Superoxide and peroxynitrite in atherosclerosis

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Communicated by Irwin Fridovich, July 26, 1993

ABSTRACT The role of reactive oxygen species in the vascular pathology associated with atherosclerosis was examined by testing the hypothesis that impaired vascular reactivity results from the reaction of nitric oxide ('NO) with superoxide (O2•–), yielding the oxidant peroxynitrite (ONOO•–). Contractility studies were performed on femoral arteries from rabbits fed a cholesterol-supplemented diet. Cholesterol feeding shifted the EC50 for acetylcholine (ACH)-induced relaxation and impaired the maximal response to ACh. We used pH-sensitive liposomes to deliver CuZn superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) to critical sites of NO reaction with O2•–. Intravenously injected liposomes (3000 units of SOD per ml) augmented ACH-induced relaxation in the cholesterol-fed group to a greater extent than in controls. Quantitative immunocytochemistry demonstrated enhanced distribution of SOD in both endothelial and vascular smooth muscle cells as well as in the extracellular matrix. SOD activity in vessel homogenates of liposome-treated rabbits was also increased. Incubation of β very low density lipoprotein with ONOO•– resulted in the rapid formation of conjugated dienes and thiobarbituric acid-reactive substances. Our results suggest that the reaction of O2•– with NO is involved in the development of atherosclerotic disease by yielding a potent mediator of lipoprotein oxidation, as well as by limiting NO stimulation of vascular smooth muscle guanylate cyclase activity.

Endothelium-dependent relaxation is impaired in vessels from atherosclerotic patients (1, 2) and hypercholesterolemic animal models (3–6), suggesting the functional modification of endothelium-derived relaxing factor (EDRF) in hyperlipidemia. The dynamic role of the endothelium in the regulation of vascular tone was established when it was observed that relaxation of isolated blood vessels by vasoactive agents, such as acetylcholine (ACH) and the calcium ionophore A23187, was dependent on an intact endothelium and a diffusible factor (EDRF) that stimulated cGMP-dependent relaxation of vascular smooth muscle cells (VSMCs; ref. 7). Nitric oxide (NO) and EDRF share similar chemical and pharmacological properties (8) and are derived from the oxidation of a terminal guanidino group of L-arginine (9, 10).

Numerous mechanisms have been suggested for the defect in vascular relaxation in atherosclerosis and hypercholesterolemic animal models. They include an increased diffusion barrier for NO due to intimal cell proliferation and lipid deposition (11), L-arginine depletion (3, 12, 13), altered endothelial cell (EC) receptor coupling mechanisms (14), and inactivation of NO by superoxide (O2•–; refs. 15 and 16). Reactive oxygen species are potent pathologic mediators in atherosclerosis as well as other vascular diseases (17, 18). Both O2•– and hydroxyl radical ('OH) contribute to the oxidative modification of low density lipoproteins [i.e., low density lipoprotein, very low density lipoprotein (LDL, VLDL); ref. 19], a critical event in development of the atherosclerotic lesion (20). In addition, reactive oxygen species have been implicated in the alterations of EC-dependent relaxation observed in atherosclerosis (21, 22).

Peroxynitrite (ONOO•–), a product of NO reaction with O2•–, has recently been defined as a potent oxidant and potential mediator of vascular tissue injury (23–27). We postulate that both the independent reactions of O2•– and NO and their reaction yielding ONOO•– are critical in the initiation and maintenance of the atherosclerotic state and contribute to the defect in vasorelaxation. The present studies suggest common mechanisms underlying lipoprotein oxidation and impaired vascular relaxation of hypercholesterolemic rabbits.

MATERIALS AND METHODS

Materials. Bovine CuZn superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) was from Diagnostic Data Inc. (Mountain View, CA). Dioleoylphosphatidyl ethanolamine and dioleoylglycerol-3-succinate were obtained from Avanti Polar Lipids. L-Arginine, ACh, indomethacin, papaverine, phenylephrine, and tetraethylammonium hydroxide were from Sigma.

