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Nitric Oxide and Low-Density Lipoprotein Oxidation

NEIL HOGG* and B. KALYANARAMAN

Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

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Nitric oxide can have both pro-oxidant and antioxidant effects on low-density lipoprotein. Nitric oxide does not appear to react directly with components of LDL. However, in the presence of oxygen (through NO_2 and N_2O_3 formation) or superoxide (through peroxynitrite formation) nitric oxide may cause oxidation of the lipid, protein and antioxidant components of LDL. Conversely, nitric oxide is a potent inhibitor of LDL oxidation when initiated by copper ions or by azo-initiators. The possible implications of these observations to vascular pathology are discussed.

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INTRODUCTION

The comprehensive and continuing investigation of low-density lipoprotein (LDL) oxidation has provided an invaluable insight into the mechanistic complexity of the lipid peroxidation chain reaction in a multi-component biological system.[1–4] The oxidation of LDL has been extensively studied, mainly because of the potential importance of this process in the early stages of atherosclerosis.[5,6] In addition to this, LDL represents a useful model in which to examine, at the molecular level, oxidative and antioxidant processes. LDL is a complex particle that consists of a phospholipid outer monolayer that enspheres a cholesterol-ester core.[1] A single molecule of apolipoprotein B (apo-B) is integrated into this lipid droplet, and has structural, recognition, and enzymatic activities.[1,7,8] In addition, LDL contains numerous lipid-soluble molecules, including vitamin E, ubiquinol and β-carotene, which have variable antioxidant properties.[1] This particle is therefore a self-contained model for the study of lipid-protein-antioxidant reactions with respect to lipid peroxidation.

Nitric oxide is an important regulator of vascular diseases. Inhibition of endothelial nitric oxide synthase (NOS) enhances atherosclerosis,[9,10] and arginine supplementation inhibits and regresses atherosclerosis,[11] presumably by enhancing NO formation. Paradoxically, atherosclerotic tissue generates more oxides of nitrogen than healthy tissue.[12] This suggests that although nitric oxide synthase activity is upregulated in atherosclerotic tissue, nitric oxide is being oxidized before it can exert its beneficial, anti-atherogenic effects. The most rapid, and most likely, scavenger of nitric oxide is

*Corresponding author.

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superoxide,[13–15] which has been shown to be elevated in atherosclerotic tissue.[15] Nitric oxide reacts with superoxide at a diffusion-controlled rate to generate peroxynitrite (Eq. (1)):[13,16]

$$\bullet NO + O_2^- \bullet \longrightarrow ONOO^- \qquad (1)$$

This review will examine the direct effects of nitric oxide and its oxidation products on LDL from the perspective of both oxidative chemistry and vascular pathology.

THE REACTIONS OF NITRIC OXIDE WITH COMPONENTS OF THE LDL PARTICLE

Nitric oxide has been reported to have multiple effects on LDL or on isolated components of the LDL particle. Wang *et al.*[17] showed evidence that nitric oxide could directly oxidize LDL lipid and fragment apo-B. Such changes were accompanied by a change in electrophoretic mobility consistent with lipid oxidation. These investigators used dissolved nitric oxide gas (125–500 μM) added, under anaerobic conditions, in a single addition or in aliquots over time. In agreement with this report, Chang *et al.*[18] demonstrated that bubbling nitric oxide gas through a solution of LDL, for 3 min, followed by 7 days incubation causes a reduction in LDL β-carotene content.

In contrast to these studies are several reports that show little, if any, oxidative activity of nitric oxide. Darley-Usmar *et al.*[19] showed that incubation of LDL with the nitric oxide donor S-nitroso-N-acetyl penicillamine (SNAP) did not result in an alteration in electrophoretic mobility. In addition Jessup *et al.*[20] demonstrated only the ubiquinone content of LDL is significantly diminished by addition of up to 200 μM solutions of nitric oxide and only above 100 μM nitric oxide was a small increase in lipid hydroperoxide observed.

