

# Antioxidants

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## FREE RADICALS AND ANTIOXIDANTS

A free radical is a molecule with one or more unpaired electrons capable of abstracting an electron from another molecule, thereby oxidizing it. Antioxidant is an ill-defined term best characterized as "any substance that, when present at low concentrations compared to an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" (1). In chemical terms, only oxidants and reductants exist. In earlier times the restriction of antioxidants to chain-breaking antioxidants such as vitamin E was used.

Free radical chemistry is now acknowledged as occurring in all biological systems serving a variety of biological functions. The redox status is known to regulate a number of enzyme activities. Moreover, free oxygen radicals and other reactive oxygen species are formed by many endogenous processes, such as metabolism and neutrophil function, as well as being a part of environmental exposures.

## LOW-DENSITY LIPOPROTEIN (LDL) OXIDATION

In atherosclerosis, interest has centered on oxidation of lipids, particularly low-density lipoprotein (LDL) particles. These are complexes containing a number of different lipid classes and distinct lipids, e.g. cholesterol, free and esterified triglycerides, phospholipids. Oxidation leads to modification of the apolipoprotein "cap" and other structural changes of the LDL particle.

It has long been known that cholesterol can be found in atherosclerotic plaques, that high LDL levels are found in accelerated atherosclerosis (cholesterol levels above 4.1 mmol/l), that LDL is protected from oxidation by lipophilic antioxidants, that titers of autoantibodies against oxidized LDL correlate with progression of carotid atherosclerosis (for detailed review see 2).

Due to the modification of the apolipoprotein B-oxidized LDL is taken up by macrophages without the feedback regulation of LDL uptake by other cells. The

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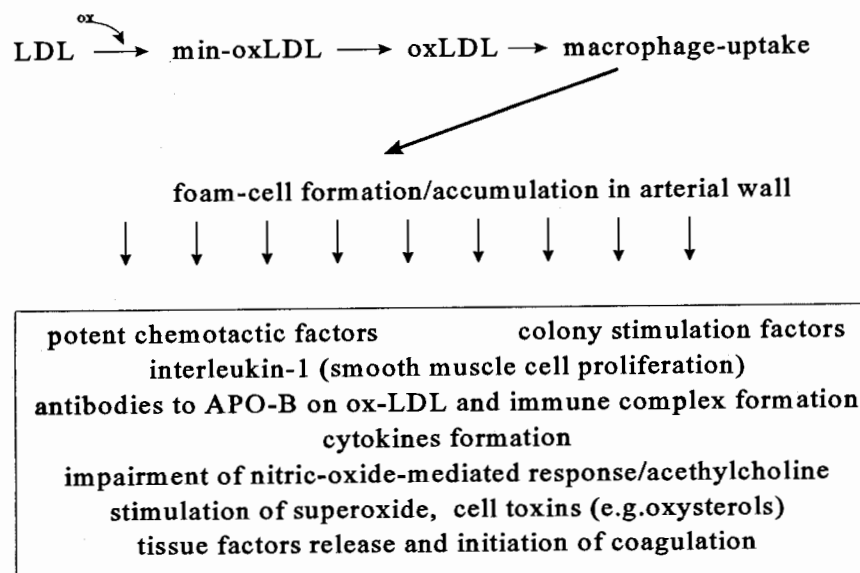


Fig. 1.

modification of apolipoprotein B can be mediated by a variety of biologically active molecules formed by oxidation, e.g. oxidized sterols, oxidized fatty acids, phospholipid and protein derivatives, malondialdehyde, 4-hydroxynoneal (e.g. 3). The unregulated uptake of oxidized LDL leads to modification of the macrophages and their accumulation as foam cells in the arterial wall and a series of other events, depicted in Fig. 1.

#### FACTORS OF IMPORTANCE FOR INITIATING/PREVENTING LDL-OXIDATION

The main single identified factor inhibiting LDL oxidation is the LDL concentration of  $\alpha$ -tocopherol, demonstrated as a positive correlation between the LDL  $\alpha$ -tocopherol concentration and the lag phase of LDL oxidation *in vitro* (2) and references therein. The lag phase determination consists of isolation of LDL or VLDL+LDL from plasma and exposure *in vitro* to a standardized oxidative system with monitoring of lipid oxidation, e.g. by conjugated diene formation or similar methods. The system most used is based on copper ions or hemin (2), or gentle oxidation with substances that spontaneously release peroxy radicals (4).

The estimation *in vitro* of "LDL oxidation lag time" is an attractive intermediate biomarker for development of atherosclerosis, and a single study has proven

a correlation between development of atherosclerosis and LDL lag time (5). It is still not sufficiently proven, however, that a increase in lag time brought about by dietary or supplementary means leads to a reduction in the rate of arteriosclerotic development, or whether such a potential effect is general on arteries or restricted to certain parts or regions of the arterial tree.

Several intervention trials are ongoing and will hopefully be able to answer such questions (6). Further discussion of this is presented later in this paper.

