

# The role of reactive oxygen and antioxidant species in periodontal tissue destruction

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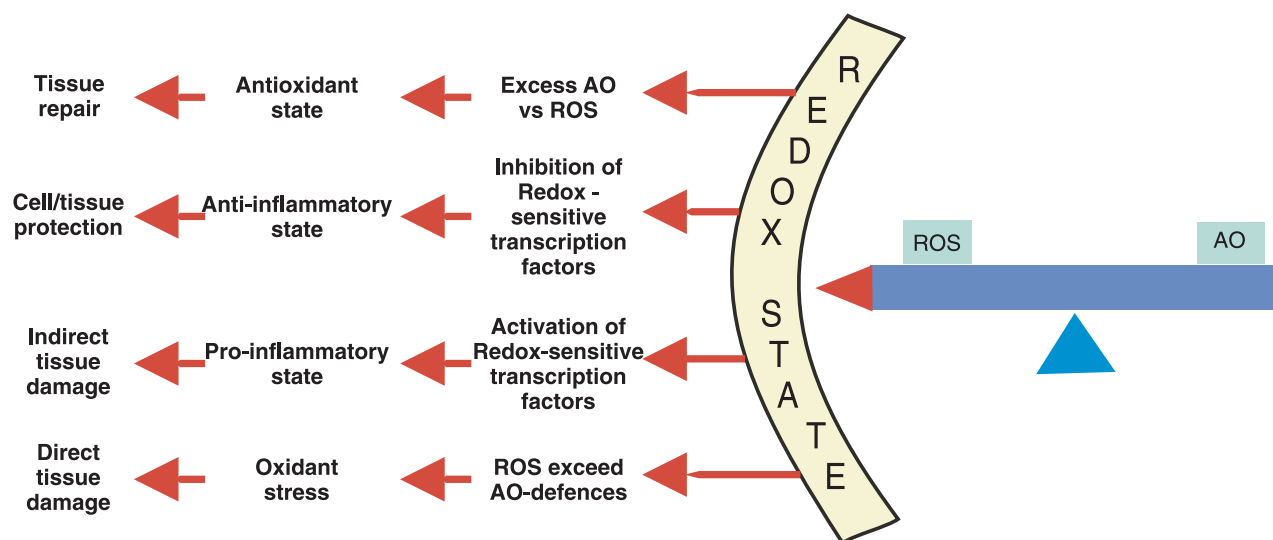
## Context of the review

Periodontitis is a term used to describe an inflammatory process, initiated by the plaque biofilm, that leads to loss of periodontal attachment to the root surface and adjacent alveolar bone and which ultimately results in tooth loss. The inflammatory and immune responses to the bacteria and also viruses (387) that colonize the periodontal and associated tissues involve the systemic circulation and ultimately the peripheral systems of the body. This creates a complex bi-directional series of host-microbial interactions involving cellular and humoral factors and networks of cytokines, chemokines, and growth factors. It is believed that while the primary etiological agent is specific, predominantly gram-negative anaerobic or facultative bacteria within the subgingival biofilm (1, 172), the majority of periodontal tissue destruction is caused by an inappropriate host response to those microorganisms and their products (239). More specifically, a loss of homeostatic balance between proteolytic enzymes (e.g. neutrophil elastase) and their inhibitors (e.g.  $\alpha_1$ -antitrypsin) and reactive oxygen species (ROS) and the antioxidant defense systems that protect and repair vital tissue, cell, and molecular components is believed to be responsible. The basis for such dysregulation is in part genetic (38–82%) (270) and in part the result of environmental factors (e.g. smoking) (311).

This review focuses predominantly on the role of ROS and antioxidant defense systems in the pathobiology of periodontitis, with a view to identifying specific therapeutic targets for future host-modulating therapies. Medline and PubMed databases were searched from 1966 to July 2005 under the following key terms: 'reactive oxygen species', 'oxygen radicals', 'free radicals', 'antioxidants', including terms for

individual antioxidants (e.g. ascorbic acid, ascorbate, vitamin C, urate, uric acid, etc.) and 'periodont\*' or 'periodontitis' or 'periodontal disease'. A total of 503 papers were identified of which 388 were written in English. We acknowledge some excellent original research published in other languages but have largely limited our review to those papers published in English and additional appropriate manuscripts from the biomedical literature to underpin this review. We were conscious that our search criteria did not identify every paper in this field (e.g. 7), and we have therefore supplemented the search with manual searching. If isolated manuscripts have been overlooked, we apologize to those authors and would greatly appreciate a reprint of their published work. We do not intend to go over old ground, which has been widely covered in previous reviews by our group (79, 80) and by our colleagues (39, 64, 434); however, some repetition is necessary to set the field in context for the reader who is new to the field.

A paradigm shift in our understanding of the importance of reactive oxygen and antioxidant species to human biology over the last decade came from the realization that vital and ubiquitous transcription factors, such as nuclear factor- $\kappa$ B and activating protein-1 were redox-sensitive. This is reflected in this review and the reader's attention is specifically drawn to an underlying principle: the smaller, more subtle, changes in intracellular redox-state trigger gene transcription events, which may lead to tissue damage secondary to the induction of a pro-inflammatory state. The larger upward shifts in the pro-oxidant/antioxidant ratio intracellularly bring about direct damage to vital biomolecules and structures, cell membrane damage and dysfunction, and cell death (by necrosis or accelerated apoptosis), and extracellularly cause direct connective tissue damage (both



**Fig. 1.** The biological effects of small and large shifts in the balance of activity between reactive oxygen species (ROS) and antioxidant (AO) species.

mineralized and unmineralized) and damage to extracellular matrices and their components (Fig. 1).

Antioxidant defense systems are therefore also discussed with respect to restoring the intracellular redox balance and their effects upon gene transcription and cell signaling. Also discussed is the ability of antioxidants to prevent the formation of reactive species and to effect their removal and repair the resultant biological tissue damage. At the end of this review, we will briefly allude to potential therapeutic strategies that are worthy of exploration in the future, to modulate the inappropriate host response currently deemed responsible for the majority of periodontal tissue damage manifest as 'periodontitis'.

## Background and terminology

Free radicals have been defined as 'any species capable of independent existence that contain one or more unpaired electrons' (175). They are, by nature, highly reactive and diverse species, capable of extracting electrons and thereby oxidizing a variety of biomolecules vital to cell and tissue function, which not only include oxygen free radicals, but also nitrogen and chlorine species. This review will predominantly focus on oxygen radicals. However, it is not possible to review ROS activities without discussing briefly collaborating species from other classes. In particular peroxynitrite ( $\text{ONOO}^-$ ) formation, derived from the reaction of nitric oxide ( $\text{NO}^\bullet$ ) and superoxide ( $\text{O}_2^{\bullet -}$ ) will be briefly discussed, given new data indicating its hitherto under-appreciated importance in the pathogenesis of inflammatory diseases (96).

ROS is a term that has become more popular because it encompasses other reactive species which are not true radicals but are nevertheless capable of radical formation in the intra- and extracellular environments. Table 1 is modified slightly from the comprehensive review by Battino et al. (39) and summarizes the major true oxygen radicals and ROS.

Antioxidants are defined as 'those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay or inhibit oxidation of that substrate' (181). In normal physiology there is a dynamic equilibrium between ROS activity and antioxidant defense capacity and when that equilibrium shifts in favor of ROS, either by a reduction in antioxidant defenses or an increase in ROS production or activity, oxidative stress results. Oxidative stress was defined by Sies (381) as 'a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage'. Given that it is estimated that between 1 billion and 3 billion reactive species are generated per cell per day, the importance of antioxidant defense systems to the maintenance of health becomes clear (19).

The redox potential is a measure (in volts) of the affinity of a substance for electrons, relative to hydrogen. Substances more strongly electronegative than hydrogen (i.e. capable of oxidizing hydrogen) have positive redox potentials and are oxidants. Substances less electronegative than (i.e. capable of reducing) hydrogen have negative redox potentials and are reducing agents. Oxidation and reduction reactions always go together and are termed redox reactions. Within the gingival crevice/pocket a low redox potential is regarded as essential for the growth

**Table 1.** True radical and reactive oxygen species (ROS) and their symbols

True radicals	Radical symbol	ROS	ROS symbols
Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	$H_2O_2$
Hydroxyl	$\bullet OH$	Hypochlorous acid	HOCl
Perhydroxyl	$HO_2^{\bullet -}$		
Hydroperoxyl	$HOO^{\bullet}$	Singlet oxygen	$^1O_2$
Alkoxy	$RO^{\bullet}$	Ozone	$O_3$
Aryloxy	$ArO^{\bullet}$		
Arylperoxyl	$ArOO^{\bullet}$		
Peroxy	$ROO^{\bullet -}$		
Acyloxy	$RCOO^{\bullet}$		
Acylperoxyl	$RCOOO^{\bullet}$		

Convention is to use  $\bullet$  to signify an unpaired electron and  $-$  or  $+$  to indicate the molecular charge, which may be +ve or -ve or indeed neutral (e.g.  $\bullet OH$ ).

and survival of subgingival anaerobes (308), whereas within cells and tissues a reducing environment (low redox potential) is protective against oxidative stress. There is therefore an apparent conflict in developing future therapeutic strategies for periodontitis which are based on redox biology, because maintaining a low redox status to protect host cells and tissues from oxidative stress is conducive to encouraging growth and survival of anaerobes. However, it is vital to the understanding of this review and the complexities of redox biology to remember that the body is compartmentalized. Bacteria are not intracellular pathogens (unlike viruses) and therefore maintaining a low redox state within a cell may not have relevance to a high redox state within the periodontal pocket/gingival crevice. A key example of compartmentalized differences in antioxidant composition was highlighted by Brock et al. (58), who demonstrated that the antioxidant composition of gingival crevicular fluid differed substantially from the plasma and saliva compartments, with reduced glutathione (GSH) being a major antioxidant within gingival crevicular fluid, whereas uric acid predominates in saliva and plasma, which also possess higher protein-antioxidant levels.

## Reactive oxygen and antioxidant species in biology and medicine

### Atomic and molecular oxygen – basic principles

Atoms have shells containing electrons ( $e^-$ ) which have energy to prevent them being sucked into the

positively charged nucleus. Each shell (numbered 1, 2, 3, 4, etc.) can have up to four orbital patterns (denoted by s, p, d, f) spinning in either direction. Each orbital only has two electrons spinning in opposite directions (the Pauli Exclusion Principle) as a pair. Figure 2 shows that atomic oxygen (O) has *shell-1* with one s-orbital ( $2e^-$ ) and *shell-2* with one s-orbital ( $2e^-$ ) and three p-orbitals ( $4e^-$ ). One of the three p-orbitals is occupied by an electron pair ( $2e^-$ ) but for complex reasons of atomic kinetics (see 444) the remaining two p-orbitals have individual electrons (hence  $8e^-$  in total). The outer  $2e^-$  have the same energy and as each occupies a separate p-orbital they spin in a parallel direction. Atomic oxygen is thus denoted  $(1s)^2 (2s)^2 (2p)^4$ . Molecular di-oxygen ( $O_2$ ) forms by the joining of two oxygen atoms and is regarded as a stable ‘bi-radical’, because it has 16 electrons (eight per atom) occupying two atomic shells, but the outer  $2e^-$  are unpaired. There are two s-orbitals in *shell-1*, two s-orbitals in *shell-2*, and the remaining  $8e^-$  occupy p-orbitals in which  $6e^-$  are paired with opposite spins and the outer  $2e^-$  occupy individual orbitals (because this takes less energy) and, like atomic oxygen, they have a parallel spin. The result is a molecule with a desire to pair up its outer unpaired electrons (therefore a powerful oxidizing agent) but because of the ‘spin restriction’ inherent in oxygen from its outer unpaired  $2e^-$ , which have a parallel spin, it cannot accept electron pairs, because  $2e^-$  do not exist in isolation with parallel spins (atomic energy conservation) to pair with the outer  $2e^-$  of oxygen. Why is this important? It becomes simple to understand the relationship between oxygen and the ROS derived

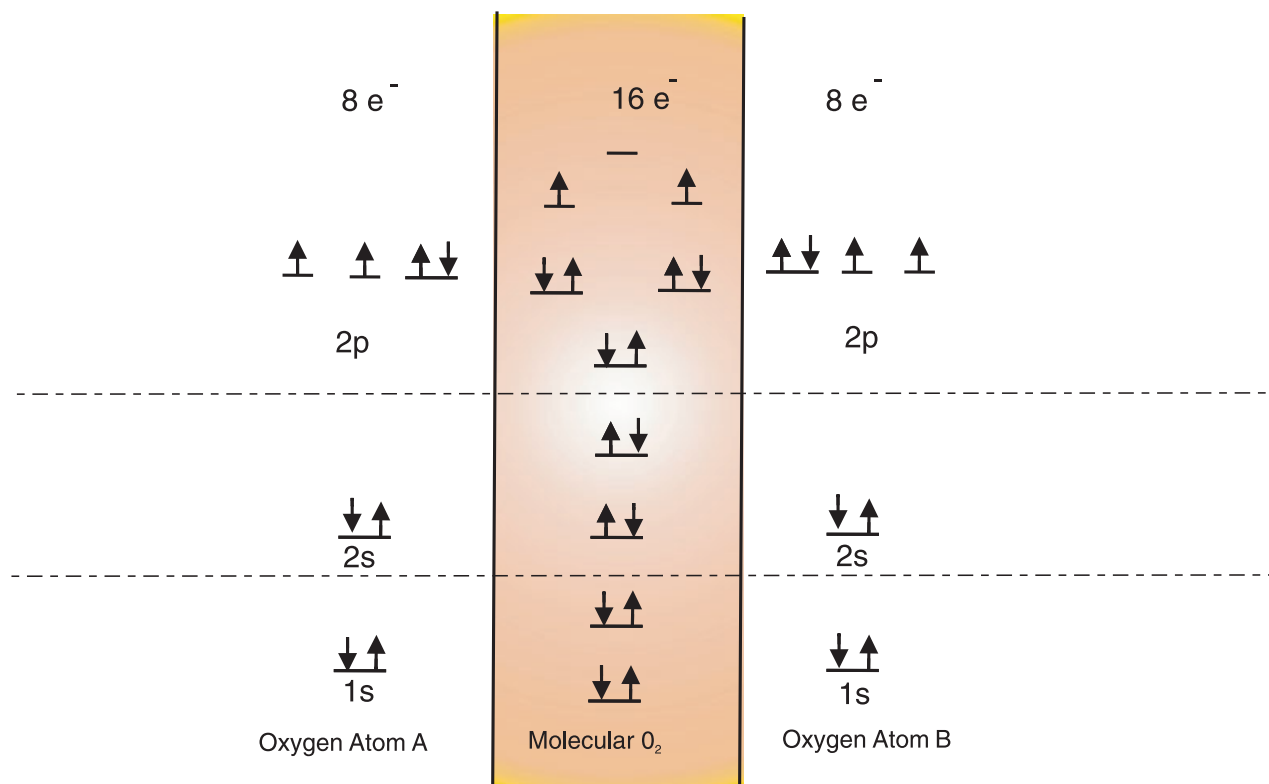
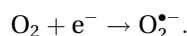
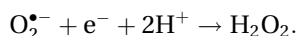


Fig. 2. The organization of electrons within the shells and orbitals of atomic and molecular oxygen. Note the outer two electrons occupy parallel spins in both atomic and molecular oxygen (spin restriction).

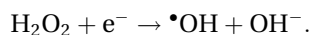
from it, in atomic terms. The addition of one  $e^-$  to oxygen results in the formation of the superoxide anion:



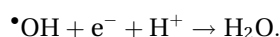
The addition of a second  $e^-$  results in the formation of the ROS hydrogen peroxide ( $H_2O_2$ ):



The addition of a third  $e^-$  results in the formation of the hydroxyl radical ( $\bullet OH$ ):



The addition of a fourth  $e^-$  results in the formation of water ( $H_2O$ ):



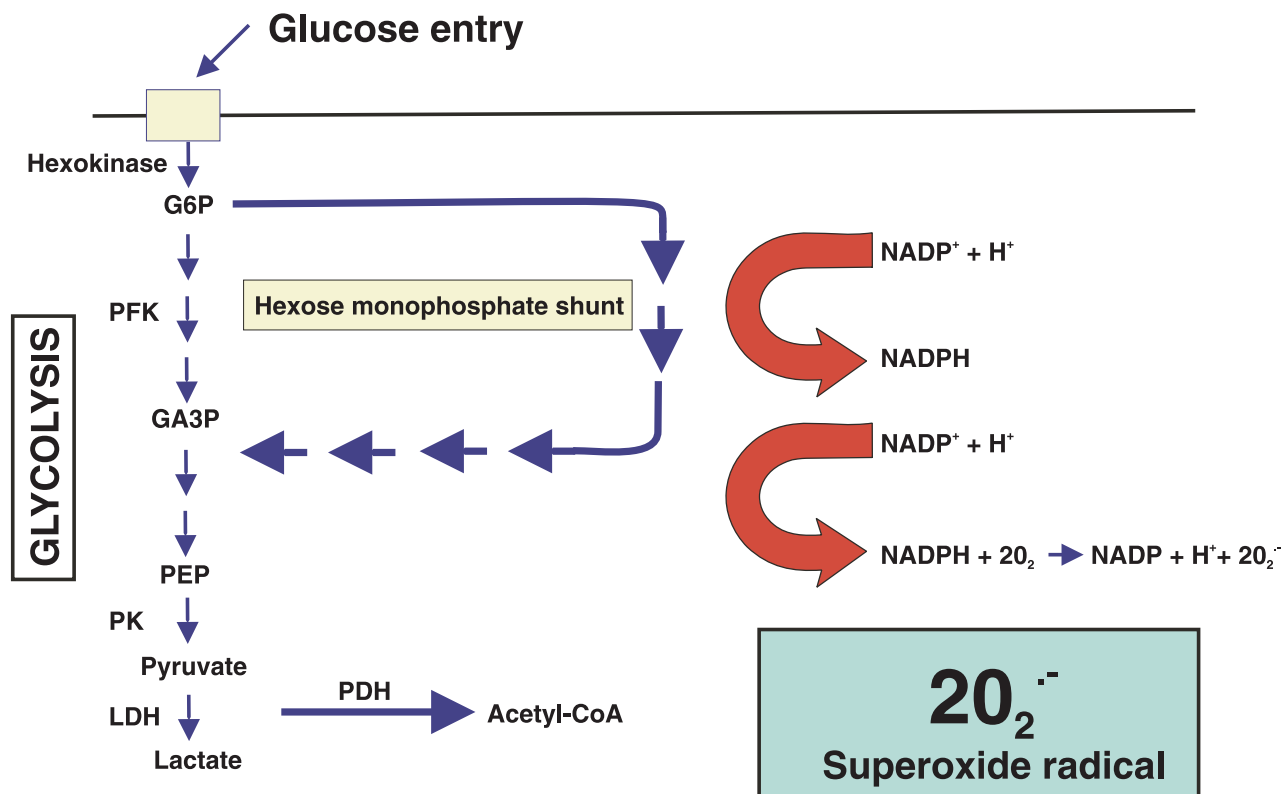
## Origins and formation of ROS and oxygen radicals

The details of oxygen radical and ROS formation and removal have been reviewed previously (see 39, 64, 80, 434). Exogenous sources include heat, trauma, ultrasound, ultraviolet light, ozone, smoking, exhaust fumes, radiation, infection, excessive exercise, and

therapeutic drugs (64, 105, 185). Endogenous sources are primarily:

- bi-products of metabolic pathways – electron leakage from mitochondrial electron transport systems forming superoxide;
- functional generation by host defense cells (phagocytes) and cells of the connective tissues (osteoclasts and fibroblasts).

Cell metabolism involves the consumption of oxygen and its utilization via glycolysis to form pyruvate (Fig. 3) within the mitochondria. The amino acid cycle follows and ATP is generated. However, electrons leak from their transporters at a constant rate, reducing oxygen to the superoxide anion. The incomplete reduction of oxygen is estimated at 1–3% of consumed oxygen (63, 300) and at a rate that exceeds the mitochondrial antioxidant scavenger's ability to remove superoxide. Superoxide dismutase 2, which is manganese-dependent, functions to remove the superoxide radicals that form. Nevertheless, mitochondrial DNA damage by ROS and reactive nitrogen species (RNS) still occurs and is a process believed to be important in certain chronic diseases and in aging (380). Mitochondria are therefore an important source of metabolism-derived ROS *in vivo* and mitochondrial DNA appears to suffer damage more readily than nuclear DNA (96) because of its



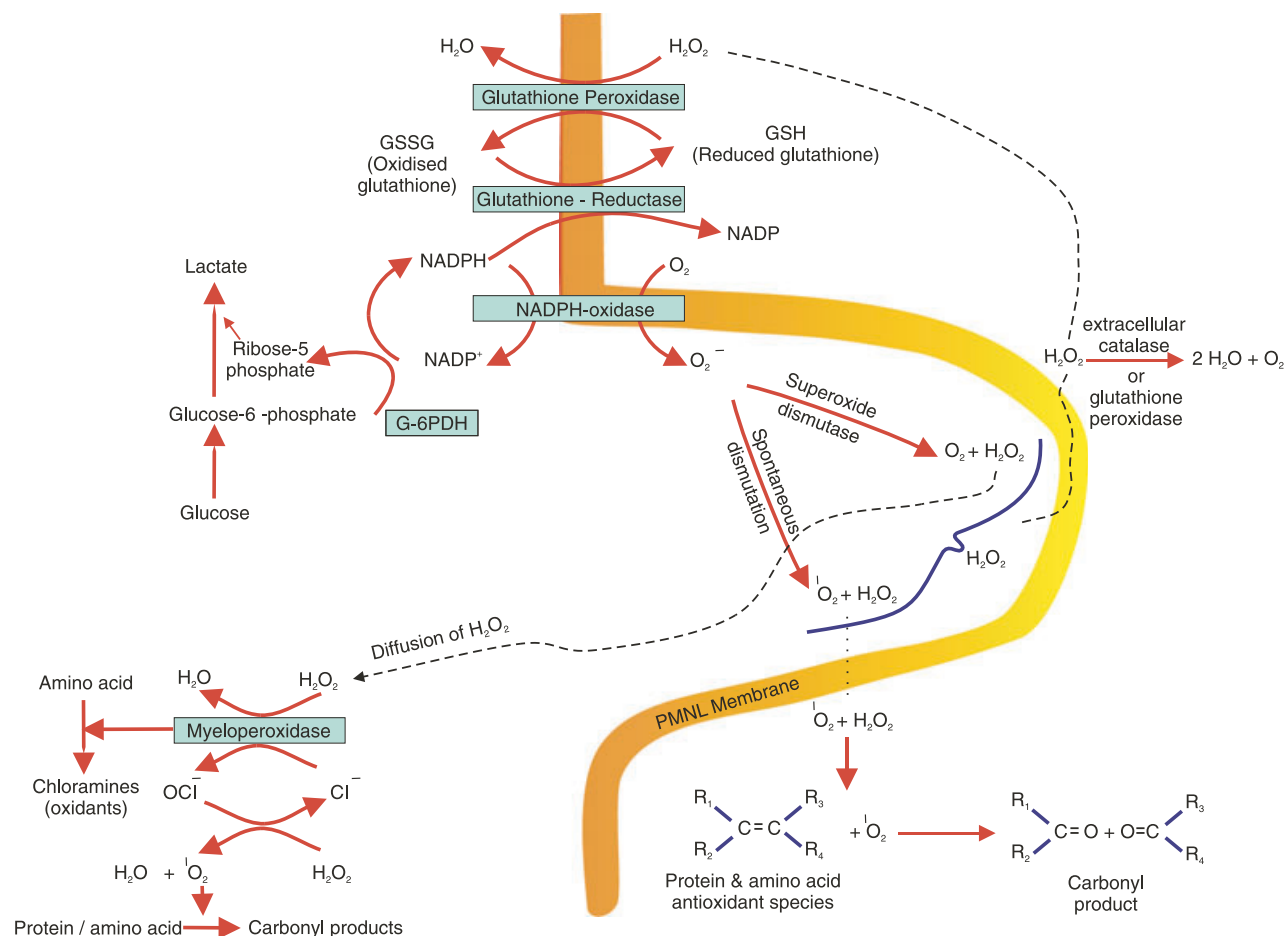
**Fig. 3.** A schematic representation of the NADPH-oxidase (hexose-monophosphate) shunt, demonstrating the use of glucose-6-phosphate (G6P) and NADPH to effect the single electron reduction of oxygen to superoxide. PFK, phos-

phor-fructokinase-1; GA3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; NADP, nicotinamide adenine di-nucleotide phosphate.

proximity to the ROS generated, the lack of histone proteins to scavenge radicals, and perhaps inefficiencies in the poly(ADP-ribose) polymerase DNA repair mechanisms which repair strand breaks (360).

Functional production of superoxide involves activation of the hexose-monophosphate (or NADPH-oxidase) shunt, which shunts glucose-6-phosphate from the glycolysis pathway (Fig. 3) and utilizes molecular oxygen and NADPH (Fig 3 and 4) to form the superoxide radical anion ( $O_2^{\bullet-}$ ). This process comprises the so-called 'respiratory burst' within neutrophilic polymorphonuclear leukocytes (neutrophils) and is stimulated by a variety of mitogens/antigens/cytokines and other mediators such as granulocyte-macrophage colony-stimulating factor. Important activators are opsonized particles which activate Fc $\gamma$  receptors (Fc $\gamma$ R), bacterial DNA which can activate Toll-like receptors (e.g. TLR-4, TLR-9) (446), small peptides from bacteria, such as fMetLeuPhe, and protein kinase C agonists, such as phorbol myristate acetate. The NADPH-oxidase has a complex structure including cytosolic sub-units (e.g. p47phox, p40phox, p67phox), and sub-units which are bound within the lipid membrane (e.g. gp91phox,

p22phox). The proximal pathways that link the cell surface receptors to the oxidase differ in temporal behavior and biochemical components, but the downstream pathways seem to converge at the cytosolic activation points of the NADPH-oxidase (372). One pathway involves phosphorylation of p47phox, following which the cytosolic sub-units re-locate to the vacuolar membrane forming the catalytically active oxidase. Superoxide is only produced in the zone of the plasma membrane that is in contact with the phagocytosed particle and until recently, no one had been able to reconcile the relatively low activity/toxicity of superoxide and hydrogen peroxide (which are also long-lived) with the efficient nature of microbial destruction following oxidase activation. A recent paper by Ahluwalia et al. (6) however, provided evidence that the ROS production process within neutrophils may destroy opsonized particles indirectly, by activation of lysosomal proteases. They demonstrated that the elevated cytosolic Ca<sup>2+</sup> levels resulting from cell surface receptor stimulation, opened Ca<sup>2+</sup>-dependent K<sup>+</sup> channels in the phagolysosome (vacuole) membrane. At the same time the NADPH-oxidase pumped superoxide ( $e^-$ ) into the



**Fig. 4.** A schematic representation of the biochemical interactions between neutrophil-superoxide (via the NADPH-oxidase) and the neutrophil-antioxidant enzyme systems, with emphasis on glutathione, its peroxidase and

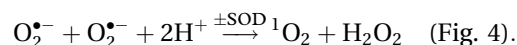
reductase. G-6PDH, glucose-6-phosphate dehydrogenase; NADPH, nicotinamide adenine di-nucleotide phosphate-H; PMNL, neutrophilic polymorphonuclear leukocyte.

vacuole (phagolysosome) from its location in the vacuolar membrane, depolarizing the membrane and generating an ionic gradient, which resulted in an inward flux of  $K^+$  ions. The hypertonic  $K^+$ -rich alkaline environment (raised pH) within the vacuole is proposed to activate the lysosomal proteases within, and those proteases bring about the microbial destruction (341).

