Free radicals and antioxidants in cardiovascular disease

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Several lines of evidence suggest that free radical-mediated oxidative damage to lipoproteins may be an important factor predisposing them to uptake into the vascular wall. This has led to interest in the factors that determine the susceptibility of lipoproteins to oxidation and their relationship to the development of coronary heart disease. Of these factors, the lipoprotein content of natural antioxidant vitamins such as vitamin E, and beta-carotene have aroused particular interest. Studies in animal models of atherosclerosis suggest that natural and synthetic antioxidants can retard the development of atheroma. Epidemiological comparisons between populations and studies within populations also support the contention that high plasma levels or dietary intake of natural antioxidant vitamins may protect against the development of coronary disease in man. Prospective randomized controlled trials of antioxidants in high risk groups are underway to test whether the theoretical promise of a beneficial role for antioxidants in coronary heart disease prevention will be fufilled.

Keywords: antioxidants, coronary artery disease, free radicals, vitamin E

Introduction

Atherosclerosis and its complications, most notably coronary heart disease (CHD), continue to be the major cause of premature death in the developed world. A variety of important risk factors for its development including hypercholesterolaemia, hypertension, smoking and diabetes have been identified. Although the outstanding pathological feature of atherosclerosis is the collection of cholesterol esters from circulating lipoproteins in the intimal layer of large arteries the concentration of cholesterol in circulating plasma alone remains a relatively poor predictor of disease. This has therefore stimulated research into the pathophysiological mechanisms underlying the development of atherosclerosis and, in particular, into the factors that facilitate the deposition of lipoproteins in the vascular wall. The first part of this article will review the accumulating evidence that free radical-induced oxidation of lipoproteins may be an important event in this process. By extension, the second part will review the evidence that dietary antioxidants which retard oxidation may slow or prevent the development of atherosclerosis and CHD *in vivo*.

Free radicals: definition and sources

A free radical can be defined as 'any species capable of independent existence that contains one or more unpaired electrons' occupying an atomic orbital by itself. This situation is energetically unstable, often making such species highly reactive and short-lived. Stability is achieved by the removal of electrons from (i.e. oxidation of) surrounding molecules to produce an electron pair. However, the remainder of the attacked molecule then possesses an unpaired electron and has therefore become a free radical (Figure 1). Subsequent events depend on the reactivity of the target radical. If high, further target molecules will be attacked and lead to propagation of a free radical chain reaction. In this way the presence of a single radical may initiate a sequence of electron transfer (redox) reactions. In the case of antioxidant targets the resultant radical has low reactivity and the chain is broken.

Most of the important free radicals in human biology are derived from oxygen. The tetravalent reduction of oxygen in the mitochondrial electron transport is necessary to generate energy. However, the reduction is not 100% efficient and some partially reduced oxygen may leak from the system in the form of the superoxide radical ($O_2^{\bullet -}$). Superoxide radicals may dismute (either spontaneously or, more rapidly, catalysed by superoxide dismutase) to form hydrogen peroxide (H_2O_2). Neither species is a particularly powerful oxidant but both may interact with transition metal ions such as Fe^{2+} and Cu^{+} to generate the highly reactive hydroxyl radical ($OH^{\bullet}$). The latter is probably responsible for much of the biological damage *in vivo*. Other sources of free radicals include the respiratory burst of leukocytes, reperfusion of previously hypoxic tissues, autoxidation of molecules such as glucose and exogenous sources such as cigarette smoke.

Free radical-induced damage: lipid peroxidation

Free radicals can disturb biological systems by damaging a variety of their constituent molecules. Lipids, proteins, carbohydrates and DNA are all potential targets for the chaotic oxidative attack of radicals produced in their vicinity.

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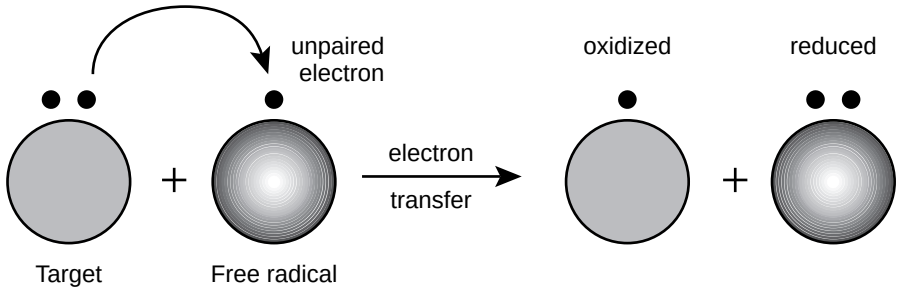

Figure 1 The reaction of a free radical with a non-radical species. The unpaired electron of the free radical is highly reactive and tends to abstract an electron from an electron pair of the non-radical target. Following electron transfer the free radical is now stable but a target radical has been created. Subsequent events depend on the reactivity of the target radical. If high further target molecules will be attacked and lead to propagation of a free radical chain reaction. In the case of antioxidant targets the resultant radical has low reactivity and the chain is broken.

Polyunsaturated fatty acid residues (that is, fatty acids with more than one carbon-carbon double bond) in lipoproteins have a chemical structure that makes them a particularly vulnerable target for free radical oxidation (lipid peroxidation) which may be of greatest relevance to atherogenesis.

Lipid peroxidation is initiated if a radical enters the lipid phase and has sufficient reactivity to abstract a hydrogen atom from a fatty acid bis-allylic methylene (-CH_2-) group (Figure 2). This process is favoured in carbon-hydrogen bonds adjacent to unsaturated double bonds since the covalent bond is weaker. Hydroxyl radicals are certainly capable of this but superoxide is apparently not and would also be unlikely to enter the hydrophobic membrane interior. In many systems the presence of metal ions such as iron seems to be essential for initiation presumably because it functions as a template for local hydroxyl formation.

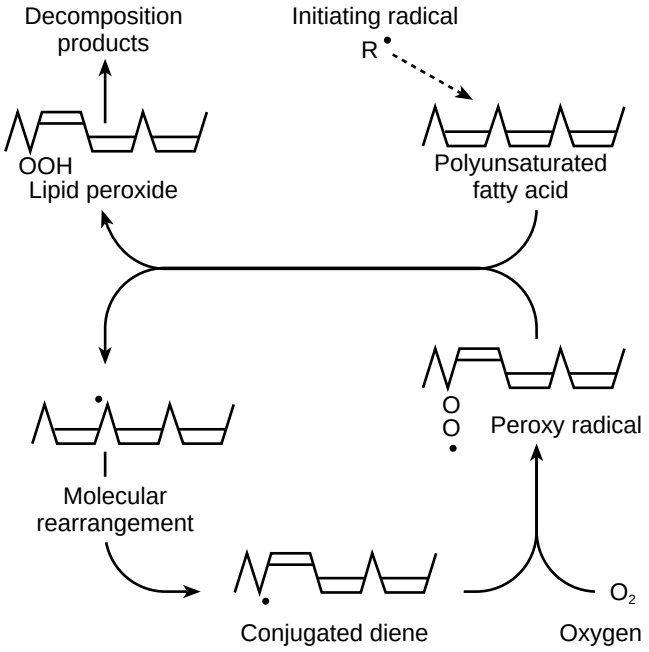

Figure 2 The initiation and propagation steps of lipid peroxidation. Oxidizing species such as hydroxyl, peroxyl and alkoxyl radicals and singlet oxygen are capable of abstracting one of the bisallylic hydrogen atoms on the carbon atom between two double bonds of an unsaturated fatty acid. The radical product undergoes molecular rearrangement to a conjugated diene which reacts rapidly with oxygen to form a peroxy radical. The latter can itself abstract a hydrogen atom from unsaturated fatty acids leading to propagation of the reaction and production of a lipid peroxide. Lipid peroxides are relatively unstable and form a variety of toxic decomposition products. If oxygen and polyunsaturated fatty acids remain available a single initiating radical can lead to free radical chain reaction involving many hundreds of lipid molecules. Chain-breaking antioxidant molecules react preferentially with peroxy radicals and prevent propagation.

