Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric oxide donors

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Abstract We have previously shown that nitric oxide donors inhibit the oxidation of low density lipoprotein (LDL) initiated by copper ions or by azo-*bis*-amidinopropane (Hogg et al., 1993. *FEBS Lett.* **334:** 170–174). In this study, the nitric oxide donors S-nitroso-N-acetylpenicillamine (SNAP), spermine NONOate, and sodium nitroprusside were tested for their ability to inhibit macrophage-dependent oxidation of LDL. SNAP and spermine NONOate inhibited macrophage-dependent oxidation of LDL in a time- and concentration-dependent manner. We propose that nitric oxide is acting as a chain-breaking antioxidant that can inhibit the progression of lipid peroxidation in cell dependent-oxidation of LDL. By this mechanism nitric oxide could be an endogenous defense against atherogenesis. In contrast, sodium nitroprusside enhanced cell-mediated oxidation of LDL by a mechanism dependent on superoxide production and transition metal ions. Sodium nitroprusside also enhanced LDL oxidation by cell culture medium alone by a similar mechanism. The use of sodium nitroprusside as a nitric oxide donor in cellular systems appears to be complicated by the release of iron leading to an enhanced oxidative stress. Thus the effects of sodium nitroprusside in such systems may be unrelated to nitric oxide release.–**Hogg, N., A. Struck, S. P. A. Goss, N. Santanam, J. Joseph, S. Parthasarathy, and B. Kalyanaraman.** Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric oxide donors. *J. Lipid Res.* 1995. **36:** 1756–1762.

Supplementary key words atherosclerosis · lipid peroxidation · macrophages

The production of free radicals by vascular cells may be related to the formation and progression of pathological states. In the case of atherosclerosis, a strong argument has been made for a causative role of free radicals in the formation of an atherosclerotic lesion (1). This hypothesis is based on the observation that the oxidative modification of low density lipoprotein (LDL), a process that may be initiated by vascular free radicals, results in the formation of a modified form of the lipoprotein that is potentially atherogenic. Such oxidative modification can be accomplished by incubation of LDL with many of the different cell types associated with the artery wall such as endothelial cells, smooth muscle cells and macrophages (2, 3).

The identification of endothelium-derived relaxing factor as nitric oxide has led to the investigation of the role of this free radical in LDL oxidation. It has been reported that the simultaneous generation of nitric oxide and superoxide causes oxidative modification of LDL, whereas, the release of either free radical alone does not (4). Macrophages, stimulated to induce nitric oxide synthase and thus generate nitric oxide, are less able to oxidize LDL than unstimulated cells (5–7). We have previously reported that nitric oxide donors can inhibit LDL oxidation initiated by either copper (II) ions or 2,2′-azo-*bis*-2-amidinopropane hydrochloride (ABAP) (8).

Malo-Ranta et al. (9) have recently shown that the nitric oxide donor GEA 3162 inhibits the oxidative modification of LDL by endothelial cells. In this study we show that nitric oxide donors are also efficient inhibitors of macrophage-mediated oxidation of LDL. One exception is sodium nitroprusside which enhances macrophage-dependent LDL oxidation by an iron-dependent process.

Abbreviations: ABAP, 2,2′-azo-*bis*-2-amidinopropane hydrochloride; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; DME-F12, Dulbecco's modified Eagle's medium nutrient mixture F12 HAM; LDL, low density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RAW cells, RAW 264.7 macrophages; SNAP, S-nitroso-N-acetylpenicillamine; SNN, spermine NONOate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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MATERIALS AND METHODS

Materials

S-nitrosoacetylpenicillamine (SNAP) was synthesized as previously described (10). LDL was isolated from human plasma as previously described (11). Bovine erythrocyte Cu/Zn superoxide dismutase (SOD) was purchased from Boehringer Mannheim (Germany). Sodium nitroprusside, butylated hydroxytoluene (BHT), and diethylenetriaminepentaacetic acid (DTPA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Spermine NONOate (SNN) was purchased from Cayman Chemicals (Ann Arbor, MI). Thiobarbituric acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypsin-EDTA, ferrozine, RPMI 1640, and Dulbecco's modified Eagle's medium nutrient mixture F12 HAM (DME-F12) were purchased from Sigma Chemical Company (St. Louis, MO). Desferrioxamine was purchased from Ciba-Geigy Corp. (Summit, NJ).