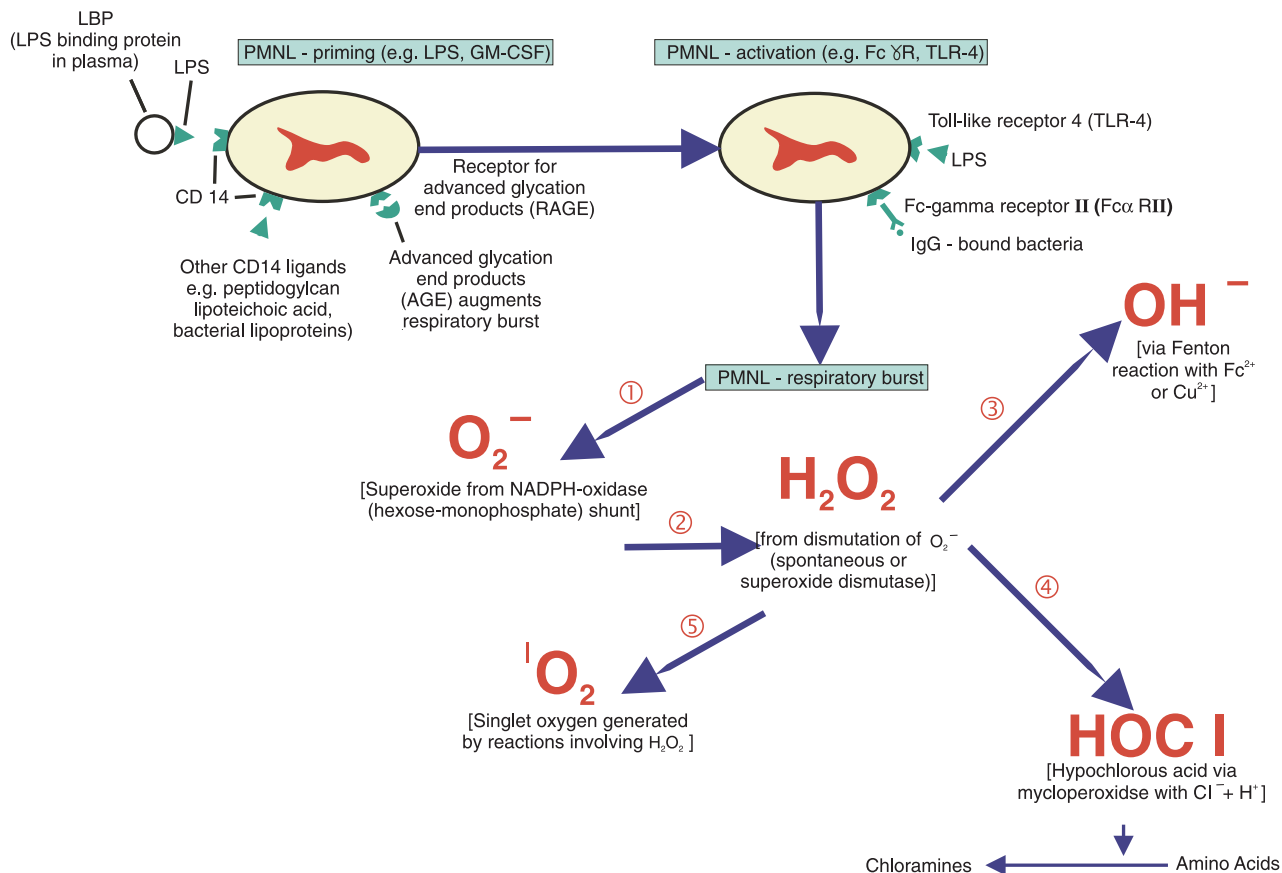
The activation of the oxidase and the subsequent generation of superoxide and the downstream ROS cascade are illustrated in Fig. 5. In healthy individuals, neutrophil priming may arise peripherally or locally within the tissues, following which neutrophil stimulation induces the oxidative burst. Superoxide forms initially and then either spontaneously dismutates to hydrogen peroxide (a rapid reaction at pH 7.4) or is actively converted to hydrogen peroxide by one of three superoxide dismutase enzyme systems. Superoxide dismutase (SOD) has been localized within human periodontal ligament (208) and may

represent an important defense mechanism within gingival fibroblasts against excess superoxide release (384). The latter activates the conversion up to 10,000 times faster than the spontaneous dismutation reaction (39):

- superoxide dismutase 1 – a  $Cu^{2+}/Zn^{2+}$ -dependent enzyme found within the cytosol;
- superoxide dismutase 2 – the  $Mn^{2+}$ -dependent enzyme located within the mitochondria;
- superoxide dismutase 3 – extracellular enzyme, found at low levels extracellularly.

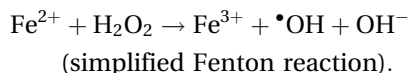


Once formed, hydrogen peroxide acts as a substrate for neutrophil myeloperoxidase, which converts it to hypochlorous acid (HOCl) another ROS. The latter possesses a huge array of biological activities, other than its microbicidal properties (447), some pro- and some anti-inflammatory (see 254 for



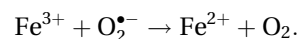
**Fig. 5.** The role of bacteria and their products in receptor-mediated neutrophil ROS production, augmented by AGE/RAGE interactions. LBP, lipopolysaccharide (LPS) binding protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; AGE, advanced glycation endproducts; RAGE, receptor for advanced glycation endproducts; PMNL, neutrophilic polymorphonuclear leukocyte.

review) forming chloramines such as taurine-*n*-monochloramine and cytotoxic aldehydes. The activity of hypochlorous acid depends upon its concentration, high concentrations being cytotoxic, but even at low concentrations (10–20  $\mu$ M) hypochlorous acid can oxidize and inactivate  $\alpha_1$ -antitrypsin (447), activate neutrophil collagenase (396), and disrupt a variety of protein functions (366). Hydrogen peroxide may also undergo 'Fenton reactions' in the presence of  $Fe^{2+}$  or  $Cu^{2+}$  ions, forming the most potent of all oxygen radicals, the hydroxyl radical ( $\bullet OH$ ).



This reaction is one of the most important in free radical biology (398). It gives rise to 'site-specific' hydroxyl radical formation (97) because the uncharged hydrogen peroxide can diffuse across lipid membranes, and Fenton reactions may therefore convert a low activity and largely weak ROS to the potent hydroxyl radical in close proximity to vital cell structures

and macromolecules (e.g. DNA). Normally soluble ferrous iron ( $Fe^{2+}$ ) is not present *in vivo*, being bound tightly to proteins, but it can be produced within cells (e.g. mitochondria or cytosol) by the action of superoxide on ferric iron ( $Fe^{3+}$ ) within iron storage proteins.



The dismutation of hydrogen peroxide also gives rise to the ROS called singlet oxygen ( $^1O_2$ ). Singlet oxygen is molecular oxygen that has received an input of energy, which in turn allows a reversal of spin direction of one of the outer two unpaired electrons to occupy a high-energy state (anti-parallel spin). This creates a situation in which it is now possible for the outer two unpaired electrons to pair up, because the acquisition of two unpaired electrons with opposite spins is possible (see earlier section 'Atomic and molecular oxygen – basic principles'). Singlet oxygen, while not a true radical, is therefore highly reactive and capable of initiating lipid peroxidation (236) from the side chains of polyunsaturated fatty acids. Eosinophils are a source of singlet oxygen (215)



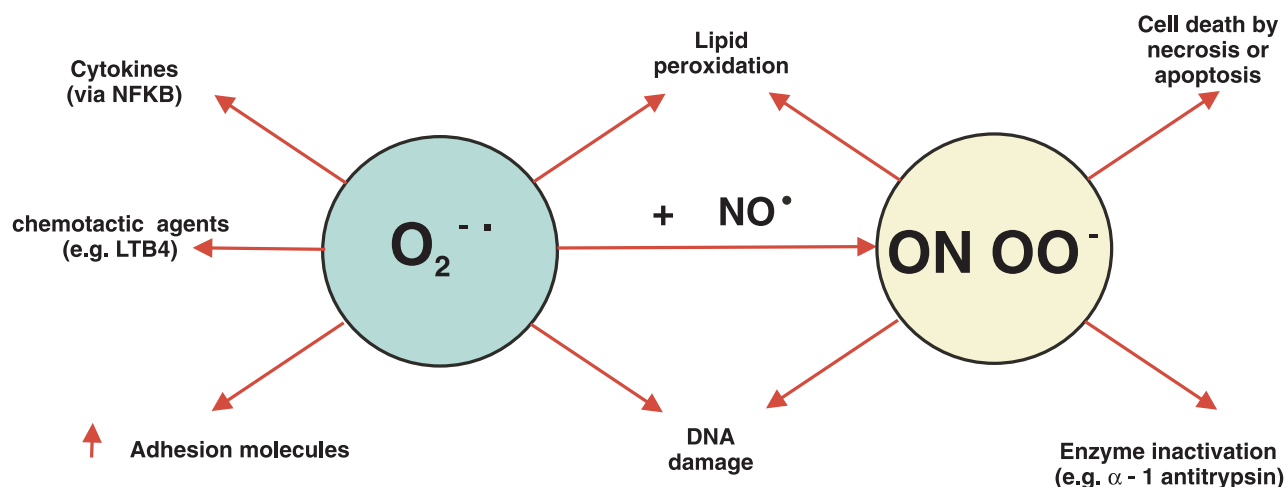


Fig. 6. The interactions between superoxide and nitric oxide to form the peroxynitrite anion and the molecular and cellular effects of these reactive species. NF-κB, nuclear factor kappa B; LTB4, leukotriene B4.

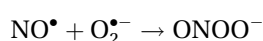
but the reaction of ozone with biomolecules may also give rise to singlet oxygen (214). Removal of singlet oxygen is achieved by carotenoid pigments, which will absorb the energy of singlet oxygen and release heat (136).

### Superoxide and nitric oxide

The formation and actions of superoxide have been discussed above and elsewhere (39, 64, 79, 82, 434), but its interaction with nitric oxide (NO) is worthy of specific mention. Nitric oxide is synthesized from L-arginine by a family of enzymes called nitric oxide synthases. There are three forms:

- type 1 nitric oxide synthase – brain enzyme (bNOS);
- type 2 nitric oxide synthase – inducible enzyme (iNOS), found in macrophages;
- type 3 nitric oxide synthase – endothelial cell enzyme (eNOS).

Endothelial cell nitric oxide synthase causes smooth muscle relaxation within blood vessels (vasodilatation) because nitric oxide is a small lipophilic molecule which enters cells readily and binds to heme-bound iron, extracting it from the porphyrin ring. This, and subsequent cytosolic interactions, leads to the sequestration of cellular calcium, a drop in intracellular calcium levels and subsequent smooth muscle relaxation (205, 279). However, macrophage-derived inducible nitric oxide synthase is of interest; when released simultaneously with superoxide it forms the reactive nitrogen species peroxynitrite anion (45).



While peroxynitrite is not a true radical it is now believed to be responsible for many of the cytotoxic

effects previously attributed to nitric oxide and superoxide (71, 401). These activities include:

- lipid peroxidation;
- glutathione depletion by oxidation;
- nitrotyrosine formation which may inhibit superoxide dismutase activity;
- DNA damage by nitrosilation, deamination and oxidation;
- high concentrations cause rapid cellular necrosis (53);
- low concentrations cause apoptosis (53, 357).

The broad range of biological activities that may arise from the interaction of nitric oxide, superoxide, and peroxynitrite were summarized by Cuzzocrea et al. (96) in their excellent review from which Fig. 6 is taken. Superoxide establishes a pro-inflammatory state in a variety of ways, such as triggering nuclear factor-κB transcription of pro-inflammatory cytokines. Other examples include:

- endothelial cell damage (120);
- increased vascular permeability (173);
- neutrophil chemotaxis via leukotriene B4 formation (104);
- lipid peroxidation (113);
- DNA strand breaks (114).

### Hydrogen peroxide

Hydrogen peroxide is a weak ROS, the potential of which to cause tissue damage is limited to its interaction with transition metal ions via Fenton chemistry or ultraviolet light, when it forms the more potent hydroxyl radical. Unless concentrations exceed  $50 \mu M$  the cytotoxicity of hydrogen peroxide is limited (187) and its biological significance is more as a cell-signaling molecule (3). Hydrogen peroxide plays a



role as a second messenger in nuclear factor- $\kappa$ B activation (see section on 'Redox-sensitive signaling pathways and periodontal disease') in some cells (33, 367) and where inflammation is present it may:

- increase adhesion molecule expression (348);
- cause cell proliferation;
- induce apoptosis (89, 190);
- modulate platelet aggregation (292).

The principal enzymes charged with removal of hydrogen peroxide are the antioxidant enzymes catalase, which predominantly acts intracellularly, glutathione peroxidase, which operates within mitochondria and extracellularly, and the thioredoxin-linked peroxidases (187). Hydrogen peroxide is also ingested at high concentrations in tea and coffee and is thought to diffuse into oral mucosal cells (186). It is also produced by oral bacteria and salivary hydrogen peroxide is used by the salivary peroxidase system to oxidize thiocyanate into antimicrobial products (68).

### The hydroxyl radical

The hydroxyl ( $\cdot\text{OH}$ ) radical and the related perhydroxyl radical ( $\text{HO}_2\cdot$ ) are the most potent species known to cause damage and destruction to an array of cellular and tissue components. Specifically, damage may affect cellular and extracellular targets. Cellular targets include:

- *lipids* – via lipid peroxidation (see below);
- *carbohydrates* – forming carbohydrate radicals or depolymerizing mucopolysaccharides;
- *protein damage* – may result from several ROS (see below), but the hydroxyl radical is the most potent culprit oxidizing aliphatic amino acids and creating hydroxylated derivatives of protein side-chains (protein hydroperoxides) (144, 233). Characteristic of hydroxyl radical activity is hydroxylation of tyrosine, tryptophan, phenylalanine (211), and also histidine (423). Subsequent Fenton reactions create aromatic hydrocarbons and aliphatic products;
- *DNA* – damage by peroxynitrite has been previously discussed, but hydroxyl radical-mediated damage to DNA and RNA is regarded as the most significant;
- *oxidation of antiproteases* – hydroxyl radical-mediated lipid peroxidation (see below) creates radical intermediates capable of inactivating  $\alpha_1$ -antitrypsin by oxidation of methionine-358 (275);
- *low molecular weight species* – the most important low molecular weight species believed to control intracellular redox potential and subsequent transcription factor activity is reduced glutathione (GSH), which, given its strategic importance to the NADPH-oxidase (Fig. 4), as well as ROS-scavenging

and gene transcription (of pro- and anti-inflammatory molecules) will be specifically discussed later.

Extracellular targets include:

- *extracellular matrix components* – in particular proteoglycan and constituent glycosaminoglycan chain-degradation (reviewed in 434);
- *collagens and structural proteins* – proline sites on collagen appear especially prone to hydroxyl radical-mediated degradation (277) and as collagen contains 16% proline residues (whereas average human proteins contain only 5.6%) (117), the type 1 collagen of periodontal ligament may be particularly sensitive to oxidative degradation (320).

## Mechanisms of tissue damage

### Protein damage

The biology of ROS-mediated protein damage is highly complex and remains poorly understood. Dean et al. (102) comprehensively reviewed the field and pointed out that some oxidized proteins are poorly handled by cells so they accumulate during aging and in chronic conditions such as diabetes. The effects of such accumulation can lead to functional inactivation, which may be reversible or non-reversible, and an increased susceptibility to degradation by proteases (175). Radical attack may affect C=C bonds creating carbon-centered radical intermediates, which can interact and create folds in proteins, thus disrupting their structure and function. The two species formed by homolytic fission of a C=C bond may take an electron each, thereby forming neutrally charged individual radical intermediates. This requires a large input of energy and normally electron abstractions give rise to the formation of one positively charged and one negatively charged radical species. Thiol ( $-\text{SH}$ ) groups are also targets on proteins for ROS activity and disulfide bridges form following hydrogen removal ( $\text{S}=\text{S}$ ), again creating cross-linkages. The effects of ROS on proteins are summarized simply in Fig. 7 (adapted from Dean et al.) (102) and include:

- protein folding or unfolding (which may or may not be reversible);
- protein fragmentation and polymerization reactions;
- protease degradation of the modified protein;
- formation of protein radicals;
- formation of protein-bound ROS;
- formation of stable end products e.g. carbonyl compounds such as oxo-acids or aldehydes (e.g. alanine to acetaldehyde) (98).

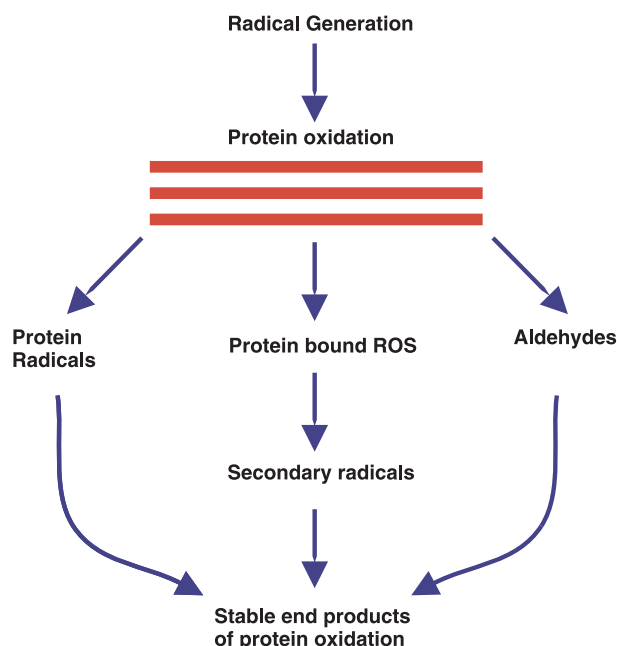


Fig. 7. A schematic view of the effects of ROS on proteins and amino acids (adapted from Dean et al.) (102).

Figure 8 is a schematic representation of some proposed interactions.

### Lipid peroxidation

Lipid peroxidation is one of the most important reactions of free radical species. Most effective at activating this process is the hydroxyl radical and also

peroxynitrite anion ( $\text{ONOO}^-$ ) as discussed earlier. Figure 9 illustrates one sequence of events, initiated by a hydroxyl radical, which gives rise to the lipid peroxidation chain reaction. Krinsky (236) describes six stages, but Halliwell (175) simplifies the reaction to three major stages:

- initiation;
- propagation;
- termination.

The hydroxyl (or peroxynitrite) radical attacks a polyunsaturated fatty acid side chain (e.g. arachidonic acid) in the lipid membrane (initiation) and abstracts a hydrogen atom, forming a carbon-centered radical ( $\text{L}^\bullet$ ). The latter may either rearrange to form a conjugated diene, or may combine with another polyunsaturated fatty acid side-chain radical to form a covalent bond, thus creating cross-linkages and disrupting the membrane structure and function (this can lead to an influx of  $\text{Ca}^{2+}$  ions and subsequent activation of  $\text{Ca}^{2+}$ -dependent proteases). However, most commonly the side chain radical reacts with oxygen forming a lipid peroxyl radical ( $\text{LOO}^\bullet$ ) which may then attack another polyunsaturated fatty acid side chain (propagation) generating another carbon-centered radical and a lipid hydroperoxide ( $\text{LOOH}$ ). The side chain radical forms another lipid peroxyl radical in the presence of oxygen, which attacks another polyunsaturated fatty acid side chain and so the chain reaction continues, forming

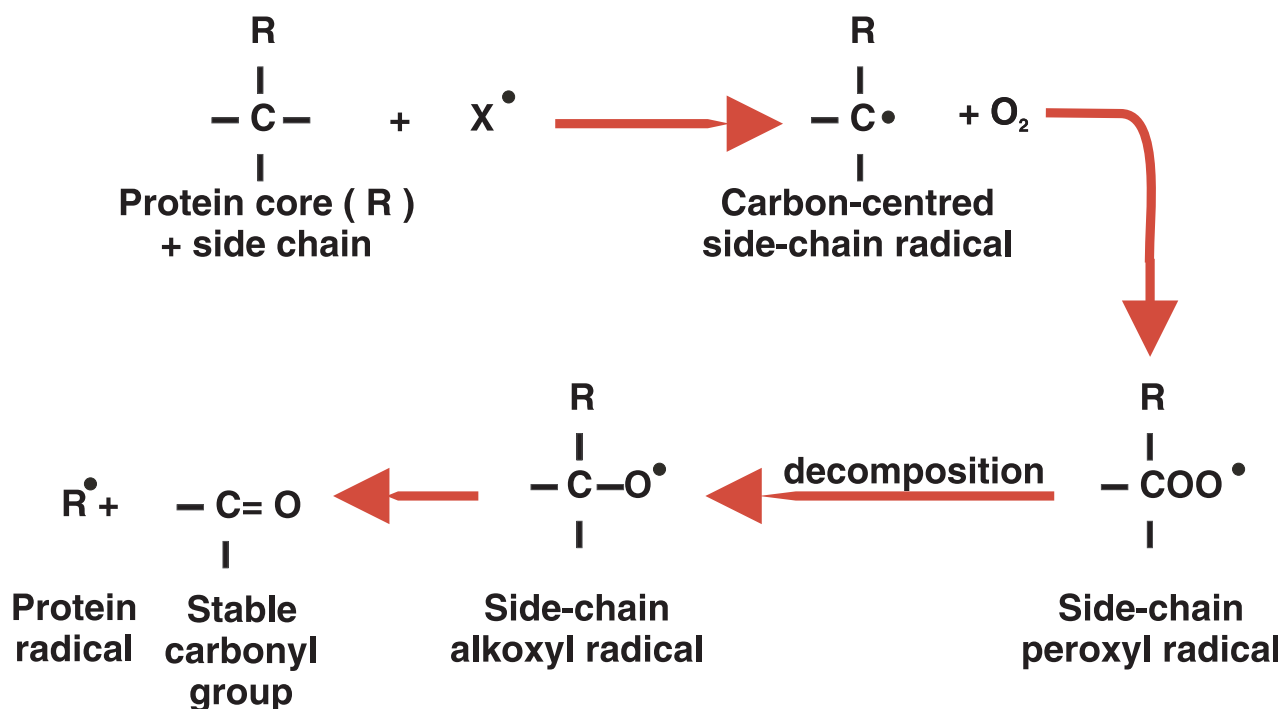


Fig. 8. Some effects of ROS attack upon protein cores and side chains, illustrating the formation of stable carbonyl groups.

**PUFAs**  
e.g. arachadonic acid

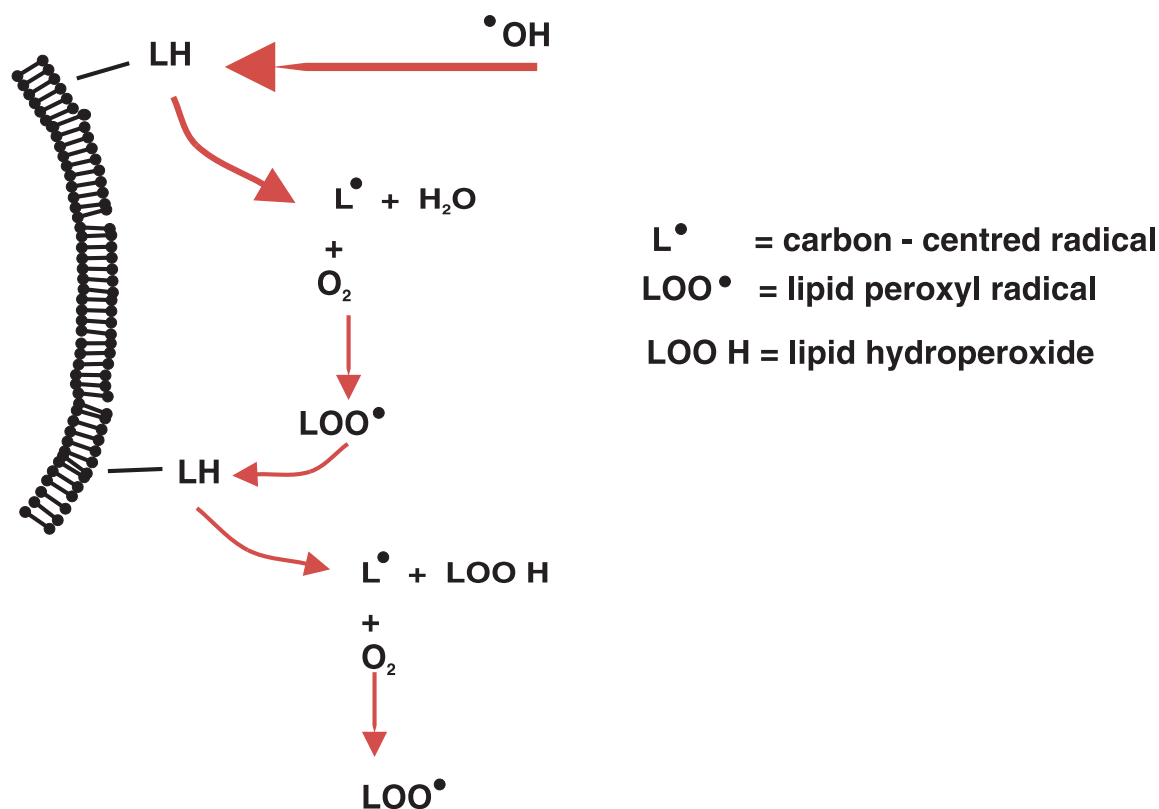


Fig. 9. The lipid peroxidation chain reaction initiated by hydroxyl radicals (see text). PUFA, polyunsaturated fatty acid.

hundreds of lipid hydroperoxides, which decompose to cytotoxic aldehydes and less toxic aldehydes (e.g. malondialdehyde). Accumulation of lipid hydroperoxides disrupts cell membrane function causing it to collapse. Termination is most effectively brought about by the lipid-soluble radical scavenger vitamin E ( $\alpha$ -tocopherol), which is vital to membrane integrity.

Products of lipid peroxidation include a variety of bioactive molecules:

- conjugated dienes;
- lipid peroxides;
- aldehydes, e.g. malondialdehyde, which is an example of a thiobarbituric acid reactive substance;
- acrolein;
- isoprostanes, e.g. F2-isoprostanes from arachidonic acid (8-*iso*-PGF<sub>2</sub>) (346);
- neuroprostanes (F4-isoprostanes);
- volatile hydrocarbons, e.g. pentane, ethane (184).

#### DNA damage

Mechanisms of DNA damage by peroxynitrite and hydroxyl radicals include:

- strand breaks;

- base pair mutations (purine and pyrimidine bases);
- conversion of guanine to 8-hydroxyguanine (55), which is measured as a marker of DNA damage as the nucleoside 8-hydroxydeoxyguanosine (17);
- deletions;
- insertions;
- nicking;
- sequence amplification.

Hydroxyl radicals cause damage to all four bases and create a characteristic 'DNA fingerprint' (179).

#### Antioxidant defense systems

The antioxidant defense systems of the human body are complex and various classification systems exist. Individual antioxidant systems have been comprehensively reviewed previously and the reader is referred to the following reviews (39, 79, 80, 96, 102, 167, 182, 236, 298, 381, 406).

Antioxidants can be categorized by several methods:

- their mode of function (Table 2);
- their location of action (intracellular, cell membrane or extracellular – Table 3);

**Table 2.** Antioxidants classified by mode of action

Mode of action	Examples
Preventative antioxidants	Enzymes: superoxide dismutase enzymes (1, 2 and 3), catalase, glutathione peroxidase, DNA repair enzymes, e.g. poly(ADP-ribose) polymerase, others
	Metal ion sequestrators: albumin, lactoferrin, transferrin, haptoglobin, ceruloplasmin, hemopexin, carotenoids, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, uric acid, polyphenolic flavenoids
Scavenging (chain breaking) antioxidants	Ascorbate (vitamin C), carotenoids (including retinol – vitamin A), uric acid, $\alpha$ -tocopherol (vitamin E), polyphenols (flavenoids), bilirubin, albumin, ubiquinone (reduced form), reduced glutathione and other thiols (free or protein bound)

**Table 3.** Examples of key antioxidants classified by location

Location	Examples
Intracellular	Superoxide dismutase enzymes 1 and 2, catalase, glutathione peroxidase, DNA repair enzymes e.g. poly(ADP-ribose) polymerase, others, reduced glutathione, ubiquinone (reduced form)
Extracellular	Superoxide dismutase enzyme 3, selenium-glutathione peroxidase, reduced glutathione, lactoferrin, transferrin, haptoglobin, ceruloplasmin, albumin, ascorbate, carotenoids, uric acid
Membrane associated	$\alpha$ -Tocopherol

NB Extracellular reduced glutathione levels are normally only 1–2  $\mu$ M (400). Selenium-glutathione peroxidase is different to the intracellular enzyme (30).

- solubility (lipid or water), although considerable interaction exists between aqueous and lipophilic antioxidants in protecting lipoproteins against oxidative damage (194, 306) (Table 4);
- their structural dependents (Table 5);
- their origin/source, e.g. dietary or non-dietary sources (Table 6).

The preventative antioxidants (Table 2) function by enzymatic removal of superoxide and hydrogen peroxide or by sequestration of divalent metal ions, preventing Fenton reactions and subsequent hydroxyl radical formation (183). Lactoferrin is probably more important than transferrin within the periodontal tissues, given the dominance of the neutrophil infiltrate (183) and the recognition of high levels of

lactoferrin within gingival crevicular fluid (5). The chain-breaking antioxidants are the most important within extracellular fluids. Low molecular weight species donate electrons before becoming oxidized, requiring subsequent reduction or replacement to maintain the body's antioxidant capacity. The lipid soluble antioxidants ( $\alpha$ -tocopherol and the carotenoids) act at the cell membrane level and protect against lipid peroxidation, whereas the water-soluble scavengers are more important within the extracellular tissue fluids.

It is important to recognize however, that several antioxidants have dual and sometimes triple actions. For example, ascorbate acts as a chain-breaking or scavenging antioxidant as well as a preventative antioxidant by virtue of its ability to recycle  $\alpha$ -tocopherol (vitamin E) from its oxidized form (296) and by its ability to bind metal ions, respectively. Similarly, the intracellular enzymes (superoxide dismutase, catalase, glutathione peroxidase) are regarded by some as preventative antioxidants (39, 298). The efficacy of an antioxidant depends upon:

- its location (intra- vs. extracellular or cell membrane bound);
- the nature of the ROS-challenge;

**Table 4.** Key antioxidants classified by solubility

Solubility	Examples
Water soluble	Haptoglobin, ceruloplasmin, albumin, ascorbate, uric acid, polyphenolic flavenoids, reduced glutathione and other thiols, cysteine, transferrin
Lipid soluble	$\alpha$ -Tocopherol, carotenoids, bilirubin, quinones (e.g. reduced ubiquinone)

**Table 5.** Antioxidants classified by structures they protect

Mode of action	Examples
DNA protective antioxidants	Superoxide dismutase enzymes 1 and 2, glutathione peroxidase, DNA repair enzymes [e.g. poly(ADP-ribose) polymerase], reduced glutathione, cysteine
Protein-protective antioxidants (102)	Sequestration of transition metals by preventative antioxidants
	Scavenging by competing substrates
	Antioxidant enzymes
Lipid-protective antioxidants	$\alpha$ -Tocopherol (vitamin E), ascorbate (vitamin C), carotenoids (including retinol – vitamin A), reduced ubiquinone, reduced glutathione, glutathione peroxidase, bilirubin

NB Carotenoids (including carotenoid-derivatives) include  $\alpha$ -,  $\beta$ - and  $\gamma$ -carotene, lycopene, lutein, cryptoxanthine, zeoxanthine, retinol (vitamin A).

**Table 6.** Some key antioxidants classified by their origin

Solubility	Examples
Exogenous antioxidants (obtained only through the diet): phytonutrients (259)	Carotenoids, ascorbic acid, tocopherols ( $\alpha$ , $\beta$ , $\gamma$ , $\delta$ ), polyphenols (e.g. flavenoids, catechins such as epigallocatechin-gallate), folic acid, cysteine
Endogenous antioxidants (synthesized by the body)	Catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, reduced glutathione, ceruloplasmin, transferrin, ferritin, glycosylases, peroxisomes, proteases
Synthetic	N-acetylcysteine, penicillinamine, tetracyclines (449)

- other antioxidant species important in co-operative interactions (176);
- other environmental conditions (e.g. pH, oxygen tension).

