in favour of the reactive species, so that oxidative damage levels increase. In some diseases, this makes a significant contribution to tissue injury, giving rise to prospects for therapeutic intervention with rationally designed antioxidant drugs.

Basic Definitions

Free radicals

In the structure of atoms and molecules, electrons usually associate in pairs, each pair moving within a defined region of space (an atomic or molecular orbital). One electron in each pair has a spin quantum number of $+1/2$, the other $-1/2$. A free radical is any species capable of independent existence (hence the term 'free') that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. The simplest free radical is an atom of the element hydrogen, with one proton and a single electron. Examples of oxygen-centred radicals (i.e. the unpaired electron is located on O) are superoxide ($O_2\bullet^-$) and hydroxyl ($OH\bullet$). Thiyl radicals ($RS\bullet$) are sulfur-centred radicals, trichloromethyl ($CCl_3\bullet$) is an example of a carbon-centred radical, and nitric oxide ($NO\bullet$), is a free radical in which the unpaired electron is delocalized between two different atoms. A dot is always used to denote free radicals.

Radicals can add together; for example, atomic hydrogen forms diatomic hydrogen (eqn [I]),

$$H\bullet + H\bullet \rightarrow H_2 \qquad [I]$$

and superoxide reacts with nitric oxide (eqn [II]).

$$O_2\bullet^- + NO\bullet \rightarrow ONOO^- \text{ (peroxynitrite)} \qquad [II]$$
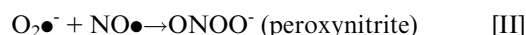
In all cases, a nonradical is formed. This is usually less reactive than the parent radicals (e.g. H_2 is less chemically reactive than $H\bullet$) but not always. For example, $ONOO^-$ is more damaging to human tissues than either $O_2\bullet^-$ or $NO\bullet$.

Most biological molecules are nonradicals. When a free radical reacts with a nonradical, a new free radical is generated. For example, $OH\bullet$ reacts with hydrocarbons (including the fatty acid side-chains of membrane lipids) to abstract $H\bullet$ and leave behind a carbon-centred radical (eqn [III]).

$$\geqslant CH + OH\bullet \rightarrow \geqslant C\bullet + H_2O \qquad [III]$$
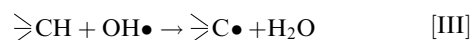
This process can start the free radical chain reaction of lipid peroxidation. A reactive free radical such as $OH\bullet$ abstracts hydrogen from a fatty acid side-chain as above. The resulting carbon-centred radicals react with oxygen (eqn [IV]).

$$\geqslant C\bullet + O_2 \rightarrow \geqslant C-O-O\bullet \qquad [IV]$$
$$\text{peroxyl radical}$$
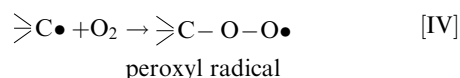
Peroxyl radicals attack membrane proteins, and can also attack adjacent fatty acid side-chains (eqn [V]).

$$\geqslant C-O-O\bullet + \geqslant CH \rightarrow \geqslant C-O-OH + \geqslant C\bullet \qquad [V]$$
$$\text{lipid hydroperoxide}$$
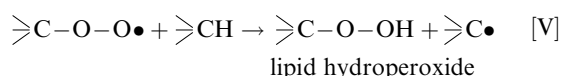
The $C\bullet$ radical reacts with O_2 to give another peroxyl radical and the chain reaction continues. Hence, attack of a single $OH\bullet$ can cause oxidation of multiple fatty acid side-chains to lipid hydroperoxides. The polyunsaturated fatty acid side-chains essential to the fluidity of membranes are

the most susceptible to free radical attack and subsequent lipid peroxidation.

As a different example, OH• adds to the purine base guanine in DNA, but again a new radical is generated, an 8-hydroxyguanine radical (eqn [VI]).

$$\text{Guanine} + \text{OH}\bullet \rightarrow [\text{8-hydroxyguanine}]\bullet \qquad [VI]$$
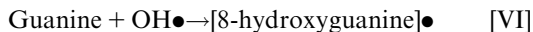
Reactive oxygen species

The term reactive oxygen species (ROS), often used in the biomedical free radical literature, is a collective term that includes not only oxygen-centred radicals such as $O_2\bullet^-$ and OH•, but also some nonradical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen $^1\Delta_g$, and hypochlorous acid (HOCl) (**Table 1**). A similar term, reactive nitrogen species, is also becoming widely used (**Table 1**). Some of these species are much less 'reactive' than others, e.g. $O_2\bullet^-$ and NO• react directly with few molecules in the human body, whereas OH• can react with anything. When generated *in vivo*, OH• will react at its site of formation.