Vessel Contraction Studies. New Zealand White rabbits (2.5–3.0 kg) were maintained on rabbit chow containing 1% cholesterol (Ralston Purina) for 6 months prior to study [cholesterol-fed (Chol-fed) group]. Age- and weight-matched controls were fed a standard diet. After exsanguination under ketamine/rompun anesthesia, vessels were isolated and changes in tension were measured in femoral artery ring segments as described (28). After maximal contraction with 70 mM KCl and recovery, phenylephrine was added to achieve 30% of maximal tone. Rings were then exposed to increasing doses of ACh; relaxation is reported as the percentage decrease in preexisting tone. After the generation of cumulative ACh dose–response curves, rings were exposed to 30 μM papaverine. In some experiments, rings from control and Chol-fed rabbits were incubated with 3 mM L-arginine for 30 min prior to administration of ACh. In other studies, vessels were treated with native bovine SOD (200 units/ml) before measuring ACh-induced relaxation. All studies were performed in the presence of 5 μM indomethacin.

Abbreviations: ACh, acetylcholine; SOD, superoxide dismutase; VLDL, very low density lipoprotein; Lip-SOD, liposome-entrapped SOD; EDRF, endothelium-derived relaxing factor; VSMC, vascular smooth muscle cell; EC, endothelial cell; LDL, low density lipoprotein; Chol-fed, rabbits fed a 1% cholesterol diet; TBARS, thiobarbituric acid-reactive substances.

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Liposome-Entrapped SOD (Lip-SOD). Liposomes were composed of dioleoylphosphatidylethanolamine and dioleoylglycerol-3-succinate (1:1). Lipids were dried under N₂ and hydrated 36 hr in 210 mM sucrose/7 mM Hepes. During hydration, pH 8.5 was maintained with tetraethylammonium hydroxide. Lipids were added to SOD and dissolved in sucrose/Hepes buffer, and the mixture was extruded through a 600-nm filter under N₂ pressure (Extruder, Lipex Biomembranes, Vancouver, BC Canada); mean lipidosome diameter was 217 nm. Final SOD concentration was 3000 units/ml. Lip-SOD was injected daily (1500 units/kg) via the marginal ear vein for 5 days before sacrifice.

Analytical Procedures. Plasma cholesterol levels were determined by an enzymatic method (29) modified for 96-well plates. Aortic SOD activity was assayed in a 10% homogenate in 50 mM KPO₄/0.1 mM EDTA/0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, pH 7.8. After centrifugation at 10,000 × g for 10 min at 4°C, supernatant SOD activity was measured by inhibition of oxime dioxygenase-mediated reduction of cytochrome c (30). Tissue DNA was measured by fluorescence (31). Aortic segments were prepared for quantitative electron microscopic immunocytochemistry as described (32). Ultrathin cryosections were incubated with rabbit anti-bovine CuZn SOD (1:100 dilution) and 10-nm gold colloid conjugated to protein A. Distribution of SOD labeling was measured by counting gold granules and tissue points of randomly selected photographic fields. Because of variable loss of endothelium during cryosectioning, EC SOD density was quantitated in selected segments with intact endothelium.

β-VLDL Oxidation. Peroxynitrite was synthesized as described (23). β-VLDL, isolated as described (33), was exposed to ONOO⁻ or 5 μM CuSO₄. Lipid oxidation was assessed by measurement of thiobarbituric acid-reactive substances (TBARS) and formation of conjugated dienes (25).

RESULTS

Plasma cholesterol was markedly elevated in rabbits fed a 1% cholesterol diet for 6 months, from 43 ± 6 to 2696 ± 292 mg/dl. Intimal thickening was apparent, with extensive plaque deposition, ≈50% of the luminal surface of the femoral artery.

