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Low-density lipoprotein oxidation, antioxidants, and atherosclerosis: a clinical biochemistry perspective

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Cardiovascular disease is the leading cause of mortality in westernized populations. An increased concentration of plasma low-density lipoprotein (LDL) cholesterol constitutes a major risk factor for atherosclerosis. Several lines of evidence support a role for oxidatively modified LDL in atherosclerosis and for its in vivo existence. Antioxidants have been shown to decrease atherosclerotic lesion formation in animal models and decrease LDL oxidation; the evaluation of LDL oxidation in vivo is therefore very important. However, there is a paucity of methods for direct measurement of LDL oxidation. Of the direct methods currently available, the preferred ones seem to be the measurement of F_2-isoprostanes, autoantibodies to epitopes on oxidized LDL, and the assessment of antioxidant status. Of the indirect measures, the most uniformly accepted procedure is examining the oxidative susceptibility of isolated LDL by monitoring conjugated diene formation.

INDEXING TERMS: cholesterol • cardiovascular disease • α-tocopherol • ascorbate • β-carotene • conjugated dienes • apolipoprotein B-100 • fatty acids • thiobarbituric acid-reactive substances • prostaglandins • isoprostanes

An increased concentration of plasma low-density lipoprotein (LDL) cholesterol constitutes a major risk factor for atherosclerosis. Clinical, epidemiological, and genetic studies convincingly demonstrate that LDL promotes atherosclerosis. However, the precise mechanism(s) by which LDL promotes the development of the early fatty-streak lesion still remains to be elucidated. Uptake of cholesterol by the classical LDL receptor pathway cannot result in appreciable cholesterol accumulation because the LDL receptor is subject to feedback inhibition by the intracellular cholesterol content *[1]*. However, modified forms of LDL such as acetyl LDL or oxidized LDL (Ox-LDL) are taken up by the scavenger receptor mechanism, resulting in cholesterol accumulation and subsequent foam cell formation, since the scavenger receptor is not regulated by the cholesterol content within the cell *[1]*.[1]

Mechanisms of LDL Oxidation

LDL oxidation is generally believed to occur mainly in the intima of the artery, in microdomains sequestered from antioxidants. Several lines of evidence observed by different groups over the years support a role for Ox-LDL in atherogenesis *[2–6]*. LDL can be oxidatively modified in a cell-free system by transition metals such as iron and copper and by all the major cells of the arterial wall such as endothelial cells, smooth muscle cells, and monocyte–macrophages. Physiologically relevant mechanisms underlying LDL oxidation in vivo are yet to be established. Various studies implicate superoxide anion as one agent that promotes oxidation of LDL lipids, mediated by smooth muscle cells and phagocytes *[7]*. A well-understood pathway is the membrane-associated NADPH oxidase of activated phagocytes. Activated human neutrophils and monocytes oxidize LDL via a pathway that is inhibited by superoxide dismutase and metal chelators *[8, 9]*. Thiols autooxidize in the presence of metal ions, forming thiyl radicals and superoxide, which promote LDL oxidation *[10]*. It has been proposed that arterial smooth muscle cells reduce disulfides to thiol intracellularly and export thiol to the extracellular medium; the thiol then autooxidizes, forming a species that can promote oxidation. LDL oxidation by thiols in a cell-free system supports this hypothesis. Certain cellular enzymes, such as 15-lipoxygenase, that convert polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides may also oxidize LDL *[11]*. Soybean lipoxyge-

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[1] Nonstandard abbreviations: Ox-LDL, oxidized LDL; PUFA, polyunsaturated fatty acid; AAPH, 2,2′-azobis(2-amidinopropane); apo, apolipoprotein; CD, conjugated diene; MDA, malondialdehyde; HNE, 4-hydroxynonenal; MM-LDL, minimally modified LDL; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; IL-1, interleukin-1; EDRF, endothelium-derived relaxation factor; BHT, butylated hydroxytoluene; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substances; GC, gas chromatography; MS, mass spectrometry; and PGF_2, prostaglandin F_2.