Antioxidant

'Antioxidant' is a term that is widely used but it is surprisingly difficult to define clearly. Food scientists use antioxidants to inhibit lipid peroxidation, a process which causes rancidity in food materials, so they often regard a good antioxidant as one that efficiently inhibits lipid peroxidation. Museum curators use 'antioxidants' to preserve organic artefacts. Polymer scientists use 'antioxidants' to control polymerization in the manufacture of rubber, plastics and paint and for the protection of clear plastics against ultraviolet light. Combustion is a free radical process: the oil industry makes extensive use of antioxidants and a knowledge of free radical mechanisms in the design of better automobile fuels and lubricating oils. All these scientists have their own views on what a good antioxidant should be.

What about living organisms? When reactive species are generated *in vivo*, many antioxidants come into play. Their relative importance depends upon which species is generated, how it is generated, where it is generated, and what biomolecular target of damage is measured. To encompass these various complexities, I use a broad definition of an antioxidant as Any substance that, when

Table 1 Reactive species

Reactive Oxygen Species (ROS)

Radicals	*Nonradicals*
Superoxide, $O_2\bullet^-$	Hydrogen peroxide, H_2O_2
Hydroxyl, OH•	Hypochlorous acid, HOCl
Peroxyl, $RO_2\bullet$ (e.g. lipid peroxyl, see text)	Hypobromous acid, HOBr
	Ozone, O_3
Alkoxyl, RO•	Singlet oxygen $^1\Delta_g$
Hydroperoxyl $HO_2\bullet$	

Reactive Nitrogen Species (RNS)

Radicals	*Nonradicals*
Nitric oxide (nitrogen monoxide), NO•	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2\bullet$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+
	(e.g. as nitryl chloride, NO_2Cl)
	Alkyl peroxynitrites, ROONO

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, O_3, $ONOO^-$, $^1O_2$, H_2O_2). RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as such nonradicals as HNO_2 and N_2O_3. $ONOO^-$ is often included in both categories. 'Reactive' is not always an appropriate term: H_2O_2, NO• and $O_2\bullet^-$ react quickly with few molecules, whereas OH• reacts quickly with almost everything. $RO_2\bullet$ RO•, HOCl, $NO_2\bullet$, $ONOO^-$ and O_3 have intermediate reactivities. HOCl and NO_2Cl could also be regarded as 'reactive chlorinating species'; HOBr as a 'reactive brominating species'.

present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.

The term 'oxidizable substrate' includes every type of molecule found *in vivo*. This definition emphasizes the importance of the damage target studied and the source of 'reactive species' used when antioxidant action is examined *in vitro*. There is no universal 'best' antioxidant; different antioxidants are needed to protect different biomolecules *in vivo*.

Free Radicals *in Vivo* – the Concept of Oxidative Stress

Formation of reactive species

Free radicals and other reactive species are constantly generated in the human body. Some are made by 'accidents of chemistry'; for example, leakage of electrons directly on to O_2 from the intermediate electron carriers of the mitochondrial electron transport chain generates a steady stream of $O_2\bullet^-$. Exposure of living organisms to ionizing radiation splits the $O-H$ bonds in water (the major constituent of living cells) to generate $OH\bullet$ and $H\bullet$. The hydroxyl radical reacts at a diffusion-controlled rate with almost all molecules in living cells. Hence, when $OH\bullet$ is formed *in vivo*, it damages whatever it is generated next to – it cannot migrate any significant distance within the cell. Indeed, the harmful effects of excess exposure to ionizing radiation on living organisms are thought often to be initiated by attack of $OH\bullet$ on proteins, DNA and lipids. For example, lipids undergo lipid peroxidation and the [8-hydroxyguanine]$\bullet$ radical can lose one electron to form the mutagenic oxidized base 8-hydroxyguanine (which produces GC→TA transversion mutations).