Mouse peritoneal macrophages

Mouse peritoneal macrophages were harvested from female, virgin, Swiss Webster mice (18 g) 3 days after peritoneal injection of 3% thioglyclolate (2.5 ml), by lavage with phosphate-buffered saline (10 mM phosphate, 125 mM NaCl, pH 7.4). Cells were plated at a density of 3 million cells per 2 cm^3 well and left overnight to adhere to RPMI 1640 containing fetal bovine serum (10%). Non-adherent cells and serum were removed by washing twice with DME-F12. All oxidation experiments were performed in DME-F12.

RAW 264.7 macrophages (RAW cells)

RAW cells were purchased from the American Type Culture Collection and grown in 75 ml tissue culture flasks in RPMI 1640 containing fetal bovine serum (10%) in an incubator regulated with 5% carbon dioxide and 95% air. Once confluent, the cells were washed twice with phosphate-buffered saline and incubated with trypsin-EDTA until the cells had become unattached (usually < 5 min). RPMI 1640 containing 10% serum (10 ml) was added and the cells were precipitated by centrifugation. After resuspension in RPMI 1640 (10% serum) the cells were plated at a density of 2 million cells/2 cm^3 well and incubated overnight before washing with DME-F12. All oxidation experiments were performed in DME-F12. Cell viability was determined using MTT as described in Sigma product literature. Incubation of RAW cells with the compounds used in this study reduced cell viability by less than 5% after 24 h incubation.

B4 endothelial cells

The B4 line of endothelial cells was grown to confluence in 60 mm dishes in Ham's F10 medium containing 10% fetal calf serum. Cells were washed with 3 × 2 ml of F10 medium and oxidation was performed in 2 ml serum-free F10.

Assays of LDL oxidation

TBARS were determined as previously described (12). Lipid hydroperoxides were determined iodometrically as previously described (13).

Iron release

Iron release was detected using ferrozine, a chelator of Fe^{2+} ions. Ferrozine was incubated with sodium nitroprusside in DME-F12 in the presence and absence of RAW cells and superoxide dismutase. The formation of the iron complex was monitored spectrophotometrically at 562 nm (ε_{562} = 27,900 $M^{-1}cm^{-2}$) (14).

Statistics

Significant difference was assessed by the Student's *t*-test.

RESULTS

LDL (100 μg/ml) was incubated with mouse peritoneal macrophages in serum-free DME-F12 for 24 h after which time the LDL was assayed for TBARS and lipid hydroperoxide (**Fig. 1**). Both TBARS and lipid hydroperoxide are products of LDL-lipid oxidation. TBARS are primarily formed from the oxidation of fatty acids with more than two double bonds (15); whereas, lipid hydroperoxides are formed from all polyunsaturated fatty acids. As the majority of polyunsaturated fatty acids in LDL are linoleate molecules (16) a greater yield of lipid hydroperoxide than TBARS is always observed. Purified LDL, before addition to cells, contained < 1 nmol/mg protein of TBARS and < 5 nmol/mg protein of lipid hydroperoxide. After 24 h incubation with mouse peritoneal macrophages, LDL showed a dramatic increase in both lipid hydroperoxide and TBARS content (Fig. 1) as has been previously reported (17). LDL incubated under identical conditions in the absence of cells showed a smaller increase in TBARS and lipid hydroperoxide. This agrees with earlier observations that LDL will slowly oxidize when incubated in medium alone (2).