Removal of a hydrogen atom produces a carbon centred radical which stabilizes by a molecular rearrangement to form a conjugated diene which shows characteristic u.v. absorption at 234nm [1]. If oxygen is available (it is several times more soluble in lipid than aqueous phases) the likely fate of this radical is to combine to produce a peroxyl radical. Once formed a peroxyl radical has sufficient reactivity to abstract a hydrogen atom from an adjacent unsaturated fatty acid to produce a further carbon-centred radical that itself reacts with oxygen to form a peroxyl radical. This is the propagation stage of lipid peroxidation. Therefore, if it is unchecked a single radical initiation can lead to long chains of lipid hydroperoxide (peroxide) formation. Lipid hydroperoxides are unstable and tend to decompose rapidly to form secondary products. These include alkanes like ethane and pentane detectable by gas chromatography and aldehydes such as malondialdehyde and 4-hydroxy-2,3-trans-nonenal. These products are themselves toxic and may be responsible for many of the deleterious effects of lipid peroxidation. In particular, aldehydes may interact with the apolipoprotein moiety of lipoproteins and prevent normal cellular recognition [2].

Antioxidant defences

A variety of antioxidant mechanisms have evolved to combat the potential threat of damage to vital biological structures from the aforementioned sources [3–5] (Table 1). An antioxidant can be considered as a molecule that, when present at low concentrations compared with those of an oxidizable substrate, significantly inhibits oxidation of that substrate. This antioxidant effect can be achieved in three different ways, as illustrated in Figure 3. Thus the intracellular environment contains enzymes such as superoxide dismutase, catalase and glutathione peroxidase that catalyse the breakdown of oxidants generated *in situ* by cellular metabolism. Preventative antioxidant proteins exist to sequester free transitional metal ions which would facilitate the production of the hydroxyl radical. These include the iron-binding protein transferrin and copper-binding proteins caeruloplasmin and albumin.

Table 1 Antioxidant mechanisma in human biology.

- *Antioxidant enzymes*
  - Superoxide dismutase
  - Catalase
  - Glutathione peroxidase/Glutathione reductase
- *Preventative antioxidants*
  - Caeruloplasmin
  - Transferrin
  - Albumin
- *Chain-breaking antioxidants*
  - *Water-soluble*
    - Urate
    - Ascorbate
    - Thiols
    - Bilirubin
    - Flavonoids
  - *Lipid-soluble*
    - Tocopherols
    - Ubiquinol-10
    - Beta-carotene

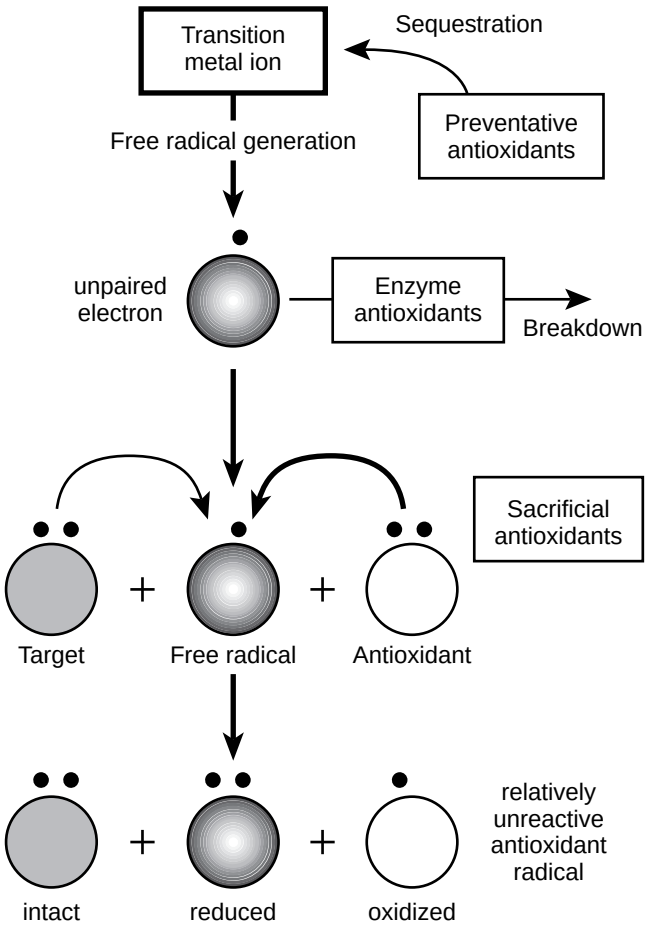

Figure 3 The interaction of free radicals and antioxidants. Enzyme antioxidants catalyse the breakdown of radical species usually in the intracellular environment. Preventative antioxidants bind transition metal ions such as iron and copper preventing their interaction with hydrogen peroxide and superoxide to produce highly reactive hydroxyl radicals. Sacrificial ('chain-breaking') antioxidants are powerful electron donors and react preferentially with free radicals before more important target molecules are damaged. In doing so the antioxidant is sacrificed (oxidized) and must be regenerated or replaced. By definition, the antioxidant radical is relatively unreactive and unable to attack further molecules [3–5].

In spite of these mechanisms free radicals are constantly generated *in vivo*. For this reason body fluids contain a rich array of low molecular weight molecules that preferentially react with (scavenge) free radicals before more important structures are damaged but are sacrificed (consumed) in the process. These can be conveniently divided into those that are water-soluble and those that are lipid-soluble and exist in environments such as lipoproteins and cell membranes to prevent the propagation phase of lipid peroxidation (chain-breaking antioxidants). Of the aqueous molecules the best known is vitamin C (ascorbate) which is the most powerful electron donor and is the first plasma antioxidant to be sacrificed upon exposure to oxidative stress. Its oxidation product dehydroascorbate can then be regenerated to ascorbate intracellularly. Man is unable to synthesize vitamin C for which fresh fruits and vegetables form the major dietary sources. Urate is also a recognized sacrificial antioxidant whose role *in vivo* is still under evaluation, whilst a more recently recognized group of antioxidants are the polyphenols (including flavonoids) which are active free radical scavengers *in vitro*. Their major dietary sources are fruits and vegetables as well as beverages such as tea and wine. Other molecules with a minor antioxidant role include bilirubin and thiol groups, which are mostly associated with albumin.

