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Inhibition of low-density lipoprotein oxidation by nitric oxide

Potential role in atherogenesis

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The effects of nitric oxide (•NO) and nitrovasodilators on the oxidation of low-density lipoprotein (LDL) have been studied. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) inhibited Cu^{2+}- and 2,2′-azobis-2-amidinopropane hydrochloride-dependent oxidation of LDL as monitored by oxygen consumption and the formation of thiobarbituric acid-reactive substances, conjugated dienes, and lipid hydroperoxides. In the case of SNP, inhibition of LDL oxidation occurred only when the incubation mixture was irradiated with visible light. SNAP, however, exerted a dose-dependent inhibition of Cu^{2+}-catalyzed oxidation of LDL even in the dark. Addition of •NO dissolved in deoxygenated buffer also inhibited the progression of LDL oxidation. Mouse peritoneal macrophages were less able to degrade LDL that had been oxidized in the presence of SNAP. Using an •NO electrode, it was estimated that a continuous production of •NO ($\leq$ 760 nM/min) could retard the progression of LDL oxidation. We propose that •NO can inhibit LDL oxidation by acting as a chain-breaking antioxidant that is capable of scavenging carbon-centered and peroxyl radicals. Biological implications of this novel •NO antioxidant property are discussed in relation to atherogenesis and contrasted to the prooxidant property of •NO when generated in the presence of superoxide.

Nitric oxide; Low-density lipoprotein; Atherosclerosis; Lipid peroxidation

1. INTRODUCTION

The oxidation of low-density lipoprotein (LDL) has been implicated in the early stages of atherosclerotic lesion formation [1–3]. The hypothesis requires that LDL, which is trapped within the arterial wall, undergoes oxidation resulting in the recruitment of macrophages to the intima. These macrophages then ingest the oxidized lipoprotein via macrophage scavenger receptors [4]. Cholesterol accumulation is not regulated by macrophage-cholesterol levels; therefore, the cells become engorged with cholesterol-ester forming the 'foam cells' observed in early atherosclerotic lesions [5].

While the in vivo mechanism of LDL oxidation remains unclear, several in vitro systems, e.g. Cu^{2+}, azo-initiators, lipoxygenases, macrophages, endothelial and smooth muscle cells, have been shown to oxidize LDL [3,6]. Recently, there has been much interest in the role of nitric oxide (•NO) in atherosclerosis because of the discovery that many of the cell types associated with atherosclerotic lesions produce •NO [7]. It has also been demonstrated that •NO-dependent vasodilation is inhibited by oxidized LDL [8].

•NO has been reported to have a dual effect on LDL oxidation. In macrophage-dependent oxidation of LDL, increased •NO production has been shown to be protective [9,10]; whereas, in systems containing LDL and superoxide, •NO exhibits prooxidant behavior. The simultaneous release of •NO and superoxide has been shown to modify LDL to an oxidized and potentially atherogenic form [11,12]. It has been postulated that the prooxidant effect of •NO in the presence of superoxide occurs through the formation of peroxynitrite ($^{-}$OONO) [13–15]. However, the mechanism by which cytokine-stimulation of •NO inhibits the macrophage-dependent oxidative modification of LDL is poorly understood. Clearly, the reaction between •NO and superoxide cannot account for the protective mechanism of •NO, as the resulting $^{-}$OONO is a more potent oxidant than either superoxide or •NO alone [12]. Recently, it has been suggested that either chelation of redox-active metal ions by •NO, formation of nitrosothiols, or reaction between •NO and intracellular iron- containing enzymes such as lipoxygenase could account for this protection [9,16].