nase and phospholipase A_2 have also been shown to stimulate LDL oxidation in the absence of cells *[11]*. 15-Lipoxygenase protein and mRNA have been found in atherosclerotic lesions *[12]*, although some groups question the role of 15-lipoxygenase in LDL oxidation. The heme protein, myeloperoxidase, secreted by activated phagocytes, may also oxidize lipoproteins by acting as a physiological catalyst. The products of myeloperoxidase action, hypochlorous acid and tyrosyl radical, promote lipoprotein oxidation *[13, 14]*. Nitric oxide and peroxynitrite are other oxidants relevant to LDL oxidation produced by endothelial cells and macrophages. It appears that peroxynitrite increases the modification of LDL *[15]*. However, stimulated macrophages producing increased nitric oxide oxidize LDL to a lesser extent than resting cells, and inhibitors of nitric oxide synthase increase LDL oxidation by activated macrophages *[16]*. Thus, LDL can be oxidatively modified by numerous different mechanisms. To date, however, there is no consensus on the predominant mechanism of LDL oxidation in vivo.

In vitro, LDL can be modified oxidatively in the presence of transition metals such as iron and copper. LDL oxidized by a cell-free system is physiochemically and biologically indistinguishable from LDL oxidized by a cellular system *[2]*. In vitro, LDL can bind copper, which can promote rapid lipid peroxidation *[17]*. LDL can be oxidized in a metal-independent system with 2,2′-azobis(2-amidinopropane) (AAPH), a water-soluble azo compound that thermally decomposes, leading to the formation of aqueous peroxyl radicals at a constant rate *[18]*.

The oxidizability of LDL also depends on its size. Subjects with a predominance of small, dense LDL exhibit a greater risk of coronary artery disease compared with individuals with a predominance of large, more buoyant LDL *[19]*. Studies from numerous laboratories have shown that small, dense LDL is more susceptible to oxidation *[20, 21]*.

Oxidative Modification of LDL

Human LDL is defined as the population of lipoproteins that can be isolated by ultracentrifugation within a density range of 1.019–1.063 kg/L *[6]*. Each LDL particle contains ~1600 molecules of cholesteryl ester and 170 molecules of triglycerides, which form a central lipophilic core. This core is surrounded by a monolayer of ~700 phospholipid molecules, consisting mainly of lecithin and small amounts of sphingomyelin and lysolecithin and 600 molecules of free cholesterol. Embedded in the outer layer is a large protein, apolipoprotein (apo) B-100, consisting of 4536 amino acid residues. The total number of fatty acids bound in different classes of an LDL molecule is ~2700, half of these being PUFAs, mainly linoleic acid. Variations in PUFA content contribute to the difference in oxidation behavior of different LDL samples. The PUFAs in LDL are protected against free radical damage by several antioxidants, the predominant one being α-tocopherol.

Oxidation of LDL is a free radical-mediated process, resulting in numerous structural changes, all of which depend on a common initiating event, the peroxidation of PUFAs in LDL. The peroxidation of a PUFA is shown in Fig. 1. Oxidation of LDL is initiated by reactive oxygen species that abstract a H• from a double bond in PUFA, which is followed by molecular

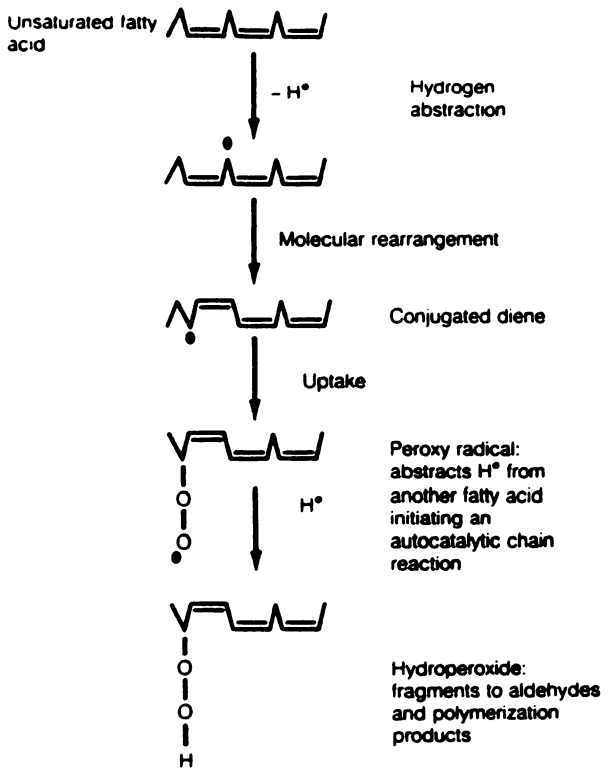

Fig. 1. Peroxidation of a PUFA.
Adapted from ref. *22*.

