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Implications of Lag Time Concept in the Oxidation of LDL

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Oxidation of low density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis. The most common technique for measuring the oxidation of lipoproteins is the continuos measurement of the formation of conjugated diene at OD 234 nm. The concept of "lag time", derived from such measurements, has been used to test the efficacy of various antioxidants for their ability to inhibit the oxidation of LDL. This review will elaborate on some of the factors that might affect the lag time.

Keywords: Oxidized LDL, atherosclerosis, conjugated diene, antioxidants

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidized low density lipoprotein

The eighties saw major advances in biomedical sciences and scientists are now equipped with powerful techniques to solve problems associated with debilitating diseases that affect mankind. Advances in molecular biology not only have led to a better understanding of the etiology of a number of diseases including cancer, AIDS, etc. but also have made gene therapy realistic and feasible. Yet, basic lipid chemistry and biochemistry, an area that lost its luster and glory in the 70s and early 80s, proved much more versatile and valuable in solving the mystery of atherosclerosis, a major manifestation of coronary artery disease. Prof. Esterbauer played a major role in elucidating the lipid oxidation pathway that has withstood a number of challenges. His concept of "lag time" of the oxidation of low density lipoprotein (LDL) is a standard by which antioxidants are discovered, evaluated, and compared. Unfortunately despite his long list of contributions to oxidative studies his lifetime was short. We would like to dedicate this article in his memory.

IS OXIDATION A RISK FACTOR?

Much of the progress made in our understanding of atherosclerosis can be traced to the identification of not one, but several, major risk factors.[1] These include elevated plasma cholesterol, diabetes, hypertension, and smoking. In addition to

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these, increased plasma Lp(a) levels, lack of physical activity, elevated plasma homocysteine levels, may also affect the progression of the disease. While these are factors that can be measured and quantified, the concept of "oxidative stress" has emerged as a novel potential risk factor for cardiovascular disease.[1] Despite the fact that it is not yet measurable or comparable among subjects, there are more studies that attempt to understand the impact of other risk factors on the basis of oxidation. Oxidation may be one of the basis for the initiation and the development of the atherosclerotic lesion, but oxidation as a risk factor remains to be established.

OXIDIZED LDL AND ATHEROSCLEROSIS

Plasma cholesterol, particularly that associated with LDL has been suggested to be an important risk factor in the development of coronary artery disease.[2] The cholesterol that accumulates in the atherosclerotic plaque is localized as cytoplasmic lipid droplets in macrophages and is derived predominantly from plasma LDL, which is internalized by cells usually via the LDL receptor.[2] However, animals and humans that lack the LDL receptor develop severe forms of atherosclerotic lesions, and *in vitro* incubations of macrophages with LDL failed to develop lipid engorged foam cells. These observations prompted Goldstein and Brown to propose the modified LDL hypothesis, which, simply stated, suggests that LDL has to undergo some type of modification before it could be internalized by macrophages via alternate receptors.[3] These alternate pathways are commonly referred to as scavenger pathways, and a number of scavenger receptors have now been identified.[4–7]

Acetylation to the degree required for scavenger receptor-mediated uptake does not occur *in vivo*, nor was such a suggestion ever made. Fogelman and associates, realizing that the formation of Schiff bases involving the amino groups of lysines and malondialdehyde (MDA), a product of lipid peroxidation, would also result in the acquisition of a net negative charge, generated MDA modified LDL and found that the modified lipoprotein was readily taken up and degraded by macrophages.[8] Fogelman and associates concluded that MDA could be generated *in vivo* during the aggregation of platelets (from the degradation of thromboxanes) or via lipid peroxidation.

A plethora of studies has suggested that oxidized LDL may be the one of such modified lipoproteins that may be physiologically important.[9] Early studies by Steinberg and associates showed that LDL incubated with endothelial cells or other cell types is internalized avidly by macrophages in contrast to LDL that was not previously exposed to cells.[10,11] They demonstrated that during this incubation, large amounts of decomposition products of lipid peroxidation are generated and antioxidants such as vitamin E, BHT, and probucol inhibited such generation of oxidized LDL.[9–12] Other investigators also showed that such modified LDL could be generated by *in vitro* oxidation of LDL using a number of different oxidation systems. Based on these observations, it was suggested that oxidized LDL might represent a biologically relevant modified lipoprotein. Currently, a number of proatherogenic properties have been attributed to both lipid and protein components of oxidized LDL.[9] There is also an overwhelming sentiment among scientists that cellular oxidation reactions, in general, are deleterious and that antioxidants may slow the cellular signaling events that mark the biochemical events accompanying the disease process (Table I).

