Antioxidants in health and disease

I S Young, J V Woodside

Abstract

Free radical production occurs continuously in all cells as part of normal cellular function. However, excess free radical production originating from endogenous or exogenous sources might play a role in many diseases. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. This article reviews the basic chemistry of free radical formation in the body, the consequences of free radical induced tissue damage, and the function of antioxidant defence systems, with particular reference to the development of atherosclerosis.

(*J Clin Pathol* 2001;**54**:176–186)

Keywords: free radicals; antioxidants; oxidative stress; coronary heart disease; atherosclerosis

Department of Clinical Biochemistry, Institute of Clinical Science, Grosvenor Road, Belfast, Northern Ireland, BT12 6BJ, UK
I S Young

Department of Surgery, Royal Free and University College London Medical School, 67–73 Riding House Street, London, W1P 7LD, UK
J V Woodside

Correspondence to:
Professor Young, Department of Clinical Biochemistry, Institute of Clinical Science, Royal Group of Hospitals, Grosvenor Road, Belfast BT12 6BJ, UK
I.Young@qub.ac.uk

Accepted for publication
5 June 2000

An antioxidant can be defined as: "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate".[1] The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. In recent years, a substantial body of evidence has developed supporting a key role for free radicals in many fundamental cellular reactions and suggesting that oxidative stress might be important in the pathophysiology of common diseases including atherosclerosis, chronic renal failure, and diabetes mellitus. The aim of this review is to consider mechanisms of free radical formation in the body, the consequences of free radical induced tissue damage, and the function of antioxidant defence systems in health and disease.

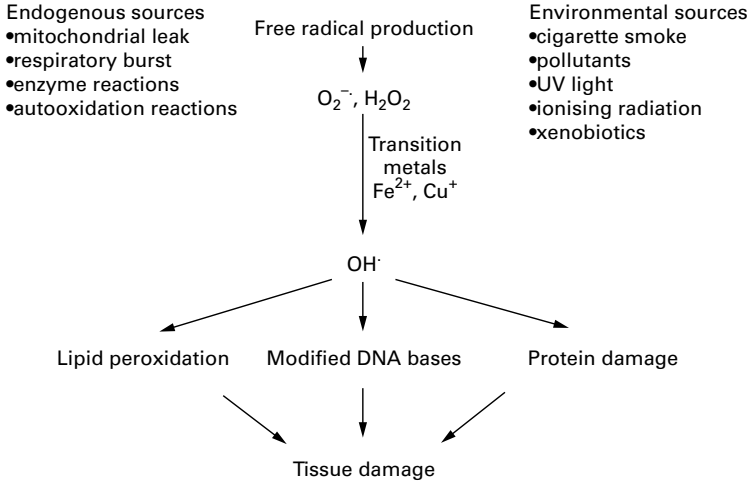

Figure 1 Major sources of free radicals in the body and the consequences of free radical damage.

Free radicals and their chemical reactions

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital.[2] The presence of an unpaired electron results in certain common properties that are shared by most radicals. Radicals are weakly attracted to a magnetic field and are said to be paramagnetic. Many radicals are highly reactive and can either donate an electron to or extract an electron from other molecules, therefore behaving as oxidants or reductants. As a result of this high reactivity, most radicals have a very short half life (10^{-6} seconds or less) in biological systems, although some species may survive for much longer.[2] The most important free radicals in many disease states are oxygen derivatives, particularly superoxide and the hydroxyl radical. Radical formation in the body occurs by several mechanisms, involving both endogenous and environmental factors (fig 1).

Superoxide ($O_2^{-\cdot}$) is produced by the addition of a single electron to oxygen, and several mechanisms exist by which superoxide can be produced in vivo.[3] Several molecules, including adrenaline, flavine nucleotides, thiol compounds, and glucose, can oxidise in the presence of oxygen to produce superoxide, and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mitochondrial membrane performs the reduction of oxygen to water. During this process free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of a few electrons into the mitochondrial matrix and this results in the formation of superoxide.[4] The activity of several other enzymes, such as cytochrome p450 oxidase in the liver and enzymes involved in the synthesis of adrenal hormones, also results in the leakage of a few electrons into the surrounding cytoplasm and hence superoxide formation. There might also be continuous production of superoxide by vascular endothelium to neutralise nitric oxide,[5 6] production of superoxide by other cells to regulate cell growth and differentiation,[7] and the production of superoxide by phagocytic cells during the respiratory burst.[8]

Any biological system generating superoxide will also produce hydrogen peroxide as a result of a spontaneous dismutation reaction. In addition, several enzymatic reactions, including those catalysed by glycolate oxidase and

D-amino acid oxidase, might produce hydrogen peroxide directly.[9] Hydrogen peroxide is not a free radical itself, but is usually included under the general heading of reactive oxygen species (ROS). It is a weak oxidising agent that might directly damage proteins and enzymes containing reactive thiol groups. However, its most vital property is the ability to cross cell membranes freely, which superoxide generally cannot do.[10] Therefore, hydrogen peroxide formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical, which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide. Therefore, hydrogen peroxide acts as a conduit to transmit free radical induced damage across cell compartments and between cells. In the presence of hydrogen peroxide, myeloperoxidase will generate hypochlorous acid and singlet oxygen, a reaction that plays an important role in the killing of bacteria by phagocytes.[11]

The hydroxyl radical ($OH^{\cdot}$), or a closely related species, is probably the final mediator of most free radical induced tissue damage.[12] All of the reactive oxygen species described above exert most of their pathological effects by giving rise to hydroxyl radical formation. The reason for this is that the hydroxyl radical reacts, with extremely high rate constants, with almost every type of molecule found in living cells including sugars, amino acids, lipids, and nucleotides. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is likely to be the transition metal catalysed decomposition of superoxide and hydrogen peroxide.[13]

All elements in the first row of the d-block of the periodic table are classified as transition metals. In general, they contain one or more unpaired electrons and are therefore themselves radicals when in the elemental state. However, their key property from the point of view of free radical biology is their variable valency, which allows them to undergo reactions involving the transfer of a single electron. The most important transition metals in human disease are iron and copper. These elements play a key role in the production of hydroxyl radicals in vivo.[13] Hydrogen peroxide can react with iron II (or copper I) to generate the hydroxyl radical, a reaction first described by Fenton in 1894:

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

This reaction can occur in vivo, but the situation is complicated by the fact that superoxide (the major source of hydrogen peroxide in vivo) will normally also be present. Superoxide and hydrogen peroxide can react together directly to produce the hydroxyl radical, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present a reaction sequence is established that can proceed at a rapid rate:

$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2$$

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

net result:

$$O_2^{-} + H_2O_2 \rightarrow OH^{-} + OH^{\cdot} + O_2$$

The net result of the reaction sequence illustrated above is known as the Haber-Weiss reaction. Although most iron and copper in the body are sequestered in forms that are not available to catalyse this reaction sequence, it is still of importance as a mechanism for the formation of the hydroxyl radical in vivo. The actual reactions, however, may be somewhat more complex than those described above and it is possible that other reactive intermediates such as the ferryl and perferryl radicals might also be formed.[12]

Approximately 4.5 g of iron can be found in the average adult man, most of which is contained in the haemoglobin molecule and other haem containing proteins. Dietary iron is absorbed preferentially from the proximal part of the small intestine in the divalent form and is transferred to the circulation in which it is carried by transferrin.[14] Under most circumstances iron remains tightly bound to one of several proteins, including transferrin, lactoferrin, haem proteins, ferritin, or haemosiderin. In addition, however, it seems likely that a small iron pool will be maintained as complexes with a variety of small molecules, such as nucleotides and citrate within the cytoplasm and subcellular organelles.[14] This pool is probably capable of catalysing an iron driven Fenton reaction in vivo. Certainly, these complexes can promote hydroxyl radical formation in vitro.[15] Redox reactive iron can be measured using the bleomycin iron assay,[16] although it remains unclear to what extent iron detected by this assay correlates with any discrete anatomical or physiological pool. In normal circumstances, no bleomycin reactive iron is detectable in plasma from healthy subjects, implying that transferrin or ferritin bound iron is not available to drive hydroxyl radical production.[17] However, transferrin will release its iron at an acidic pH, particularly in the presence of small molecular weight chelating agents such as ADP, ATP, and citrate.[15] Such conditions are found in areas of active inflammation and during ischaemia reperfusion injury,[18] and it is therefore likely that hydroxyl radicals contribute to tissue damage in these settings. Iron is released from ferritin by reducing agents including ascorbate and superoxide itself,[19 20] and hydrogen peroxide can release iron from a range of haem proteins.[21] Therefore, although the iron binding proteins effectively chelate iron and prevent any appreciable redox effects under normal physiological conditions, this protection can break down in disease states. The role of copper is analogous to that described above for iron.[22 23]

