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Review

Blood Radicals

Reactive Nitrogen Species, Reactive Oxygen Species, Transition Metal Ions, and the Vascular System

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Free radicals, such as superoxide, hydroxyl and nitric oxide, and other "reactive species", such as hydrogen peroxide, hypochlorous acid and peroxynitrite, are formed *in vivo*. Some of these molecules, e.g. superoxide and nitric oxide, can be physiologically useful, but they can also cause damage under certain circumstances. Excess production of reactive oxygen or nitrogen species (ROS, RNS), their production in inappropriate relative amounts (especially superoxide and $NO^{\cdot}$) or deficiencies in antioxidant defences may result in pathological stress to cells and tissues. This oxidative stress can have multiple effects. It can induce defence systems, and render tissues more resistant to subsequent insult. If oxidative stress is excessive or if defence and repair responses are inadequate, cell injury can be caused by such mechanisms as oxidative damage to essential proteins, lipid peroxidation, DNA strand breakage and base modification, and rises in the concentration of intracellular "free" Ca^{2+}. Considerable evidence supports the view that oxidative damage involving both ROS and RNS is an important contributor to the development of atherosclerosis. Peroxynitrite (derived by reaction of superoxide with nitric oxide) and transition metal ions (perhaps released by injury to the vessel wall) may contribute to lipid peroxidation in atherosclerotic lesions.

KEY WORDS: oxygen radical; nitric oxide; peroxynitrite; oxidative stress; vascular system; reactive nitrogen species.

INTRODUCTION: THE BASIC TERMINOLOGY

A *free radical* is any species capable of independent existence (for however brief a period) that contains one or more unpaired electrons[4] (1). A superscript dot is used to denote a free radical. Examples include the oxygen-centered radicals superoxide ($O_2^{\cdot -}$) and hydroxyl ($OH^{\cdot}$), the glutathione radical ($GS^{\cdot}$, a sulphur-centred radical), trichloromethyl ($CCl_3^{\cdot}$, a carbon-centred radical), ubisemiquinone radical (important in mitochondrial electron transfer) and nitric oxide ($NO^{\cdot}$), a physiologically-important radical (2) in which the unpaired electron is delocalized between both atoms. The best known free radical produced by the vascular system is $NO^{\cdot}$, which plays a key role in controlling vasodilation and platelet adherence/aggregation (2). Nitric oxide is produced not only by vascular endothelial cells, but also by multiple other cell types, including phagocytes, and has a plethora of important physiological functions (2).

The term *reactive oxygen species* is widely used by biologists to include oxygen radicals and a number of related species, such as H_2O_2, that do not themselves contain unpaired electrons but are often involved in the generation of free radicals (Table 1). A similar term, *reactive nitrogen species,* is beginning to enter the literature (Table 1). Ironically, $O_2^{\cdot -}$ and $NO^{\cdot}$ radicals are among the least reactive of the molecules generally included under these headings. Indeed, $O_2^{\cdot -}$ (like $NO^{\cdot}$) has useful physiological roles (3–7); the best known is probably its participation (probably as a precursor of more reactive species) in the killing of bacteria by phagocytes (4). However, a large excess of $O_2^{\cdot -}$ can damage some biological targets (3). For example, superoxide has been shown to be capable of inactivating some bacterial enzymes containing iron-sulphur clusters, such as aconitase (3, 8). *In vitro* experiments suggest that superoxide can also inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain, perhaps by a similar mechanism (9). Nitric oxide can be directly toxic by inhibiting the enzyme ribonucleoside diphosphate reductase, apparently by direct reaction with a tyrosine radical at the enzyme active site (10). At physiological pO_2, $NO^{\cdot}$ can reversibly inhibit mitochondrial respiration (11): this may be important in the pathology of ischaemia-reperfusion and, conceivably, in the control of normal respiration. Excess $NO^{\cdot}$ can also result in damage to mitochondrial iron-sulphur proteins, oxidation of essential cellular -

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[4] Atomic and molecular orbitals contain a maximum of two electrons. If only one electron is present, it is said to be *unpaired.*

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Table 1.

Reactive Oxygen Species (ROS)	
Radicals	Non-radicals
Superoxide, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\cdot}$	Hypochlorous acid, HOCl[a]
Peroxyl, $RO_2^{\cdot}$	Ozone, O_3
Alkoxyl, $RO^{\cdot}$	Singlet oxygen $^1\Delta g$
Hydroperoxyl, $HO_2^{\cdot}$	Peroxynitrite, $ONOO^-$[b]
Reactive Nitrogen Species	
Radicals	Non-radicals
Nitric oxide, $NO^{\cdot}$	Nitrosyl, NO^+
Nitrogen dioxide, $NO_2^{\cdot}$	Nitroxide, NO^-
	Nitrous acid, HNO_2
	Dinitrogen trioxide, N_2O_3
	Dinitrogen tetroxide, N_2O_4
	Nitronium ion, NO_2^+
	Peroxynitrite, $ONOO^-$[b]
	Alkyl peroxynitrites, ROONO

[a] Could equally well be called a "reactive chlorine species".
[b] Peroxynitrite is often included under both categories.
Note: Reactive is a relative term: $NO^{\cdot}$ and $O_2^{\cdot -}$ have limited reactivity whereas $OH^{\cdot}$ reacts with everything. The other species have intermediate reactivities. $NO^{\cdot}$ and $O_2^{\cdot -}$ are similar in other respects: they both have important physiological actions but are toxic in excess, often by generating other oxidants (including $OH^{\cdot}$ or species resembling it). Ironically, superoxide is one of the few molecules with which $NO^{\cdot}$ reacts quickly (and vice versa).

SH groups and efflux of intra-mitochondrial Ca^{2+}, but it may be reaction products of $NO^{\cdot}$ (such as peroxynitrite) that are responsible for such effects rather than $NO^{\cdot}$ itself (12–14).

It is well established that $O_2^{\cdot -}$ and H_2O_2 are produced within most, if not all, aerobic cells (1, 3). In addition, some cells release them extracellularly, especially activated phagocytes (4) but also (but to lesser extent) fibroblasts (5, 6) and lymphocytes (7). There are also claims that endothelial cells release $O_2^{\cdot -}$ and H_2O_2 (see below).

By contrast to $O_2^{\cdot -}$ and $NO^{\cdot}$, the hydroxyl ($OH^{\cdot}$) radical reacts fast with almost all molecules found *in vivo* (Table 1). Hydroxyl radical is produced in living organisms by several mechanisms. One is the splitting of covalent bonds in water by low-wavelength electromagnetic radiation [15]:

$$H_2O \xrightarrow{\text{energy}} H^{\cdot} + OH^{\cdot} \quad (1)$$

Another is the reaction of H_2O_2 with transition metal ions such as iron or copper (1). It is frequently argued that the reactive species formed in these systems is not $OH^{\cdot}$, but much evidence suggests that it is (reviewed in (16)).

