Chronic Treatment With Polyethylene-Glycolated Superoxide Dismutase Partially Restores Endothelium-Dependent Vascular Relaxations in Cholesterol-Fed Rabbits

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The endothelium-derived relaxing factor is rapidly inactivated by superoxide radicals, and atherosclerotic vessels generate excess radical species. We tested the hypothesis that an imbalance between intrinsic superoxide dismutase (SOD) activity and the generation of superoxide radicals in atherosclerotic arteries may result in augmented inactivation of endothelium-derived relaxing factor. Vascular SOD was increased in normal and cholesterol-fed (1% cholesterol for 4 months) rabbits approximately twofold by treatment with polyethylene-glycolated SOD (PEG-SOD; 41,000 units/kg/day i.m.) for 1 week. Aortic rings from these animals and nontreated control and atherosclerotic rabbits subsequently were studied in organ chambers. Endothelium-dependent relaxations to acetylcholine and the calcium ionophore A23187 were improved by PEG-SOD in atherosclerotic but not in normal rabbits. PEG-SOD pretreatment did not alter endothelium-independent relaxations to nitroprusside. Thus, treatment with PEG-SOD can partially restore impaired endothelium-dependent relaxation of atherosclerotic arteries. We conclude that generation of oxygen-derived radicals likely contributes to endothelial dysfunction of atherosclerotic arteries. (Circulation Research 1991;69:1293–1300)

Endothelium-dependent relaxation is impaired in cholesterol-fed rabbits,1–3 primates,4 and atherosclerotic human coronary arteries.5,6 Bioassay studies have suggested that the mechanism underlying this impairment of endothelial function is reduced production of endothelium-derived relaxing factor (EDRF) or release of a defective EDRF.7,8 Recent studies have shown that the production of nitrogen oxides (NO and one-electron oxidation products) from hypercholesterolemic and atherosclerotic rabbit aorta is markedly enhanced rather than impaired.9 These findings indicate that the enzyme responsible for production of NO and related compounds is not impaired by hypercholesterolemia and that there is not a deficiency of substrate or cofactors necessary for NO production, but that the endothelium-derived NO is released in an inactivated, non-vasoactive form. This observation raises the possibility that the endothelial dysfunction in cholesterol-fed rabbits may be related to augmented degradation of EDRF.

There is excess generation of reactive radical species including oxygen-derived free radicals in atherosclerotic arteries.10,11 EDRF in physiological buffer solution is destroyed rapidly by radical-generating systems such as xanthine/xanthine oxidase and is protected by superoxide dismutase (SOD), suggesting that EDRF is vulnerable to superoxide radicals.12,13 Inhibition of endothelial copper-zinc SOD, which scavenges superoxide anions14 and is part of the cellular antioxidant defense mechanism, also inactivates EDRF.15 Thus, one mechanism for abnormal endothelium-dependent vascular relaxation in
atherosclerosis may relate to an imbalance between intrinsic SOD activity and the generation of superoxide radicals. To test this hypothesis, we increased vascular tissue levels of SOD in normal and cholesterol-fed rabbits by treating the animals with polyethylene-glycolated SOD (PEG-SOD). After treatment, we studied endothelium-dependent and endothelium-independent relaxations of rabbit aorta in vitro. We anticipated that PEG-SOD might improve endothelium-dependent relaxation in atherosclerotic rabbits.

Materials and Methods

Animals

New Zealand White rabbits of either sex (2.5–3.0 kg) were fed standard rabbit chow (Continental Grain, Chicago) (n = 16) or an identical chow enriched with 1% cholesterol for 4 months (n = 16). As shown previously, this feeding protocol produces typical vascular lesions (fatty streaks with intimal thickening).1–3,7,8 Eight rabbits of each group were chosen at random and received PEG-SOD injections (1,000 units/kg/day i.m.) for 1 week. The dose of PEG-SOD corresponds to 41,000 units/kg/day measured by our nitroblue tetrazolium (NBT) SOD assay. The last injection was given approximately 20 hours before the beginning of the study.

Vascular Responses

Rabbits were killed with sodium pentobarbital (80 mg/kg i.v.). To prevent blood coagulation, heparin (1,000 units i.v.) was administered simultaneously with the barbiturate. The entire aorta from the aortic arch to the iliac bifurcation was excised and immediately placed in aerated Krebs' buffer of the following millimolar composition: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, EDTA calcium 0.026, and glucose 11.1, pH 7.40. Arterial blood for measurement of cholesterol and triglyceride levels was obtained by cardiac puncture (drawn in tubes containing EDTA). The isolated aorta was cleaned of perivascular tissue, and eight rings (3–4 mm long) were cut from the thoracic portion beginning at the aortic arch. The rest of the aorta was used for the determination of SOD activity.

