Oxidants and antioxidants in atherogenesis: an appraisal

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Abstract   Oxidized low density lipoprotein (Ox-LDL) has a plethora of components that are not present in native LDL. Their presence and quantity depends on the nature, type, and extent of oxidation. Lipids esterified to oxidized fatty acids are the major components formed during the early phase of oxidation and these show a number of proatherogenic properties in in vitro cell culture systems. Recently, evidence has been forthcoming to suggest that some of these oxidized lipids also could elicit "antioxidant–antiatherogenic" responses from cells. Moreover, some of the cellular effects of Ox-LDL that were previously interpreted as atherogenic could also be reinterpreted to suggest an antiatherogenic cellular response. In addition to the above, the antioxidants that are carried in lipoproteins could have anomalous behavior attributable to their metabolism, ability to be internalized by arterial cells, and the presence of oxidative systems that could render them prooxidants.■ In conclusion, there are numerous contributing factors that need to be studied and understood before antioxidant therapy becomes an option for the treatment for cardiovascular diseases.—Parthasarathy, S., N. Santanam, S. Ramachandran, and O. Meilhac. **Oxidants and antioxidants in atherogenesis: an appraisal.** *J. Lipid Res.* **1999.** 40: **2143–2157.**

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It is now generally accepted that oxidation of low density lipoprotein (LDL) plays a significant pathogenic role in atherosclerosis (1, 2). More than 1,000 papers dealing directly or indirectly with the oxidation of LDL were published between January 1991 and the end of 1998. More details, supporting circumstantial evidence, and interpretations have been added to the hypothesis, and yet the hypothesis is currently at an impasse. Little, if any, has changed during the past few years regarding the answer to the question: do antioxidants prevent the progression of atherosclerotic lesions in humans? Despite a recent article sponsored by the American Heart Association concurring that there is no conclusive evidence to suggest that a daily supplement of antioxidant nutrients can prevent atherosclerosis (3), claims of beneficial actions of antioxidants have left both the public and the medical professionals confused. On the other hand, the success of basic research makes one wonder whether one needs to wait for

the "right antioxidant," e.g., a lipoxygenase inhibitor, trial in humans to advance the hypothesis. For example, several years ago there were studies that proposed that 12,15-lipoxygenase initiates the oxidative process (4–6). Recently, Cyrus et al. (7) showed that apoE$^{-/-}$/L-12O$^{-/-}$ double-knockout mice (apoE and 12,15-lipoxygenase) developed less severe lesions than either the heterozygote mice or the parent apoE-deficient mice.

In this review we will briefly discuss the current status of the basic research and rephrase the question: how far are we beyond cholesterol to suggest that oxidized components of LDL add to the atherogenicity of the particle? We will direct our questions to the presence of the key components, i.e., oxidized lipids, that make oxidized-LDL (Ox-LDL) more atherogenic.

Ox-LDL has been suggested to have a plethora of pro-atherogenic effects (1, 2) that can be predominantly attributed to its lipid components. Ox-LDL was originally conceptualized to account for the enhanced uptake by macrophages by way of scavenger receptor (8, 9). Products generated from the decomposition of peroxidized lipids, such as aldehydes, were supposed to modify apolipoprotein B-100 (apoB-100) to a more electronegative form capable of interacting with the macrophage scavenger receptor (s) (10, 11). This feature of the protein appeared to be one of the few distinguishing characteristics of Ox-LDL that was touted as an obligatory feature for its interaction with cell surface receptors. Based on this, a number of cell surface proteins were identified as putative receptors for Ox-LDL (8, 12–16). Recently, based on the ability of the lipids of the Ox-LDL to compete for the uptake and degradation of Ox-LDL by macrophages, the presence of potential ligands for the scavenger receptors in the lipid phase was suggested (17). However, these studies do not exclude proteolipids (lipophilic protein-lipid components) that could be present in the lipid phase as potential competitors.


NATIVE LDL THAT IS OFTEN USED IN IN VITRO STUDIES MAY NOT REPRESENT NATIVE LDL IN PLASMA

Before one discusses the biological effects of Ox-LDL, it is important to define the terminology and describe the potential forms of Ox-LDL. Theoretically, the native LDL particle as it circulates in the plasma, shown schematically in **Fig. 1A**, should contain unmodified apoB-100 and no associated peroxides or aldehydes. It should be rich in antioxidants and polyunsaturated fatty acids. Most of the studies on defining the effects of Ox-LDL on cells compare the effects of "native LDL" with in vitro Ox-LDL. How native these particles are would depend on the limitations of currently available methods of isolation, detection of oxidized lipids and their degradation products. LDL is extremely oxidation-prone and labile. **Figure 2** illustrates the vulnerability of LDL to oxidation even during ultracentrifugal isolation. After a single centrifugation of