Although free radical production occurs as a consequence of the endogenous reactions described above and plays an important role in normal cellular function, it is important to remember that exogenous environmental factors can also promote radical formation. Ultraviolet light will lead to the formation of singlet oxygen and other reactive oxygen species in the skin.[24] Atmospheric pollutants such as ozone and nitrogen dioxide lead to radical formation and antioxidant depletion in the bronchoalveolar lining fluid, and this may

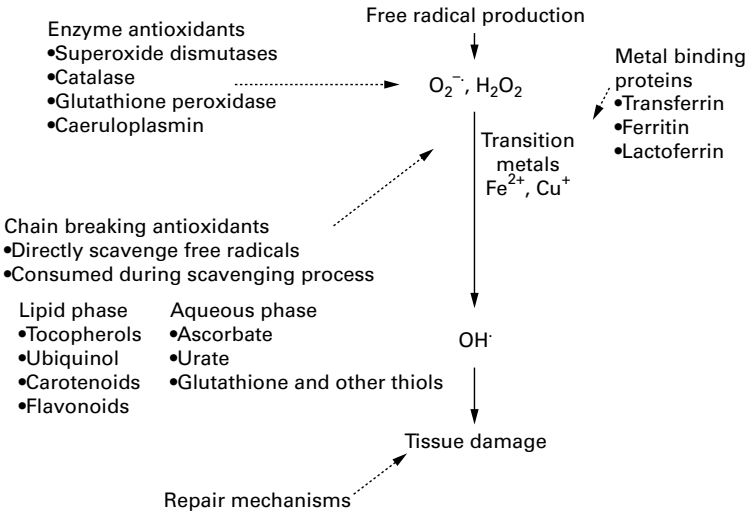

Figure 2 Antioxidant defences against free radical attack. Antioxidant enzymes catalyse the breakdown of free radical species, usually in the intracellular environment. Transition metal binding proteins prevent the interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide producing highly reactive hydroxyl radicals. Chain breaking antioxidants are powerful electron donors and react preferentially with free radicals before important target molecules are damaged. In doing so, the antioxidant is oxidised and must be regenerated or replaced. By definition, the antioxidant radical is relatively unreactive and unable to attack further molecules.

exacerbate respiratory disease.[25–27] Cigarette smoke contains millimolar amounts of free radicals, along with other toxins.[28]

Various xenobiotics also cause tissue damage as a consequence of free radical generation, including paraquat,[29] paracetamol,[30] bleomycin,[31] and anthracyclines.[32]

Antioxidant defence systems

Because radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, an extensive range of antioxidant defences, both endogenous and exogenous, are present to protect cellular components from free radical induced damage. These can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins[2] (fig 2).

THE ANTIOXIDANT ENZYMES

Catalase

Catalase was the first antioxidant enzyme to be characterised and catalyses the two stage conversion of hydrogen peroxide to water and oxygen:

catalase–Fe(III) + H_2O_2 → compound I

compound I + H_2O_2 → catalase–Fe(III) + $2H_2O$ + O_2

Catalase consists of four protein subunits, each containing a haem group and a molecule of NADPH.[33] The rate constant for the reactions described above is extremely high (~10^7 M/sec), implying that it is virtually impossible to saturate the enzyme in vivo. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The amount of catalase in cytoplasm and other subcellular compartments remains unclear, because peroxisomes are easily ruptured during the manipulation of cells. The greatest activity is present in liver and erythrocytes but some catalase is found in all tissues.

Glutathione peroxidases and glutathione reductase

Glutathione peroxidases catalyse the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide[34]:

ROOH + 2GSH → GSSG + H_2O + ROH

Other peroxides, including lipid hydroperoxides, can also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidases require selenium at the active site, and deficiency might occur in the presence of severe selenium deficiency.[35] Several glutathione peroxidase enzymes are encoded by discrete genes.[36] The plasma form of glutathione peroxidase is believed to be synthesised mainly in the kidney.[37] Within cells, the highest concentrations are found in liver although glutathione peroxidase is widely distributed in almost all tissues. The predominant subcellular distribution is in the cytosol and mitochondria, suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular compartments. The activity of the enzyme is dependent on the constant availability of reduced glutathione.[38] The ratio of reduced to oxidised glutathione is usually kept very high as a result of the activity of the enzyme glutathione reductase:

GSSG + NADPH + H^+ → 2GSH + $NADP^+$

The NADPH required by this enzyme to replenish the supply of reduced glutathione is provided by the pentose phosphate pathway. Any competing pathway that utilises NADPH (such as the aldose reductase pathway) might lead to a deficiency of reduced glutathione and hence impair the action of glutathione peroxidase. Glutathione reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution to glutathione peroxidase.[39]

Superoxide dismutase

The superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide:

$O_2^- + O_2^- + 2H+ \rightarrow H_2O_2 + O_2$

The hydrogen peroxide must then be removed by catalase or glutathione peroxidase, as described above. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.

(1) Copper zinc superoxide dismutase (CuZnSOD): CuZnSOD is found in the cytoplasm and organelles of virtually all mammalian cells.[40] It has a molecular mass of approximately 32 000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom.

(2) Manganese superoxide dismutase (MnSOD): MnSOD is found in the mitochondria of almost all cells and has a molecular mass of 40 000 kDa.[41] It consists of four protein subunits, each probably containing a single manganese atom. The amino acid sequence of MnSOD is entirely dissimilar to that of CuZnSOD

and it is not inhibited by cyanide, allowing MnSOD activity to be distinguished from that of CuZnSOD in mixtures of the two enzymes.

(3) Extracellular superoxide dismutase (EC-SOD): EC-SOD was described by Marklund in 1982[42] and is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above. EC-SOD is synthesised by only a few cell types, including fibroblasts and endothelial cells, and is expressed on the cell surface where it is bound to heparan sulphates. EC-SOD is the major SOD detectable in extracellular fluids and is released into the circulation from the surface of vascular endothelium following the injection of heparin.[43] EC-SOD might play a role in the regulation of vascular tone, because endothelial derived relaxing factor (nitric oxide or a closely related compound) is neutralised in the plasma by superoxide.[44]

THE CHAIN BREAKING ANTIOXIDANTS

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then react with other targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralised by a chain breaking antioxidant.[45] Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts.[46] In general, the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing the further propagation of the chain reaction. Such antioxidants can be conveniently divided into aqueous phase and lipid phase antioxidants.