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

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^{\cdot} + OH^- \quad (3)$$

A third is the reaction of $O_2^{\cdot -}$ with hypochlorous acid (HOCl) (17)

$$HOCl + O_2^{\cdot -} \rightarrow O_2 + OH^{\cdot} + Cl^- \quad (4)$$

and a fourth may be the decomposition of peroxynitrite (see below).

Attack of hydroxyl radical upon biological molecules usually sets off free radical chain reactions (1, 15, 16). Because free radicals are formed *in vivo*, organisms have evolved antioxidant defence systems. Some ROS/RNS escape these defences, however, and repair systems are needed to prevent the accumulation of oxidatively damaged molecules (discussed in refs. 1, 18, 19).

PRODUCTION OF ROS AND RNS BY VASCULAR ENDOTHELIUM

Vascular endothelial cells make $NO^{\cdot}$ for essential physiological functions (2). Like most (or probably all) cells, they make intracellular $O_2^{\cdot -}$ and H_2O_2 (20, 21). Endothelial cells obtained from several species, including humans, have also been shown to *release* $O_2^{\cdot -}$ and H_2O_2 (20–32) but it is not clear if they do this all the time, or only after exposure to cytokines or after an insult, such as ischaemia/reperfusion (or, in the case of studies on cultured cells, the trauma of the cell isolation process or the exposure to ambient pO_2). Suggested sources of the extracellular $O_2^{\cdot -}$ produced by vascular endothelial cells (Figure 1) include the enzyme xanthine oxidase (although its significance in human cells has been questioned (33)), NADH oxidase enzymes (e.g. in bovine coronary artery

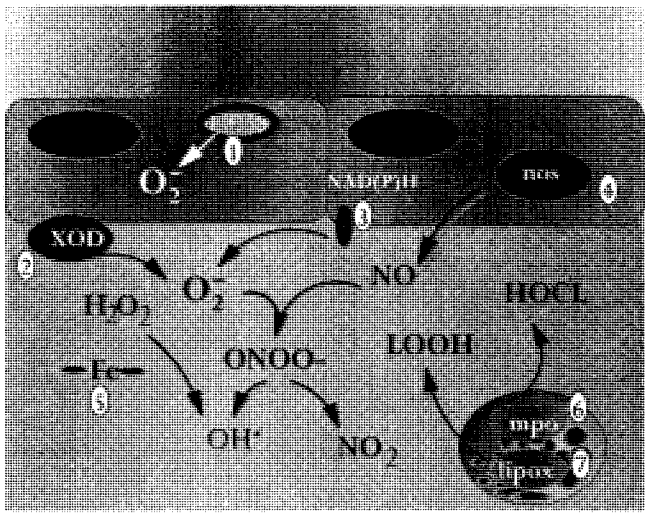

Fig. 1. Formation of ROS/RNS in the vasculature. In this scheme some of the potential sources of ROS/RNS derived from inflammatory or endothelial cells in the vessel wall are shown. Key: 1: Mitochondrial formation of superoxide. 2: Xanthine oxidase forms both hydrogen peroxide and superoxide. 3: NADPH or NADH oxidases may catalyse the extracellular formation of superoxide by the endothelium. 4: Nitric oxide is synthesised by nos enzymes present in both the endothelium and other cell types. The formation of peroxynitrite and its subsequent decomposition to form hydroxyl radicals and nitrogen dioxide, $NO_2^{\cdot}$ (or species resembling them) are shown. In addition peroxynitrite can directly modify proteins, lipids and nucleic acids. 5: "Catalytic" iron may promote the formation of hydroxyl radicals ($OH^{\cdot}$); both this and heme iron can stimulate lipid peroxidation. 6: The enzyme myeloperoxidase catalyses the formation of hypochlorous acid (HOCl) which may oxidise antioxidants such as thiols and ascorbate and possibly lipoproteins. 7: Lipoxygenase enzymes catalyse the insertion of lipid hydroperoxides in lipids; hydroperoxide breakdown in the presence of transition metals can propagate lipid peroxidation. N.B. For the sake of clarity the equations shown are not balanced and do not show all the reactants/products.

endothelium (34)) and NADPH oxidases (35, 36). An intriguing aspect of xanthine oxidase is that release of this enzyme into the circulation (whatever the source) can lead to its binding to the lumenal surface of endothelial cells (37), rendering these cells susceptible to damage. In humans, circulating xanthine oxidase has been reported to be elevated in patients with rheumatoid arthritis (38) and after aortic cross-clamping (39). This raises the possibility that damage to organs rich in xanthine dehydrogenase/oxidase, e.g. the gut, may result in free radical-dependent vascular dysfunction at sites distant from the primary tissue injury.

If endothelium normally generates $NO^{\cdot}$ (2), but produces extracellular $O_2^{\cdot -}$ and H_2O_2 only after an insult, we would anticipate that the "normal" vasculature experiences much more $NO^{\cdot}$ than $O_2^{\cdot -}$, i.e. the "physiological" $NO^{\cdot}/O_2^{\cdot -}$ ratio is high. Such a high ratio may be beneficial because one of the few fast reactions of $O_2^{\cdot -}$ is that with $NO^{\cdot}$, to give peroxynitrite (40, 41):

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^- \qquad (5)$$

One consequence of reaction (5) is that $O_2^{\cdot -}$ antagonises the biological actions of $NO^{\cdot}$. For example, if excess $O_2^{\cdot -}$ is made it can act as a vasoconstrictor, and this may have deleterious effects in some clinical situations, e.g. causing pathological vasoconstriction and hypertension (42, 43). Hypercholesterolaemia has been reported to increase endothelial $O_2^{\cdot -}$ generation in rabbits (31).