rearrangement, leading to the formation of conjugated double bonds referred to as conjugated dienes (CD) *[6]*. During this initiation phase of LDL oxidation, the rate of oxidation is suppressed by the presence of endogenous antioxidants within the LDL particle, which results in the lag phase of oxidation. The lag phase is followed by a rapid propagation phase, which occurs when the antioxidants are depleted and involves the abstraction of another H• by a PUFA–peroxyl radical (LOO•) from another PUFA, resulting in the formation of lipid peroxides. A typical time course of copper-catalyzed LDL oxidation, depicting both lag and propagation phases, including measures of both lipid and protein oxidation, is shown in Fig. 2. These indices of oxidation will be discussed in detail later. Cholesterol in LDL can be oxidized to oxysterols such as 7-ketocholesterol *[23]*. The propagation phase is followed by a decomposition or degradation phase, during which there is cleavage of double bonds, resulting in the formation of aldehydes. The major aldehydes produced include malondialdehyde (MDA), 4-hydroxynonenal (HNE), and hexanal, which can cross-link with amino groups on apo B-100.

Changes in the protein moiety also occur during the oxidation of LDL *[6, 24]*. After oxidation, there is an increase in the negative charge on the LDL particle, possibly due to the derivatization of positively charged amino groups through the formation of a Schiff base with aldehydes. Also, after oxidation, apo B-100 undergoes oxidative scission, leading to fragmentation.

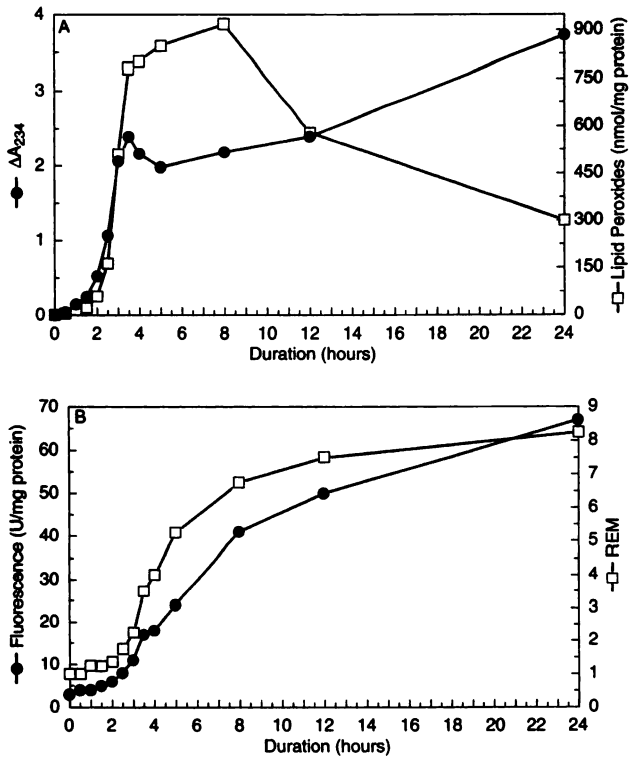

Fig. 2. Typical time course of LDL oxidation showing lag, propagation, and decomposition phases: (*A*) CD and lipid peroxide formation; (*B*) measurement of apo B fluorescence during LDL oxidation and relative electrophoretic mobility (REM) of oxidized LDL.

LDL (200 mg/L protein) was incubated with 5 μmol/L copper in phosphate-buffered saline for 8 h.

Biological Effects of Ox-LDL

Ox-LDL exerts several biological effects that may contribute to the initiation and progression of the atherosclerotic process *[2–6]*. A schema depicting the role of Ox-LDL in atherogenesis is shown in Fig. 3. During the oxidation of LDL, initially, minimally modified LDL (MM-LDL) is formed in the subendothelial space. MM-LDL is typified by mild lipid peroxidation and uptake by the classical LDL receptor. MM-LDL can induce leukocyte–endothelial adhesion and secretion of monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) by the endothelium *[25]*. This results in monocyte binding and recruitment to the endothelium and subsequent migration into the subendothelial space, where M-CSF promotes their differentiation into tissue macrophages. Macrophages in turn can modify MM-LDL into a more oxidized form. Ox-LDL is no longer recognized by the LDL receptor; instead it is taken up by the scavenger receptor on the monocyte–macrophages, and this uptake is not regulated by intracellular cholesterol content. This results in appreciable cholesterol accumulation within the macrophages, resulting in foam cell formation. Ox-LDL is a potent chemoattractant for monocytes and a potent inhibitor of macrophage motility, thereby promoting retention of macrophages in the arterial wall. Ox-LDL is cytotoxic, which could promote endothelial dysfunction and the evolution of the fatty streak into a more advanced lesion. Ox-LDL could also promote atherogenesis by altering expression of other genes in the arterial wall. In addition to leukocyte adhesion molecules M-CSF and MCP-1, Ox-LDL can stimulate interleukin-1 (IL-1) release from macrophages *[26]*. IL-1b has been shown to induce smooth muscle cell proliferation and endothelial adhesiveness to leukocytes *[27]*. In addition, IL-1b mRNA has been found in atherosclerotic lesions.