Goss *et al.*[21] attempted to reconcile these discrepancies by demonstrating that bolus addition of NO (500 μM) to LDL under aerobic conditions can cause a mild oxidation that is enhanced by contaminating transition metal ions in the medium. In the presence of the transition metal ion chelator DTPA the enhancement is not observed. Under strict anaerobic conditions nitric oxide does not oxidize LDL lipid, and oxidation is likely due to nitrogen dioxide and dinitrogen tetraoxide. It has previously been shown that nitrogen dioxide will oxidize unsaturated fatty acids.[22]

Reports that nitric oxide can directly react with isolated components of the LDL particle[23] are also likely due to the oxidative chemistry of nitrogen dioxide. For example, α-tocopherol is not oxidized by nitric oxide, but is oxidized to the quinone by nitrogen dioxide.[24] Interestingly, an apparently direct reaction between β-carotene and nitric oxide has been demonstrated to form a nitroxide by addition of nitric oxide to the conjugated diene moiety.[25] However, nitric oxide, when slowly released into solution does not result in significant oxidation of β-carotene.[21]

REACTIONS OF PEROXYNITRITE WITH LDL

The first reports demonstrating an oxidative reaction of peroxynitrite towards LDL used the sydnonimine SIN-1.[19,20] This compound simultaneously releases nitric oxide and superoxide, which rapidly react to form peroxynitrite.[26,27] Incubation of LDL with SIN-1, resulted in the depletion of LDL antioxidants and the formation of both lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), indicating lipid peroxidation was occurring.[19] In addition, SIN-1 decreased the concentration of free amino groups of apo-B and increased the net negative charge of the lipoprotein.[19] These changes are similar to those observed when LDL is oxidized by copper(II) ions.

Later studies using chemically synthesized peroxynitrite indicated that peroxynitrite-treated LDL is a scavenger for the macrophage scavenger

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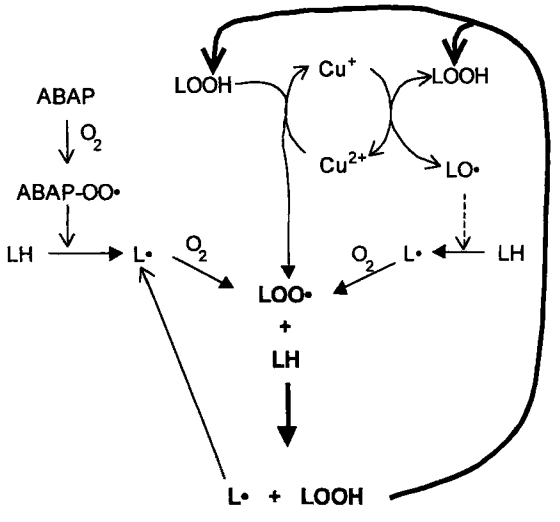
receptor and is therefore potentially atherogenic.[28] One of the earliest reactions to occur during the oxidation of LDL by SIN-1 is the oxidation of α-tocopherol to α-tocopheryl quinone.[29] This reaction also occurs in rat liver microsomes[30] and in organic solution.[31] The mechanism of reaction appears to be a direct two-electron oxidation of α-tocopherol to the α-tocopheryl cation, followed by nucleophillic addition of OH^- to the 8a position.[31] γ-tocopherol, a minor component of vitamin E, reacts with peroxynitrite to form a nitrated product (4-nitro -γ-tocopherol) and the orthoquinone, tocored.[32,33] The difference in chemistry between α- and γ-tocopherol is related to the absence of a methylgroupatthe4positionofγ-tocopherol.More recently, peroxynitrite has been shown to oxidize apo-B cysteinyl thiols by a lipid peroxidation-dependent pathway.[34]

INHIBITION OF LDL OXIDATION BY NITRIC OXIDE

The oxidative modification of LDL is thought to be a critical step in the formation of an atherosclerotic lesion.[5] It is hypothesized that LDL that migrates to the arterial intima is subject to an oxidative stress. This causes oxidation of LDL lipid, which ultimately results in a decrease in net negative charge of apo-B. Once apo-B is modified, LDL is taken up by interstitial macrophages, via the scavenger receptor, to form the foam cells that are characteristic of early lesion development. The subendothelial environment is constantly exposed to nitric oxide from the basal activity of endothelial nitric oxide synthase. Consequently any interactions between nitric oxide and LDL may have profound importance to the biological chemistry of this lipoprotein.

