Natural antioxidants and atherosclerosis

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The precursors of fibrous atherosclerotic plaques are fatty streaks, characterized by accumulation of fat-laden macrophages beneath an intact endothelium. These macrophages are derived from monocytes in the circulating blood and the lipid is derived from plasma low density lipoprotein (LDL). But LDL is poorly taken up by monocytes/macrophages in vitro unless it has been oxidatively modified. Hence the phypothesis has developed that one determinant of atherosclerosis is whether LDL becomes oxidized by free radicals in the subendothelial space. An epidemiological study of 12 European sub-populations which all have about the same plasma cholesterol concentration but quite different incidences of coronary heart disease (CHD) showed a significant inverse correlation of plasma α-tocopherol with CHD. In several animal models, vitamin E or some other antioxidants attenuate experimental atherosclerosis. Each particle of LDL contains about 8–12 molecules of tocopherol, 0.5 to 1 molecule of ubiquinol-10 and small amounts of carotenoids but other antioxidants in the extracellular fluid, notably (water-soluble) ascorbate protect against oxidative damage in in vitro experiments with human blood plasma. The ascorbate presumably acts by regeneration α-tocopherol from its one-electron oxidation product, the α-tocopheroxyl radical. The author found that the small amounts of ubiquinol present in LDL offer important protection against oxidation. Unlike vitamins C and E, ubiquinol is biosynthesized by humans but it is also obtained from the diet (some fatty fish are the richest sources). The ubiquinol content of plasma LDL was increased 4-fold by giving volunteers ubiquinone. Their plasma LDL was subsequently found more resistant against oxidation.

Background

Atherosclerosis represents a major form of ischaemic heart disease (IHD), the leading cause of death in western countries. Elevated levels of low density lipoprotein (LDL) cholesterol are an important risk factor for atherosclerosis. This can be concluded from clinical observations on patients with familial hypercholesterolemia, a single, well-defined gene defect involving the LDL receptor. The premature atherosclerosis observed in these patients must be, directly or indirectly, related to their elevated levels of LDL in plasma. While undoubtedly important, hypercholesterolemia is however clearly not the single causative factor for atherosclerosis. Other variables contribute (and probably interact) with hypercholesterolemia in determining the overall risk for atherosclerosis.

Among the additional risk factors, lifestyle and nutrition are of interest. Epidemiological evidence suggests that the age-adjusted IHD mortality rate can vary substantially in populations of different countries. For example, northern Europeans have a much higher rate of IHD than people in Italy, Spain or France. It has been suggested that antioxidants present in the fruit- and vegetables-rich Mediterranean-type diet provides some protection against heart disease. Indeed, supporting evidence for this comes from the WHO Monica project in which the plasma concentrations of cholesterol and antioxidants in European sub-populations were measured and correlated to their risk of IHD incidents. Among 12 sub-populations with similar cholesterol levels, the plasma concentrations of α-tocopherol (TOH) strongly and inversely correlated with the mortality rate[1]. A negative correlation was also observed with plasma vitamin C; however, it was weaker than that observed for TOH and disappeared when smoking was introduced as an additional, separate risk factor[1]. Plasma levels of vitamin A and β-carotene did not correlate with the mortality rate. Additional evidence for a beneficial effect of dietary antioxidants (vitamins C and E and β-carotene) on cardiovascular disease in humans has been reviewed recently[2].

Animal studies further support the idea that antioxidant nutrients have a beneficial effect on atherosclerosis. Some 40 years ago, vitamin C-deficient guinea pigs were used as an animal model of atherosclerosis[3]. It was reported that in these deficient animals the ground substance of the sub-endothelial space was disturbed and lipid deposited in the sub-endothelial space[3]. The latter could be reversed by providing the animals with vitamin C in the diet[4]. The same group of scientists also reported that human post-mortem lesion material was deficient in vitamin C[5,6]. Other groups using guinea pigs as an animal model for atherosclerosis failed to confirm that simple vitamin C deficiency was associated with lipid deposition in the sub-endothelial space[7,8]. It was observed however, that a combination of vitamin C deficiency and a high fat diet caused lipid deposition in the sub-endothelial space of guinea pigs that were attenuated by vitamin C supplementation[8].