Control and Chol-fed groups exhibited no differences in the maximal tone generated with 70 mM KCl or in the phenylephrine concentrations required to achieve submaximal contraction of vessels. Chol-fed rabbits manifested a significant shift in the EC₅₀ to ACh-induced relaxation: 1.73 μM vs. 0.0521 μM (P < 0.01) as well as a 33% reduction in maximal response (Fig. 1A). Incubation of ring segments with L-arginine failed to augment ACh-induced relaxation in Chol-fed rabbits (Fig. 1B). Pretreatment with native CuZn SOD had a minor effect on the ACh dose–response profile in control but not Chol-fed rabbits (Fig. 2A). Due to the limited cellular uptake of native SOD, we delivered SOD in vivo via pH-sensitive liposomes. Vessels isolated from both control and Chol-fed rabbits treated for 5 days with Lip-SOD demonstrated enhanced ACh-induced relaxation (Fig. 2B). Lip-SOD restored ACh-induced relaxation in Chol-fed rabbits close to control values (EC₂₀, 0.165 μM). In addition, the ΔEC₂₀ for ACh-induced relaxation was greater (P < 0.01) in the Chol-fed group than in the control group. Furthermore, Lip-SOD treatment enhanced the maximum relaxation of segments to ACh in Chol-fed rabbits. Treatment of both control and Chol-fed rabbits with empty pH-sensitive liposomes had no effect on ACh responses.

There was no difference between groups in the response to papaverine, an endothelial-independent vasodilator; vessels relaxed below the initial vessel tone measured before phenylephrine administration, 114.52% ± 3.13% (control) vs. 116.35% ± 6.51% (Chol-fed). Lip-SOD had no effect on responses to papaverine in either group.
Table 1. Vascular enzymatic activity and tissue distribution of CuZn SOD delivered via pH-sensitive liposomes

<table>
<thead>
<tr>
<th>Measurement</th>
<th>- SOD liposomes</th>
<th>+ SOD liposomes</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Chol-fed</td>
</tr>
<tr>
<td>Enzymatic activity</td>
<td>0.92 ± 0.13</td>
<td>1.06 ± 0.43</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelium</td>
<td>0.70 ± 0.02</td>
<td>1.53 ± 0.27**</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>0.99 ± 0.08</td>
<td>1.88 ± 0.11**</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>0.40 ± 0.12</td>
<td>1.18 ± 0.14**</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.31 ± 0.06</td>
<td>1.16 ± 0.32**</td>
</tr>
<tr>
<td>Elastin</td>
<td>1.95 ± 0.16</td>
<td>2.43 ± 0.33</td>
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SOD activity is expressed as mean ± SD of aortae from three animals from each group. Morphometric data are means ± SD from analysis of four sites from each treatment group. Because there was no difference in SOD distribution between control and Chol-fed groups, the groups were combined and analyzed for treatment with and without Lip-SOD. *, P < 0.01; **, P < 0.05.

1Unit(s) of SOD per µg of DNA.
2Point density of colloidal gold particles × 10−2 (relative ratio of no. of immunogold particles per tissue point).

Quantitative electron microscopic immunocytochemistry assessed the distribution of SOD in the blood vessel wall. Lip-SOD localized not only in the endothelium but also in the subendothelial space and in underlying VSMCs (Table 1; Fig. 3). A small degree of crossreactivity was evident between rabbit anti-bovine SOD and endogenous rabbit SOD. However, multivariate analysis of labeling densities showed significant increases in labeling in sections from Lip-SOD-injected rabbit vessels compared with controls (Table 1). SOD activity was similar in vessel homogenates from control

FIG. 3. Electron micrographs of aortic segments from control and Lip-SOD-treated rabbits. (a) A small degree of crossreaction of the anti-bovine CuZn SOD to rabbit CuZn SOD was observed in the ECs from control rabbits. (b) Labeling of ultrathin cryosections with anti-bovine SOD (arrowheads) demonstrates enhanced SOD distribution in the endothelium, smooth muscle, and extracellular matrix of the Lip-SOD-treated animal. m, Mitochondria; d, desmosome; Col, collagen fibrils; El, elastin. (Bars = 0.5 µm.)
and Chol-fed rabbits (Table 1). Lip-SOD led to an almost 2-fold increase in vessel SOD activity in both groups.