#### ANTIOXIDANT FUNCTIONS IN LDL

Although the fat soluble  $\alpha$ -tocopherol (vitamin E is a term that refers to several tocopherols) is considered the foremost antioxidant preventing LDL oxidation, other antioxidant vitamins and endogenous substances have been identified as important. Interactions between the various antioxidants are also considered important. The water-soluble vitamin C (ascorbate) can effectively eliminate the tocopheryl radical arising from oxidation of  $\alpha$ -tocopherol, the process occurring in the aqueous-lipid transition phase and generating the aqueous ascorbyl radical and thus preventing LDL oxidation (7, 8). The effect of  $\beta$ -carotene on LDL oxidation is controversial, as is the literature, which tends to conclude that  $\beta$ -carotene is an ineffective antioxidant in LDL (9).

A high content of protectant antioxidants vitamins is assumed to inhibit the deleterious effects of oxidized LDL: endothelia cytotoxicity, uncontrolled LDL uptake by macrophages, activation of endothelial adhesiveness, monocyte chemotaxis, auto-antibody generation towards oxidized LDL. It must be emphasized, however, that presently there are no available methods for direct estimation of LDL oxidation *in vivo* or to identify circulating oxidized LDL.

#### ANTIOXIDANTS, NO FORMATION AND NO RESPONSE

Details on NO formation and function are given elsewhere in this book. It has been suggested for some time that the ubiquitous intracellular antioxidant glutathione has some influence on, for example, the formation of NO from nitroglycerin. This was in part supported by the findings that the drug N-acetyl cysteine, a glutathione-stimulating agent, enhanced the response to nitroglycerin in animals (10) and in man (11). However, more detailed investigations revealed a complex and not fully understandable role of glutathione in the action of nitroglycerin (12, 13). Preliminary investigations have shown that vitamin C has a similar effect to that of N-acetyl cysteine (14) and an effect of vitamin C on endothelial vasomotor dysfunction has also been demonstrated in smokers (15) and in patients with coronary disease (16).

TABLE I  
Evidence for and against the oxidation hypothesis of atherosclerosis

Research area	Finding	Ref.
Pathology (1910)	cholesterol found in atherosclerotic plaques	(23)
Clinical science (1954)	high LDL in early and severe atherosclerosis	(24, 25)
Pathology 1975	foam cells can be derived from monocytes	(26)
Cell biology (1983)	oxidized LDL is toxic to human skin fibroblasts	(27)
Cell biology (1986)	oxidized LDL is recognized by macrophage scavenger receptor	(28)
Epidemiology (1987)	inverse correlation between ischaemic heart disease and plasma antioxidants	(29)
Clinical science (1988)	early oxidative modification of LDL identified in human plasma	(30)
Immunology (1990)	arteriosclerotic lesions contain oxidized LDL (epitopes characteristic for oxidized LDL)	(31, 32)
Clinical science (1992)	correlation between progression of atherosclerosis and oxidized LDL autoantibody titer	(33)
Epidemiology (1993-4)	large epidemiological studies (cohort) show reduced relative risk associated with diet high in antioxidant vitamins and supplements	(34, 35, 36)
Epidemiology (1996)	low flavonoid intake is associated with higher risk of coronary disease	(5)
Epidemiology (1996)	association between intake of food with high intake of vitamin E and coronary heart disease, but not between vitamin E supplements and coronary heart disease	(37)
Controlled trial (1994)	no effect of $\beta$ -carotene/vitamin E on ischemic heart disease	(38)
Controlled trial (1995)	probucol lowers HDL <sub>2b</sub> and therefore fails to affect atherosclerosis	(39)
Controlled trial (1995)	pravastatin reduces carotid atherosclerotic progression by increasing LDL+VLDL lag time	(20)
Controlled trial (1995)	vitamin E supplementation reduces the rate of non-fatal myocardial infarction in patients with symptomatic coronary atherosclerosis	(40)
Controlled trial	no effect of $\beta$ -carotene plus vitamin A on death from cardiovascular disease	(21)
Controlled trial	no effect of $\beta$ -carotene on death from cardiovascular disease	(19)

The mechanism by which antioxidants, e.g. N-acetyl cysteine/glutathione or vitamin C, interact with NO is unclear. One attractive possibility is that in states with oxidative stress, e.g. smoking, NO is eliminated by reaction with superoxide anions producing peroxynitrite. Along these lines it is intriguing that NO-liberating agents can lead to formation of hydroxylated DNA bases (17), usually formed from reactive oxygen species, presumably including peroxynitrite. A second possi-

bility is that antioxidants could regulate important enzyme or transporter mechanisms by changing redox status (18).

#### PROVING THE EFFECTS OF ANTIOXIDANTS IN PREVENTION IN ATHEROSCLEROSIS

The establishment of methodologies for estimating the primary prevention of human disease is a challenging scientific task. Among many difficulties a prominent feature relates to the decades of pathophysiological development characterizing diseases such as cardiovascular diseases and cancer. At present there has been only one controlled intervention study with preventive measures performed for more than 10 years (19).