Several groups have shown that in different rabbit models of atherosclerosis, administration of vitamin E attenuates the progression of the disease (eg[9-11]). Treatment with synthetic antioxidants such as probucol[12,13] or butylated hydroxytoluene[14] also inhibits the development and progression of atherosclerosis in various animal models. Interestingly however, in all these cases (except that with butylated hydroxytoluene) antioxidant administration also had a significant hypolipaemic activity.

Low density lipoprotein oxidation

How can the beneficial role indicated above for antioxidants in atherosclerosis be rationalized? It is now widely accepted that the precursors of fibrous plaques and more complicated atherosclerotic lesions are the fatty streaks, ie the accumulation of fat-laden monocytes/macrophages (referred to as foam cells) beneath an intact endothelial cell layer (reviewed in [15]). These phagocytes are derived from the circulation, following adherence to and penetration of the arterial endothelium. Like other circulating macromolecules, LDL transverses endothelial cells and accumulates in the subendothelial space. However, before LDL is taken up by monocytes/macrophages at sufficiently high rates to cause foam cell formation, the lipoprotein must undergo some form of 'modification'. While a number of chemical 'modifications' have been shown to have this effect, there is mounting evidence that 'oxidative modification' is biologically important[15]. The presence of highly effective antioxidant defences in human blood (see below) has led to the 'assumption' that oxidative modification of LDL associated with atherogenesis takes place in the subendothelial space[15], even though there is surprisingly little *direct* evidence for this. The beneficial effect of antioxidants on atherosclerosis is most often attributed to their protective action on LDL oxidation[2,15]. While not discussed here, other explanations can not be excluded (see eg[16]); also, oxidative LDL modification

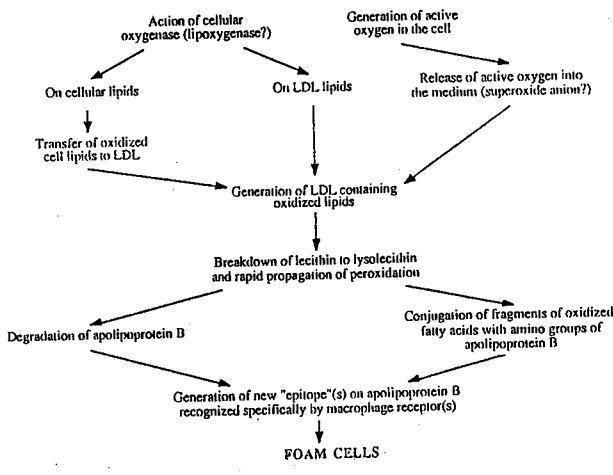

Figure 1. Mechanisms thought to lead to oxidative modification of LDL by cells (Steinberg et al. 1989).

resulting in an atherogenic lipoprotein may be achieved by non-radical oxidants such as hypochlorite, against which antioxidants offer little protection[17].

The precise mechanism by which LDL oxidation proceeds in the subendothelial space is still unclear, and a number of free radical-mediated mechanisms have been proposed[15] (Figure 1). These include the generation of reactive oxygen species (ROS) by cells of the subendothelial space causing (per)oxidation of LDL lipids, the possible involvement of transition metals for the catalysis of highly reactive radicals derived from the primary ROS[18], and the action of cellular lipoxygenases on either LDL lipids directly[19], or on cellular lipids with subsequent transfer of oxidized lipids to LDL[15] (but see [20]). Following initiation, LDL lipid peroxidation is thought to propagate, leading to massive lipid oxidation, with breakdown of oxidized lipids to reactive moieties that can modify amino acid residues on the protein in LDL, thereby generating new epitope(s) recognized by specific macrophage receptors[15].