The body is also compartmentalized with respect to antioxidant species. For example, superoxide dismutase enzymes, catalase and glutathione peroxidase contribute little to the removal of superoxide and hydrogen peroxide in the extracellular environment (180) and Brock et al. (58) demonstrated a very different antioxidant profile for gingival crevicular fluid than for saliva or plasma compartments.

#### Ascorbic acid (vitamin C)

The role of vitamin C as an antioxidant in inflammatory diseases has been discussed in previous reviews (39, 79, 182). Its activities may be summarized as:

- scavenging water-soluble peroxy radicals;
- scavenging superoxide and perhydroxyl radicals;
- prevention of damage mediated by hydroxyl radicals on uric acid;
- scavenger of hypochlorous acid;
- decreases heme breakdown and subsequent  $\text{Fe}^{2+}$  release thereby preventing Fenton reactions;
- scavenger of singlet oxygen and hydroxyl radicals;
- re-forms  $\alpha$ -tocopherol from its radical;
- protects against ROS-release from cigarette smoke.

Other relevant effects recently reported include the ability to reduce C-reactive-protein-mediated expression of monocyte adhesion molecules (340, 443) and the ability to decrease pro-inflammatory gene expression via effects on the nuclear factor- $\kappa$ B transcription factor (159). Vitamin C is an essential nutrient and plasma levels are approximately 30–60  $\mu\text{M}$  (351), being reduced in smokers (51, 134). The reasons for the latter finding relate in part to reduced vitamin C consumption (473), but are also the result of decreased absorption and increased destruction (262). Gingival crevicular fluid levels are reported to be three-fold higher than plasma levels (269) and vitamin C has been shown to prevent activation of neutrophil collagenase (396). Vitamin C is an essential nutrient with a recommended daily intake of 40–60 mg (247).

Ascorbate is converted by radical attack to the ascorbyl radical, which then breaks down to dehydroascorbate (48). Dehydroascorbate can be converted back to ascorbate directly by reduced GSH or by the NAD-semi-dehydroascorbate reductase enzyme system, which also utilizes GSH. These systems are intracellular and thus ascorbate within the

extracellular fluids is rapidly depleted (oxidized) in conditions of oxidative stress (143) unless adequate GSH levels are present (however extracellular GSH levels are normally only 1–2  $\mu\text{M}$ ) (400).

### $\alpha$ -Tocopherol (vitamin E)

Vitamin E is generally regarded as the most important and effective lipid-soluble antioxidant *in vivo*, vital to maintaining cell membrane integrity against lipid peroxidation (Fig. 9) by peroxy radical scavenging (168). Its antioxidant behavior is the result of a single phenolic OH group, which when oxidized gives rise to the vitamin E (tocopheryl) radical. The latter requires other antioxidant species to re-constitute vitamin E, the most effective being the reduced form of co-enzyme Q10 (ubiquinol) in the lipid environment and ascorbic acid in the aqueous phase (298). Vitamin E is a term actually used to describe seven tocopherol sub-species, which behave in a similar manner to  $\alpha$ -tocopherol (440). Vitamin E possesses anti-inflammatory as well as antioxidant properties and these were reviewed by Brock (57):

- inhibition of protein kinase C and subsequent platelet aggregation;
- inhibition of nitric oxide production by vascular endothelium;
- inhibition of superoxide production by macrophages and neutrophils (32), by inhibition of p47phox phosphorylation during NADPH oxidase activation (see earlier).

The limitations of vitamin E's efficacy as an antioxidant are the result of:

- its limited mobility within cell membranes (296);
- its lack of water solubility (many ROS are generated in the aqueous phase).

As with vitamin C,  $\alpha$ -tocopherol levels in plasma are significantly compromised in smokers (249).

### Carotenoids

Carotenoids are tetraterpenes with over 600 variants (406). These include among others (see Table 5):

- lycopene;
- $\alpha$ -carotene;
- $\beta$ -carotene;
- lutein;
- cryptoxanthine;
- retinol (vitamin A<sub>1</sub>);
- dehydroretinol (vitamin A<sub>2</sub>).

Derived only from the diet (green vegetables, tomatoes, fruits), lycopene predominates in plasma (148), with tomatoes being the main dietary source in humans (other sources include red grapefruits and water melon). Carotenoids are lipophilic and higher

plasma concentrations have been shown to protect against various inflammatory and malignant diseases (406). Like many other extracellular antioxidants,  $\beta$ -carotene levels (85, 417), and intake (364) are reduced in smokers, whereas others such as lycopene appear unaffected by smoking.  $\beta$ -carotene is efficient at scavenging singlet oxygen ( $^1\text{O}_2$ ) and other carotenoid antioxidant activities include the scavenging of peroxy radicals (395). Vitamin A is controversial as an antioxidant because its behavior depends upon the oxygen tension of the immediate environment. At the low partial oxygen pressures found in most tissues  $\beta$ -carotene acts as an antioxidant but this initial activity is followed by pro-oxidant behavior at higher oxygen tensions, associated with substantial detrimental effects upon the surrounding tissues (196, 305).

### Co-enzyme Q10

Co-enzyme Q10 exists in an oxidized form (ubiquinone or CoQ) and a reduced form (ubiquinol or CoQH<sub>2</sub>), both of which possess antioxidant activity (409). Considerable data support powerful antioxidant activities. Battino et al. (39) briefly reviewed over 15 animal and human studies performed both in their laboratories and in those of co-workers, which demonstrate substantial and robust evidence for an important antioxidant role for co-enzyme Q10 in free radical-mediated neurodegenerative diseases. Interestingly, co-enzyme Q10 deficiency has been demonstrated in the gingival tissues of periodontitis subjects (192, 248), but there is currently a lack of intervention studies in human periodontitis to substantiate clinical therapeutic benefit; these are needed.

### Uric acid

Uric acid is one of the major radical scavengers within plasma, urine, and saliva (58, 177, 281). Its antioxidant activities include:

- scavenger of singlet oxygen (18);
- scavenger of hydroxyl radicals (18);
- scavenger of hypochlorous acid (452);
- protection of  $\alpha_1$ -antitrypsin when combined with ascorbate (257);
- binding of divalent metal ions preventing Fenton chemistry (101).

Normally uric acid is oxidized to allantoin enzymatically or by hydroxyl radicals but the enzymatic route does not occur in humans, therefore allantoin formation is used as a marker of urate oxidation by ROS (measured as allantoin:urate ratio) (161) and allantoin is believed to be important to the antioxidant capacity of urate *in vivo* (177).

## Polyphenols

The polyphenolic flavenoids have been reviewed by Battino et al. (39) and are absorbed following dietary intake of, in particular, vegetables, red wine, and tea (343). There are over 4,000 known flavenoids (327), the most researched being the water-soluble catechin, epigallocatechin gallate, and the polyphenol, quercetin, which has over 140 derivatives. Polyphenols function by:

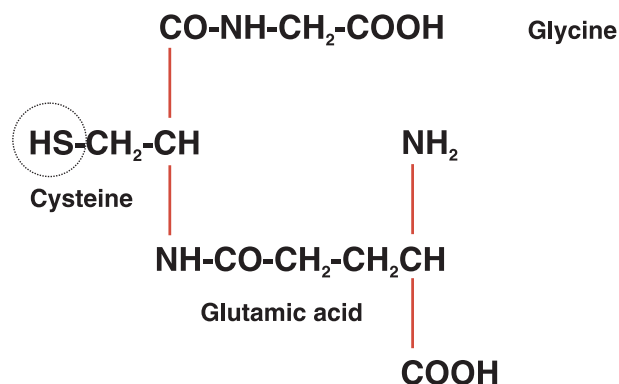
- radical scavenging;
- terminating lipid peroxidation;
- iron chelation;
- sparing vitamin E;
- restoration of vitamin C.

There are currently no data, however, on the effects of high-dose dietary polyphenols on inflammatory diseases.

## Glutathione

Glutathione is a non-essential tri-peptide (Fig. 10) in that it can be synthesized within the cell; however, its constituent amino acids are 'essential' and obtained through the diet. Glutathione exists in oxidized (GSSG) and reduced (GSH) forms and GSH is a ubiquitous thiol that plays a major role in human physiology and pathology, for several reasons:

- it is one of the most vital intracellular antioxidant scavengers;
- it is essential to the glutathione peroxidase antioxidant enzyme system, which removes hydrogen peroxide by converting two GSH molecules to one GSSG molecule and water (Fig. 4; reviewed in 80, 169, 333);
- it plays a major role in maintaining the intracellular redox balance and thus regulating signaling pathways which are affected by oxidative stress;

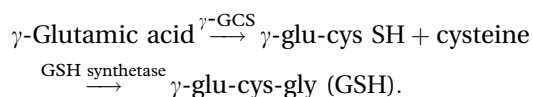


**Fig. 10.** The structure of the tri-peptide reduced glutathione (GSH) illustrating its constituent amino acids and the strategic importance of cysteine.

- it acts as a neurotransmitter governing neuro-immune–endocrine functions;
- it is important to the preservation and restoration of other antioxidant species, e.g. vitamin C and vitamin E;
- it regulates the expression/activation of redox sensitive transcription factors such as nuclear factor- $\kappa$ B and activating protein-1, thereby controlling inflammatory cytokine production and other activities (Table 8).

The latter role is complex (see later), because GSH synthesis itself can also be regulated by cytokines (170, 169).

Dietary GSH and the central amino acid responsible for most of GSH's biological activities, cysteine, are absorbed intact in the small intestine and will increase GSH levels in plasma and tissues (408). However, by contrast, GSH is not efficiently transported into most mammalian cells; there are some exceptions such as lung alveolar epithelial cells (106), a property that is vital to GSH-based therapeutic management of inflammatory lung diseases. The reasons behind the poor uptake of GSH by most cells lie in its intracellular synthetic pathway. The assembly of GSH from cysteine,  $\gamma$ -glutamic acid, and glycine requires two intracellular enzymes and one membrane-bound enzyme.



Cysteine is the rate-limiting substrate and  $\gamma$ -glu-cys-synthetase ( $\gamma$ -GCS) is the rate-limiting enzyme in GSH synthesis. The conversion of  $\gamma$ -glu-cys to  $\gamma$ -glu-cys-gly (GSH itself) is rapid and involves glutathione synthetase. It is thought that 80% of  $\gamma$ -glu-cys-synthetase is bound to GSH within the cytosol and is inactive. When cytosolic GSH levels are depleted, the  $\gamma$ -glu-cys-synthetase is released and synthesizes more GSH (201). The third enzyme is membrane located and called  $\gamma$ -glutamyl-transpeptidase and this enzyme breaks down extracellular GSH (from the diet or released following cell death) to its constituent amino acids (158). The cysteine can be transported across the cell membrane and this triggers GSH synthesis. Unfortunately, dietary administration of cysteine is not possible because it is neurotoxic and rapidly oxidized to cystine, which does not cross the cell membrane (216). The synthetic drug (and paracetamol overdose rescue agent) *N*-acetyl-cysteine is used to deliver cysteine to cells because it will reduce cystine to cysteine (333).

Given the importance of GSH in physiological homeostasis there are other ways of increasing



intracellular levels besides synthesis. GSH forms the substrate for the antioxidant enzyme glutathione peroxidase, but is re-constituted from GSSG by glutathione reductase (Fig. 4). These cycling reactions are not only vital to the cell redox status but also directly to the NADPH-oxidase. Physiologically therefore, the reductase reaction drives strongly in favor of GSH creating a 90% intracellular ratio of GSH:GSSG.

Intracellular GSH levels are usually high (1–10 mM) accounting for 90% of intracellular non-protein thiols (265, 358) and extracellular levels are low (1–4  $\mu$ M in plasma) (94, 400). It is therefore interesting that the epithelial lining fluid in lung alveoli contains 200–400  $\mu$ M GSH and this is reduced in chronic lung diseases and elevated as a protective mechanism in chronic smokers (reviewed in 333). The discovery of millimolar levels of GSH in gingival crevicular fluid (83) and high levels contributing to the total antioxidant status of the cervical epithelium (93), has led to the hypothesis that GSH may represent an innate and fundamental defense strategy at exposed epithelial surfaces (79, 83). Interestingly, some periodontal pathogens (certain *Fusobacteria*, *Peptostreptococcus micros*, and *Treponema denticola*) metabolize GSH and convert it to the cytotoxic hydrogen sulfide (69, 87, 256, 318), and recently, distinct metabolic pathways underlying this process in *T. denticola* were reported (86).

It has also been reported that the smoking of a single cigarette is capable of inducing a significant reduction of salivary glutathione concentration (469, 470) and similar data exist for plasma (333). Circulating polymorphonuclear lymphocytes from cigarette smokers have been shown to release more superoxide (334). The detrimental effects of smoking on cell and tissue GSH levels have been reviewed (333). Similar data exist for periodontitis with a dose-dependent reduction of periodontal ligament GSH reported as a result of smoking (77) and GSH has been shown to protect against the cytotoxic actions of nicotine in periodontal ligament fibroblasts (76).

### Global antioxidant defense

The body's antioxidant systems are highly integrated and complex and while the study of individual systems and species greatly improves our understanding of their role in human diseases, it ignores their co-operative activities and may present a picture that does not accurately represent the *in vivo* situation. As a result of their cellular and extracellular ubiquity and rapid rates of sacrificial oxidation the free radical scavengers confer substantial protection on vital macromolecules (182). They also work in concert

through redox cycling reactions, regenerating each other from their respective radical species (79, 407). Figure 11 illustrates this process, GSH regenerating  $\alpha$ -tocopherol and vitamin C from their radicals, preventing further lipid peroxidation and cellular damage (131, 312, 382). Several assays of global antioxidant defense or 'total antioxidant capacity' have therefore been developed to reduce the costly and time-consuming task of measuring individual antioxidant species. Such assays also provide information about the combined effectiveness of individual species (the total antioxidant capacity may be greater than the sum of individual antioxidants) and may also account for the influence of antioxidant substances that are as yet undiscovered or are technically difficult to assay. These assays provide an overview of the biological interactions between individual antioxidant species and provide a measure of the capacity of biological systems to withstand oxidative attack and will be discussed later. There are limitations to assays of total antioxidant capacity, the main one being that they provide limited information on specific mechanisms of radical removal and hence the contribution of individual antioxidant species to the pathogenesis of disease.

Halliwell (178) highlighted the problems associated with establishing definitive involvement of ROS and reduced antioxidant activities in several human diseases. He also pointed out that the oral environment offered opportunities to explore free radical and antioxidant biology more easily than other body compartments/systems. In particular, the ability to apply antioxidants topically and create significant concentrations locally within the tissues offers exciting opportunities for novel host-modulating therapies. It would seem appropriate to expand this activity, in view of the importance of periodontal health to the health of the UK population (178).

### Dietary antioxidants vs. individual supplementation

The role of nutrition in the inflammatory lesion of periodontitis was briefly reviewed by Ritchie and Kinane (344) and aspects of malnutrition and periodontal diseases have been comprehensively reviewed by Enwonwu and others (129, 130, 293). As shall become apparent, early studies of individual vitamin supplementation in the 1970s and 1980s were limited at best in their success in improving periodontal outcomes and most failed to achieve any clinical benefit. These data are consistent with those for other chronic inflammatory diseases and there is an argument that man-made 'neutraceuticals' do not provide the necessary co-factors that

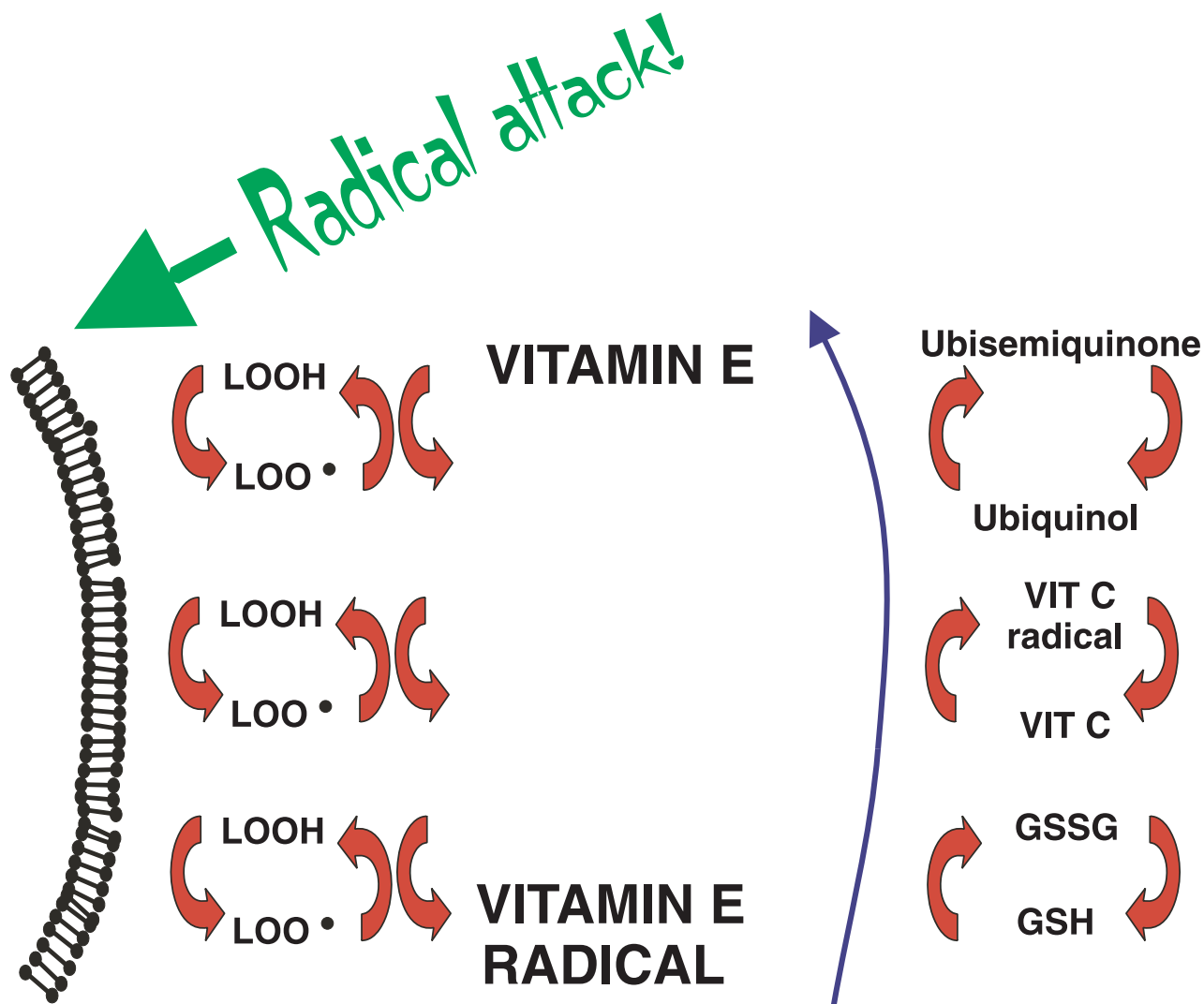


Fig. 11. The role of vitamin E in protecting cell membranes against lipid peroxidation and the reconstitution of vitamin E from its radical by co-operative antioxidant

reactions in both the lipid- and water-soluble phases. Note role of GSH as a chain-breaking antioxidant species.

whole food nutrition does. Indeed, some can provide serious adverse reactions as highlighted earlier, as a result of the formation of pro-oxidants *in vivo*. Given the co-operative behavior of antioxidants, it may take a cascade of antioxidants to decrease ROS-mediated damage, something that individual supplements may not achieve. If deficiencies in individual phytonutrients (nutritional chemicals) (259) can be definitively linked to specific conditions then individual supplements may be beneficial. This is true for GSH in managing diabetic neuropathy (424) and cysteine/GSH in managing HIV disease (118). This conflict became evident when attempts to reduce oxidative stress as a result of lipid peroxide formation with five different doses of vitamin E showed no benefit (263), but whole food concentrates of fruit and vegetable reduced lipid peroxides

by 75% overall and to undetectable levels in a third of subjects (453).

### Measuring ROS and antioxidants *in vivo*

There are currently no gold standard methods for measuring antioxidant capacity or ROS-mediated tissue damage in human biology. All systems utilize different indices of measurement and the specificity of the biomarker employed will dictate the measurement obtained, which differs between assays and between different biological samples and their components. However, one must start somewhere and the next section briefly reviews the state of the discipline and the limitations of the respective assay systems.

## Measuring ROS and oxidative stress/damage in biological samples

Free radicals and other reactive species have extremely short half-lives *in vivo* ( $10^{-6}$ – $10^{-9}$  s) and simply cannot be measured directly. *In vitro* systems called ‘spin traps’ are used to measure radical species but there are currently no suitable spin traps/probes available for *in vivo* measurement of ROS production in the human, because of their unknown toxicity. *Ex vivo* spin traps can be used and these would include:

- ascorbic acid – which forms semi-dehydroascorbate when it traps a radical (see earlier);
- aromatic traps – such as salicylates and phenylalanine. However, such traps lack specificity for the hydroxyl radical and peroxynitrite and quantification is not deemed possible;
- urate – which is oxidized to allantoin, which can be measured in various fluids (e.g. plasma, urine, cerebrospinal fluid) in diseases whose onset and course are associated with oxidative stress.

The majority of clinical studies employ biomarkers of oxidative stress or tissue damage to vital macromolecules, rather than spin traps. Halliwell and Whiteman (184) comprehensively reviewed these assays recently and this section provides a brief overview of the field, based on their review. Those wishing to learn more about measuring ROS activity and tissue damage are strongly advised to read Halliwell and Whiteman’s excellent review.

As previously mentioned under ‘mechanisms of tissue damage’, the main sources of biomarkers of ROS activity are:

- lipid peroxidation;
- protein/amino acid oxidation;
- carbohydrate damage;
- DNA damage.

### Biomarkers of lipid peroxidation

Lipid peroxidation is not currently thought to result from superoxide, hydrogen peroxide or nitric oxide activity because these reactive species are deemed to be too weak to cause lipid damage. Biomarkers commonly employed are:

- conjugated dienes;
- thiobarbituric acid reactive substances (notably malondialdehyde);
- isoprostanes;
- ethane/pentane and other volatile hydrocarbons.

Thiobarbituric-acid-reactive substances have become obsolete as a measure of ROS damage (184), because they lack specificity for ROS activity, being formed by mechanisms other than lipid peroxidation.

Malondialdehyde can be measured directly or by high-pressure liquid chromatography and is more specific to ROS activity than thiobarbituric-acid-reactive substances. Another aldehyde formed by lipid peroxidation is acrolein, which is more cytotoxic than malondialdehyde and may be a better biomarker (422).

Isoprostanes form following peroxidation of the polyunsaturated fatty acid side chains of lipids (Fig. 9) and are currently regarded as the best biomarkers of lipid peroxidation (e.g. F2-isoprostanes) (346). Either mass spectroscopy or enzyme-linked immunosorbent assays can be used for quantification, although there are question marks against the reliability of some enzyme-linked immunosorbent-assays (328). Specific isoprostanes (e.g. 8-*iso*-PGF<sub>2α</sub>) can be measured in plasma as markers of ‘whole body’ oxidative stress, or more site-specifically within tissues such as synovial fluid (38), breath condensate (332), and cerebrospinal fluid (280). Indeed, isoprostanes have been used as biomarkers to demonstrate a strong association between oxidative stress and obesity and hypercholesterolemia (184). However, isoprostanes are metabolized rapidly when formed and attempting to assess correlations between markers of lipid damage and those of DNA or protein oxidation is pointless given the different time courses of their removal *in vivo*.

Ethane and pentane may be useful biomarkers of oxidative stress, particularly ethane (99), but their collection and measurement is cumbersome and technically challenging.

### Biomarkers of DNA damage

Products of hydroxyl radical attack on DNA bases (purines and pyrimidines) and carbohydrate moieties (deoxyribose) can be measured by various methods (high-pressure liquid chromatography: gas or liquid), liquid chromatography or antibody methods (115). No individual reaction product should be used as the sole index of DNA damage (184), but despite this, 8-hydroxydeoxyguanosine is frequently used in this manner (92); 8-hydroxydeoxyguanosine can be formed during DNA preparation and analysis by artifactual means and it remains a controversial biomarker.

The comet assay (121, 132) can be used to measure DNA strand breaks by direct application to single cell preparations. In the assay a small number of treated cells suspended in a thin agarose sandwich are lysed and electrophoresed at alkaline pH, before staining with a fluorescent DNA binding dye. Broken/dam-

aged DNA fragments migrate at different rates according to their size and thus to the extent of damage, and they form comet tails. Cells are classified by tail length, however enzymatic cleavage of DNA during apoptosis and DNA repair enzyme activity can also create comets.

### Biomarkers of protein damage

Protein damage can affect biological homeostasis in a variety of ways:

- altered protein function (due to folding);
- creation of secondary radicals (carbon-centered radicals);
- inactivation of important protease inhibitors, allowing protease activity to go largely unchallenged;
- creation of immunologically active by-products;
- damage to DNA repair enzymes.

Figure 8 illustrates how peroxy and alkoxy radicals can form from ROS attack on amino acid or protein structures. The ingestion of cooked foods can complicate the interpretation of data from human tissues because cooking itself will oxidize amino acids. Furthermore, oxidized proteins are rapidly removed by the proteasome, again complicating interpretation of data from biomarker studies.

The carbonyl assay measures protein carbonyl groups formed as relatively stable end products of protein oxidation by ROS. Both enzyme-linked immunosorbent assays and spectrophotometric assays exist (62, 246), but again carbonyls are not specific biomarkers of ROS damage because protein-bound aldehydes and glycated proteins are also measured (184). Indeed, acrolein is a protein-bound aldehyde that has been widely used to measure oxidative damage. At best, carbonyl levels provide average measures of protein damage by ROS in human tissues and fluids.

### Measuring antioxidant status of biological samples

Different antioxidant species partition themselves within different compartments of the body. Fat-soluble antioxidants associate with the lipid moieties of cells, tissues, or fluids (e.g. tocopherols, carotenoids), hydrophilic antioxidants associate with water-soluble fractions (e.g. uric acid, proteins), and some can bridge both compartments (e.g. urate, ascorbate). Different assays measure different antioxidants, some lipophilic and some hydrophilic; some assays assess preventative antioxidant systems and others assess scavenging antioxidants. Assays also vary with respect

to their sensitivity towards different species within a compartment. For example in the TRAP assay (total radical trapping antioxidant parameter) protein antioxidants contribute 10–50% of the total antioxidant capacity (442), whereas the enhanced chemiluminescence total antioxidant capacity assay (82, 448) is more sensitive to the efficient non-protein radical scavengers. Great care is therefore required when interpreting data from different assays, which employ different indices of oxidative damage. Some of the systems available were recently reviewed (465).

While important associations can be established between disease status and individual antioxidants in biological systems, there are major drawbacks to this type of approach. First, antioxidant systems behave co-operatively and not in isolation (see section on ‘Global antioxidant defense’ and Fig. 11); the sum of the individual antioxidant activities does not represent their global capacity to remove ROS and effect tissue repair. Second, interactions between hydrophilic and lipophilic antioxidants are not taken into account, and third, hitherto undiscovered antioxidant species are ignored. For these reasons, assays of total antioxidant capacity have been developed and this review will discuss these systems rather than the various systems for measuring individual antioxidant species or enzyme antioxidants. In broad terms, assays measure either lipid-soluble or water-soluble antioxidants, although more recently assays have been developed that measure antioxidants from both compartments, and also appear to assess co-operation between lipid and aqueous-phase antioxidants (9).

### Assays for water-soluble antioxidants

Yeum et al. (465) described two broad approaches to measuring hydrophilic antioxidant species. The first approach utilizes a hydrophilic pro-oxidant or radical-inducing species such as 2,2'-azobis(2,4-amidinopropane)dihydrochloride, which spontaneously decomposes at body temperature to produce peroxy radicals (via interaction with carbon-centered radical species). Various substrates can be used as ‘reporters’ for the water-soluble peroxy radical activity and common examples would be:

- R-Pe (dichlorofluorescein-diacetate, phycoerythrin) – a fluorescent protein (65);
- DCFH (2',7'-dichlorodihydrofluorescein) – providing a fluorescent signal, e.g. total radical trapping antioxidant parameter assay (149, 427) and ORAC (oxygen radical absorbance capacity) assay (65);
- crocin – produces a bleaching reaction when exposed to peroxy radicals, which can be measured as an absorbance change (418).

The oxidation of the reporter is inhibited by the antioxidant system that is exposed to the radical-generating reaction and the antioxidant capacity is determined by calculating the delay or profile (e.g. area under curve) of the reaction. The greater the antioxidant capacity, the greater the delay in reporter activity and therefore signal generation.

The second approach uses systems that generate free radical chain reactions *in vitro* using an oxidant (e.g. hydrogen peroxide rather than a pro-oxidant) and an oxidizable substrate (e.g. luminol) and then assesses the ability of an antioxidant system to scavenge the radicals produced. The decay in signal or the period of time for which the signal is absent is used as the measure of antioxidant status and a standard antioxidant species is used as the calibrant. The most frequently used calibrant is the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Examples would be:

- ferric reducing ability of plasma (FRAP) assay, which measures the production of a colored complex produced by the reduction of trivalent iron (ferric) ions to divalent (ferrous) ions (46);
- Trolox equivalent antioxidant capacity (TEAC) assay, measures the scavenging/quenching of the ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation (271);
- enhanced chemiluminescence assay (ECL) (82, 448), which measures the ability of the antioxidant system to inhibit the enhanced light signal produced following the oxidation of the chemiluminescent substrate luminol by hydrogen peroxide, using horseradish peroxidase as the catalyst. Trolox is used as the calibrant as indeed it is for the total radical trapping antioxidant parameter and Trolox equivalent antioxidant capacity assays.

