

Review Article

Free radicals, antioxidants and international nutrition*

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The oxidative degradation of polyunsaturated fatty acids is the primary factor in limiting the shelf-life of most manufactured foods. Free radical mechanisms are implicated in the pathogenesis of human diseases and in the process of ageing. This has led to the suggestion that antioxidants, and plant diet-derived antioxidants in particular, might have health benefits as prophylactic agents. Delineating the *in vivo* contribution of plant extracts and/or plant-derived antioxidants (the pure active principles in plant extracts with antioxidant indications) to the modulation of the pathological consequences of oxidative stress in the human body is complicated by the fact that antioxidant actions may be achieved through more than one mechanism. The interest in the health promoting qualities of plant foods may be ascribed to the observation that various compounds present in these foods possess antioxidant properties *in vitro*. From a food stability perspective, one would be interested in the integrity of the food and the effects of storage on the molecular components of the food. For humans, the emphasis is on the importance of nutritional antioxidants in health and disease management.

Key words: free radicals, antioxidants, flavonoids, DNA damage, isoprostanes, phytochemicals, protein damage, atherosclerosis, lipid peroxidation, oxidative stress.

Free radical chemistry: An introduction

A free radical is any chemical species capable of independent existence and possessing one or more unpaired electron, an unpaired electron being one that is alone in an orbital. Radicals, often denoted by the insertion of the superscript dot (\cdot), are generally less stable than non-radicals, although their reactivities vary. The rate and selectivity of reactions of this type depends on high concentrations of the radicals, delocalization of the single electron of the radical (thus increasing its life time), and on the absence of weak bonds in any other molecules present with which the radical could interact. Most biological molecules, however, are non-radicals containing only paired electrons. Much of the work in physical and organic chemistry¹⁻³ relating to free radicals gathered momentum following the demonstration of the existence of the triphenylmethyl radical ($\text{Ph}_3\text{C}\cdot$).⁴ Gerschman *et al* proposed 'that oxygen poisoning and radiation injury have at least one common basis of action, possibly the formation of oxidizing free radicals'.⁵ This pioneering idea soon began to capture the imagination of scientists. In the early 1960s, superoxide was found to be associated with a number of enzymes, including xanthine oxidase. In 1968 it was discovered that superoxide was secreted into solution, allowing superoxide to mediate cellular toxicity.⁶⁻⁷

From an environmental perspective, photochemical reactions involving reactive oxygen species are attractive for cleaning up pollution given that many 'self-repair' processes in the atmosphere and natural waters are driven by light.⁸ Because electronically excited states of molecules may be both better oxidizing and/or reducing agents compared with their ground state counterparts, electron transfer processes can generate highly reactive species, which can be used to chemically decompose a pollutant into harmless end products.

Suggestions that oxidative stress play a role in human diseases have led to the proposal that health might be improved by increased dietary intake of antioxidants.⁹⁻¹³ Drugs (prescription only medicines and over the counter medicines) with antioxidant indications, have a functional relationship between health status and disease state.¹⁴⁻¹⁵ The role that food and drugs might play in the management of health is shown in Fig. 1.

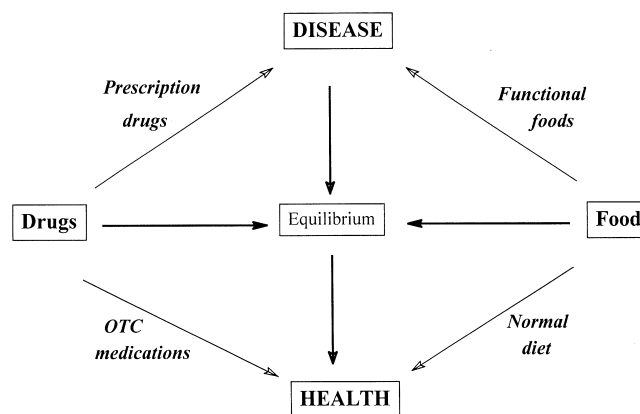


Figure 1. Functional relationships between health status and disease state and the role that food and drugs might play in the management of health.

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Reactive oxygen species

Free radicals of importance in living organisms include hydroxyl (OH·), superoxide (O₂⁻), nitric oxide (NO·) and peroxy (RO₂·). Peroxynitrite (ONOO⁻), hypochlorous acid (HOCl), hydrogen peroxide (H₂O₂), singlet oxygen¹Δg (often written as ¹O₂) and ozone (O₃) are not free radicals but can easily lead to free radical reactions in living organisms. The term 'reactive oxygen species' (ROS) is often used to include both the radical and non-radical species. Oxidative stress is the term referring to the imbalance between the generation of reactive oxygen species and the activity of the antioxidant defenses. Severe oxidative stress can cause cell damage and death. It has been implicated in numerous human diseases including cancer, atherosclerosis, iron overload, rheumatoid arthritis, Parkinson's disease, motor neurone disease, diabetes, malaria, and in HIV infection and AIDS.¹⁶⁻¹⁹ The importance of oxidative stress injury is dependent on the molecular target, the severity of the stress and the mechanism by which the oxidative stress is imposed, that is, drug induced, Fenton chemistry, trauma, enzyme activation (e.g., nitric oxide synthase activity) and the cellular transduction mechanisms which may affect the expression of certain proteins including the DNA repair enzymes.^{20,21} Brief comments on nitric oxide, peroxy radicals, hydroxyl radicals and hypochlorous acid will be made to illustrate the complexity of the mechanism of reactions involving ROS.