Peroxynitrite is a cytotoxic species, e.g. it oxidizes methionine residues and -SH groups on proteins (reviewed in (44)). In addition, peroxynitrite rapidly protonates at physiological pH and then decomposes to form a range of cytotoxic products, that can hydroxylate and nitrate aromatic rings and cause other biological damage (Figure 2). One of these products resembles

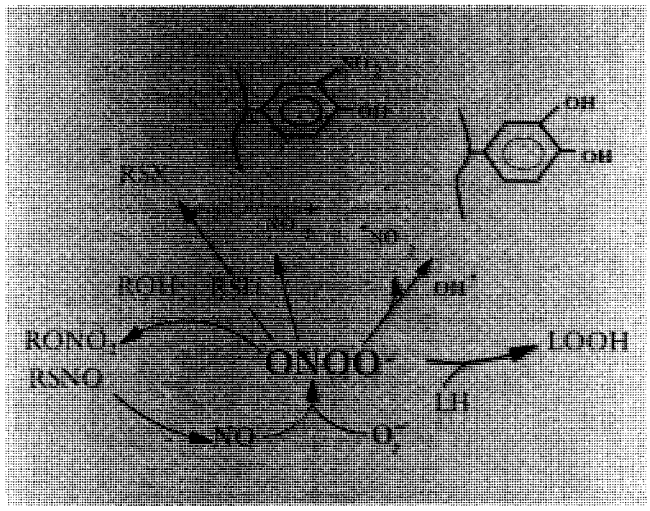

Fig. 2. Possible products from the interaction of superoxide and nitric oxide. Nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) react to form peroxynitrite, $ONOO^-$. LH represents an unsaturated lipid and LOOH the corresponding lipid hydroperoxide. RSH represents a thiol such as glutathione (GSH) which reacts with $ONOO^-$ to form higher oxidation products (RSX) or, at low yields, S-nitrosothiols (RSNO). A similar reaction proceeds with the alcohol functional group (ROH) to form an organic nitrate ($RONO_2$). The nitrosonium cation NO_2^+ may be formed, which may then nitrate tyrosine. An alternative route to tyrosine nitration involves the formation of nitrogen dioxide ($^{\cdot}NO_2$). Exposure to peroxynitrite can also lead to tyrosine hydroxylation.

$OH^{\cdot}$ in its reactivity (44–46). Some reactions of $ONOO^-$ with biological molecules generate low yields of $NO^{\cdot}$ donors, such as nitrosothiols and organic nitrates/nitrites (47, 48).

ANTIOXIDANT DEFENCES

Removal of excess $O_2^{\cdot -}$ by intracellular superoxide dismutase (SOD) enzymes is an important physiological antioxidant defence mechanism (3). The walls of blood vessels additionally contain an "extracellular SOD" enzyme (EC-SOD), which could conceivably serve to modulate the $O_2^{\cdot -}/NO^{\cdot}$ interaction (49). Mice lacking a functional EC-SOD gene appear normal, but are more sensitive to hyperoxia (50).

The action of all types of SOD enzymes generates H_2O_2:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (6)$$

Vascular endothelial cells can generate additional H_2O_2 by the action of several oxidase enzymes (Figure 1), including xanthine oxidase (32), which makes both $O_2^{\cdot -}$ and H_2O_2 during its catalytic cycle. If phagocytes adhere to endothelium and become activated, the H_2O_2 that they produce can readily diffuse into endothelial cells (20), since H_2O_2 easily crosses membranes (1). In endothelial cells, both catalase and glutathione peroxidase enzymes are involved in removal of H_2O_2 (51, 52). Large amounts of H_2O_2 are cytotoxic to all cell types, and endothelial cells are no exception. Some of this damage is direct, e.g. one target of direct damage (oxidation of an essential -SH group) by excess H_2O_2 is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (53). In addition, H_2O_2 can generate $OH^{\cdot}$ within endothelial cells, apparently by reaction with intracellular iron ions as shown in equation 2 (1, 32, 54, 55).

How Could Iron and Copper Catalytic for Free Radical Reactions Arise in Endothelial Cells?

Transition metal ions, especially iron and copper, are powerful promoters of oxidative damage in endothelial and other cells, because they can accelerate lipid peroxidation (see below) and catalyze $OH^{\cdot}$ formation. But how do they become available in "catalytic" forms: organisms usually take great care in the handling of iron (16, 56), using both transport (such as transferrin) and storage (such as ferritin and haemosiderin) proteins so as to minimize the amount of "catalytic" iron within cells and in extracellular fluids? The same is true of copper (1, 16). This careful sequestration of transition metal ions is an important contribution to antioxidant defence (1, 16).

However, one consequence of excess formation of ROS or RNS is the release of catalytic iron and copper ions. Thus, $O_2^{\cdot -}$ can mobilize iron from the storage protein ferritin (57), although the amount of superoxide-releasable iron is small, and so ferritin-bound iron is much safer than an equivalent amount of 'free' iron (16, 58). H_2O_2 can degrade haem proteins to release iron and free haem (59–61), both of which can catalyze lipid peroxide decomposition (1), generating free radicals that propagate this process (Figure 1). Haem may be particularly effective in promoting oxidative damage within endothelial cells, since it is quickly taken up (62). The internalized haem is degraded by haem oxygenase enzymes, and the iron released is stored in ferritin (63). Indeed, transfection of the human haem oxygenase gene into rabbit endothelial cells protected them against haem and haemoglobin toxicity (64).

It is not only ROS that can cause metal ion liberation. High levels of $NO^{\cdot}$ (directly or perhaps via $ONOO^-$) lead to iron release from iron-sulphur proteins within cells (8, 12). Cell destruction itself causes liberation of intracellular iron and copper ions into the surrounding tissues, spreading and accelerating oxidative damage (16, 65, 66). Degradation of copper-containing proteins, such as caeruloplasmin, releases "catalytic" copper (67–69). For example, peroxynitrite readily displaces copper from the plasma protein caeruloplasmin (69).

LIPID HYDROPEROXIDES AND METALS: A CYCLE OF DESTRUCTION

Metal ions not only exacerbate the toxicity of H_2O_2 (equations 2 and 3) but also that of *lipid peroxides*. Lipid hydroperoxides are formed as the by-product of a number of biological processes and in the absence of metals they are, like H_2O_2, of limited reactivity (1). Such peroxides may be metabolised by glutathione peroxidase enzymes without the release of free radical intermediates (52). However, if they encounter "catalytic" transition metals such as iron or copper ions, decomposition of lipid peroxides can occur (1, 16) to form alkoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals

$$\underset{(Cu^{2+})}{Fe^{3+}} + LOOH \rightarrow \underset{(Cu^{+})}{Fe^{2+}} + LOO^{\cdot} + H^{+} \quad (7)$$

$$\underset{(Cu^{+})}{Fe^{2+}} + LOOH \rightarrow LO^{\cdot} + OH^{-} + \underset{(Cu^{2+})}{Fe^{3+}} \quad (8)$$

Haem and certain metalloproteins can also catalyze lipid peroxide decomposition. For example, O'Donnell and Burkitt (70) showed that human endothelial cells decompose butyl hydroperoxide to free radicals, apparently by interaction with mitochondria. "Catalytic" metal ions were also released within the cells and contributed to cell injury by the peroxide.