Ox-LDL can adversely affect the coagulation pathway by inducing tissue factor *[28]* and plasminogen activator inhibitor-1 synthesis *[29]*; also, products of Ox-LDL can impair expression of inducible genes such as tumor necrosis factor and platelet-derived growth factor *[30]*. Ox-LDL inhibits endothelium-derived relaxation factor (EDRF)-mediated vasorelaxation *[31]*. EDRF appears to be crucial in maintaining coronary vasodilation, and its activity is impaired in hypercholesterolemia and atherosclerosis. Another atherogenic property of modified LDL is its immunogenicity. MDA-modified LDL has been shown to stimulate formation of autoantibodies, and immune complexes of LDL aggregates are efficiently internalized by macrophages via Fc receptors *[32]*. This could promote further cholesterol accumulation.

Several lines of evidence support the in vivo existence of Ox-LDL *[2–6]*. LDL extracted from human atherosclerotic lesions exhibits many immunological, physicochemical, and biological properties of LDL oxidized in vitro, such as cross-reactivity with antibodies to MDA–lysine conjugates, presence of oxidized lipid and apo B fragments, increased electrophoretic mobility, increased uptake by macrophages, and chemotactic activity towards monocytes *[5]*. Oxidatively modified apo B has also been isolated from plasma of healthy subjects and patients with atherosclerosis *[33]*. Antibodies against epitopes on Ox-LDL recognize material from atherosclerotic lesions but not from healthy arteries *[34]*. Ox-LDL has also been demonstrated in plasma of Watanabe heritable hyperlipidemic rabbits and humans *[34]*. The presence of autoantibodies against Ox-LDL has been positively correlated with the progression of atherosclerosis, as manifested by carotid artery stenosis *[35]*. Also, the oxidative susceptibility of LDL varied with severity of coronary atherosclerosis as evaluated by angiography *[36]*. The oxidative susceptibility of LDL appears to be increased with established coronary artery disease risk factors such as diabetes, smoking, hypertension, and hyperlipidemia *[37–40]*. Finally, antioxidants such as probucol, α-tocopherol, butylated hydroxytoluene (BHT), and N,N′-diphenyl phenylenediamine have been shown to decrease the degree of LDL oxidation and atheromatous lesions in animal models of atherosclerosis.

Measurement of LDL Oxidation

The evaluation of LDL oxidation in vivo is fraught with difficulties. One of the main problems is that lipoprotein oxidation is likely to occur in the mileu of the artery wall, rather than in the general circulation. Even if some lipoproteins are oxidized in the circulation, the concentrations of these modified lipoproteins may be difficult to detect and may not reflect the extent of oxidation occurring in the arterial wall. Also, extensively modified lipoproteins are rapidly cleared from the circu-