Heinecke *et al.*[13] described that modification of LDL by smooth muscle cells also was related to lipid peroxidation. Most of the earlier studies were focused on the generation of a modified LDL by an oxidative process that resulted in an increased uptake by macrophages. Since these initial studies, a number of cell types including, fibroblasts, neutrophils, monocytes,

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TABLE I Biological effects of oxidized LDL

1	Chemotactic for human T lymphocytes, monocytes/macrophages, arterial smooth muscle cells etc.
2	Activates phospholipase D
3	Inhibits endothelium-dependent relaxation of aorta
4	Native and ox-LDL enhance production of PDGF receptors in human smooth muscle cells
5	Causes DNA fragmentation and apoptosis of lymphoblastoid cells
6	Leads to delayed and sustained rise of cytosolic calcium in bovine aortic endothelial cells
7	Increases adhesive properties of endothelial cells
8	Induces MAP kinase pathway, ceramide production
9	Enhances procoagulatory activity of human monocytes/macrophages, *in vitro*
10	Reduces pinocytic activity in cultured endothelial cells
11	Inhibits migration of aortic endothelial cells
12	Delays endothelial wound healing causing dysfunction
13	Induces cytotoxicity and causes endothelial and aortic smooth muscle cell injury
14	Induces apoptosis in cultured smooth muscle cells and endothelial cells
15	Stimulates collagen production, MAP kinase pathway, ceramide production
16	Stimulates growth of smooth muscle cells
17	Increased uptake and deposition of cholesteryl esters by macrophages and induces foam cell formation
18	Enhances platelet aggregation
19	Cause spasms of the aorta and has arrythmogenic effects
20	Stimulates the growth of macrophages
21	Inhibits L-CAT: reverse cholestrol transfer mechanism
22	Increases tissue factor expression
23	Stimulate plasminogen activator inhibitor, stimulates G proteins
24	Suppress activation of NF kappa B in the macrophages,
25	Suppress endothelin-1 secretion
26	Induces Heat shock proteins
27	Decreases the expression of nitric oxide synthase in human platelets
28	Stimulates prostacyclin production

macrophages, and others have been shown to oxidize LDL. However, serious doubts remain whether most of these cell types are indeed capable of initiating the oxidation of LDL. Even though it was generally assumed that the actual uptake process was due to the modification of the apoprotein, it was not until several years later that studies by Parthasarathy *et al.*[14] using lipid-free, solubilized apoprotein established that the protein moiety was responsible for the recognition by macrophages. For this reason most of the earlier studies referred the oxidized LDL as oxidatively modified LDL. The term "modification" which originally referred to protein alterations, in later years included lipid alterations as well. Now modified LDL may simply refer to any LDL particle that has properties different from that of native, plasma LDL.

THE CONJUGATED DIENE

One of Esterbauer's pioneering contributions to the study of oxidized LDL is the technique of measurement of oxidation of LDL on a continuous basis. Unsaturated fatty acids have 1,4-pentadienyl system of double bonds and when they undergo oxidation there is a migration of double bonds with the formation of a 1,3-butadienyl system of conjugated double bonds. The conjugated double bonds have a characteristic absorption maximum at around 234 nm. Esterbauer utilized this property to monitor the progression of oxidation by continuously recording the increasing absorption at 234 nm. Esterbauer also utilized the free radical chemistry of oxidation of unsaturated fatty acids and correctly surmised that as long as the LDL is protected by antioxidants, the rate of increase at 234 nm or the rate of formation of conjugated dienes will be very low.[15] Upon depletion of antioxidants the rate is expected to increase in proportion to the initiating radicals and when all the substrates, that is polyunsaturated fatty acids, have been utilized, the rate is suggested to plateau. Graphically this will be represented by the curve as shown in Figure 1, for the copper-mediated oxidation of LDL. The point at which the initial slope representing the initiation rate and the point at which the propagation slope intersect was arbitrarily chosen to depict the time point at which the antioxidant are depleted from LDL. Esterbauer designated this point as "lag time", which has formed the basis of over 200 scientific publications. Although initially the focus was on the content of antioxidants associated with LDL in determining the lag time,

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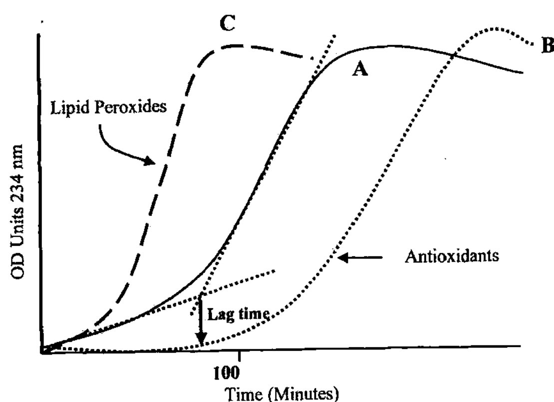

FIGURE 1 Oxidation of LDL by copper. A. Oxidation of LDL by copper system. B. Inhibition of oxidation by antioxidants as indicated by a greater lag time. C. Lipid peroxides increase the oxidation of LDL thereby decreasing the lag time.