In the enhanced chemiluminescence assay, utilizing a point in the light recovery curve (which signals gradual exhaustion of the antioxidants within the sample added) as the end point can provide different measures of total antioxidant capacity. The convention is to use an early end point, such that only the most efficient radical scavengers are measured; however, utilization of a later end point would also include less efficient scavengers and produce a different index of total antioxidant capacity. In the total radical trapping antioxidant parameter assay, ABAP (2,2'-azobis[2-amidinopropane]hydrochloride) spontaneously decomposes to liberate water-soluble peroxy radicals, which induce lipid peroxidation. The delay in induction of lipid peroxidation (after adding an antioxidant-containing solution) is measured by

oxygen uptake and compared to a Trolox standard. The total radical trapping antioxidant parameter assay is complex to perform, requiring dedicated expertise, and is also time-consuming and sensitive to the detection of protein antioxidant species. The assay has been modified to employ chemiluminescence to detect peroxy radicals (266). The enhanced chemiluminescence assay described by our group is rapid and cheap to run but requires synthesis of a stock of enhancer, has not been comprehensively assessed for its ability to measure lipid-soluble antioxidant species and is most sensitive to non-protein antioxidant species.

### Assays for lipid-soluble antioxidants

Niki (297) introduced a pro-oxidant radical inducer called 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) to generate lipid-soluble peroxy radicals rather than 2,2'-azobis(2,4-amidinopropane)dihydrochloride, which generated water-soluble peroxy radicals (see earlier). Lipid peroxidation (and its inhibition by lipid-soluble antioxidants) was then measured by assessing the production of conjugated dienes, where substrates included DCFH or diphenyl-1-pyrenylphosphine (471).

The excellent review of antioxidant assays by Yeum et al. (465) highlights modifications made to the Trolox equivalent antioxidant capacity and oxygen radical absorbance capacity assays, to better incorporate lipid-soluble antioxidants into the outcome measures of these indices of antioxidant activity. The same group (9) have also reported the development of a total antioxidant capacity assay that measures both the lipophilic and hydrophilic antioxidant compartments of plasma, and which also measures the interaction between these compartments.

In summary, there is currently a complex array of assays of global antioxidant activity, all with specificities for different biological molecules in different tissue/fluid compartments of the body. Those that are based upon hydrophilic radical species and substrates (probes) measure predominantly water-soluble antioxidants and those based upon lipophilic radicals and probes will measure predominantly fat-soluble antioxidants. More recently, systems have been developed which will measure both water- and fat-soluble antioxidants but their relative sensitivities to individual species within those compartments are still largely unexplored. Moreover, most assays depend upon *in vitro* models of oxidative stress (generating radicals in an assay tube) and the extent of oxidative stress, its time of onset, or indeed the duration of its inhibition by antioxidant-containing

samples, is utilized as the measure of total antioxidant activity. Different assays produce different measures and they also differ in their sensitivity to the known major antioxidants, as well as in their ability to detect currently uncharacterized antioxidant species. There is little doubt that assays of total antioxidant capacity offer the advantage of assessing more holistically a biological system's ability to withstand oxidative stress, and account to some degree for the long recognized co-operative antioxidant interactions, but different oxidative stresses produce different results. It is imperative when attempting to analyze data from studies of total antioxidant capacity that the reader is clear which assay has been used and what the sensitivity of that assay is to lipophilic and hydrophilic antioxidants. The latter may dramatically affect the conclusions drawn, which can impact on our understanding of pathogenic processes and potential future therapeutic strategies.

## Evidence for the presence and role of ROS in periodontal tissue damage

The idea that ROS are associated with the pathogenesis of a variety of inflammatory diseases and have a role (direct or indirect) in tissue damage has become a major area of research over the last decade as demonstrated by electronic searches of the literature. However, supporting evidence for their role in tissue damage is often indirect and circumstantial. Indeed, few reports fulfill any, or all, of 'Halliwell's Postulates', those being the criteria required to be fulfilled before ROS can be concluded to be key mediators of tissue injury in a given disease (178, 184). The four criteria proposed by Halliwell, similar to those proposed by Robert Koch in 1884 (231) to establish a causal relationship between an organism and a disease, are:

- ROS or the oxidative damage caused must be present at site of injury;
- the time course of ROS formation or the oxidative damage caused should occur before or at the same time as tissue injury;
- direct application of ROS over a relevant time course to tissues at concentrations found *in vivo* should reproduce damage similar to that observed in the diseased tissue;
- removing or inhibiting ROS formation should decrease tissue damage to an extent related to their antioxidant action *in vivo*.

This section will review the literature reporting studies that provide evidence for the role of ROS in the pathogenesis of the periodontal diseases and that aim to fulfill one or any combination of the first three criteria. The majority of studies supporting the last criterion will be reviewed in the next section (Studies on antioxidants and periodontal disease) together with studies reporting changes in antioxidant status where reduced levels may imply an increased presence of ROS.

The four criteria proposed by Halliwell have an underlying assumption that ROS are generated at a level that results in direct damage to tissue components. In this sense they are limited because not all could be fulfilled if low concentrations of ROS were important in causing indirect tissue damage via activation of redox-dependent signaling pathways within cells. Therefore, some evidence implicating ROS as a potential indirect mediator of tissue damage in the periodontal diseases will also be reviewed in a separate section ('Redox-sensitive signaling pathways and periodontal disease').

## ROS production by neutrophils and other cells in periodontal disease

Current evidence indicates that periodontal disease occurs in predisposed individuals with an aberrant inflammatory/immune response to microbial plaque. Because neutrophils are the predominant inflammatory cell in gingival connective tissue, pocket epithelium, and within the gingival crevice (273), a large number of studies have been performed investigating possible associations between neutrophil function and periodontal disease. It has long been known that defects in neutrophil function predispose to some forms of periodontal disease, for example in individuals with Chediak-Higashi syndrome (52) or chronic neutropenia (304). However, such defects are less well defined in the more common chronic and aggressive forms of the disease not associated with an underlying systemic condition. Chronic inflammatory conditions are generally thought to be associated with increased oxidative stress, with phagocytes (particularly the neutrophil) being implicated in disease pathogenesis because of the generation of the oxidative burst during phagocytosis and killing. Originally, ROS were thought to be directly microbicidal but recent evidence indicates that their role is to establish an environment in the phagocytic vacuole suitable for killing and digestion by enzymes released into the vacuole from cytoplasmic granules (371; see

'Origins and formation of ROS and oxygen radicals'). However, ROS are normally generated as part of the physiological functioning of all cells and their role as mediators in cell signaling is now seen to be crucial for maintaining health (119). Although this section will concentrate on reviewing the data on ROS generation and release by neutrophils in the periodontal diseases, some data implicating other cells in contributing to oxidative stress will be included for completeness.

### ***In vivo* conditions required for ROS production by neutrophils**

Significant ROS generation by neutrophils requires a minimum oxygen tension of about 1% and a pH of 7.0–7.5 (12, 145). Both these conditions are found within periodontal pockets (123, 267), indicating that chronic or excess ROS production is possible at this important site of periodontal tissue damage. This, together with a variety of studies demonstrating reduction in local levels of chain-breaking antioxidants and antioxidant enzyme systems in disease (7, 58, 126), suggests that any ROS generated could accumulate and cause additional damage. Furthermore, oxidation products produced locally by neutrophil ROS (e.g. oxidized low-density lipoprotein) could further amplify neutrophil ROS generation directly as well as up-regulating adhesion molecules (244, 363, 413). The latter could increase the time taken for neutrophils to transit the tissues effectively increasing the local oxidative load. In addition, factors present at high levels at diseased sites may enhance ROS production by neutrophils locally. For example, polyamines are found at high levels within the diseased periodontium (240, 437) and have the capacity to enhance ROS generation by neutrophils (339, 438). On the other hand, neutrophils have an ability to withstand the hostile environment of the periodontal pocket. Thus, neutrophils are able to function and initiate respiratory burst activity in the presence of sulfide at the toxic levels found at diseased sites (88).

### **Environmental and methodological aspects of ROS generation by neutrophils**

The published data on ROS production by neutrophils in periodontal disease are, with few exceptions (50, 163, 250), derived from studies based on the analysis of peripheral blood neutrophils (see Table 7). Thus, any differences detected suggest a systemic rather than a local effect. Furthermore, most of the studies are based upon luminol-dependent chemiluminescence, which gives an assessment of total (intracellular and extracellular) ROS generation because

luminol has the capacity to cross cell membranes. While it is considered to be one of the most effective chemiluminescent substrates for detecting ROS generation by neutrophils (234), luminol does not detect superoxide directly, but must first be oxidized by removal of one electron by species such as hydroxyl radicals, peroxynitrite, and hydrogen peroxide (in the presence of peroxidase) (184). Isoluminol, used in only one study to date (142), is similar to luminol but more hydrophilic and is therefore is not able to cross cell membranes. It is the chemiluminescent substrate of choice for detecting the extracellular generation of ROS (234, 253).

Results obtained with these chemiluminescent methods for detecting ROS can show considerable day-to-day variation, making the inclusion of an age- and gender-matched control, whose neutrophils are analyzed simultaneously with those from the patient, important if consistent and comparable results are to be obtained (24, 25, 50, 462). A variety of methodological factors can also affect results (49). In particular, the presence of divalent cations in the assay buffers can abolish the priming effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on Fc $\gamma$ R-mediated activation of neutrophils and increase light output (28, 142).

The other methods used to study neutrophil ROS production in periodontal disease are based on dichlorofluorescein diacetate, which yields a highly fluorescent product after deacetylation and reaction with ROS (particularly hydroxyl radicals and peroxynitrite rather than hydrogen peroxide as often quoted in papers) (375), indirect detection of superoxide by lucigenin-dependent chemiluminescence and a colorimetric assay for superoxide involving superoxide dismutase-inhibitable reduction of ferricytochrome c.

### ***In vitro* ROS generation by neutrophils in periodontal health and disease**

Studies of ROS generation by peripheral blood neutrophils in periodontal disease have used a variety of patient groups, different pathways of activation, and methods of ROS detection (Table 7). It is therefore not surprising that overall there is no agreement as to whether ROS generation is altered in periodontal disease. Most of the early studies (1984–1992) investigated juvenile periodontitis patients and used bacteria or zymosan, with and without opsonization with autologous or heterologous serum. Most data supported the view that neutrophil ROS generation was associated with disease but one study (197) suggested that the effect was the result of opsonic activity of the patient's serum rather than a function of the cells themselves. However, while there is evidence that



**Table 7.** Studies investigating ROS production from *in vitro* activated peripheral neutrophils

Reference	Disease status <sup>1</sup> and numbers	Assay method <sup>2</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs. health)				
				Autologous serum opsonized	Non- autologous serum opsonized	Ig or histone II* opsonized	fMLP	PMA or PDB*
Åsman et al. 1984 (24)	JP; 8 M, 6 F with age/ sex-matched controls	LDCL <sup>3</sup> using HBSS Peak signal	100:1	Latex ↔ <i>S. aureus</i> ↑				
Ellegaard et al. 1984 (125)	JP; 12 CP; 10 Controls; 22, age/ sex-matched	Cytochrome c reduction using HBSS			Zymosan ↔ Zymosan ↔			↔
Henry et al. 1984 (197)	JP <sup>4</sup> ; 26 Asian subjects with age/sex-matched controls	LDCL <sup>3</sup> using HBSS Peak signal	5 mg/10 <sup>6</sup> cells (1.1 mg/ml)	Zymosan ↑ Zymosan <sup>5</sup> ↔	Zymosan ↔			
Åsman et al. 1986 (25)	JP <sup>4</sup> ; 5M, 8F with age/ sex-matched controls	LDCL <sup>3</sup> Peak signal	200:1	<i>S. aureus</i> ↑		<i>S. aureus</i> ↑		
Van Dyke et al. 1986 (429)	LJP; 14 patients and 17 controls	Cytochrome c reduction	6.7 mg/ml		Zymosan ↔			
Åsman 1988 (22)	JP; 6M, 6F (after successful treatment) with age/sex-matched controls	LDCL <sup>3</sup> using HBSS in FMLP assay only % CL based on peak signal	200:1 12.5 nM fMLP	<i>S. aureus</i> ↑		<i>S. aureus</i> ↑	↔	
Åsman et al. 1988 (26)	JP; 3M, 7F with age/ sex-matched controls	LDCL <sup>3</sup> using HBSS peak signal	200:1	<i>S. aureus</i> ↓				
Whyte et al. 1989 (450)	Young CP; n = 8 with age-matched controls Old CP; 8 patients with age-matched controls	LDCL using HBSS Peak signal Subject PMNL assayed individually with PMNLs from a 'standard' donor	100 µl of OD <sub>625</sub> 1.0 suspension/10 <sup>6</sup> cells	<i>F. nucleatum</i> ↑ <i>E. coli</i> ↑ <i>F. nucleatum</i> ↓ <i>E. coli</i> ↓	<i>F. nucleatum</i> ↑ <i>E. coli</i> ↑ <i>F. nucleatum</i> ↓ <i>E. coli</i> ↓			
Mattout et al. 1990 <sup>6</sup> (261)	JP	NK	NK				↓	↔

Table 7. Continued

Reference	Disease status <sup>1</sup> and numbers	Assay method <sup>2</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs. health)				
				Autologous serum opsonized	Non- autologous serum opsonized	Ig or histone II* opsonized	fMLP PMA or PDB*	None
Guarnieri et al. 1991 (163)	CP; 14 patients with 16 controls Gingival crevicular fluid PMNLs Peripheral PMNLs	Cytochrome c reduction	NK					
Shapira et al. 1991 (376)	RPP; 4 M, 1 F with age-matched controls	Cytochrome c reduction using HBSS LDCL using HBSS Cpm at 5 min	0.5–1 µg/ml PMA Bacteria NK			<i>Streptococcus</i> ↑*		↔
Zafriopolous et al. 1991 (468)	RPP; 19 patients JP; 10 patients CP; 10 patients All with age/sex- matched controls	LDCL Peak signal	0.35 mg/1.4 × 10 <sup>5</sup> cells (0.35 mg/ml)	Zymosan ↔ Zymosan ↔ Zymosan ↔		<i>Streptococcus</i> ↑*		↔
Åsman and Bergstrom 1992 (23)	JP; 4 patients with controls JP; 3 patients with controls	LDCL <sup>3</sup> % CL based on peak signal	200:1	<i>S. aureus</i> (↔) <i>S. aureus</i> or <i>A. actinomycetemcomitans</i> or <i>P. gingivalis</i> (↔)	<i>S. aureus</i> (↔) <i>S. aureus</i> or <i>A. actinomycetemcomitans</i> or <i>P. gingivalis</i> (↔)			↔
Kimura et al. 1993 (227)	LJP; 15 patients GJP; 13 patients CP; 52 patients Unmatched controls; 30, age = 30.5 ± 0.9	Dichlorofluorescein- diacetate oxidation and flow cytometry with whole blood	100 ng/ml				(↔) (↔) (↔)	↔ ↔ ↔
Gomez et al. 1994 (153)	RPP; 3 or 5 patients JP; 3 or 4 patients CP; 3 or 4 patients All with age/sex- matched controls	LDCL <sup>3</sup> using HBSS Peak signal and total CL	0.32 mg/ml 5 × 10 <sup>-6</sup> M PDB	Zymosan ↔ Zymosan ↓ Zymosan ↔			↔* ↔* ↔*	↔ ↔ ↔

Table 7. Continued

Reference	Disease status <sup>1</sup> and numbers	Assay method <sup>2</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs. health)				
				Autologous serum opsonized	Non- autologous serum opsonized	Ig or histone II* opsonized	fMLP or PDB*	None
Leino et al. 1994 (245)	LJP; 3M, 6F with age/ sex-matched controls	LDCL using $\text{Ca}^{2+}/\text{Mg}^{2+}$ - free HBSS and leukocytes Peak signal	50 $\mu\text{g}/10^5$ cells (100 $\mu\text{g}/\text{ml}$ ) 76 nM fMLP 80 nM PMA	Unopsonized zymosan $\uparrow$ (females only)	Zymosan <sup>7</sup> ( $\uparrow$ ) Zymosan $\uparrow$ (females only)	Zymosan ( $\uparrow$ ) Zymosan $\uparrow$ (females only)	$\uparrow$ ( $\uparrow$ )	( $\uparrow$ )
Mouynet et al. 1994 (286)	G; 8 patients CP; 8 patients EOP; 17 patients Unmatched controls; 7	LDCL on PMNL and whole blood Peak signal and CL index	NK	Zymosan $\leftrightarrow$ Zymosan $\leftrightarrow$ Zymosan $\leftrightarrow$	Zymosan $\leftrightarrow$ Zymosan $\leftrightarrow$ Zymosan $\leftrightarrow$			$\leftrightarrow$ $\leftrightarrow$ $\leftrightarrow$
Gustafsson and Åsman 1996 (164)	CP; 9M, 5F with age/ sex-matched controls	LDCL <sup>3</sup> using PBS with 0.25% human albumin Peak signal expressed as ratio to paired control	300:1		<i>S. aureus</i> $\uparrow$			
Gustafsson et al. 1997 (165)	As above: Gustafsson and Åsman 1996 (164)	As above	300:1 as above with priming <sup>8</sup>		<i>S. aureus</i> $\uparrow$ after priming			
Fredriksson et al. 1998 (139)	CP <sup>9</sup> ; 8M, 9F with age/ sex-matched controls	LDCL using PBS with 0.25% human albumin Peak signal Dichlorofluorescein-diacetate oxidation and flow cytometry	300:1 $\pm$ priming with TNF- $\alpha$ and LPS		<i>S. aureus</i> $\uparrow$ $\pm$ priming  <i>S. aureus</i> $\leftrightarrow$ $\pm$ priming			
Hurtia et al. 1998 (204)	LJP; 5F (4 in maintenance treatment phase) with age/sex-matched controls	LDCL using $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS Peak signal	50 $\mu\text{g}/10^5$ cells (100 $\mu\text{g}/\text{ml}$ )	Unopsonized zymosan ( $\uparrow$ )				
Biasi et al. 1999 (50)	GEOP; 6M, 9F with age/ sex-matched controls	Cytochrome c reduction in HBSS with 0.2% human albumin plus $\text{Ca}^{2+}/\text{Mg}^{2+}$	0.1 mg/ml $10^{-7}$ M fMLP 10 ng/ml PMA	Zymosan $\leftrightarrow$			$\downarrow$ $\leftrightarrow$ $\leftrightarrow$	$\leftrightarrow$
Fredriksson et al. 1999 (141)	CP; 8M, 9F with age/sex-matched controls	LDCL using PBS with 0.25% human albumin Peak signal	300:1		<i>S. aureus</i> $\uparrow$			

Table 7. Continued

Reference	Disease status <sup>1</sup> and numbers	Assay method <sup>2</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs. health)				
				Autologous serum opsonized	Non- autologous serum opsonized	Ig or histone II* opsonized	fMLP or PDB*	PMA None
Fredriksson et al. 1999 (140)	CP, non-smoking; 21 and 29 controls CP, smoking; 19 and 14 controls	LDCL using PBS with 0.25% human albumin Peak signal	300:1			<i>S. aureus</i> ↑  <i>S. aureus</i> (↑)		
Gainet et al. 1999 (146)	RPP; 6M, 4F LJP; 4M, 4W CP; 5M, 3F Unmatched controls; 14	Dichlorofluorescein-diacetate oxidation and flow cytometry using whole blood	10 <sup>-6</sup> M fMLP			↑ ↓ <sup>10</sup> ↔ ↔ <sup>10</sup> ↔ ↔ <sup>10</sup>	↑ <sup>10</sup> ↔ <sup>10</sup> ↔ <sup>10</sup>	↑ ↔ ↔
Gustafsson et al. 2000 (166)	CP; 20 (8 smokers, 12 non-smokers) with 18 unmatched controls (6 smokers, 12 non-smokers)	LDCL using PBS with 0.25% human albumin using leukocytes Peak signal	300:1			<i>S. aureus</i> (↑)		
Fredriksson et al. 2003 (142)	CP (successfully treated, periodontally healthy), non-smoking; 10M, 5F with age/sex-matched controls	LDCL using PBS with 0.25% human albumin iLDCL using HBSS with divalent cations and 0.25% albumin Peak signal	300:1 <i>S. aureus</i> 2 mg/ml zymosan 2 pmol/ml PMA	Zymosan <sup>11</sup> (↑) <i>S. aureus</i> ↑  <i>S. aureus</i> ↑			(↑)	
Gronert et al. 2004 (160)	LAP; 4 Family members without LAP; 4 CP; 6 Unmatched controls; 10	Cytochrome c reduction, no experimental details given	LTB4, IL-8, fMLP, PMA Amounts NK			↑ ↔ ↔	↑ <sup>12</sup> ↔ <sup>12</sup> ↔ <sup>12</sup>	
Zekonis and Zekonis 2004 (472)	CP (untreated?); 9 M, 13 F with 16 unmatched controls	LDCL using buffy coat cells and HBSS. Cell number assayed variable; CL data retrospectively adjusted for cell number	0.2 ng/ml LPS <sup>13</sup> 6 × 10 <sup>6</sup> <i>E. coli</i> /ml	<i>E. coli</i> LPS↑ <i>E. coli</i> ↓				↑

Table 7. Continued

Reference	Disease status <sup>1</sup> and numbers	Assay method <sup>2</sup>	Stimulus to cell ratio or concentration	Stimulants and result (periodontal disease vs. health)				
				Autologous serum opsonized	Non- autologous serum opsonized	Ig or histone II* opsonized	fMLP	PMA or PDB*
Sadzeviciene et al. 2005 (355)	Type 1 diabetes and severe periodontitis; 38 with 27 unmatched controls	Lucigenin-dependent CL using buffy coat cells and HBSS. Cell number assayed variable; CL data retrospectively adjusted for cell number	$6 \times 10^6$ <i>S. aureus</i> or <i>E. coli</i> ↑↑	<i>S. aureus</i> ↑ <i>E. coli</i> ↑↑				None

Response compared with control cells: ↑ and ↓, large and small increase; ↓ and ↓, large and small decrease; ↔, no difference. Arrows in brackets indicate trends that were not statistically significant or based on small sample numbers. ND = not detected.

PMNL, neutrophilic polymorphonuclear leukocyte (neutrophil); LPS, lipopolysaccharide; TNF, tumor necrosis factor; PDB, Phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; NK, not known; LDCL, luminol-dependent chemiluminescence; HBSS, Hanks' balanced salt solution; RGDS, Arg-Gly-Asp-Ser peptide.

<sup>1</sup>JP, juvenile periodontitis; LJP, localized JP; GJP, generalized JP; RPP, rapidly progressing periodontitis; CP, chronic periodontitis; GEOP, generalized early-onset periodontitis; LAP, localized aggressive periodontitis; G, gingivitis.

<sup>2</sup>Using isolated peripheral blood neutrophil and phosphate-buffered saline or other buffer without divalent cations unless stated. Unmodified Hanks' balanced salt solution usually contains  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Divalent cations can affect luminal-dependent chemiluminescence (142) and interactions between zymosan and CR3 (245).

<sup>3</sup>Patient and matched control cell pairs assayed at the same time.

<sup>4</sup>Young patients (18–35 years old) with localized and generalized disease.

<sup>5</sup>Opsonized with heat-inactivated serum (56° for 30 min).

<sup>6</sup>Data taken from Fredriksson et al. (142).

<sup>7</sup>Zymosan opsonized with complement using immunoglobulin-depleted serum.

<sup>8</sup>Priming agents used were tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), RDGS, lipopolysaccharide (LPS) and MetLeuPhe at levels that did not cause activation.

<sup>9</sup>Treated patients that had  $\geq 5$  mm attachment loss at six or more sites.

<sup>10</sup> $\text{H}_2\text{O}_2$  production after treatment with IL-8 (50 ng/ml) followed by MetLeuPhe (fMLP); interleukin-8 (IL-8) alone had no effect. Other studies demonstrated that  $\text{H}_2\text{O}_2$  production did not differ between any of the patient groups and controls after treatment with tumor necrosis- $\alpha$  (TNF- $\alpha$ ) (100 U/ml) or lipopolysaccharide (LPS) (5  $\mu\text{g}/\text{ml}$ )  $\pm$  MetLeuPhe (fMLP).

<sup>11</sup>Zymosan opsonized with guinea-pig complement.

<sup>12</sup>Similar results found with interleukin-8 and leukotriene- $\text{B}_4$  treatment.

<sup>13</sup>Compared with controls, patient's cells showed a significant greater than fourfold increase in response to *E. coli* lipopolysaccharide.

'priming factors' may be increased in the serum in some forms of periodontal disease (e.g. lipopolysaccharide-binding protein in rapidly progressive periodontitis) (377, 391), this cannot be the whole explanation because neutrophils from juvenile periodontitis patients still exhibited greater ROS production than matched controls after stimulation with *Staphylococcus aureus* opsonized with a commercially available purified immunoglobulin preparation (22, 23, 28). Since these early studies, opsonization using a purified immunoglobulin preparation rather than patient or control serum has normally been used in Fc $\gamma$ R stimulation experiments with bacteria (normally *S. aureus*; Table 7). These studies, essentially by a single research group in Sweden, have consistently demonstrated a small but significantly higher level of luminol-dependent chemiluminescence generation by Fc $\gamma$ R-stimulated peripheral neutrophils isolated from chronic periodontitis patients compared to age- and gender-matched controls (139, 140–142, 164, 165). Control stimulations with unopsonized *S. aureus* did not elicit detectable luminol-dependent chemiluminescence. Interestingly, although only very low Fc $\gamma$ R-stimulated ROS generation can be detected in the extracellular compartment using isoluminol, a similar difference between patients and controls was found (142). Recent results from our laboratory, on both total and extracellular Fc $\gamma$ R-stimulated ROS production in chronic periodontitis patients, agree with those discussed above (459). Thus neutrophils in both chronic and juvenile periodontitis show a hyperreactive phenotype with respect to luminol-dependent chemiluminescence after Fc $\gamma$ R stimulation using immunoglobulin G-opsonized bacteria.

A recent study investigating the effects of 'unopsonized' bacteria (*S. aureus* and *Escherichia coli*;  $6 \times 10^6$ /ml) on lucinogen-dependent chemiluminescence in patients with type 1 diabetes and severe periodontitis detected significant ROS generation by peripheral blood leukocytes which was eight- to 90-fold higher in patients than unmatched controls (355). However, the assays, performed in the presence of fresh autologous plasma, which would contain opsonins, are not equivalent to the unopsonized *S. aureus* controls discussed above. Essentially, the *S. aureus* results are similar to those previously published for periodontitis in the absence of systemic disease (23, 25, 26, 450). A similar problem exists in the interpretation of the findings obtained for 'unopsonized' *E. coli* and *E. coli* lipopolysaccharide in chronic periodontitis patients (472; Table 7).

The underlying basis for the hyperreactive phenotype of peripheral neutrophils in respect of Fc $\gamma$ R-stimulated ROS generation seen in periodontitis is unclear. Several studies have failed to detect differences in membrane expression of Fc $\gamma$ R III/CD16 (23, 29, 164, 245) or associations with Fc $\gamma$ R polymorphisms (142, 229). Similarly, the hyperreactive phenotype does not appear to be affected by *in vitro* priming with a variety of agents (TNF- $\alpha$ , lipopolysaccharide, fMetLeuPhe, Arg-Gly-Asp-Ser peptide) (139, 165) or to be the result of the method of neutrophil preparation (141). Localized juvenile/aggressive periodontitis may be associated with a constitutional defect in diacylglycerol kinase activity (160, 203, 421). Early data investigating Fc $\gamma$ R-stimulated ROS generation in successfully treated (i.e. periodontally healthy) patients with juvenile periodontitis demonstrated that hyperreactivity was present before and after treatment, supporting the presence of such a constitutional defect (26). More recent data on patients with chronic periodontitis after successful treatment further suggest that Fc $\gamma$ R-stimulated ROS hyperresponsiveness is constitutional rather than reactive (142). The only other treatment-related study suggested that baseline ROS generation by neutrophils in patients with diabetes and periodontitis was reduced 12 weeks after non-surgical therapy (8). However, details of how the chemiluminescence assays were performed are lacking and matched control assays were not performed. Thus, the results for before and after comparisons are based on actual light output, which is known to show considerable variation with time and within an individual (24, 25, 462). Preliminary data from our laboratory suggest that hyperresponsiveness, in terms of total luminol-dependent chemiluminescence produced after Fc $\gamma$ R stimulation, may be partially reduced by therapy but that neutrophils from patients with chronic periodontitis exhibit increased baseline, unstimulated extracellular ROS release (isoluminol-dependent chemiluminescence) that is not affected by therapy (460). Furthermore, unstimulated peripheral neutrophils from patients with chronic generalized periodontitis also exhibit a distinct molecular phenotype (461). Further studies at the gene expression level will be necessary to characterize the underlying processes responsible.

The data on ROS generation in periodontitis using zymosan, unopsonized or opsonized using serum (autologous and heterologous) or complement (human and guinea-pig), is harder to interpret. Six of the nine reports found in the literature (Table 7) essentially find no difference in ROS generation between

patient and control neutrophils over a range of zymosan concentrations (0.1–6.7 mg/ml; serum opsonized) in both chronic and aggressive forms of periodontal disease. Two other studies have reported higher luminol-dependent chemiluminescence using unopsonized and complement-opsonized zymosan on neutrophils from a small group ( $n = 5$ ) of juvenile periodontitis patients (204) and on 15 patients with chronic disease (142), respectively, but the findings were not statistically significant. The only study apparently showing a hyperreactive neutrophil phenotype after stimulation with unopsonized as well as serum, complement and IgG-opsonized zymosan is that of Leino and co-workers in patients with juvenile periodontitis (245). However, this study was performed on a peripheral blood leukocyte preparation rather than isolated neutrophils. Thus, currently available data suggest that ROS generation by neutrophils via zymosan/CR3 (complement receptor-3) stimulation is not altered in periodontal disease.