Exposure of β-VLDL to ONOO− resulted in rapid generation of lipid oxidation products as indicated by both TBARS and conjugated diene formation (Fig. 4). Control experiments demonstrated that up to 100 μM NO alone did not stimulate lipid peroxidation.

**DISCUSSION**

The present studies suggest that defective EC-dependent relaxation in a rabbit model of hypercholesterolemia is due in part to the endothelial depletion of EDRF/NO with O2. Numerous mechanisms have been postulated for the impaired EC-dependent relaxation of hypercholesterolemic blood vessels. Intimal thickening as a barrier to EDRF does not appear to be crucial, as cessation of a high cholesterol diet restores EC-dependent relaxation in Cynomolgus monkeys despite the continued presence of thickened intima (34).

Vessels from Chol-fed rabbits did not differ in their response to an EC-independent vasodilator, in agreement with other investigators (21), suggesting that the ability of VSMCs to relax is intact. Other reports propose that impaired EC-dependent relaxation is due to L-arginine depletion, the substrate for NO synthesis (3, 12, 13). However, we and others (35) were unable to restore vascular relaxation in arterial segments pretreated with arginine. Further evidence against L-arginine depletion includes chemiluminescent techniques demonstrating that the basal release of NO and its metabolites are increased in the aorta of hypercholesterolemic rabbits (36). This suggests that EDRF/NO synthesis is elevated and that impaired relaxation may be due to enhanced inactivation of EDRF/NO, leading to a reduction in its vasoactive properties.

Reactive oxygen species have been implicated in both the pathogenesis and altered physiologic responses of atherosclerosis. Oxidation of LDL, a critical event in atheroma formation, is associated with enhanced cellular production of O2− (37, 38). The efficacy of probucol in the prevention of intimal lesion development in Watanabe hyperlipidemic rabbits is related to its antioxidant properties (39). In addition, the presence of oxidized LDL in atheromatous plaques correlates with progression of atherosclerotic carotid disease (40). Oxidized LDL has also been shown to directly inhibit EC-dependent vasorelaxation (41, 42). Generation of O2− in situ reduces the endothelial-dependent relaxation in normal vessels (16, 43) and may be involved in the abnormal EC-dependent relaxation in atherosclerosis (22). Indirect measures of excess production of reactive oxygen species in hypercholesterolemia have been reported, including elevated levels of cholesterol oxides and oxidant-modified proteins (44, 45). However, the precise role of oxidants and their critical reaction sites in the initiation of lipoprotein oxidation and impairment of vascular function in atherosclerotic processes remains unclear.

We suggest that a cholesterol-enriched environment enhances vascular production of O2−. This excess O2− can then react with NO, reducing the vasoactive levels of NO and diminishing the response to EC-dependent vasodilators. In this case, normal or even elevated levels of SOD may be insufficient to effectively scavenge the excess O2−. This is suggested by the slightly higher endogenous levels of SOD measured in Chol-fed animals. The hypercholesterolemic state may lead to increased expression of vessel SOD (45, 46), but in our studies it was not significantly different from controls. Impaired EC-dependent relaxation may then be due to enhanced reaction of NO with O2−, yielding ONOO− (23). This reaction is rapid, with a rate constant of k = 6.7 × 109 M−1s−1, faster than both the SOD-catalyzed and spontaneous dismutation of O2− to H2O2 (47). At physiological pH, ONOO− is protonated to peroxynitrite acid (ONO2H), yielding nitrogen dioxide (NO2) and a molecule with OH-like reactivity (23, 48). We have observed that ONOO− is a less potent activator of VSMC guanylate cyclase than NO (unpublished observations) and suggest that EC-dependent relaxation is impaired in our model due to diminished rates of cGMP formation. Alternatively, the impaired relaxation may be the result of ONOO−-derived OH, which stimulates guanylate cyclase activity to a lesser extent than NO (49).