The basic science and epidemiological findings are strongly in favor of antioxidant vitamins having a protective effect on LDL oxidation as well as a protective effect of high dietary intake of foodstuffs with a high antioxidant vitamin content. There are also intervention trials where a single substance increases LDL oxidation lag time and protects against atherosclerotic progression (20). In a randomized controlled trial conducted for 12 years where 11 036 persons were randomized to  $\beta$ -carotene 50 mg every other day and 11 035 to placebo, there was no effect on death from cardiovascular disease or overall mortality (19). In a shorter trial,  $\beta$ -carotene plus vitamin A administration did not have any effect (21).

At the 1995 international conference on  $\beta$ -carotene and antioxidant vitamins it was put forth that effects of primary intervention of these substances would have to be judged from a combined evaluation of laboratory and epidemiologic evidence (22).

#### CONCLUSIONS

The free radical theory links oxidation of circulating LDL particles to the development of atherosclerosis. Substantial evidence has accumulated from laboratory data and clinical investigations. The hypothesis is further substantiated by epidemiologic observations of slower atherosclerotic development in people with high antioxidant intake from diet. Controlled primary prevention trials of  $\beta$ -carotene and vitamin E have been negative. However, the optimum dosage and observation time may not have been used. Moreover, the conditions of such controlled trials, 30 000 of subjects or more and many years of observation, may not allow identification the effective doses of what were probably combinations of antioxidant substances in various doses and combinations.

Recent evidence points at interesting interactions between NO and antioxidants

that might be important in the explanation of antioxidant effects, and might help in the design of future clinical trials.

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## DISCUSSION

YELLON: Can I play devil's advocate? Morris Brown's recent study actually demonstrated an increase in mortality with vitamin E. Is it possible that by scavenging free radicals you prevent preconditioning?

POULSEN: Clinical trials are not very good for testing hypotheses about underlying mechanisms. I referred to some of the probucol trials that were negative. But some aspects of those trials might still be relevant. The negative  $\beta$ -carotene trials have had adverse commercial impact, but have also reduced support for further research, which may be a mistake.

W SCHAPER: You stated that most of the reactive oxygen species come from normal mitochondrial oxidation, and that 1-5% of the oxygen is converted into reactive oxygen species. Would cells with high oxygen uptake and oxidation be the first to come to reactive oxygen species? Endothelium, which has almost no mitochondria, has a very low oxygen consumption and is one of the few tissues that produces lactate in the presence of oxygen. Would this source of reactive oxygen species be particularly low in the endothelium?

POULSEN: Production is not the only determinant of free radical levels available in the cell without defense systems. Oxidation rates of DNA correlate well with oxygen consumption, at least on a whole body basis.

ENGLER: The superoxide leak of the mitochondria is linearly proportional to the flux and the  $PO_2$ . A  $PO_2$  four times higher in the endothelial cell will result in the same superoxide production if it has one quarter the flux.

HEARSE: I frequently hear this figure of 3-5% being quoted. Has anybody independently confirmed it?

HARRISON: I think the mitochondria production of superoxides is irrelevant. The superoxide probably does not make it out of the mitochondria to interplay with NO or to damage lipids that are nearby. There is a large amount of manganese SOD in the mitochondria, so if anything makes it out, it might be hydrogen peroxide, but the problem with this is that there is also a large amount of glutathione peroxidase in the mitochondria. So, the only conditions



in which you might see this are conditions in which there are genetic abnormalities of the mitochondria. I presented data on Tuesday in which we showed that when you homogenize either endothelial cells or smooth muscle cells, far and away the greatest source of superoxide is this membrane NADH oxidase, which is not a mitochondrial oxidase. I believe the role of the mitochondria as a source of superoxide for the rest of the cell is quite overblown. Mitochondrial superoxide is quite likely important for various facets of mitochondrial function.

POULSEN: If you are talking about endothelial cells, I would agree.

HARRISON: And smooth muscle cells?

POULSEN: Yes.

HARRISON: In the macrophage and the neutrophil ...?

POULSEN: Yes, but they are specialized for that.

ENGLER: I agree completely with Dr. Harrison when mitochondria are normal, but following ischemia-reperfusion in myocytes, mitochondria could be important.

HARRISON: Noone has ever shown that the mitochondria can release superoxide into the cytoplasm.

LUCCHESI: You have shown some very convincing evidence regarding the toxic effects of tobacco smoke. I would like to consider another xenobiotic agent, alcohol, and its metabolites, acetyl-aldehyde, and their ability to affect cardiac metabolism and free radical production in the heart.

POULSEN: Small molecules like alcohol that are metabolized by cytochrome B452E1 have quite a co-production of hydrogen peroxide. This was once used as an assay for B450 activity.

FURCHGOTT: Dr. Harrison, you brought up the fact that your findings show NADPH induction at the membranes of endothelial cells and smooth muscle

cells? Does that enzyme deliver superoxide intracellularly, extracellularly or both ways?

HARRISON: I don't think it is extracellular. I think in a microsomal fraction it is intracellular. So it is probably not releasing superoxide on the surface.