Nitric oxide

Nitric oxide plays a significant role in the regulation of cell function and tissue viability: this includes the recognized ability to mediate signal transduction via stimulation of guanylate cyclase-mediated cGMP synthesis.²²⁻²⁷ The role of NO· has been demonstrated with relation to malaria, whereby NO· appears to be partially involved in resistance to malaria infection, in cardiovascular disease, acute inflammation, cancer, neurodegenerative diseases and diabetes.²³ The reaction between NO· and O₂⁻ leads to DNA oxidative damage due to the formation of peroxynitrite, which may have OH-like potential leading to the formation of nitroguanine and other loose products.²⁸⁻³² It has been suggested that peroxynitrite formed by the reaction between NO· and O₂⁻ mediates NO-dependent toxicity. In addition to the DNA base nitration mentioned above, ONOO⁻ potentiates endothelial-dependent activation of guanylate cyclase, bactericidal activity, trypanocidal activity, conversion of low density lipoprotein (LDL) to a form that may be recognized by the macrophage scavenger receptor, induction of peroxidation of lipids, oxidation of methionine and SH residues in proteins, depletion of antioxidants (e.g. ascorbate, glutathione), nitration of tyrosine residues and inactivation of α₁-antiproteinase (a major inhibitor of serine proteases *in vivo*).

Reaction of peroxynitrite with antioxidants

The addition of ONOO⁻ to biological fluids leads to the nitration of tyrosine residues: the presence of these appears to be a 'marker' of ONOO⁻-dependent damage *in vivo*. Peroxynitrite inactivates α₁-antiproteinase, the major inhibitor of serine proteases such as elastase, in human body fluids. Thus, ONOO⁻ generation *in vivo* can facilitate both oxidative and proteolytic damage.^{22,38} The protein α₁-antiproteinase (α₁AP) is an especially sensitive target of damage, so the

antioxidant's protective action might be even greater *in vivo* depending, of course, on the precise location of the antioxidant in relation to the site of ONOO⁻ generation. Although inactivation of α₁AP may involve a direct attack on methionine residues by ONOO⁻ nitration of tyrosine by ONOO⁻ is a complex reaction that may involve such species as NO₂·, NO₂⁺ and CO₂·. Activated human polymorphonuclear neutrophils have been shown to convert NO· into NO₂Cl and ·NO₂ through myeloperoxidase pathway, a reaction that may contribute to cellular dysfunction.³⁹ The reaction of peroxynitrite with tyrosine (in proteins) and phenolic 'antioxidant' compounds and its inactivation of the proteolytic inhibitor α₁-antiproteinase are good assays for determining putative antioxidant activity.^{22,33-37,40-42}

Peroxy radicals

These are formed during lipid oxidation chain reactions, such as the oxidation of polyunsaturated fats resulting in deterioration of lipid-containing foods. Lipid peroxidation may be initiated by any species that has sufficient reactivity to abstract hydrogen from a polyunsaturated fatty acid side chain (e.g. those of arachidonic acid and linolenic acid) in membrane lipids. End-products of lipid peroxidation could also have profound effects on vascular function because of their ability to mimic or antagonize the actions of some of the stereospecific products formed by cyclooxygenase and lipoxygenase enzymes. For example, the F₂-isoprostanes are generated by the peroxidation of arachidonic acid via the generation of peroxy radical isomers which undergo endocyclization to prostaglandin-like compounds. Their formation *in vivo* appears to be enhanced under conditions of oxidative stress, such as smoking or exposure to xenobiotics, and under pathological conditions associated with inflammation. The mechanism of LDL oxidation possesses the general characteristics of the free radical reaction of lipid peroxidation.⁴³⁻⁴⁵

Hypochlorous acid

Hypochlorous acid is produced by the neutrophil-derived enzyme myeloperoxidase at sites of inflammation and when activated neutrophils infiltrate reoxygenated tissue. The enzyme oxidizes chloride (Cl⁻) ions in the presence of H₂O₂.⁴⁶⁻⁴⁹ Hypochlorous acid is a potent chlorinating and oxidizing agent.⁵⁰ Cholesterol forms chlorohydrins that could disrupt cell membranes, leading to cell lysis and death.⁵¹ Cholesterol chlorohydrins may become potential biomarkers for oxidative damage associated with neutrophil/monocyte activation. Hypochlorous acid can attack many other biological molecules. For example, the proteolytic inhibitor α₁AP is the major inhibitor in human plasma of proteolytic enzymes such as elastase. The α₁AP protein accounts for approximately 90% of the elastase-inhibitory capacity of the human serum.⁵² Thus, its inactivation by HOCl might greatly potentiate tissue damage because elastase is also released from activated neutrophils. Hypochlorous acid attacks primary amines and sulfhydryl (SH) groups in proteins, and chlorinates purine bases in DNA.^{50,53,54} Physiological levels of HOCl can cause protein fragmentation of collagenase and prevent collagen gelation.⁵⁵ Reactions of HOCl/OCl⁻ react with endogenous amines to form N-chloramines, which exhibit a lower oxidizing potential

than HOCl.^{50,56} Hypochlorous acid reacts with substituted aryl amine-aniline, 1-naphthylamine and 1-naphthol to form long-lived products that bind DNA and that are suggested to be genotoxic to human cells⁵⁷