Several reports have been published investigating the effect of the chemotactic peptide  $f$ MetLeuPhe on ROS generation in both chronic and aggressive forms of periodontal disease. The two studies involving patients with chronic periodontitis found no difference in ROS generation compared to unmatched controls (146, 160). Data obtained from analysis of neutrophils from patients with aggressive periodontitis (juvenile periodontitis, localized juvenile periodontitis, rapidly progressive periodontitis and generalized early-onset periodontitis) are inconsistent, with increased, decreased, and similar levels of ROS production being reported (Table 7). These findings are against a background of evidence demonstrating defects in neutrophil function including locomotion, adherence, and phagocytosis (399, 428) as well as altered expression of  $f$ MetLeuPhe surface receptor (317, 429). Evidence suggests that a diacylglycerol kinase activity, an enzyme that controls cellular levels of diacylglycerol which is important in the control of many cellular responses including the respiratory burst, is altered in peripheral neutrophils in localized juvenile periodontitis (160, 421). More recently, it has been shown that blocking diacylglycerol kinase activity in neutrophils from healthy donors significantly increased respiratory burst activity after  $f$ MetLeuPhe stimulation. Moreover, only three of five localized juvenile periodontitis patients studied appeared to have a diacylglycerol kinase defect (203), suggesting that the variability in some studies of  $f$ MetLeuPhe-stimulated ROS generation in disease may be the result of such individual patient variation. A similar inconsistency in results is also

evident in studies investigating agents that directly activate protein kinase c (phorbol myristate acetate and phorbol-12,13-dibutyrate; Table 7). However, except for one report (163), all the studies indicate either no difference in phorbol myristate acetate/phorbol-12,13-dibutyrate-stimulated ROS generation or a slightly higher (but usually statistically insignificant) level of production in patients compared to controls.

While control unstimulated cells have normally been included in all these studies, reports demonstrating detectable 'spontaneous' ROS generation are normally based upon non-chemiluminescent methods of detection (Table 7). In those studies where detectable levels of unstimulated ROS generation have been assessed ( $n = 6$ ) there are three reports suggesting higher levels in disease (146, 163, 472). Whether these reports represent the *in vivo* situation or are a consequence of neutrophil preparation and assay conditions is unknown.

Thus, the most consistent finding from studies on peripheral neutrophils in periodontitis is that disease is associated with a heightened ROS response to  $Fc\gamma R$  stimulation. Preliminary studies also indicate that peripheral neutrophils from patients with chronic periodontitis exhibit a low level of extracellular ROS production that is significantly higher than that of controls (459). Whether such a difference is present in neutrophils within the gingival crevice is unknown. The only investigations of crevicular neutrophils have examined phorbol myristate acetate-induced ROS production in chronic periodontitis (163, 250). While crevicular neutrophils of patients demonstrated a heightened response compared to those from healthy controls (163), comparison of cell responses isolated from blood and from diseased, treated and healthy sites within patients suggested that both baseline ROS generation and phorbol myristate acetate responses were lowest in diseased sites (250). Treatment appeared to increase both baseline ROS production and response to phorbol myristate acetate, restoring a phenotype similar to that found peripherally. These data could indicate that neutrophils isolated from diseased sites are either inhibited from responding or that *in vivo* activation and ROS generation have reduced their ability to respond *in vitro*.

#### **Factors that may affect ROS generation by neutrophils at sites of disease**

Plaque bacteria and their products are an obvious source of factors that could stimulate neutrophils infiltrating the periodontal tissues. Enhanced ROS generation by peripheral neutrophils from patients



with both chronic and aggressive disease can be stimulated with opsonized bacteria associated with periodontal disease (*Fusobacterium nucleatum*, *Actinobacillus actinomycetemcomitans*) in a similar way to the studies using *S. aureus* (23, 450). This finding suggests that the hyperreactive phenotype of peripheral neutrophils could have local tissue-damaging consequences. Further studies have shown that several isolates of two strains of fusobacteria (*F. nucleatum* and *F. necrophorum*) can stimulate significant ROS generation (in the presence of plasma), cytokine [interleukin-1 $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$ , IL-8] and elastase production by neutrophils isolated from healthy individuals (378). This group has also shown that *F. nucleatum*, in the absence of plasma, could also stimulate large amounts of ROS production and induce lipid peroxidation *in vitro* (379). That *F. nucleatum* might be pivotal in neutrophil-dependent, ROS-induced tissue damage within the periodontium is also supported by the finding that phagocytosis of *F. nucleatum* induces significantly greater ROS generation than phagocytosis of *Porphyromonas gingivalis* or *A. actinomycetemcomitans* (219). Interestingly, although *P. gingivalis* may have an inhibitory effect on neutrophil generation of ROS (467), its ability to cleave transferrin and release iron or iron-containing peptide fragments may contribute to tissue destruction by catalyzing the formation of the toxic hydroxyl radical via Fenton reactions (156; see 'Origins and formation of ROS and oxygen radicals').

Disease sites will also be associated with increased levels of a variety of cytokines and chemokines produced by inflammatory cells (including neutrophils) and the normal resident cell population within the periodontal tissues. A variety of pro-inflammatory cytokines (TNF- $\alpha$ , granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, IL-8, IL-1, IL-6), growth factors (e.g. platelet-activating factor), and lipopolysaccharide have been shown to have a priming effect on the human neutrophil oxidative burst both *in vitro* and *in vivo* (124, 224). Furthermore, certain combinations of cytokines (e.g. granulocyte-macrophage colony-stimulating factor and TNF- $\alpha$ ) are synergistic in their priming activity for fMetLeuPhe-stimulated ROS generation (224). Although potentially important in local ROS production, such priming effects are not thought to be the cause of the Fc $\gamma$ R hyperresponsive phenotype of peripheral neutrophils in chronic periodontal disease (139, 165). TNF- $\alpha$  can prime for ROS generation by neutrophils from patients with chronic and aggressive periodontitis as well as periodontally healthy individuals (28, 139, 146, 165). By contrast,

IL-8 can prime for fMetLeuPhe-stimulated neutrophil ROS production in health, chronic disease, and localized juvenile periodontitis but not in rapidly progressive disease (146). These data suggest that cytokines can modulate the respiratory burst activity of neutrophils and have a role in determining oxidative stress locally within the tissues.

### Effects of tobacco smoking on ROS production by neutrophils

Although smoking is a known risk factor for the development of periodontitis and cigarette smoke contains a mixture of reactive species, tobacco use has only recently been taken into consideration in studies of neutrophil ROS production. As the effect of smoking on the immune response in periodontal disease is the subject of a companion review to this (353), only those papers investigating the neutrophil oxidative burst and smoking directly will be reviewed. *In vitro* studies have shown that exposure of neutrophils, from medically and periodontally healthy donors, to cigarette smoke reduced phorbol myristate acetate-stimulated ROS generation (ROS detected by ferricytochrome c and dichlorofluorescein diacetate/flow cytometry methods). By contrast baseline, unstimulated ROS production was increased by smoke exposure, although there appeared to be two distinct cell populations (high and low ROS producers) (354). A recent luminol-dependent chemiluminescence study of fMetLeuPhe and opsonized zymosan-stimulated neutrophils isolated from smokers and never smokers found similar results, with smokers having significantly lower levels of ROS production but higher chemotaxis (392). While the deficiency in ROS response was restored by a period of 20 days abstinence, chemotactic activity remained high. By contrast to these reports, a study of 82 healthy males, 41 chronic cigarette smokers and 41 non-smokers, has demonstrated an increased rate of ROS generation by phorbol myristate acetate-stimulated peripheral neutrophils (cytochrome c reduction) from smokers, together with elevated GSSG and reduced GSH levels in plasma (373). This study also suggested that serum from smokers caused necrosis of neutrophils *in vitro*.

In a study of the Fc $\gamma$ R-stimulated luminol-dependent chemiluminescence response of peripheral blood leukocytes, no differences were detected between smokers and non-smokers, irrespective of the presence of periodontal disease (140). However, peripheral blood leukocytes from periodontally healthy control patients who smoked displayed a higher median level of luminol-dependent chemi-

luminescence (not significant) than those from non-smokers. This difference reduced the level of Fc $\gamma$ R-stimulated hyperreactivity when comparing patients and controls in the smoking group because patients who smoked had similar levels of peripheral blood leukocyte luminol-dependent chemiluminescence to those that did not. Further studies by the same group have confirmed this finding and shown that TNF- $\alpha$  has a greater priming effect for Fc $\gamma$ R-induced ROS generation by peripheral blood leukocytes from smokers than non-smokers (166).

The data discussed are inconsistent and difficult to reconcile. It is possible that methodological differences such as validation of smoking status, level of smoking, assay, and method of ROS stimulation could account for the disparity in results. Further studies in this area related to the periodontal diseases are warranted.

### Neutrophil-derived myeloperoxidase in periodontal disease

Myeloperoxidase is released into the phagosome and extracellularly during phagocytosis and activation of neutrophils when it is important for generating hypochlorous acid and other ROS (20, 228), which have potential for tissue damage (see 'Origins and formation of ROS and oxygen radicals'). A variety of opsonized oral bacteria have been shown to stimulate the release of all three myeloperoxidase isoforms (274) and raised levels in gingival crevicular fluid from diseased sites have been reported in gingivitis (66), chronic periodontitis (60, 445, 454), rapidly progressive periodontitis (307), localized juvenile periodontitis (397), and aggressive periodontitis (59, 61). These studies have demonstrated a relationship between the gingival crevicular fluid level of myeloperoxidase and clinical measures of disease (61, 445, 454) and that levels reduce after successful treatment (59–61, 397, 454). While the presence of heightened levels of myeloperoxidase at disease sites is a potentially useful marker of neutrophil infiltration into the tissues, its presence inevitably suggests local generation of hypochlorous acid and other ROS, potentially increasing the oxidative load and promoting tissue damage.

### Other cellular sources of ROS

Although all cells produce ROS during normal physiological functions (119), it is mononuclear and neutrophilic polymorphonuclear phagocytes that produce high levels to facilitate the killing and destruction of microbes (371). In consequence, most investigations into the role of ROS in tissue damage

have concentrated on the activity of such cells. However, there is evidence that other cells normally resident within periodontal tissues may contribute to local oxidative stress and a few relevant findings will be reviewed in this section. Fibroblasts, which represent the largest population of cells in healthy gingiva and periodontal ligament, are able to spontaneously release detectable levels of ROS in culture media containing Ca<sup>2+</sup> (289, 384). The time course of this spontaneous ROS production appears to be comparable in form and magnitude to that detected in unstimulated neutrophils and endothelial cells (289). Stimulation studies indicated that a variety of agents increase superoxide generation by both skin and gingival fibroblasts, with IL-1 $\beta$ , transforming growth factor- $\beta$ , phorbol myristate acetate, and the calcium ionophore A23187 being weak stimulators and *E. coli* lipopolysaccharide, fMetLeuPhe, Group A streptococcal cell walls and TNF- $\alpha$  inducing significant ROS production. Generation of superoxide by stimulated fibroblasts appears to be well regulated in that the levels produced are equivalent to those that stimulate fibroblast cell growth and proliferation (289). Furthermore, stimulated cells respond by increasing their levels of mitochondrial superoxide dismutase thus protecting them from ROS-induced damage (384). Interestingly, the level of superoxide dismutase induction differed between stimulants, suggesting that *in vivo* fibroblasts could be stimulated to produce damaging levels of ROS by plaque-derived factors.

Another aspect of this work that may be important *in vivo* is the necessity of Ca<sup>2+</sup> for detectable ROS production by fibroblasts. Calcium levels are high in Howship's lacunae (383) and osteoclastic activity at the alveolar crest could result in a local increase in Ca<sup>2+</sup> levels promoting ROS production by gingival fibroblasts in a region potentially exposed to plaque bacteria and their components.

Junctional and crevicular epithelium are at the 'front line' in terms of defense against the entry of plaque bacteria and their products into periodontal tissues. While there is a growing body of evidence demonstrating that epithelial cells actively participate in immune/inflammatory responses by producing a variety of cytokines, their direct contribution to oxidative stress has only recently been appreciated. Both skin and gingival epithelial cell lines express a heme-flavoprotein NADPH oxidase (Nox), distinct from the Phox isoform of phagocytes. Although the activity of this NADPH oxidase is 20-fold lower than those reported for the phagocyte oxidase, chronic production of superoxide by epithelium within the

crevice/pocket could represent a significance source of local ROS (73).

### Local presence of ROS in periodontal disease

The majority of tissue destruction in periodontitis is considered to be the result of an aberrant inflammatory/immune response to microbial plaque adjacent to the gingival margin and to involve prolonged release of neutrophil enzymes and ROS. While there are data in the literature about the presence of the former at diseased sites (425), there have been no published studies investigating directly the presence and levels of ROS in periodontal tissues, gingival crevicular fluid, saliva or blood in periodontal health and disease. Although not surprising, given the difficulties in detecting ROS directly (reviewed in 187), possibilities do exist for local detection of ROS using endogenous molecular 'spin traps' such as urate. Allantoin is one of urate's oxidation products that has been shown to be elevated in conditions associated with oxidative stress and periodontal disease such as diabetes (47), lung disease in pre-term infants (303), rheumatoid arthritis (161, 463), and chronic heart failure (116). Of particular significance is that allantoin has been successfully detected by high-pressure liquid chromatography in small samples (4 µl) of cerebral microdialysis fluid (258), making the analysis of gingival crevicular fluid in health and disease a tangible goal.

A second potential avenue of enquiry is the direct detection of hydrogen peroxide, a relatively stable ROS, by sampling the air within the oral cavity. Studies have shown that hydrogen peroxide can be detected in exhaled air and breath condensate, and that levels appear to correlate with inflammation (reviewed in 332).

Because of the difficulties inherent in detecting and quantifying ROS directly in any disease, most research has concentrated on measuring the products (biomarkers) generated by ROS reacting with lipids, DNA or proteins within the body. There are a variety of generally accepted biomarkers, a number of which have been studied in the context of the periodontal diseases. The following section is based upon the literature searches outlined in the 'Context of the review' plus subsidiary searches using PubMed with 'Periodont\*' as the search term individually linked to all the biomarkers of oxidative damage (n = 33) discussed by Halliwell and Whiteman (184). It is important to remember that none of these markers is absolutely specific for ROS damage (i.e. they can be

generated by means other than reacting with ROS) or specific to the periodontal tissues or periodontitis.

### Systemic and local presence of the products of ROS reaction with biomolecules

Most published work in the periodontal literature has focused on markers of ROS reactions with lipids. Of the three main markers of lipid peroxidation in common use, (thiobarbituric acid reactive substances, malondialdehyde, isoprostanes – see 'Biomarkers of lipid peroxidation'), the isoprostanes are considered the best available (133, 346). To date only thiobarbituric acid reactive substances and malondialdehyde have been investigated in chronic periodontitis (314, 416, 419). Importantly, due to the potential confounding effects of tobacco use because of the large amount of oxidative species contained within cigarette smoke (329), smokers were excluded from the latter two studies.

All the published studies have suggested that patients with chronic periodontitis have higher levels of lipid peroxidation than periodontally healthy controls. Thus thiobarbituric acid reactive substances were raised in patients both systemically in plasma and erythrocytes (314) and locally in tissue homogenates (314, 419). More importantly, bearing in mind the inadequacy of thiobarbituric acid reactive substances as an acceptable marker of lipid peroxidation (184), malondialdehyde was also found to be raised in gingival crevicular fluid and saliva of patients compared to controls (416). Both studies investigating thiobarbituric acid reactive substances also measured other parameters that lend support to the contention that the thiobarbituric acid reactive substance results are related to inflammation. Thus, thiobarbituric acid reactive substances at sites refractory to phase-1 periodontal treatment correlated with the total amount of IL-1 $\beta$  in gingival crevicular fluid from those sites (419). Again, more significantly, both salivary and gingival crevicular fluid levels of malondialdehyde decreased in subjects after clinically successful phase-1 therapy (416). Interestingly, the gingival crevicular fluid concentrations of malondialdehyde/4-hydroxyalkanal reported by Tsai et al. (416) were 200- to 400-fold higher than the respective saliva concentrations, and while the authors do not discuss this issue, it seems likely to reflect a substantially higher amount of ROS activity (thus lipid peroxidation) in gingival crevicular fluid than saliva, given that the two biological media do not differ greatly in total antioxidant capacity (58). This raises questions about

the appropriateness of saliva as a medium for assessing ROS activity/effects within the periodontium.

Studies on the ligature-induced 'periodontitis' model in rats using the malondialdehyde assay support the human studies and have demonstrated increased lipid peroxidation in the serum (388) and gingival tissue homogenates (108, 110) of experimental rats compared to those from sham-operated controls. Interestingly, development of ligature-induced inflammation and the increase in malondialdehyde could be significantly reduced using an inducible nitric oxide synthase inhibitor (aminoguanidine) (108) or a superoxide dismutase mimetic that specifically removes superoxide anions (110).

There are a variety of studies investigating lipid peroxidation in conditions related to periodontal disease that also provide indirect support for a role of ROS in disease pathogenesis. For example, in a study of a family affected by different degrees of Papillon-Lefèvre syndrome, plasma hydroperoxide levels (measured by the ferrous ion oxidation xylene-2 (FOX-2) assay) (301) were high compared with reference values (40). However, while there appeared to be a positive relationship between hydroperoxide levels and phenotypic manifestations of the disease, caution is needed because of the small number of cases and the variability of results from the ferrous ion oxidation xylene-2 assay with sample storage (389). Of greater significance, two of the most important risk factors for the development of periodontitis, smoking (311) and diabetes (264), can be linked to alterations in gingival cells and lipid peroxidation levels. It has been known for some time that components of cigarette smoke are associated with inhibition and killing of periodontal fibroblasts (e.g. 291, 324, 411). More recently it has been observed that nornicotine, a metabolite found in high concentrations in the plasma of smokers, not only causes the development of advanced glycation end products (72, 112) but also induces an increase in the expression of the receptor for the advanced glycation end products by human gingival fibroblasts *in vitro* (220). Furthermore, expression of the receptor for advanced glycation end products by gingival endothelium and epithelium has been demonstrated immunohistochemically in diseased sites from periodontitis patients with and without type 2 diabetes (221). These findings together with previous studies in mice showing that infusion of advanced glycation end product albumin results in advanced glycation end product deposition and increased generation of thiobarbituric acid reactive substances in various tissues including the gingivae (365), provide a

mechanistic link between diabetes, smoking, and periodontal disease based upon oxidative stress.

A novel and potentially useful method for estimating lipid peroxidation that may have application to the periodontal diseases is the analysis of exhaled, or oral cavity sampled, air for volatile hydrocarbons (332). Some work has been reported from Russia (432; paper in Russian) suggesting differences in oral levels of short chain fatty acids and aldehydes between periodontitis patients and controls. The methodologies for assessment of volatile organic compounds within the oral cavity have been developed in the field of oral malodor and suggest that the predominant volatile organics are alkanes and methylated alkanes which could represent the end products of lipid peroxidation (322).

Information on markers of ROS reaction with DNA and proteins in periodontitis is limited. The majority of published data on oxidative damage to DNA has been reported by a Japanese group who investigated 8-hydroxydeoxyguanosine levels in saliva by enzyme-linked immunosorbent-assay. These studies demonstrated that levels of 8-hydroxydeoxyguanosine in samples from subjects with chronic periodontitis were significantly higher than those from periodontally healthy controls (403). In patients, salivary 8-hydroxydeoxyguanosine levels correlated with clinical attachment loss and age but not smoking, bleeding on probing or probing depth. Levels did, however, decrease after successful initial periodontal therapy. Further data from this group suggest that salivary 8-hydroxydeoxyguanosine levels correlate with polymerase chain reaction-detectable levels of *P. gingivalis* (362) and that 8-hydroxydeoxyguanosine can be detected in gingival crevicular fluid from some sites associated with periodontally involved teeth with hopeless prognosis (404). Levels of 8-hydroxydeoxyguanosine excreted in the urine are known to be related to smoking and gender (251) but not age (35–65 year range) (325). As the published data on saliva have not been stratified for these variables, further studies in this area are warranted.

Information on protein oxidation in human periodontal disease is limited to the cohort study of Sculley and Langley-Evans (370). Subjects ( $n = 129$ ) were classified using a modification of the Community Periodontal Index of Treatment Need (CPITN) score that was taken to indicate disease (defined as a combined score for all sextants of greater than 6) or severity of disease. The results indicate that protein carbonyls are significantly higher in the tertile of patients with an average total CPITN score of  $\geq 13$  compared to that with a score of  $\leq 10$ . The reverse

**Table 8.** Representative genes\* under the control of the redox-sensitive transcription factors AP-1 and NF- $\kappa$ B

Target gene activity	Activating protein-1 (AP-1)	Target gene activity	Nuclear factor $\kappa$ B (NF- $\kappa$ B)
Inflammatory cytokines	IL-2	Inflammatory cytokines	IL-1 $\beta$
	IL-8		IL-2
	TNF- $\alpha$		IL-6
Cell proliferation	Cyclin D	Chemokines	IL-8
	Cyclin A		IL-12
	Cyclin E		IL-17
	p53		TNF- $\alpha$
	p21 <sup>Cip1</sup>		MIP-1 $\alpha$
	p16 <sup>Ink4</sup>		MIP-2
	p19 <sup>ARF</sup>	Acute phase proteins	CRP
Epithelial cell differentiation	TGF- $\beta$		LBP
	Keratins 1, 5 and 14	Growth factors	G-CSF
	Filaggrin		GM-CSF
	Involucrin		VEGF
	Transglutaminase 1	Tissue remodeling	MMP-1
Tissue remodeling	TGF- $\beta$		MMP-3
	MMP-1		MMP-9
	MMP-3	Cell adhesion	E-selectin
Cell adhesion	MMP-9		VCAM-1
	E-selectin		ICAM-1
	$\alpha_2$ and $\alpha_6$ integrins		
	$\beta_4$ integrin		
	Laminin $\alpha_3A$		

IL, interleukin; MMP, matrix metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor; MIP, major intrinsic protein; CRP, C-reactive protein; LBP, lipopolysaccharide-binding protein; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; VCAM, vascular cell adhesion molecule; ICAM, intracellular adhesion molecule; NF $\kappa$ B, nuclear factor- $\kappa$ B.

\*Data taken from Makarov (255), Matt (260) and Yates and Rayner (464). This is an illustrative list and does not contain all gene targets for these transcription factors. Furthermore, because the promoters and enhancers of the dependent genes often contain binding sites for other transcription factors, interactions between these factors increase or decrease their ability to initiate transcription. The level of regulatory control is often further increased by the requirement for co-activators such as CREB-binding protein (260).

was found for urate and total antioxidant (ferric reducing ability of plasma assay) flow rates. The only other data available on protein oxidation is based on immunohistochemical localization of nitrotyrosine in tissues obtained from rats subjected to ligation-induced periodontitis (108–110, 252). Nitrotyrosine can be formed by peroxynitrite (see ‘Origins and formation of ROS and oxygen radicals’ – ‘Superoxide and nitric oxide’) and is usually interpreted as evidence of protein oxidation. Although often thought to be a specific marker for peroxynitrite activity, nitrotyrosine can be formed from tryrosine by a range of reactive nitrogen species (184). All four of the animal

studies demonstrated an increased presence of inflammatory cells and nitrotyrosine-positive cells in tissues associated with ligated teeth compared to controls. These changes were significantly reduced, as were tissue malondialdehyde levels, by an inducible nitric oxide synthase inhibitor (aminoguanidine) (108) or a superoxide dismutase mimetic that specifically removes superoxide anions (110). In two of these ligation studies immunohistochemical staining for poly(ADP-ribose) was performed as an indicator of poly(ADP-ribose) polymerase activation of DNA repair. Positive staining was consistently detected in tissues associated with ligated teeth,

which was significantly reduced by treatment with aminoguanidine (108) and Tempol (a membrane-permeable radical scavenger) (109) suggesting that oxidative damage to DNA is also a factor in this animal model. It is clear from the above that additional studies in human periodontal disease are required to elucidate the extent and nature of protein and DNA oxidation in periodontal disease.

## Effects of ROS on periodontal tissues and components

The direct action of ROS on connective tissue components has been extensively studied in relation to a variety of inflammatory diseases and has been the subject of recent reviews (64, 434). Furthermore, the enumeration of possible biomarkers of the collagenous and non-collagenous components of connective tissues in periodontal disease has been recently reviewed (128, 151, 285). This section will concentrate on reviewing the literature that directly relates to oxidative alterations of periodontal tissues with a view to highlighting aspects of pathology that require investigation and that might suggest future therapeutic targets and/or markers for assessing development, progression or regression of periodontitis. There are a number of reports investigating the direct effects of ROS on gingival cells and connective tissue components *in vitro*. These studies often depend upon artificial systems of ROS generation which substitute for the neutrophil-generated ROS that are implicated in tissue damage *in vivo*.

The direct damaging effects of ROS on gingival cells have received little attention. Gingival fibroblasts and epithelial cell monolayers exposed to unstimulated neutrophils from medically and periodontally healthy subjects experience minimal detachment or damage (14, 103). Neutrophils stimulated with phorbol myristate acetate disrupted epithelial cell detachment without lysis by a mechanism involving proteolysis rather than any direct effect of ROS. By contrast, ROS generated using a neutrophil myeloperoxidase, chloride, glucose, and glucose oxidase system caused lysis of epithelial targets that could be inhibited by azide and catalase (14). This observation has important implications for disease pathogenesis but evidence that *in vivo* levels of ROS production by neutrophils in periodontitis cause such an effect are lacking. Similarly, there appear to be no studies comparing ROS-mediated damage to cells or extracellular matrix components by neutrophils isolated from patients and periodontally healthy controls, despite the growing evidence

base that neutrophils in periodontitis exhibit a hyperactive/reactive phenotype in respect of ROS production (see 'In vitro ROS generation by neutrophils in periodontal health and disease').

Although the effects of ROS on bone resorption have not been studied in periodontal disease it has been shown that certain ROS (superoxide and hydrogen peroxide) activate osteoclasts (44, 174) and promote osteoclast formation (147). Furthermore, osteoclasts produce ROS at the ruffle border/bone interface, suggesting a more direct role in resorption (223, 393). Such a direct role in bone resorption in periodontitis is supported by the finding that hydroxyl radicals and, to a lesser extent, hydrogen peroxide can degrade alveolar bone proteoglycans *in vitro* (284).

Much of the data available on ROS-mediated degradation of the extracellular matrix of connective tissues has focused on investigating the pathology and pathogenesis of rheumatoid arthritis (reviewed in 434). Thus, many of the studies relate to components of the synovium, particularly the non-collagenous components of synovial fluid and cartilage (i.e. proteoglycans). ROS, artificially generated or produced by neutrophils stimulated *in vitro*, have been shown to preferentially degrade the protein components (core and link) of proteoglycans resulting in component glycosaminoglycans being attached to smaller peptide fragments. In addition, the glycosaminoglycans themselves can be degraded, but differ in their susceptibilities to attack with sulfated glycosaminoglycans being more resistant than non-sulfated molecules. Studies specifically on the extracellular matrix components of periodontal tissues have demonstrated the *in vitro* ability of ROS to degrade proteoglycans extracted from porcine gingivae as well as within intact frozen sections of tissue (37). More recent studies by Moseley and co-workers have investigated the effects of a range of ROS on glycosaminoglycans and proteoglycans present in the soft and calcified tissues of the periodontium. Their findings demonstrated that all glycosaminoglycans undergo a variable degree of chain depolymerization and residue modification (especially in the presence of hydroxyl radicals) and that sulfated glycosaminoglycans were more resistant to ROS degradation than the non-sulfated glycosaminoglycan hyaluronan (282, 283). Furthermore, it was shown that chondroitin sulfate proteoglycans from alveolar bone were particularly susceptible to damage by hydroxyl radicals, which caused degradation of both the core proteins and glycosaminoglycan chains (284). By contrast, hydrogen peroxide caused more selective

damage with core proteins being more susceptible than glycosaminoglycan chains. A similar pattern of ROS damage is said to occur with proteoglycans isolated from gingival soft tissue (434).

The accumulated evidence clearly suggests that ROS at physiological levels can selectively damage proteoglycans associated with both the soft periodontal tissues and alveolar bone. Furthermore, that these extracellular matrix components are degraded in periodontal disease is supported by data from a large number of studies based on the analysis of gingival crevicular fluid and tissue extracts for their degradation products (reviewed in refs 128, 434). What is less clear is whether the degradation of proteoglycans in periodontal disease is, at least in part, the result of oxidative damage. The proteoglycan components found in gingival crevicular fluid from cases of advanced periodontitis appear to originate from alveolar bone, rather than the periodontal soft tissues (433). They are partial degradation products in which cleavage of the core proteins has occurred without significant alteration of the glycosaminoglycan chains. This finding is consistent with the pattern of oxidative damage to alveolar bone proteoglycans seen *in vitro* (284). Similarly, biochemical analysis of gingival proteoglycans in inflamed gingivae demonstrates a similar pattern of degradation of the core proteins with retention of relatively intact sulfated glycosaminoglycan chains (36, 127, 330). In contrast to its sulfated counterparts, the ubiquitous non-sulfated glycosaminoglycan hyaluronan, found to be most susceptible to ROS damage *in vitro* (282, 283), is completely degraded in inflamed gingival tissue (36, 127, 330). Thus, the pattern of proteoglycan and glycosaminoglycan degradation seen in periodontitis reflects the *in vitro* data on the effects of ROS and is consistent with a role for oxidative damage to non-collagenous components of both the hard and soft tissues of the periodontium.