Inclusion of the nitric oxide donors, SNAP (500 μM) and sodium nitroprusside (500 μM), had variable effects on cell-dependent and cell-independent oxidation of LDL (Fig. 1). SNAP inhibited the accumulation of TBARS and lipid hydroperoxide in the presence and absence of mouse peritoneal macrophages, suggesting that this nitric oxide donor is able to prevent the oxidation of LDL. The inclusion of sodium nitroprusside resulted in a modest but significant increase in lipid hydroperoxide formation in both the presence and

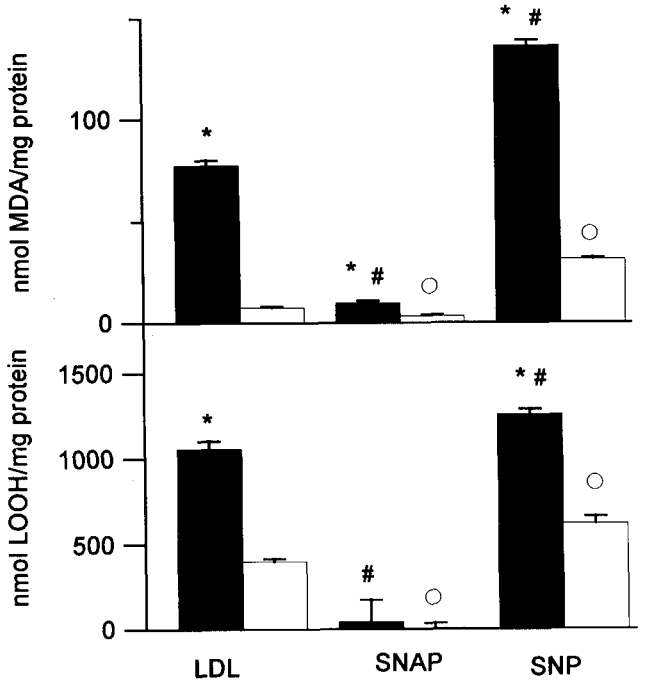

Fig. 1. The effect of nitric oxide donors on the oxidation of LDL by mouse peritoneal macrophages. LDL (100 μg/ml) was incubated with mouse peritoneal macrophages (■) or without mouse peritoneal macrophages (□) for 24 h at 37°C in DME-F12, in the presence of SNAP (500 μM) or sodium nitroprusside (SNP, 500 μM). Samples were quenched with BHT (20 mM) and DTPA (1 mM) and assayed for either TBARS or lipid hydroperoxide. Data represent mean ± SD (n = 3). (*P < 0.005 vs. cell free, #P < 0.005 vs. LDL with mouse peritoneal macrophages and ○P < 0.005 vs. LDL without mouse peritoneal macrophages using the Student's t-test).

absence of mouse peritoneal macrophages. However, sodium nitroprusside caused a significant increase in TBARS formation in both cell-mediated and cell-free oxidation of LDL (Fig. 1). SNAP, SNN, and sodium nitroprusside had no intrinsic TBARS nor did they affect the TBARS assay performed using either authentic malondialdehyde or auto-oxidized LDL.

Incubation of LDL with RAW cells, a mouse macrophage cell line, also resulted in the formation of TBARS indicating that these cells are also able to stimulate LDL oxidation (**Fig. 2A**). SNAP and SNN inhibited the formation of TBARS in a concentration-dependent manner. A concentration of 400 μM SNAP and 300 μM SNN was required to completely inhibit TBARS production. At lower concentrations SNAP, but not SNN, enhanced LDL oxidation. The mechanism for this effect is as yet unclear. Neither N-acetylpenicillamine (500 μM) nor spermine (500 μM) affected macrophage-dependent LDL oxidation. As with mouse peritoneal macrophages, the inclusion of sodium nitroprusside resulted in a dramatic stimulation of TBARS production (Fig. 2A). This stimulation was concentration-dependent with a maximum effect at 200 μM. The levels of TBARS were higher than would be expected for complete oxidation of LDL (usually between 80–150 nmol mg/LDL) indicating that under these conditions it is possible that cellular lipids are contributing to the observed TBARS formation. LDL is, however, important in this process as incubation of sodium nitroprusside with cells alone did not result in any appreciable TBARS formation (data not shown). It is interesting to note that incubation of sodium nitroprusside with LDL in the absence of cells also resulted in TBARS formation (Fig. 2B). Thus the mechanisms responsible for sodium nitroprusside-