Reactions of antioxidants with hypochlorous acid

Assays for hypochlorous acid that could be performed with ease to test the ability of an antioxidant to react with the molecule include the elastase assay;^{58,59} assay with catalase;⁶⁰ inhibition of taurine-chloramine formation;⁶¹ and the oxidation of 5-thio-2-nitrobenzoic acid (TNB).⁶² An antioxidant protecting against damage by HOCl might do so not only by scavenging HOCl but also by inhibiting myeloperoxidase. Thiols that are good scavengers of HOCl might also act as competing substrates for myeloperoxidase enzyme and therefore slow down HOCl formation.^{63–65} Interestingly, several phenolic compounds including flavonoids react quickly with HOCl and can protect α_1 -antiproteinase and other susceptible targets against damage *in vitro*.^{66,67}

Hydroxyl radicals

The hydroxyl radical (OH·) is a highly reactive oxygen-centred radical with an estimated half life in cells of only 10⁻⁹ s. One feature of the hydroxyl radical is that it begets another radical when it reacts with a molecule: the result is the formation of another radical species. The resulting species usually has lower reactivity than the OH·. Hydroxyl radical attacks all proteins, DNA, polyunsaturated fatty acids in membranes and almost any biological molecule it touches. In the case of OH· generation by Fenton-type chemistry,^{68,69} the extent of OH· formation is largely determined by the availability and location of the metal ion catalyst.

Copper ions are more reactive in causing DNA damage in the presence of H₂O₂ compared with equimolar iron ions *in vitro*.⁷⁰ Metal-dependent carcinogenesis is widely discussed in the literature.⁷¹ Iron ions are absorbed from the gut and transported to iron requiring cells by the protein transferrin. Iron specifically bound to transferrin does not participate in free radical reactions.⁷² Excess iron is stored as ferritin and haemosiderin in an attempt to keep the iron pool as small as possible. Hydroxyl radical generation can take place when the homeostasis is altered. For example, copper and iron ions released into perfusates can cause ischemia–reperfusion injury.⁷³ Tissue injury can itself cause ROS generation (e.g. by causing activation of phagocytes or releasing transition metal ions from damaged cells), which may, or may not depending on the situation, contribute to a worsening of the injury. Traumatic brain injury and stroke may involve a postinjury stimulation of iron ion-dependent free radical reactions. Parkinson's disease is caused by the death of cells in the substantia nigra. Lysis of dead cells could cause iron ion release. Thus, patients with Parkinson's disease may be under oxidative stress and free radical reactions are probably contributing to the degeneration of the substantia nigra.⁷⁴ Data from the assessment of oxidative DNA damage in the brain have shown that DNA damage is higher in the temporal lobe compared with other brain regions in Alzheimer's disease.⁷⁵

Protection against ROS-induced damage

The phagocytes (i.e. neutrophils, monocytes, macrophages, eosinophils) provide protection against foreign organisms. They generate O₂⁻, H₂O₂ and, in the case of neutrophils, HOCl as one of their mechanisms for killing foreign organisms.^{46,47} This essential defence mechanism, however, can go wrong; several diseases, such as rheumatoid arthritis and inflammatory bowel disease, are accompanied by excessive phagocyte activation and resulting tissue damage, to which ROS contribute. The interrelationship between ROS and antioxidants in humans is very complex.¹⁵ Of the known antioxidant enzymes, superoxide dismutases (SOD)^{7,76} remove the superoxide radical (O₂⁻) by accelerating its conversion to H₂O₂. Human cells have a SOD enzyme which contains manganese at its active site (MnSOD) in the mitochondria. A SOD with copper and zinc at the active site (Cu,Zn-SOD) is also present but largely in the cytosol. Mutations to the SOD-1 have been associated with the pathology of the degenerative disease amyotrophic lateral sclerosis (ALS).⁷⁷ Catalases in the peroxisomes convert H₂O₂ into water and O₂ and help to dispose of H₂O₂ generated by the action of oxidase enzymes located in these organelles. However, the most important H₂O₂-removing enzymes in human cells are glutathione peroxidases (GSHPX).⁷⁸ These enzymes require selenium, as selenocysteine at the active site, for their action. Glutathione peroxidases enzymes remove H₂O₂ by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, an FAD-containing enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. A variety of radical-scavenging antioxidants, including GSH, uric acid, α -tocopherol (vitamin E) (Fig. 2) and ascorbic acid (vitamin C) exist. α -Tocopherol delays lipid peroxidation by reacting with chain-propagating peroxy radicals faster than these radicals can react with proteins or fatty acid side-chains.⁷⁹ In theory, β -carotene (Fig. 3) has remarkable antioxidant chemistry, a function that has been difficult to demonstrate in a beneficial manner in biological systems. Excellent accounts on vitamin E and β -carotene may be found in several reports.^{79–84} Thus, scavenging enzymes and antioxidants can inhibit free radical production by chelating the transition metal catalysts, breaking chain reactions, reducing concentrations of ROS, and by scavenging initiating radicals.