Vitamin E is a lipid-soluble antioxidant and refers to alpha-tocopherol and other related tocotrienols. Important dietary sources are vegetable oils, cereal grains, egg yolk, liver and milk. It is the major antioxidant of lipoproteins and membranes where it acts as a powerful chain-breaking antioxidant. The efficient function of vitamin E *in vivo* depends upon its regeneration following oxidation to the tocopheryl radical. This is achieved by a direct electron transfer with vitamin C at the lipid-water interface of lipoproteins (and membranes) [6]. Ubiquinol-10 (reduced co-enzyme Q_{10}) is present in much lower concentration than vitamin E but is also an efficient free radical scavenger and may also contribute to the regeneration of tocopherol. Finally, beta-carotene is the most abundant carotenoid and circulates in lipoproteins. Its major dietary sources are fruits and vegetables. Beta-carotene quenches singlet oxygen but is not a particularly active radical scavenger other than at low oxygen tensions; the precise pathophysiology of its antioxidant function however remains to be defined.

Oxidative-modification hypothesis of atherosclerosis

Basic research has now advanced our understanding of some of the biochemical events that lead to the development of the atherosclerotic plaque [7]. Most of the cholesterol in the mature lesion originates from circulating low-density lipoprotein (LDL) particles which have been ingested by subendothelial macrophages. However, when exposed to high concentrations of native LDL macrophages in culture do not accumulate cholesterol due to down-regulation of LDL receptor-mediated uptake in the cholesterol-replete state [8]. In contrast, oxidatively modified forms of LDL are much more avidly taken up by cultured macrophages which

become converted to cholesterol-laden 'foam cells', a characteristic histological feature of the atherosclerotic lesion. The rapid uptake of the oxidatively modified LDL has been attributed to a new receptor known as the 'scavenger receptor' which is not down-regulated by cholesterol accumulation [9] (Figure 4). Recognition of LDL by the scavenger receptor depends on derivatization of lysine residues of apolipoprotein B by aldehydes produced by the spontaneous decomposition of lipid hydroperoxides [2].

Although oxidation of LDL is not likely in the circulation, all of the cells of the vessel wall—endothelial cells, smooth muscle cells and macrophages—can modify LDL *in vitro* [10–12]. The mechanisms by which LDL particles become oxidized are, as yet, incompletely understood but are likely to involve exposure to free radicals in the intimal layer of the vascular wall, possibly generated by transition metal ions [13]. However, LDL can be oxidized by all of the major cell types found in the arterial wall and also by exposure to transition metal ions such as copper *in vitro*. Furthermore, the oxidation of LDL is facilitated by binding to local intimal proteoglycans [14].

Oxidized LDL may also be atherogenic by mechanisms other than its rapid uptake into macrophages via the scavenger receptor. Oxidized forms of LDL are a chemoattractant for macrophages and smooth muscle cells [15] and facilitate monocyte adhesion to endothelium and entry into the subendothelial space [16]. They are also cytotoxic towards arterial endothelial cells [17] and inhibit the release of nitric oxide and the resulting endothelium-dependent vasodilatation [18].

The presence of oxidized LDL *in vivo* has been well-established. Oxidatively modified forms of LDL have been

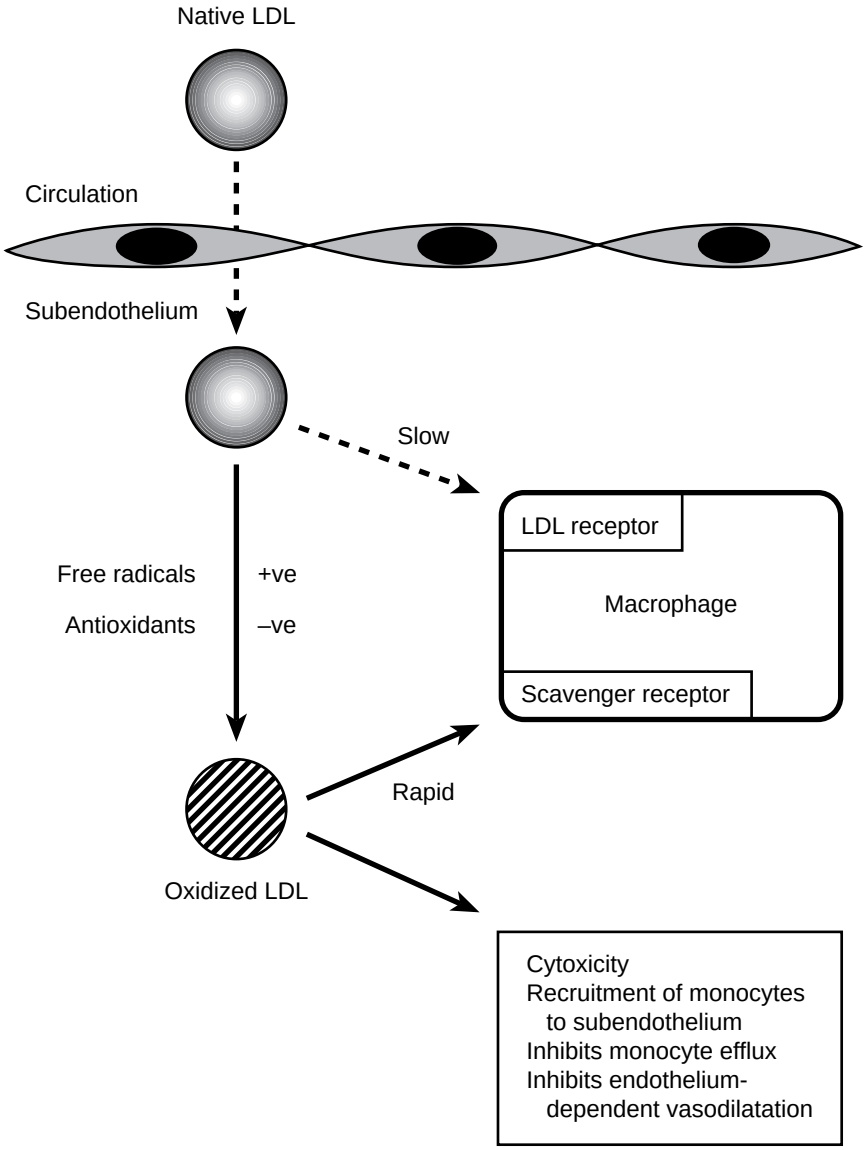

Figure 4 The oxidative-modification theory of atherosclerosis. Intermittently, circulating LDL particles cross the endothelium into the intima of blood vessels. In their native form they are unfavourable for uptake into intimal macrophages and most return to the circulation. However, some particles may be oxidized *in situ* by local cells possibly facilitated by the presence of transition metal ions and binding to proteoglycans. After oxidative modification the LDL particles are rapidly taken up into macrophages via the scavenger receptor. Subsequent loading with cholesteryl esters forms so-called 'foam cells' the earliest histological evidence of atherosclerosis. Oxidized LDL has other deleterious biological activities including cytotoxicity towards local cells.

identified in human atherosclerotic plaques by extraction [19] or immunochemical detection [20] and circulating antibodies that recognize epitopes on oxidized LDL can be found *in vivo* [21]. The titres of LDL auto-antibodies have also been correlated with the progression of atherosclerosis. With these discoveries the oxidative-modification hypothesis of atherosclerosis was established and further research directed towards the factors that influence the susceptibility of the human LDL particle to oxidation (Table 2).

