The Effect of Nitric Oxide Release Rates on the Oxidation of Human Low Density Lipoprotein*

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The chronic inhibition of nitric oxide synthase (NOS) accelerates atherosclerosis in cholesterol-fed rabbits (1, 2). Supplementation of L-arginine, the substrate for NOS, has been shown to decrease fatty streak formation in rabbits and correct endothelial dysfunction in hypercholesterolemic humans (3–5). These data suggest that basal production of NO from the vascular endothelium can suppress the changes associated with early atherosclerotic lesion formation. Moreover, augmentation of NO production can partially counteract the effects of elevated cholesterol levels.

The mechanisms by which NO affects atherosclerotic lesion formation are unclear; however, there are many possibilities. NO has been shown to inhibit inflammatory cell adhesion to the endothelium (6) and suppress smooth muscle cell proliferation (7). In later stages of atherosclerosis, the anti-thrombotic properties of NO (8) may also be relevant.

It has frequently been proposed that the initiation of the atherosclerotic process is oxidative in nature. Free radical-mediated oxidative damage to both the vascular endothelium and low density lipoprotein (LDL) has been proposed to be a vital component of the atherosclerotic mechanism (9, 10). We have recently shown that NO suppresses the oxidative modification of LDL (11–13) and inhibits the toxicity of oxidized LDL to endothelial cells (14). We have proposed that the way in which NO inhibits oxidative processes is by scavenging propagatory lipid peroxyl radicals, thus suppressing the lipid peroxidation chain reaction (11–14). This mechanism will generate lipid/NO adducts that have recently been identified (15, 16).

Many biological effects of NO appear to be related to the rate of NO formation (15, 17, 18). Bolus delivery of high concentrations of NO may result in limited LDL oxidation that can be augmented by Cu2+ (12). Slow release of NO from the donor spermine NONOate, however, suppresses LDL oxidation at concentrations of 2–10 μM (12).

In this investigation, we have examined the effect of NO release rate on the oxidative modification of LDL using four structurally related NO donor compounds (NONOates) with a wide range of NO release rates (see Fig. 1 for structures). We conclude that an optimal rate of NO release is required for maximal suppression of LDL oxidation, and that this optimal rate depends upon the rate and the mechanism of LDL oxidation.

This conclusion is supported by a kinetic model of LDL oxidation in the presence of NO.

EXPERIMENTAL PROCEDURES

Materials—Copper(II) sulfate was purchased from Fisher. 2,2′-Azobis-2-amidinopropane hydrochloride (ABAP) was obtained from Polysciences, Inc. (Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl)amino]]diazene-1-ium-1,2-diolate (MNN), (Z)-1-[N-(3-aminoisopropyl)-N-ammoniohexyl]diazene-1-ium-1,2-diolate (MNN), (Z)-1-[N-(3-aminoisopropyl)-N-ammoniohexyl]diazene-1-ium-1,2-diolate (MNN), and 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium (SIN-1) were purchased from Cayman Chemical Co. Spermamine, 1,1,3,3-tetramethoxystyrylamine, and thiobarbituric acid were purchased from Sigma. Diethylenetriaminopentaacetic acid (DTPA) was obtained from Aldrich. LDL was isolated from human plasma as described previously (19). All concentrations of LDL are given in terms of LDL protein as measured by the Lowry assay (20).

Measurement of LDL Oxidation—LDL was incubated in phosphate-buffered saline (PBS) at 37 °C, either alone or in the presence of MNN, MNN, SNN, or DDTA. Oxidation was initiated with copper(II) sulfate, ABAP, or SIN-1. Addition of decayed NONOate had no effect upon oxidation at the concentrations used in these experiments. Oxidation was monitored using the following assays.

Thiobarbituric Acid-reactive Substances (TBARS) Measurements—LDL (10–20 μg) was incubated with thiobarbituric acid (0.5%, w/v, in H2SO4, 50 mM) for 30 min at 100 °C. Samples were centrifuged for 5 min, and the difference in the absorbance was determined between 532-542 nm.
and 580 nm. TBARS concentration was calculated as malondialdehyde (MDA) equivalents using a MDA standard curve. MDA was generated by the acid hydrolysis of 1,1,3,3-tetramethoxypropane. SNN (500 μM) had no effect upon the standard curve.