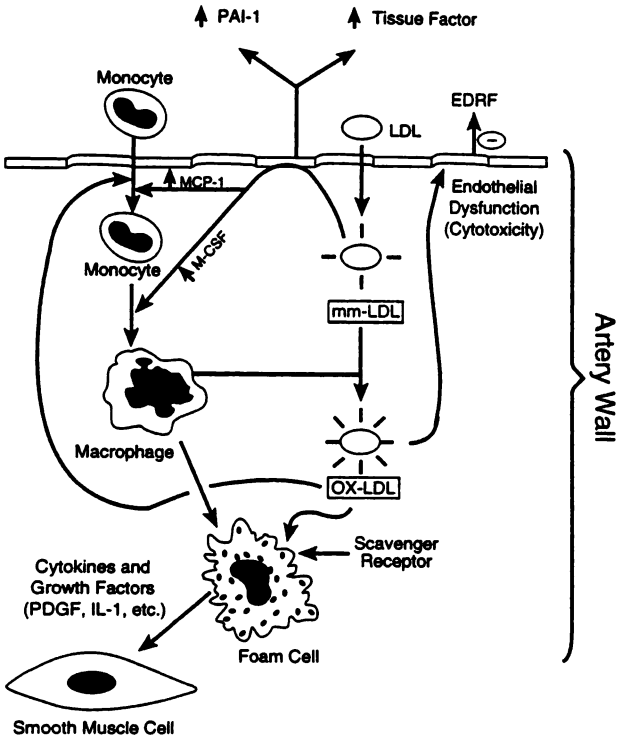

Fig. 3. Schema depicting role of Ox-LDL in atherogenesis.

lation by scavenger receptors, and therefore their residency in the plasma may be too short-lived and the concentrations too low for easy measurement. In experimental animals, samples of arterial tissue can be obtained to examine the amount of oxidative modification; however, in humans, limited samples (blood, urine, and expired air) are available. In view of the increasing interest in the role of LDL oxidation in the pathogenesis of atherosclerosis, there is clearly a need for improved methods to evaluate lipoprotein oxidation, especially in vivo, particularly at extravascular sites. Because of the difficulties encountered in obtaining tissue samples in humans, several indirect measures of lipoprotein oxidation and antioxidant potential in vivo must be used and are discussed below. Recently, we described various measures of quantifying LDL oxidation *[41]*.

LDL is isolated from plasma in EDTA (1 g/L) either by sequential ultracentrifugation in NaBr solutions *[42]* or by rapid vertical spin gradient ultracentrifugation *[43]*. The isolated LDL is extensively dialyzed agianst NaCl-EDTA, pH 7.4, filtered, and stored at 4 °C after purging with nitrogen. To eliminate EDTA before the oxidation experiments, LDL is dialyzed overnight against two changes of phosphate-buffered saline (PBS) at pH 7.4, in the dark at 4 °C or passed through a Sephadex G-25 column (Pharmacia, Piscataway, NJ). For oxidation of LDL by copper, filtered LDL (200 mg/L) is incubated at 37 °C in PBS for 8 h in a time-course experiment, with 5 μmol/L copper. Oxidation is stopped at the various time points by BHT-EDTA, followed by refrigeration.

CONJUGATED DIENES

One of the most widely used methods for monitoring LDL oxidation in vitro has been the measurement of CD *[44]*. This method is rapid and easily performed. Oxidation of PUFA side chains of LDL is accompanied by the formation of dienes that absorb ultraviolet light at 234 nm. Since Ox-LDL remains fully soluble in buffer, the increase of 234-nm diene absorption can be measured directly in solution, without extraction of LDL lipids. The typical time course of copper-mediated LDL oxidation (Fig. 2) shows a lag phase, in which diene absorption shows only a slight increase, followed by a propagation phase in which 234-nm absorption rapidly increases. In succession, the 234-nm absorption decreases, then increases again in the decomposition phase, because the aldehydes formed also absorb in the 210–240-nm region. Currently, this appears to be the best index of LDL oxidizability and is clearly the most popular.

SPECTROPHOTOMETRIC ASSAY FOR LIPID PEROXIDES

This rapid and simple method is based on the oxidation of iodide to iodine by lipid peroxides formed during oxidation of LDL *[45]*. However, the disadvantage is that the lower detection limit is similar to the reagent blank and the method measures other peroxides as well.

Another iodometric assay deals with the problem of specificity by hydrolyzing esterified lipids and extracting them with ethyl acetate before determination of their hydroperoxide content *[46]*. The ferrous ion oxidation assay *[47]*, in which ferrous ions are oxidized in the presence of xylenol orange, also seems to be relatively specific for lipid peroxides. The most specific and sensitive assay for determination of lipid peroxides in biological fluids is HPLC with isoluminol chemiluminescence detection *[48]*. However, this assay is time consuming and is not readily adaptable to the clinical laboratory setting.

THIOBARBITURIC ACID-REACTIVE SUBSTANCES (TBARS) ASSAY

The most commonly used assay in LDL oxidation studies, both in the presence and absence of cells, is the TBARS test *[49]*. In this test, the chromogen is formed by the reaction of one molecule of MDA with two molecules of TBA. The method involves heating the sample with TBA under acidic conditions and reading the absorbance of the MDA-TBA adduct formed at 532 nm. This test is not specific for MDA, since sugars and amino acids may also form TBA adducts; furthermore, a significant amount of peroxides are formed during the heating step of the assay. Various modifications of the TBA test have been proposed, involving differences in sample treatment, acid concentration, heating time, and presence or absence of antioxidants.