Similarly, studies have demonstrated that ROS have a variety of effects on type I collagen *in vitro* including direct fragmentation and polymerization as well as producing oxidative modifications, rendering the molecule more prone to proteolysis (287, 434). The structure of collagen, with its high proline/hydroxyproline content, is particularly susceptible to damage by ROS. Superoxide anions and hydroxyl radicals are able to cleave collagen into small peptides at proline and hydroxyproline residues, liberating hydroxyproline-containing peptides (276). In many tissues, including blood vessels, heart, lungs, kidney, and placenta, collagen is thought to be pro-

tected from the action of superoxide by the expression of high levels of extracellular superoxide dismutase which binds to heparin and type I collagen (320). Loss of extracellular superoxide dismutase activity contributes to a number of diseases associated with tissues that are associated with high levels of constitutive enzyme expression (e.g. atherosclerosis, hypertension, coronary artery disease, and diabetic vasculopathy) (302). Although periodontal disease is associated with increased levels of superoxide dismutase-1 (found in the cytoplasm and nuclei of cells) in gingival extracts, there have been no studies of the extracellular isoenzyme (superoxide dismutase-3) in periodontitis (7, 314). Increased local breakdown of collagen in periodontal disease has been suggested from investigating gingival crevicular fluid for collagen metabolites such as hydroxyproline (193), *N*-propeptide (54), and collagen cross-links (pyridinoline and deoxypyridinoline) (150, 313). While the presence of these collagen metabolites in gingival crevicular fluid is likely to be the result of a combination of proteolysis by host and bacterial collagenases, oxidative damage may make a direct or indirect contribution to their production.

While collagen may be susceptible to direct attack by ROS, it has been shown that collagen, along with other proteins, can interact with lipid peroxidation products such as malondialdehyde (386). In normal circumstances collagen and other components of the extracellular matrix are important in controlling movement of cells and connective tissue cell function (356). However, modification of collagen and serum proteins indirectly by ROS, via interaction with lipid peroxidation products such as malondialdehyde, can significantly alter fibroblast functions such as adhesion, proliferation, and longevity (345). Such alterations of *in vivo* fibroblast function should be expected in periodontal disease because of the documented increase in lipid peroxidation within the gingival tissues (reviewed above).

The glycation and glyoxidation of proteins to produce advanced glycation end products is dependent on oxidative processes (209) and occurs in diabetes (295) and smoking (72, 112), two major risk factors for periodontitis. In connective tissues, collagens are particularly susceptible to glycation. In the case of type I collagen, glycation modifies its structural properties (410) and alters its interactions with surrounding extracellular matrix molecules and cells, including pre-osteoblasts (217) and neutrophils (278). Of special significance to the pathogenesis of periodontal disease is the finding that *in vitro* glyoxidation of type I collagen significantly increased neutrophil



adhesion and chemotaxis of neutrophils as well as having a priming effect on subsequent stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (278). These results suggest that oxidation-dependent changes in collagen within the periodontal connective tissues could retard neutrophil migration through the tissues and increase their potential to produce ROS, two factors that may be important in the pathogenesis of periodontal disease.

Other studies on the effects of ROS on plasma have suggested that superoxide *in vitro* can modify a chloroform-extractable factor bound to serum albumin rendering plasma, and its chromatographically purified albumin, highly chemotactic to neutrophils *in vitro* and *in vivo* (321). ROS-mediated modification of tissue fluid albumin within the periodontal tissues could thus contribute to the influx of neutrophils seen in disease. Furthermore, the imbalance of metalloproteinases and their tissue inhibitors in gingival crevicular fluid and in tissues associated with disease (326, 390, 420) could be the result of direct damage of tissue inhibitor of matrix metalloproteinases by ROS (171) or ROS-induced alterations in matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression by cells within the periodontium (189, 222, 361). In any event, ROS-related increases in matrix metalloproteinase activity could play a significant role in the destruction of both the native or oxidatively altered proteins within extracellular matrix.

While the actions of ROS on DNA are well documented, there appears to be only one published report investigating DNA damage in gingival tissues in periodontal health and disease (394). The results, based upon polymerase chain reaction analysis of total DNA extracted from gingivae, found deletions within mitochondrial DNA only in samples from periodontitis patients. It is well known that mitochondrial DNA mutations, such as deletions resulting from oxidative damage, are associated with aging and several chronic diseases (309). Furthermore, once damaged, oxidative stress within the cell can be amplified because of decreased expression of proteins critical for electron transport, leading to cell death (430).

## Redox-sensitive signaling pathways and periodontal disease

Traditionally, ROS production by phagocytes has been associated with the defense of the body to infection as they are essential for the efficient killing of microbes. By contrast, ROS at high levels, or chronically produced, can cause oxidative stress

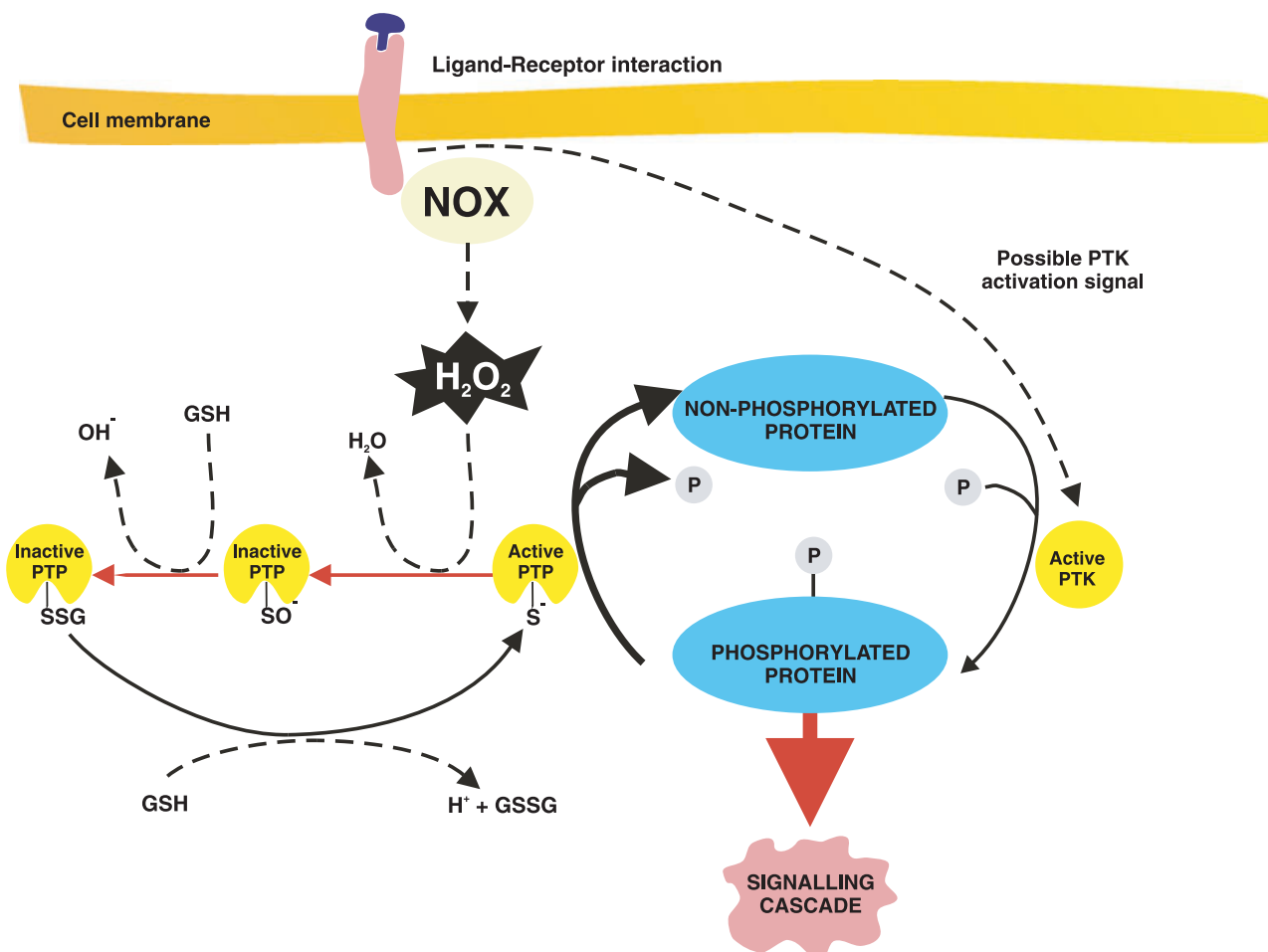
within tissues and result in direct damage to cells and the extracellular matrix. Subsequently, products of this oxidative damage, such as advanced glycation end products and lipid peroxide-modified proteins, can lead to further ROS-induced damage by their priming and chemotactic actions on neutrophils (see 'Effects of ROS on periodontal tissues and components'). However, it is now apparent that ROS play a major role in the normal physiological functioning of all cells and are involved in a variety of receptor-mediated signaling pathways where they activate or inhibit phosphatases and kinases involved in signal transduction as well as directly affecting the ultimate binding of some transcription factors to their DNA targets (119, 210, 260, 349). In particular there are two 'redox-sensitive' transcription factors of potential importance in the pathogenesis of periodontal disease, namely nuclear factor- $\kappa$ B and activator protein 1. They can be activated by a variety of stimuli, including bacterial products, viral proteins, cytokines, growth factors, radiation, ischemia/reperfusion, and oxidative stress (255). After activation, they regulate the transcription of genes important in inflammation, tissue remodeling, cellular proliferation, apoptosis, and repair (Table 8). The levels of both endogenous GSH and exogenous thiol-containing antioxidants can alter cellular responses to inflammatory stimuli (e.g. 155, 232) indicating a potentially fruitful avenue of research for both the prevention and treatment of inflammatory disease such as periodontitis. The following section will briefly discuss the physiological role of ROS and glutathione in signaling pathways generally before considering activating protein-1 and nuclear factor- $\kappa$ B and their potential role in the pathogenesis of the periodontal diseases.

## Redox signaling

Under normal physiological conditions the cellular response to a ligand binding to its receptor within the cell membrane initiates a cascade of events often resulting in altered gene expression. Exogenous or receptor-stimulated ROS have been shown to alter numerous signaling pathways, including, for example all the mitogen-activated protein kinases (415) which phosphorylate protein substrates. Normal regulation of membrane receptor signaling cascades are thought to depend upon local generation of ROS which, possibly via the oxidation and reduction of glutathione, cause post-translational modification of proteins (e.g. phosphorylation) resulting in altered function (e.g. allowing the protein to perform the next step in a cascade of reactions or allowing/

enhancing a protein transcription factor to bind to DNA). The precise targets for ROS in this process are largely unknown, but probably include the thioredoxins, peroxiredoxins and protein tyrosine phosphorylase which have a cysteine residue in the thiolate (i.e. ionized;  $S^-$ ) form at their active sites. Thiolates, unlike thiols (SH), are highly reactive with hydroperoxides under normal physiological conditions, after which they can participate in disulfide exchange reactions with, for example, GSH (137). A possible mechanism whereby ROS produced at the site of receptor–ligand interaction could regulate (i.e. turn on and turn off) a specific signaling pathway involving phosphorylation of protein intermediates is illustrated in Fig. 12. In the unstimulated cell, the

signaling intermediate is kept in its non-phosphorylated form because protein tyrosine phosphatase activity is greater than protein tyrosine kinase activity (i.e. pathway switched off). On ligand–receptor interaction, locally produced ROS inactivate the target protein tyrosine phosphorylase by oxidizing a reactive thiolate (protein tyrosine phosphatase- $S^-$ ) within the active site, producing a sulfenate (protein tyrosine phosphatase- $SO^-$ ) which subsequently forms a mixed disulfide with GSSG (protein tyrosine phosphatase-SSG; the formation of a mixed disulfide is often called ‘glutathionation’) (35). The enzyme is then reactivated by another exchange reaction with GSH. Thus, in the presence of ROS, phosphorylase activity is inhibited allowing production of the



**Fig. 12.** Possible model of redox signaling [adapted from Forman et al. (137)]. In the unstimulated state the protein intermediate is non-phosphorylated because of the greater activity of the phosphatase (protein tyrosine phosphatase (PTP)) vs. the kinase (protein tyrosine kinase (PTK)): *pathway turned OFF*. Ligand binding causes receptor-associated NADPH oxidase (NOX) to generate hydrogen peroxide that inactivates PTP resulting in formation of the phosphorylated protein intermediate by

PTK: *pathway turned ON*. Switching on may also involve a second signal from the ligand–receptor interaction that activates PTK. The pathway switches off when hydrogen peroxide is no longer produced by receptor-associated NOX and active PTP dephosphorylates the protein intermediate faster than it is being produced. Again, this may be further helped by the activity of PTK being reduced because the ligand–receptor activation signal stops. GSSG, oxidised glutathione; GSH, reduced glutathione.

phosphorylated protein intermediate that can trigger the next signaling event in the pathway (i.e. pathway switched on). On cessation of ROS production, the phosphorylase returns to its normal active form and restores the protein intermediate to its non-phosphorylated state, effectively switching off the signal. A number of such signaling events are thought to be important in the activation of redox-sensitive transcription factors such as nuclear factor- $\kappa$ B and activating protein-1 (119, 137, 210).

From the above it should be clear that receptor signaling cascades in normal cell function are controlled by local changes in redox balance and involve the oxidation of GSH. Evidence for this role, as opposed to the normally quoted role of GSH within the cell as an antioxidant protecting the cell against oxidative stress, is now accumulating (137, 138, 336). However, the overall redox balance within the cell is maintained by glutathione and the ratio of GSH:GSSG plays the major role in maintaining the reduced state of most molecules within cells. The GSH:GSSG ratio is regulated by glutathione peroxidase, glutathione disulfide reductase, new synthesis plus conjugation and exchange reactions (see Fig. 4) (119, 137). Oxidative stress, as a result of excessive generation of ROS within a cell or exposure to exogenous sources of ROS, has the capacity to activate redox-sensitive signaling pathways in the absence of specific ligand–receptor interaction and, in extreme circumstances, to cause irreversible damage to cellular proteins by overcoming the stabilizing effect of intracellular GSH. Thus, in periodontal disease, it is likely that redox-dependent pathways such as those culminating in the activation of nuclear factor- $\kappa$ B and activating protein-1 will involve both true redox signaling (via interaction with cytokines and growth factors with their specific receptors) as well as non-enzymatic, potentially irreversible reactions induced by oxidative stress.

### Activator protein-1 and nuclear factor- $\kappa$ B

As these families of transcription factors have been the subjects of many reviews where their structure and activating pathways have been described (e.g. refs 210, 255, 464), only brief details will be included here.

The nuclear factor- $\kappa$ B series of transcription factors comprise homo- and hetero-dimers of proteins belonging to the Rel family. In the non-activated state, nuclear factor- $\kappa$ B is found in the cytoplasm bound to an inhibitory protein I $\kappa$ B. Binding of ligand to a variety of cell surface receptors (e.g. TNF-receptor-1, TNF-receptor-2 and TLR-4, TLR-9)

(235, 260, 439), or oxidative stress (e.g. hydrogen peroxide) (138), activates I $\kappa$ B kinase, which phosphorylates two critical serine residues in I $\kappa$ B, marking it for rapid ubiquitination and degradation by I $\kappa$ B ubiquitin ligase and 26S proteasome respectively. The resulting free nuclear factor- $\kappa$ B rapidly translocates to the nucleus where it binds to sites in the promoter/enhancer regions of target genes. While this may be sufficient for induction of gene transcription, many stimuli (e.g. IL-1, TNF- $\alpha$ ) require interactions with other transcriptional co-activators (e.g. CREB-binding protein) (260) regulated by other kinases (e.g. protein kinase A, p38 mitogen-activated protein kinase) (255).

Activating protein-1 is a heterogeneous group of dimeric transcription factors consisting of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), or activating transcription factor (ATF2, ATF3, B-ATF) proteins. Unlike nuclear factor- $\kappa$ B, activating protein-1 activity is regulated via an increase in transcription of Jun and Fos genes as well as by post-translational phosphorylation of Jun and Fos proteins by mitogen-activated protein kinases, protein kinase A, and protein kinase C. Phosphorylation alters the DNA-binding capacity of the proteins and modulates their transcriptional activity (464).

There are two important points to understand when considering the activation of transcription factors and the functional outcome (i.e. what genes are transcribed and the level of transcription). First, in the case of activating protein-1 and nuclear factor- $\kappa$ B, there are multiple points in the activation cascade that can be under redox control and ROS generation may be required at different sites within the cell (210). Second, it is possible that a single stimulus will cause the activation of several signaling pathways and activate several different transcription factors. For example, receptor binding of TNF- $\alpha$  (TNF receptors 1 and 2) or lipopolysaccharide–TLR interaction can induce activation of both nuclear factor- $\kappa$ B and activating protein-1 (260). Furthermore, the regulatory elements (promoter and enhancer) of most genes contain binding sites for several transcription factors, whose combined activities will ultimately determine gene transcription (255).

### Presence and role of redox-sensitive transcription factor activation in periodontal disease

The published literature on nuclear factor- $\kappa$ B alone has grown exponentially since its discovery some 15 years ago. Its potential importance in periodon-

titis has been alluded to in a review on activation of nuclear factor- $\kappa$ B in atherogenesis, where it has been suggested that ROS production and oxidation of low-density lipoprotein is a major etiological factor in fatty streak formation (294). Therefore, this section will review recent literature specifically investigating the presence and role of the redox-sensitive transcription factors activating protein-1 and nuclear factor- $\kappa$ B in periodontal disease and/or concerning responses of cells normally found within the periodontium to plaque-associated components. Cells directly involved in innate or specific immunity (i.e. neutrophils, macrophages, dendritic cells, and lymphocytes) will not be included because the available data would constitute several reviews in their own right. Furthermore, the accumulating body of evidence linking both bone resorption and cartilage damage to activation of nuclear factor- $\kappa$ B and activating protein-1 via the receptor activator of nuclear factor- $\kappa$ B (RANK) and its ligand (RANKL) will not be included because it has been reviewed elsewhere (290, 405). Suffice to say that current data demonstrate an important role for nuclear factor- $\kappa$ B signaling pathways in the induction of osteoclasts and osteoclastic activity by lipopolysaccharide and receptor activator of nuclear factor- $\kappa$ B ligand both *in vitro* and *in vivo* (213, 466). Furthermore, these studies indicate that bone resorption can be inhibited by a synthetic peptide that interferes with the I $\kappa$ B-kinase complex (213) and a naturally occurring nuclear factor- $\kappa$ B inhibitor found in medicinal herbs (466). Such findings are not only important for our understanding of the mechanisms controlling bone resorption in periodontitis but also indicate exciting new potential therapies.

To our knowledge there appears to be only one report on the presence and distribution of nuclear factor- $\kappa$ B in gingival tissues in health and disease (16). This immunohistological study indicates a higher incidence of nuclear nuclear factor- $\kappa$ B (p50 and p65) staining in the suprabasal layers of epithelium (site unspecified) in the gingiva from patients (87.5% positive) compared to controls (17.5% positive). Conversely, the level of cytoplasmic I $\kappa$ B staining was lowest in tissues from patients (5% of specimens were positive compared to 35% of control specimens). The high rate of control samples found to be negative for I $\kappa$ B suggests a sensitivity or antigen retrieval problem, as I $\kappa$ B should be present within the cytoplasm of cells within all control specimens (i.e. it is only degraded when nuclear factor- $\kappa$ B is activated). Nuclear fac-

tor- $\kappa$ B reactivity was also found in cells within the lamina propria and although not stated, presumably associated with inflammatory cells. These results are similar to those found in lichen planus (oral and cutaneous) (359) and suggest that periodontitis is associated with epithelial activation of nuclear factor- $\kappa$ B.

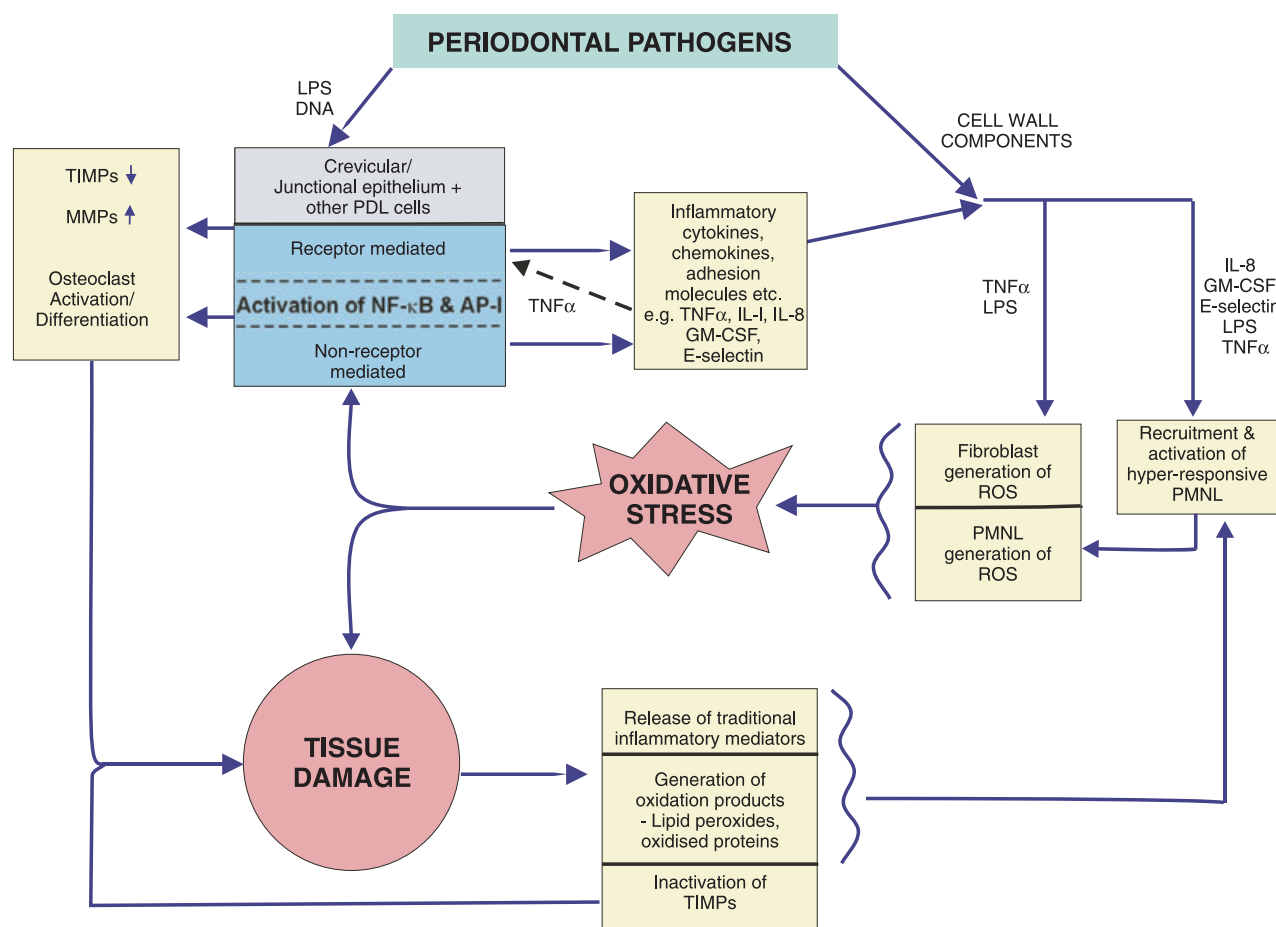
That activation of nuclear factor- $\kappa$ B in crevicular and junctional epithelial cells that are adjacent to plaque containing periodontal pathogens is likely is supported by *in vitro* experiments on the responses of oral epithelial cells (primary cultures and cell lines). Experiments on oral epithelial cell lines demonstrated epithelial cell activation of nuclear factor- $\kappa$ B, probably via TLR-2, and IL-8 production after challenge with *S. aureus* peptidoglycan, *P. gingivalis* sonic extract, *P. gingivalis* fimbriae, a fimbrial peptide and *N*-acetylmuramyl-L-alanyl-D-isoglutamine (a common component of peptidoglycans in parasitic bacteria) (21, 238). Interestingly, infection of primary oral keratinocyte cultures with *P. gingivalis* resulted in up-regulation of c-Jun kinase (JNK) and down-regulation of protein kinase-ERK1/2, no activation of nuclear factor- $\kappa$ B and lack of IL-8 secretion (100, 441). Furthermore, stimulation of primary oral epithelial cell cultures with a cell wall extract of *F. nucleatum* results in TNF- $\alpha$  production, via activation of nuclear factor- $\kappa$ B, and  $\beta$ -defensin-2 production via activation of activating protein-1 (237). These results illustrate the point that crevicular/junctional epithelium can have different and/or multiple responses to stimuli dependent upon their nature. A bacterial extract, cell wall component or whole dead bacterial cells can all cause different epithelial cell responses to each other, none of which may be the same as that induced by the living organism.

In addition to the studies on epithelial cells, there are a number of reports investigating the transcription factor responses of periodontal ligament cells and gingival fibroblasts to periodontal bacterial challenge. Periodontal ligament cells appear to respond, using the nuclear factor- $\kappa$ B pathway, to stimulation with major surface proteins of oral spirochetes (*Treponema* spp.) and *A. actinomycetemcomitans* lipopolysaccharide to produce IL-6, IL-8, intercellular adhesion molecule-1 (241), and active matrix metalloproteinase-2 (412), respectively. Other studies have demonstrated that gingival fibroblasts produce IL-6 and TNF- $\alpha$  in an nuclear factor- $\kappa$ B-mediated response to a lipoprotein extract of *Tannerella forsythia* involving TLR-2 (195), similar to the epithelial cell responses mentioned previously.

By contrast, *P. gingivalis* lipopolysaccharide appears to cause activation of both activating protein-1 and nuclear factor- $\kappa$ B pathways in gingival fibroblasts, via CD14 and TLR-4, and production of inflammatory cytokines (439). As indicated earlier, activation of activating protein-1 and nuclear factor- $\kappa$ B can be the result of a variety of stimuli, including products whose genes are controlled by the transcription factors themselves (e.g. IL-1, TNF- $\alpha$ ). Thus, cytokines produced by overlying epithelium in response to bacteria, might be important in stimulating cells such as fibroblasts and endothelial cells deeper in the tissues. For example, IL-1 $\beta$  can stimulate collagenase gene expression and protein production via activation of activating protein-1 in gingival fibroblasts (188). In such a situation, collagen degradation would be the product of two different receptor–ligand interactions in two physically separated cell types both using redox-sensitive signaling pathways. It seems possible therefore, that antioxidant therapy to

prevent collagen degradation in this example could act at two different sites, namely within the tissues or within the gingival crevice.

Similar stimulation studies have been performed using human umbilical vein endothelial cells to investigate the potential responses of the periodontal vasculature to infection with *P. gingivalis* (84, 230, 436). All three studies demonstrated activation of nuclear factor- $\kappa$ B and production of inflammatory chemokines, adhesion molecules, and osteoprotegerin. The latter study demonstrated that *P. gingivalis* stimulated endothelial cell production of monocyte chemoattractant protein-1 was associated with activation of both activating protein-1 and nuclear factor- $\kappa$ B. Interestingly, pretreatment of the cells with *N*-acetyl-L-cysteine (10 mM) or GSH (10 mM) significantly reduced monocyte chemoattractant protein-1 gene expression and protein production. Treatment with a NADPH oxidase inhibitor reduced gene and protein expression to below unstimulated baseline



**Fig. 13.** Simplified diagram illustrating a central role of ROS in generating chronic inflammation and tissue damage in response to periodontal pathogens. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; NF- $\kappa$ B, nuclear factor kappa B; AP-1,

activating protein-1; PDL, periodontal ligament; TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; ROS, reactive oxygen species.

levels (i.e. ROS required for redox signaling was 'neutralized', not allowing efficient signaling to occur (Fig. 12). These results indicate the potential for using antioxidants to inhibit receptor-mediated activation of redox-sensitive transcription factors in disease.

That excessive endogenous ROS, produced by hyperresponsive neutrophils or fibroblasts (see 'ROS production by neutrophils and other cells in periodontal disease'), within the periodontal tissues could activate activating protein-1 and nuclear factor- $\kappa$ B with the resulting pro-inflammatory and tissue damaging effects seen in periodontal disease has not been shown. However, that such events *could* occur as a result of such oxidative stress (Fig. 13) and might be inhibited by thiol compounds is amply supported by the literature (e.g. refs 2, 31, 137, 138, 368). Similarly, exogenous factors, such as smoking, could lead to oxidative stress within periodontal tissues and initiate or potentiate tissue damage, especially as nicotine, at physiological levels ( $0.8 \mu\text{M}$ ), has been shown to activate protein kinase-ERK-2 in lung cancer cells (198) and cause ROS production and nuclear factor- $\kappa$ B activation in a colon adenocarcinoma cell line (95).

## Evidence for the presence and role of antioxidants in periodontal tissue protection/repair

The traditional view of the strategic importance of antioxidants to the maintenance of cell and tissue homeostasis and viability has been related to their ability to prevent and repair ROS-mediated damage. While this remains a vitally important property, defining antioxidants by this role alone is a dated concept. The role of antioxidants in controlling redox-regulated gene transcription is likely to be as important, or indeed more so, to the pro- or anti-inflammatory status of cells and tissues. Indeed, where there is a proven and substantive role for ROS in the pathogenesis of a disease, in particular diseases associated with 'hyper-inflammation' such as inflammatory lung diseases, neurodegenerative diseases, type 2 diabetes, cardiovascular disease, adverse pregnancy outcome, rheumatoid arthritis, and periodontitis, the impact of antioxidant-regulated gene transcription factors is likely to be of paramount importance (see above).