the plasma for the isolation of LDL (18), LDL is clearly seen as an orange band. Even in sealed centrifuge tubes stored at 4°C or at 25°C for as little as 4 h, the orange-yellow carotenoids are bleached out, which is attributable to oxidative changes. However, it is unlikely that any measurable degree of oxidation of lipids has occurred during this time, but any loss of antioxidants is sure to affect further in vitro oxidation of the particle making it more labile. This will be particularly true when very small amounts of lipoproteins are isolated and compared in vitro for oxidative susceptibility. Of course, no one leaves LDL in the centrifuge tube, but it is a common practice to dialyze LDL in large volumes for several hours after isolation to remove ethylenediaminetetraacetic acid (EDTA) for oxidation studies. Changes similar to those seen in undisturbed centrifuge tubes are likely to occur under these conditions. Thus the EDTA-removed "native LDL" would represent, not the LDL that was in circulation, but the LDL that has already been conditioned to have increased
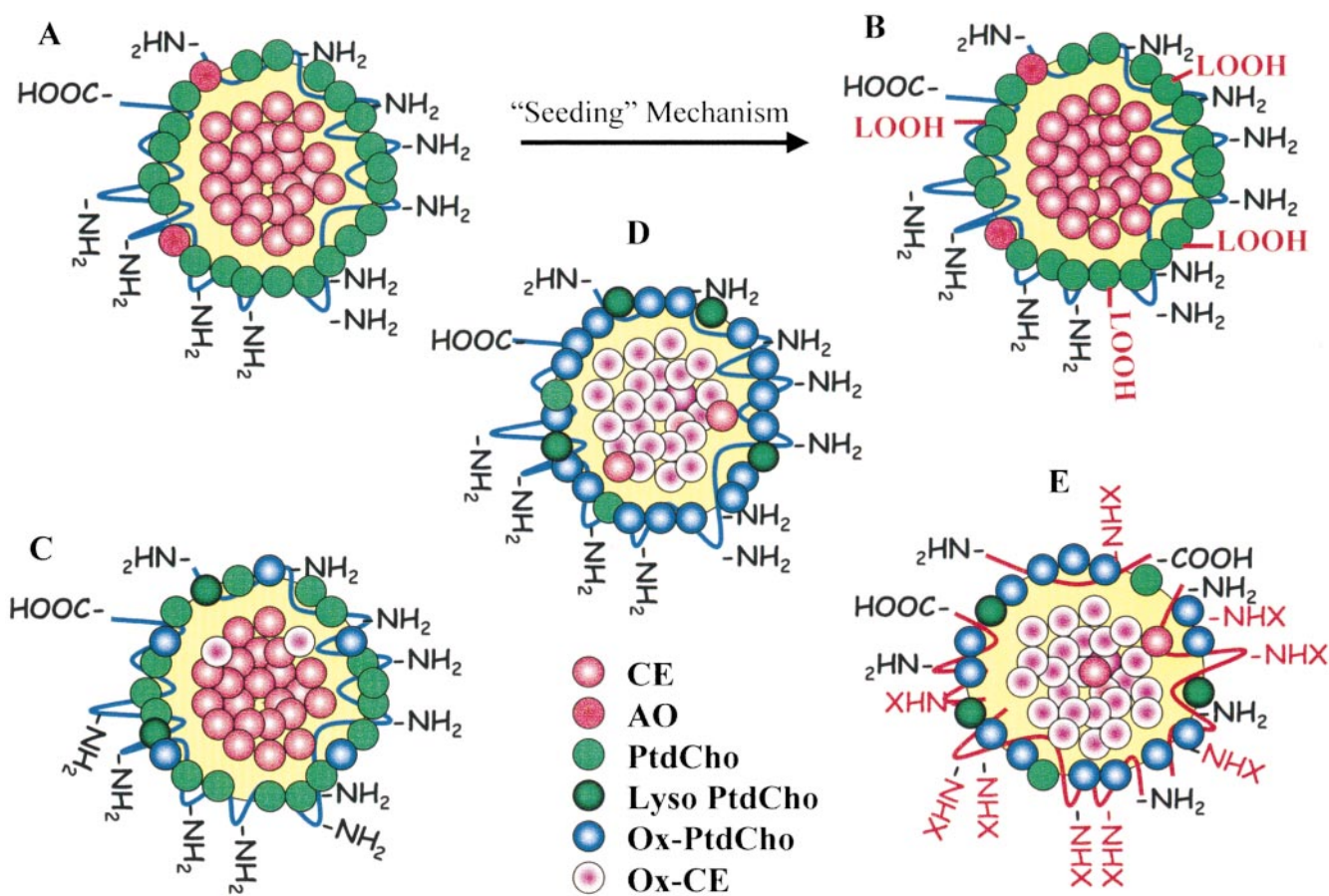
Fig. 1. Ox-LDL: different forms. 2A-Native LDL: Contains one intact polypeptide (apoB-100), no lipid peroxides or aldehydes, enriched in PUFA and antioxidants. Cleared by way of LDL receptor. 2B-Seeded LDL: Contains one intact polypeptide (apoB-100), enriched in PUFA and antioxidants. Cleared by way of LDL receptor. Peroxidized lipids and other proteins generated elsewhere might become associated with the circulating LDL. 2C-Minimally Ox-LDL: Contains one intact polypeptide (apoB-100), decreased amounts of PUFA and antioxidants. Some of the lipids of LDL themselves might be oxidized. Biologically active. Cleared by way of LDL receptor. 2D-Extensively Ox-LDL: Contains degraded and crosslinked polypeptides. Enormously decreased amounts of PUFA and antioxidants. Extensive oxidation of lipids of LDL. Massive amounts of lipid peroxides and new lipids such as lyso PtdCho. Presence of degraded lipids – core lipid aldehydes. 2E-Oxidatively modified LDL: Contains degraded and crosslinked polypeptides. Alterations in amino acid composition. Loss of lysine due to covalent modifications. Enormously decreased amounts of PUFA and antioxidants. Presence of degraded lipids–core lipid aldehydes and lyso PtdCho.
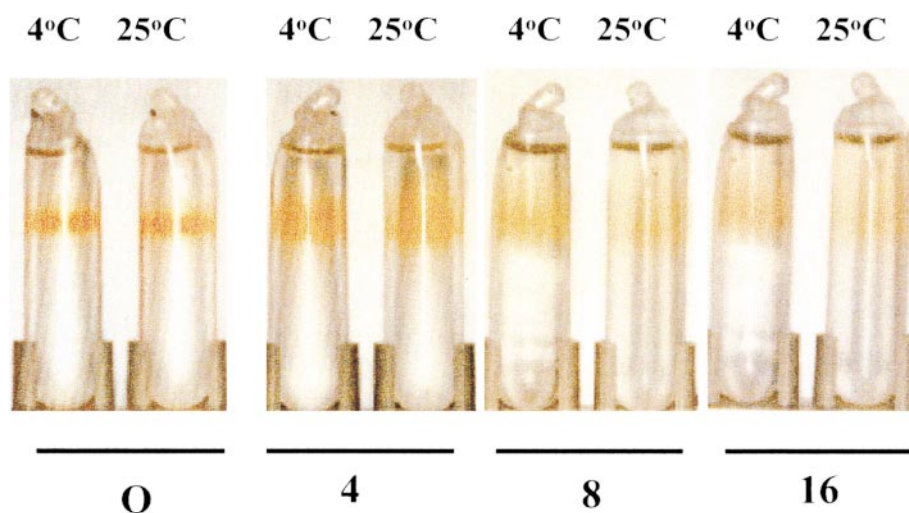
Fig. 2. Potential Oxidation of LDL during isolation. LDL was isolated from heparinized plasma of normal human donors using a TL-100 Beckman table-top ultracentrifuge. A single-spin gradient isolation was performed adjusting 2 ml of plasma to a d 1.31 g/ml with potassium bromide layered with saline and spun at 100,000 rpm for 1 h. The isolated sample was respun at d 1.063 g/ml to concentrate and purify LDL from any albumin contamination. The LDL was left at 4° and 25°C in the sealed centrifuge tubes at the time periods specified.