Lipid phase chain breaking antioxidants

These antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E.[47] Vitamin E occurs in nature in eight different forms, which differ greatly in their degree of biological activity. The tocopherols (α, β, γ, and δ) have a chromanol ring and a phytyl tail, and differ in the number and position of the methyl groups on the ring. The tocotrienols (α, β, γ, and δ) are structurally similar but have unsaturated tails. Both classes of compounds are lipid soluble and have pronounced antioxidant properties.[48] They react more rapidly than polyunsaturated fatty acids with peroxyl radicals and hence act to break the chain reaction of lipid peroxidation. In addition to its antioxidant role, vitamin E might also have a structural role in stabilising membranes.[49] Frank vitamin E deficiency is rare in humans, although it might cause haemolysis[50] and might contribute to the peripheral neuropathy that occurs in abetalipoproteinaemia.[51]

The absorption, transport, and regulation of plasma concentrations of vitamin E in humans has been reviewed by Kayden and Traber,[52] although in general the metabolism of vitamin E is not well described. In cell membranes and lipoproteins the essential antioxidant function of vitamin E is to trap peroxyl radicals and to break the chain reaction of lipid peroxidation.[53] Vitamin E will not prevent the initial formation of carbon centred radicals in a lipid rich environment, but does minimise the formation of secondary radicals. α-Tocopherol is the most potent antioxidant of the tocopherols and is also the most abundant in humans. It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. This resonance stabilised radical might subsequently react in one of several ways. α-Tocopherol might be regenerated by reaction at the aqueous interface with ascorbate[54] or another aqueous phase chain breaking antioxidant, such as reduced glutathione or urate.[55] Alternatively, two α-tocopheroxyl radicals might combine to form a stable dimer, or the radical may be completely oxidised to form tocopherol quinone.

The carotenoids are a group of lipid soluble antioxidants based around an isoprenoid carbon skeleton.[56] The most important of these is β-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen,[57] but can also trap peroxyl radicals at low oxygen pressure with an efficiency at least as great as that of α-tocopherol. Because these conditions prevail in many biological tissues, the carotenoids might play a role in preventing in vivo lipid peroxidation.[58] The other important role of certain carotenoids is as precursors of vitamin A (retinol). Vitamin A also has antioxidant properties,[59] which do not, however, show any dependency on oxygen concentration.

Flavonoids are a large group of polyphenolic antioxidants found in many fruits, vegetables, and beverages such as tea and wine.[60–62] Over 4000 flavonoids have been identified and they are divided into several groups according to their chemical structure, including flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). Epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases such as coronary heart disease (CHD).[63–65] However, little is currently known about the absorption and metabolism of flavonoids and epidemiological associations might be a consequence of confounding by other factors. Available evidence suggests that the bioavailability of many flavonoids is poor,[66–68] and plasma values very low, although there is some evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage.[68 69] Apart from flavonoids, other dietary phenolic

compounds might also make a small contribution to total antioxidant capacity.[70]

Ubiquinol-10, the reduced form of coenzyme Q10, is also an effective lipid soluble chain breaking antioxidant.[71] Although present in lower concentrations than α-tocopherol, it can scavenge lipid peroxyl radicals with higher efficiency than either α-tocopherol or the carotenoids, and can also regenerate membrane bound α-tocopherol from the tocopheryl radical.[72] Indeed, whenever plasma or isolated low density lipoprotein (LDL) cholesterol is exposed to radicals generated in the lipid phase, ubiquinol-10 is the first antioxidant to be consumed, suggesting that it might be of particular importance in preventing the propagation of lipid peroxidation.[73] However, work to clarify further its role has been hampered by the ease with which ubinquinol-10 becomes oxidised during sample handling or analysis.

Aqueous phase chain breaking antioxidants

These antioxidants will directly scavenge radicals present in the aqueous compartment. Qualitatively the most important antioxidant of this type is vitamin C (ascorbate).[74] In humans, ascorbate acts as an essential cofactor for several enzymes catalysing hydroxylation reactions. In most cases, it provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity. Its best known role is as a cofactor for prolyl and lysyl oxidases in the synthesis of collagen. However, in addition to these well defined actions, several other biochemical pathways depend upon the presence of ascorbate.[75] In addition to its role as an enzyme cofactor, the other major function of ascorbate is as a key chain breaking antioxidant in the aqueous phase.[76] Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals, and singlet oxygen. During its antioxidant action, ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate. The semidehydroascorbyl radical is relatively stable owing to dispersion of the charge associated with the presence of a single electron over the three oxygen atoms, and it can be readily detected by electron spin resonance in body fluids in the presence of increased free radical production.[77] Dehydroascorbate is relatively unstable and hydrolyses readily to diketogulonic acid, which is subsequently broken down to oxalic acid. Two mechanisms have been described by which dehydroascorbate can be reduced back to ascorbate; one is mediated by the selenoenzyme thioredoxin reductase[78] and the other is a non-enzyme mediated reaction that uses reduced glutathione.[79] Dehydroascorbate in plasma is probably rapidly taken up by red blood cells before recycling, so that very little, if any, dehydroascorbate is present in plasma.[80]

Apart from ascorbate, other antioxidants are present in plasma in high concentrations. Uric acid efficiently scavenges radicals, being converted in the process to allantoin.[81] Urate might be particularly important in providing protection against certain oxidising agents, such as ozone.[82] Indeed, it has been suggested that the increase in life span that has occurred during human evolution might be partly explained by the protective action provided by uric acid in human plasma.[83] Part of the antioxidant effect of urate might be attributable to the formation of stable non-reactive complexes with iron, but it is also a direct free radical scavenger. Albumin bound bilirubin is also an efficient radical scavenger,[84] and it has been suggested that it might play a particularly crucial role in protecting the neonate from oxidative damage,[85] because deficiency of other chain breaking antioxidants is common in the newborn.

The other major chain breaking antioxidants in plasma are the protein bound thiol groups. The sulphydryl groups present on plasma proteins can function as chain breaking antioxidants by donating an electron to neutralise a free radical, with the resultant formation of a protein thiyl radical. Albumin is the predominant plasma protein and makes the major contribution to plasma sulphydryl groups, although it also has several other antioxidant properties.[86] Albumin contains 17 disulphide bridges and has a single remaining cysteine residue, and it is this residue that is responsible for the capacity of albumin to react with and neutralise peroxyl radicals.[87] This property is important in view of the role albumin plays in transporting free fatty acids in the blood. In addition, albumin has the capacity to bind copper ions and will inhibit copper dependent lipid peroxidation and hydroxyl radical formation. It is also a powerful scavenger of the phagocytic product hypochlorous acid, and provides the main plasma defence against this oxidant.[88]

Because albumin itself is damaged when it acts as an antioxidant, it has been viewed as a sacrificial molecule that prevents damage occurring to more vital species.[86] The high plasma concentration of albumin and a relatively short half life mean that any damage suffered is unlikely to be of biological importance. However, in vitro work has shown that protein thiyl radicals can themselves act as a potential source of reactive oxidants. The thiyl radical can abstract an electron from a polyunsaturated fatty acid to initiate the process of lipid peroxidation,[89] a reaction that can be inhibited by ascorbate and retinol. The antioxidant effects of albumin and other proteins have been shown to decrease at high concentrations and it has been suggested that this is because thiyl radicals can oxidatively damage other molecules. The importance of these findings to the antioxidant role of albumin in vivo remains unclear.

Reduced glutathione (GSH) is a major source of thiol groups in the intracellular compartment but is of little importance in the extracellular space.[90] GSH might function directly as an antioxidant, scavenging a variety of radical species, as well as acting as an essential factor for glutathione peroxidase (discussed above). Thioredoxin might also function as a

key intracellular antioxidant, particularly in redox induced activation of transcription factors.[91]

INTERACTIONS BETWEEN CHAIN BREAKING ANTIOXIDANTS

Although the actions of chain breaking antioxidants have been considered separately above, it is vital to remember that in vivo complex interactions between antioxidants are likely to occur. For instance, it is likely that ascorbate will recycle the tocopheryl radical at the aqueous–lipid interface, so regenerating tocopherol.[54] This might be crucial in ensuring that tocopherol concentrations are maintained in lipoproteins and membranes. In a similar manner, glutathione can regenerate ascorbate from dehydroascorbate. A complex interplay is therefore likely to exist between antioxidants, making it difficult to predict how antioxidants will function in vivo. It therefore becomes meaningless to ask which antioxidant is most important: the answer will depend on the circumstances existing in a particular microenvironment at a specific time, and on the nature of the oxidant injury taking place.