Whereas $OH\bullet$ is probably always harmful, other (less reactive) free radicals may be useful *in vivo*. Thus, $NO\bullet$ is synthesized from the amino acid L-arginine by vascular endothelial cells, phagocytes, and many other cell types. Nitric oxide has multiple functions: for example, it helps to regulate blood pressure and may be involved in the killing of parasites by macrophages. Superoxide radical ($O_2\bullet^-$), the one-electron reduction product of oxygen, is produced by phagocytic cells and helps them to kill bacteria. Evidence is accumulating to suggest that smaller amounts of extracellular $O_2\bullet^-$ may be generated, perhaps as intercellular signal molecules, by several other cell types, including endothelial cells, lymphocytes and fibroblasts. Superoxide may also be involved in the 'sensing' of blood O_2 levels by the carotid body.

Much $O_2\bullet^-$ generated *in vivo* probably undergoes a dismutation reaction, represented by the overall equation [VII],

$$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{[VII]}$$
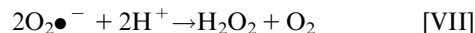
in which hydrogen peroxide (H_2O_2), a nonradical, is formed.

H_2O_2 resembles water in its molecular structure and is very diffusible within and between cells. As well as arising from $O_2\bullet^-$, H_2O_2 can be produced by the action of several oxidase enzymes in cells, including amino acid oxidases and xanthine oxidase. Like $O_2\bullet^-$, H_2O_2 can have useful metabolic functions. For example, H_2O_2 is used by the enzyme thyroid peroxidase to help make thyroid hormones, and H_2O_2 is sometimes used as an intracellular signal molecule. For example, activation of nuclear factor κB by tumour necrosis factor α in some cell types may require H_2O_2, and H_2O_2 can inhibit protein phosphatases and so increase net protein phosphorylation. H_2O_2 is poorly reactive but can combine with iron or copper ions to generate highly-reactive $OH\bullet$, (e.g. eqn [VIII]).

$$H_2O_2 + Fe^{2+} \text{ (or } Cu^+) \rightarrow OH\bullet + OH^- + Fe^{3+} \text{ (}Cu^{2+})$$

$$\text{[VIII]}$$
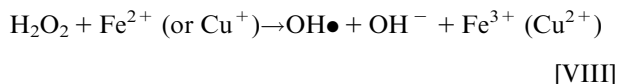
Antioxidant defences

Antioxidant defence systems scavenge, and minimize the formation of, reactive oxygen species but they are not 100% effective. Hence, repair systems exist to deal with molecules that have been oxidatively damaged. Damage to DNA by $OH\bullet$ appears to occur in all aerobic cells and is thought by many to be a significant contributor to the age-dependent development of cancer. The antioxidant network is complex, and includes both endogenous and diet-derived molecules. Superoxide dismutase enzymes (SODs) remove $O_2\bullet^-$ by accelerating its conversion to H_2O_2 (eqn [VII]). Human cells have a SOD enzyme containing manganese at its active site (MnSOD) in the mitochondria. A SOD with copper and zinc at the active site (CuZnSOD) is also present, but largely in the cytosol. Catalase enzymes convert H_2O_2 to water and O_2, but more important H_2O_2-removing enzymes in human cells are the glutathione peroxidases (GSHPX), one of the few classes of human enzymes that require selenium for their action. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Organisms are also careful to keep iron and copper safely protein-bound whenever possible, so that the reaction shown in equation [VIII] is prevented.

In addition to enzymes, low-molecular mass free radical scavengers exist. GSH can scavenge various reactive species (e.g. HOCl and $ONOO^-$) directly, as well as being

a substrate for GSHPX enzymes. α-Tocopherol (derived from the diet, as vitamin E) is the most important free radical scavenger within membranes. It can inhibit lipid peroxidation by scavenging peroxyl radical intermediates (eqn [IV]) and so halting the chain reaction. Several other antioxidants are present in the diet, including ascorbate (vitamin C) and flavonoids.

Antioxidant defences exist as a balanced coordinated system. Thus, although SOD is important to normal cell function, an excess of SOD in relation to activities of peroxide-metabolizing enzymes can be deleterious. This has been shown by transfecting cells with human cDNAs encoding SOD. The consequences of excess SOD may be relevant to the clinical condition known as Down syndrome, in which trisomy of chromosome 21 leads to elevated levels of CuZnSOD, the gene encoding which is located on this chromosome.