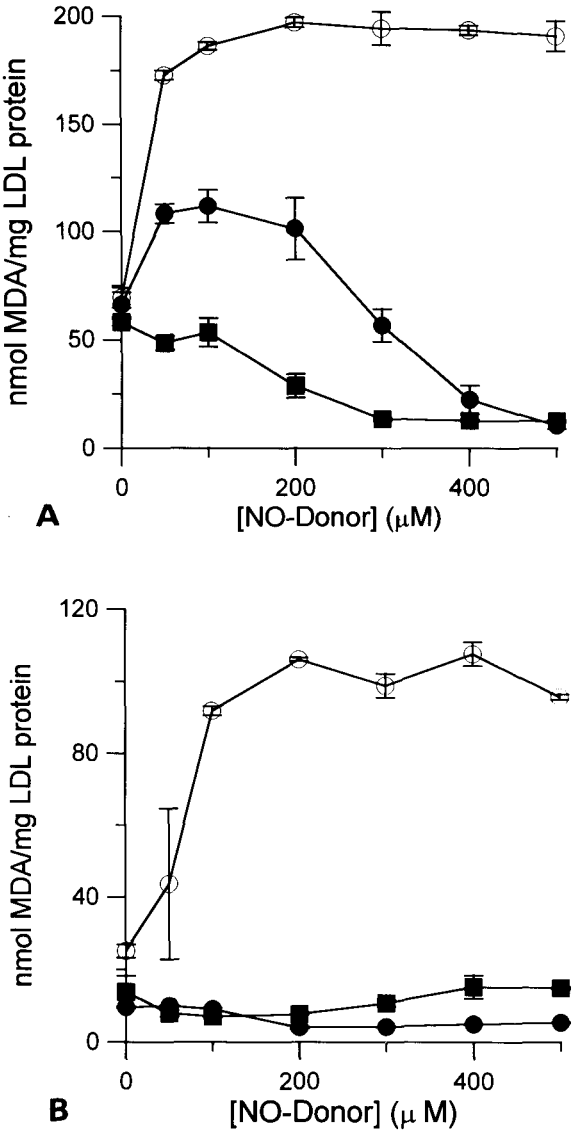

Fig. 2. The effect of nitric oxide donors on the oxidation of LDL by RAW cells. LDL (100 μg/ml) was incubated with (A) or without (B) RAW cells at 37°C for 24 h in DME-F12 in the presence of SNAP (●), spermine NONOate (■), or sodium nitroprusside (○). Samples were quenched with BHT (20 mM) and DTPA (1 mM) and assayed for TBARS. Data represent mean ± SD (n = 3).

stimulated LDL oxidation occur in the cell culture medium alone.

The oxidation of LDL by B4 endothelial cells was inhibited by SNAP (92% inhibition with 1 mM) and SNN (76% inhibition with 1 mM). This is in agreement with the previous observation that GEA 3162, a nitric oxide releasing agent, inhibited LDL oxidation by endothelial cells (9). Sodium nitroprusside increased LDL oxidation by B4 endothelial cells (208% of control at 1 mM) indicating that the stimulatory effect of sodium nitroprusside is independent of cell type.

Figure 3 shows the kinetics of TBARS formation during LDL oxidation by RAW cells. After a lag-time of about 9 h, oxidation of LDL lipid occurred. TBARS production reached a maximum at 18 h. The inclusion of SNAP (200 μM) extended the lag-time to 15 h after which oxidation occurred. SNN (200 μM), however, inhibited oxidation for 21 h. The presence of sodium nitroprusside (100 μM) decreased the lag-time to about 3 h after which extensive oxidation occurred. Maximum oxidation was greater in the presence of sodium nitroprusside than in its absence. The data in Fig. 3 have been corrected for cell-free experiments and for this reason oxidation appears less than in Fig. 2.