A second important property of chain breaking antioxidants is their ability to act as pro-oxidants. In certain circumstances, the presence of an antioxidant might paradoxically lead to increased oxidative damage. For instance, it has been reported that the administration of vitamin C can sometimes lead to an increase in oxidative damage, particularly if iron is also administered.[92] Similarly, it has been clearly shown in vitro that tocopherol might promote LDL oxidation in the absence of an aqueous phase antioxidant such as ascorbate.[93] Whether these reactions are important in vivo is as yet unclear. However, the possibility that antioxidants may have pro-oxidant effects in vivo must be considered when designing and interpreting the results of clinical trials of antioxidant supplementation.

THE TRANSITION METAL BINDING PROTEINS

As discussed above, transition metal binding proteins (ferritin, transferrin, lactoferrin, and caeruloplasmin) act as a crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein, caeruloplasmin, might also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron.[94]

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

Fe^{2+} is the form of iron that drives the Fenton reaction and the rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect.

Consequences of oxidative damage

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defences, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Lipoprotein particles or membranes characteristically undergo the process of lipid peroxidation, giving rise to a variety of products including short chain aldehydes such as malondialdehyde or 4-hydroxynonenal, alkanes, and alkenes, conjugated dienes, and a variety of hydroxides and hydroperoxides.[45] Many of these products can be measured as markers of lipid peroxidation. Detailed discussion of this complex issue is outside the scope of this review, but the measurement of isoprostanes by gas chromatography mass spectroscopy is probably the most specific marker of free radical damage to lipids.[95] Oxidative damage to proteins and nucleic acids similarly gives rise to a variety of specific damage products as a result of modifications of amino acids or nucleotides.[45] Such oxidative damage might also lead to cellular dysfunction, and it is this that might contribute to the pathophysiology of a wide variety of diseases.

Oxidative stress and disease

A role for oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory conditions,[96] certain cancers,[97] and the process of aging.[98] In many cases, this follows the observation of increased amounts of free radical damage products, particularly markers of lipid peroxidation, in body fluids. It is important to remember, however, that lipid peroxidation is an inevitable accompaniment of cell death from any cause. In most cases peroxidation is a secondary phenomenon, and this does not therefore directly indicate an important role for oxidative stress in the disease concerned. If a primary role for oxidative stress in a particular setting is to be sustained, there should be a plausible mechanism by which increased free radical production or a decrease in antioxidant defences might occur. In addition, evidence of oxidative stress should be detectable before the onset of tissue damage and augmentation of antioxidant status at an early stage should either prevent or greatly reduce tissue damage.

Atherosclerosis can be taken as an example of a process for which there is substantial evidence of a role for oxidative stress. Hypercholesterolaemia is universally accepted as a major risk factor for atherosclerosis. However, at any given concentration of plasma cholesterol, there is still great variability in the occurrence of cardiovascular events. One of the major breakthroughs in atherogenesis research has been the realisation that oxidative modification of LDL might be a crucially important step in the development of the atherosclerotic plaque.[99 100] The formation of foam cells from monocyte derived macrophages in early atherosclerotic lesions is not caused by native LDL but only after the modification of LDL by various chemical reactions such as oxidation. Oxidation of LDL is a process initiated and propagated by free radicals or by one of several enzymes,[101] and is believed to occur mainly in the arterial wall in a microenvironment where antioxidants may become depleted. All the cells of the vessel wall—endothelial cells, smooth muscle cells, macrophages, and lymphocytes—

can modify LDL in vitro.[102–104] Several mechanisms are likely to be involved, including transition metal ion mediated generation of hydroxyl radicals, the production of reactive oxygen species by enzymes such as myeloperoxidase and lipoxygenase, and direct modification by reactive nitrogen species. Because oxidation of LDL is primarily a free radical mediated process that is inhibited by antioxidants, antioxidant depletion might be a risk factor for cardiovascular disease (CVD).

Evidence for LDL oxidation in vivo is now well established. In immunocytochemical studies, antibodies against oxidised LDL stain atherosclerotic lesions but not normal arterial tissue.[105] LDL extracted from animal and human lesions has been shown to be oxidised and is rapidly taken up by macrophage scavenger receptors.[106] In young survivors of myocardial infarction (MI), an association has been demonstrated between increased susceptibility of LDL to oxidation and the degree of coronary atherosclerosis,[107] whereas the presence of ceroid, a product of lipid peroxidation, has been shown in advanced atherosclerotic plaques.[108]

Oxidised LDL has several properties that promote atherogenesis, apart from its rapid uptake into macrophages via the scavenger receptor. Oxidised forms of LDL are chemotactic for circulating macrophages and smooth muscle cells and facilitate monocyte adhesion to the endothelium and entry into the subendothelial space.[109] Oxidised LDL is also cytotoxic towards arterial endothelial cells[110] and inhibits the release of nitric oxide and the resulting endothelium dependent vasodilation.[111] Therefore, there is a potential role for oxidised LDL in altering vasomotor responses, perhaps contributing to vasospasm in diseased vessels. In addition, oxidised LDL is immunogenic; autoantibodies against various epitopes of oxidised LDL have been found in human serum.[112 113] and immunoglobulin (IgG) specific for epitopes of oxidised LDL can be found in lesions.[114] Oxidised LDL can induce arterial wall cells to produce chemotactic factors, adhesion molecules, cytokines, and growth factors that have a role to play in the development of the plaque.[115 116]

Apart from the atherogenic consequences of LDL oxidation, it is increasingly recognised that reactive oxygen and nitrogen species directly interact with signalling mechanisms in the arterial wall to regulate vascular function.[117] The activities of oxidant generating enzymes in the arterial wall are regulated by both receptor activation and by non-receptor mediated pathways. The effects of antioxidants on these processes are complex but provide alternative mechanisms by which antioxidant supplementation might ameliorate vascular pathology, for instance by improving endothelial function.

Evidence that antioxidant micronutrients potentially reduce the risk of CHD comes from four major sources. First, studies of antioxidant supplementation in animal models of atherosclerosis have generally shown a reduction in disease.[118 119] Second, many studies have now shown that antioxidant supplementation in healthy subjects or patients with CHD can reduce levels of free radical damage products and protect LDL against oxidation.[120 121] Vitamin E appears to be the most effective antioxidant; both β-carotene and vitamin C have produced extensions in lag time to oxidation only in a few studies, although it remains possible that they might have a beneficial effect in individuals with poor baseline status. Third, large scale epidemiological studies generally show that low intakes of antioxidants are associated with increased cardiovascular risk after correcting for other risk factors.[122–125] The epidemiological evidence is strongest in the case of vitamin E. In particular, two large longitudinal studies in the USA examined the association between antioxidant intake and the risk of CHD. In a group of 39 910 male health professionals, men who took vitamin E supplements in doses of at least 100 IU/day for over two years had a 37% lower relative risk of CHD than those who did not take vitamin E supplements, after adjustment for age, coronary risk factors, and intake of vitamin C and β-carotene.[126] In the nurses' health study of 87 245 female nurses, women who took vitamin E supplements for more than two years had a 41% lower relative risk of major coronary disease.[127] This effect persisted after adjustment for age, smoking, obesity, exercise, blood pressure, cholesterol, and the use of postmenopausal oestrogen replacement, aspirin, vitamin C, and β-carotene. High vitamin E intakes from dietary sources were not associated with a significant decrease in risk, although even the highest dietary vitamin E intakes were far lower than intakes among supplement users.