In the healthy human body, there is an approximate balance between production of reactive species and antioxidant defences. Indeed, low levels of products of free radical attack on biomolecules are found even in healthy tissues (**Table 2**). Antioxidant defences do not completely prevent attack by reactive species, so that repair systems are also needed to minimize damage levels. Repair of oxidized DNA (e.g. removal of 8-hydroxyguanine residues) is especially important.

Oxidative stress

The term oxidative stress refers to the situation of serious imbalance between production of reactive species and antioxidant defence. Sies, who introduced the term from the title of the book he edited in 1985, introduced a definition in 1991 in the introduction of the second edition as 'a disturbance in the prooxidant–antioxidant balance in favour of the former, leading to potential damage'.

In principle, oxidative stress can result from:

1. Diminished antioxidants, e.g. mutations affecting antioxidant defence enzymes (such as CuZnSOD, MnSOD and GSHPX) or toxic agents that deplete such defences. For example, many xenobiotics are metabolized by conjugation with GSH; high doses can deplete GSH and cause oxidative stress even if the xenobiotic is not itself a generator of reactive species. Depletions of dietary antioxidants and other essential dietary constituents can also lead to oxidative stress.
2. Increased production of ROS/RNS, e.g. by exposure to elevated levels of toxins that are themselves reactive species (e.g. nitrogen dioxide gas, $NO_2\bullet$) or are metabolized to generate such species, or by excessive activation of 'natural' ROS/RNS-producing systems (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases, such as rheumatoid arthritis and ulcerative colitis).

Table 2 Evidence that damage by reactive oxygen (ROS) and nitrogen species (RNS) occurs *in vivo*

Target of damage	Evidence
DNA	Low levels of oxidative base damage products are present in DNA isolated from all aerobic cells; levels often increase in patients with chronic inflammatory diseases or subjected to oxidative stress, e.g. from smoking. Some base damage products are excreted in urine, presumably resulting from DNA repair processes. Smokers and rheumatoid arthritis patients excrete more 8-hydroxydeoxyguanosine (8-OHdG). Elevated 8-OHdG concentrations are frequently observed in animals treated with carcinogens or other toxins.
Protein	Attack of ROS upon proteins produces carbonyls and other amino acid modifications (e.g. methionine sulfoxide, valine hydroxides, 2-oxohistidine, protein peroxides, hydroxylation of tyrosine to DOPA, formylkynurenine). Low levels of carbonyls and certain other products (e.g. *ortho*-tyrosine, valine oxidation products) have been detected in healthy animal tissues and body fluids. Nitrotyrosines, products of attack on tyrosine by RNS, have been detected in atherosclerotic lesions, human plasma and urine; concentrations are higher in body fluids/tissues from patients with chronic inflammatory diseases. Bityrosine has been detected in urine and atherosclerotic lesions.
Lipid	Accumulation of 'age pigments' in tissues. Lipid peroxidation in atherosclerotic lesions. Presence of specific end products of peroxidation (e.g. isoprostanes) in body fluids (including urine); levels increase in plasma during oxidative stress, e.g. in smokers, in CCl_4 treatment of animals, and in premature babies.
Uric acid	Attacked by several ROS to generate allantoin, cyanuric acid, parabanic acid, oxonic acid and other products, which are present in human body fluids. Levels of these products increase in chronic inflammatory/metal overload diseases.

Mechanism 2 is usually thought to be more relevant to human diseases and is frequently the target of attempted therapeutic intervention, but the antioxidant nutritional status of sick patients may often be compromised.

Consequences of oxidative stress

Oxidative stress can result in:

1. Adaptation: for example by upregulation of antioxidant defence systems. For example, if adult rats are gradually acclimatized to elevated O_2, they can tolerate pure O_2 for much longer than control rats, apparently owing to increased synthesis of antioxidant defence enzymes and of GSH in the lung. Ischaemic preconditioning provides another example. A brief period of ischaemia in pig hearts leads to depression of contractile function, and administration of antioxidants to the animals offers protection. However, repeated periods of ischaemia lead to quicker return of contractile function on reperfusion, but this adaptive response is blocked in the antioxidant-treated animals. Hence ROS produced by ischaemia are initially damaging, but also lead to a response protective against subsequent insult.