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Abbreviations: ABAP, 2,2′-azobis(2-amidinopropane hydrochloride); EDRF, endothelium-derived relaxing factor; L•, lipid radical; LDL, low-density lipoprotein; LO•, lipid alkoxyl radical; LOO•, lipid peroxyl radicals; LOOH, lipid hydroperoxide; MDA, malondialdehyde; •NO, nitric oxide; $^{-}$OONO, peroxynitrite; PBS, phosphate-buffered saline; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

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In this communication, we show that a continuous generation of •NO can totally inhibit LDL oxidation and propose a novel antioxidant mechanism of action for •NO. We have used sodium nitroprusside (SNP)/light, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and authentic •NO solution during the oxidation of LDL by Cu^{2+}, and 2,2′-azobis(2-amidinopropane hydrochloride) (ABAP). We show that, under these conditions, •NO acts only as an antioxidant.

2. MATERIALS AND METHODS

2.1. *Materials*

SNP, copper (II) sulfate, and butylatedhydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO). ABAP was obtained from Polyscience, Inc. (Worrington, PA). SNAP was synthesized from *N*-acetyl-D,L-penicillamine as described by Field et al. [17]. Stock solutions of SNAP were prepared in 50 mM H_2SO_4. Solutions of •NO were prepared from NO gas (99% purity) obtained from Matheson Gas Co. (Madison, WI). NO was bubbled through a saturated solution of NaOH before addition to a sealed vessel containing deionized water. The vessel had previously been evacuated to remove other gasses from solution. Human LDL was isolated from plasma as described previously [18]. Resident peritoneal macrophages were obtained from female Swiss–Webster mice as described previously [19].

2.2. *LDL oxidation*

LDL (100 μg protein/ml) was incubated with either Cu^{2+} (10 μM) or ABAP (1 mM) alone or in the presence of SNP, SNAP, or •NO. All incubations were performed in Dulbecco's phosphate-buffered saline (PBS) at 37°C in open vessels that were continually stirred. Aliquots were removed at the indicated time points for measurement of thiobarbituric acid-reactive substances (TBARS) or lipid hydroperoxide (LOOH). Irradiation was performed using a Viewlex projector through a clear glass window in the side of the incubator chamber.

2.3. *TBARS measurement*

LDL (10–20 μg) was incubated with thiobarbituric acid (TBA, 0.5% w/v) in H_2SO_4 (50 mM) for 30 min in a boiling water bath and then centrifuged at 2000 rpm. TBARS concentrations were calculated as malondialdehyde (MDA) equivalents using the extinction coefficient of 150 $mM^{-1} \cdot cm^{-1}$ at 532 nm.

2.4. *Conjugated diene formation*

Oxidation of LDL (100 μg/ml) was monitored continuously at 234 nm [20]. Oxidation was initiated by the addition of Cu^{2+} (5 μM) to the sample cuvette.

2.5. *Iodometric measurement*

Total LOOH were determined by a microiodometric method according to Girotti et al. [21] with minor variations. Quantitation was based on an absorption coefficient of 22.5 $mM^{-1} \cdot cm^{-1}$, as determined with enzymatically standardized *t*-butyl hydroperoxide.

2.6. *•NO measurement*

•NO was measured using a commercially available •NO meter (Iso-NO, World Precision Instruments, Inc., Sarasota, FL). The electrode was calibrated by the method outlined in the product literature (see [22]).

2.7. *Macrophage degradation of LDL*

The uptake and degradation of [^{125}I]LDL by mouse peritoneal macrophages was measured as the appearance of trichloroacetic acid-soluble radioactivity. This non-iodide radioactivity was formed by cells and excreted in the medium following a 5 h incubation of oxidized [^{125}I]LDL with the cells [19].

3. RESULTS

3.1. *Effects of SNP/light on Cu^{2+}-catalyzed oxidation of LDL*

SNP releases •NO when exposed to visible light [23,24]. Incubation of LDL and Cu^{2+} at 37°C in PBS resulted in the generation of both TBARS (Fig. 1A) and LOOH (Fig. 1B). The largest change in both parameters was observed during the first hour of incubation. This corresponds well with previous reports of Cu^{2+}-dependent oxidation of LDL if it is assumed that the lag-period of the oxidation process is complete within the first hour [3]. In the presence of SNP where light was strictly excluded from the reaction vessel, a slight inhibition of the generation of both TBARS (Fig. 1A) and LOOH (Fig. 1B) was observed. However, after a 4-h incubation, levels of both oxidation products were similar to control levels. When the incubation mixture was irradiated continuously, a dramatic inhibition of LDL oxidation was observed (Fig. 1A and 1B). This suggests that •NO released from SNP during irradiation is responsible for the observed inhibition.