TABLE II Systems that oxidize LDL *in vitro*

Addition of micromolar amounts of copper or iron
Addition of free radical generators, such as AAPH, AMVM
Hypochlorous acid
Peroxynitrite
UV radiation
Photooxidation
Hemin, heme, and other iron containing proteins
Ceruloplasmin
Various peroxidases
Lipoxygenase
Cholesterol oxidase and other miscellaneous oxidases
Cell systems that may contain more than one oxidative enzyme system

currently a number of other parameters have been designated to contribute to the lag time. Also, the concept of lag time has been extended not only to the formation of conjugated dienes, but also to the formation of thiobarbituric acid products, modification of the apoprotein, and a number of other parameters related to the oxidation of LDL. A number of parameters have been known to contribute to the oxidation of LDL *in vitro*. Before one goes further, I would like to remind the readers that neither the factors that contribute to the oxidation of LDL nor the factors that initiate the oxidation of LDL *in vivo*, have yet been determined.

Systems that are capable of oxidizing LDL *in vitro* are listed in Table II. These systems are not alike, either in the ability or in the mechanisms by which they oxidize LDL. For example, the mechanism by which copper initiates the oxidation of LDL is still unknown. It is generally believed that preexisting peroxides react with copper and generate peroxy radicals that initiate further oxidation of LDL. However, it is the experience of several investigators that, even LDL that is very carefully isolated, is readily oxidized by copper even when there is no evidence of peroxides associated with LDL. Furthermore, a number of investigators have documented the presence of binding sites for copper on the apoprotein molecule, which may play a role in initiating oxidation. For example, it has been suggested that thiol groups associated with the apoprotein may initiate the formation of radicals in the presence of copper.[16,17] This may explain why copper is able to initiate the oxidation of high-density lipoprotein also. While it was believed that even minute quantities of serum may be able to prevent the oxidation of isolated LDL, recent studies suggest that copper could effectively initiate the oxidation of diluted serum.[18]

In contrast to the oxidation of LDL by copper, oxidation by free radical generators such as AAPH, AMVN, etc are fairly straightforward. These radical initiators generate peroxy radicals at a constant rate that readily initiate the oxidation of LDL. The mechanism by which peroxynitrite could initiate the oxidation of LDL is also subject to speculation. Hydroxyl radical generated from peroxynitrite may initiate the oxidation of LDL. Studies using butylated hydroxy toluene and superoxide dismutase have been shown to inhibit such reactions.[19,20] Other mechanisms are also feasible that might depend on the generation of tyrosine radical on protein or even direct action of peroxynitrite on lipids generating a lipid radical.

Some of the antioxidants that have been shown to decrease lag time are shown in Table III.

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TABLE III Antioxidants that increase the lag time of isolated LDL

1	Vitamin E and other tocopherol derivatives
2	Probucol and its derivatives
3	Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and other diisopropyl phenols
4	Ascorbic acid
5	Lipoic acid
6	Spin traps such as phenylbutylnitrone (PBN)
7	21-Aminosteroids (Lazaroids.)
8	RU 486, Onapristone and other synthetic steroids
9	Estradiol and related compounds
10	Phytoestrogens
11	Tamoxifen, raloxifene
12	Nitric oxide donors
13	β-Carotene and other carotenoids
14	Thiols and other thiol derivatives, such as captopril
15	Dithiocarbamates
16	Aminoguanidine
17	Miscellaneous amino acids
18	Quercetin
19	Smoke extracts
20	Dehydroepiandrasterone
21	Dipyridamole
22	Diphenylphenylenediamine
23	Curcumin, caffeic acid, and other plant phenols
24	Fibric acid derivatives
25	HMG-CoA reductase inhibitors
26	Ferritin
27	Monounsaturated fatty acids, plasmalogens
28	Carvedilol, Nifedipine, and other antihypertensive agents
29	EDTA and other metal chelators
30	Catechins and other green tea constituents