Conjugated Diene Measurements—LDL (200 μg/ml) incubated in PBS at 37 °C, was continuously monitored at 234 nm to detect the formation of conjugated dienes as described previously (21).

Apolipoprotein B (ApoB) Modification—Oxidative modification of LDL results in an increase in net negative charge that can be detected using agarose electrophoresis (Paragon LIPO lipoprotein electrophoresis kit). Relative electrophoretic mobility (REM) was calculated as the ratio of the electrophoretic mobilities of the samples to that of native LDL (22).

α-Tocopherol Measurements—α-Tocopherol was extracted into heptane and assayed by high performance liquid chromatography as described previously (23).

Kinetic Modeling—Kinetic simulations were performed using software written by Frank Neese, Facultät für Biologie, Universität Konstanz, Konstanz, Germany. Integration of the equations shown under “Appendix,” using the rate constants defined in Table II, was performed using the Stiff Euler algorithm.

RESULTS

Verification and Characterization of NO Release from NONOate Compounds—Each NO donor compound was analyzed under our experimental conditions to determine the kinetics of NO release. The decay rate was determined by monitoring the decrease in absorbance at 250 nm in PBS at 37 °C (24). The decay of each compound followed first order kinetics with rates of MNN > PNN > SNN >> DNN in agreement with the known rates of decay. The presence of both LDL and copper(II) sulfate had no significant upon the kinetics of decay (data not shown). The calculated half-life and kobs are listed for each donor compound are listed in Table I. Both ESR spin-trapping (25) and electrochemical (26) techniques were used to verify that NO is released during the breakdown of each compound (data not shown).

The Effect of NO Release Rate upon Copper-mediated LDL Oxidation—LDL (200 μg/ml) was incubated with copper(II) sulfate (20 μM) in PBS at 37 °C, in the presence or absence of each NONOate compound (10 μM). Oxidation was assayed by monitoring the formation of TBARS. In the presence of copper(II) sulfate, TBARS formation increased over time reaching a maximum of 80 ± 1.4 nmol of MDA/mg of LDL protein (Fig. 2). Addition of each NONOate compound led to an inhibition of LDL oxidation for approximately 2 h, while DNN, with a t1/2 of 21.9 ± 0.7 h, inhibited oxidation for 1 h. This indicates that there is an optimal rate of NO production for maximal suppression of Cu2+-dependent LDL oxidation.

The oxidation of LDL has previously been shown to cause an increase in the net negative charge of the LDL particle (22, 27). This is thought to result from the modification of lysine residues of the ApoB protein by aldehydes formed during lipid peroxidation. When LDL (200 μg/ml) was incubated with copper(II) sulfate (20 μM) for 4 h in PBS at 37 °C, an increase in the electrophoretic mobility of the LDL particle was observed (Fig. 3A). The presence of each NONOate compound (10 μM) had a time-dependent inhibitory effect upon this change in mobility (Fig. 3B). Both the fastest releasing (MNN) and slowest releasing (DNN) NO donors (see Table I) were much less effective...
inhibitors than PNN and SNN.

The formation of conjugated dienes is a sensitive and direct measurement of lipid peroxidation. LDL (200 μg/ml) was incubated in PBS at 37 °C in the presence of copper(II) sulfate (20 μM). After an initial lag period, there was a rapid increase in the rate of conjugated diene formation. As shown in Fig. 4A, the length of the lag time was calculated by determining the time at which the absorbance reached half of the maximum absorbance. As previously demonstrated (12), addition of SNN (4 μM) led to an increase in the length of the lag time by approximately 2.5 h (Fig. 4A). The concentration dependence of the inhibition of conjugated diene formation for each of the four NONOates is shown in Fig. 4B. The presence of all NONOate compounds led to an increase in the lag time of LDL oxidation. The extent of inhibition exhibited a biphasic relationship to the NONOate decay rate (see Table I).