Peroxidation of Low-density Lipoproteins (LDL)

An important physiological substrate for lipid peroxidation is lipoproteins, especially LDL, which may become trapped in the arterial intima and are then at risk of oxidation (71, 72). The biological characteristics of the different classes of lipoproteins are key factors in understanding their potential contribution to atherosclerosis. For example, the accumulation of macrophage-derived foam cells in the artery wall by uptake of oxidatively-modified LDL is a key event in atherosclerosis (71–73). Indeed, high levels of LDL are associated with increased risk of developing coronary heart disease. High density lipoproteins (HDL), on the other hand, mediate reverse cholesterol transport and appear to be anti-atherogenic. There is much evidence in favour of the "oxidative hypothesis" for the development of atherosclerosis. Major points include:

1. The presence of decomposition products of lipid peroxides, such as the aldehydes 4-hydroxynonenal and malondialdehyde, conjugated to proteins in human atherosclerotic lesions (71–74).
2. LDL extracted from human and rabbit lesions resembles LDL that has been oxidised *in vitro* (71).
3. Antibodies against oxidised LDL have been found in serum from patients with coronary artery disease and the titre of these auto-antibodies is correlated with progression of carotid atherosclerosis (75).
4. Epidemiological data are consistent with a protective action of dietary chain-breaking antioxidant inhibitors of lipid peroxidation (especially α-tocopherol) against atherosclerosis (76, 77). Direct evidence for the importance of these antioxidants is provided by studies showing that dietary supplementation with α-tocopherol decreases the incidence of myocardial infarction (e.g. ref (78)). Dietary supplementation with α-tocopherol (or the synthetic lipid-soluble antioxidant probucol) increases the resistance of LDL subsequently isolated from the blood to oxidation promoted *in vitro* by copper (79–84).

Sensitivity of LDL to Oxidation as a Risk Factor for Developing Coronary Heart Disease

A prediction of the oxidative hypothesis for atherosclerosis is that the lower the resistance of plasma LDL to oxidation the greater the chance that it will become oxidised in the vessel walls and contribute to the development of the disease. Indeed, by using the sensitivity of LDL to oxidation in the presence of Cu^{2+} *in vitro* it has been shown that subjects at risk of developing coronary heart disease have LDL which is more readily oxidised than controls (82, 83). What factors affect the oxidisability of LDL when measured by this approach? The experimental parameter defined by most investigators using Cu^{2+}-stimulated LDL oxidation is the"lag phase", i.e. the time taken before peroxidation of a given sample of LDL subjected to oxidative challenge begins to accelerate (Figure 3), calculated as described in (84). Factors which render LDL more resistant to oxidation include:

1. A lipid composition containing a greater proportion of saturated and mono-unsaturated fatty acids, such as oleic acid. For example, LDL isolated from rabbits fed on a diet highly enriched in oleic acid showed a marked resistance to oxidation *in vitro* (85).
2. Dietary supplementation with antioxidants which intercept lipid peroxyl radicals, such as α-tocopherol or probucol; both usually increase the length of the lag phase (79–81).
3. A decrease in the content of "seeding" lipid peroxides in the LDL particle (86–91) (Figure 4).

Conversely, increasing the levels of polyunsaturated fatty acids and lipid peroxides results in decreased resistance of LDL to oxidation (86–90). The response to supplementation with antioxidants is complex and the concentration of α-tocopherol in LDL does not correlate well with the length of the lag phase (84). For example, while increased LDL α-tocopherol usually decreases copper dependent oxidation, the extent of this effect is highly variable between individuals (86–91). However, when the oxidisability of the same samples was assessed by inducing lipid peroxidation using a mechanism independent of the presence of pre-formed lipid hydroperoxides little or no variation was observed (91).

These data support the hypothesis that the endogenous lipid peroxide content of LDL isolated from different individuals is an important variable (90, 91). Indeed, the action of α-tocopherol is highly affected by treatments which alter the concentration of "seeding peroxides" within LDL (86–89). If LDL isolated from plasma truly reflect the state of LDL in the artery wall (this is, of course, open to argument), then it is possible that the endogenous LDL content of lipid peroxides is an addi-

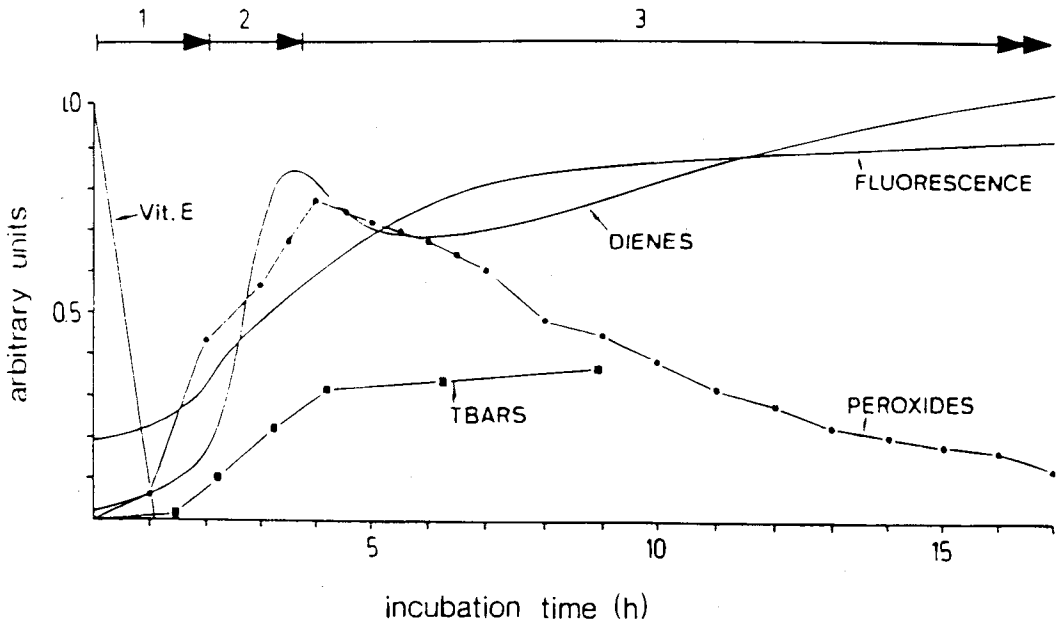

Fig. 3. Sequence of events during copper ion-stimulated oxidation of low-density lipoprotein (LDL). Usually, α-tocopherol is first consumed and the "peroxide pool" is filled, whereupon peroxidation accelerates. Peroxidation can be measured as formation of conjugated dienes at 234 nm, fluorescence at 430 nm, formation of lipid hydroperoxide, or production of TBARS (thiobarbituric acid-reactive material), although TBARS alone is not a good index of LDL oxidation. Period 1 (top) is the lag phase, period 2 is the propagation phase. In phase 3, peroxides decompose to aldehydes and other products. Data provided by courtesy of Prof. Dr. H. Esterbauer.

tional risk factor for the development of atherosclerosis. As yet, the precise levels of LDL "seeding peroxides" *in vivo,* and their origin (oxidized lipids in the diet? lipoxygenase enzymes? attack by ROS/RNS?) is uncertain. The role of vascular lipoxygenase enzymes continues to be debated (93, 94): they may not be a major mechanism of total LDL oxidation, but they could help to provide the"seeding peroxides".