The evidence linking the water soluble vitamin C with CVD is less strong than for vitamin E. In the physicians' follow-up study, a high intake of vitamin C was not associated with a lower risk of CHD in men, whereas in women from the nurses' health survey, an initial effect was attenuated after adjustment for multivitamin use. Only one prospective study involving 11 348 adults demonstrated an inverse relation between vitamin C intake and overall cardiovascular mortality.[128] This effect resulted largely from the use of vitamin C in supplements and might have been caused by other antioxidant vitamins in multivitamin preparations. A prospective population study of 1605 healthy men aged 42, 48, 54, or 60 years in Finland has recently shown that men who had vitamin C deficiency had a relative risk of MI of 2.5 compared with men with higher plasma vitamin C concentrations, after adjustment for other risk factors.[129] There is also some indication that increased dietary intake of β-carotene is associated with reduced risk of CHD, although again the evidence is less convincing than that for vitamin E. In the prospective nurses' health survey, consumption of vitamins A and β-carotene in food and supplements weakly predicted the incidence of CHD; Gaziano and Hennekens calculated a 22% risk reduction for women in the highest quintile of β-carotene compared with those in the lowest.[130]

Thus, there is a plausible case supported by experimental studies, animal experiments, and epidemiology linking oxidative stress and atherosclerosis. The key test of such a hypothesis is whether increased antioxidant intake can be shown to prevent the clinical manifestations of atherosclerosis in humans. Several published randomised studies have now considered this issue, and others are currently ongoing. Early results have not been encouraging.

The α-tocopherol, β-carotene cancer prevention trial (ATBC), conducted among 29 133 male heavy smokers in Finland, found no reduction in CHD morbidity or mortality during five to eight years of treatment with vitamin E (50 mg daily) or β-carotene (20 mg daily).[131] Those assigned vitamin E had no significant decrease in deaths from ischaemic heart disease (IHD), but a 50% excess of deaths from cerebral haemorrhage, whereas those assigned to β-carotene experienced an 11% increase in deaths from IHD. In a further analysis, a subgroup of the original subjects who had suffered a previous MI were considered.[132] The endpoint of this substudy was the first major coronary event after randomisation. The proportion of major coronary events did not decrease with either α-tocopherol or β-carotene supplements. In fact, β-carotene conferred an excess of fatal IHD (75% increase in risk). There was a beneficial effect of vitamin E on non-fatal MI with a risk reduction of 38%. By contrast, in the Chinese cancer prevention study conducted among 29 584 poorly nourished residents of Linxian, China, those randomised for 5.25 years to a combined regimen of 15 mg/day β-carotene, 30 mg/day vitamin E, and 50 μg/day selenium had a significant 9% reduction in total mortality, a significant 21% decrease in stomach cancer deaths, and a non-significant 10% decrease in cerebrovascular mortality.[133] However, the wisdom of generalising these findings to well nourished populations remains uncertain.

The β-carotene and retinol efficacy trial (CARET), designed to test the effects of a combined supplement of 30 mg β-carotene and 25 000 IU retinol daily among 18 314 cigarette smokers and individuals with occupational asbestos exposure, was ended early when researchers recognised a raised risk of death from lung cancer in those receiving β-carotene and, again, no beneficial effect on CVD was found.[134] For CVD mortality, there was a non-significant 26% increase in the treated group ($p = 0.06$).

The physicians' health study followed more than 22 000 US male doctors treated with 50 mg β-carotene or placebo every other day for an average of 12 years. The trial appears to have been conducted meticulously and its results seriously question any beneficial effect with such supplementation on CVD in well nourished populations. There were no significant effects on individual outcomes, or on a combined endpoint of non-fatal MI, non-fatal stroke, and cardiovascular death, for which the relative risk was 1.0 (95% confidence interval, 0.91 to 1.09).[135] There was also no evidence of harm (or benefit) among the 11% of participants who were current smokers at baseline, although small effects could not be ruled out.

Greenberg *et al* studied the effect of β-carotene supplementation (50 mg/day) in 1720 male and female subjects for a median period of 4.3 years with a median follow up of 8.2 years.[136] Subjects whose plasma values of β-carotene were in the highest quartile at the beginning of the study had the lowest risk of death from all causes compared with those in the lowest quartile. However, supplementation had no effect on either all cause or cardiovascular mortality. Thus for β-carotene supplementation, it would appear that there are no overall benefits among those individuals with a good nutritional status who are at low or average risk of developing CHD. The situation might be different, however, for those with a previous history of such disease.

Hodis *et al* have shown a reduction in CAD progression (as measured angiographically) in men given 100 IU vitamin E daily, although no benefit was found for vitamin C.[137] Singh *et al* found that a combination of vitamins A, C, E, and β-carotene administered within a few hours after acute MI and continued for 28 days led to significantly fewer cardiac events and a lower incidence of angina pectoris in the supplemented group.[138] The Cambridge heart antioxidant study (CHAOS), a trial of vitamin E supplementation on 2002 patients with angiographic evidence of coronary disease, was carried out with a mean treatment duration of 1.4 years.[139] It was found that this short term supplementation with α-tocopherol (268 or 537 mg/day) reduced CHD morbidity in patients, in that patients had a significantly (77%) decreased risk of subsequent non-fatal MI. However, no benefit was found in terms of cardiovascular mortality, with a non-significant excess among vitamin E allocated participants.

The GISSI-P study randomised 11 324 men surviving a myocardial infarction to 300 mg vitamin E, 1 g n-3 polyunsaturated fatty acids (PUFAs), both, or neither in a randomised, placebo controlled trial.[140] Results suggested a beneficial effect of n-3 PUFAs but no benefit with vitamin E ($p = 0.07$). However, further analysis of secondary endpoints suggested some beneficial effects of vitamin E. In addition, the effect of vitamin E might have been ameliorated by the Mediterranean diet of the subjects. Neither of these qualifications holds true for the HOPE study,[141] which recruited over 9000 subjects likely to be eating a typical northern European diet, who were at high risk for cardiovascular events because they had CVD or diabetes in addition to one other risk factor. Subjects were randomly assigned according to a two by two factorial design to receive either 400 IU of vitamin E daily from natural sources or matching placebo, and either an angiotensin converting enzyme inhibitor (ramipril) or matching placebo for a mean of 4.5 years. Vitamin E supplementation had no effect on primary or secondary cardiovascular endpoints.

Thus, for vitamin E in Western populations, the only available trial data in primary prevention are from the ATBC trial, which show no effect. In secondary prevention, the accumulating trial data for vitamin E are less consistent, although not particularly encouraging. The CHAOS study was positive, although it suffers from design limitations. The GISSI-P study gave a borderline result, whereas the HOPE study was unequivocally negative.

How should we interpret the discordance between data from cohort studies and the results so far available from clinical trials? In general, it might be that the duration of clinical trials is too short to show a benefit, and that antioxidant intake over many years is required to prevent atherosclerosis. Thought needs to be given to trial design, with dose, duration of treatment and follow up period, initial antioxidant values and dietary intake, and extent and distribution of existing atherosclerosis being taken into consideration. Animal models have nearly always tested the effects of antioxidants on the early atherosclerotic lesions. Whether or not antioxidants have inhibitory effects on the later stages remains to be seen. In addition, the complex mixture of antioxidant micronutrients found in a diet high in fruit and vegetable intake might be more effective than large doses of a small number of antioxidant vitamins. It could be that several of these compounds work together but have no effect individually, or that other dietary components (such as trace elements) might be effectors of antioxidant action. The trial evidence available so far relates only to α-tocopherol and β-carotene. Although effective at protecting against lipid peroxidation, these antioxidants have little effect on arterial endothelial function. Ascorbate, in contrast, seems more effective in improving endothelial function, although there is less epidemiological support for a protective effect of ascorbate.

Alternatively, the significant results linking antioxidant intake with CHD risk observed in cohort studies might be the result of confounding with other lifestyle behaviours. Slattery *et al* examined dietary antioxidants and plasma lipids in the coronary artery risk development in young adults (CARDIA) study and found that a higher intake of antioxidants was associated with other lifestyle factors such as physical activity and non-smoking.[142] Plasma concentrations of antioxidants are linked with social class, being higher in more affluent groups. Although these variables can be individually controlled for in analyses, it might be that a complex lifelong behaviour pattern needs to be studied before conclusions regarding antioxidants and CHD can be made. For example, passive smoking has recently been shown to have an atherogenic effect on LDL,[143] yet exposure to smoke is a difficult lifestyle variable to control for in cohort analyses.