The Effect of NO Release Rate on ABAP-mediated LDL Oxidation—ABAP, a peroxyl radical generator, has been shown to oxidize LDL by initiating lipid peroxidation (28). Addition of ABAP (1 mM) to LDL (200 μg/ml) led to a time-dependent increase in TBARS formation over a period of 10 h after a 2-h lag period (Fig. 5). MNN had no effect on ABAP-dependent LDL oxidation, PNN increased the lag period by 2 h, and both SNN and DNN inhibited LDL oxidation over the full 10-h period (Fig. 5). The NO donor compounds also inhibited the change in REM of LDL during ABAP-dependent oxidation as shown in Fig. 6. The inhibitory effect was again of the order SNN > DNN > PNN > MNN.

The rate of ABAP-dependent LDL oxidation can be sensitively measured by following the decrease in α-tocopherol levels. In the absence of NO donors, α-tocopherol was consumed over a 2-h period at an approximately linear rate (Fig. 7). MNN had no effect on α-tocopherol decomposition, in agreement with the observation that this compound did not inhibit lipid peroxidation (Fig. 5) and protein modification (Fig. 6). PNN inhibited α-tocopherol depletion for approximately 1 h and SNN for approximately 2 h (Fig. 7). Surprisingly, although DNN inhibi-
EDU, α-tocopherol oxidation (Figs. 5 and 6), it had little inhibitory effect on α-tocopherol depletion (Fig. 7), indicating that the sparing of α-tocopherol is not an absolute requirement for the inhibitory effect of NO.

The Effect of NO Release Rates on LDL Oxidation

SIN-1, which releases NO and O$_2^-$ to generate peroxynitrite (29), has been shown to oxidize LDL (30) and cause a stoichiometric conversion of α-tocopherol to α-tocopherol quinone (24, 31, 32). To determine the effect of NO release on SIN-1-dependent LDL oxidation, LDL was incubated with SIN-1 (1 mM) in PBS at 37 °C. Reactions were quenched with DTPA (100 μM) and BHT (20 μM), and α-tocopherol levels (A) and α-tocopherol quinone levels (B) were determined. All reaction mixtures contained DTPA (100 μM). Data points represent mean ± S.D. (n = 3).

DISCUSSION

The Effect of NO Release Rate on LDL Oxidation—Several effects of NO have been shown to be dependent on the rate of NO delivery. For example, NO is more toxic to Chinese hamster ovary cells when slowly infused through a sylastic membrane than when added as a bolus (18). Previous work with NONOate compounds has shown that slower rates of NO release are much more effective at inhibiting DNA synthesis and the proliferation of smooth muscle cells (33). NO has also been shown to have both oxidant and antioxidant effects, depending
on the rate of formation (15). If 'NO is neither added to LDL in bolus amounts (12) or continuously bubbled through a solution of LDL (34), oxidation may occur. 'NO may also cause LDL oxidation in the presence of superoxide (30). If 'NO is slowly released from donor compounds, it is an efficient antioxidant, preventing LDL oxidation.

The mechanism by which 'NO inhibits lipid peroxidation is thought to be the scavenging of lipid radicals that propagate the peroxidative chain reaction. The reaction between the lipid peroxyl radical and 'NO creates LOONO, an organic peroxynitrite. It has been suggested that such compounds decay via homolytic cleavage to yield an alkoxyl radical (LOO') and 'NO₂ (35). If this were true of LOONO in LDL, the action of 'NO may be expected to be pro-oxidant, as both LOO' and 'NO₂ are able to oxidize LDL lipid. It is likely, therefore, that LOONO decays by internal re-arrangement to LOONO₂, a stable organic nitrite. Alternatively, homolysis of LOONO could be followed by rapid re-association of LOO' and 'NO₂, within the solvent cage, to form LOONO₂. The mechanistic details of how 'NO inhibits lipid peroxidation have yet to be clearly established.

Here we have investigated the effect of different rates of 'NO release on copper(II) sulfate, ABAP and peroxynitrite-dependent LDL oxidation. Oxidation of LDL by copper(II) sulfate was inhibited most effectively by PNN and SNN, the order of effectiveness being SNN > PNN > DNN > MNN (Figs. 2–4). This indicates a biphasic relationship between the rate of 'NO production and the efficacy of 'NO as an antioxidant. This relationship was observed at all concentrations of the NONOate compounds tested (Fig. 4). It is interesting to note that when concentrations as high as 12 μM of MNN and DNN were used, they were still less effective than 4 μM SNN.