Figure 4 is a diagramatic representation of the redox interactions between copper ions (4A) and the haem protein myoglobin (Figure 4B), both of which can decompose lipid hydroperoxides, and LDL in the presence of a chain breaking antioxidant. The first step with both pro-oxidants is the metal-dependent decomposition of the lipid hydroperoxide to form peroxyl or alkoxyl radicals. The peroxyl radicals formed may be eliminated by reaction with an antioxidant, AH (such as α-tocopherol) but the lipid peroxide formed will still contribute to the peroxide pool.

α-Tocopherol: Antioxidant or Pro-oxidant?

The lipid-soluble chain-breaking antioxidant α-tocopherol usually slows lipid peroxidation by scavenging intermediate peroxyl radicals

$$LO_2^{\cdot -} + TH \rightarrow LO_2H + T^{\cdot} \quad (9)$$

and there is considerable epidemiological evidence supporting an inverse association between lipid-standardized plasma α-tocopherol levels and cardiovascular disease (76–78). Intervention trials also show that consumption of excess α-tocopherol decreases the incidence of myocardial infarction (78). Hence α-tocopherol is usually regarded as the most important antioxidant in LDL. However, α-tocopherol is a reducing agent and it has been shown that it can reduce copper(II) ions during copper-dependent oxidation of lipids, including LDL (89,95)

$$TH + Cu^{2+} \rightarrow T^{\cdot} + Cu^{+} \quad (10)$$

It is possible for α-tocopheryl radical formed by such a reaction to *promote* lipid peroxidation by abstracting a hydrogen atom from an unsaturated fatty acid (96). This has been called a "pro-oxidant" effect of α-tocopherol (96) and is shown in Figure 4C. Fortunately, the rate of reaction of α-tocopheryl ($T^{\cdot}$) radicals with fatty acids is orders of magnitude lower than the reaction of peroxyl radicals with PUFAs (97) and appears only to assume any significance when the "seeding peroxide" content is low (89). As soon as peroxides are present, the scavenging of peroxyl radicals by α-tocopherol will give it a net antioxidant role.

In addition, $\alpha T^{\cdot}$ radicals probably have a very short lifetime in LDL *in vivo.* The $\alpha T^{\cdot}$ radical is quickly recycled by ascorbic acid

$$T^{\cdot} + \text{ascorbate} \rightarrow TH + \text{ascorbate}^{\cdot} \quad (11)$$

and ubiquinol

$$CoQH_2 + T^{\cdot} \rightarrow CoQH^{\cdot} + TH \quad (12)$$

Reaction (11) is probably far more important, since the amount of ubiquinol in human LDL appears small (much less than one molecule per LDL particle (90)), which suggests that reaction (12) is not physiologically essential. The addition of ascorbate or ubiquinol prevents the apparent "pro-oxidant" effect of α-tocopherol in LDL *in vitro* (96).

The Pharmacological and Toxicological Properties of Lipid Decomposition Products

Metal ion-catalyzed decomposition of lipid hydroperoxides forms a wide range of carbonyl compounds, including

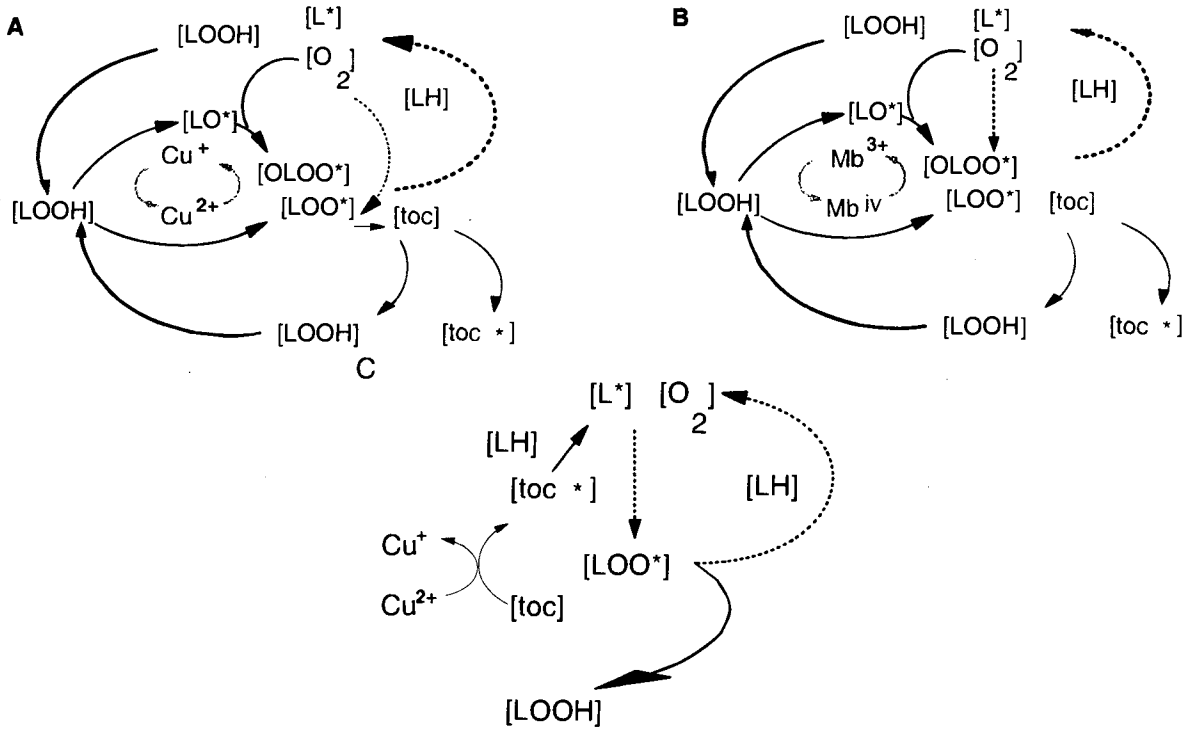

Fig. 4. The copper and metmyoglobin-dependent decomposition of lipid peroxides and the role of antioxidants. Panels A and B depict simplified reaction schemes for the Cu^{2+} or metmyoglobin (metMb)-dependent oxidation of LDL. Panel C shows the reactions consistent with α-tocopherol mediated peroxidation. The initial reactions described in panels A and B are the decomposition of the "seeding peroxide" (LOOH) in the LDL particle by Cu^{2+} or by metMb to form peroxyl ($LOO^{\cdot}$) and alkoxyl ($LO^{\cdot}$) radicals. These can react with any α-tocopherol (toc) present to form the tocopheryl radical (toc*), thereby generating 1 mol of lipid peroxide