That ascorbate (vitamin C) may serve as an important antioxidant *in vivo* is widely claimed.⁸⁵ Ascorbic acid and its derivatives have useful functions in the food industry, where they are used during processing to enhance food stability.⁸⁶ The ability of ascorbic acid to show antioxidant properties is related to the fact that the dehydroascorbate radical is less reactive than are many of the radicals that can be scavenged by ascorbate.⁸⁷ Intracellular enzymic systems exist *in vivo* to reduce this radical back to ascorbate using NADH (the NADH-semidehydroascorbate reductase enzyme) or GSH (the dehydroascorbate reductase enzyme) as sources of reducing power. Ascorbic acid is often rapidly depleted in human extracellular fluids under conditions of oxidative stress.⁸⁸

Evaluating the role of free radicals in disease pathology and establishing a logical basis for the therapeutic use of antioxidants requires the use of validated biomarkers (Table 1). Numerous antioxidant supplementation studies for the

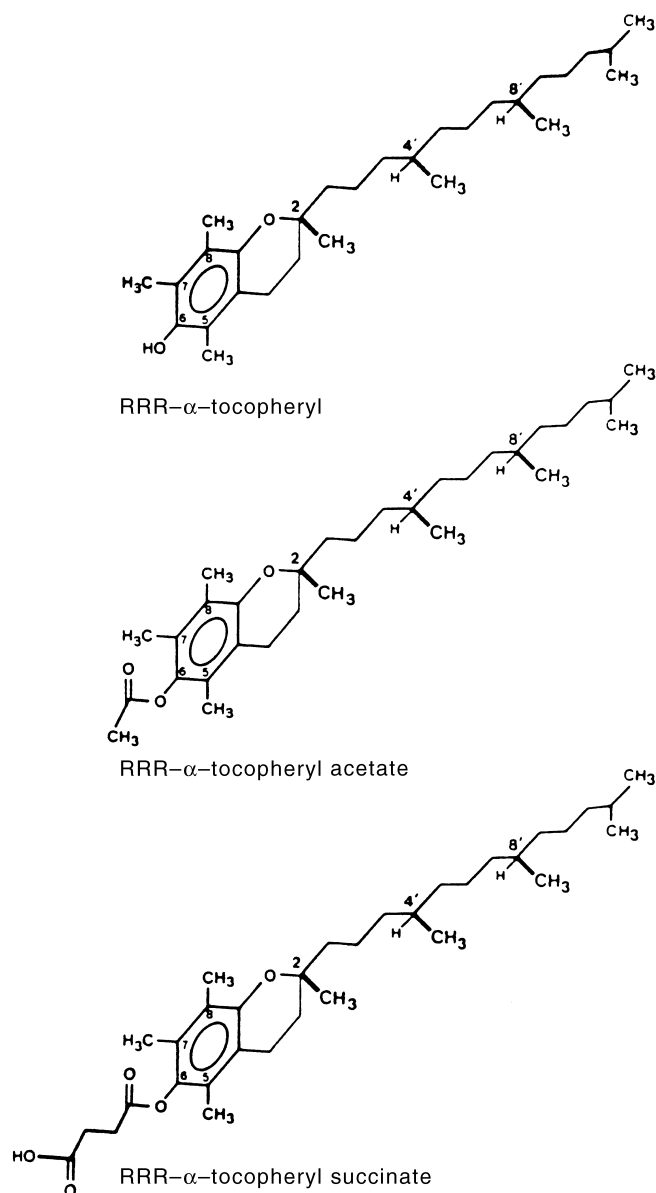


Figure 2. Chemical structure of tocopherols.

primary prevention of chronic diseases have been undertaken. In each case, the principal endpoint has been 'incidence' of the respective disease, that is, incidence of cancer or cardiovascular disease. In a departure from this, the extent of oxidative DNA damage in Scottish men aged between 50 and 59 years was investigated by Duthie *et al.*⁸⁹ Their result suggests that long-term antioxidant supplementation can decrease both endogenous and exogenous oxidative DNA damage in lymphocytes. Future supplementation studies in order to evaluate the pharmacology of antioxidants (drug-derived or plant-derived antioxidants) should balance the use of *in vivo* biomarkers with the choice of population, formulation and dose of antioxidants being used, the expected outcome variables, and the pathologic viables. Figure 4 suggests one such rationale. The global direction would be for food and drug antioxidants to be evaluated for their inherent properties using *in vitro* models. Assessment of their protective effects in human health and disease should then consider how the steady state levels of markers of oxidative damage are affected by the antioxidants.