One of the most important factors appears to be lipid-soluble antioxidant vitamin content, particularly vitamin E (alpha- and gamma-tocopherol), ubiquinol-10 and beta-carotene in order of abundance [12]. These antioxidant vitamins exist in the hydrophobic environment of the lipoprotein particle where they protect vulnerable polyunsaturated fatty acids from free radical oxidation (Figure 5). In this way they prevent the production of the toxic products that produce the altered biological properties of oxidized LDL. *In vitro* measurements of the appearance of these products in LDL subjected to oxidative stress suggest that not until all of the vitamin E is oxidized can lipid peroxidation begin. When LDL is exposed to oxidative stress *in vitro*, lipid peroxidation can only proceed after the sequential loss of its antioxidants in the order ubiquinol-10, alpha-tocopherol, gamma-tocopherol, lycopene and beta-carotene [1, 22, 23]. Accordingly, LDL supplemented with vitamin E *in vitro* [1, 24] or *in vivo* [25] is much harder to oxidize *in vitro*.

In addition, several other factors also influence the susceptibility LDL to oxidation. Polyunsaturated fatty acids appear to be the most vulnerable moiety following the application of oxidative stress. Therefore, it is not surprising that particles of varying fatty acid content show different behaviour in such circumstances. For example, LDL enriched in linoleic acid was more susceptible to oxidation while that enriched in monounsaturated fatty acids was more resistant [26, 27]. Small dense LDL particles are also more easy to oxidize than more buoyant particles [28]. This may explain the apparent risk for CHD conferred by the presence of an excess of these particles. Finally, the binding of LDL particles to proteoglycans, glycation and the presence of preformed lipid peroxides (which greatly facilitate subsequent metal-induced oxidation) are also important.

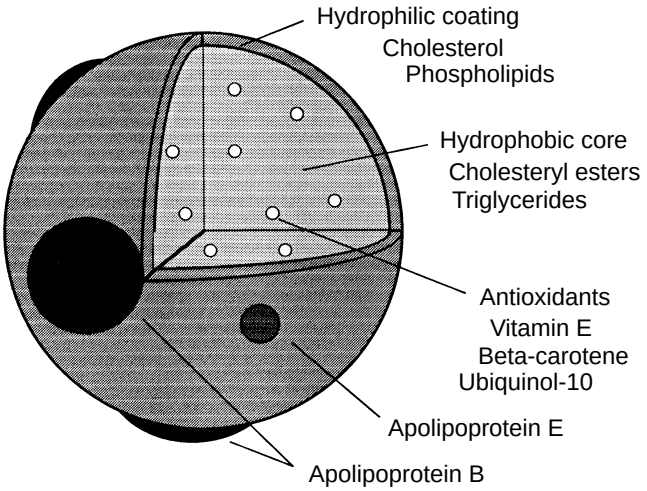

Figure 5 The structure of the LDL particle. The particle has two distinct phases. The hydrophobic core consists of cholesteryl esters (1600) and triglycerides (170) many of which contain vulnerable polyunsturated fatty acid moieties. Within the lipid-soluble environment there are a variety of antioxidant molecules. The most common is alpha-tocopherol (vitamin E, 5–10 per LDL) but other molecules such as ubiquinol-10 (0.33), beta-carotene (0.27) and lycopene (0.21) exist at lower concentration. The edge of the hydrophobic region is a monolayer of free cholesterol (600) and phospholipids (700) which forms an interface with the surrounding aqueous environment. Floating within the core is the large apolipoprotein B molecule (m.w. 510 000) which accounts for most of the protein content and is important for particle recognition by the LDL receptor. Total particle m.wt. = 2 500 000, density range 1.019–1.063 g ml^{-1} and diameter 19–25 nm. Other lipoprotein particles have similar basic structures but differ in their apolipoprotein content, density, lipid-protein ratio and antioxidant content [12].

Evidence for a protective role of antioxidants *in vivo*

Evidence comes from studies of antioxidant intervention in animals and epidemiological observations in man (Table 3).

Animal studies

The potential beneficial role for both natural and synthetic antioxidants in the prevention of atherosclerosis has been investigated in a variety of animal models [29]. Trials of

Table 2 Factors influencing the susceptibility of LDL to oxidation.

- *Antioxidant content*
  - *Intrinsic (lipid-soluble)*
    - Vitamin E (alpha-tocopherol)
    - Ubiquinol-10 (co-enzyme Q_{10})
    - Beta-carotene?
  - *Extrinsic (water-soluble)*
    - Vitamin C (ascorbate)
    - Urate
    - Flavonoids and other polyphenols
- *Polyunsaturated fatty acid content*
- *Particle size*
- *Proteoglycan binding*
- *Protein glycation?*
- *Lipid peroxides?*

Table 3 Evidence for the protective role of antioxidants against the development of coronary heart disease (CHD).

- *Animal studies*
  - —antioxidant supplementation retards atherosclerosis in animal models
- *Epidemiological studies*
  - —inter-population epidemiological studies
  - —intra-population epidemiological studies
  - (blood antioxidant levels or dietary antioxidant intake)
- *Case-control studies*
  - —case-control studies of CHD
  - low blood antioxidant levels are an independent risk factor in cases
- *Prospective randomized controlled trials*
  - —secondary prevention of CHD
  - —primary prevention of CHD
  - —antioxidants positively influences other risk factors

vitamin E supplementation for prevention of the development of aortic atherosclerotic lesions in a variety of animal models have yielded conflicting results. Although the majority of investigators have seen benefits [30–32], some have found the vitamin either ineffective or deleterious [33]. Plasma cholesterol levels in these studies were extremely high (15–30 mmol l^{-1}) and vitamin E supplements in some studies very high (1000–10,000 mg kg^{-1}).

By contrast, investigations of the impact of vitamin C on atherogenesis have suggested in the main that vitamin C deficient diets are associated with increased aortic accumulation of cholesterol [34]. Although this effect may be due to deficient antioxidant defences, vitamin C deficiency may also have an adverse effect on lipid metabolism and synthesis of structural components in the arterial wall [29].

Most trials of the antioxidant drug probucol have suggested that the development of atherosclerosis is inhibited [35]. Although this effect has been attributed to a decrease in susceptibility of LDL to oxidation [36], probucol may alter a variety of other physiological processes involved in atherosclerosis, for example, lipid levels. Butylated hydroxytoluene also inhibits the susceptibility of LDL to oxidation *in vitro* and retards the progression of atherosclerosis in animal models [37].

The interpretation of the studies is further complicated by the fact that vitamin E and probucol may have other physiological effects. None of the animal studies have demonstrated unequivocally that the above substances are acting entirely by an 'pure' antioxidant mechanism. However, the generally promising results of small trials with antioxidants in animal models of atherosclerosis has stimulated a search for epidemiological evidence that antioxidant intake might influence the rate of atherosclerotic complications in human populations.

Epidemiological studies

Inter-population epidemiology A number of investigators have attempted to correlate rates of CHD in different populations with their dietary intake of antioxidants (mostly in fruits and vegetables) or blood antioxidant levels. For example, Armstrong *et al.* [38] reported a strong inverse association of regional consumption of fresh fruit and vegetables and CHD in nine UK regions, with correlation coefficients of $r=-0.83$ for men ($P<0.05$) and $r=-0.91$ for women ($P<0.05$). By contrast, other authors have also tried to link the temporal variation of cardiovascular disease with antioxidant intake. A notable example is the decline in CHD rates in the United States in the 1960s and 1970s which has been ascribed to the concomitant increases in per capita intake of fruits and vegetables [39] or the production of vitamin C supplements [40].