When oxidation was initiated with ABAP, the order of antioxidant efficiency was SNN > DNN > PNN > MNN (Figs. 5 and 6), again showing a biphasic relationship between the rate of 'NO release and its antioxidant efficacy. A comparison between Figs. 2 and 5 indicates that copper(II) sulfate-dependent oxidation is a more rapid process than ABAP-dependent oxidation. When oxidation occurred at a slow rate (i.e. with ABAP), a slower rate of 'NO release was most effective at inhibiting LDL oxidation. In contrast, when oxidation occurred more rapidly (i.e. with copper(II) sulfate), a faster 'NO release rate was most effective. This suggests that there is an optimal rate of 'NO production for maximal suppression of LDL oxidation, and that this optimal rate is dependent upon the rate of oxidation.

The Effect of 'NO Release Rate on α-Tocopherol Depletion—The depletion of α-tocopherol is a sensitive measurement of oxidation. ABAP-dependent depletion of α-tocopherol was inhibited by SNN and PNN but not by MNN and DNN (Fig. 7). Although DNN did not inhibit α-tocopherol depletion, it was a potent inhibitor of LDL oxidation (Figs. 5 and 6). This suggests that the level of 'NO generated by DNN is unable to compete with α-tocopherol as a peroxyl radical scavenger but is still sufficiently high to inhibit LDL oxidation after α-tocopherol has been consumed.

Kinetic Model of α-Tocopherol Depletion—The effect of 'NO on ABAP-dependent α-tocopherol depletion can be modeled by the following set of reactions (see Table II for k values).

\[
\begin{align*}
&k_1 & (\text{ABAP} - \text{initiating radical (LOO')}} \\
&k_2 & (\text{NONOate} - 2' \text{NO}) \\
&k_3 & (\text{LOO}’ + ' \text{NO} - \text{LOONO}) \\
&k_4 & (O_2 + ' \text{LOO}’ + LH - \text{LOO} + ' \text{LOOH}) \\
&k_5 & (\text{LOO}’ + ' \text{TH - LOOH} + ' \text{TH}) \\
&k_6 & (\text{LOO} + ' \text{TH - products}) \\
&k_7 & (\text{'NO - ?})
\end{align*}
\]

**REACTIONS 1–7**

Reaction 1 describes the formation of radicals that are responsible for the initiation of oxidation. k₁ is a pseudo-zero order rate constant that was experimentally determined from the rate of α-tocopherol depletion in the absence of 'NO (36). This constant includes both the initial rate of ABAP decomposition and the inefficiencies in peroxynitrite radical generation. The decay of NONOate is represented in Reaction 2, where one molecule of NONOate generates two molecules of 'NO. The inhibition of lipid peroxidation by 'NO is most likely due to the diffusion limited reaction between 'NO and LOO’, shown in Reaction 3. Inhibition may also occur via a direct reaction between 'NO and the ABAP-derived peroxyl (Reactions 1 and 3); such reactions have been observed by electron paramagnetic resonance (37).

The propagation of lipid peroxidation (Reaction 4) is the sum of both the rate-limiting hydrogen abstraction from an unsaturated lipid and the rapid reaction between the lipid radical and oxygen. Reactions 5 and 6 show the peroxyl radical trapping reactions of both α-tocopherol and the α-tocopheroxyl radical. Reaction 7 encompasses all of the additional reactions, which result in the removal of 'NO from the solution, such as reactions with oxygen, or partitioning into the atmosphere. The values used for each rate constant are shown in Table II.