The first suggestions that nitric oxide might inhibit LDL oxidation came from two reports[20,35] that endotoxin-stimulated macrophages were less able to oxidize LDL than unstimulated cells. Endotoxin induces the synthesis of nitric oxide synthase, leading to enhanced nitric oxide production.[36] In addition, the attenuated ability of endotoxin-stimulated macrophages to oxidize LDL was reversed by L-N-monomethyl arginine, a specific inhibitor of NOS. There are several chemical models for the oxidative modification of LDL. The two most commonly employed use either a copper(II) salt or an 'azo-initiator' to initiate lipid oxidation. One fundamental difference between these two methods is that initiation of oxidation by copper(II)-dependent is autocatalytic whereas initiation by azo-initiators is effectively linear. The mechanisms by which these oxidants initiate lipid peroxidation are shown in Scheme 1.

Catalytic quantities of copper(II) ions will oxidize LDL lipid with characteristic kinetics (Figure 1).[37] The change in absorbance at 234 nm is an easy, and continuous, way to monitor the oxidation process. The absorbance change arises from the rearrangement of the fatty acid double



SCHEME 1 *Initiation of lipid peroxidation by copper ions and ABAP.* ABAP decays to give a peroxyl radical (ABAP-OO•) which can abstract a hydrogen atom from an unsaturated lipid (LH) to give a lipid radical (L•). This radical feeds into the lipid peroxidation chain reaction. Copper ions react with lipid hydroperoxide to generate lipid peroxyl and alkoxyl radicals that can also feed into the peroxidation chain reaction. Copper-dependent oxidation is auto-catalytic as lipid hydroperoxide, the product of lipid peroxidation, feeds back to copper and re-initiates a chain of oxidation.

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bond arrangement, from a non-conjugated diene to a conjugated diene, that occurs during lipid peroxidation (Scheme 2). The kinetic profile of conjugated diene formation can be divided into three regions (Figure 1).[37] During the first period, referred to as the lag period, a low level of oxidation occurs. The lag time is due both to the endogenous antioxidants, which are consumed during this period, and to the autocatalytic nature of the reaction. Following the lag period, oxidation accelerates to reach a rapid and fairly linear rate, in what is often called the propagation phase. The maximum rate of the propagation phase occurs when the rate of initiation equals the rate of termination. The end of the propagation phase occurs as a result of depletion of either substrate or oxygen, and is followed by a complex third phase. During this last phase, lipid hydroperoxides are broken down to a complex mixture of products including aldehydes and hydrocarbons.[38]

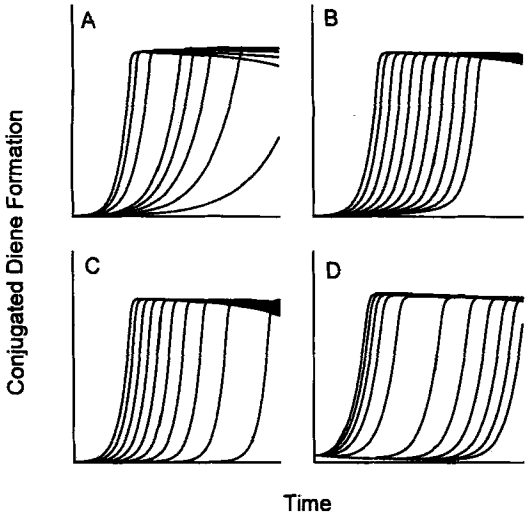
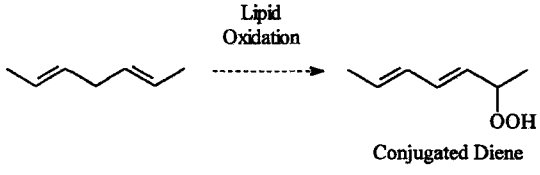


FIGURE 1 *Kinetics of LDL oxidation in the presence of different classes of inhibitors.* The kinetics of copper-dependent LDL oxidation was modeled using kinetic simulation software (see Ref. [21] for details). Simulations were performed in the presence of increasing concentrations of four different classes of antioxidant: (A) a metal ion chelator, (B) a hydrogen donator, (C) a peroxyl radical scavenger and (D) nitric oxide.



SCHEME 2 Conjugated diene formation during lipid peroxidation.