Sodium nitroprusside-stimulated oxidation of LDL by RAW cells was completely inhibited by DTPA (100 μM) and desferrioxamine (100 μM) indicating that oxidation

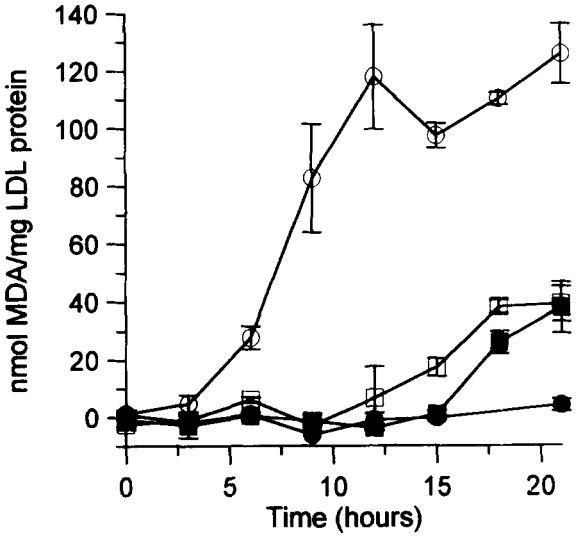

Fig. 3. The kinetics of RAW cell-dependent LDL oxidation in the presence of nitric oxide donor compounds. LDL (100 μg/ml) was incubated with or without RAW cells in DME-F12 in the absence (□) or presence of spermine NONOate (200 μM, ●), SNAP (200 μM, ■), or sodium nitroprusside (○, 100 μM). The medium was removed at the appropriate time point and the oxidation was quenched by the addition of BHT (20 mM) and DTPA (1 mM). TBARS concentration was measured and results were calculated as nmol MDA/mg protein. Data have been corrected for oxidation in the absence of cells, by subtraction, and represent mean ± SD (n = 3).

was dependent on transition metal ions. After the incubation of LDL with RAW cells in the presence of desferrioxamine and sodium nitroprusside, it was observed that the medium became strongly yellow in color. The chromophore had a maximum absorbance at 410 nm and was identified as the Fe^{3+}-desferrioxamine complex. In incubations containing superoxide dismutase, the color was not observed (data not shown). Incubation of RAW cells and sodium nitroprusside (0.1 mM) and LDL (100 μg/ml) in the presence of ferrozine, a chelator of Fe^{2+}, resulted in the formation of the ferrozine-iron complex. The time course for the formation of the ferrozine-iron complex is shown in **Fig. 4A.** Incubation of RAW cells with sodium nitroprusside (100 μM) resulted in the release of 25 μM Fe^{2+} after 21 h. Release of Fe^{2+} was also observed during the incubation of sodium nitroprusside in medium alone. SOD (100 μg/ml) partially inhibited both cell-dependent and cell-independent iron release. Oxidation of LDL under these conditions is shown in Fig. 4B. As before, sodium nitroprusside dramatically stimulates LDL oxidation after a lag period of only 3 h. However, SOD (100 μg/ml) inhibits LDL oxidation at all time points. Incubation of RAW cells with sodium nitroprusside (100 μM) in the presence of ferrozine resulted in no detectable iron release, indicating that nitric oxide production was not responsible for the observed iron release in this system. However, the cells cannot be completely discounted as an additional source of iron when incubated with sodium nitroprusside.

DISCUSSION

Macrophages stimulated with lipopolysaccharide and interferon-γ were shown to have a reduced ability to oxidize LDL (6, 7). This observation was attributed to the induction of nitric oxide synthase and the formation of nitric oxide as the nitric oxide synthase inhibitors MDL 100,248 and N^G-monomethyl-L-arginine were shown to reverse this effect. In the present study we show that the addition of various nitric oxide donors to unstimulated macrophages also prevented these cells from oxidizing LDL. This inhibition was observed with three different nitric oxide donors that exhibited different mechanisms of nitric oxide release. Spermine NONOate will spontaneously release nitric oxide in aqueous solution (18). Each molecule of spermine NONOate liberates two molecules of nitric oxide and a molecule of spermine. The half life of this compound is about 40 min at 37°C and pH 7.4 (19) and yet it was able to inhibit LDL oxidation for 24 h. This suggests either that the compound is potent at low concentrations or that the presence of the compound early in the time course of oxidation is inhibitory to later oxidation.