An alternative approach is to measure plasma levels of various antioxidants. For example, the World Health Organisation MONICA project compared the risk of ischaemic heart disease mortality with mean plasma antioxidant levels in random samples of 100 individuals from 12 European populations with normal cholesterol levels (5.7–6.2 mmol l^{-1}) [41]. Both absolute and lipid-standardized vitamin E concentrations showed a strong inverse correlation with CHD mortality rates ($r^2=0.63$, $P=0.002$ and $r^2=0.73$, $P=0.0004$ respectively). A similar inverse correlation was found for vitamin C ($r^2=0.41$, $P=0.03$) but not for beta-carotene ($r^2=0.21$, $P=0.14$). By contrast, Riemersma *et al.* [42] found an inverse association between cholesterol-adjusted vitamin E and cardiovascular mortality in four European populations (Southwest Finland, North Karelia, Scotland and Italy) although this failed to achieve statistical significance.

Such comparative studies are limited by potential confounding factors. Much of the dietary evidence relates to the intake of fresh fruit and vegetables which may contain non-antioxidant elements that protect against CHD. Similarly, such diets are usually lower in saturated fats and are consumed by individuals displaying other beneficial including lifestyle characteristics e.g. regular exercise, non-smoking. Furthermore, they do not take into account likely genetic variability.

Intra-population epidemiology Prospective cohort studies within populations identify antioxidant status (dietary intake or plasma levels) at the beginning of the study and monitor the cohort for subsequent events. This methodology reduces the possible impact of recall or selection bias found in case-control studies (see below) and also removes the possible influence of CHD on antioxidant levels.

Two large studies published in 1993 examined the relationship between dietary intake of antioxidant vitamins and coronary heart disease. Stampfer *et al.* [43] followed a cohort of 87 245 nurses who were free of coronary disease at entry to the study; during a follow-up period of 8 years 552 coronary events were identified in the cohort. In comparison with the lowest quintile of vitamin E intake, the top quintile had a relative risk (RR) of 0.66 (95% CI, 0.50–0.87) after adjustment for age and smoking. Most of the risk reduction could be attributed to the use of vitamin E supplements (>100IU day^{-1}) with no relationship existing when dietary intake alone was considered. Adjustment for the intake of the other antioxidant vitamins beta-carotene and ascorbate (which was higher in the supplement using subjects) reduced the apparent impact of vitamin E (RR, 0.69; confidence interval, 0.49–0.97). However, at least 2 years of supplement usage was required to have any benefit suggesting that its effect was due to an impact on a chronic disease process such as atherosclerosis. The relative risk between highest and lowest quintile of beta-carotene was 0.78 (95% CI 0.59–1.03) with a significant trend across quintiles ($P=0.02$), but Vitamin C intake had no apparent protective effect. However, the vitamin users as a group in this study were more health conscious with a lower body mass index, a lower intake of saturated fats and a higher intake of fibre. They also smoked less, had more users of hormone replacement therapy and took more vigorous exercise. These factors were adjusted for but other associated but unmeasured confounding factors might have had an important influence.

Rimm *et al.* [44] conducted a similar study amongst 39 910 male health professionals aged 40–75 years who were followed up for 4 years after completing baseline questionnaires. In this group 667 CHD events (myocardial infarction or revascularizations) were identified, and men who took 100IU day^{-1} or more of vitamin E supplements had a

relative risk of 0.63 (95% CI, 0.47–0.84) compared with those who took no supplements. Although the dietary vitamin E intake showed a weak non-significant association with the risk of CHD ($P=0.11$), dietary beta-carotene intake was associated with a reduced risk of CHD in smokers (relative risk, 0.30; 95% CI, 0.11–0.82) and ex-smokers (relative risk, 0.60; 95% CI, 0.38–0.94).

Enstrom *et al.* [45] studied the vitamin C intake of 11 349 men and women in the United States by dietary recall questionnaire. They found that the cardiovascular mortality rate in the highest third of vitamin C intake was significantly lower than the lowest ($r^2=0.66$; 95% CI = 0.53–0.82). This study was limited by the limitations associated with the use of a dietary recall technique and lack of information about vitamin supplements other than vitamin C which may have confounded the result. By contrast, an earlier 12 year study of 1462 Swedish women found no protective effect for vitamin C on cardiovascular mortality [46].

The Massachusetts Elderly Cohort Study examined beta-carotene intake by personal interview in 1299 participants who were subsequently followed up for an average of 5 years [47]. Within the study group 151 individuals died from CHD related causes of which 47 were fatal myocardial infarctions. After controlling for confounding variables the relative risk of cardiovascular death from lowest to highest quartiles of beta-carotene intake was 1.00, 0.75, 0.65, 0.57 respectively (P for trend = 0.016). The similar figures for fatal myocardial infarction alone were 1.00, 0.77, 0.59 and 0.32 (P for trend = 0.02).

Finally, one cohort study reported that self-administered vitamin E supplements above 100IU day^{-1} reduced the rate of progression of mild and moderate coronary lesions (< 50% stenosis) on serial coronary angiography over a 2 year period [48]. This therefore provided some evidence that antioxidant vitamin intake reduced progression of coronary artery atherosclerosis.

To date few dietary studies have concentrated on the potential benefits of dietary flavonoids which, among other properties, are powerful antioxidants and can protect LDL from oxidation *in vitro* [49]. The Zutphen Elderly Study recorded the dietary intake of flavonoids in a cohort of 805 elderly Dutch men [50], with the major dietary sources of flavonoids being tea, onions and apples; during follow-up 43 men died from CHD, with the relative risk of CHD death in the highest compared to the lowest tertile of flavonoid intake being 0.42 (95% CI 0.20–0.88; P value for trend = 0.015). This significant relationship persisted after controlling for the intake of other antioxidants.

Rather than assessing dietary intake, some prospective studies measured plasma antioxidant status at baseline. The Basel Prospective Study [51] examined a cohort of 2974 men in a population with relatively high vitamin E levels (median 35 $\mu mol\ l^{-1}$). After a 12 year follow-up there was a slighly increased risk of death from CHD among those subjects in the lowest quartile of plasma levels of beta-carotene (RR = 1.53; 95% CI 1.07–2.20) or vitamin C (RR = 1.25; 95% CI 0.77–2.01) at entry compared with those in the highest quartile independent of vitamin E and other risk factors; low levels of both vitamin C and beta-carotene increased the risk further (RR = 1.96; 95% CI 1.10–3.50). However, Vitamin E alone was not a predictor of CHD in this study. Similarly, the Lipid Research Clinic Coronary Primary Prevention Trial measured plasma carotenoid content at baseline [52], and after 14 years of follow-up, carotenoid levels were inversely related to risk of myocardial infarction after adjustment for age, smoking and cholesterol levels.

In summary, data from prospective cohort studies suggest that higher dietary intake of vitamin E (primarily from supplements) is associated with a significant reduction in risk of CHD. Both dietary and plasma measurement studies suggest a protective effect of beta-carotene especially among smokers. The data for vitamin C are less convincing. The role of dietary flavonoids is still under evaluation.

Whilst epidemiological studies do suggest some role for antioxidants in CHD prevention, spurious associations, rather than cause and effect, cannot be excluded [53], because vitamin supplementation may be linked to a generally healthier lifestyle. These epidemiological studies also suggest that prevention of CHD requires large amounts of some vitamins that are difficult if not impossible to obtain from dietary sources alone.