The spectrophotometric test of TBARS *[50]* includes precipitation of protein with trichloroacetic acid. The assay is conducted in the presence of BHT-EDTA to minimize peroxide formation due to heating.

To increase sensitivity, the MDA-TBA adduct can be extracted into an organic solvent (butanol) and measured fluorometrically *[51]*. Although this test is widely used to assess lipid

peroxidation, it lacks specificity and should not be the only measure used.

RELATIVE ELECTROPHORETIC MOBILITY

LDL has a negatively charged surface and migrates to the anode in agarose gel electrophoresis under nondenaturing conditions. Oxidation renders LDL more negatively charged, possibly because of derivatization of lysine residues of apo B-100 by some reactive aldehydes formed during oxidation, and accordingly its electrophoretic mobility increases (Fig. 2). Another possibility for the increase of negative charge on LDL during oxidation is that reactive oxygen species generated convert histidine and proline residues to negatively charged aspartate or glutamate. A measurable index of this is the relative electrophoretic mobility, which is the ratio of migration distance of oxidized to native LDL. This is a very reliable way to quantify LDL oxidation in vitro but it clearly lacks the sensitivity of an in vivo test. Other aldehydic modifications will also alter the electrophoretic mobility of LDL.

APO B-100 FLUORESCENCE

The oxidative modification of LDL also generates fluorophores, which fluoresce strongly at 430 nm with excitation at 360 nm, owing to derivatization of apo B-100 lysine residues by reactive aldehydes *[52]*. A typical time course of apo B fluorescence is shown in Fig. 2. This assay has the same problems as discussed for measuring electrophoretic mobility. Although it is a very reliable index of protein modification of LDL during oxidation, the assay is not sensitive enough to measure basal LDL oxidation.

FATTY ACID CONTENT

Since oxidative modification of LDL is essentially a free radical-mediated process involving oxidation of PUFAs in LDL, measurement of the fatty acid content could be an indication of the oxidative susceptibility of the LDL particle. About half of the fatty acids in LDL are PUFAs, mainly linoleic acid and minor amounts of arachidonic and docosahexaenoic acids. Dietary habits confer a large degree of interindividual variability in the LDL fatty acid composition. Therefore, it is useful to monitor the disappearance of these three main fatty acids. Fatty acids are measured by gas chromatography (GC) after extraction and transmethylation *[53]*. However, this instrumentation is not generally available in clinical laboratories and the method is very time consuming.

ALDEHYDES

It has been proposed that aldehydes such as MDA or HNE, generated by lipid peroxidation from PUFAs in LDL, interact with apo B and specifically modify lysine residues *[54]*. For measurement of aldehydic lipid peroxidation products, LDL is derivatized with dinitrophenyl hydrazine, and the hydrazones are extracted with dichloromethane, separated by thin-layer chromatography, and analyzed by HPLC with an ODS column (e.g., Ultrasphere absorbance Spherisorb column; Waters, Milford, MA) and eluted with acetonitrile: water (9:10, by vol). The effluent is monitored at 223 nm and HNE can be identified *[55]*. This is a good measure of LDL oxidation but is not easily adaptable to the routine laboratory.

OXYSTEROLS

Oxidative modification of LDL can also be assessed by measuring oxidation products of cholesterol, oxysterols *[56]*. Although several are produced, 7-ketocholesterol has been identified as the main oxysterol produced during copper-catalyzed and cell-mediated oxidation of LDL and can be measured by GC or GC–mass spectrometry (MS) methods. Measurement of oxysterols in human plama can become a test of LDL oxidation. However, not much data have been reported in this regard.

F_2-ISOPROSTANES

It was recently discovered that a series of structurally unique prostaglandin F_2 (PGF_2)-like compounds (F_2-isoprostanes) are produced in vivo in humans by a noncyclooxygenase mechanism involving free radical-catalyzed peroxidation of arachidonic acid. Of these, 8-epi-PGF_2-α, the major component, is a potent vasoconstrictor *[57]*. The release of PGF_2 is increased in LDL oxidized by macrophages, endothelial cells, or copper and can be measured by a solid-phase extraction procedure, followed by GC-MS *[57]*.The formation of F_2-isoprostanes is induced in plasma and LDL exposed to oxidative stress in vitro *[58]*. Also, as was recently shown, F_2-isoprostanes and their metabolites are increased in plasma and urine of smokers *[59]*. However, although this is a measure of LDL oxidation, measurement in urine reflects whole-body oxidation rather than LDL oxidation. Since this novel method offers a lot of promise, research should be directed at developing a plasma assay that can be adapted to the clinical laboratory.