$$LOO^{\cdot} + TH \rightarrow T^{\cdot} + LOOH$$

Alternatively, $LOO^{\cdot}$ (and $LO^{\cdot}$, possibly via molecular rearrangement to form $LOO^{\cdot}$) can propagate peroxidation by attack upon adjacent lipid (LH) to form a lipid alkyl radical (L*). In the complete absence of lipid peroxide the initial sequence of reactions may be that depicted in panel C. In this case Cu^{2+} is reduced to Cu^{+} by α-tocopherol and the resulting tocopheryl radical abstracts a H atom from a lipid molecule (albeit at a very slow rate) to promote lipid peroxidation and so create a peroxide pool. As soon as this occurs, the cycles shown in panels A and B will presumably begin to contribute to the oxidation of LDL.

noxious aldehydes such as malondialdehyde and 4-hydroxynonenal (98). These can react with lysine residues on the surface of the LDL apo-B protein to convert LDL into a ligand for the macrophage scavenger receptor (73). In addition, lipid derived radicals may react directly with the protein to cause fragmentation and modification of amino acids (72).

The adducts formed by reaction of aldehydes with lysine residue form highly immunogenic epitopes and antibodies have been prepared that recognise malondialdehyde and 4-hydroxynonenal conjugated LDL (71, 72). These antibodies cross react with material in atherosclerotic lesions but not normal vessel wall tissue (see above).

The most important detoxification pathway for these aldehydes is probably their conjugation with reduced glutathione (GSH), catalyzed by glutathione S-transferase enzymes (98). Macrophages exposed to oxidized LDL can respond by increased GSH synthesis, which greatly decreases the cytotoxicity of oxidised LDL to these cells (99). If GSH synthesis is compromised, this appears to enhance cholesteryl ester deposition (100). These responses may be relevant *in vivo* since hypercholesterolaemia in rabbits is associated with increased levels of free thiol groups, probably GSH, and increased activity of glutathione-dependent enzymes in blood vessels (101).

Other end-products of lipid peroxidation could 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 F_2-isoprostanes are generated by the peroxidation of arachidonic acid via the generation of peroxyl radical isomers which undergo endocyclization to prostaglandin-like compounds (102). Their formation *in vivo* appears to be enhanced under conditions of oxidative stress, such as smoking, exposure to xenobiotics, and pathological conditions associated with inflammation (103–106). F_2 isoprostanes are formed in lipoproteins subjected to oxidative stress, e.g. by exposure to transition metals, haem proteins or peroxynitrite (107).

ENDOTHELIAL OXIDATIVE STRESS

In healthy individuals, the generation of ROS/RNS seems approximately in balance with antioxidant defences, although there appears to be a slow cumulative oxidative damage that contributes to the ageing process and to age-related diseases such as cardiovascular disease and cancer (108–110). A serious imbalance between ROS/RNS and antioxidant defences in favour of the former can create a state of *oxidative stress* (111).

Most cells can tolerate mild oxidative stress, which often leads to increased synthesis of antioxidant defence systems (1, 111). For example, exposure of endothelial cells to haem induces the synthesis of haem oxygenase and ferritin (63). Exposure of aortic smooth muscle cells to oxidized LDL also induced haem oxygenase (112). However, severe oxidative stress produces major derangements of cell metabolism, including DNA strand breakage, rises in intracellular 'free' Ca^{2+}, damage to membrane ion transporters and/or other proteins, depletion of NAD^+ and ATP, and sometimes peroxidation of lipids (1, 110, 111, 113). All these events have been described in vascular endothelial cells subjected to severe oxidative stress (114–119). Damage can be direct, e.g. if H_2O_2 oxidizes essential thiol groups on glyceraldehyde-3-phosphate dehydrogenase or membrane ion transport proteins, or if $OH^{\cdot}$ is formed by reaction of H_2O_2 with DNA-bound metal ions, leading to DNA strand breakage and base modification (120). Damage can also be indirect. For example, excessive rises in "free" Ca^{2+} can activate proteases (which may attack the cytoskeleton) and nucleases, providing an additional method of causing DNA strand breakage (113).

However, lower levels of oxidative stress provoke more subtle molecular changes in endothelial cells, some of which may have regulatory significance. Changes reported in endothelial cells in response to low levels of $O_2^{\cdot -}$, H_2O_2 or other oxidants include activation of the cytoplasmic gene transcription factor NF-κB, increased synthesis of adhesion molecules promoting platelet and phagocyte attachment, changes in ion transport and PAF synthesis, increased prostacyclin release, and activation of phospholipases and adenylate cyclase (121–132). The exact responses may often depend on the type of endothelial cell examined (species, and site in the vascular network) and possibly on how long it has been in culture and how gently it was isolated. Protein kinases, thiol-containing proteins (especially thioredoxin) and cell surface receptors can all respond to oxidative stress in ways that affect cell metabolism. Peroxynitrite nitrates tyrosine residues in proteins (Figure 2) which would be expected to affect tyrosine phosphorylation/dephosphorylation reactions involved in signal transduction.

Hence, there is a fine balance between regulatory properties of ROS/RNS (e.g. at sites of acute 'controlled' inflammation) and damaging properties, depending on the amount generated, the cellular target of damage, the activity of antioxidant defences and the presence or absence of transition metal ions. Indeed, what is measured as "damage" may often be a physiological response (e.g. induction of adhesion molecules). Of particular importance are the possible regulatory and/or deleterious consequences of ROS and RNS produced by activated phagocytes adhering to endothelial cells (133, 134). Activated human neutrophils generate not only $O_2^{\cdot -}$ and H_2O_2 (and possibly sometimes $NO^{\cdot}$ (135)), but also hypochlorous acid (HOCl), which can oxidize protein -SH groups and methionine residues, deplete antioxidants and lead to chlorination of tyrosine residues (with possible effects on signal transduction) (136–138). As discussed earlier, HOCl also reacts with $O_2^{\cdot -}$ to make $OH^{\cdot}$ (equation 4).

INITIATION OF ATHEROSCLEROSIS: THE ROLE OF ROS AND RNS

The free radical chain reaction of LDL oxidation plays an important role in the progression of atherosclerosis, but could ROS/RNS play a role in *initiation* of this process?