propensity to undergo further oxidation, e.g., antioxidant depletion. Such isolation and dialysis are often justified with the statement that no peroxides could be detected in such "native" LDL. However, the vulnerability of such isolated "native" LDL to undergo further oxidation in vitro might depend on innumerable factors, including its concentration during storage.

CONCEPTUALIZED FORMS OF OX-LDL

Seeded LDL

Depending on the dietary lipids, and pathological conditions, it is very likely that circulating LDL may have associated oxidized lipids, such as lipid hydroperoxides (LOOH) and other degradation products (Fig. 1B), derived from peripheral tissues including the liver and the intestine (19). This LDL could represent the "seeded" LDL, which might have an increased propensity to undergo further oxidation. However, no polyunsaturated fatty acid (PUFA) or antioxidants are lost from the LDL when LDL is "seeded" as the particle itself has not interacted with an oxidant. The association of proteins that may have "antioxidant" functions could counterbalance the seeding process. For example, enzymes such as platelet-activating factor-acetyl hydrolase (20) and other hydrophobic proteins may also associate with the lipoproteins and contribute/inhibit the oxidative process.

Mildly oxidized LDL

De novo oxidation of intrinsic LDL lipids might occur under a wide variety of normal and pathological conditions contributed by xanthine oxidase (21), peroxynitrite (22, 23), myeloperoxidase (18, 24), and other oxidative

processes (25) (Fig. 1C). When such oxidation is carried out to a minimal degree, the resultant particle might represent the "minimally Ox-LDL" or "mm-LDL" and, as such, might be physically indistinguishable from the native lipoprotein, except for the expected loss of polyunsaturated fatty acids and antioxidants. ApoB-100 is intact and little protein damage or modification has been detected. On the other hand, the lipids, particularly phospholipids are enormously affected (26–28). Studies by Berliner et al. (29) have contributed a wealth of knowledge towards the understanding the chemistry, physiology, biochemistry, and atherogenicity of these lipids.

There is little evidence to support that cells internalize these mildly oxidized lipoproteins by any pathways other than the LDL receptor-mediated pathway (30). On the other hand, there is evidence to support that these particles might undergo a more rapid oxidation when subjected to oxidation in vitro. For example, because of their increased peroxide content and decreased antioxidant levels, they are more readily oxidized by copper as compared to native LDL (31–33). When LDL oxidized to a different extent are intravenously injected into animals, they are cleared more rapidly from circulation as compared to native LDL. Steinbrecher et al. (34) observed years ago that the in vivo clearance of Ox-LDL depended on the extent of oxidation of the particle. This would suggest that either the components of LDL generated during milder oxidation had a direct role on their uptake by LDL receptor or that they accelerate the oxidation process resulting in more extensive oxidation and greater uptake by the liver scavenger receptor. To our knowledge, there is no evidence that mildly or moderately Ox-LDL is cleared more readily by the LDL receptor. A third alternative, that they are cleared faster as compared to native LDL by the

liver scavenger receptor(s) but not as fast as the more extensively modified LDL, is also possible. It is generally believed that plasma is an antioxidant-rich milieu and if so, the third alternative is more likely to account for the enhanced removal of the mildly or moderately Ox-LDL.