Low density lipoprotein antioxidation

Non-proteinaceous antioxidants surrounding lipoproteins

As it is difficult to gain access to extracellular material surrounding LDL in the sub-endothelial space, most studies on antioxidant defences in extracellular fluids have used human blood plasma as a model. A number of proteinaceous defences that are present in plasma are designed primarily to eliminate transition metals from participating in unwanted redox reactions leading to deleterious effects (reviewed in [21,22]). Extracellular fluids also contain non-proteinaceous antioxidants[21,22]. Among them, ascorbate (AH-, the reduced form of vitamin C) appears to be the most efficient aqueous antioxidant.

In an attempt to assess qualitatively the relative contribution of the various non-proteinaceous antioxidants in extracellular fluids, we exposed freshly obtained human plasma to a constant chemical source of aqueous radical oxidants and examined the temporal consumption of antioxidants and formation of oxidatively damaged lipids[23]. We observed that AH- was oxidized first, followed by protein-thiols, bilirubin, uric acid and α-tocopherol (TOH, the most active form of vitamin E). Aqueous (peroxyl) radical-induced peroxidation of lipoprotein lipids in plasma was detectable only following complete consumption of AH-, despite the presence of normal amounts of TOH and other antioxidants[23]. Considering the generally accepted view of TOH's action[24], it was surprising to find formation of significant amounts of lipid hydroperoxides (LOOH, a primary form of oxidized lipids) in the presence of vitamin E. Additional work on the antioxidant properties of AH- suggests that this antioxidant represents the first and most efficient line of antioxidant defence in human plasma against some (but not all) aqueous, radical oxidants (reviewed in [25]). We have recently obtained similar efficient antioxidation by AH- in rat thoracic lymph, another extracellular fluid (D. Mohr, Y. Umeda, T. Redgrage, R. Stocker, unpublished). It therefore seems reasonable to assume that AH- plays a similarly important role as an aqueous antioxidant in the extracellular fluid of the sub-endothelial space in humans.

Low density lipoprotein-associated antioxidants

To understand the mechanism of how LDL lipids

become oxidized to generate an atherogenic lipoprotein, information on how LDL itself is protected against oxidation is important. LDL contains a surface coat consisting of a monolayer made up of phospholipids, free cholesterol and a protein (referred to as apoprotein B-100). This surface coat surrounds a core containing the neutral cholesterylesters and triglycerides. The composition of human LDL is given in Table 1. As can be seen, each LDL particle contains about 8–12 molecules of TOH, 0.5–1 molecules of ubiquinol-10 (QH_2) and small amounts of carotenoids. Thus, TOH is by far the most abundant antioxidant associated with human LDL.

Table 1. Composition of human LDL.

Components		Relative mass (%)	Molecule/ Particle
Protein	Apo B-100	23.4	1
Lipids	Phospholipids	20.6	800
	Cholesterol	9.0	500
	Cholesterylesters	41.7	1500
	Triglycerides	5.3	180
Antioxidants	α-Tocopherol		8–12
	Ubiquinol-10		0.5–1.0
	Lycopene		0.7
	β-Carotene		0.4

Because TOH is the major lipid-soluble antioxidant in LDL, it is generally also regarded as the most important lipid-soluble antioxidant in this lipoprotein[26] as well as in human plasma[27]. In direct contradiction to this however, various groups have reported that the amount of TOH in LDL does not correlate well with the lipoproteins resistance against oxidation[28-30].