Case-control studies

Case control studies compare antioxidant status or intake in identified cases of CHD and appropriately matched controls.

The European Community Multicentre (EURAMIC) Study examined the relationship between adipose tissue levels of vitamin E and beta-carotene in 683 people with a first myocardial infarction and in 727 hospital-based control individuals in 12 centres [54]; the age and centre-adjusted risk of myocardial infarction in the lowest quintile of beta-carotene compared with the highest was 2.62 (95% CI, 1.79–3.83), but after controlling for body mass index and smoking the odds ratio was reduced to 1.78 (95% CI, 1.17–2.71) with a significant trend across quintiles (P for trend = 0.001). The increased risk was confined mainly to smokers in whom the adjusted odds ratio was 2.39 (95% CI, 1.35–4.25). However, a low vitamin E level was not associated with an increased risk of myocardial infarction but the inverse correlation of beta-carotene was strongest at high vitamin E levels. Vitamin E supplement users were excluded from this sample.

Riemersma *et al.* [55] studied 6000 men aged 35–54 years in Edinburgh and identified 110 patients with angina by the WHO Chest Pain questionnaire; their plasma antioxidant levels were compared with 394 controls randomly selected from the sample. The unadjusted risk of angina was significantly higher in the lowest quintile compared with the highest for vitamin E (odds ratio 2.51, 95% CI 1.24–5.10), vitamin C (odds ratio 2.35, 95% CI 1.16–4.78) and beta-carotene (odds ratio 2.64, CI 1.32–5.29). The relationship with vitamin E (but not vitamin C or beta-carotene) remained significant after controlling for smoking, cholesterol, blood pressure, weight and age (odds ratio 2.98, CI 1.07–6.70). Since smoking reduces vitamin C levels it was therefore not surprising that its inclusion in a multivariate analysis might diminish the impact of vitamin C.

However, leukocyte levels are probably a better indicator of total body vitamin C content than levels in plasma which may fluctuate. Ramirez & Flowers [56] therefore measured

leukocyte vitamin C levels in a case-controlled study of CHD, and found levels to be significantly lower in cases with angiographically proven CHD when compared with matched controls ($P<0.001$).

Whilst such case-control study methods offer useful data about the impact of antioxidants on CHD incidence they do have significant limitations. Firstly, the results are very dependent on the selection of the cases and controls, which are usually small groups that are intended to be an adequate representation of larger populations; this generalisability may be far from true. The cases and controls may also differ in their recall of important confounding factors such as smoking. Finally, they do not take into account the fact that the index disease (i.e. CHD) itself may adversely influence antioxidant status.

However, some of these objections have been overcome by adopting a nested case-control design. In such studies the blood samples are taken at the beginning of the study and at the end of follow-up antioxidant levels are measured only in the cases and appropriately matched controls. This does has the advantage of allowing large cohorts to be followed for CHD events but limiting the number of sample measurements required. The major problem however is that reliable storage of antioxidants, especially vitamin C, is difficult. Using a nested case-controlled study, Kok *et al.* [57] followed up individuals for 6–9 years and matched 168 controls against 84 subjects who died from cardiovascular disease; those in the lowest tertile of serum vitamin E (frozen at -20° C) had a relative risk of 1.5, with wide confidence intervals that included zero (95% CI 0.6–3.5). Salonen *et al.* [58] also found no significant association with serum vitamin E (frozen at -20° C) in 92 cases and 92 controls selected from a cohort of 12 000. By contrast, Street *et al.* [59] found a significant inverse relationship between plasma beta-carotene at baseline and risk of subsequent myocardial infarction in 125 cases, 125 community-based and 125 hospital-based controls, using samples that had been frozen at -70° C for up to 15 years.

Prospective randomized controlled trials

The foregoing discussion provides powerful (but not conclusive) evidence that antioxidants may have some beneficial effects in preventing atherosclerosis. However, observational studies are limited by the possibility of confounding factors, i.e. the dietary intake or blood level of an antioxidant may merely represent a marker for other protective dietary factors of lifelstyle traits. These potential benefits of antioxidants can only be rigorously tested in prospective intervention studies that randomly allocate at risk populations to antioxidant or placebo treatment.

Preliminary data is available from the Physicians' Health Study, a randomized, placebo-controlled, double-blind, 2×2 factorial design trial of aspirin and beta-carotene in the prevention of cardiovascular disease and cancer in 22 071 male US physicians aged 40–84 years originally recruited in 1982 [60]. A history of angina pectoris or revascularization prior to randomization was reported in 333 doctors at entry to the study. Of these 160 were assigned to beta-carotene and 173 to placebo. After a mean follow-up of 60.2 months, a significant reduction in CHD events was seen in the actively treated group (relative risk = 0.49, 95% CI = 0.29–0.88).

The ATBC Cancer Prevention Study Group [61] performed a randomized trial of the impact of vitamin E (50 mg day^{-1}), beta-carotene (20 mg day^{-1}) or both in a group of 29 133 Finnish smokers aged 50–69 years who were treated for 5 to 8 years with the primary aim of preventing lung cancers. Alpha-tocopherol was associated with a 5.8% reductiuon in CHD deaths (658 *vs* 704) but this was apparently offset by an increase in deaths from cancer and haemorrhagic strokes, such that the overall mortality was actually increased by 2% ($P=0.6$). In this study, the mortality was in fact 8% higher in those given beta-carotene ($P=0.02$) which was a combination of increased deaths from CHD (11.4%) and cancer. This study therefore suggests that beta-carotene alone is not protective and that carotene may even represent a marker of diets rich in fruit and vegetables which may contain another protective component or be a marker for a healthy lifestyle. However, the vitamin E dose was relatively low, and previous epidemiological studies have suggested that significant benefits required 100IU day^{-1} [44], although in the Nurses Health Study [43] even multivitamin preparations containing Vitamin E 30IU day^{-1} were beneficial (RR, 0.78; 95% CI, 0.64–0.96).

The Cambridge Heart Antioxidant Study (CHAOS) was a double-blind, placebo-controlled study with stratified randomization of 2002 patients with angiographically proven CHD to placebo or vitamin E (800IU day^{-1} for the first 546 patients, 400IU day^{-1} for the remainder) [62]. After a median followup of 510 days, vitamin E treatment significantly reduced the combined primary endpoints of cardiovascular deaths and non-fatal myocardial infarction (RR 0.53; 95% CI, 0.34–0.83; $P=0.005$). The risk of nonfatal myocardial infarction was particularly reduced (RR, 0.23; 95%CI, 0.11–0.47), with beneficial effects apparent after 1 year of treatment. All cause mortality was increased slightly in the group allocated vitamin E. However, this study was probably underpowered to examine mortality and outcome was not examined in relation to baseline plasma vitamin E concentrations in the placebo group. Another unusual feature in CHAOS was the reduction in vitamin E dosage half-way through the trial; whilst the study was not designed to test a dose-response relationship, the initial dosage was based on epidemiological observations [43, 44]. Based on the current available evidence, the role of vitamin E supplementation is therefore particularly promising in CHD prevention, although the precise mechanisms of its action remain uncertain and further studies are required to ascertain patient groups for whom the benefits would be greatest (Table 4).