Extensively oxidized LDL

Can there be an oxidized but not "modified" LDL? The biological relevance of extensively Ox-LDL, which practically does not have any oxidizable fatty acids left, remains unexplored. When LDL is oxidized, for example by copper, it undergoes oxidation after an initiation, propagation and termination sequence as suggested by Esterbauer et al. (35). When oxidation plateaus, all the oxidizable fatty acids are consumed and the particle should be enriched in oxidized fatty acids (Fig. 1D). It should be noted that transition from a mildly oxidized particle to a fully oxidized particle (along the theoretical propagation phase) might occur rapidly (at least in the presence of copper), whereas the modification of the apolipoprotein by lipid peroxides and their decomposition products to generate the oxidatively modified LDL might be slower. In other words, theoretically there might be a stage when the particle is rich in peroxidized lipids but not yet fully modified to be able to be recognized by the scavenger receptor(s). We predicted that CD36 could be one such receptor(s) that could recognize the oxidized lipids of the Ox-LDL (36) based on the finding that moderately Ox-LDL was more readily recognized than extensively Ox-LDL by this receptor (12, 37). More recent studies showed that CD36 indeed recognizes the oxidized lipids of Ox-LDL (38). The interaction of Ox-LDL containing intact oxidized lipids with the cells that express CD36 in circulation and the endothelium is likely and cannot be excluded at present (12). Again, whether such a particle could really exist might depend on factors that are involved in the detoxification of the peroxides to hydroxides (39, 40), decomposition of lipid peroxides into aldehydes, and their ability to modify lysines of the apoprotein.

Extensively oxidized and modified LDL

It is ironic that the protein component of the extensively oxidized and modified LDL (protein-modified LDL, Fig. 1E) as defined by the early studies of Steinbrecher and associates (41) has not yet been fully characterized. Extensive proteolysis, oxidation of amino acids, extensive cross links, and chemical nature of the modification of amino groups by various products of oxidized lipids make characterization of the protein moiety a formidable challenge that very few laboratories could undertake. Ironically, like the true native LDL, the true oxidatively modified LDL, as depicted in Fig. 1E, is unlikely to occur even in the atherosclerotic artery. Such a particle would be completely devoid of PUFA, monounsaturated fatty acid (MUFA), and antioxidants (even MUFA will undergo co-oxidation under such conditions). Such particles are very likely to be cleared from plasma by the liver, even if they are generated in the plasma compartment. In the fatty streak lesions, it is more likely that macrophages would have

TABLE 1. Biological properties of the Ox-LDL protein

1. Recognized by macrophage scavenger receptor(s) (SR-AI/II) resulting in an enhanced uptake (13). Such uptake might account for foam cell formation.
2. Recognized by unknown proteins (receptors, e.g., LOX-1) on cell surface (143, 144). Such recognition might explain the signaling pathways activated by components of Ox-LDL.
3. Antigenic and results in the generation of autoantibodies. Has multiple antigenic domain (145). The antigenicity might play a major role in immune clearance as suggested by Calara and coworkers (146).
4. Induces the expression of Interleukin-1 by foam cell macrophages (147).
5. May result in ceroid accumulation in macrophage (148). Ceroids are autofluorescent lipid–protein complexes which accumulate in degenerative tissues including macrophage foam cells.
6. May be chemotactic to monocyte/macrophages (acetyl LDL and other modified LDL forms are chemotactic) (149).

cleared moderately oxidized particles long before such extensively oxidized and modified particles are generated. Currently, very few biological properties have been attributed to the protein component of the extensively Ox-LDL as summarized in **Table 1**. However, none of these properties are unique to the protein. For example, oxidized lipids are also antigenic (42–44), can also induce IL-1 synthesis by macrophages (45, 46), are recognized by the scavenger receptor(s) (17), and are chemotactic for monocyte/macrophages (47), in addition to the oxidatively modified apolipoprotein. One is left to wonder then, whether oxidative changes in apoB-100 have any relevance to atherosclerosis other than their potent antigenicity.

MAJOR CHARACTERISTICS OF NATIVE AND OX-LDL

The lipids and the protein component of Ox-LDL differ substantially from those of the native LDL (**Table 2**). In contrast to the single long chain polypeptides (apoB-100) of native LDL, the Ox-LDL has numerous proteolytic fragments derived from apoB-100. It is unlikely that these are linear polypeptides derived from simple non-enzymatic proteolysis (48) of apoB-100. It is more likely that these represent proteolyzed and randomly cross-linked fragments. In addition to the expected oxidized products, the lipid fraction of the Ox-LDL also contains enzymatic breakdown products such as lysophosphatidylcholine (lysoPtdCho). It may also contain phospholipids and cholesteryl esters in which the oxidized fatty acid moiety has been shortened. These oxidatively "tailored" lipids might represent "core aldehydes", whose contribution to the modification process is yet to be determined (49, 50). In summary, there is very little in common among these two LDL forms other than the fact that the former is derived from the latter.

If the contribution of oxidatively modified apoB-100 to the atherosclerotic process is minimal and if the oxidized lipids play a major role, then why is the oxidative modification of other lipoproteins ignored? They also contain

TABLE 2. Lipids and proteins of native and Ox-LDL

Native LDL	Ox-LDL
Presence of intact, single apolipoprotein B-100. Non-antigenic in the same species	Proteolyzed, fragmented, oxidized, cross-linked apolipoprotein with a different amino acid composition. The protein is also covalently modified by lipid peroxidation products that include intact core aldehydes derived from esterified lipid (48). New functional groups (protein carbonyls, sulfonic acids, carboxylic acids, etc.) might be present on the protein. Chlorination and nitration might occur (150). Highly antigenic (145)
Contains abundant amounts of PUFA and antioxidants	PUFA and antioxidants are depleted (35)
Free of lipid peroxides and their degradation products such as aldehydes and ketones	Contains massive amounts of lipid peroxides and their degradation products
Very low levels of oxidized cholesterol	Increased amounts of oxidized cholesterol product (151). May also contain large amounts of cholesterol ester core aldehydes (49)
No unusual lipids	Chlorinated and nitrated lipid species might be present (152, 153).
May contain very low levels of lyso PtdCho	Contains increased levels of lyso PtdCho. Might also contain oxidatively tailored phospholipids, which are intact phospholipids with fragmented fatty acid chain

unsaturated lipids, which are expected to undergo oxidation. There is no evidence to date to suggest that oxidized lipids on one type of lipoprotein are different from their presence in another type of lipoprotein.