It is widely believed that atherosclerosis is initiated by damage to the vascular endothelium (139). The injury may be physical, such as the turbulent blood flow over the endothelium that occurs at bifurcations in the arteries. It may be exacerbated by metabolic stresses such as hypercholesterolemia; it has been reported that in hypercholesterolemic rabbits $O_2^{\cdot -}$ formation is increased in endothelial cells and this may originate from xanthine oxidase (31, 140). Monocytes and macrophages play an important role in the development of atherosclerotic lesions. Activation of these phagocytes in or upon blood vessel walls could injure endothelial cells by several mechanisms, including the secretion of $O_2^{\cdot -}$, H_2O_2, and $NO^{\cdot}$ to an extent that could overwhelm local antioxidant defences. Arterial endothelial cells are among the cell types that have been shown to be capable of oxidizing LDL *in vitro* so that macrophages will internalize it faster. This LDL modification could involve generation of extracellular $O_2^{\cdot -}$ and H_2O_2 by endothelial cells, which could then interact with transition metal ions and thiol compounds in the media used, to form damaging species such as $OH^{\cdot}$ and thiyl radicals (141–143). We need more information about the *in vivo* significance of such reactions, and whether they are relevant to the pro-atherogenic effects of certain thiols, such as homocysteine.

The uptake of peroxidized LDL by the macrophage scavenger receptors could be regarded as a protective mechanism, if it sequesters peroxidized LDL within the arterial wall, preventing further injury to endothelial cells. However, it could also result in activation of macrophages to secrete factors (such as platelet-derived growth factor) that promote the progression of atherosclerosis by stimulating proliferation of other cell types. Thus, LDL that has undergone some peroxidation, but is not yet recognized by the macrophage scavenger receptors, might be especially deleterious to endothelial cells, both directly and indirectly, e.g. by encouraging adhesion of monocytes (144–147). Low concentrations of peroxides might acclerate cyclooxygenase and lipoxygenase-catalyzed reactions in endothelium, leading to enhanced formation of eicosanoids (148, 149). Oxidized LDL might also stimulate the production of eicosanoids by macrophages (150).

Availability of transition metal ions, which catalyse the decomposition of lipid peroxides into aldehydes that can modify apoprotein B and cause recognition by the scavenger receptors, may be an important factor in determining whether or not an LDL particle that has undergone peroxidation is actually taken up. It has been shown that advanced human atherosclerotic lesions contain iron and copper in forms that can accelerate free radical reactions, including LDL peroxidation (151–154). Evans *et al.* (155) showed that mechanical disruption of normal human arterial wall releases "catalytic" iron and copper ions. Although this is a somewhat crude model, it illustrates the potential of vessel wall injury to create conditions favouring lipid peroxide decomposition and conversion of H_2O_2 to $OH^{\cdot}$.

But how does LDL oxidation *begin* in atherosclerotic lesions? How are the "seeding peroxides" generated? Lipoxygenases may be involved (93, 94). Some LDL peroxides might conceivably originate from consumption of peroxidized food lipids. The gut appears efficient in peroxide metabolism, but traces of peroxides might get through in a high-fat pro-atherogenic diet. Peroxynitrite may also play an important role. Addition of $ONOO^-$ to LDL causes lipid peroxidation and the oxidized LDL is recognized by macrophage scavenger receptors (156). Peroxynitrite can displace "catalytic" copper ions from

caeruloplasmin, favouring more LDL oxidation (69). Preliminary evidence (based on the presence of nitrated tyrosine [Figure 2]) in a few lesion samples) has been presented that $ONOO^-$ can be generated in human atherosclerotic lesions, even in fatty streaks (157). This deserves further exploration.

NITRIC OXIDE: GOOD OR BAD IN ATHEROSCLEROSIS?

Nitric oxide could act as a pro-oxidant by generating $ONOO^-$, which oxidises LDL (156). However, things are not so simple. Several groups have shown that $NO^\cdot$ can have antioxidant properties, e.g. by binding to ferrous ions and heme proteins and diminishing their pro-oxidant effects (158, 159) and by removing $O_2^{\cdot -}$ (160). Thus in a $O_2^{\cdot -}$-dependent injury system, $NO^\cdot$ could be protective (160, 161), provided that the peroxynitrite produced does not aggravate injury, e.g if it is dissipated harmlessly by reaction with components in its environment, including such extracellular antioxidants as urate and ascorbate (162). Just as $NO^\cdot$ reacts quickly with $O_2^{\cdot -}$, it also reacts fast with other radicals, including peroxyl radicals (163).

$$LO_2^{\cdot -} + NO^\cdot \rightarrow LOONO \qquad (13)$$

Hence $NO^\cdot$ can scavenge the peroxyl radicals that propagate lipid peroxidation in LDL (163, 164). $NO^\cdot$ may also deter phagocyte adherence to endothelium (165). Is reaction (13) good or bad? By analogy with the way in which $ONOO^-$ can decompose to toxic species, LOONO *might* break down to alkoxyl radicals (equation 14). Preliminary data suggest, however, that LOONO species might be stable under physiological conditions (166).

$$LOONO \rightarrow LO^\cdot + NO_2^{\cdot -} \qquad (14)$$

It has recently been suggested that myeloperoxidase (in addition to its ability to oxidize Cl^- to HOCl in the presence of H_2O_2) may contribute directly to LDL oxidation in atherosclerotic lesions (167). This enzyme uses H_2O_2 to oxidize tyrosine into tyrosyl radicals, which can apparently cause LDL oxidation (168). Although the relative significance of these various MPO-dependent reactions remains to be clarified, the ability of $NO^\cdot$ to react at an almost diffusion-controlled rate with tyrosyl radicals (169) might also contribute to the antioxidant effects of this small, diffusible free radical.

Consistent with a net *protective* effect of $NO^\cdot$ *in vivo* are observations that inhibition of $NO^\cdot$ synthesis by murine peritoneal macrophages increased the ability of these cells to oxidize LDL *in vitro,* whereas treatment of the cells with interferon-γ and tumor necrosis factor-α (TNF-α) to increase $NO^\cdot$ synthesis diminished LDL oxidation (170, 171). Addition of an $NO^\cdot$ donor to endothelial cells *in vitro* inhibited LDL oxidation (172), and addition of extra L-arginine (the precursor of $NO^\cdot$) to the diet of hypercholesterolaemic rabbits (or normal rabbits after balloon angioplasty) decreased the development of atherosclerosis (173, 174). Elevation of the e-NOS level of rat carotid artery endothelium by genetic manipulation decreased the extent of restenosis after balloon injury (175).