3.2. *Effects of SNAP on Cu^{2+}-catalyzed LDL oxidation*

SNAP has been shown to produce •NO spontaneously at physiological pH [17]. Fig. 2A shows the effect of SNAP on the formation of conjugated dienes during Cu^{2+}-catalyzed oxidation of LDL. Cu^{2+}-dependent oxidation of LDL exhibits characteristic kinetics as shown

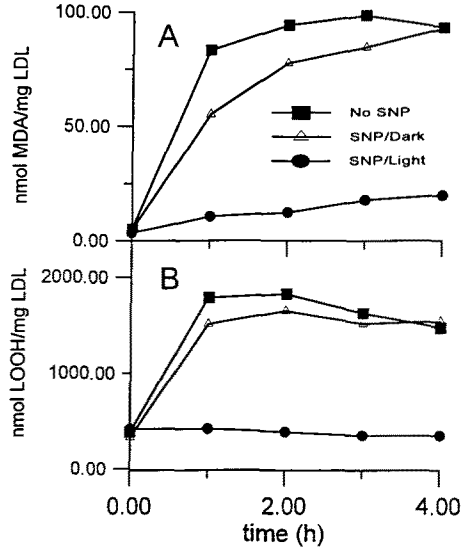

Fig. 1. Effects of SNP on Cu^{2+}-catalyzed oxidation of LDL. LDL (100 μg/ml) was incubated with Cu^{2+} (10 μM) at 37°C in PBS. Experiments were performed in the absence of SNP (■), in the presence of SNP (100 μM) in the dark (△) and in the presence of SNP (100 μM) and light (●). All experiments were performed in duplicate using the same preparation of LDL. Oxidation was measured by the formation of both (A) TBARS and (B) LOOH. Data points represent means of duplicate measurements. The average error was approximately ± 1 nmol/mg for TBARS and ± 100 nmol/mg for LOOH.

in Fig. 2A (trace 1). The time course consists of a slow rate of oxidation (the lag period) followed by a rapid rise in the rate of formation of conjugated dienes [20]. As shown in Fig. 2A, SNAP increased the length of the lag-period of LDL oxidation in a concentration-dependent manner. In the presence of 2.5 mM SNAP, there were no detectable increases in the levels of conjugated dienes for up to 10 h. However, after 24 h, this sample also had a greater amount of conjugated diene formation (data not shown) suggesting oxidation had occurred. The length of the lag-period lay between 10 and 24 h. As with conjugated diene formation, SNAP addition also results in an increase in the length of the lag-period of oxidation as measured by TBARS formation (Fig. 2B). Consistent with these results, Cu^{2+}-catalyzed oxidation of LDL in the presence of SNAP (1 mM) exhibited a lower propensity toward macrophage degradation (1.1 μg/5 h/mg protein) than LDL oxidized in the absence of SNAP (6.7 μg/5 h/mg protein).