ATHEROGENIC EFFECTS OF OX-LDL

A substantial body of evidence suggests that most, if not all, of the atherogenic effects of "Ox-LDL" are derived from the oxidized lipid components (51). The "active" lipids include both esterified and unesterified peroxidized lipids, lysoPtdCho, cholesterol oxidation products, aldehydes derived from breakdown of both esterified and unesterified oxidized fatty acids, and perhaps proteolipids that may have peroxidized lipids bound to fragmented apoB-100. These products are abundant in mildly or moderately Ox-LDL and have a longer half-life in plasma as compared to the more extensively Ox-LDL (41). Every single aspect of atherogenesis is likely to be affected by one or more of these components. For example, the induction of adhesion molecules for monocytes at the endothelial cell surface and the chemotactic process itself are affected by oxidized fatty acid (52) and oxidized phospholipids, respectively (53, 54). LysoPtdcho is chemotactic to both monocytes and T-lymphocytes (53, 54). Oxidized fatty acids affect the proliferation of smooth muscle cells profoundly (55–57). Even the formation of foam cells, which once was attributed to the interaction of the oxidatively modified protein with the scavenger receptor(s), could be partly attributed to the lipids of Ox-LDL (38). **Table 3** summarizes some of the proatherogenic properties of components of Ox-LDL.

The adhesion and entry of monocytes into the intima is an early event in atherogenesis (1, 58). LysoPtdCho, 13-hydroperoxy linoleate (13-HPODE), and a number of products derived from oxidized PtdCho are suggested to induce the adhesion and chemotactic recruitment of monocytes by way of activation of endothelial cell adhesion molecules and specific chemotactic factors (52, 59–61). Some of these (lysoPtdCho) are also direct chemotaxins for monocytes and T-lymphocytes (53, 54). Ox-LDL affects the differentiation of monocytes into macrophages and induces the expression of putative receptors for its uptake. Its ability to induce lipid accumulation has been well documented in early literature (1). Cell culture studies have demonstrated the proliferative and signaling effects of Ox-LDL on smooth muscle cells which has been the basis to suggest its role in smooth muscle cell migration into the intima and in restenosis (56, 62, 63). Oxidized lipids and oxidants appear to activate matrix-digesting proteinases which might play a role in plaque instability and rupture (64, 65). Components of Ox-LDL have also been suggested to affect vascular endothelium-dependent relaxation and promote pro-coagulatory responses from both the endothelium and the platelets (66–69). One can note from the above description that the effects cover what one would surmise from the presence of Ox-LDL in circulation as well as from its presence in the intima.

Table 3 also suggests that lysoPtdCho affects a variety of cells in a proatherogenic manner. Earlier studies showed that during the oxidation of LDL, there is extensive hydrolysis of PtdCho that generates lysoPtdCho (41, 70). Unfortunately, many investigators assume that the presence of lysoPtdCho is synonymous with the oxidation of LDL. LysoPtdCho is a normal metabolite of PtdCho and its levels can be elevated due to increased lecithin:cholesterol acyltransferase activity or any phospholipase A reaction (71). Previous studies have also shown that lysoPtdCho levels in β-VLDL are increased in rabbits upon cholesterol feeding (72), without any obvious changes in oxidation. Thus, properties attributed to lysoPtdCho need not reflect the biological effects of Ox-LDL. In fact, studies by Kume, Cybulsky, and Gimbrone (59) showed clearly that lysoPtdCho

TABLE 3. Proatherogenic effects of components of Ox-LDL

Biological Effect	Component	Ref.
1. Cell adhesion and recruitment		
Induction of endothelial cell adhesion molecules	Lyso PtdCho	59, 154, 52
Intercellular Adhesion Molecule-1 (ICAM-1),	Oxidized fatty acids	
Vascular Cell Adhesion Molecule-1 (VCAM-1)		
Monocyte and T-lymphocyte chemotactic activity	Lyso PtdCho	53
Inhibition of macrophage chemotaxis	Unknown lipid	149, 155
Induction of interleukin-8	Unknown components	156
	Oxysterols	157
Increased monocyte adhesion to endothelium	Oxidized phospholipid(s)	158, 26
Induction of granulocyte/monocyte colony stimulating factor (GM–CSF)	Unknown lipid	159, 160, 161
Induction of monocyte chemotactic protein-1	Unknown components	162, 163, 164
Chemotactic activity for smooth muscle cells	Unknown components	165
2. Cell proliferation		
Induction of cell proliferation	Unknown components	166, 56, 167, 168, 169
Induction of growth promoting activity	Lyso PtdCho	170, 171, 172
	Unknown components	173
3. Procoagulant effects		
Induction of tissue factor	Unknown components	174, 175, 176
Inhibition of protein C	Unknown components	174, 177
Induction of plasminogen activator inhibitor-II (PAI-II)	Unknown components	176
Induction of platelet aggregation	Unknown components	178, 179, 180
4. Endothelium dysfunction/cytotoxicity		
Inhibition of endothelium-dependent relaxation	Oxysterols	68
	LysoPtdCho	66, 181, 182, 183
Cellular cytotoxicity	Oxysterols	151, 184, 185
Inhibition of endothelial cell migration	Lipid peroxides	186
Induction of heat shock proteins	Unknown components	187, 188
5. Miscellaneous		
Increased interleukin-1 (IL-1) production	Hydroxy linoleic acid (13- and 9-HODEs), modified proteins	45, 147
Increased antigenicity	Modified proteins, modified lipids	189, 190, 191, 192, 193, 194
	Lyso PtdCho	

and not Ox-LDL increased the adhesion molecules on the endothelial surface.