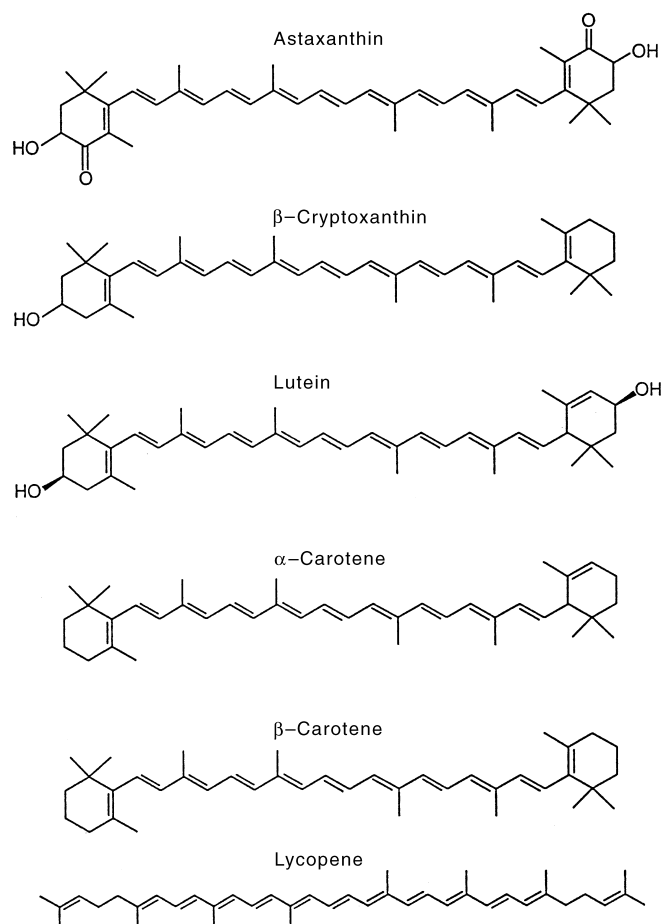


Figure 3. Typical carotenoid structures, including xanthophylls (oxygenated carotenoids) and carotenes (hydrocarbons).

Table 1. Measurement of oxidative damage in humans.

There are several indicators of the extent of oxidative damage in humans. Some of the most common include measuring:

Oxidative DNA damage

- GC/MS/SIM detection of oxidized base products
- HPLC-based assays for oxidized base products
- Single gel electrophoresis assay (Comet assay)

Oxidative damage to lipids

- Measurement of conjugated dienes
- Measurement of isoprostanes
- Measurement of hydroperoxides
- Measurement of thiobarbituric acid reactive materials by HPLC

Assessment of the levels of antioxidant enzymes

- Catalase, superoxide dismutase and glutathione peroxidase

Assessment of protein damage

- Steady state protein damage can be quantified in terms of the numbers of protein carbonyls and modified tyrosine residues.
- Total ongoing (repaired) protein damage can be indicated by the concentration of modified tyrosines and fluorescent bityrosines in the urine.

Assessment of levels of low molecular weight antioxidants and vitamins

- Uric acid/allantoin, glutathione, flavonoids, vitamin E and C, β -carotene

Measurement of oxidative DNA damage

Oxidative damage to DNA appears to occur continuously *in vivo*, in that low levels (presumably a 'steady state' balance between DNA damage and repair) have been detected in

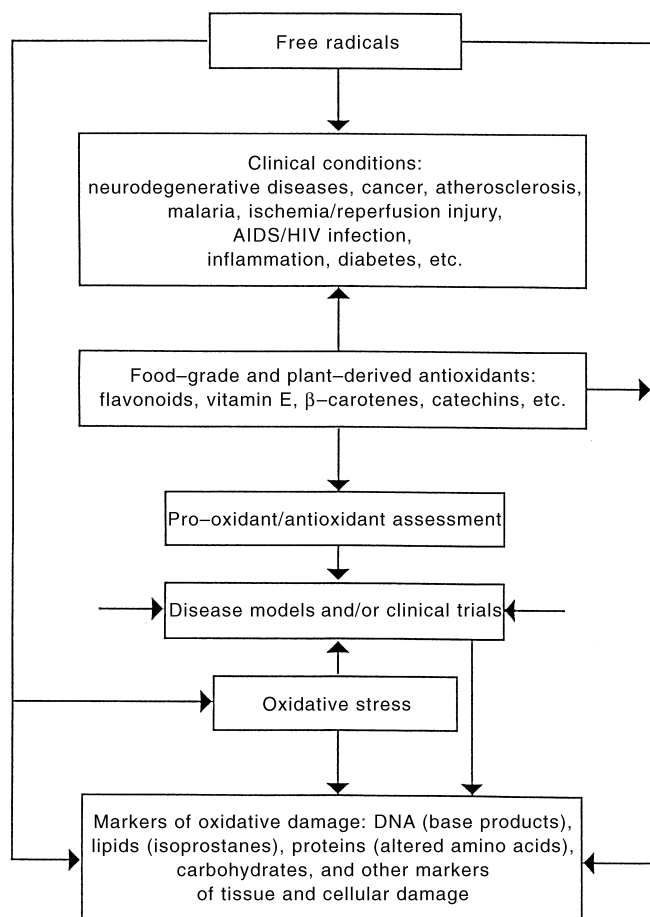


Figure 4. Human antioxidant strategy.

DNA isolated from human cells and tissues.^{32,90–92} Background radiation may be one source but radiation-generated $\text{OH}\cdot$ is formed over the whole cell and only a small fraction hits DNA.⁹³ Other potential sources of $\text{OH}\cdot$ or $\text{OH}\cdot$ -like species include the decomposition of ONOO^- , the reaction of O_2^- with HOCl , and HOCl itself, which can attack DNA bases generating chlorinated products. The greatest interest has been in reactions of transition metal ions with H_2O_2 as a source of $\text{OH}\cdot$.^{70,71,94–96} Oxidative stress and cell death can liberate metal ions from their normal sequestration sites and they might then bind to DNA, a powerful metal ion chelator. Several DNA base damage products are excreted in human urine, including the nucleoside 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxyadenine and 7-methyl-8-hydroxyguanine, but the one most exploited is 8-OHdG, which is usually measured by a method involving HPLC with electrochemical detection. The level of 8-OHdG in urine is probably not affected by the diet since nucleosides are not absorbed from the gut. It is also possible that some or all of the 8-OHdG excreted in urine may arise not from DNA but from deoxyGTP (dGTP) in the DNA precursor pool of nucleotides. An enzyme has been described which hydrolyzes dGTP containing oxidized guanine to prevent its incorporation into DNA.^{97,98} The need to validate measurements of DNA base products as markers of oxidative damage *in vivo* is critical.⁹⁹