Other randomized trials of antioxidant supplements are currently underway. The Physician's Health Study is testing beta-carotene (50 mg on alternate days) in 20 000 healthy US physicians with a 12 year follow-up [63]. The Women's Health Study is testing beta-carotene (50 mg on alternate days), vitamin E (600IU on alternate days) and aspirin in 40 000 healthy US female nurses [64]. In Britain, the combined Medical Research Council and British Heart Foundation Heart Protection Study has begun and will test the effects of a combined antioxidant preparation as well as

Table 4 Possible mechanisms for the protective role of vitamin E against the development of coronary heart disease (CHD).

- *Stabilization of coronary lesions*
  —by reducing lipid peroxidation, vitamin E accumulation in the lipid core of an atherosclerotic plaque could reduce macrophage recruitment and rupture of macrophage-rich plaques. However, it may be difficult for significant amounts of vitamin E to reach target tissues [54].
- *Inhibition of LDL oxidation and prevention of fatty streak formation*
  —if so, it could take years for an effect to become noticeable.
- *Reduction of prothrombotic factors*
  —preliminary data from the CHAOS investigators suggest a beneficial reduction of plasma fibrinogen in patients taking vitamin E 800IU/day (but not 400IU/day) [65]. Vitamin E also reduces platelet adhesion and aggregation, and its oxidized moiety Vitamin E-quinone inhibits vitamin K-dependent clotting factors [66–68].
- *Improvement of nitric oxide dependent vasodilatation*
  —Vitamin E inhibits oxidized-LDL mediated stimulation of endothelin production and inhibition of nitric oxide production [19].

an HMG CoA reductase inhibitor in a 2×2 factorial design in preventing coronary disease in high risk groups. The HOPE Study will examine the impact of vitamin E (400IU day^{-1}) and the ACE inhibitor ramipril in a 2×2 factorial design in patients at high risk for vascular events. The Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.M.AX) trial will examine the impact of a mixture of vitamin E, vitamin C, beta-carotene, selenium and zinc in 15 000 healthy men and women.

Whilst the outcome from further prospective trials are awaited with interest, the role of vitamin E is particularly promising. However, the efficacy of different antioxidant vitamins in CHD prevention could reflect differences in solubility, absorption from diet and incorporation into very-low-density lipoproteins. These prevention trials with high doses of antioxidants (often 40–80 times the usual dietary intake) are important but can never tell us whether our habitual daily dietary antioxidant intake can prevent CHD disease.

Conclusions

Data from *in vitro* studies is strongly suggestive that oxidation of LDL is an important factor leading to its cell-mediated uptake into the vascular wall as well as a variety of other adverse effects. Oxidation of lipoproteins has now been demonstrated to occur *in vivo*. Epidemiological observations are suggestive that high dietary intakes or plasma levels of antioxidant vitamins are protective against the development of atherosclerosis. The results of further randomized prospective controlled trials of antioxidant therapy will soon be available. The role of vitamin E, the major LDL-associated antioxidant, however, is particularly promising.

Nevertheless, the effects of any antioxidant intervention can be expected to require several years of treatment since their impact is on a chronic slowly progressive condition. Therefore, it is important that such trials also demonstrate the safety of long term antioxidant treatment. A significant cardioprotective effect might also have major cost implications for the routine use of such agents.

References

1 Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Rad Res Commun* 1989; **6**: 67–75.
2 Steinbrecher UP, Lougheed M, Kwan WC, Dirks M. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *J Biol Chem* 1989; **264**: 15216–15223.
3 Halliwell B. How to characterize a biological antioxidant. *Free Rad Res Commun* 1990; **9**: 1–32.
4 Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. *Arch Biochem Biophys* 1990; **280**: 1–8.
5 Stocker R, Frei B. Endogenous antioxidant defences in human blood plasma. In *Oxidative stress: oxidants and antioxidants*. ed Sies H. London: Academic Press, 1991; 213–242.
6 Packer JE, Slater TF, Wilson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979; **278**: 737.
7 Steinberg D, Parsatharathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989; **320**: 915–924.
8 Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann Rev Biochem* 1977; **46**: 897–930.
9 Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Ann Rev Biochem* 1983; **52**: 223–261.
10 Morel DW, DiCorleto PE, Chisholm GM. Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. *Arteriosclerosis* 1984; **4**: 357–364.
11 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 USA* 1981; **78**: 6499–6503.
12 Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine* 1992; **13**: 341–390.
13 Smith C, Mitchinson MJ, Aruoma OI, Halliwell B. Stimulation of lipid peroxid-ation and hydroxyl radical generation by the contents of human atherosclerotic lesions. *Biochem J* 1992; **286**: 901–905.
14 Hurt-Camejo E, Camejo G, Rosengren B, Lopez F, Ahlström C, Fager G, Bondjers G. Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. *Arteriosclerosis Thrombosis* 1992; **12**: 569–583.
15 Quinn MT, Parthasarathy S, Fong LG, Steinberg D. Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/ macrophages during atherogenesis. *Proc Natl Acad Sci USA* 1987; **84**: 2995–2998.
16 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**: 2039–2046.