Conclusions

There is overwhelming evidence that oxidative stress occurs in cells as a consequence of normal physiological processes and environmental interactions, and that the complex web of antioxidant defence systems plays a key role in protecting against oxidative damage. These processes appear to be disordered in many conditions, and a plausible hypothesis may be constructed implicating oxidative stress as a cause of tissue damage. However, as illustrated by the example of CHD, attempts to intervene therapeutically by using antioxidant supplements have so far been largely unsuccessful. A more complete understanding of the biochemical events occurring at a cellular level to influence oxidative damage is required to guide future therapeutic advances.

1 Halliwell B, Gutteridge JC. The definition and measurement of antioxidants in biological systems. *Free Radic Biol Med* 1995;**18**:125–6.
2 Halliwell B; Gutteridge JM. *Free radicals in biology and medicine*, 2nd ed. Oxford: Clarendon Press, 1989.
3 Halliwell B, Gutteridge JC. Biologically relevant metal ion-dependent hydroxyl radical generation—an update. *FEBS Lett* 1992;**307**:108–12.
4 Becker LB, Vanden Hoek TL, Shao ZH, *et al*. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am J Physiol* 1999;**277**:H2240–6.
5 Barbacanne MA, Margeat E, Arnal JF, *et al*. Superoxide release by confluent endothelial cells, an electron spin resonance (ESR) study. *J Chim Phys Phys Chim Biol* 1999;**96**:85–92.
6 Tsao PS, Heidary S, Wang A, *et al*. Protein kinase C-epsilon mediates glucose-induced superoxide production and MCP-1 expression in endothelial cells. *FASEB J* 1998;**12**: 512.
7 Masters CJ. Cellular signalling: the role of the peroxisome. *Cell Signal* 1996;**8**:197–208.
8 Curnutte JT, Babior BM. Chronic granulomatous disease. *Adv Hum Genet* 1987;**16**:229–45.
9 Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979;**59**:527–605.
10 Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990;**186**:1–85.
11 Tatsuzawa H, Maruyama T, Hori K, *et al*. Singlet oxygen as the principal oxidant in myeloperoxidase-mediated bacterial killing in neutrophil phagosomes. *Biochim Biophys Res Commun* 1999;**262**:647–50.
12 Lloyd RV, Hanna PM, Mason RP. The origin of the hydroxyl radical oxygen in the Fenton reaction. *Free Radic Biol Med* 1997;**22**:885–8.
13 Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* 1995;**18**:321–36.
14 Goldenberg HA. Regulation of mammalian iron metabolism: current state and need for further knowledge. *Crit Rev Clin Lab Sci* 1997;**34**:529–72.
15 Aruoma OI, Halliwell B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. *Biochem J* 1987; **241**:273–8.
16 Gutteridge JM, Rowley DA, Halliwell B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. *Biochem J* 1981;**199**:263–5.
17 Gutteridge JC, Quinlan GJ, Evans TW. Transient iron overload with bleomycin detectable iron in the plasma of patients with adult respiratory distress syndrome. *Thorax* 1994;**49**:707–10.
18 Pepper JR, Mumby S, Gutteridge JC. Sequential oxidative damage, and changes in iron binding and iron oxidizing plasma antioxidants during cardiopulmonary bypass surgery. *Free Radic Res* 1994;**21**:377–85.
19 Gutteridge JMC, Quinlan GJ. Malondialdehyde formation from lipid hydroperoxides in the thiobarbituric acid test: the role of lipid radicals, iron salts and metal chelators. *J Appl Biochem* 1983;**5**:293–9.
20 O'Connell M, Halliwell B, Moorhouse C, *et al*. Formation of hydroxyl radicals in the presence of ferritin and haemosiderin. Is haemosiderin formation a biological protective mechanism? *Biochem J* 1986;**234**:727–31.
21 Benatti U, Morelli A, Guida L, *et al*. The production of activated oxygen species by an interaction of methemoglobin with ascorbate. *Biochem Biophys Res Commun* 1983;**111**: 980–7.
22 Swain J, Gutteridge JM. Prooxidant iron and copper, with ferroxidase and xanthine oxidase activities in human atherosclerotic material. *FEBS Lett* 1995;**368**:513–15.
23 Koizumi M, Fujii J, Suzuki K, *et al*. A marked increase in free copper levels in the plasma and liver of LEC rats: an animal model for Wilson disease and liver cancer. *Free Radic Res* 1998;**28**:441–50.
24 McCaughan JS. Photodynamic therapy—a review. *Drugs Aging* 1999;**15**:49–68.
25 Cross CE, Van der Vielt A, O'Neill C, *et al*. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994;**102**:185–91.
26 Kelly FJ, Mudway I, Krishna MT, *et al*. The free radical basis of air pollution focus on ozone. *Respir Med* 1995;**89**:647–56.