3.3. *Effect of SNP/light on ABAP-induced oxidation of LDL*

To ascertain whether the inhibitory effect of SNP/light is restricted to Cu^{2+}-catalyzed oxidation of LDL, ABAP was used as a free radical initiator. Fig. 3 shows the effect of SNP/light on the formation of TBARS during ABAP-induced oxidation of LDL. Clearly, SNP/light greatly suppressed ABAP-induced LDL oxidation. SNP did not affect ABAP-dependent oxidation of LDL in the dark (results not shown). It was observed that

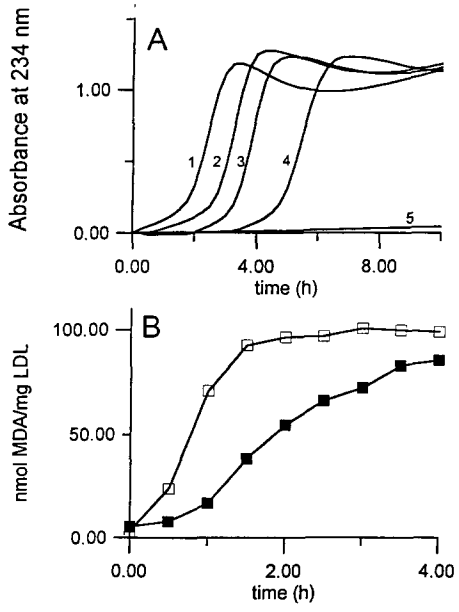

Fig. 2. Effect of SNAP on Cu^{2+}-catalyzed oxidation of LDL. LDL (100 μg/ml) was incubated with Cu^{2+} (10 μM) at 37°C in PBS. (A) conjugated diene formation: traces 1–5 contained 0, 0.1, 0.5, 1.0 and 2.5 mM SNAP, respectively. (B) TBARS formation in the presence (■) and absence (□) and of SNAP (1 mM). Data represent means of duplicate determinations.

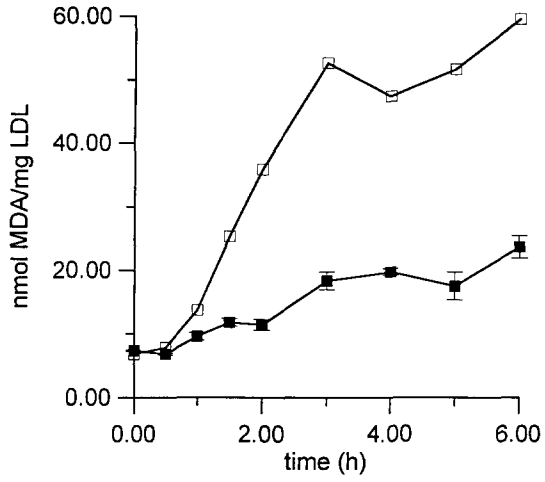

Fig. 3. Effect of SNP on ABAP-induced oxidation of LDL. LDL (100 μg/ml) was incubated with ABAP (1 mM) in PBS at 37°C in the presence (■) and absence (□) of SNP (100 μM). Samples were continually irradiated and aliquots were taken for TBARS analysis. Error bars represent mean ± S.E.M. (n = 3).

ABAP oxidation of LDL is accelerated in the presence of light, thus preventing direct comparison between reactions performed in the dark.

3.4. *Measurement of •NO formed during SNP/light and SNAP decomposition*

•NO released from the decomposition of SNAP and SNP/light was measured using the •NO-electrode (Fig. 4). The initial rate of •NO from SNP (100 μM)/light was observed to be 760 nM/min. This rate of •NO production completely inhibits LDL oxidation as shown in Fig. 1. This concentration of SNP continues to generate •NO for several hours (Fig. 4A), and inhibition can be sustained over this time period (Fig. 1). Assuming that the decay of SNP is first order and that LDL does not affect •NO production, it can be estimated that the rate of •NO production after 4 h will be 120 nM/min. This rate of •NO production is still able to inhibit LDL oxidation (Fig. 1). •NO generation from SNAP is spontaneous and exhibits markedly different kinetics (Fig. 4B). SNAP (40 μM) releases all of its •NO within the first 20 min; after which time, the •NO concentration decays, presumably due to reaction with oxygen. This implies that although the absolute amount of •NO released by either SNP/light or SNAP may be similar, SNP will have a much more pronounced inhibitory effect due to the slower release of •NO. This effect is seen in Figs. 1 and 2.