Peroxynitrite exhibited potent oxidative effects on β-VLDL, the principal carrier of cholesterol in this model. When compared to 5 μM Cu2+, a commonly used in vitro lipoprotein oxidant (50), ONOO− rapidly generated greater quantities of lipid peroxidation products. Similar results have been observed using the syndomin, SIN-1, a compound yielding NO and O2−, which react to form ONOO− (51). Thus, the product of NO and O2− reaction may play a critical role in the initiation and extension of atherosclerotic lesions as well as the altered vascular reactivity associated with the disease.

These studies also demonstrate that pH-sensitive liposomes are effective vectors for the delivery of antioxidant enzymes to the wall of both normal and atherosclerotic vessels. Pharmacologic efficiency of Lip-SOD, as indicated by enzymatic analysis and changes in vascular function, contrasts with the minimal effect of native SOD. It is not unexpected that native SOD did little to restore EC-dependent relaxation. The 32-kDa SOD is electrostatically repelled from cell surfaces at pH 7.4 and is thus excluded from intracellular compartments, where significant extents of both the production and reactions of O2− and NO will occur.
pH-sensitive liposomes facilitated delivery of SOD to internal sites of O$_2^-$ production, thus lowering steady state O$_2^-$ concentration and limiting O$_2^-$ reaction with NO to yield ONOO-". Once injected intravenously, liposomes gain direct contact with the blood vessel wall and become incorporated via endocytosis. In the acidic environment of the endosome (pH 5.0), the liposomal membrane undergoes a phase transition (52), promoting liposome--endosome fusion and release of liposomal contents to the cytosol. An increase in SOD content was demonstrated in both control and Chol-fed liposome-injected rabbits. Parallel immunocytochemical quantitation of SOD distribution reveals that SOD gained access not only to ECs but to VSMCs and the interstitium as well. These locales are all critical sites of excess production of oxidants associated with the development and maintenance of atherosclerosis. Thus, Lip-SOD delivery is particularly effective in raising tissue levels of the enzyme. Polyethylene glycol-derivatized SOD (PEG-SOD) to enhance vascular endothelial antioxidant enzyme levels has been previously used (53). However, higher doses of PEG-SOD (41,000 units·kg$^{-1}$·d$^{-1}$ vs. 1500 units·kg$^{-1}$·d$^{-1}$) were used to achieve similar results in an animal model of atherosclerosis (54).

In summary, our findings suggest that an imbalance in vascular O$_2^-$ and NO arises in response to a hyperlipidemic environment. Impaired vascular relaxation results in part from the reaction of NO with O$_2^-$. The ONOO- formed as a product of this reaction can then participate in the pathogenesis of the atherosclerotic lesion in two ways. First, impaired vascular reactivity results from the production of ONOO-, which acts as a relatively weak stimulus for guanylate cyclase activity in VSMCs. Second, ONOO- can initiate lipoprotein oxidation, which then contributes to the production of the fatty streak and subsequent plaque formation characteristic of the atherosclerotic lesion, and may also contribute to altered EC-dependent vascular relaxation.

We wish to thank Drs. J. S. Beckman and M. Winn for their technical expertise. This work was supported by National Institutes of Health Grants PO1-HL 48676, RO1-HL 41180, and T32-HL 07457 and by grants from the Alabama Affiliate, American Heart Association. C.R.W. is the recipient of a Harriet P. Dustan fellowship from the Alabama Affiliate, American Heart Association; M.M.T. is the recipient of a Parker B. Francis Fellowship for Pulmonary Research.