When considering antioxidant approaches to the management of (periodontal) diseases a number of factors must be considered (Box 1):

### Box 1. Factors to account for when contemplating antioxidant approaches to therapy

1. There should be evidence of excess ROS production associated with the presence of disease (ideally locally to the diseased tissues);
2. There should be evidence of ROS-mediated tissue damage either by:
  - (a) Direct effects of ROS activity (biomarkers) measured locally;
  - (b) Indirect effects of ROS activity via hyper-inflammation as a result of local redox-sensitive transcription factor activity and subsequent imbalances of pro- and anti-inflammatory cytokine behavior;
3. There should be clear mechanistic links between oxidative stress, the observed tissue damage and the mode of activity of the candidate antioxidant;
4. Supplementation with the antioxidant should reduce the incidence of disease at the affected site or tissues;
5. Supplementation with the antioxidant should reduce disease recurrence;
6. Subjects with the disease should have a demonstrable local deficiency of the antioxidant, or of total antioxidant capacity;
7. Subjects without disease should have no antioxidant deficiency;
8. Restoration of the antioxidant level locally should improve clinical measures of disease;
9. Adjunctive use of the antioxidant with traditional therapies should provide improved treatment outcomes over non-surgical therapy alone;
10. Markers of local ROS activity should decrease with antioxidant therapy.

*To consider prophylactic use of antioxidant in periodontitis patients criteria 1–5 should be satisfied.*

*To consider use of antioxidants in active periodontal therapy criteria 5–10 should be satisfied.*

## Association studies

Association studies are important to help establish relationships between disease and a particular risk factor, but they are limited because they do not allow conclusions to be drawn concerning 'causation'. Longitudinal studies help to establish temporal relationships between the occurrence of a disease and a

particular risk factor, but ultimately intervention studies enable us to determine the value of a particular therapeutic approach. Studies that have explored associations between individual and total antioxidant activities and periodontitis are summarized in Table 9. It is important when interpreting these data, to appreciate:

- the likelihood of reporter bias, i.e. negative associations are not reported as frequently as positive associations;
- the interpretation of the study outcome with respect to the biological fluid/tissue investigated. As discussed earlier, the antioxidant profiles of different body compartments vary considerably and deficiencies in plasma antioxidants may have very different implications to deficiencies within periodontal tissues, where local ROS activities predominate. Gingival crevicular fluid studies should be regarded as significantly more pertinent to periodontal disease pathogenesis than those involving saliva and ideally studies involving gingival biopsies should carry the greatest weight. However, given the ethical implications of collecting controlled biopsy samples, the difficulties associated with choice of biopsy site and the problems linked to tissue handling to prevent oxidation of labile antioxidant species, some caution must also be exercised in the interpretation of data from biopsies.

### Studies of plasma antioxidant status

Early studies of individual antioxidant micronutrients were unconvincing in their associations between dietary antioxidant intake and periodontitis, (11, 67, 352, 435). However, many of these studies relied upon dietary questionnaires rather than plasma biochemistry and questionnaires were less sophisticated than those currently in use, which have been extensively validated for major epidemiological studies (e.g. National Health and Nutrition Examination Surveys – NHANES in the U.S.A., National Diet and Nutrition Survey in the UK – NDNS). Epidemiological and case-control studies (426) support the finding that serum vitamin C intake is reduced in periodontitis subjects, in particular in smokers (299) and older patients (15). Furthermore, it has been suggested that vitamin C deficiency is an etiological factor contributing to periodontal disease in diabetes (10). In order to determine whether serum antioxidant concentrations were associated with altered relative risk for periodontitis, we employed multiple logistic regression for dual case definitions (both mild and severe disease) of periodontitis in an analysis of 11,480 NHANES III adult participants (> 20 years) (81). Mild disease (1567 cases)

was defined according to the criteria of Tomar and Asma (414) and severe disease (609 cases) as  $\geq 2$  mesiobuccal sites with clinical attachment loss of  $\geq 5$  mm and one or more mesiobuccal sites with probing depth of  $\geq 4$  mm (modified from CDC Working Group proposal). Serum concentrations of vitamin C, bilirubin and total antioxidant capacity (TAOC) were inversely associated with periodontitis, the association being stronger in severe disease. Vitamin C and TAOC remained protective in never-smokers. In the subpopulation of never-smokers the protective effect was more pronounced, 0.38 (0.26, 0.63, vitamin C) and 0.55 (0.33, 0.93, TAOC). When re-analysis was performed for never smokers, significant inverse relationships only remained for vitamin C and bilirubin. In summary, the few studies that have explored individual antioxidant scavengers in serum or plasma have shown only mildly compromised levels in periodontitis subjects relative to healthy controls, except where smoking is a co-factor, where derived TAOC and vitamin C levels in particular are further compromised.

Only three studies to our knowledge have investigated total antioxidant capacity in serum/plasma from periodontitis patients and controls and one of those was in dogs (316). All demonstrated significantly lower total antioxidant capacity in serum and plasma samples from periodontitis subjects (58, 83, 316). Brock et al. (58) found that the reduced serum total antioxidant capacity concentration in periodontitis did not quite reach significance, whereas differences in plasma levels did, which may reflect differences in preparation methods for serum and plasma (serum is prepared at higher centrifugal forces and is more prone to oxidation) or the effects of clotting factor removal, or indeed relatively small sample numbers. Interestingly, plasma total antioxidant capacity was significantly lower than serum total antioxidant capacity and this was found to be entirely the result of the lower plasma total antioxidant capacity in the periodontitis subjects relative to paired serum samples. Panjamurthy et al. (314) found lower plasma vitamin C, vitamin E, and GSH in periodontitis patients even after adjusting for protein levels, whereas antioxidant enzyme levels were raised, the authors attributing this to a protective response to oxidative stress (thiobarbituric acid reactive substance levels were raised in periodontitis subjects). In contrast, Sobaniec and Sobaniec-Lotowska (388) found that serum antioxidant enzyme levels were lowered during ligature-induced periodontitis in a rat model. Interestingly, Pussinen et al. (331) found an inverse relationship between serum vitamin C concentrations and antibody levels to *P. gingivalis*.

**Table 9.** Association studies investigating individual antioxidants and total antioxidant capacity and periodontal disease

Biological samples	Reference	Study design	Subject numbers	Outcome
<i>Studies on serum/plasma</i>				
Serum vitamin E levels and periodontal disease	Slade et al. 1976 (385)	Case-control study	12 periodontitis cases and 12 controls	No difference in serum vitamin E levels between cases and controls
Gingival crevicular fluid vitamin C levels in health	Meyle and Kapitzka 1990 (269)	Convenience sample	21 healthy volunteers	GCF vitamin C levels at healthy sites in healthy volunteers (207.3 µM) > plasma (72 µM)
Prevalence of pocketing and periodontal disease in patients with low and high plasma vitamin C levels	Vaananen et al. 1993 (426)	Case-control study	75 dentate subjects with low vitamin C ( $\leq 25$ µM) and 75 matched controls with plasma vitamin C > 50 µM	↑ prevalence of pockets > 4 mm in cases (60%) vs. controls (37%). 5% of cases and 18% of controls had healthy periodontal tissues
Plasma vitamin C levels and <i>P. gingivalis</i> serology	Pussinen et al. 2003 (331)	Dual population study (Finland and Russia)	431 males	-ve correlation between <i>P. gingivalis</i> antibody levels and vitamin C concentration
Serum vitamin C levels in elderly Japanese and periodontitis	Amarasena et al. 2005 (15)	Population study	413 subjects	Inverse relationship between serum vitamin C and clinical attachment loss
Ligature-induced periodontitis and AO enzymes	Sobaniec and Sobaniec-Lotowska 2000 (388)	Animal model	Study in rats	Reduced superoxide dismutase, glutathione peroxidase, glutathione reductase and increased malondialdehyde in serum
Co-enzyme Q and vitamin E and hydroperoxide levels in an extended Papillon-Lefèvre syndrome family	Battino et al. 2001 (40)	Case report	1 extended family	Proband had low co-enzyme Q and α-tocopherol (mother had low co-enzyme Q) and highest hydroperoxide levels were in proband and mother
Co-enzyme Q and vitamin E and hydroperoxide levels in a Papillon-Lefèvre syndrome family	Battino et al. 2003 (42)	Case report	1 family group	Raised hydroperoxide levels, lowered antioxidant levels, altered co-enzyme Q and α-tocopherol levels. Low polyunsaturated fatty acid and high mono-saturated fatty acid levels
Polymorphisms in glutathione-S-transferase gene	Kim et al. 2004 (226)	Case-control study	115 periodontitis and 126 controls	Increased risk among glutathione-S-transferase-M1 <sup>+</sup> smokers for periodontitis (odds ratio 3.1, CI 1.5-6.6) & near significant moderate risk among M1 <sup>+</sup> non-smokers (odds ratio 1.8, CI 1.0-3.1)
Total antioxidant capacity of plasma periodontitis and controls	Chapple et al. 2002 (83)	Case-control pilot (non-smokers)	10 advanced periodontitis cases and 10 controls	Periodontitis subjects had lower plasma total antioxidant capacity than healthy controls



**Table 9.** Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
Total antioxidant capacity of plasma and serum periodontitis and controls	Brock et al. 2004 (58)	Case-control study (non-smokers)	17 periodontitis cases and 17 controls	Higher serum and plasma total antioxidant capacity for healthy controls than periodontitis cases, only significant for plasma
Total antioxidant capacity of serum in dogs	Pavlica et al. 2004 (316)	Cohort study in dogs	41 dogs	Total antioxidant capacity in periodontitis was lower than in health/gingivitis. -ve correlation between total antioxidant capacity and periodontal parameters
Antioxidant-enzyme and scavenger levels and thiobarbituric acid reactive substance	Panjamurthy et al. 2005 (314)	Case-control study	25 periodontitis subjects and 25 controls	Periodontitis patients had ↑ thiobarbituric acid reactive substances and enzyme antioxidant levels. However, scavenging antioxidants were lower in periodontitis subjects vs. controls
<i>Saliva studies</i>				
Uric acid correlates with total antioxidant capacity of saliva	Meucci et al. 1998 (268)	Cohort study	Non-periodontitis subjects pre- and post-dialysis	Uric acid concentration of saliva in hemodialyzed patients correlates with saliva total antioxidant capacity
Effect of smoking single cigarette on salivary GSH	Zappacosta et al. 2002 (470)	Convenience sample of smokers	20 volunteer smokers	Smoking single cigarette compromises protective role of glutathione against smoke-derived chemicals (smoke-derived aldehydes)
Saliva total antioxidant capacity in periodontitis and health	Moore et al. 1994 (281)	Case-control study	7 periodontal disease and 28 control subjects	Total antioxidant capacity of saliva does not appear compromised in periodontal disease patients. Uric acid contributes 70% of total antioxidant capacity of saliva
Pilot study of saliva total antioxidant capacity in periodontitis	Chapple et al. 1997 (82)	Case-control pilot study	18 periodontitis cases and 16 controls	Saliva total antioxidant capacity (concentration) reduced in cases vs. controls. However, no difference in production of total antioxidant capacity ( $\mu\text{M}/\text{min}$ ) between groups
Saliva total antioxidant capacity	Sculley and Langley-Evans 2003 (370)	Cohort study – periodontal health as continuous variable and logistic regression used	129 patients	Total antioxidant capacity lower in women than men. Periodontitis patients had lower total antioxidant capacity ( $\mu\text{M}$ ), flow rates and higher protein carbonyls (oxidative stress) than controls
Total antioxidant capacity in stimulated saliva health vs. periodontitis	Diab-Ladki et al. 2003 (111)	Case-control study	17 periodontitis cases and 20 controls	Total antioxidant capacity significantly decreased in patients vs. controls but albumin, uric acid and ascorbic acid no different between groups

**Table 9.** Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
Total antioxidant capacity in stimulated and unstimulated saliva periodontitis vs. health	Brock et al. 2004 (58)	Case-control study	17 periodontitis cases and 17 controls	Total antioxidant capacity of saliva (concentration or rate of production) no different between cases and controls
<i>GCF AO Studies</i>				
Lactoferrin and transferrin in GCF	Adonogianaki et al. 1994 (5)	21-day experimental gingivitis study	6 healthy volunteers	PMNL-derived lactoferrin increases with inflammation
Vitamin C and vitamin E levels – effect of smoking	Seri et al. 1999 (374)	Cohort study – non-periodontitis subjects	41 students (16 non-smokers and 25 smokers)	Vitamin C reduced in smokers and vitamin A non-significantly lower. No differences in vitamin E
Glutathione levels in periodontitis and health	Chapple et al. 2002 (83)	Case-control pilot (non-smokers)	10 advanced periodontitis cases and 10 controls	Reduced glutathione levels in GCF in millimolar range relative to $\mu\text{M}$ in serum. GCF levels of reduced, oxidized and total glutathione lower in periodontitis patients than controls
Glutathione-peroxidase levels in periodontitis and health	Huang et al. 2000 (202)	Cohort study	23 patients with periodontitis	Glutathione peroxidase levels negatively correlated with pocket depth and CAL. Glutathione peroxidase increased with periodontal therapy
Superoxide release and AO capacity in GCF of periodontitis subjects and controls	Guarnieri et al. 1991 (163)	Case-control study	14 periodontitis patients and 16 controls	Spontaneous release of superoxide in GCF of periodontitis subjects. No difference in antioxidant capacity between patients and controls
Total antioxidant capacity of GCF in periodontitis and health	Brock et al. 2004 (58)	Case-control study	17 periodontitis cases and 17 controls	Total antioxidant capacity of GCF is qualitatively and quantitatively different from plasma and saliva. Total antioxidant capacity reduced in periodontitis vs. controls
Total antioxidant capacity of GCF in dogs	Pavlica et al. 2004 (316)	Cohort study in dogs	41 dogs	Total antioxidant capacity in periodontitis was lower than in health/gingivitis. Negative correlation between total antioxidant capacity and periodontal parameters
Glutathione peroxidase and lactoferrin in GCF in periodontitis and health	Wei et al. 2004 (445)	Case-control	19 periodontitis subjects and 8 controls	Glutathione peroxidase and lactoferrin levels higher in periodontitis vs. controls
Superoxide dismutase activity in GCF and gingival tissues in periodontitis patients and controls	Akalin et al. 2005 (7)	Case-control study	26 periodontitis subjects and 16 controls	No differences in GCF superoxide dismutase activity in patients vs. controls

Table 9. Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
<i>Biopsy studies</i>				
Glutathione levels in periodontal disease	Giorgi et al. 1992 (152)	Case-control study	18 marginal gingivitis subjects and 16 controls	Showed higher glutathione in tissues of gingivitis subjects vs. controls
Metallothionein levels in smokers and non-smokers with advanced periodontitis	Katsuragi et al. 1997 (218)	Cohort study	33 patients with advanced periodontitis, 22 smokers and 11 non-smokers	Metallothionein (a scavenging and preventative antioxidant) levels increased in the gingivae of smokers vs. non-smoker periodontitis patients
Superoxide dismutase and catalase levels	Ellis et al. 1998 (126)	Cohort study	44 patients scheduled for extractions	Showed that superoxide dismutase and catalase levels decrease in tissues as pocket depth increases
Heme-oxygenase-1 expression in gingival fibroblasts	Chang et al. 2005 (78)	Cohort study	20 periodontitis patients (10 smokers and 10 non-smokers)	Heme-oxygenase-1 antioxidant enzyme levels were higher in smokers with periodontitis vs. non-smokers
Antioxidant-enzyme and scavenger levels and thiobarbituric acid reactive substance	Panjamurthy et al. 2005 (314)	Case-control study	25 periodontitis subjects and 25 controls	Periodontitis patients had higher thiobarbituric acid-reactive substance and enzyme antioxidant levels. However, scavenging antioxidants were lower in periodontitis subjects vs. controls
Superoxide dismutase in GCF and gingival tissues in periodontitis patients and controls	Akalin et al. 2005 (7)	Case-control study	26 periodontitis subjects and 16 controls	Superoxide dismutase activity in patients' tissues significantly higher than controls
<i>Dietary AO intake studies</i>				
Ascorbate intake and periodontal disease in the U.S.A.	Ismail et al. 1983 (206)	Case-control study	NHANES I	Intake of ascorbic acid above RDA did not seem to be associated with improved periodontal health
Dietary vitamin C intake and risk for periodontal disease	Nishada et al. 2000 (299)	Case-control study	NHANES III – multiple logistic regression	Dietary vitamin C intake showed weak but significant inverse relationship with periodontitis (clinical attachment loss) in current and former smokers
Serum carotenoid (Lycopene) levels and mean tomato intake (source of Lycopene) in periodontitis and congestive heart failure	Wood and Johnson 2004 (455)	Population study	NHANES III – various statistical methods	Relationship found between periodontitis and congestive heart failure but high tomato intake reduces relative risk of heart failure in periodontitis subjects
Oral morbidity in older adults and impaired diet	Bailey et al. 2004 (34)	Cohort study	22 with oral problems and 125 without	Increased oral morbidity with lower intakes of vitamins A and B <sub>6</sub>
<i>Other AO studies</i>				
Antioxidant activity from tannins isolated from <i>Vaccinium vitis-idaea</i> L.	Ho et al. 1999 (200)	<i>In vitro</i> study	6 tannins	Scavenging and anti-superoxide activities demonstrated for tannins from <i>Vaccinium vitis-idaea</i> L.

GCF, gingival crevicular fluid; NHANES, National health and nutrition examination survey; PMNL, neutrophilic polymorphonuclear leukocyte (neutrophil).

Overall, the balance of evidence supports an antioxidant compromise in the plasma of periodontitis patients, but whether this reflects a response to the demonstrated peripheral blood neutrophil hyperreactivity (see earlier) or is the result of reduced intake of dietary antioxidants, malabsorption or metabolic compromise because of polymorphisms in key redox-regulating enzymes (226) remains unclear. Furthermore, the biological relevance of such a peripheral antioxidant compromise to the antioxidant-defense systems within the periodontal tissues themselves is unknown. Changes in antioxidant enzyme systems in plasma are likely to lack relevance or significance given their low concentrations and rates of activity, relative to the antioxidant scavengers.

Battino et al. have reported abnormally high hydroperoxide levels and compromises in serum co-enzyme Q10 and vitamin E in Papillon-Lefèvre syndrome subjects, suggesting substantial oxidative stress in these subjects and a potential role for specific antioxidant therapies (40, 42). Interestingly, retinoids have been used for managing hyper-keratoderma in Papillon-Lefèvre syndrome (402) and retinol has been shown to correct defective CD3-induced human T-lymphocyte activation *in vitro* in Papillon-Lefèvre syndrome patients (13). Retinoids may directly regulate cathepsin-C (*CTSC*) gene expression as retinoic acid response elements have been identified in cathepsin-C gene promoter regions (338). This also raises the possibility of dietary modification as a potential treatment strategy in Papillon-Lefèvre syndrome.

### Studies of salivary antioxidant capacity

Studies of salivary antioxidants (Table 9) require careful interpretation because methods of saliva collection differ and there is confusion over what represents 'unstimulated saliva'. Saliva stimulation may result from physical stimulation of minor or major glands by food, speech, and muscle activity or by sensory stimulation mediated via the parasympathetic nervous system (via smell, taste, thought, etc.) The long held view of 'unstimulated' saliva collection within the field of oral biology/physiology, is that the patient should be seated in a dark room at constant temperature, with no external visual or audible stimulation and the saliva should be collected by drooling (no use of oral musculature). This is frequently ignored today and the majority of 'unstimulated' saliva samples are in fact stimulated and their composition is likely to be radically different because of the stimulation process. It is essential to

determine saliva flow rates and to express delivery of antioxidants per unit time as well as overall concentrations. Our group illustrated this point in a pilot study, where differences were found between periodontitis cases and controls in salivary total antioxidant capacity expressed in  $\mu\text{M}$  (lower in cases) but no differences were found in total antioxidant capacity-delivery ( $\mu\text{M}/\text{min}$ ) (82). Stimulation increased the release of antioxidants per unit time, but the concentration was lowered by stimulation because of dilution effects. Other variables include whole/mixed saliva vs. individual gland secretions and minor gland secretions.

Data on salivary total antioxidant capacity are conflicting. Moore et al. (281) were the first to explore salivary 'total capacity and concentration' and found no differences between cases and controls. However, only seven periodontally diseased patients were examined and one of the criteria for disease diagnosis was the fact that patients had been referred by their general practitioner to a specialist unit. Healthy patients were not defined and the authors acknowledged that some 'healthy' subjects had gingival inflammation. Nevertheless, Brock et al. (58) found no differences between saliva total antioxidant capacity expressed as  $\mu\text{M}/\text{l}$  and as  $\mu\text{M}/\text{min}$  in a tightly controlled case-control study of non-smokers (though there was a trend towards higher values in healthy subjects), using an enhanced chemiluminescence assay. The data conflicted with those from an earlier study by the same group (82), but the latter study had not stratified subjects for smoking habit. Both Chapple et al. (82) and Brock et al. (58) found that total antioxidant capacity delivery ( $\mu\text{M}/\text{min}$ ) increased with stimulation of saliva flow, but that total antioxidant capacity concentration ( $\mu\text{M}/\text{l}$ ) decreased. A larger cohort study by Sculley and Langley-Evans (370) found lower total antioxidant capacity (by ferric reducing ability of plasma assay) delivery rates in stimulated saliva samples from women than men and also in periodontitis subjects vs. controls. The gender differences were confirmed by Brock et al. (58) for saliva and also for serum and plasma total antioxidant capacity. In the Sculley and Langley-Evans study, women had significantly lower saliva flow rates than men, which may explain the gender-specific differences in total antioxidant capacity. Urate levels were also lower in women than men, which given the large contribution of urate to saliva total antioxidant capacity (281) may also contribute to the lower overall salivary total antioxidant capacity in females. The categorization of disease in this study was obscure and not in line with current systems. The ter-

tiles of CPITN scores were used to grade disease; the authors stated desire being to consider periodontal health as a continuous variable. However, oxidative stress measured by protein carbonyl assay was higher in the diseased patients than the healthy subjects, consistent with their total antioxidant capacity data. Diab-Ladki et al. (111) found similar results to Sculley and Langley-Evans (370) in a small case-control study, the lower saliva total antioxidant capacity in periodontitis subjects being independent of salivary uric acid, ascorbate and albumin levels, which did not differ between groups.

Moore et al. (281) determined that the predominant antioxidant component of saliva was uric acid (> 70% of antioxidant activity), data that are consistent with subsequent reports (268) and also with data for serum antioxidant components. Chapple et al. (83) and Brock et al. (58) demonstrated that gingival crevicular fluid total antioxidant capacity was qualitatively and quantitatively different from saliva and plasma and confirmed earlier findings that GSH was the most dominant radical scavenger in gingival crevicular fluid (79, 82). They also demonstrated that salivary total antioxidant capacity was lower than paired serum, plasma and gingival crevicular fluid samples. Interestingly, Zappacosta et al. (470) demonstrated that smoking a single cigarette compromised the total glutathione content of saliva and emphasized the importance of glutathione in protection against cigarette smoke-derived toxins. Tsai et al. (416) investigated glutathione concentrations in saliva in an association study ( $n = 22$ , Table 9) and also assessed the effect of stage-1 therapy at 1-month post-treatment on 21 subjects with periodontitis. They discussed 'GSH' levels, but their assay actually measured total glutathione and not GSH. They found that salivary glutathione concentrations were significantly reduced in periodontitis subjects relative to controls and that treatment increased glutathione concentrations. These data are very difficult to interpret because saliva flow rates were not assessed and differential levels of GSH and GSSG were not reported.

Overall the relevance of saliva as a medium for assessing surrogate markers of reactive oxygen and antioxidant species in periodontitis patients must be open to question, given its microbiological and constitutional differences from gingival crevicular fluid. Moreover, saliva contains gingival crevicular fluid and the contribution of gingival crevicular fluid antioxidants to saliva will vary according to the degree of saliva stimulation (83).

### Studies of gingival crevicular fluid antioxidant capacity

Only three studies have investigated gingival crevicular fluid total antioxidant capacity in periodontitis subjects, two in humans (58, 163) and one in poodle dogs (316). Guarnieri et al. (163) demonstrated spontaneous generation of superoxide in the gingival crevicular fluid of periodontitis subjects, but found no differences in antioxidant scavenging capacity between cases and controls. However, they collected gingival crevicular fluid samples by a crevice washing method, which oxygenates samples and the samples were stored at  $-20^{\circ}\text{C}$ , conditions shown to result in the rapid loss of scavenging antioxidants (82). Brock et al. (58) demonstrated a significantly lower total antioxidant capacity (by their enhanced chemiluminescence assay) in periodontitis subjects relative to age- and sex-matched controls. Samples were collected as fasting samples and stored under liquid nitrogen before assay. Gingival crevicular fluid total antioxidant capacity was significantly greater than in paired serum and plasma samples in healthy subjects, but this difference was not seen in periodontitis subjects. Moreover, there was no gender bias in gingival crevicular fluid total antioxidant capacity, unlike paired saliva and serum/plasma samples, reflecting substantial differences in the gingival crevicular fluid compartment relative to salivary or plasma compartments. Pavlica et al. (316) confirmed the above data in their study of miniature poodle dogs and found significant negative correlations between serum and gingival crevicular fluid total antioxidant capacity and gingival inflammation.

Lactoferrin levels in gingival crevicular fluid have been shown to be increased in periodontitis (5, 445) as indeed have glutathione peroxidase levels (445). However, Huang et al. (202) found that within periodontitis subjects, glutathione peroxidase levels correlated negatively with pocket depth and attachment loss and increased post-therapy. Akalin et al. (7) investigated gingival crevicular fluid levels of superoxide dismutase in 26 periodontitis subjects and 16 controls and found no significant differences between groups. This is unsurprising given that superoxide dismutase is predominantly an intracellular antioxidant enzyme system, the very low extracellular levels (Akalin et al. (6) found levels 40-fold and 160-fold lower in gingival crevicular fluid than paired tissues from control and periodontitis subjects respectively) being regarded as of little significance to extracellular antioxidant defenses (182). ROS scavengers are more effective than preventative anti-

oxidant systems in extracellular tissues and fluids and we have demonstrated that GSH is the most important antioxidant in gingival crevicular fluid (83) with levels 1,000-fold higher than paired plasma samples and significantly reduced in periodontitis relative to matched control subjects. Seri et al. (374) demonstrated that gingival crevicular fluid vitamin C (and non-significantly vitamin A) levels were reduced in periodontally healthy smokers, with no differences detected for vitamin E.

Gingival crevicular fluid is the most appropriate fluid to sample when investigating periodontal status, because it passes through the tissues and accumulates biomarkers of tissue events. Data from gingival crevicular fluid support the conclusion that local antioxidant scavenging defenses are compromised in periodontitis, but whether this represents a predisposition to disease or results from the inflammatory lesion, requires longitudinal studies of periodontal therapy to be performed.

#### Studies of periodontal tissue antioxidant capacity

Only five studies of gingival biopsies have investigated antioxidant levels within the periodontal tissues. In 1992 Giorgi et al. (152) investigated what they termed 'GSH levels' in biopsies from gingivitis and healthy subjects. However, the assay used in this study only measured total glutathione and GSSG and such assays are inaccurate to determine GSH levels by subtraction unless the GSH:GSSG ratio is very high, otherwise the best that can be achieved is a non-specific measure of total glutathione. The details of tissue sampling and preparation were absent from their report and their data are therefore very difficult to interpret. Katsuragi et al. (218) investigated the radical scavenging and preventative antioxidant metallothionein in biopsy samples from 22 cigarette smokers and 11 non-smokers during periodontal flap surgery. They found increased levels of metallothionein in the tissues of smokers and concluded that this was a protective response to the increased inflammation in these patients' gingivae. Unfortunately, they did not measure any indices of oxidative stress to evaluate the balance of pro-radical and anti-radical activities within their tissue samples. Increases in the antioxidant enzyme heme oxygenase-1 were demonstrated in periodontitis subjects who smoked relative to non-smoking diseased controls (78), consistent with the theory of Katsuragi et al. By contrast Ellis et al. (126) demonstrated decreases in tissue levels of catalase and superoxide dismutase with increasing pocket depth in their cohort study. Most recently, an ambitious case-control study by

Panjamurthy et al. (314) demonstrated increases in oxidative stress (thiobarbituric acid reactive substances) and antioxidant enzymes in periodontitis tissues relative to healthy control tissues, but antioxidant scavengers were significantly lower in periodontitis than control tissues. A study of superoxide dismutase activity within gingival tissues confirmed these data, with significantly higher activity found in the tissues of periodontitis subjects relative to that of control subjects (7).

Biopsy studies are difficult to implement for ethical and technical reasons, but the limited data so far confirm the presence of more significant oxidative stress in periodontitis tissues relative to control tissues and the apparent up-regulation of antioxidant enzyme systems. The latter are largely insignificant in their efficacy in the extracellular environment and this is reflected in the finding that their concentrations reduced with increasing pocket depth. The most important radical scavenging systems appear reduced in periodontitis, consistent with gingival crevicular fluid data, but again the temporal relationship between these findings and the development and progression of the periodontitis lesions is unclear and requires intervention studies.

#### Intervention studies

While the medical literature has comprehensively evaluated the effects of individual antioxidant interventions in randomized controlled trials upon systemic diseases (cancer and ROS-mediated inflammatory diseases), the periodontal literature is deficient in such studies. Table 10 documents intervention studies performed on animal and human subjects as well as *in vitro* studies based upon cell culture and other model systems. Four abstracts are also discussed, given the paucity of data available, but these are not included in Table 10.