Copper-dependent lipid peroxidation is thought to proceed through the reactions shown in Eqs. (2)–(6). Redox cycling of copper ions results in the

$$Cu^{2+} + LOOH \longrightarrow Cu^{+} + LOO\bullet + H^{+} \quad (2)$$

$$Cu^{+} + LOOH \longrightarrow Cu^{2+} + LO\bullet + OH^{-} \quad (3)$$

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \quad (4)$$

$$L\bullet + O_2 \longrightarrow LOO\bullet \quad (5)$$

$$2L\bullet/LOO\bullet \longrightarrow \text{Products} \quad (6)$$

decomposition of lipid hydroperoxides (low levels of which are always present in LDL) to form lipid alkoxyl and peroxyl radicals (Eqs. (2) and (3)) which both feed into the lipid peroxidation chain reaction (Eqs. (4) and (5)). In the absence of antioxidants, chain termination occurs through radical–radical reactions (Eq. (6)).

Inhibitors of lipid peroxidation affect the shape of the conjugated diene

$$LOO\bullet + AH \longrightarrow LOOH + A\bullet \quad (7)$$

curve (Figure 1).[39,40] Inhibitors can be divided into three main classes: (i) copper chelators, (ii) hydrogen atom donors and (iii) peroxyl radical scavengers. As shown in Figure 1A, antioxidants that work through copper chelation, such as EDTA, cause an increase in the length of the lag period of oxidation and a decrease in the rate of oxidation during the propagation phase. The plot of lag time vs. concentration for a copper chelator is shown in Figure 2. As the concentration of free copper approaches zero, the lag period approaches infinity. Figure 1B shows the typical effect of hydrogen donators (AH), such as α-tocopherol on conjugated diene formation.

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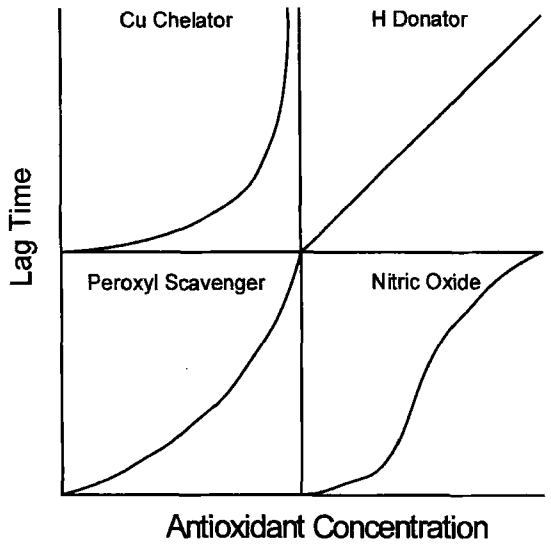

FIGURE 2 *Effect of different classes of antioxidant on the lag time of LDL oxidation.* Lag times, calculated from Figure 1, were plotted as a function of antioxidant concentration for the four classes of antioxidant.

These compounds donate a hydrogen atom to the peroxyl radical thus breaking the chain of propagation (Eq. (7)). As the net change in lipid peroxide, due to this reaction, is zero once the antioxidant has been consumed oxidation proceeds as before. Consequently the length of the lag time is a linear function of antioxidant concentration (Figure 2). Variations from this ideal situation can occur if the antioxidant radical has a low ability to initiate peroxidation reaction, as has been suggested for α-tocopherol.[3]

Peroxyl radical scavengers act by reacting with and removing peroxyl radicals (Eq. (8)). This type of activity is most likely an attribute of

$$LOO\bullet + A\bullet \longrightarrow LOOA \qquad (8)$$

stable free radicals. The α-tocopheroxyl radical, generated from hydrogen donation reactions (Eq. (7)), has been suggested to be a peroxyl radical scavenger, consequently each α-tocopherol molecule inhibits two chains of oxidation.[41] As true peroxyl radical scavengers do not regenerate lipid peroxide (cf Eqs. (7) and (8)), the inhibition profile for these compounds is expected to be that in Figure 1C. The non-linear dependence of the lag-time on the antioxidant concentration (Figure 2) arises from the fact that the hydroperoxide concentration is reduced during this time. Consequently, LDL becomes more resistant to oxidation by copper during the lag time.