While the oxidation of LDL by copper or many of the *in vitro* oxidation systems may be readily inhibited by antioxidants, one of the systems that has gained enormous importance *in vivo* is the oxidation of LDL by peroxidase. Studies by Weiland, Parthasarathy and Steinberg were the first to suggest that peroxidase such as horseradish peroxidase might be able to initiate the oxidation of LDL.[21] They observed that incubation of LDL either in the presence of pre-existing lipid peroxide or hydrogen peroxide together with horseradish peroxidase resulted in the formation of a LDL species that possessed increased electrophoretic mobility and was avidly degraded by macrophages. On the basis of the observation that a direct action of peroxidase failed to initiate the oxidation of lipids, they

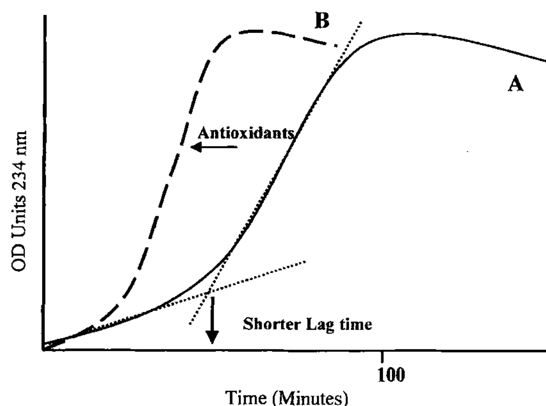

FIGURE 2 Oxidation of LDL by peroxidases. A. Oxidation of LDL by peroxidase system. B. Activation of oxidation by low concentrations of antioxidants thereby shortening the lag time.

suggested that antioxidants such as vitamin E might play an intermediate role in the oxidation of LDL. Savenkova *et al.*, demonstrated that myeloperoxidase (MPO), an enzyme present in neutrophils and monocytes is capable of oxidizing LDL in the presence of tyrosine.[22] They also suggested that intermediate formation of tyrosine radical is an obligatory requirement in the oxidation of LDL. These studies could be interpreted to suggest that antioxidants that are simple phenols might fail to protect LDL against oxidation by peroxidase by actually forming intermediates in the oxidation of LDL. This is not surprising as Stocker and associates demonstrated that vitamin E radical may be intermittently associated with propagation of oxidation within the LDL particle thus assuming a prooxidant role.[23] Studies by Santanam and Parthasarathy also suggested that in the presence of peroxidase, simple phenols assume a paradoxical prooxidant role as opposed to the traditional antioxidant role.[24] This is not an exclusive property of phenols as even amino compounds, such as diphenylphenylenediamine (DPPD) shortened the lag time of oxidation (Figure 2). Substituted phenols or phenols in which the phenoxy radicals are buried by other larger groups, failed to promote the oxidation of LDL, suggesting that the simple phenoxy radicals may

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readily react with the lipid that is generating a lipid radical. These results suggest that the concept of lag time might provide a greater insight in accessing the ability of LDL to undergo oxidation in the presence of peroxidase, that is, LDL that is deficient in antioxidants may actually undergo a less rapid oxidation as opposed to LDL that is enriched with an antioxidant.

In addition to antioxidants, the peroxide content of LDL might play major role in determining lag time of oxidation. Studies by O'Leary and associates showed that the addition of free fatty acid peroxides or increase in the concentration of LDL associated peroxides, greatly reduced the lag time.[25,26] Such an observation was also confirmed by the study of Santanam and Parthasarathy.[24,27] This could be interpreted to suggest that the presence of lipid peroxides may react with copper generating peroxy radicals thereby consuming the antioxidant at a greater rate as compared to LDL that is deficient in initial peroxides.[27] Thus, not only the antioxidant content, but also the pre-existing lipid peroxides play a major role in determining the lag time of a given LDL to undergo oxidation. Higher the peroxide content of the LDL, greater is its susceptibility to undergo oxidation and shorter is the lag time (Figure 1). Consequently, LDL that is pretreated with enzymes, such as glutathione peroxidase or its mimic, ebselen is expected to undergo slower rate of oxidation with a greater lag time.[28–30] In contrast, LDL that was seeded with peroxide, for example, by cells that are enriched in 15-lipoxygenase reaction, is oxidized more rapidly as compared to LDL that was incubated with control cells.[31] This concept was recently put to test in studies by Wetzstein *et al.* who showed LDL isolated from athletes who consumed daily supplements of beta carotene was oxidized at the same rate as LDL isolated from un-supplemented exercisers.[32] However, when these two groups were subjected to acute bouts of exercise, the LDLs isolated from those who were supplemented with beta carotene were more resistant to oxidation as compared to LDL samples isolated from control subjects. Since the content of vitamin E was similar in both subjects, the results were interpreted to suggest that the presence of beta carotene prevented the formation of initial peroxides thereby increasing the lag time of oxidation as compared to those who did not consume beta carotene. In these controlled subjects the acute bout of exercise was suggested to initiate oxygen radical formation and seed LDL with peroxides as a result of such oxidative stress. Since the LDL was isolated from plasma it was also assumed that the initial seeding of peroxides were lipid peroxides in nature and not free hydrogen peroxides or other radicals.