Although the conditions under which human neutrophils, monocytes and macrophages generate $NO^\cdot$ remain to be fully established, it is well known that they produce $O_2^{\cdot -}$ and H_2O_2 and that vascular endothelial cells generate $NO^\cdot$. Hence it is very likely that these species come into contact in human atherosclerotic lesions. Whether $NO^\cdot$ is then good or bad may be critically dependent on the *ratio* of $NO^\cdot$ to such species as $O_2^{\cdot -}$, H_2O_2, $LO_2^\cdot$ and $LO^\cdot$ (166, 176, 177).

POTENTIAL THERAPEUTIC INTERVENTIONS

There are, of course, many therapeutic interventions that can be applied to the treatment of cardiovascular disease which do not directly involve free radicals, metals or oxidative stress. Lowering plasma cholesterol is a popular and effective approach; in the context of the oxidative hypothesis of atherosclerosis one might simply argue that it is decreasing the level of LDL particles that could be oxidized in the vessel wall. Hypertension is a risk factor: oxidative stress may again be involved in its pathology (42, 178). If oxidation is important in vascular disease then antioxidants should be therapeutically effective, but only if they can be successfully directed at the appropriate target in the necessary concentrations (179). Below we outline some of the "radical" approaches which are either currently available or in development for the treatment of vascular disease. Some of the key points are shown schematically in Fig. 5.

Nitric Oxide Synthase Inhibitors

If the *balance* between $NO^\cdot$ and ROS (especially $O_2^{\cdot -}$) at sites of injury can affect the net outcome then its manipulation may be beneficial therapeutically. For example, under conditions where excess generation of $NO^\cdot$ is contributing to the tissue damage, inhibition of NOS would seem to be a reasonable

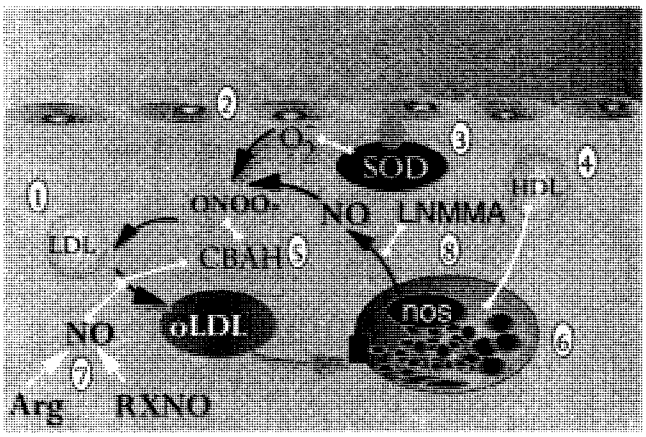

Fig. 5. Therapeutic approaches to vascular oxidant stress. ROS/RNS formation is depicted by the solid arrows and points of intervention by the white bars. Key: 1: LDL may be oxidised by several processes in the artery wall; decreasing its concentration by the use of lipid lowering agents should decrease oxLDL production. 2: Loss of functional $NO^\cdot$ is an early event in the atherosclerotic process and $NO^\cdot$ may be restored by scavenging superoxide with SOD (3). LDL oxidation can be inhibited by chain breaking antioxidants (5; CBAH). 6: Macrophages are involved in the detoxification of oxidised LDL and this process may be enhanced by increasing macrophage oxidative defenses or 4: through increasing reverse cholesterol transport via high density lipoprotein (HDL). 7: Nitric oxide possesses antioxidant and anti-inflammatory properties and restoring an appropriate level may be achieved with either arginine or $NO^\cdot$ donors (RXNO). 8: Inhibition of $NO^\cdot$ synthase (nos) with compounds such as LNMMA (N-monomethyl L-arginine).

therapeutic strategy [Figure 5]. However, given the widespread functions of NO· in the body, selective inhibition of the specific isozyme of NOS which is contributing to the disease process would seem to be the preferred approach. Considerable progress has been made in the development of such compounds (180–182). Potent selective inhibitors of iNOS are being considered for treatment of life threatening hypotension in septicaemia and selective nNOS inhibitors may be valuable in treating stroke. Other less selective NOS inhibitors may be useful in an intensive care setting, where potential side effects such as hypertension can be carefully monitored (183).

Nitric Oxide Donors

In some cases, NO· may be lacking. Nitrovasodilators such as glycerol trinitrate are metabolised by vascular smooth muscle cells to release NO· They provide immediate relief from the pain of angina by relaxing the constricted coronary vasculature. Their effects are short-term because they induce tolerance (ironically, a process in which enhanced vascular $O_2^{\cdot -}$ generation may be involved (184)). There is scope for the development of compounds which may act through a similar mechanism but are not tachyphylactic. New generations of NO· donors may allow selective release of NO· in different tissues and local restoration of the balance between RNS and ROS. For example, nitrosoglutathione appears to show platelet selective effects when administered *in vivo* (185). The clinical indications of such compounds may be much broader than angina and could include prevention of the re-occlusion of coronary arteries after angioplasty, and arresting premature labour (186, 187). Direct application of NO· itself is being used in the treatment of pulmonary hypertension. Arginine administration may also have beneficial effects by enhancing NO· formation in the vasculature (187).

Scavenging of $O_2^{\cdot -}$

Despite the fact that $O_2^{\cdot -}$ itself is inefficient in the direct oxidation of lipids (1), it can act as a precursor to much more reactive species such as OH· and $ONOO^-$. Its removal would seem a straightforward therapeutic approach (188, 189) but this is complicated by the extreme rapidity of the reaction between NO· and $O_2^{\cdot -}$ (40). Localisation of the SOD enzyme or $O_2^{\cdot -}$ scavenger to the vascular endothelium may be necessary (188, 190), e.g. by the use of recombinant EC-SOD or SOD constructs containing a heparin-binding domain (188). Alternatively, the source of superoxide in the vasculature, which has not been identified with any degree of certainty in humans, could be inhibited directly.

Chain Breaking Antioxidants

One advantage of this approach is that identification of the pro-oxidant mechanism is not necessary since lipid peroxidation is propagated by the same species, the peroxyl radical, which can be intercepted by peroxyl radical scavengers. However, such compounds may be relatively ineffective in a metal/lipid peroxide dependent oxidation mechanism unless present at high concentrations. In experimental models of atherosclerosis probucol has been effectively used to inhibit the development of atherosclerotic lesions at a dosage level of 0.1% of the diet (191). Such high dosing regimens appeared to be necessary for an anti-atherosclerotic effect and might be inappropriate if converted to the equivalent dosage for humans (192).

CONCLUSION

Free radicals are formed constantly in the vascular system. Often they are useful (especially NO·), but in excess they can be dangerous. In the case of atherosclerosis, there is good evidence that NO· and ROS contribute to its development. Since atherosclerosis begins in the first decade of life, learning how it starts and how to prevent it would make a major impact upon human health.

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