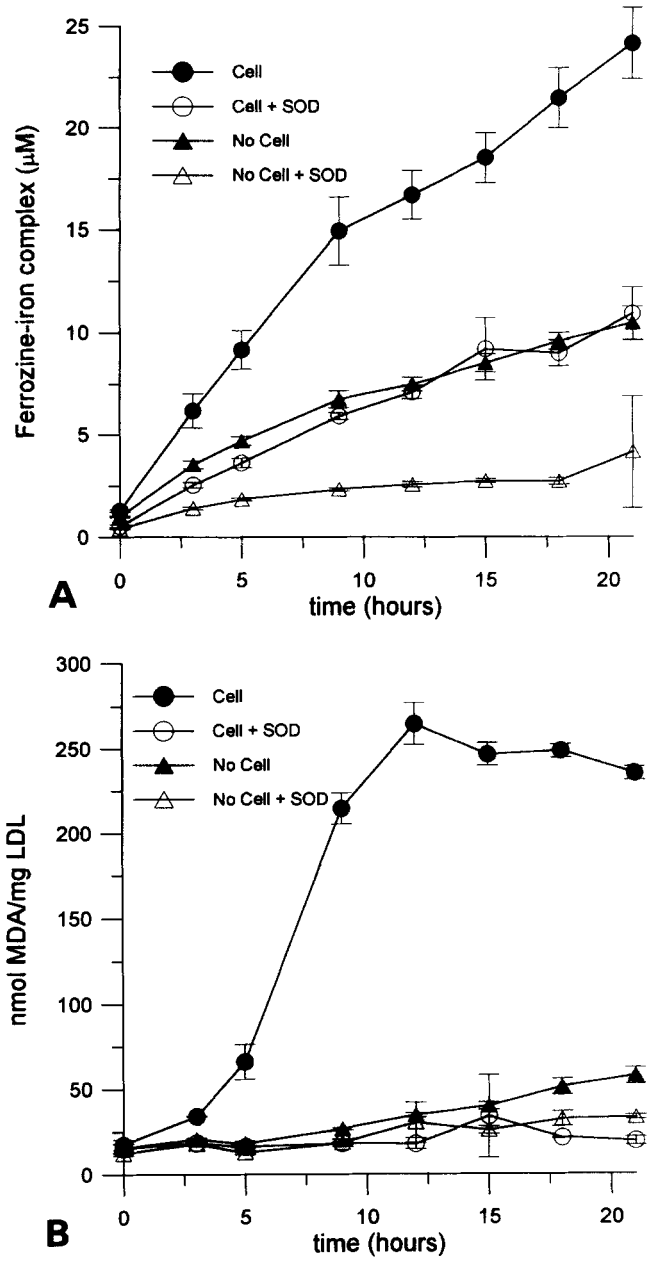

Fig. 4. The effect of SOD on sodium nitroprusside-stimulated LDL oxidation. LDL (100 μg/ml) was incubated with (circles) or without (triangles) RAW cells at 37°C in DME-F12 medium containing sodium nitroprusside (100 μM) in the presence (open symbol) and absence (closed symbol) of SOD (100 μg/ml). For iron detection, an identical experiment was performed in the presence of ferrozine (1 mM). Samples were quenched with BHT (20 mM) and DTPA (1 mM, TBARS only) and assayed for ferrous iron (top) or TBARS (bottom). Data represent mean ± SD (n = 3).

SNAP will not liberate nitric oxide spontaneously but requires the presence of trace amounts of transition metal ions (20, 21). These are present as an intrinsic part of DME-F12 and so SNAP will liberate nitric oxide in both the presence and absence of cells.

We have previously shown that compounds that liberate nitric oxide are able to inhibit copper(II) ion- and ABAP-mediated oxidation of LDL and suggested that nitric oxide can directly scavenge peroxyl radicals and is thus able to stop the propagation of lipid peroxidation (8). We show here that cell-dependent oxidation of LDL, which is absolutely dependent on the propagation of lipid peroxidation, is inhibited by the presence of nitric oxide donors. Therefore, we suggest that nitric oxide is also acting as a peroxyl radical scavenger in this system.