The ability of preexisting peroxide to modulate the anti- and pro-oxidant nature of thiols is of great interest. Previous studies by Parthasarathy,[16] Sparrow and Olzewski,[17] and by Heinecke *et al.*[33] showed that the addition of thiols greatly enhanced the oxidation of LDL. These studies were extended to suggest that cells might oxidize LDL by a thiol-dependent mechanism. However, more recent studies by Santanam and Parthasarathy provided evidence to suggest that in fresh LDL that contained very little peroxides, thiols, as expected, were powerful antioxidants and inhibited the oxidation of LDL.[27]

While antioxidants and preexisting lipid peroxides may contribute to the shortening or lengthening of lag time, the fatty acids themselves play a major role in determining the susceptibility of LDL to oxidation. Studies by Parthasarathy, Reaven and associates have shown that LDL enriched in monounsaturated fatty acids was resistant to oxidation as opposed to LDL enriched in polyunsaturated fatty acids.[34,35] This not only reflected in the overall decrease in the amount of conjugated diene formed, but also in apparent increase in lag time suggesting that lipid peroxy radicals themselves are efficient in propagating the oxidation of LDL. This propagation was shown not only to result in the oxidation of another fatty acid but also in the oxidation of another double bond within the

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same fatty acid molecule. Such a reaction has not been described before and Fruebis, Parthasarathy and Steinberg described the superoxide generating reaction in the presence of lipophilic amino compounds which resulted in the oxidation of another double bond, that is, intermolecular propagation resulting in the formation of fluorescence adducts involving amino groups of proteins or other lipophilic compounds.[37] Such a reaction would explain the ability of superoxide dismutase to quench the oxidation process as observed by many investigators. This will also explain the formation of fluorescence compounds other than dityrosine during the oxidation of LDL by copper.

As explained earlier, the oxidation of LDL by peroxidase may involve utilization of antioxidants such as vitamin E or tyrosine or other amino acids to initiate lipid peroxidation. As one would expect, the lag time measured by the increase in conjugated diene formation during the peroxidase catalyzed oxidation of LDL would be much shorter as compared to the oxidation of LDL by copper. Consequently, addition of low amounts of antioxidants that would further generate radicals would increase the rate of oxidation and further diminishing of lag time as anticipated. However, when the concentration of antioxidants would exceed to interact with fatty acid radical there will be a separation of oxidation as expected. Furthermore, when the antioxidant radicals abound, self quenching is also expected as might be the case in the oxidation of LDL by MPO in the presence of tyrosine. As Santanam and Parthasarathy demonstrated, the addition of tyrosine to LDL in the MPO catalyzed oxidation of LDL, enormous amounts of dityrosine were formed but as expected there was a complete inhibition of the formation of conjugated dienes.[24]

MPO protein and products of MPO reaction have been reported in the atherosclerotic artery[37–39] and there is an overwhelming sentiment among scientists that MPO may be involved in the oxidation of LDL *in vivo*. If such were the case, one has to be cautious in the interpretation of studies involving "antioxidant supplementation" as a prevention of atherosclerosis. While animal studies might validate the oxidation hypothesis, these studies also utilize mega doses of antioxidants, often exceeding the concentration of cholesterol in the diet. The plasma concentrations of antioxidants are also substantially high under these conditions. In humans, such doses are often not feasible and transport systems, such as those responsible for the secretion of vitamin E might prevent the availability of large concentrations of antioxidants in the plasma. Under such conditions, it would be impossible to predict the concentration at which prooxidant nature changes into antioxidant benefits.

A number of investigators have attempted to use the formation of conjugated diene as a simple measurement of the amount of oxidized lipids present in LDL. However, such measurements are of questionable significance as a number of chemicals and drugs can absorb at 234 nm and a simple measurement at one wavelength at any given time does not ensure the presence of conjugated dienes. Until more simple, reliable, and predictable methods are available, the determination of lag time of isolated LDL (or diluted serum samples) might be the best alternative. Recent advances in the isolation of LDL from frozen plasma, the use of multiple analysis and multiple methods for the oxidation of LDL should provide the next best approach to the analysis of arterial LDL samples. To this effort, we owe to Prof. Herman Esterbauer for providing us with a simple tool for measuring the oxidation of LDL.

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