#### Vitamin C

Vitamin C has been investigated predominantly in relation to gingivitis, though studies have focused on subjects who were not deficient in vitamin C and the relatively small numbers of volunteers limit the power of these investigations. Studies of megadose supplementation of periodontally healthy and non-vitamin-C-deficient young subjects demonstrated no benefit from vitamin C supplementation on measures of gingival inflammation. Studies designed to provide periods of vitamin C depletion and repletion in periodontally healthy non-smokers have demonstrated beneficial effects upon gingival bleeding only,

**Table 10.** Intervention studies investigating antioxidants and antioxidant micronutrients in periodontal therapy

Biological samples	Reference	Study design	Subject numbers	Outcome
<i>Vitamin C studies</i>				
Ascorbic acid megadoses and effects upon clinical parameters/blood/gingival tissues	Woolfe et al. 1984 (456)	7 week follow-up, parallel design adjunctive vitamin C and non-surgical therapy	10 non-deficient subjects (5 supplemented and 5 controls)	No significant differences between groups suggesting no benefit from megadose vitamin C in non-deficient subjects
Ascorbic acid megadoses (twice RDA) in 21-day experimental gingivitis study	Vogel et al. 1986 (431)	Double blind parallel design during 4-week experimental gingivitis	24 periodontally healthy and non-deficient dental students. 2 phases with mega-dose vitamin C given in phase 2	Significant increases in plasma vitamin C in supplemented group. No differences in responses to experimental gingivitis
Ascorbic acid depletion and supplementation (alternating)	Leggott et al. 1986 (242)	3-month prospective – alternating 7-day diet for 3 months (cross-over)	11 healthy non-smoking males (19–28 years)	No effects on plaque or pocket depths. Gingival inflammation affected by ascorbate status, especially crevice bleeding (↑ with depletion and ↓ with replenishment)
Ascorbic acid normal and high doses vs. depletion – effects on gingivitis	Jacob et al. 1987 (207)	Cross-over dietary intake study	Male subjects	Normal and high ascorbate doses reduced gingival inflammation and crevice bleeding relative to deficient doses
Ascorbic acid depletion and replenishment (alternating)	Leggott et al. 1991 (243)	13-week prospective cross-over design. Used 6 index teeth only for assessment	12 healthy non-smokers (25–43 years). 32 days depletion and 8 of 12 completed 56-day replenishment. Periodontitis-free subjects	No changes in plaque accumulation pocket depth or CAL throughout. Gingival bleeding ↑ with ascorbate depletion and ↓ with replenishment. No change in specific periodontal pathogens ( <i>A. actinomycetemcomitans</i> , <i>P. gingivalis</i> , <i>B. forsythus</i> , <i>C. rectus</i> , <i>E. sputigena</i> , <i>E. corrodens</i> )
Effect of vitamin C on soluble ICAM-1, neopterin (monocyte activation marker) and PMNL elastase	Scott et al. 2005 (369)	Randomized double-blind placebo controlled trial. Not periodontally diseased	20 smokers and 20 non-smokers (age and sex-matched)	Soluble ICAM-1 elevated at baseline in smokers vs. non-smokers. No effect of vitamin C on circulating levels of sICAM-1, neopterin, leukocyte elastase
<i>Vitamin E studies</i>				
Effects of diets with varying vitamin E supplements on ligature-induced periodontitis in rats	Parrish et al. 1977 (315)	Parallel (3 groups) fed diet low, medium and high in vitamin E for 8 weeks, then 6 weeks of ligature-induced periodontitis	36 male adult albino rats	No differences histologically between groups in this acute model
Effect of vitamin E on gingival wound healing in rats	Kim and Shklar 1983 (225)	Parallel designed study of gingivectomy wound healing in rats	60 male/female Sprague-Dawley (albino) rats. 4 groups, gingivectomy ± vitamin E (n = 20 at 7 days each), no gingivectomy ± vitamin E (n = 10 each)	More rapid healing of gingivectomy wounds in supplemented rats with complete healing at 7 days

Table 10. Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
Effect of topical 5% vitamin E gel, placebo and chlorhexidine gel on plaque and periodontitis	Cohen et al. 1991 (91)	Parallel, double-blind controlled study with adjunctive scaling and root planing in 2 quadrants	48 adults	No differences detected between all three groups except for chlorhexidine, which reduced plaque significantly
Effect of stress, periodontal status and vitamin E on disease progression	Cohen et al. 1993 (90)	Longitudinal intervention study – parallel design	32 rice rats subjected to stress (test) or no stress (controls) and split between vitamin E high and low diets prior to stress	Vitamin E supplementation had significant protective effect against bone loss. Effect was most pronounced at sites most susceptible to bone loss
Effect of vitamin E and selenium on collagen breakdown in subcutaneous sponge granulation tissue model	Åsman et al. 1994 (27)	Parallel intervention study	2 Sprague–Dawley rats in each of 2 test and 1 control groups	The vitamin E/selenium combination reduced collagen degradation in experimental granulation tissue. Suggested protective effects against ROS-induced collagen degradation
Effect of vitamin E on protection of oral epithelial cell lines from ROS damage	Royack et al. 2000 (350)	<i>In vitro</i> parallel design with 5 groups	2 primary human oral epithelial cell lines	H <sub>2</sub> O <sub>2</sub> produced hydroxyl radicals and altered cell cycle. Vitamin E pre-treatment of cells lowered hydroxyl radical concentrations but no effect seen on cell cycle changes induced by hydroxyl radicals
<i>CO-enzyme Q10 studies</i>				
Effect of topical co-enzyme Q10 ± subgingival debridement on clinical outcome measures	Hanioka et al. 1994 (191)	Split mouth placebo-controlled intervention study	10 subjects – 20 test sites (co-enzyme Q10 application) and 10 controls (soyabean oil). All subjects had subgingival debridement	Significant improvements from debridement at all sites. Improved gingival redness and bleeding and lower bacterial peptidase activities at co-enzyme Q10 sites vs. controls
<i>Antioxidant enzymes</i>				
Effect of superoxide dismutase on measures of inflammation induced by peritoneal and foot pad injections of periodontal bacteria	Misaki et al. 1990 (272)	Controlled and parallel design intervention study	115 Wistar rats. 3 test groups (n = 54, 26, 26) and 1 control group (n = 9)	Model system in rats which demonstrated 'curative effect' of superoxide dismutase on <i>B. gingivalis</i> -induced inflammation and periodontal wound healing
Local delivery of liposome-encapsulated superoxide dismutase and catalase and ligature periodontitis in beagles	Petelin et al. 2000 (319)	Split-mouth design for 4 Tx modalities – supra-scaling and enzymes, supra- and sub-scaling and enzymes, scaling and root planing and enzymes, enzymes only	15 Beagle dogs. 3 groups of 5 dogs – grp 1 = superoxide dismutase, grp 2 = catalase and grp 3 = both agents in liposomes. 4 treatment strategies per group as listed in adjacent cell	Greatest suppression of inflammation, reduction in PPD and gain in attachment was with scaling and root planing + local superoxide dismutase administration. Also greatest bone formation by digital subtraction radiography in scaling and root planing + superoxide dismutase group (catalase provided no additional benefit over superoxide dismutase)



**Table 10.** Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
<i>Antioxidant mouthrinses and toothpastes</i>				
Effect of chlorhexidine on chemiluminescence from peripheral blood neutrophils	Goulttschin and Levy 1986 (157)	<i>Ex-vivo</i> study of neutrophils stimulated by polyhistidine $\pm$ chlorhexidine	Unspecified number of healthy volunteers	Non-toxic concentrations of chlorhexidine (0.1–1 $\mu$ g/ml) inhibited superoxide production by neutrophils
Effect of various mouthwashes on lipid peroxidation	Firatli et al. 1994 (135)	Studies on bovine brain extracts. <i>In-vitro</i> study on Malondialdehyde levels in presence Ox-brain extracts of different mouthwashes		Doxycycline had best antioxidant activity at low levels, but similar effects achieved with high levels of Sanguinarine and Listerine and to lesser extent for tetracyclines. No effects for chlorhexidine or cetyl-pyridium-chloride
Total antioxidant capacity of mouthrinses assessed in-vitro by cell-free and cell-dependent assay systems	Battino et al. 2002 (41)	<i>In-vitro</i> study of mouthrinse inhibition of ROS activity and DNA damage (comet assay)	12 commercially available mouthrinses and their individual components. Effects on HFL-1 fibroblast cultures	Listerine had greatest antioxidant activity with this assay system. Effects attributed to methylsalicylate content. No antioxidant effect of chlorhexidine in this system
Antioxidant effect of toothpastes with antioxidant additives in cell-free assay system and comet assay for effects on keratinocytes	Battino et al. 2005 (43)	<i>In vitro</i> study with cell free antioxidant assay and comet assay for keratinocyte cell line	Commercially available toothpastes	Only toothpastes containing sodium ascorbyl phosphate and 50–80 mg toothpaste per ml water demonstrated antioxidant activity. Concluded that antioxidant activity of toothpaste components should be considered when arranging new toothpaste formulations
<i>Other intervention studies</i>				
Effect of methylene blue on microbiological and clinical indices in chronic periodontitis	Wilson et al. 1992 (451)	Split mouth design using 6–8 diseased sites. Half treated with methylene blue and the other with sterile water for 7 days	7 patients with periodontitis (50 sites in total)	Methylene blue reduced the proportions of gram-negative anaerobes, spirochaetes and motile organisms and decreased GCF flow. No changes seen in terms of BOP and PPD
Tetracycline inhibition of hypochlorous acid (HOCL)-induced PMNL-pro-collagenase activation	Ramamurthy et al. 1993 (337)	<i>In vitro</i> study with osteoblastic osteosarcoma cell line	Cell line study	Doxycycline and modified tetracyclines significantly inhibited collagenase activity in presence of HOCL. Tetracyclines inhibit HOCL-induced collagenase activation

**Table 10.** Continued

Biological samples	Reference	Study design	Subject numbers	Outcome
Effect of taurine in chitosan film on healing of experimental bone defects	Ozmeric et al. 2000 (310)	<i>In vitro</i> experimental bone defects induced and healing response assessed in chitosan and chitosan/taurine groups	6 dogs	Increased PMNL and macrophage cell counts concluded to be beneficial to tissue repair/healing and effects of taurine more significant than chitosan alone
Effect of nutritional and plant-derived nutraceuticals on periodontal outcomes	Munoz et al. 2001 (288)	2-cell randomized placebo-controlled parallel clinical trial – 60 days	63 patients – 32 test (multi-vitamin tablet) and 31 control (placebo) as home care adjunctive	Significant improvements in PPD and gingival index in supplement group (not for CAL or bleeding index). Concluded that multi-vitamin nutritional supplements beneficial adjunct to established periodontal therapies
Effect of local delivery green tea catechin in slow release system on clinical outcomes and black-pigmented gram-negative rods	Hirasawa et al. 2002 (199)	Split mouth design with 2 deep pockets bilaterally. One treated with catechin and the other with placebo	6 volunteers with advanced periodontitis	PPD and black-pigmented gram-positive rods and peptidase activity reduced significantly at test sites over baseline, in adjunctive catechin group but not in controls. Beneficial effects of green tea catechin used as adjunct
Effects of aminoguanidine on markers of oxidative stress and tissue damage in 8-day ligature rat model	Di Paola et al. 2004 (108)	8-day ligature-induced periodontitis model – placebo-controlled. Histological/chemical study	40 rats; 20 with ligature, 20 sham operated. In each group 10 received aminoguanidine and 10 received placebo	Aminoguanidine reduced inducible nitric oxide synthetase activity, myeloperoxidase activity, tyrosine nitration and tissue malondialdehyde levels and also histological and radiographic bone loss
Effects of M40403 (superoxide dismutase mimetic) on markers of oxidative stress and tissue damage in 8-day ligature rat model	Di Paola et al. 2005 (110)	As above	20 male Sprague–Dawley rats (n = 10 received M40403 and n = 10 received placebo)	M40403 reduced myeloperoxidase activity, tyrosine nitration and tissue malondialdehyde levels and also histological and radiographic bone loss
Effects of Tempol on markers of oxidative stress and tissue damage 8-day ligature rat model	Di Paola et al. 2005 (109)	As above	20 male Sprague–Dawley rats (n = 10 received Tempol and n = 10 received placebo)	Tempol reduced myeloperoxidase activity, tyrosine nitration and poly(ADP-ribose) polymerase and also histological and radiographic bone loss

RDA, recommended daily amount; PMNL, neutrophilic polymorphonuclear leukocyte (neutrophil); ICAM, intracellular adhesion molecule; sICAM, soluble intercellular adhesion molecule; CAL, clinical attachment loss; PPD, probing pocket depth; Tx, treatment; GCF, gingival crevicular fluid; BOP, bleeding on probing.

although these effects seem likely to be the result of ascorbate's role in collagen metabolism within small vessel walls, rather than direct antioxidant effects. While studies of periodontally healthy volunteers may provide some basic physiological pointers as to the value of vitamin C in periodontal tissues, it seems unlikely that they will impact significantly on therapeutic management strategies, when key risk factors for ascorbate levels in human tissue and plasma relate to:

- The effects of cigarette smoking;
- Increasing age;
- Subjects who are vitamin C-deficient.

A recent study by Scott et al. (369) investigated the effects of ascorbate supplementation on soluble intercellular adhesion molecule-1 expression, neopterin (a marker of monocyte activation) and leukocyte elastase levels in 20 smokers and 20 age- and sex-matched controls. While the subjects were not periodontally diseased and were young, at least a rationale was evident for the study (smoking status) and a randomized double-blind controlled design was used. Vitamin C supplementation demonstrated no effects upon the measured outcomes. There are many reasons for the above findings. First, in healthy young subjects with no periodontitis the effects of supplementation may be insufficient to confer benefit, when all other relevant biological systems are functioning normally in 'non-risk' individuals. Second, there is good evidence from the medical literature that monocyte-bound adhesion molecule expression is up-regulated (intercellular adhesion molecule-1) by acute-phase proteins such as C-reactive protein (458). Interestingly, ascorbate supplementation normalizes monocyte-endothelial adhesion in such subjects (457). Given the evidence for raised plasma C-reactive protein levels in periodontitis patients (122), it may be that any beneficial effects of vitamin C supplementation will only be evident in periodontitis subjects and may indeed be independent of the antioxidant activities of ascorbic acid. In summary, there is currently insufficient evidence to support or refute the beneficial effects of vitamin C supplementation in periodontitis subjects.

### Vitamin E

Of the few intervention studies performed with vitamin E, three were on rats, which do not naturally develop periodontal disease but in which an acute version of the disease can be ligature-induced. Accelerated gingival wound healing was demonstrated in a rat model by Kim and Shklar (225) with

vitamin E supplementation and Goodson and Bowles (154) demonstrated that patients with periodontitis who rinsed their mouths with vitamin E daily for 21 days experienced a significant decrease in gingival crevicular fluid flow compared with an unsupplemented control group. Two studies by Cohen and co-workers (91, 90) provided an insight into the potentially differing effects of vitamin E, with different study designs. In a human study (91) there was no observable benefit from a 5% vitamin E gel used as an adjunct to therapy. However, in a study on rats, vitamin E had a significant protective effect against alveolar bone loss and this effect was most pronounced at the sites most 'at risk' of bone loss. Åsman et al. (27) demonstrated in a rat model that the combination of vitamin E and selenium was protective against ROS-induced collagen degradation. Most recently, Royack et al. (350) demonstrated that vitamin E pre-treatment of oral epithelial cells reduced the production of hydroxyl radicals (derived from hydrogen peroxide). In summary, as with vitamin C, there is insufficient evidence within the periodontal literature to draw any conclusions about vitamin E supplementation and periodontitis, other than that the effects of supplementation may differ between prophylactic regimens and those aimed at adjunctive corrective therapy.

### Co-enzyme Q10

A study by Hanioka et al. (191) reported upon the efficacy of adjunctive topical co-enzyme Q10 application to periodontal pockets in a split-mouth placebo-controlled study. Improvements in gingival redness and bleeding and lower bacterial peptidase activities were demonstrated at co-enzyme Q10 treated sites over those that received mechanical therapy alone. In a double-blind placebo-controlled cross-over pilot study Denny et al. (107) assessed the antioxidant and anti-inflammatory effects of 90 mg/day co-enzyme Q10 in 10 non-smoking periodontally healthy volunteers. They also demonstrated reductions in gingival bleeding after 28 days of supplementation but found no changes in gingival crevicular fluid total antioxidant capacity, indicating that the potentially beneficial effects of co-enzyme Q10 may be independent of its antioxidant activity.

### Antioxidant enzymes

Two studies have reported the effects of superoxide dismutase in animal models. In a rat model Misaki et al. (272) proposed a 'curative effect' of superoxide

dismutase on *P. gingivalis*-induced inflammation and enhanced wound healing. Petelin et al. (319) used a complex four-treatment modality study design on 15 Beagle dogs (three groups of five dogs) and demonstrated that adjunctive superoxide dismutase provided significantly better reductions in probing pocket depth, clinical attachment loss and suppression of inflammation than scaling and root planing alone. Increased bone density was also demonstrated in superoxide dismutase groups and catalase appeared to offer no additional benefit. It is interesting to speculate whether human studies would show benefit from superoxide dismutase supplementation, given the slow rate of activity of this largely intracellular enzyme relative to the radical scavenging species of the extracellular environment.

### Mouth rinses and toothpastes

Given the rapidly growing evidence base supporting a significant role for ROS-mediated tissue damage and antioxidant deficiency in periodontitis patients, interest has re-emerged recently in the potential antioxidant effects of mouth rinses and toothpastes. An initial study by Goultschin and Levy (157) demonstrated that chlorhexidine demonstrated antioxidant properties in a bio-assay of superoxide release by peripheral blood neutrophils. This was confirmed by Roberts et al. (347) in a cell-free enhanced chemiluminescence antioxidant assay, where the ranking of commercially available mouthwashes (highest to lowest) was, Listerine® (benzoic acid), Oraldine® (Hexitidine), Plax® (Triclosan), Veadent® (Sanguinarine) Corsodyl® (0.2% chlorhexidine), Oral-B anticavity rinse® (cetyl-pyridium-chloride) and Tatum Verde® (Benzidamine HCl). Firatli et al. (135) found similar results using an assay for lipid peroxidation. Battino et al. (41) looked at individual components of mouth rinses, in addition to the whole rinse and found no antioxidant activity from Corsodyl®, using a spectrophotometric cell free assay and also the comet assay to measure DNA fragmentation by hydrogen peroxide on HFL-1 fibroblasts. They also found the antioxidant activity of Listerine® to be high, but this was because of its methylsalicylate content (a phenolic compound capable of scavenging hydroxyl radicals) (4). The study by Battino et al. serves to remind us of two important issues that must be accounted for when interpreting the results of assays in antioxidant biology:

- Different assay systems yield different results, depending upon the index of anti-radical activity being used (e.g. bias towards protein antioxi-

dants vs. low molecular weight scavengers, or lipid soluble antioxidants vs. water-soluble species);

- It is essential to explore the antioxidant activity of individual components of compound preparations to determine the active antioxidant species. At the same time it is important to recognize that the biological activities of individual compounds within complex formulations may change (increase or decrease) as the result of interactions with other compounds in the formulation.

A recent study has addressed the antioxidant activity of toothpaste formulations (43) and found that pastes containing sodium ascorbyl phosphate displayed clear antioxidant activity *in vitro*. The antioxidant activities of ZnCl and NaF were significant, which was deemed to be because of the ability of Zn<sup>2+</sup> to protect thiol groups and prevent hydrogen peroxide and superoxide formation by transition metals (56) and the ability of F<sup>-</sup> to complex divalent iron ions (41). In summary, studies of mouth rinses or toothpastes should account for different indices of antioxidant activity, as well as the individual components of the rinse/paste. Antioxidant benefit needs to be demonstrated for the compound preparation as well as individual components and *in vivo* studies are needed which demonstrate antioxidant activity, as well as anti-gingivitis/periodontitis benefits.

### Other intervention studies (nutritional and other supplements)

Munoz et al. (288) investigated the adjunctive effects of a multi-vitamin phytonutrient supplement used as part of a home-care regimen in 63 periodontal patients and found significant improvements in probing pocket depth and gingival indices in the supplemented group relative to the placebo group. Hirasawa et al. (199) demonstrated beneficial effects from a slow-release locally applied green tea catechin system using a split mouth design in six subjects (Table 10). The antioxidant activities of green tea catechins were also demonstrated *in vitro* by Ho et al. (200) and a study by Bailey et al. (34) reported that oral health problems in community-dwelling older rural adults were associated with impaired intake of certain foods and nutrients. In a randomized controlled trial involving 75 smokers with periodontal disease (162), significant improvements in attachment levels were found for those taking single-dose vitamin C/vitamin E/grape seed extract (1100 mg vitamin C, 135 mg vitamin E, 42 mg grape seed) and

double-dose (2200 mg vitamin C, 270 mg vitamin E, 84 mg grape seed) supplements vs. placebo, providing evidence that antioxidant supplementation may have therapeutic benefit in smokers with periodontitis.

Recently Di Paola et al. (109) investigated the effects of the radical scavenger Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a water-soluble low molecular weight and neutrally charged piperidine nitroxide, that is capable of crossing cell membranes. They used a daily 10 mg/kg dose (vs. vehicle control) injected intraperitoneally into male Sprague-Dawley rats that had an 8-day ligature-induced acute periodontitis affecting one side of the mouth and matched non-ligature teeth on the contralateral side. They demonstrated that Tempol reduced myeloperoxidase activity, vascular permeability, nitrotyrosine formation and poly(ADP-ribose) polymerase activity, as well as reducing histological and radiographic bone loss. Other studies by this group using the same rat model have shown similar beneficial effects using aminoguanidine, an inducible nitric oxide synthase inhibitor (108), and a superoxide dismutase mimetic (110). While they used an acute periodontitis model within the rat for investigating a chronic disease of humans, their data did demonstrate a potential role for ROS and oxidative stress in the periodontal tissue damage obtained with their model, and that antioxidant therapy was protective against such damage.

In summary, there is currently a lack of human intervention studies to establish a body of evidence supporting the utility of antioxidant therapies in the management of periodontitis. Those studies available to date, do provide evidence for significantly beneficial effects but mechanistic links between individual antioxidant species and pathogenic mechanisms are needed (point 3 of box 1), along with studies involving whole food nutrition. Given the demonstrable contribution of excess ROS activity and associated damage, the antioxidant deficiencies at a local and systemic level in periodontitis patients, along with the dual antioxidant and anti-inflammatory activities of some antioxidant species and their proven benefit in medical conditions that are associated with oxidative stress, this represents an exciting and important area of enquiry for the future.

## The role of glutathione

The importance of reduced glutathione (GSH) to intracellular redox status and as a chain-breaking radical scavenger has been discussed earlier. The

millimolar levels measured within gingival crevicular fluid in health (83) are intriguing given that extracellular GSH concentrations normally fall within the 1–5  $\mu\text{M}$  range. This, along with the significantly reduced GSH levels in gingival crevicular fluid from non-smoking periodontitis patients, poses a number of important questions.

- Why are GSH levels so high in gingival crevicular fluid?
- What are the reasons for their reduction in periodontitis?
- What is the relationship between GSH in periodontal cells and tissues to disease activity and progression?

There are already answers to the second question within the periodontal literature. Studies of betel nut chewing and periodontitis have demonstrated that the areca nut alkaloid 'arecoline' inhibits gingival fibroblast attachment, spreading, and migration in a dose-dependent manner and that these changes are associated with a depletion of intracellular GSH (212). Chang et al. (75) confirmed that arecoline-induced gingival fibroblast toxicity was the result of depletion of intracellular thiols and they demonstrated that a synergistic effect was achieved with nicotine (74). They concluded that arecoline-induced thiol depletion in periodontal ligament fibroblasts may render them more susceptible to the effects of nicotine. The same group (76) demonstrated that nicotine was cytotoxic to periodontal ligament cells from explant cultures and reduced cell proliferation and protein synthesis in a dose-dependent manner. They investigated the abilities of catalase, superoxide dismutase and GSH to protect against these cytotoxic effects using inhibitors and promoters of GSH and found that GSH protection negated nicotine cytotoxicity, whereas catalase and superoxide dismutase had no effect. Chang et al. (77) subsequently demonstrated that cigarette smoke decreased periodontal ligament fibroblast GSH levels in a dose-dependent manner and stimulated stress-specific genes. Such data clearly link cigarette smoking and nicotine cytotoxicity to GSH depletion within periodontal ligament and gingival fibroblasts and are consistent with the huge body of data from the inflammatory lung disease literature (333, 335, 336).

A series of studies have also demonstrated that certain periodontal pathogens possess specific enzyme pathways for GSH metabolism and use GSH to produce hydrogen sulfide, removing a protective antioxidant species and forming a cytotoxic by-product. Persson et al. (318) were the first to demonstrate that out of 75 species, *Peptostreptococcus*, *Eubacterium*, *Selenomonas*, *Centipeda*, *Bacteroides*

and *Fusobacterium* metabolized cysteine to produce volatile sulfur compounds. Carlsson et al. (69, 70) demonstrated that two sub-species of *P. micros* and five *Fusobacteria* spp. used GSH to generate hydrogen sulfide. It has been proposed that the enzyme  $\gamma$ -glutamyl-transpeptidase, which breaks GSH down to cys-gly in human cell membranes, is also present in *T. denticola* outer cell envelopes and that  $\gamma$ -glutamyl-transpeptidase may play a role in the propagation of *T. denticola* within inflamed periodontal tissues (256). Chu et al. (86, 87) confirmed that GSH could be used by *T. denticola* to synthesize hydrogen sulfide, confirming that GSH removal could be an important mechanism in the virulence expression of *T. denticola*. The evidence-base supports depletion of GSH by certain periodontal pathogens as well as cigarette smoke.

Recent research has identified a significantly increased risk of periodontitis in subjects with the polymorphic glutathione-S-transferase-M1 allele (226). Glutathione-S-transferase is a xenobiotic metabolizing enzyme vital to detoxification reactions (323) and utilizes free GSH to achieve this. Chemical inducers of glutathione-S-transferase gene expression generate ROS by metabolic processes or modify thiols causing a depletion of free intracellular GSH and activating redox-sensitive transcription factors. Kim and co-workers (226) identified an increased risk among glutathione-S-transferase-M1<sup>+</sup> smokers for periodontitis (odds ratio 3.1, CI 1.5–6.6) and a near significant moderate risk even among M1<sup>+</sup> non-smokers (odds ratio 1.8, CI 1.0–3.1). They concluded that smoking and glutathione-S-transferase-M1 genotype were independent risk indicators for periodontitis. This is the first evidence that GSH depletion in periodontitis subjects may have a genetic basis.

The high levels of GSH within gingival crevicular fluid may be the result of increased synthesis by cells of the periodontal tissues, or of active release mechanisms, or passive release secondary to protease activity on gingival epithelial cells (342). Moreover, the activities of certain periodontal pathogens and cigarette smoking have been shown to lower GSH levels within periodontal cells and oxidative stress further depletes GSH, a consequence being the activation of redox-sensitive transcription factors and creation of a pro-inflammatory state (Fig. 12). Whether an additional genetic basis exists for reduced GSH levels in periodontally diseased tissues, via polymorphisms in the genes encoding the various enzyme systems responsible for GSH synthesis, oxidation/reduction or utilization within cells, remains

to be determined. However, given the growing body of evidence linking reduced levels of GSH to periodontal inflammation, we postulate that increasing or preserving intracellular GSH levels within the cells of the periodontal tissues is likely to provide a novel adjunctive antioxidant and anti-inflammatory strategy to traditional periodontal therapies.

## Conclusions and the future

Oxidative stress lies at the heart of the periodontal tissue damage that results from host–microbial interactions, either as a direct result of excess ROS activity/antioxidant deficiency or indirectly as a result of the activation of redox-sensitive transcription factors and the creation of a pro-inflammatory state (Fig. 13). A body of literature supports peripheral blood neutrophil hyperactivity in chronic and aggressive forms of periodontitis, with respect to total Fc $\gamma$ -receptor-mediated ROS generation. On balance, currently available data suggest that this hyperactivity has a constitutional element rather than being entirely the result of peripheral priming (e.g. by cytokines or lipopolysaccharide). Furthermore, it seems possible that baseline hyperactivity (i.e. low-level extracellular ROS release in the absence of exogenous stimulus) is also a constitutional property of peripheral neutrophils from periodontitis patients. This, together with the evidence for compromised plasma antioxidant capacity, independent of smoking, suggests an underlying environment of oxidative stress, within periodontitis patients. In addition to this albeit subtle systemic compromise, considerable evidence has emerged over the last 2 years that oxidative stress and depressed antioxidant function are features of periodontal tissues and fluids in periodontitis subjects. As illustrated in Fig. 13, several avenues of enquiry now exist for the development of novel antioxidant-based approaches to periodontal therapy. Moreover, specific pathways, such as the glycation and glyoxidation of proteins to produce advanced glycation endproducts (and increased receptor for advanced glycation end product expression), are dependent on oxidative mechanisms and are highly prevalent in type 2 diabetes and smokers, the two major risk factors for periodontitis. Such oxidation products increase neutrophil adhesion, chemotaxis and priming and in hyper-active/reactive neutrophils, may augment the damaging effects of periodontal bacteria-mediated increases in ROS and oxidative stress, providing one explanation for the increased risk of periodontitis in type 2 diabetes and

smokers. Besides the largely unexplored traditional routes of increasing the antioxidant capacity of periodontal tissues and extracellular fluids to manage or prevent the excess ROS-mediated tissue damage associated with periodontal hyper-inflammation (e.g. from the activities of neutrophils and fibroblasts), newer routes based upon the modulation of redox-sensitive transcription factors have recently emerged. Such transcription factors (nuclear factor- $\kappa$ B and activating protein-1) are not only activated by receptor-ligand initiated pathways, but also by non-receptor-mediated shifts in intracellular redox state, largely involving GSH depletion (oxidation/glutathionylation) and ROS (e.g. hydrogen peroxide) as metabolic triggers. This array of pathways provides opportunities to develop novel antioxidant therapies that target extracellular, membrane-bound or intracellular processes (Fig 12 and 13) and which function not only as antioxidants in the traditional sense but also as powerful anti-inflammatory agents.

This review has attempted to bring together 40 years of periodontal research on reactive oxygen and antioxidant species and more recent work on redox biology, and to set this against the huge body of evidence within the biomedical literature, to highlight common mechanistic pathways and exciting new opportunities for the future development of host modulation therapies in periodontology.

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