COULD OXIDATION PRODUCTS OF ANTIOXIDANTS AFFECT ATHEROGENESIS?

Forgotten among these products are the products of oxidation that are derived from the metabolism of antioxidants such as vitamin E and carotenoids. The oxidation products from the latter, namely apocarotenals and apocarotenoic acids, could have significant impact on the target cells, perhaps interacting via the retinoic acid receptor(s) which has been suggested to be involved in the regulation of scavenger receptor expression in macrophages (73). Previous studies have suggested that the induction of the acetyl-LDL during the differentiation of monocytes into macrophages could be inhibited by retinoic acid (73). This receptor plays a major role in cell differentiation and is involved in the "cross-talk" with peroxisomal proliferator receptors (74) which have recently been shown to interact with an oxidized fatty acid 13-HPODE and Ox-LDL. While this might appear a farfetched speculation, one has to remember that carotenoids are the first to disappear during oxidation and currently there is no explanation as to why carotenoids have conflicting effects on experimental atherosclerosis (75–77). It should be mentioned that a direct antioxidant action of carotenoids in preventing the oxidation of LDL has been a topic of intense criticism (78, 79). As seen later, radicals derived from vitamin E and other phenols could become prooxidants.

ARE OXIDIZED LIPIDS ATHEROGENIC?

From the impressive list of proatherogenic effects, one could jump to the conclusion that oxidants, such as oxidized lipids, are atherogenic. There is little information in the literature to suggest that direct oxidative stress in vivo is a risk and is atherogenic. The closest studies that would suggest a link between oxidative stress and the potential for increased risk for atherosclerosis come from dietary studies by Staprans et al. (19, 80) on rabbits consuming oxidized lipids. These studies showed that oxidized (heated) fat is absorbed, incorporated into lipoproteins secreted by the liver, and enhances the in vitro oxidizability of isolated LDL (19). The suggestion that oxidized lipids are atherogenic came about even before the Ox-LDL hypothesis was formulated. The atherogenicity of dietary cholesterol has long been attributed to the presence of oxysterols in the diet (81).

IS CONSUMPTION OF ANTIOXIDANTS BENEFICIAL?

A corollary hypothesis to the oxidation theory is that the consumption of antioxidants is beneficial in the prevention of cardiovascular disease (2, 77, 82). The literature is divided and ambiguous on support of this conclusion. Three types of studies are generally cited in support of the hypothesis.

Epidemiological studies

Epidemiological studies based on dietary information indicated a negative correlation between the presence of antioxidants in the plasma of subjects and the incidence of coronary heart disease (CHD) (83–85). This finding has led to the speculation that specific dietary ingredients such as red wine, olive oil, green tea, licorice, etc., might have "antioxidant" components that could be responsible for protection against CHD (86–91). The lower incidence of CHD in individuals who consume the "Mediterranean diet" was attributed to the antioxidant effects of both the MUFA and polyphenolic components of olive oil in such diets (88, 92, 93). However, to date, while polyphenolic extracts or MUFA from these sources effectively prevented the in vitro oxidation of LDL (94, 95), the potential benefit of MUFA in preventing atherosclerosis in animals or humans is yet to be established (96, 97). On the other hand, recent studies by Hayek and coworkers (95) have shown that red wine and polyphenols retarded the development of atherosclerosis in apoE-deficient animals.

Antioxidants inhibit the oxidation of LDL in vitro

An a priori assumption is made that antioxidants that inhibit the oxidation of LDL in vitro may have the potential to inhibit the progression of the disease in vivo. Over the past 15 years, the number of chemicals that inhibit the oxidation of LDL in vitro has grown enormously (**Table 4**). The list does not include the myriad of chemicals tested by the pharmaceutical industries. Of those tested for their ability to inhibit the oxidation of LDL in vitro, very few have been tested for their efficacy in vivo. More importantly, little information is available to suggest whether these compounds are absorbed in sufficient quantity to prevent oxidation in vivo. A mere in vitro demonstration of an antioxidant effect is not enough to suggest that a pharmacological agent might be beneficial in vivo. Those who are familiar with the "Lazaroids" might recollect that despite their in vitro antioxidant effects, none of these compounds became a viable "drug". Are antioxidants associated with LDL in the circulation? It is currently believed that lipophilic antioxidants that are enriched in LDL might be more important in preventing the oxidation of LDL. It is therefore necessary that an orally given antioxidant be enriched in plasma LDL in order to have an effect. Are antioxidants available in the sub-endothelial intima to prevent the oxidation of LDL? Recent studies from Fruebis et al. (98) have shown that a mere antioxidant effect in plasma is insufficient to have an effect on atherosclerosis. They provided probucol and an analog of probucol to animals along with an atherosclerotic diet and demonstrated that LDL isolated from both groups resisted in vitro oxidation of LDL. However, only probucol prevented the progression of the lesion while the analog did not have an effect. This was attributed to the poor availability of the analog in the intima. These points need to be tested in suitable animal models to determine the efficacy of potential antioxidants in preventing the disease.