Investigating in detail the early stages of LDL lipid oxidation, we started to unravel the above-mentioned anomalies. We discovered that the small amounts of QH_2 present in LDL offer outstanding protection against oxidation[31]. Thus, exposing freshly isolated LDL to a constant flux of aqueous radicals, LDL lipid peroxidation is inhibited substantially in the presence of QH_2, which is the first of LDL's antioxidants to be consumed. Following consumption of QH_2, lipid peoxidation proceeds at high rates and in a radical chain process: despite the presence of normal amounts of TOH, up to 20–40 molecules of oxidized lipid can be formed for each oxidant 'hitting' an LDL particle[31]. Peroxidation of LDL lipids via a chain reaction process in the presence of TOH has also been observed by Niki and co-workers[32], in line with our previous observations in human blood plasma[23]. Adding AH- to the oxidizing LDL completely prevents lipid oxidation, demonstrating that this vitamin is indeed a very efficient antioxidant[31]. The observed complete prevention of LDL lipid peroxidation by AH- is most likely due to the ability of this antioxidant to regenerate TOH from its one-electron oxidation product, α-tocopheroxyl radical (TO) (see below); AH- is unable to regenerate QH_2[33].

Unlike the vitamins C and E, QH_2 can be synthesized by humans, and part of the QH_2 in our body is derived from such biosynthesis, the other from dietary sources. Certain species of fish (eg sardines and mackerels) are the richest sources of QH_2. We therefore tested whether LDL's content of QH_2 can be increased by dietary supplementation and whether such QH_2-supplemented LDL is more resistant towards oxidation. As QH_2 in foods is present primarily in its (two electron) oxidized form ubiquinone-10 (Q, which itself is *not* an antioxidant), we administered volunteers with 3 × 100 mg Q per day. Such supplementation resulted in an approximately 4-fold increase in the levels of QH_2 (the reduced form of Q that is antioxidant-active), but did not alter the content of other antioxidants in LDL[34]. More importantly, QH_2-supplemented LDL was significantly more resistant against oxidation compared to the non-supplemented LDL[34]. These results strongly support the efficacy and importance of QH_2 in LDL antioxidation.

Dietary supplementation with TOH also results in an increase in its concentration in LDL, although it appears that high doses have to be used to achieve a similar relative increase compared with Q supplementation[30,34]. We have made similar observations with α-tocotrienol, another form of vitamin E that is present in certain foodstuff and is as antioxidant-active as TOH[35]. As alluded to earlier however, the relationship between TOH supplementation and LDL oxidizability is weak, in contrast to the situation with QH_2. Other groups have studied the protective effect of antioxidant nutrients on in vitro LDL oxidizability. Table 2 shows that in most cases the oxidizability of LDL is inhibited completely or reduced in the presence of supplemented antioxidants and using different types of oxidizing conditions. A word of caution appears appropriate here regarding potential problems with in vitro studies using lipid-soluble antioxidants. Due to their physical properties, 'physiologically correct' incorporation of these lipophilic substances when added in vitro (eg in ethanolic solution) is not guaranteed. We have observed significantly different behaviour of in vitro versus in vivo QH_2-enriched LDL[34], and this may explain why one group observed a protective activity of relatively large amounts of added β-carotene on LDL oxidation in vitro[36]. In our hands, β-carotene is *not* an efficient in vitro lipid antioxidant. In any case, it is commendable to substantiate in vitro findings with appropriate in vivo supplementation studies.

Pro-oxidant activity of α-tocopherol

Examining the precise role of TOH in LDL lipid antioxidation in the absence of AH- and following QH_2 consumption, we observed that under conditions where LDL was exposed to a constant flux of aqueous or lipophilic radicals, the rate of lipid hydroperoxide formation actually *decreased* as TOH was consumed[37]. Furthermore, LDL supplemented with TOH peroxidized at a higher rate than the corresponding control, non-supplemented lipoprotein[37]. In fact, the rate at which cholesteryllinoleate, the major single substrate for oxidation in LDL (Table 1), becomes oxidized is directly proportional to the lipoprotein's content of TOH[38]. These findings clearly demonstrate that TOH *alone* is not an efficient antioxidant for LDL; rather it can act as a potent pro-oxidant in the isolated lipoprotein.