17 Hessler JR, Robertson AL Jr., Chisolm GM. LDL cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cell culture. *Atherosclerosis* 1979; **32**: 213.
18 Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelial-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990; **344**: 160–162.
19 Ylä-Herttuala S, Palinski W, Rosenfield 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**: 1086–1095.
20 Palinski W, Rosenfeld ME, Ylä-Herttuala S, *et al*. Low-density lipoprotein undergoes oxidative modification *in vivo*. *Proc Natl Acad Sci USA* 1989; **86**: 1372–1376.
21 Salonen JT, Ylä-Herttuala S, Yamomoto R, *et al*. Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet* 1992; **339**: 883–887.
22 Jessup W, Rankin SM, De Whalley CV, Hoult JRS, Scott J, Leake DS. Alpha-tocopherol consumption during low-density-lipoprotein oxidation. *Biochem J* 1990; **265**: 399–405.
23 Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low-density lipo-protein more efficiently against lipid peroxidation than does alpha-tocopherol. *Proc Natl Acad Sci USA* 1991; **88**: 1646–1650.
24 Esterbauer H, Puhl H, Dieber-Rotheneder M, Waeg G, Rabl H. Effect of antioxidants on oxidative modification of LDL. *Ann Med* 1991; **23**: 573–581.
25 Reaven PD, Khouw A, Beltz WF, Parthasarathy S, Witztum JL. Effect of dietary antioxidant combinations in humans. Protection of LDL by vitamin E but not by beta-carotene. *Arteriosclerosis Thrombosis* 1993; **13**: 590–600.
26 Reaven PD, Parthasarathy S, Grasse BJ, Miller E, Steinberg D, Witztum JL. Effects of oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolaemic subjects. *J Clin Invest* 1993; **91**: 667–668.
27 Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D. Low density lipoprotein rich in oleic acids is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. *Proc Natl Acad Sci USA* 1990; **87**: 3894–3898.
28 Tribble DL, Holl LG, Wood PD, Krauss RM. Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. *Atherosclerosis* 1992; **93**: 189–199.
29 Lynch SM, Frei B. Antioxidants as antiatherogens: animal studies. In *Natural antioxidants in human health and disease* Academic Press Inc 1994; 353–358.
30 Wilson RB, Middleton CC, Sun GY. Vitamin E, antioxidants and lipid peroxidation in experimental atherosclerosis of rabbits. *J Nutrition* 1978; **108**: 1858–1867.
31 Wojcicki J, Rozewicka L, Barcew-Wiszniewska B, *et al*. Effect of selenium and vitamin E on the development of experimental atherosclerosis in rabbits. *Atherosclerosis* 1991; **87**: 9–16.
32 Williams RJ, Motteram JM, Sharp CH, Gallagher PJ. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis* 1992; **94**: 153–159.
33 Keaney JF Jr., Gaziano JM, Xu A, *et al*. Low-dose alpha-tocopherol improves and high-dose alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. *J Clin Invest* 1994; **93**: 844–851.
34 Ginter E. Marginal vitamin C deficiency, lipid metabolism and atherogenesis. *Adv Lipid Res* 1978; **16**: 167–220.
35 Carew TE, Schwenke DC, Steinberg D. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect. Evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks slowing the progression of atherosclerosis in the WHHL rabbit. *Proc Natl Acad Sci USA* 1987; **84**: 7725–7729.
36 Regnström J, Walldius G, Carlson A, Nilsson J. Effect of probucol treatment on the susceptibility of low density lipoprotein isolated from hypercholesterolaemic patients to become oxidatively modified *in vitro*. *Atherosclerosis* 1990; **82**: 43–51.
37 Björkhem I, Henriksson-Freyschuss A, Breuer O, Dietztalusy V, Berglund L, Henriksson P. The anti-oxidant butylated hydroxytoluene protects against athero-sclerosis. *Arteriosclerosis Thrombosis* 1991; **11**: 15–22.
38 Armstrong BK, Mann JL, Adelstein AM, Eskin F. Commodity consumption and ischaemic heart disease mortality with special reference to dietary practices. *J Chronic Dis* 1975; **36**: 673–677.
39 Verlangieri AJ, Kapeghian JC, El-Dean S, Bush M. Fruit and vegetable consumption and cardiovascular disease mortality. *Med Hypotheses* 1985; **16**: 7–15.
40 Ginter E. Decline of coronary mortality in the United States and vitamin C. *Am J Clin Nutr* 1979; **32**: 511–512.
41 Gey KF, Puska P, Jordan P, Moser UK. Inverse correlation between plasma vitamin E and mortality from ischaemic heart disease in cross-cultural epidemiology. *Am J Clin Nutr* 1991; **53** (Suppl.1): 326S–334S.
42 Riemersma RA, Oliver M, Elton MA, *et al*. Plasma antioxidants and coronary heart disease: Vitamins C and E and selenium. *Eur J Clin Nutr* 1990; **44**: 143–150.
43 Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett C. Vitamin E consumption and the risk of coronary artery disease in women. *N Engl J Med* 1993; **328**: 1444–1449.
44 Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and risk of coronary artery disease in men. *N Engl J Med* 1993; **328**: 1450–1456.
45 Enstrom JE, KaniM LE, Klein MA. Vitamin C intake and mortality among a sample of the United States population. *Epidemiology* 1992; **3**: 194–202.
46 Lapidus L, Anderson H, Bengtson C, Bosceus I. Dietary habits in relation to cardiovascular disease and death in women: a 12 year follow-up of participants in the study of women in Gothenberg, Sweden. *Am J Clin Nutr* 1986; **44**: 444–448.
47 Gaziano JM, Hennekens CH. The role of beta-carotene in the prevention of cardiovascular disease. *Ann NY Acad Sci* 1993; **691**: 148–155.
48 Hodis HN, Mack WJ, LaBree L, Cashin-Hemphill L, Sevanian A, Johnson R, Azen SP. Serial coronary angiographic evidence that antioxidant vitamin intake reduces progression of coronary artery atherosclerosis. *JAMA* 1995; **273**: 1849–1854.
49 Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 1993; **341**: 454–457.
50 Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly study. *Lancet* 1993; **342**: 1007–1011.
51 Gey KF, Stahelin HB, Eicholzer M. Poor plasma status of carotene and vitamin C is associated with higher mortality from ischaemic heart disease and stroke: Basel prospective study. *Clin Invest* 1993; **71**: 3–6.
52 Morris DL, Kritchevsky SB, Davis CE. Serum carotenoids

and coronary heart disease in the Lipid Research Clinics Primary Prevention Trial. *Circulation* 1993; **87**: 2.
53 Jha P, Flather M, Lonn E, Farkouh M, Yusuf S. The antioxidant vitamins and and cardiovascular disease: a critical review of epidemiologic and clinical trial data. *Ann Intern Med* 1995; **123**: 860–872.
54 Kardinaal AFM, Kok FJ, Ringstad J, *et al*. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC study. *Lancet* 1993; **342**: 1379–1384.
55 Riemersma RA, Wood DA, MacIntyre CC, Elton R, Gey KF, Oliver MF. Risk of angina pectoris and plasma concentrations of vitamins A, C, E and carotene. *Lancet* 1991; **337**: 1–5.
56 Ramirez J, Flowers NC. Leukocyte ascorbic acid and its relationship to coronary heart disease in man. *Am J Clin Nutr* 1980; **33**: 2079–2087.
57 Kok FJ, de Bruijn AM, Vermeeren R, *et al*. Serum selenium, vitamin antioxidants and cardiovascular mortality : A 9 year follow-up study in the Netherlands. *Am J Clin Nutr* 1987; **45**: 462–468.
58 Salonen JT, Salonen R, Penttila I, *et al*. Serum fatty acids, apolipoproteins, selenium and vitamin antioxidants and risk of death from coronary artery disease. *Am J Cardiol* 1985; **56**: 226–231.
59 Street DA, Comstock GW, Salkeld RM, Shuep W, Klag M. A population based case-control study of serum antioxidants and myocardial infarction. *Am J Epidemiology* 1991; **134**: 719–720.
60 Gaziano JM, Manson JE, Ridker PM, Buring JE, Hennekens CH. Beta-carotene therapy for chronic stable angina pectoris. *Circulation* 1990; **82**: Suppl.III 202 (Abstract).
61 The 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**: 1029–1035.
62 Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; **347:** 781–786.
63 Hennekens CH, Eberlain K. A randomised trial of aspirin and beta-carotene among US physicians. *Preventative Med* 1985; **14**: 165–168.
64 Buring JE, Hennekens CH. Randomised trials of primary prevention of cardiovascular disease in women. An investigators view. *Ann Epidemiol* 1994; **4**: 111–114.
65 Stephens NG, Parsons A, Cheeseman K, Kelly F, Schofield PM, Mitchinson MJ, Brown MJ. Anticoagulant effects of vitamin E treatment in patients with coronary disease. *Eur Heart J* 1996; **17**(Abstract Suppl): 536.
66 Steiner M. Influence of Vitamin E on platelet function in humans. *J Am Coll Nutr* 1991; **10**: 466–473.
67 Wu HP, Tai TY, Chuang LM, Lin BJ, Wang JD, Teng CM. Effect of tocopherol platelet aggregation in non-insulin-dependent diabetes mellitus: *ex vivo* and *in vitro* studies. *J Formosan Med Assoc* 1992; **91**: 270–275.
68 Dowd P, Zheng ZB. On the mechanism of the anticlotting action of vitamin E quinone. *Proc Natl Acad Sci USA* 1995; **92**: 8171–8175.
69 Boulanger CM, Tanner FC, Bea ML, Hahn AW, Werner A, Luscher TF. Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and procine endothelium. *Circ Res* 1992; **70**: 1191–1197.

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