27 Kelly FJ, Tetley TD. Nitrogen dioxide depletes uric acid and ascorbic acid but not glutathione from lung lining fluid. *Biochem J* 1997;**325**:95–9.
28 Pourcelot S, Faure H, Firoozi F, *et al*. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine and 5- (hydroxymethyl) uracil in smokers. *Free Radic Res* 1999;**30**:173–80.
29 Wilhelm J, Frydrychova M, Vizek M. Hydrogen peroxide in the breath of rats: the effects of hypoxia and paraquat. *Physiol Res* 1999;**48**:445–9.
30 Kapiotis S, Sengoelge G, Hermann M, *et al*. Paracetamol catalyzes myeloperoxidase-initiated lipid oxidation in LDL. *Arterioscler Thromb Vasc Biol* 1997;**17**:2855–60.
31 Weijl NI, Cleton FJ, Osanto S. Free radicals and antioxidants in chemotherapy-induced toxicity. *Cancer Treat Rev* 1997;**23**:209–40.
32 Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999;**57**:727–41.
33 Kirkman HN, Galiano S, Gaetani GF. The function of catalase-bound NADPH. *J Biol Chem* 1987;**262**:660–5.
34 Takahashi K, Cohen HJ. Selenium-dependent glutathione peroxidase protein and activity: immunological investigations on cellular and plasma enzymes. *Blood* 1986;**68**:640–6.
35 Nakane T, Asayama K, Kodera K, *et al*. Effect of selenium deficiency on cellular and extracellular glutathione peroxidases: immunochemical detection and mRNA analysis in rat kidney and serum. *Free Radic Biol Med* 1998;**25**:504–11.
36 Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* 1999;**27**:951–65.
37 Roxborough HE, Mercer C, McMaster D, *et al*. Plasma glutathione peroxidase activity is reduced in haemodialysis patients. *Nephron* 1999;**81**:278–83.
38 Holben DH, Smith AM. The diverse role of selenium within selenoproteins: a review. *J Am Diet Assoc* 1999;**99**:836–43.
39 Gibson DG, Hawrylko J, McCay PB. GSH-dependent inhibition of lipid peroxidation: properties of a potent cytosolic system which protects cell membranes. *Lipids* 1985;**20**:704–10.
40 Liou W, Chang L-Y, Geuze HJ, *et al*. Distribution of CuZn superoxide dismutase in rat liver. *Free Rad Biol Med* 1993;**14**:201–7.
41 Weisiger RA, Fridovich I. Mitochondrial superoxide dismutase: site of synthesis and intramitochondrial location. *J Biol Chem* 1973;**248**:4793–6.
42 Marklund S. Human copper-containing superoxide dismutase of high molecular weight. *Proc Natl Acad Sci U S A* 1982;**79**:7634–8.
43 Karlsson K, Sandstrom J, Edlund A, *et al*. Pharmacokinetics of extracellular superoxide dismutase in the vascular system. *Free Radic Biol Med* 1993;**14**:185–90.
44 McIntyre M, Bohr DF, Dominiczak AF. Endothelial function hypertension—the role of superoxide anion. *Hypertension* 1999;**34**:539–45.
45 De Zwart LL, Meerman JN, Commandeur JM, *et al*. Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med* 1999;**26**:202–26.
46 Halliwell B. How to characterize an antioxidant: an update. *Biochem Soc Symp* 1995;**61**:73–101.
47 Esterbauer H, Dieber-Rotheneder M, Striegl G, *et al*. Role of vitamin E in preventing the oxidation of low-density lipoprotein. *Am J Clin Nutr* 1991;**53**:314s–21s.
48 Horwitt MH. Data supporting supplementation of humans with vitamin E. *J Nutr* 1991;**121**:424–9.
49 Urano S, Inomori Y, Sugawara T, *et al*. Vitamin-E—inhibition of retinol-induced hemolysis and membrane-stabilizing behavior. *J Biol Chem* 1992;**267**:18365–70.
50 Swann IL, Kendra JR. Anaemia, vitamin E deficiency and failure to thrive in an infant. *Clin Lab Haematol* 1998;**20**:61–3.
51 Sokol RJ. Vitamin E deficiency and neurologic disease. *Annu Rev Nutr* 1988;**8**:351–73.
52 Kayden HJ, Traber MG. Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. *J Lipid Res* 1993;**34**:343–58.
53 Burton GW, Ingold KU. Vitamin E: applications of the principles of physical organic chemistry to the exploration of its structure and function. *Acc Chem Res* 1986;**19**:194–201.
54 May JM, Qu ZC, Mendiratta S. Protection and recycling of alpha-tocopherol in human erythrocytes by intracellular ascorbic acid. *Arch Biochem Biophys* 1998;**349**:281–9.
55 Kagan VE, Tyurina YY. Recycling and redox cycling of phenolic antioxidants. Towards prolongation of the healthy life span. *Ann N Y Acad Sci* 1998;**854**:425–34.
56 Cooper DA, Eldridge AL, Peters JC. Dietary carotenoids and certain cancers, heart disease, and age-related macular degeneration: a review of recent research. *Nutr Rev* 1999;**57**:201–14.
57 Fukuzawa K, Inokami Y, Tokumura A, *et al*. Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes. *Lipids* 1998;**33**:751–6.
58 Chaudiere J, Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. *Food Chem Toxicol* 1999;**37**:949–62.
59 Keys SA, Zimmerman WF. Antioxidant activity of retinol, glutathione, and taurine in bovine photoreceptor cell membranes. *Exp Eye Res* 1999;**68**:693–702.
60 Rice-Evans CA, Miller NJ, Paganga G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 1996;**20**:933–56.
61 Hertog MGL, Hollman PCH, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J Agric Food Chem* 1992;**40:**2379–83.
62 Hertog MGL, Hollman PCH, Putte B. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J Agric Food Chem* 1993;**41**:1242–6.
63 Hertog MGL, Feskens EJM, Hollman PCH, *et al*. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 1993;**342**:1007–11.
64 Hertog MGL, Kromhout D, Aravanis C, *et al*. Flavonoid intake and long-term risk of coronary-heart-disease and cancer in the 7 countries study. *Arch Intern Med* 1995;**155**:381–6.
65 Rimm EB, Katan MB, Ascerio A, *et al*. Relation between intake of flavonoids and risk of coronary heart disease in male health professionals. *Ann Intern Med* 1996;**125**:384–9.
66 Hollman PCH, Vries JHM, Leeuwen SD, *et al*. Absorption of dietary quercetin glycosides and quecetin in healthy ileostomy volunteers. *Am J Clin Nutr* 1995;**62**:1276–82.
67 McAnlis GT, McEneny J, Pearce J, *et al*. Absorption and antioxidant effects of quercetin from onions in man. *Eur J Clin Nutr* 1999;**53**:92–6.
68 Serafini M, Ghiselli A, Ferro-Luzzi A. In vivo antioxidant effect of green and black tea in man. *Eur J Clin Nutr* 1996;**50**:28–32.
69 Stein JH, Keevil JG, Wiebe DA, *et al*. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation* 1999;**100**:1050–5.
70 Day AP, Kemp HJ, Bolton C, *et al*. Effect of concentrated red grape juice consumption on serum antioxidant capacity and low-density lipoprotein oxidation. *Ann Nutr Metab* 1997;**41**:353–7.
71 Shi HL, Noguchi N, Niki E. Comparative study on dynamics of antioxidative action of alpha-tocopheryl hydroquinone, ubiquinol, and alpha-tocopherol, against lipid peroxidation. *Free Radic Biol Med* 1999;**27**:334–46.
72 Lass A, Sohal RS. Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes. *Arch Biochem Biophys* 1998;**352**:229–36.
73 Thomas SR, Neuzil J, Stocker R. Cosupplementation with coenzyme Q prevents the prooxidant effect of alpha-tocopherol and increases the resistance of LDL to transition metal-dependent oxidation initiation. *Arterioscler Thromb Vasc Biol* 1996;**16**:687–96.
74 Levine M, Rumsey SC, Daruwala R, *et al*. Criteria and recommendations for vitamin C intake. *JAMA* 1999;**281**:1415–23.
75 Levine M. New concepts in the biology and biochemistry of ascorbic acid. *N Engl J Med* 1986;**314**:892–902.
76 Jialal I, Vega GL, Grundy SM. Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis* 1990;**82**:185–91.
77 Pietri S, Seguin JR, Darbigny P, *et al*. Ascorbyl free-radical—a noninvasive marker of oxidative stress in human open heart surgery. *Free Radic Biol Med* 1994;**16**:523–8.
78 May JM, Cobb CE, Mendiratta S, *et al*. Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase. *J Biol Chem* 1998;**273**:23039–45.
79 May JM, Qu ZC, Whitesell RR, *et al*. Ascorbate recycling in human erythrocytes: role of GSH in reducing dehydroascorbate. *Free Radic Biol Med* 1996;**20**:543–51.
80 Koshiishi I, Mamura Y, Liu J, *et al*. Evaluation of an acidic deproteinization for the measurement of ascorbate and dehydroascorbate in plasma samples. *Clin Chem* 1998;**44**:863–8.
81 Grootveld M, Halliwell B. Measurement of allantoin and uric acid in human body fluids. A potential index of free-radical reactions in vivo? *Biochem J* 1987;**243**:803–8.
82 Cross CE, Reznick AZ, Packer L, *et al*. Oxidative damage to human plasma proteins by ozone. *Free Radic Res Commun* 1992;**15**:347–52.
83 Ames BN, Cathcart R, Schwiers E, *et al*. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer. *Proc Natl Acad Sci U S A* 1981;**78**:6858–62.
84 Frei B, Stocker R, Ames BN. Antioxidant defences and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci U S A* 1988;**85**:9748–52
85 Gopinathan V, Miller NJ, Milner AD, *et al*. Bilirubin and ascorbate antioxidant activity in neonatal plasma. *FEBS Lett* 1994;**349**:97–200.
86 Halliwell B. Albumin—an important extracellular antioxidant? *Biochem Pharmacol* 1988;**37**:569–71.
87 Stocker R, Frei B. Endogenous antioxidant defences in human blood plasma. In: Rice-Evans C, ed. *Oxidative stress: oxidants and antioxidants*. London: Academic Press, 1991:213–43.
88 Hu ML, Louie S, Cross CE, *et al*. Antioxidant protection against hypochlorous acid in human plasma. *J Lab Clin Med* 1993;**121**:257–62.
89 Riedl A, Shamsi Z, Anderton M. Differing features of proteins in membranes may result in antioxidant or prooxidant action: opposite effects on lipid peroxidation of alcohol dehydrogenase and albumin in liposomal systems. *Redox Rep* 1996;**2**:35–40.
90 Sastre J, Pallardo FV, Vina J. Glutathione, oxidative stress and aging. *Age* 1996;**19**:129–39.