The aortic rings were suspended in individual organ chambers (20 ml) filled with Krebs' buffer. The solution was aerated continuously with 95% O2–5% CO2 and maintained at 37°C. Tension was recorded with a linear force transducer (model FTO3c, Grass Instrument Co., Quincy, Mass.). Over a period of 1 hour, the resting tension was gradually increased and the artery was frequently exposed to 80 mM KCl until the optimal tension for generating force during isometric contraction was reached. The vessels were left at this resting tension throughout the remainder of the study. To prevent the synthesis of vascular prostaglandins, we performed all experiments in the presence of 10 μM indomethacin.

Vessels were preconstricted with L-phenylephrine (1 μM). After a stable contraction plateau was reached (at approximately 10 minutes), all rings were exposed cumulatively to acetylcholine (0.01–3 μM). The vessels were washed three times with fresh buffer and allowed to equilibrate for 30 minutes. At the end of each experiment, three rings were randomly selected to be exposed to the calcium ionophore A23187 (0.01–1 μM), three others to sodium nitroprusside (0.01–3 μM), and two rings to phenylephrine (0.01–10 μM).

Histological Procedures

At the end of the organ bath study, the rings were immersed in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) without their being dismounted from the force transducer and were maintained in fixative overnight. After fixation, the vessels were cut longitudinally to expose the intimal surface, were postfixed with 1% osmium tetroxide, and were prepared for scanning electron microscopy (Hitachi, S-4000). Vessel segments from cholesterol-fed animals were randomly selected (at least two from each animal) and were examined at ×600 magnification (5.0 keV). Scanning electron microscopy revealed a generally intact endothelium in both normal and atherosclerotic vessels. Although there were occasional areas of endothelial denudation, probably because of experimental handling of the vessels, these were present to similar extents in normal and atherosclerotic aortas.

For the assessment of atherosclerosis, one vessel ring from each animal that was not used in organ bath studies was immersed in 7% buffered formaldehyde (pH 7.4) for 24 hours and stained with hematoxylin and eosin or according to Verhoeff and van Gieson for light microscopy.

Determination of Vascular Superoxide Dismutase Activity

The segment of aorta not used for the organ chamber studies was placed in 1 ml ice-cold 0.05 M phosphate buffer (pH 7.8). The adventitia was stripped off, and the aorta was homogenized for 30 seconds with a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio) and then was sonicated for 30 seconds with a Vibra-Cell sonicator. Homogenates were centrifuged (700g, 10 minutes, 4°C). Supernatant was stored at −28°C until use. Protein was measured with the method of Bradford.16

SOD activity was determined by the method of Spitz and Oberley.17 Briefly, xanthine/xanthine oxidase was used to generate superoxide anions that are detected by the reduction of NBT to blue formazan. The assay spectrophotometrically (DU-70, Beckman Instruments, Fullerton, Calif.) measured the rate of blue formazan formation in the presence of tissue homogenates. Addition of increasing amounts of tissue inhibited the reduction of NBT. One unit of SOD activity is defined in this assay as that amount of protein that causes half-maximal inhibition of NBT.
reduction. Manganese SOD activity was measured by including 5 mM NaCN (30 minutes) in the assay mixture. Copper-zinc SOD activity was calculated by subtracting the manganese SOD activity value from the total SOD value.

**Immunoblotting**

Immunoblotting was carried out as described by Spitz et al.\textsuperscript{18} Briefly, equal amounts of protein were applied to 15% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, the protein was transferred to nitrocellulose sheets by the method of Towbin et al.\textsuperscript{19} Additional protein binding sites on the nitrocellulose sheets were blocked by incubation overnight in 10% fetal calf serum and 2% bovine serum albumin. The blots then were incubated with whole rabbit antisera that had been raised in our laboratory against bovine liver copper-zinc SOD. Bands were visualized by incubation with a second antibody directed against rabbit IgG that was conjugated to alkaline phosphatase and developed in the presence of NBT and 5-bromo-4-chloro-3-indolyl phosphate.

**Determination of Plasma Cholesterol and Triglyceride Levels**

Blood samples obtained from cardiac puncture were promptly centrifuged for measurement of plasma cholesterol and triglyceride levels (automatic analyzer, Abbott Spectrum, Abbott Laboratories, Chicago).

**Materials**

Acetylcholine, calcium ionophore A23187, sodium nitroprusside, xanthine, xanthine oxidase, catalase, and NBT all were obtained from Sigma Chemical Co., St. Louis, Mo. Diethylenetriaminepentaacetic acid and disodium bathocuproine disulfonic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis. PEG-SOD from bovine erythrocytes was obtained from Sigma (2,800 units/mg protein). A23187 was dissolved in dimethyl sulfoxide, the final concentration of which did not exceed 0.1%. Stock solutions of phenylephrine (0.01 M) were prepared in 0.001N HCl containing ascorbic acid (1 mg/ml). Sodium nitroprusside was protected from light. Dilutions were made in Krebs’ buffer immediately before the experiments. Indomethacin was dissolved in phosphate buffer and diluted in Krebs’ solution.