Lipid oxidation and its measurement

Lipid peroxidation is important *in vivo* and for the stability of processed foods. It contributes to the development of cardiovascular diseases such as pre-eclampsia and atherosclerosis, and the end-products of this process, particularly cytotoxic aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), can cause damage to proteins and to DNA. Peroxidation causes impairment of biological membrane functioning; for example, it decreases fluidity, inactivates membrane bound enzymes and receptors, and it may change non-specific calcium ion permeability.^{100,101} The more unsaturated a fatty acid side-chain, the greater its propensity to undergo lipid peroxidation.

The ability to measure levels of isoprostanes and hydroxyeicosatetraenoic acids (HETE) represent important developments in attempts to measure clinically relevant oxidative lipid damage. The hydroperoxides HETE and isoprostanes are biologically active. Hydroxyeicosatetraenoic acids are chemotactic for neutrophils and have been shown to facilitate calcium uptake and protein kinase C mobilization.^{102,103} The 12-HETE are involved in adrenocorticotropin and parathyroid secretion, modulation of mitogenic processes and lymphocyte function,^{104–106} while the 15-HETE may inhibit neutrophil migration across cytokine-activated endothelium.¹⁰⁷

Isoprostanes are a series of prostaglandin-like compounds formed during peroxidation of arachidonic acid.¹⁰⁸ Similar products are probably formed from other polyunsaturated fatty acids (PUFA) as they are structurally similar to prostaglandin $F_{2\alpha}$, the compounds are collectively referred to as F_2 -isoprostanes. The majority of plasma isoprostanes are esterified to phospholipids, but some are 'free'. One of the isoprostanes, 8-*iso* $\text{PGF}_{2\alpha}$ is a powerful renal vasoconstrictor which is known to cause decreased kidney blood flow and glomerular filtration rate at low nanomolar concentrations.¹⁰⁸ Elevated circulating concentrations of F_2 -isoprostanes may contribute to the pathology of hepatorenal syndrome, an almost uniformly fatal disorder characterized by the development of kidney failure in patients with severe liver disease. Urinary excretion of isoprostanes is elevated in patients with scleroderma and in smokers. Isoprostanes and their metabolites can be measured in human urine and this may prove to be a valuable assay of whole body lipid peroxidation if a confounding effect of diet can be ruled out. These developments are discussed in a number of studies.^{43,108–114}

In a study by Bachi *et al.*, the levels of 8-epi- $\text{PGF}_{2\alpha}$ excretion in non-smokers tended to be constant, with relatively low interindividual variations, suggesting that individual 'normal level' in the absence of oxidant injury such as smoking may also result from physiological or biochemical event(s) occurring at a constant rate with by-production of free radicals and oxidants (see Fig. 5).¹¹⁵ Given that basal 8-epi- $\text{PGF}_{2\alpha}$ production *in vivo* may result from lipid peroxidation triggered by a chain of chemical events starting with the reaction of endothelium-derived peroxynitrite, measurement of isoprostanes may provide a superior novel approach to assessing lipid peroxidation *in vivo*. In Fig. 5 we can imagine that the plots for non-smokers and smokers represent normal and disease states. The efficacy of antioxidants in helping to maintain the baseline 'healthy' levels of endogenous oxidative lipid damage could easily be monitored by comparing

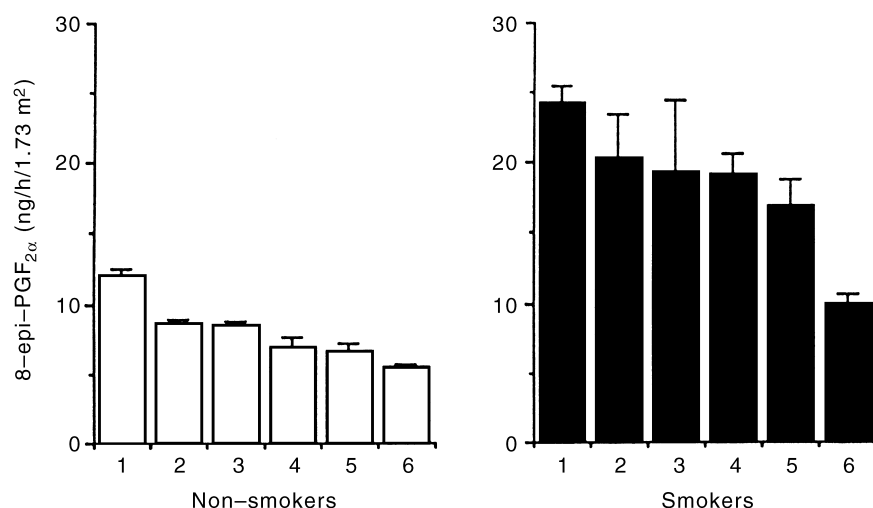


Figure 5. Levels of 8-epiPGF_{2α} in smokers. Reprinted with permission from reference 116, Elsevier Science Inc.

the rise and fall of the levels of isoprostanes. Alternatively, reducing the tendency of a given disease condition to worsen and bring patients to the state of normal health is fundamental in disease management (Fig. 1). Thus, strategies to reduce observed increases in isoprostane level can be used to monitor the efficacy of antioxidant prophylactic agents. Currently, the lack of validated assays for isoprostane other than those that make use of mass spectrometry has greatly curtailed the general use of this approach to assess oxidant injury. There is, therefore, a need to develop an inexpensive assay for isoprostanes. The availability of a simpler (e.g. immunoassays) and more reliable method for the measurement of isoprostanes could provide new and exciting insights into the role of free radicals and lipid peroxidation in human diseases.