91 Arrigo AP. Gene expression and the thiol redox state. *Free Radic Biol Med* 1999;**27**:936–44.
92 Suh J, Zhu BZ, Frei B. Anti- and pro-oxidant effects of ascorbate on iron-mediated oxidative damage to bovine serum albumin. *Free Radic Biol Med* 1999;**27**:305s1.
93 Neuzil J, Thomas SR, Stocker R. Requirement for, promotion, or inhibition by alpha-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. *Free Radic Biol Med* 1997;**22**:57–71.
94 Atanasiu RL, Stea D, Mateescu MA, *et al*. Direct evidence of caeruloplasmin antioxidant properties. *Mol Cell Biochem* 1998;**189**:127–35.
95 Liu T, Stern A, Roberts LJ, *et al*. The isoprostanes: novel prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid. *J Biomed Sci* 1999;**6**:226–35.
96 Rosenfeld ME. Inflammation, lipids, and free radicals: lessons learned from the atherogenic process. *Semin Reprod Endocrinol* 1998;**16**:249–61.
97 Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst* 1999;**91**:1194–210.
98 Ashok BT, Ali R. The aging paradox: free radical theory of aging. *Exp Gerontol* 1999;**34**:293–303.
99 Witztum JL, Steinberg D. Role of oxidized LDL in atherogenesis. *J Clin Invest* 1991;**88**:1785–92.
100 Witztum JL, Horkko S. The role of oxidized LDL in atherogenesis: immunological response and anti-phospholipid antibodies. *Ann N Y Acad Sci* 1997;**811**:88–99.
101 Heinecke JW. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr Opin Lipidol* 1997;**8**:268–74.
102 Morel DW, DiCorleto PE, Chisholm GM. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis* 1984;**4**:357–64.
103 Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by the receptor for acetylated low density lipoproteins. *Proc Natl Acad Sci U S A* 1981;**78**:6499–503.
104 Folcik VA, Nivar-Aristy RA, Krajewski LP, *et al*. Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. *J Clin Invest* 1995; **96**:504–10.
105 Itabe H, Yamamoto H, Imanaka T, *et al*. Sensitive detection of oxidatively modified low-density lipoprotein using a monoclonal antibody. *J Lipid Res* 1996;**37**:45–53.
106 Yla-Herttuala S, Palinski W, Rosenfeld ME, *et al*. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989;**84**:1086–95.
107 Regnstrom J, Nilsson J, Tornvall P, *et al*. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 1992;**339**:1183–6.
108 Ball RY, Carpenter KL, Mitchinson MJ. What is the significance of ceroid in human atherosclerosis? *Arch Pathol Lab Med* 1987;**111**:1134–40.
109 Navab M, Imes S, Hama S, *et al*. Monocyte transmigration induced by modifications of LDL in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein I synthesis and is abolished by HDL. *J Clin Invest* 1991;**88**:2039–46.
110 Hessler JR, Robertson AL, Chisolm GM. LDL cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cell culture. *Atherosclerosis* 1979;**32**:213.
111 Kugiyama K, Kerns SA, Morrisett JD, *et al*. Impairment of endothelial-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990;**344**:160–2.
112 Salonen JT, Yla-Herttuala S, Yamamoto R, *et al*. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* 1992;**339**:883–7.
113 Bergmark C, Wu R, de Faire U, *et al*. Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. *Arterioscler Thromb Vasc Biol* 1995;**15**:441–5.
114 Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest* 1991;**64**:5–15.
115 Kita T, Kume N, Ishii K, *et al*. Oxidized LDL and expression of monocyte adhesion molecules. *Diabetes Res Clin Pract* 1999;**45**:123–6.
116 Huang YH, Schafer-Elinder L, Wu R, *et al*. Lysophosphatidylcholine (LPC) induces proinflammatory cytokines by a platelet-activating factor (PAF) receptor-dependent mechanism. *Clin Exp Immunol* 1999;**116**:326–31.
117 Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol* 2000;**20**:1430–42.
118 Bjorkhem I, Henrikssonfreyschuss A, Breuer O, *et al*. The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler Thromb* 1991;**11**:15–22.
119 Verlangieri AJ, Bush MJ. Effects of d-alpha-tocopherol supplementation on experimentally induced primate atherosclerosis. *J Am Coll Nutr* 1992;**11**:131–8.
120 Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low-density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. *Proc Natl Acad Sci U S A* 1991;**88**:1646–50.
121 Esterbauer H, Puhl H, Dieber-Rotheneder M, *et al*. Effect of antioxidants on oxidative modification of LDL. *Ann Med* 1991;**23**:573–81.
122 Reaven PD, Khouw A, Beltz WF, *et al*. Effect of dietary antioxidant combinations in humans. Protection of LDL by vitamin E but not by beta-carotene. *Arterioscler Thromb* 1993;**13**:590–600.
123 Gey KG, Puska P, Jordan P, *et al*. Inverse correlation between vitamin E and mortality from ischaemic heart disease in cross-cultural epidemiology. *Am J Clin Nutr* 1991;**53**:S326–34.
124 Riemersma RA, Wood DA, MacIntyre CCA, *et al*. Risk of angina pectoris and plasma concentrations of vitamins A, C and E and carotene. *Lancet* 1991;**337**:1–5.
125 Salonen JT, Salonen R, Seppanen K, *et al*. Relationship of serum selenium and antioxidants to plasma lipoproteins, platelet aggregability and prevalent ischaemic heart disease in eastern Finnish men. *Atherosclerosis* 1988;**70**:155–60.
126 Rimm EB, Stampfer MJ, Ascherio A, *et al*. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 1993;**328**:1450–6.
127 Stampfer MJ, Hennekens CH, Manson JE, *et al*. Vitamin E consumption and the risk of coronary heart disease in women. *N Engl J Med* 1993;**328**:1444–9.
128 Enstrom JE, Kanim LE, Klein MA. Vitamin C intake and mortality among a sample of the United States population. *Epidemiology* 1992;**3**:194–202.
129 Nyyssonen K, Parviainen MT, Salonen R, *et al*. Vitamin C deficiency and risk of myocardial infarction: prospective population study of men from eastern Finland. *BMJ* 1997;**314**:634–8.
130 Gaziano JM, Hennekens CH. The role of beta-carotene in the prevention of cardiovascular disease. *Ann N Y Acad Sci* 1993;**691**:148–55.
131 Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 1994;**330**:1029–35.
132 Rapola JM, Virtamo J, Ripatti S, *et al*. Randomised trial of α-tocopherol and β-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet* 1997;**349**:1715–20.
133 Blot WJ, Li JY, Taylor PR, *et al*. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst* 1993;**85**:1483–92.
134 Omenn GS, Goodman GE, Thornquist MD, *et al*. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* 1996;**334**:1150–5.
135 Hennekens CH, Buring JE, Manson JE, *et al*. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* 1996;**334**:1145–9.
136 Greenberg ER, Baron JA, Karagas MR, *et al*. Mortality associated with low plasma concentration of beta-carotene and the effect of oral supplementation. *JAMA* 1996;**275**:699–703.
137 Hodis HN, Mack WJ, LaBrec L, *et al*. Serial coronary angiographic evidence that antioxidant vitamin intake reduced progression of coronary artery atherosclerosis. *JAMA* 1995;**273**:1849–54.
138 Singh RB, Niaz MA, Rastogi SS, *et al*. Usefulness of antioxidant vitamins in suspected acute myocardial infarction. *Am J Cardiol* 1996;77:232–6.
139 Stephens NG, Parsons A, Schofield PM, *et al*. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge heart antioxidant study (CHAOS). *Lancet* 1996;**347**:781–6.
140 Valagussa F, Franzosi MG, Geraci E, *et al*. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet* 1999;**354**:447–55.
141 Yusuf S, Phil D, Dagenais G, *et al*. Vitamin E supplementation and cardiovascular events in high- risk patients. *N Engl J Med* 2000;**342**:154–60.
142 Slattery ML, Jacobs DR, Dyer A, *et al*. Dietary antioxidants and plasma lipids: the CARDIA study. *J Am Coll Nutr* 1995;**14**:635–42.
143 Valkonen M, Kuusi T. Passive smoking induces atherogenic changes in low-density lipoprotein. *Circulation* 1998;**97**:2012–16.