Outcome from clinical trials with antioxidants using animal and human models

The third line of evidence is suggested by the limited success of animal and human clinical trials. Currently,

TABLE 4. Antioxidants that inhibit the oxidation of LDL in vitro

Group	Antioxidants
Phenols	Tocopherol derivatives, probucol, BHT, butylated hydroxy anisole, glabridin, quercetin, catechin, propyl gallate, tyrosine, caffeic acid, boldine, ubiquinol, nordihydroguairetic acid, polyphenols from plant extracts, green tea extracts, red wine extracts, licorice extract, onion extract
Thiols	Cysteine, glutathione, S-allyl cysteine, N-acetyl cysteine, lipoic acid
Metal chelators	EDTA, pyrollidine diamine thiocarbamide (PDTC), lazaroids
Hormones and antihormones	Estradiol, esterified estradiol, phytoestrogens, RU-486, onapristone, tamoxifen, ethinylestradiol
Vitamins	Vitamin E, vitamin C, carotenoids, riboflavin
Ca^{2+}-channel blockers	Nifedipine, captopril, carvedilol
Miscellaneous	DPPD, MUFA, ebselen, spintraps, nitric oxide, selenium, statins, phenothiazine derivatives, thiocarbamates, amino acids (specifically L-arginine and L-lysine), aminoguanidine, licorice extracts, plasmalogens, curcumin, caffeic acid, cigarette smoke extract, leuteolin-rich artichoke extracts, uric acid, lipoxygenase inhibitors, fibrates, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase inhibitors
Antioxidant enzymes	Catalase, SOD, paraoxanase, platelet activating factor-acetyl hydrolase, phospholipid-glutathione peroxidase
Synthetic antioxidants	Innumerable synthetic antioxidants have been reported to inhibit the oxidation of LDL

probucol, butylated hydroxytoluene (BHT), vitamin E, a 15-lipoxygenase inhibitor, and diphenylphenylenediamine (DPPD) have been shown to retard the progression of atherosclerosis in various animal models (99–106). There is limited but encouraging information to suggest that vitamin E might protect against intimal thickening of coronary arteries in humans as determined by ultrasound techniques (107–109). However, there are major areas of concern. Despite the long list of antioxidants that inhibit the oxidation of LDL in vitro, the short list of five compounds, of which two are definitely toxic (BHT and DPPD), and one with multiple modes of action (probucol), is disappointing. Considering that these studies were carried out several years ago, it is intriguing that additional antioxidants have not been tested in vivo. Considering the recent finding that the overexpression of superoxide dismutase (SOD) had no inhibitory effect on atherosclerosis (110), is it possible that many antioxidants have been tested but the negative results were not reported?

ANTIOXIDANTS AS PROOXIDANTS

Recent evidence suggests that antioxidants could be prooxidants under certain conditions (18, 111, 112). It is possible that the prooxidant nature of antioxidants could not only be important in physiology but could even be beneficial. Studies by Thomas, Neuzil, and Stocker (113), Witting, Mohr, and Stocker (114) and those by our own laboratory (18) have suggested that vitamin E could be a prooxidant in the oxidation of LDL, particularly in the presence of peroxidases. It appears that a delicate balance exists between the vitamin E radical generation and the concentration of vitamin E as suggested by Yamamoto and Niki (112) and Niki (115). The plasma concentration of vitamin E does not rise in proportion to dietary dose and, in humans, no more than a 3-fold increase could be achieved (82). While this increase might be sufficient to prolong the lag time during the in vitro oxidation of isolated LDL by copper, it is difficult to forecast its effect in vivo. More importantly, whether the observed effects could be attributed to pro- or antioxidant effects could not be established. The probability of a phenolic antioxidant to become a prooxidant is well illustrated by estradiol (116). Not only does it have the ability to become a prooxidant in the presence of peroxidases, its increased presence is also associated with an increased neutrophil activation and release of myeloperoxidase (MPO) (117). In addition to MPO, it also induces other tissue peroxidases (118), thereby increasing the odds of an increased lipid tissue peroxidation. Estrogen-induced lipid peroxidation is well documented in the literature (119). The contribution of the "prooxidant" effect of estrogens needs to be carefully evaluated. Estradiol might "help" to oxidize and clear LDL rapidly from plasma, induce antioxidant enzymes such as nitric oxide synthase in the endothelium in the arterial cells, and might have other effects (116, 120). The recent HERS report has alerted us to the potential negative consequences of estrogen replacement therapy for menopausal women with potential for atherogenesis (121, 122).

While the prooxidant effects are worrisome, studies by Bird and associates (123) and Zhang and coworkers (124) have also provided startling evidence that even probucol, an antioxidant that has been used as a yardstick for measuring the antioxidant/antiatherogenic compound, failed to reduce atherosclerosis in a mouse model of atherosclerosis. Yet other findings from Bird et al. (123) and Fruebis et al. (125) that two very similar antioxidants, probucol and a close demethylated derivative, had opposing effects on atherosclerosis even in the same experimental animals, despite comparable levels of plasma antioxidant protection, raise considerable doubts regarding the measurements of oxidants and oxidation parameters in the plasma. These results pose a number of questions. *a)* Are prooxidant effects of antioxidants relevant in atherosclerosis and, if so, which antioxidants are likely to become prooxidants and under what circumstances? *b)* Are antioxidants species-specific? Do humans and animal models of atherosclerosis metabolize a given antioxidant similarly? *c)* Do antioxidants metabolized by oxidative pathways pose an additional oxidative stress? *d)* Is an antioxidant tissue-specific? If two antioxidants partition into LDL to comparable extents, why should one enter the intima more readily? Are the antioxidants absorbed independently of LDL? *e)* Do intracellular antioxidants play a greater role than extracellular antioxidants?