These findings led to a detailed theoretical and experimental analysis of the molecular action of TOH in LDL. To our surprise, the results obtained with isolated LDL do not support the conventional mode of action of TOH, whereby this antioxidant scavenges a lipid radical

Table 2. Antioxidant nutrients and LDL's oxidizability.

Nutrient	Mode	Supplementation Dose (mg/d)	Supplementation Duration (days)	Fold Increase	Oxidizing conditions	Oxidizability	Reference
Vitamin C	In Vivo	–	–	–	ROO•/PMN	Inhibited	Stocker et al. 1991
	In Vivo	1500	28	2	Smoking	Reduced	Harats et al. 1990
Vitamin E	In Vitro	–	–	3	Cu2+	Reduced	Esterbauer et al. 1991
	In Vivo	1450	3	2.5	Maurine MØ	Reduced	Jessup et al. 1990
	In Vivo	600	28	n.d.	Smoking	Reduced	Harats et al. 1990
	In Vivo	100–800	21	3	Cu2+	Reduced	Esterbauer et al. 1991
	In Vivo	667	7	2.4	Cu2+	Reduced	Princen et al. 1992
β-Carotene	In Vitro	–	–	13	Cu2+	Reduced	Jialal et al. 1991
	In Vivo	40	14	16	Cu2+	Not altered	Princen et al. 1992
$CoQ_{10}H_2$	In Vivo	300	10	4	ROO•	Reduced	Mohr et al. 1992

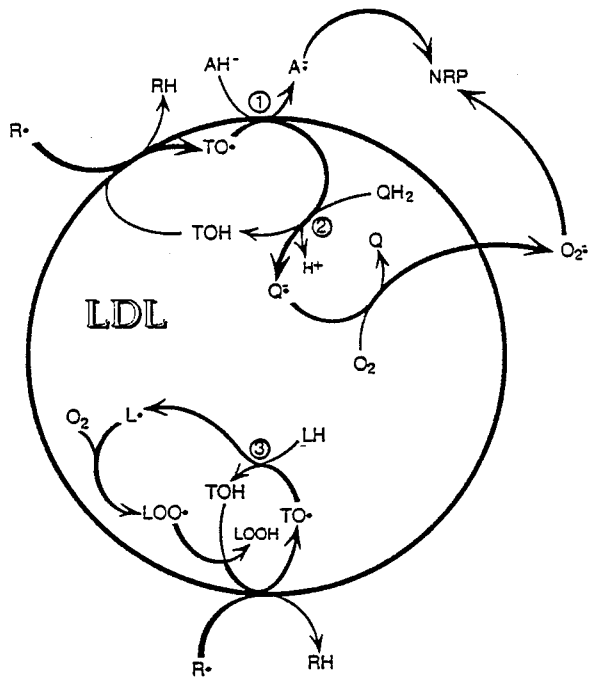

Figure 2. Anti- and pro-oxidation of LDL lipids by α-tocopherol.

produced when a peroxidation-initiating (aqueous) radical reacts with lipids, and where the resulting TO• is eliminated by reaction with another lipid or initiating radical. In this conventional mode of action, at least one molecule of LOOH is formed for each molecule of TOH consumed. In contrast, we observed that TOH in fact acts as both a *phase transfer* (ie it captures and thereby transfers aqueous radicals into the lipid phase) and *chain transfer* agent (ie it produces the lipid peroxidation chain-carrying radical) during radical-mediated LDL oxidation[38]. We have termed this novel mode of TOH's action as tocopherol-mediated peroxidation (TMP). TMP, rationalized and explained explicitly in [38], and its role in LDL antioxidation is illustrated in Figure 2.