2. Tissue injury: oxidative stress can cause damage to all molecular targets; DNA, proteins and lipids (lipid peroxidation). Often, it is not clear which is the first point of attack, since injury mechanisms overlap widely. Indeed, depending on the tissue under study, the primary cellular target of oxidative stress can vary. For example, when H_2O_2 is added to many mammalian cells, increased DNA strand breakage occurs before detectable lipid peroxidation or oxidative protein damage. This DNA damage appears to involve conversion of H_2O_2 into $OH\bullet$ in the cell nucleus, by reaction with transition metal ions (eqn [VIII]).

3. Cell death: this can occur by essentially two mechanisms, necrosis and apoptosis. Both can result from oxidative stress. In necrotic cell death, the cell swells and ruptures, releasing its contents into the surrounding area and affecting adjacent cells. Contents can include antioxidants such as catalase or GSH, and pro-oxidants such as copper and iron ions. Hence even if a cell dies by mechanisms other than oxidative stress, necrotic cell death can lead to oxidative stress in the surrounding environment. In apoptosis, the cell's own intrinsic 'suicide mechanism' is activated; apoptosing cells do not release their contents and so apoptosis does not, in general, cause damage to surrounding cells. Apoptotic cell death may be accelerated in certain diseases, such as some of the neurodegenerative diseases, and oxidative stress has been implicated.
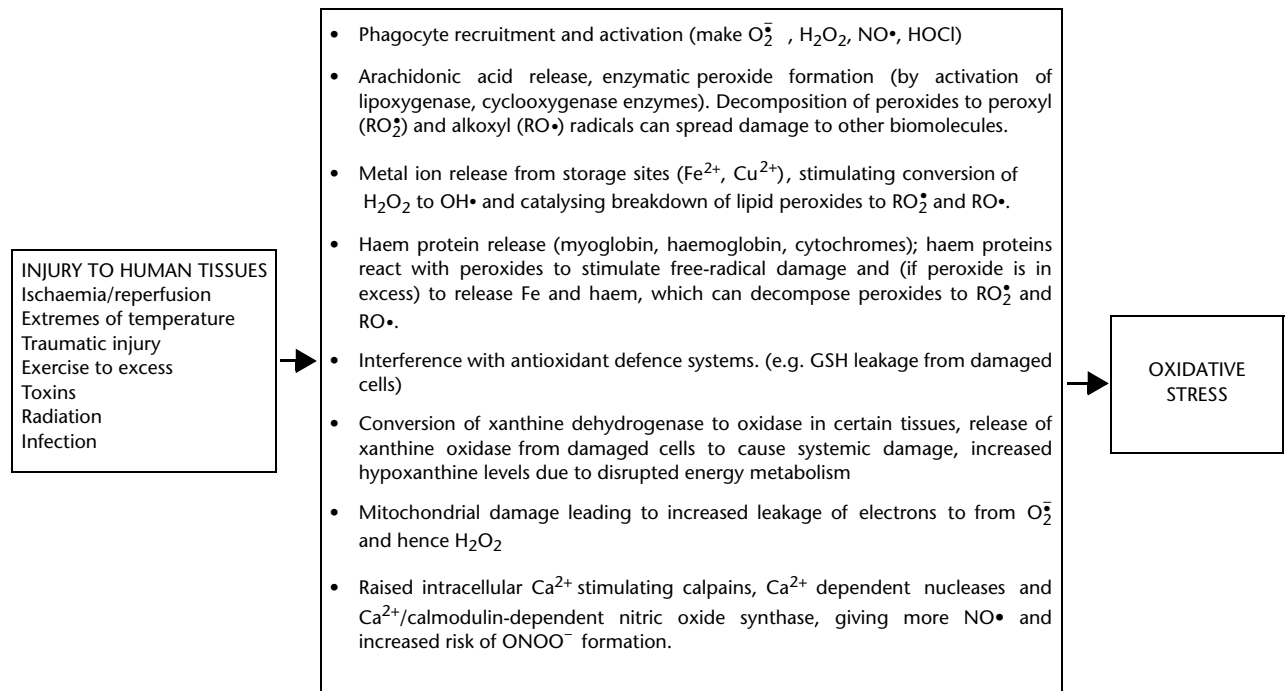