The set of differential equations generated from Reactions 1–7 (see "Appendix") can be solved using numerical methods to give the time course for each species in the reaction scheme. Fig. 9 shows a plot of the time required for total α-tocopherol depletion as a function of k₂ (the rate constant for NONOate decay). This model agrees with the experimental data, if k₂ is set to 2.5 s⁻¹, and shows a biphasic dependence on the rate of 'NO formation. The rate of 'NO formation at which maximal inhibition of α-tocopherol depletion is observed (k₂max) depends strongly on the rate of oxidation (k₁). The model predicts a linear relationship between k₁ and the optimal value for k₂max (Fig. 10). As the rate of oxidation increases, the rate at which 'NO is most effective at inhibiting oxidation also increases.

Therefore, one can predict the extent of the antioxidant effect of any 'NO donor compound by determining the rate of oxidation and the rate of 'NO release from the donor. Many spontaneous 'NO donor compounds, such as NONOate and NOR (39) compounds, are commercially available with easily determined rates of release.

An interesting prediction of this model is that the maximal inhibition of lipid hydroperoxide formation will occur at a k₂ lower than k₂max (Fig. 9). This provides a kinetic explanation for the inhibition of lipid peroxidation by DNN (Fig. 5) when DNN...
LDL oxidation varied with the rate of oxidation. This indicates the rate of oxidation of LDL. The extent of the antioxidant effect, however, NONOate compounds conferred an inhibitory effect upon the ABAP-mediated LDL oxidation. SIN-1 decomposes to form stoichiometric amounts of NO and superoxide in the presence of oxygen (29), effectively making it a peroxynitrite donor. Fig. 8 shows that SIN-1 oxidized α-tocopherol to α-tocopherol quinone, the two-electron oxidation product (31–32). NO inhibited SIN-1-dependent α-tocopherol oxidation, and this inhibition had a biphasic dependence on the rate of NO release. Our data are consistent with a direct reaction between NO and peroxynitrite. In support of this, NO was shown to react with peroxynitrite, with a rate constant of 9.1 × 10^8 M^(-1) s^(-1) (40).

CONCLUSIONS

This investigation has shown that the release of NO from all NONOate compounds conferred an inhibitory effect upon the oxidation of LDL. The extent of the antioxidant effect, however, exhibited a biphasic dependence upon the rate of NO release. The rate of NO formation that was most effective at inhibiting LDL oxidation varied with the rate of oxidation. This indicates that there is an optimal rate of NO production for maximal suppression of LDL oxidation, and that this optimal rate is dependent upon the rate of oxidation. We conclude that the antioxidant capability of NO is critically dependent not only on the rate of its release from NO donors, but also upon the relative rate of lipid oxidation. The slow generation of NO by the vascular endothelium may represent a continuous source of antioxidant, playing an integral role in suppressing oxidative reactions within the vasculature. Impairment of NO generation or acceleration of the rate of oxidation may be a critical component in both the early stages and the development of atherosclerosis.

APPENDIX

The following differential equations were constructed from Reactions 1–7. This set of differential equations is not analytically soluble. Therefore we have used numerical methods to calculate the kinetics of each species. Initial concentrations were: NONOate, 10 μM; αTH, 0.9 μM.

\[
\frac{d[LOO]}{dt} = k_1 - k_2[NO][LOO] - k_3[LOO][αTH] - k_4[LOO][αT] \quad (Eq. 1)
\]

\[
\frac{d[NO]}{dt} = -2k_2[NONOate] - k_3[LOO][NO] - k_4[NO] \quad (Eq. 2)
\]

\[
\frac{d[TH]}{dt} = -k_4[LOO][TH] \quad (Eq. 3)
\]

\[
\frac{d[LOONO]}{dt} = k_4[LOO][NO] \quad (Eq. 4)
\]

\[
\frac{d[LOOH]}{dt} = k_3[LOO][αTH] + k_6[LOO][αT] + k_7[LOO][αT] \quad (Eq. 5)
\]

\[
\frac{d[αTH]}{dt} = -k_3[LOO][αTH] \quad (Eq. 6)
\]

\[
\frac{d[αT]}{dt} = k_7[LOO][αT] - k_6[LOO][αT] \quad (Eq. 7)
\]

\[
\frac{d[αT]}{dt} = k_7[LOO][αT] \quad (Eq. 8)
\]

\[
\frac{d[NONOate]}{dt} = -k_2[NONOate] \quad (Eq. 9)
\]

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