Some of these antioxidants have actually been tested in vivo with mixed results. Upon careful analysis of the literature, one is left with the impression that *a)* an antioxidant that is effective in vitro may have little, if any, effect in vivo; *b)* an antioxidant that is effective in one model system may fail to have any effect in another; *c)* very similar antioxidants may have different effects in the same experimental system; and *d)* conditions that do not favor oxidation in vitro might actually have no effect in vivo; conversely, conditions that promote oxidation in vitro might actually prove to be beneficial in vivo.

CAN PROOXIDANTS BE BENEFICIAL?

In contrast to the proatherogenic effects described for the oxidized lipids (Table 3), there is an equally impressive list of potentially anti-atherogenic effects of peroxidized lipids (**Table 5**). First, lipid peroxides can induce the synthesis of nitric oxide (126), a molecule that not only inhibits the oxidation of LDL profoundly (127), but also has potent vasoactive properties (128). In 1990, Minor et al. (129) described an increased synthesis of nitric oxide by the atherosclerotic artery. Not only lipid peroxides, but also dihydric phenols such as nordihydroguaiaretic acid, a catechol (which could spontaneously release superoxide radicals) can induce the synthesis of nitric oxide (130). The induction can be argued as evidence for increasing the potential for the formation of the powerful prooxidant, peroxynitrite, a product of superoxide and nitric oxide. However, it is not just the synthesis of nitric oxide

TABLE 5. Antiatherogenic effects of oxidized lipids

Oxidized lipid	Gene Induced	Antiatherogenic Effect
Hydroperoxy linoleic acid (13-HPODE),13-HODE, Ox-LDL	VCAM-1(52)	s-VCAM-1, the soluble form of VCAM-1 is an angiogenic factor that would promote neovascularization (195)
13-HPODE	NO synthase (126)	Potent vasodilator and antioxidant (196)
13-HPODE	Heme Oxygenase (133)	Generates antioxidant products such as bilirubin, biliveridin, and carbon monoxide (197)
Lyso PtdCho		Causes cholesterol efflux from cells (198) Generated in vast quantities by LCAT and might promote de novo HDL synthesis by liver (199)
Cholesterol oxidation products		Inhibit cholesterol synthesis (200)
		Could potentially inhibit smooth muscle cell growth?
Unknown components of Ox-LDL	Mn-SOD (131)	Quench superoxide radicals, which may be involved in the oxidation of LDL
Unknown components of Ox-LDL		Increased glutathione synthesis (137)

alone, but lipid peroxides also similarly activate other antioxidant enzymes. For example, the induction of manganese superoxide dismutase (Mn-SOD) by oxidants is well known and is considered to be the result of activation of cellular defenses (131, 132). Recently, the induction of heme oxygenase (which would generate potent antioxidants such as bilirubin and biliveridin) (133–135), catalase (136) (O. Meilhac, unpublished observations) and glutathione (137, 138) by vascular cells under oxidative stress has been described. These findings raise an inescapable conclusion that a mild oxidative stress might itself lead to the body's antioxidant defense, a conclusion that might be supported by circumstantial studies from in vivo studies (116, 139, 140). These studies indicated that beginning exercisers suffer an oxidative stress whereas conditioned exercisers are well protected, as determined by the susceptibility of isolated LDL to oxidation (140). Needless to say, exercise and the consumption of PUFA and fish oil-derived fatty acids, which potentially can impose an oxidative stress, are well-documented deterrents of CHD in comparison to the consumption of antioxidants.

Proatherogenic oxidation of LDL has been suggested to occur in vivo in the plasma and in the intima (141, 142). When this occurs, one would expect the initial recruitment of the mononuclear phagocytes to scavenge and clear the Ox-LDL. The cells affected by the presence of the oxidized components would be expected to recuperate and replenish compromised antioxidant defenses. That might be the case in the in vitro studies, where cells are exposed to oxidants for a single time and allowed to respond. Atherosclerosis that develops over a prolonged period of time might involve repeated oxidative stress where recovery of cells and tissues is compromised. The oxidized components are not permanently scavenged and cleared, but are likely to be present as intracellular components that might be released providing a constant and persistent oxidative stress. Clearly more studies are needed to establish the long-term cellular effects of oxidized lipids.

Summary

In summary, it is prudent to assume that we do not know enough about oxidants and antioxidants to arrive at the conclusion that they will have predicted effects in various experimental models of atherosclerosis. At the same time it is not feasible to test a variety of antioxidants, both synthetic and natural, on humans without an understanding of the metabolism and availability of the antioxidant in the arterial intima. Considering the induction of antioxidant enzymes by oxidative stress and the circumstantial evidence based on our recent studies on exercisers and estradiol as outlined earlier, it might be advisable to activate the in vivo antioxidant defense, when the oxidation of low density lipoprotein is clinically important. Antioxidant supplementation might prove more useful for those who fail to respond with an increased antioxidant defense when oxidatively challenged.

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