As the most reactive component, TOH becomes oxidized (to TO•) when LDL encounters an oxidation initiating radical (R•). The TO• formed can undergo three reaction pathways, all of which result in regeneration of TOH. Pathways 1 and 2 represent *anti-oxidation*, whereas pathway 3 represents *pro-oxidation* of TOH. In the antioxidation pathways, TO• reacts with either aqueous AH- or QH_2. The ascorbyl- (A•) or semiquinone- (Q•) radicals formed decay (directly or indirectly) giving rise to non-radical products (NRP) in the aqueous phase while eliminating the harmful R•. In the absence of AH- *and* QH_2 the pro-oxidant pathway is followed, where the long-lived TO• is forced to react with LH producing a carbon-centered lipid radical (L•). Under aerobic conditions, L• will add to O_2 producing LOO• that propagates lipid peroxidation by reacting with another TOH (shown) or LH (not shown) to form (LOOH).

Nutritional implication of tocopherol-mediated peroxidation

From our model (Figure 2)[38] it follows that in most, if not all mammalian tissues and fluids, (where AH- and QH_2 are ubiquitous) TOH in fact is a *better* antioxidant than previously assumed; LOOH are not formed during normal action of TOH (pathways 1 and 2). It is clear however, that for such efficient LDL antioxidation, TOH requires 'partner' molecules that either reduce (eliminate) TO• and/or eliminate the radical character from the isolated LDL particle[37,38]. In light of this and the fact that LDL from healthy people is already rich in TOH, I consider dietary attempts aimed exclusively at further increasing the lipoprotein's concentration of TOH unlikely to be most efficient. Since QH_2 and AH- are the only natural compounds known at present to eliminate TO• and therefore TMP, a more promising strategy seems to be supplementation with these two antioxidants, *in combination with* TOH. There may be other compounds in extracellular fluids (eg the reduced form of vitamin K_1, albumin-bound bilirubin) that can also reduce and/or eliminate TO• in LDL, and the concentration of some of which may be increased by appropriate dietary means. I consider supplementation with Q as a particularly attractive and exciting possibility because the 'normal' circulating levels of this highly efficient antioxidant are low (see Table 1), and its concentration can easily be elevated to more than one molecule per LDL particle[34]. Achieving the latter would not only guarantee efficient elimination of TO•, but also enhance the possibility of additional LDL antioxidation through regeneration of QH_2[39].

Acknowledgements — The work and contribution of the colleagues and co-workers from the author's laboratory

is gratefully acknowledged. The work was supported in part by the Australian National Health & Medical Research Council grant 910284 to R.S.

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Asia Pacific J Clin Nutr (1993) 2, Suppl 1, 15–20

天然的抗氧化劑與動脉粥樣硬化

摘　要

纖維性的動脉硬化斑塊的前身是充滿脂類的巨噬細胞，　位于完整血管内皮下的脂肪紋（FATTY STREAK）。這些巨噬細胞是從血循環中的單核細胞衍生而來，　而脂類則從血漿低密度脂蛋白（LDL）衍生而來的。　LDL除非已氧化變性，很少被單核細胞或巨噬細胞攝取。　因此作者假定LDL在血管内皮下被氧化是動脉粥樣硬化的一個决定因素。

作者從１２個歐洲人群進行流行病學研究，發現這些人群的血漿膽固醇大致相同，但冠心病的發病率則不同，顯示冠心病與血漿α－生育酚呈明顯負相關。在幾種動物模型實驗中，亦發現維生素E與某些抗氧化劑可減少實驗性動脉粥樣硬化。

每一LDL顆粒含有８－１２個分子生育酚，０.５－１個分子泛醇－１０（UBIQUINOL-10）和小量類胡蘿卜素，但其他抗氧化劑則在細胞外液，值得注意的是，用人血漿做體外試驗，發現抗壞血酸（水溶性）對氧化性損害有保護作用。　抗壞血酸可能促使一個電子的氧化產物　α－TOCOPHEROXYL自由基再生成α－生育酚。作者發現，　LDL中的少量泛醇起重要的抗氧化作用。不象維生素　C和　E，泛醇不但可在體内合成，而且可在膳食中（某些肥胖魚類是豐富來源）獲取。當給予志願者泛醌可將血漿泛醇提高４位，最后他們血漿中的LDL亦發現有更好的氧化作用。