The addition of authentic •NO at concentrations (5–100 μM) increased the lag-period of Cu^{2+}-catalyzed LDL oxidation by approximately 30 min (data not shown). This indicates that the effective concentration of •NO is the same regardless of the initial concentration; presumably, due to the fact that excess •NO is rapidly degraded by oxygen.

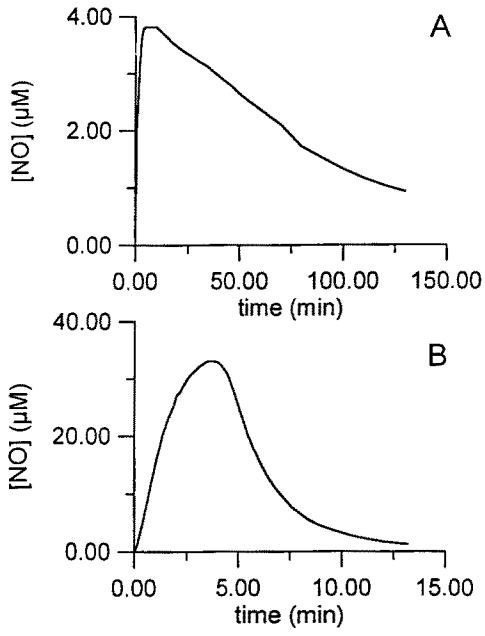

Fig. 4. Measurement of •NO released during SNP/light and SNAP decomposition. •NO was monitored using an •NO electrode. Either (A) SNP (100 μM) or (B) SNAP (40 μM) was added to PBS in an open stirred vessel maintained at 37°C. In the case of SNP, the sample was continuously illuminated during the course of the experiment.

4. DISCUSSION

4.1. *Mechanisms of Cu^{2+}-catalyzed oxidation of LDL*

The oxidation of LDL by Cu^{2+} is thought to occur as a result of the decomposition of endogenous LOOH associated with LDL as follows [3,25]:

$$Cu^{2+} + LOOH \rightarrow Cu^{+} + LOO^{\bullet} + H^{+} \quad [1]$$
$$Cu^{+} + LOOH \rightarrow Cu^{2+} + LO^{\bullet} + OH^{-} \quad [2]$$
$$LO^{\bullet} + LH \rightarrow L^{\bullet} + LOH \quad [3]$$
$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad [4]$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad [5]$$

This mechanism involves lipid peroxyl radicals (LOO•), lipid alkoxyl radicals (LO•) and lipid radicals (L•) as intermediates. The proposed mechanism is autocatalytic, and the time course of oxidation will depend critically on the level of endogenous LOOH [25,26]. LDL also contains several antioxidants, in particular α-tocopherol, that are able to scavenge LOO•, thus inhibiting LDL oxidation [3]:

$$LOO^{\bullet} + \text{vit E} \rightarrow \text{vit E}^{\bullet} + LOOH \quad [6]$$
$$LOO^{\bullet} + \text{vit E}^{\bullet} \rightarrow LOO - \text{vit E} \quad [7]$$

Figs. 1–3 clearly demonstrate that SNP/light, SNAP, and •NO solution can inhibit the progress of both Cu^{2+}- and ABAP-dependent oxidation of LDL. There are at least three mechanisms by which •NO can suppress Cu^{2+}-dependent oxidation of LDL. These are: (i) chelation of Cu^{2+} by •NO to form an inactive complex, (ii) removal of LOOH within the LDL particle, and (iii) scavenging of L• and LOO• by •NO, which will prevent both initiation (Reactions 1 and 2) and propagation (Reactions 3 and 5). Mechanism (i), namely chelation of Cu^{2+} by •NO, is unlikely as inhibition occurs when ABAP is used as the initiating agent. The kinetics of Cu^{2+}-dependent oxidation in the presence of SNAP also suggests that Cu^{2+} chelation is not the mechanism of antioxidant action by •NO. Copper(II) chelators, in general, increase the length of the lag-period, but also decrease the rate of LDL oxidation during the faster propagation phase. This behavior is not observed in Fig. 2. Also, ABAP-dependent oxidation, unlike Cu^{2+}, does not depend on the presence of endogenous LOOH. We propose, therefore, that •NO generated from the decomposition of SNAP and SNP/light inhibits LDL oxidation by scavenging L• and LOO• formed during lipid peroxidation.