Nitric oxide will react with organic peroxyl radicals with a rate constant of $1\text{–}3 \times 10^9$ $M^{-1}s^{-1}$ (22). The reaction between peroxyl radicals and α-tocopherol has a rate constant of 5×10^5 $M^{-1}s^{-1}$ (23). Thus, nitric oxide could be as effective an inhibitor of lipid peroxidation as α-tocopherol at a concentration in the order of 10^4 times lower. Furthermore, nitric oxide is a hydrophobic molecule and will partition favorably into LDL-lipid. Presumably, being freely diffusible within the LDL particle, nitric oxide will be amenable to all peroxyl radicals regardless of their orientation with respect to the antioxidant functional group or their location within the LDL particle. It is conceivable that the inhibition of macrophage-dependent LDL oxidation by nitric oxide donors occurs at the site of the LDL particle by the same mechanism as the inhibition of cell-free oxidation. It is, however, possible that nitric oxide donors are acting to prevent the initiation of LDL oxidation by either scavenging cell-derived oxidants or by inhibiting their formation as discussed by Jessup (24). A careful analysis of lipid oxidation products in the presence of nitric oxide will help our understanding of this process. The production of adducts between nitric oxide and linolenic acid during lipid peroxidation has recently been described by Rubbo et al. (25).

Sodium nitroprusside (or sodium pentacyanonitrosylferrate(II) dihydrate) is a complex of ferrous iron with cyanide and nitric oxide. Sodium nitroprusside does not spontaneously liberate nitric oxide, but requires either the presence of light or a reducing agent (26). We have previously shown that sodium nitroprusside will not affect LDL oxidation by copper(II) ions unless it is illuminated, in which case it is inhibitory (8). We show here that in the presence of cells sodium nitroprusside exhibits a dramatic pro-oxidant effect. This effect is inhibited by superoxide dismutase, DTPA, and desferrioxamine suggesting that the mechanism of oxidation involves superoxide and transition metal ions (e.g., iron). Our data suggest that superoxide released from the macrophage plays an essential role in liberating iron from sodium nitroprusside which can be chelated in both the Fe^{2+} and Fe^{3+} forms. The iron is then able to oxidize LDL by a mechanism that probably involves endogenous hydroperoxides of LDL and/or hydroper-

oxides generated by the cells. Superoxide dismutase partially inhibits the production of nitrite from sodium nitroprusside in DME-F12 in the presence and absence of RAW cells. This implies a role for superoxide in the liberation of nitric oxide from sodium nitroprusside under these conditions and it is plausible that nitric oxide release and superoxide formation occur at a similar rate. Thus nitric oxide released from sodium nitroprusside could combine with superoxide generated by the cells to generate peroxynitrite which then initiates LDL oxidation (4). This mechanism, however, seems unlikely to be a major factor as peroxynitrite oxidation of LDL is not dependent on transition metal ions. However, peroxynitrite has been shown to sensitize LDL to transition metal ion-dependent oxidation (27). The ability of sodium nitroprusside to significantly enhance LDL oxidation raises questions about the specificity of the use of this compound as a nitric oxide donor. It is likely that the addition of sodium nitroprusside as a nitric oxide donating agent (see refs. 28, 29) is complicated by an increased oxidative stress due to the accumulation of iron.

In conclusion, we show that two structurally unrelated nitric oxide donors with different mechanisms of nitric oxide release are able to inhibit the oxidation of LDL by macrophages. We postulate that this inhibition is a result of the scavenging of lipid peroxyl free radicals. The ability of nitric oxide to inhibit LDL oxidation is highly relevant to atherosclerosis as one of the first manifestations of atherosclerosis is a reduction in endothelium-derived relaxing factor activity (30). This may mean that nitric oxide is no longer available as a vascular antioxidant. Consequently, it is possible that oxidative modification of LDL is enhanced leading to a progression of the atherosclerotic lesion. In support of this, it has been recently demonstrated that chronic administration of nitric oxide synthase inhibitors exacerbates lesion development in animal models of atherosclerosis (31, 32). Moreover, L-arginine, the biological precursor of nitric oxide, has been shown to alleviate endothelial dysfunction in hypercholesterolemic humans (33) and to possess antiatherogenic properties in vivo (34).

This research was supported by National Institutes of Health grants RR01008 and HL47250 from the National Heart, Lung, and Blood Institute.

Manuscript received 21 November 1994 and in revised form 1 May 1995.

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