INJURY TO HUMAN TISSUES
Ischaemia/reperfusion
Extremes of temperature
Traumatic injury
Exercise to excess
Toxins
Radiation
Infection

→

- Phagocyte recruitment and activation (make $O_2^{\bullet-}$, H_2O_2, NO•, HOCl)

- Arachidonic acid release, enzymatic peroxide formation (by activation of lipoxygenase, cyclooxygenase enzymes). Decomposition of peroxides to peroxyl ($RO_2^\bullet$) and alkoxyl (RO•) radicals can spread damage to other biomolecules.

- Metal ion release from storage sites (Fe^{2+}, Cu^{2+}), stimulating conversion of H_2O_2 to OH• and catalysing breakdown of lipid peroxides to $RO_2^\bullet$ and RO•.

- Haem protein release (myoglobin, haemoglobin, cytochromes); haem proteins react with peroxides to stimulate free-radical damage and (if peroxide is in excess) to release Fe and haem, which can decompose peroxides to $RO_2^\bullet$ and RO•.

- Interference with antioxidant defence systems. (e.g. GSH leakage from damaged cells)

- Conversion of xanthine dehydrogenase to oxidase in certain tissues, release of xanthine oxidase from damaged cells to cause systemic damage, increased hypoxanthine levels due to disrupted energy metabolism

- Mitochondrial damage leading to increased leakage of electrons to from $O_2^{\bullet-}$ and hence H_2O_2

- Raised intracellular Ca^{2+} stimulating calpains, Ca^{2+} dependent nucleases and Ca^{2+}/calmodulin-dependent nitric oxide synthase, giving more NO• and increased risk of $ONOO^-$ formation.

→

OXIDATIVE STRESS

Figure 1 Some of the reasons why tissue injury leads to oxidative stress.

Reactive Species and Human Disease

Free radicals and other reactive species have been implicated in the pathology of over 100 human diseases, ranging from ulcerative colitis and haemorrhagic shock to cystic fibrosis and AIDS. Some human diseases may be caused by oxidative stress. For example, ionizing radiation generates OH● by splitting water molecules and many of the biological consequences of excess radiation exposure are probably due to oxidative damage to proteins, DNA, and lipids. The symptoms produced by chronic dietary deficiencies of selenium (e.g. Keshan disease) or of tocopherols (neurological disorders seen in patients with defects in intestinal fat absorption) may also be mediated by oxidative stress since selenium is an essential cofactor for GSHPX enzymes and vitamin E is an important protector against lipid peroxidation. Persistent damage to DNA by ROS/RNS may play a role in the initiation of some human cancers, by creating mutagenic lesions such as 8-hydroxyguanine.