Nitric oxide reacts with peroxyl radicals at near diffusion controlled rates[42] and is therefore likely to act as a peroxyl radical scavenger (Eq. (8)). However, a study of lag-time vs. nitric oxide donor concentration gives the profile shown in Figure 1D.[21] Although at low concentrations nitric oxide appears to act as a peroxyl radical scavenger, higher concentrations of nitric oxide are less effective than would be expected, giving a sigmoidal dependence (Figure 2). This can be explained if it is assumed that the product of the antioxidant reaction (putatively LOONO[43]) is unstable and slowly decays to a hydroperoxide or a peroxyl radical.[21] Consequently, after the nitric oxide source has been depleted re-initiation occurs from the slow breakdown of the LOONO adduct. Goss *et al.*[21] demonstrated that the sigmoidal dependence could be left-shifted by increasing the original peroxide content of the LDL. In addition maximal inhibition occurs at a rate of nitric oxide release that matches the rate of initiation.[44] Consequently a low, but continuous rate of nitric oxide formation is a more potent inhibitor of oxidation than a burst of nitric oxide release.

Nitric oxide will also inhibit lipid oxidation initiated by azo-initiators such as ABAP. This was first demonstrated in LDL where photoloysis of sodium nitroprusside was observed to inhibit LDL oxidation.[45] Recently O'Donnell *et al.*[46] demonstrated, using azo-initiators, that two nitric oxide molecules are consumed per chain reaction terminated. This stoichiometry suggests that the primary product of the reaction between nitric oxide and lipid is unstable and breaks down to a product that can also be scavenged by nitric oxide.

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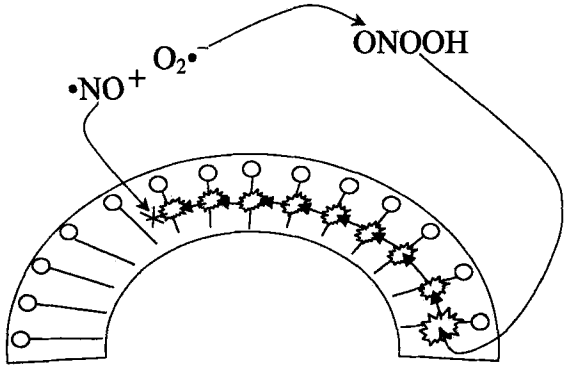
CONSEQUENCES FOR VASCULAR PATHOLOGIES

Oxidative stress and free radical formation is a feature of many cardiovascular pathologies. The elevation of vascular superoxide formation has been linked to hypertension[47] and atherosclerosis.[12] The mechanisms for such elevation have been associated with many atherosclerotic risk factors such as diabetes,[48] hyperhomocystienemia,[49] and hypercholesterolemia.[12,47,50,51] In addition, nitric oxide has been shown to be a potent antiatherogenic agent that suppresses many elements of the atherosclerotic processes.[52] Such observations have led to the hypothesis that alteration of the balance between nitric oxide and superoxide generation by vascular cells may contribute to atherogenesis.[52–54]

This hypothesis may be particularly relevant to the biological oxidative modification of LDL. As reviewed here, nitric oxide is a potent inhibitor of LDL oxidation. The low continuous flux of nitric oxide generated by the vascular endothelium would be expected to suppress such reaction *in vivo*. Only after removal of this antioxidant is sub-endothelial LDL oxidation likely to occur. In support of this are the observations that L-nitroarginine methyl ester, an inhibitor of NOS, accelerates the atherosclerotic process in animal models. The diffusion limited reaction between nitric oxide and superoxide removes the antioxidant activity of nitric oxide and generates the potent LDL oxidant, peroxynitrite (Scheme 3). The formation of peroxynitrite usually results in protein tyrosyl residue nitration. It is of interest to note that nitrotyrosine has been observed in atherosclerotic lesions[55,56] and peroxynitrite has been implicated in the pathology of atherosclerosis.[57]



SCHEME 3 In the absence of superoxide, nitric oxide will inhibit lipid oxidation. In the presence of superoxide, nitric oxide will be scavenged to form peroxynitrite, which will initiate lipid oxidation. The balance between the levels of nitric oxide and superoxide becomes a critical determinant of whether nitric oxide exhibits pro-oxidant or anti-oxidant behavior.

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