Oxidation of LDL is a free-radical-mediated process that results in numerous structural and functional changes. The initiation of LDL oxidation occurs by the peroxidation of the polyunsaturated fatty acids (PUFA) in LDL. Oxidation of LDL is initiated by hydrogen abstraction from a double bond in PUFA, followed by molecular rearrangement that leads to the formation of conjugated dienes (CD). During this process, the rate of oxidation is dependent on endogenous antioxidants in LDL, accounting for the lag phase of oxidation. The lag phase is followed by a rapid propagation phase that occurs after depletion of endogenous antioxidants and involves abstraction of another H· by a PUFA-peroxyl radical (LOO[·]) from another PUFA, resulting in the formation of lipid peroxides.^{44,45} The propagation phase is followed by a decomposition or degradation phase in which there is cleavage of double bonds, resulting in the formation of aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE) and hexanal, that can crosslink with amino groups on apo B-100. Monoclonal antibodies for the detection of 4-hydroxynonenal modified proteins, which is selective for HNE bound to histidine with some cross reaction to HNE bound to lysine and cysteine, have been described by the late Hermann Esterbauer and his group at the University of Graz, Austria (Waeg *et al.*¹¹⁶). Changes in the protein moiety of LDL also occur during oxidation.¹¹⁷ Oxidation is followed by an increase in the negative charge on the LDL, possibly due to derivatization of positively charged amino groups through the formation of Schiff base with aldehydes. Furthermore, following oxidation the apo-B protein undergoes oxidative scission

leading to fragmentation. Oxidized LDL inhibits endothelium derived relaxing factor (EDRF) and may activate T-lymphocytes in atherosclerotic lesions and stimulate proliferation of smooth muscle cells. The induction of the expression of the gene coding for the A-chain of platelet derived growth factor may cause disturbances of eicosanoid homeostasis and aggregation of platelets in support of the atherogenicity of oxidized LDL.^{118,119}

Protein oxidation and its measurement

Oxidative damage to proteins *in vivo* may affect the function of receptors, enzymes, transport proteins, etc., and perhaps generate new antigens that provoke immune responses. Products of oxidative protein damage can contribute to secondary damage to other biomolecules, for example, inactivation of DNA repair enzymes and loss of fidelity of DNA polymerases in replicating DNA. The chemical reactions resulting from attack of ROS upon proteins are complex. Free radical attack upon proteins generates radicals from amino acid residues, and electrons can be transferred between different amino acids. The levels of any one or, preferably, of more than one of these products in proteins could in principle be used to assess the balance between oxidative protein damage and the repair of, or more likely the hydrolytic removal of, damaged proteins. The molecular biology of free radical attack on proteins and its implications in certain pathology is extensively reviewed in Dean *et al.*¹²⁰ and Fu *et al.*¹²¹

Characterization of antioxidant actions

An antioxidant may be defined in a number of ways. For example, as a substance which, when present at low concentrations compared with those of an oxidizable substrate, such as fats, proteins, carbohydrates or DNA, significantly delays or prevents the oxidation of the substrate.¹²² Acidic compounds (including phenols) usable in foods which can readily donate an electron or a hydrogen atom to a peroxyl or alkoxy radical to terminate a lipid peroxidation chain reaction or to regenerate a phenolic compound, or which can effectively chelate a pro-oxidant transition metal,^{123,124} are also antioxidants. A pro-oxidant is a chemical species capable of increasing the oxidative burden of a system by increasing the synthesis of reactive oxygen, nitrogen, chlorine and sulfur species and by modulating the gene expression of the antioxidant enzymes. A compound might exert antioxidant

actions *in vivo* or in food by inhibiting generation of ROS, or by directly scavenging free radicals. Additionally, *in vivo* an antioxidant might act by raising the levels of endogenous antioxidant defenses (e.g. by upregulating expression of the genes encoding SOD, catalase or glutathione peroxidase).

It is increasingly being suggested that natural antioxidants from plants may be applied in the preservation of food materials. Simple experiments (Fig. 6) can be performed to examine direct antioxidant ability *in vitro* and to test for possible pro-oxidant effects on different molecular targets. This 'screening' approach can be used to rule out direct antioxidant activity *in vivo*: a compound that is poorly effective *in vitro* will not be any better *in vivo*. Measurements of lipid peroxidation should be the first line of tests to establish the potential antioxidant action of dietary antioxidant compounds. An antioxidant index based on the ability to scavenge peroxy radicals may then provide support for antioxidant efficacy in *in vitro* systems.¹²⁴⁻¹²⁶ Antioxidants that protect lipids against free radical damage may actually accelerate damage to other molecules such as DNA, carbohydrates and proteins under certain conditions. This points to the need to examine suggested antioxidant activity or the activity of components with a proposed 'antioxidant cocktail' using assays involving a variety of substrates. The deoxyribose assay allows the determination of rate constants of reactions with OH· radicals, the assessment of abilities to exert pro-oxidant action, and the assessment of abilities to chelate metal iron. Assays involving DNA damage have also been developed for assessing pro-oxidant actions. These assays have unique features. The positive pro-oxidant actions in the deoxyribose system rely on the ability of the compounds to interact with metal ions (i.e. to promote reduction of Fe³⁺ to Fe²⁺ chelates) and hence, to promote OH· formation in the presence of H₂O₂. The assays involving DNA rely on the ability to reduce the metal ions in the iron-bleomycin-DNA or the copper-1,10-phenanthroline-DNA complex.¹²⁴