However, in few diseases are reactive species the primary cause of the condition. More often, their increased formation is a consequence of the disease pathology, for the reasons summarized in **Figure 1**. In many cases, the resulting oxidative stress makes a significant further contribution to tissue injury. For example, injury to vascular endothelium by turbulent blood flow, viral infections or circulating toxins can initiate the process of atherosclerosis. Recuitment of monocytes to the injured vessel wall and their development into macrophages, followed by the free radical-mediated peroxidation of low-density lipoproteins within the vessel wall, plays a key role in the development of atherosclerotic lesions. Antioxidant inhibitors of lipid peroxidation, such as the drug probucol, have a significant anti-atherosclerotic effect in animals and humans, and low dietary intake of vitamin E is a risk factor for the development of cardiovascular disease. Other diseases in which oxidative stress may play an important role in tissue injury include rheumatoid arthritis, inflammatory bowel disease, acute respiratory distress syndrome (ARDS), emphysema and cancers related to chronic inflammation.
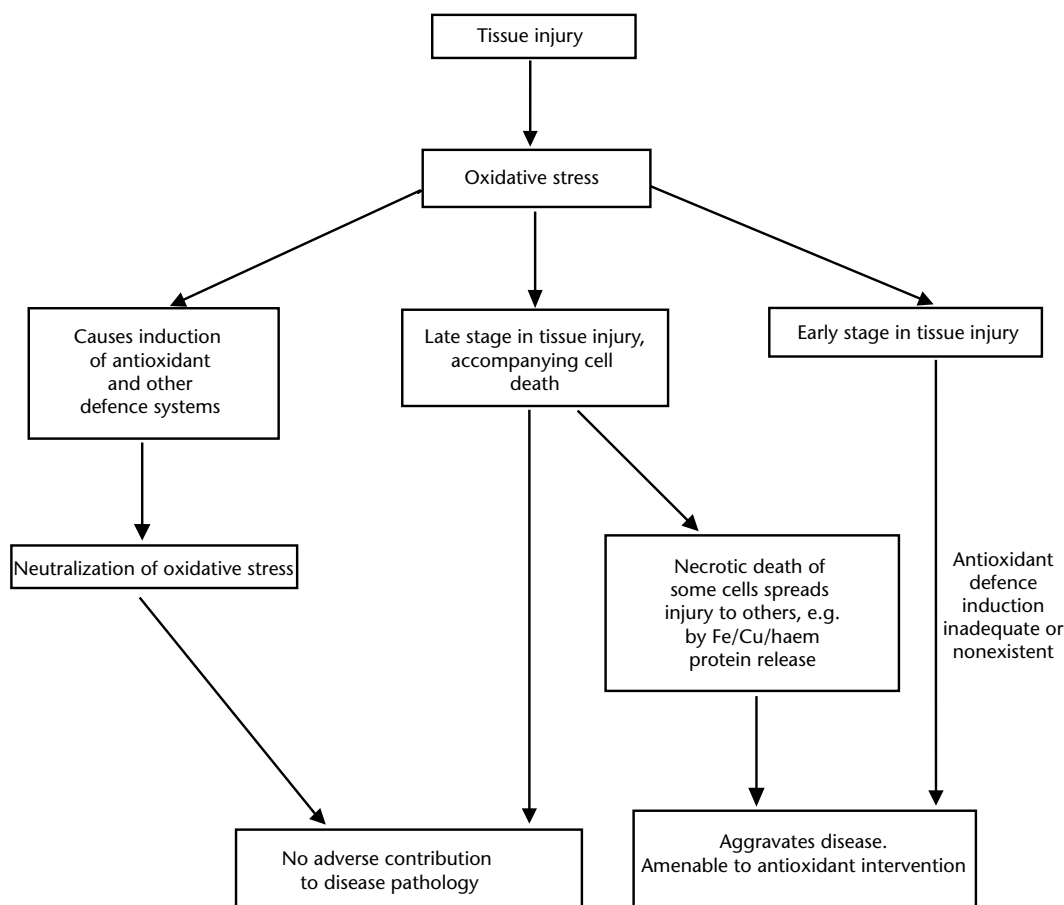

Figure 2 Free radicals: cause, consequence, or no consequence.

Consequences of oxidative stress in human disease

Even though oxidative stress occurs in probably all diseases, it is not necessarily important in the disease pathology. It may be a late stage in tissue injury, accompanying cell death rather than causing it. It may lead to sufficient induction of antioxidant defence mechanisms to protect the tissue (**Figure 2**). The following criteria should be met before concluding that reactive species are important in a given disease;

1. It should be possible to demonstrate their presence at the site of injury, either directly and/or by measuring specific, validated 'biomarkers' of oxidative damage to biomolecules
2. The time course of ROS/RNS formation and/or of oxidative damage should precede or parallel that of tissue injury
3. Direct application of ROS/RNS or their generation within the tissue should reproduce most or all of the tissue damage observed in the disease
4. Scavengers of ROS/RNS, or other antioxidants, should decrease tissue injury to an extent related to their scavenging action or ability to prevent oxidative damage (as indicated by the levels of appropriate biomarkers).

The development of novel therapeutic antioxidants by several companies is under way, especially for the treatment of cardiovascular and neurodegenerative disorders.

Summary

Free radicals and other reactive oxygen and nitrogen species are generated *in vivo*, some by accidents of chemistry and others for useful metabolic purposes. Their levels are modulated by a network of antioxidant defence systems, assisted by repair systems. Tissue injury in human disease is accompanied by an imbalance in the oxidant/antioxidant status, creating oxidative stress. The resulting increased oxidative damage to biomolecules may play an important role in the pathology of several human diseases and is amenable to therapeutic intervention with appropriate antioxidants.

Further Reading

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