Specific fluorescence patterns can be produced when certain amino acids react with lipid peroxides. Previously, dityrosine fluorescence was shown to be associated with oxidation of linoleic acid *[60]*. Phagocytes generate myeloperoxidase to kill invading bacteria; this may convert tyrosine to a radical catalyst that cross-links proteins. The stable oxidized product of the tyrosyl radical is dityrosine; its stability and intense fluorescence may allow it to also act as a marker for oxidatively damaged proteins in lesions. This method could prove useful in evaluating the role of specific protein modifications that occur during lipoprotein oxidation.

Antioxidant Status

ANTIOXIDANTS AND LDL OXIDATION

The antioxidant content of LDL is critical for its protection. In theory, if sufficient lipophilic antioxidants were present, LDL would be protected from even profound oxidant challenge. The balance between the prooxidant challenge and the presence of antioxidants determines the extent of arterial wall modification of LDL. Antioxidants such as probucol, *N,N'*-diphenyl phenylenediamine, and BHT have been shown to decrease the degree of oxidation and the extent of atheromatous lesions in animal models of atherosclerosis *[61]*, but have side effects. Thus, dietary antioxidants such as α-tocopherol, β-carotene, and ascorbic acid become attractive alternatives. We have reviewed

extensively in previous reports the role of antioxidants in relation to atherosclerosis [5].

α-Tocopherol. α-Tocopherol (vitamin E) is the principal lipid-soluble antioxidant in plasma and in the LDL particle [62]. It is a chain-breaking antioxidant and traps peroxyl free radicals. Several studies have associated low α-tocopherol concentrations with the development of atherosclerosis. A cross-sectional study of 16 European populations has shown a significant correlation between α-tocopherol concentrations and mortality from coronary artery disease [63]. A previous study has shown an inverse correlation between plasma vitamin E concentrations and the risk of angina pectoris [64]. Vitamin E supplementation has been shown to reduce the risk of coronary artery disease in men and women [65, 66], but not in middle-aged smokers from Finland who were receiving 50 mg/day of α-tocopherol for 5 years. The failure of the Finnish study may be attributed to the long period during which the study population was at risk, the relatively late time point when supplementation was initiated, and probably too low a dose of vitamin E administered to inhibit LDL oxidation. Some animal studies [67, 68] have also shown that dietary α-tocopherol can retard the progression of atherosclerosis.

α-Tocopherol has been shown to inhibit LDL oxidation in vitro, and supplementation of human volunteers with α-tocopherol has been shown to decrease the susceptibility of their LDL to oxidation [5]. A recent dose–response study shows that at least 400 IU/day is required to significantly decrease the susceptibility of LDL to oxidation [69].

α-Tocopherol may also have additional benefits for cardiovascular disease. α-Tocopherol, alone or in combination with ascorbate and β-carotene, has been shown to reduce platelet adhesion [70]. Physiological concentrations of α-tocopherol also inhibit smooth muscle proliferation, protein kinase C activity [71], and agonist-induced monocyte adhesion to cultured human endothelial cells [72]. Low-dose supplementation with α-tocopherol has been shown to preserve endothelium-dependent vasodilation in hypercholesterolemic rabbits [73].

Ascorbate. Ascorbate (vitamin C) is a water-soluble, chain-breaking antioxidant that regenerates α-tocopherol from its chromanoxyl radical form [74]. Low plasma and tissue concentrations of ascorbate have been identified as a risk factor for atherosclerosis. Plasma ascorbate has been shown to be inversely correlated with coronary disease mortality [61]. Further, the concentrations of ascorbate in atheromatous aortas are lower than in control vessels [75]. Smokers, diabetics, and patients with coronary artery disease all have lower concentrations of plasma ascorbate [76–78].

Physiological concentrations of ascorbate can inhibit LDL oxidation by copper or cultured macrophages [79], and can also inhibit oxidation by activated neutrophils or U937 cells or AAPH [80]; both systems lack metal catalysts. Dietary ascorbate supplementation has been shown to prevent LDL oxidation induced by acute cigarette smoking [81].