During *in vitro* testing, it is essential to examine the action of a compound over a concentration range that is relevant to its intended use. For example, if the compound is present *in vivo* at low concentrations (less than 1 µmol), its ability to inhibit lipid peroxidation only at high millimolar concentrations is irrelevant unless there is good reason to suspect that it concentrates at a particular site *in vivo*. The same is true if the compound exerts a pro-oxidant effect at high concentrations *in vitro* and is only present at low concentrations and exerts antioxidant action to different species.

Future perspective

When endogenous antioxidant defenses are inadequate for the purpose of scavenging the ROS completely, ongoing oxidative damage to DNA, lipids, proteins and other molecules can be demonstrated. Although ongoing oxidative damage *in vivo* is implicated in the pathology of several human diseases, it is important to appreciate that they may not be the primary cause of these diseases. Although diet-derived and drug-derived antioxidants may be particularly important in protecting against a number of human diseases, and assuming that the active components become bioavailable, the physiological relevance of the compounds, their ability to upregulate defense antioxidants and to modulate gene expression (with respect to synthesis of DNA repair enzymes), affect the cellular transduction mechanisms. With respect to free radical research, how the *in vivo* markers of oxidative stress are affected by antioxidants still needs to be fully evaluated.

In order to exploit the significance of antioxidants, it is critical to understand the features of the molecular components of free radical biology and the interrelationships of these components in mediating tissue injury. Free radicals and oxidants are part of normal human metabolism but when produced in excess, they can cause tissue injury. Tissue injury can itself cause ROS generation, for example, by causing activation of phagocytes or releasing transition metal ions from damaged cells, which may (or may not, depending on the situation) contribute to a worsening of the injury. One could argue that careful use of a range of antioxidants, plant-extracts, plant-derived antioxidants, drug-derived antioxidants, and/or antioxidant vitamins (β-carotene, vitamins E and C) supplements, combined with new methods for measuring oxidant generation in humans, would enable the unequivocal delineation of the exact contribution of ROS generation to disease pathology and to the maintenance of health.⁹ Although the global recommendation of the exact amount of vitamin E, C, β-carotene and/or flavonoids derived from the human diet (rich in fruits and vegetable or from supplements) required for optimal health must await the results of scientific endeavours directed to the issues. In this context, the ultimate goal in the 21st century is to maintain health through nutrition.

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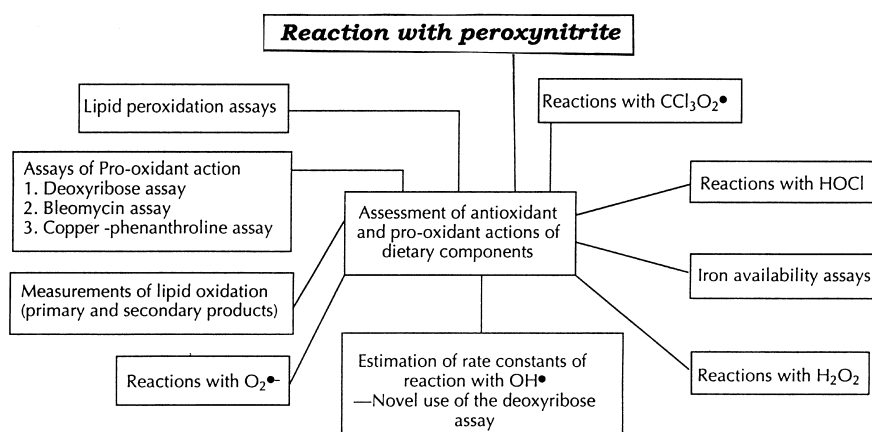


Figure 6. Experimental approaches for the characterization of potential antioxidant and pro-oxidant actions.

Free radicals, antioxidants and international nutrition

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自由基, 抗氧化物以及國際營養

摘要

多不飽和脂肪酸的氧化降解是局限大多數的半成品食品銷售有效期的主要因素。人類的老化過程以及疾病的形成與自由基的機制相關聯。因此建議使用抗氧化物, 特別是來源于植物性食品的抗氧化物來達到保持健康的預防物質。事實上, 抗氧化作用是通過多種途徑而獲得的。因而植物提取物或來源于植物性食品的抗氧化物在人體內的作用(貢獻)描述以及對人體內氧化壓力的病理結果的調節變得更加繁雜。健康促進之所以建議植物食品的質量是因為在人體外觀察到的各種成分在這些植物中的氧化特性。從食品的穩定性來說, 即要有食品的完整性, 又要保證食品分子成分的儲存效果。對人類來講, 應強調營養抗氧化物質在健康和疾病治療方面的重要性。

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