4.2. *Reaction between •NO and LDL-lipid derived radicals*

Much evidence already exists in the literature for the facile reaction between nitroxides (compounds containing >N–O• groups) and carbon-centered radicals [27–32]. Nitroxides have long been used as an antioxidant in polymerization reactions [30]. Nitroxides also were shown to inhibit lipid peroxidation in microsomes [33]. The rate constant for scavenging of the carbon-centered radicals by different nitroxides range from 10^{10} $M^{-1} \cdot s^{-1}$–10^{8} $M^{-1} \cdot s^{-1}$. The typical reaction involves the formation of alkoxylamines. Nitroxides, however, do not react rapidly with peroxyl radicals [30].

In contrast to nitroxides where the electron density is distributed on the nitrogen and oxygen atom, the electron density distribution in •NO is localized predominantly on the nitrogen. As a result, •NO can react with carbon-centered, alkoxyl, and peroxyl radicals at a rapid rate [27–32]. Thus, the following reactions are possible:

$$L^{\bullet} + {}^{\bullet}NO \rightleftharpoons LNO \quad [8]$$
$$LO^{\bullet} + {}^{\bullet}NO \rightleftharpoons LONO \quad [9]$$
$$LOO^{\bullet} + {}^{\bullet}NO \rightleftharpoons LOONO \quad [10]$$

Such radicals are formed during the lipid peroxidation process (see Reactions 1–5). •NO is a hydrophobic gas and will diffuse into the LDL particle. The local concentrations of both •NO and oxygen will determine whether Reaction 8 is able to compete with Reaction 4.

Reaction of •NO with LO• is unlikely due to both the rapid rate of reaction of LO• with unsaturated fatty acid and to the presumably very high local concentration of these fatty acids. A more likely reaction of •NO is that with LOO• (Reaction 10) because of their relatively low reactivity toward unsaturated lipids. The effect of SNAP in increasing the lag-period of LDL oxidation

(Fig. 2) is consistent with the hypothesis that •NO is acting as a scavenger of LOO•. Other known LOO• scavengers affect the kinetics of LDL oxidation identically. Characterization of the lipid nitroso products (Reactions 8–10) may shed light on the mechanistic details of the inhibition of LDL oxidation by •NO.

4.3. *Biological implications*

•NO has many properties that are believed to be antiatherogenic. These include the inhibition of platelet aggregation, the inhibition of leukocyte adhesion, and the inhibition of vascular smooth muscle proliferation [34]. One of the early events during hypercholesterolemia is a reduction in the ability of vessels to respond to endothelium-derived relaxing factor (EDRF), now known to be •NO. It was observed that oxides of nitrogen were augmented in atherosclerotic tissue [8,35] and recently demonstrated that the reduction in EDRF activity is due to an increase in superoxide formation and not a decrease in •NO synthesis in the endothelium [35–37]. The reaction between •NO and superoxide has been shown to produce $^{-}$OONO, a potent oxidant [13].

We show here that •NO exhibits potent antioxidant activity with respect to LDL oxidation. It has previously been reported that the simultaneous generation of •NO and superoxide as well as $^{-}$OONO itself, can modify LDL to a potentially atherogenic form [11,15]. Thus, the reaction between •NO and superoxide in the vasculature, may have the combined effect of removing an antioxidant, namely •NO, generating a pro-oxidant, $^{-}$OONO, and inhibiting endothelial-dependent relaxations. This may enhance the oxidative stress level experienced by LDL within artery wall resulting in modification of LDL, thereby leading to foam cell formation.

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