β-Carotene. β-Carotene, a hydrophobic member of the carotenoid family, is carried in the blood, mainly in LDL. Esterbauer et al. [62] have shown that carotenoids provide auxiliary antioxidant defenses with respect to LDL after α-tocopherol. Jialal et al. [82] have shown that properly dissolved β-carotene can inhibit LDL oxidation in vitro induced by copper or by macrophages. Preincubation of cocultures of endothelial cells and smooth muscle cells with β-carotene prevented LDL modification and its induction of monocyte transmigration [83]. The supplementation studies with β-carotene have been disappointing with respect to the protection of LDL from oxidation, in contrast to the studies with α-tocopherol.

Other antioxidants. Flavonoids are plant-derived compounds that inhibit in vitro copper-catalyzed, ultraviolet-induced, macrophage-mediated LDL oxidation [84, 85]. The inhibition of oxidation of human LDL by consumption of red wine or tea has been attributed to the presence of antioxidants such as flavonoids and other polyphenols in red wine and catechin in tea. Ubiquinol-10 is another effective lipid-soluble antioxidant that inhibits LDL oxidation due to aqueous or lipid-phase peroxyl radicals [86]. However, further studies, especially in humans, are required to validate the role of these antioxidants in inhibiting LDL oxidation.

MEASUREMENT OF ANTIOXIDANTS

A good measure of the antioxidant capacity of LDL can be derived from its antioxidant content. The lag phase of oxidation is directly proportional to the antioxidant content of LDL and is related by the equation $y = a + kx$, where y = lag time in minutes, a = other antioxidants present in LDL such as β-carotene or ubiquinol, k = efficiency constant of α-tocopherol, and x = amount of α-tocopherol (mol/mol) in the LDL [6]. Measurement of α-tocopherol, retinol, and five carotenoids (lutein, cryptoxanthin, lycopene, and α- and β-carotene) can be performed by reversed-phase HPLC. Simultaneous determination of these antioxidants is possible after ethanol precipitation and hexane extraction of plasma or LDL. The hexane phase is evaporated, reconstituted in ethanol, passed on a HPLC reversed-phase C_{18} column, and eluted with acetonitrile: dichloromethane:methanol (70:20:10 by vol) [87]. Retinol is measured at 325 nm, α-tocopherol at 292 nm, and the carotenoids at 450 nm.

Another index of the antioxidant status of plasma is the total radical-trapping antioxidant parameter [88]. This is a total estimate of antioxidants present in plasma, such as ascorbate, urate, sulfhydryls, and α-tocopherol. The activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase is also determined. However, this assay does not give information on the individual antioxidants.

Other Measures of Oxidation

Another potential way to evaluate lipoprotein oxidation is by measurement of autoantibodies against epitopes on oxidized LDL [89] by ELISA. A recent study has shown that the titer of these antibodies is an independent predictor of the progression

of carotid atherosclerosis in patients with accelerated atherosclerosis *[90]*.

Nuclear magnetic resonance analysis of oxidized lipoproteins *[91]* could provide interesting details concerning the structural aspects of these modified lipoproteins. Breakdown products of lipid peroxides are exhaled in breath as volatile hydrocarbons. The volatile oxidation products of n-6 and n-3 fatty acids, pentane and ethane, appear in breath and can be measured by GC *[92]*. This method is sensitive and noninvasive; however, it is tedious and one must purify the inspired air because of the high background created by exogenous sources such as motor vehicles and cigarette smoke. Also, it is a measure of whole-body oxidation and not LDL oxidation specifically.

In conclusion, it has become increasingly evident that oxidation of LDL is a key step in atherogenesis. Although there are various ways to measure oxidative modification of LDL, each method has its limitations. The easiest measure of oxidation that can be adapted to the clinical laboratory is the measurement of CD by continuous absorption spectrophotometry. This can be monitored during copper-catalyzed oxidation of isolated LDL. The measurement of isoprostanes and ethane and methane in breath will provide an index of whole-body oxidation.The most direct test so far that documents oxidation, e.g., LDL oxidation, is the demonstration of increased titers of autoantibodies to Ox-LDL. Thus, there is still need for a single rapid and specific measure of LDL oxidation that could become part of the laboratory repertoire in the diagnosis and management of atherosclerosis.

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