**Calculations and Statistics**

Data are presented as mean±SEM. Responses to vasodilators in the organ bath studies were expressed as percent relaxation from the maximal effect of phenylephrine. Responses from vascular segments were averaged for one animal, and the number of experiments refers to the number of animals. Statistical significance was tested using unpaired \( t \) test. A value of \( p<0.05 \) was considered significant.

**Results**

At the time of the study, rabbits pretreated and untreated with PEG-SOD were not different with respect to body weight, plasma cholesterol, or plasma triglyceride levels (Table 1). Plasma cholesterol and triglyceride levels were significantly higher in atherosclerotic rabbits than in rabbits fed a standard diet (Table 1).

**Effect of PEG-SOD Treatment on Vascular Superoxide Dismutase**

In normal animals treated with PEG-SOD, the amount of copper-zinc SOD identified by immunoblotting was qualitatively increased compared with untreated controls (Figure 2). Immunoblotting for copper-zinc SOD in the atherosclerotic rabbit aortas revealed an increase in an additional immunoreactive band as compared with normal animals.

The total SOD activity in the homogenate of aortic tissue was 120±32 units/mg protein for normal (\( n=8 \)) and 123±33 units/mg protein for cholesterol-fed rabbits (\( n=8, p>0.05 \)). Treatment with PEG-SOD for 1 week increased total SOD activity approximately twofold in both normal and cholesterol-fed animals (Figure 3).

Total SOD activity is reported because manganese SOD activity was very low or undetectable in most samples, and increases in total SOD activity in PEG-SOD–treated animals were due to an increase in the copper-zinc form of SOD.

**Vascular Responses In Vitro**

Endothelium-dependent relaxations in response to acetylcholine and the calcium ionophore A23187 were markedly impaired in atherosclerotic rabbits (Figures 4 and 5). Maximal relaxations in response to acetylcholine and A23187, respectively, were 80.3±2.0% (\( n=6 \)) and 89.8±2.3% (\( n=5 \)) in normal rabbits, and 43.3±6.6% (\( n=8 \)) and 54.3±6.3% (\( n=8 \)) in atherosclerotic rabbits. Treatment of atherosclerotic rabbits with PEG-SOD improved the maximal relaxation in response to acetylcholine (65.4±2.0%, \( n=6, p<0.05 \)) and to A23187 (73.1±3.2%, \( n=6, p<0.05 \)) as compared with untreated atherosclerotic

### Table 1. Body Weight and Plasma Cholesterol and Triglyceride Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (kg)</th>
<th>Plasma cholesterol (mg/dl)</th>
<th>Plasma triglyceride (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard diet</td>
<td>4.0±0.2</td>
<td>57±13</td>
<td>47±12</td>
</tr>
<tr>
<td>Standard diet, PEG-SOD</td>
<td>4.1±0.1</td>
<td>59±12</td>
<td>41±12</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td>3.6±0.2</td>
<td>1,859±236*</td>
<td>302±96*</td>
</tr>
<tr>
<td>Cholesterol-fed, PEG-SOD</td>
<td>3.8±0.1</td>
<td>2,071±177*</td>
<td>384±98*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. PEG-SOD, polyethylene-glycolated superoxide dismutase.

\( *p<0.01 \) vs. rabbits fed with standard diet (\( n=8 \) for each group).
FIGURE 1. Cross section of thoracic aorta from normal rabbit (left panel) and rabbit fed 1% cholesterol diet for 4 months (right panel). Verhoeff and van Gieson stain, ×40. Marked intimal thickening is present in cholesterol-fed rabbits involving approximately 40% of the vessel circumference.

animals. In contrast, treatment of normal rabbits with PEG-SOD did not alter the response to either acetylcholine or A23187 (Figures 4 and 5).

Endothelium-independent relaxation in response to nitroprusside was not different in normal and atherosclerotic animals. PEG-SOD pretreatment did not alter the response to nitroprusside (Figure 6).

The vasoconstrictor response to phenylephrine was slightly reduced in atherosclerotic rabbits (Figure 7). Interestingly, this impaired relaxation to phenylephrine was not observed in atherosclerotic rabbits treated with PEG-SOD (Figure 7).

Discussion

The present study shows that treatment of cholesterol-fed rabbits with PEG-SOD for 1 week improves the impaired endothelium-dependent vasorelaxation in response to acetylcholine and A23187. Importantly, similar treatment had no effect on endothelium-dependent vascular relaxations in normal animals. This finding has important implications regarding the mechanism of endothelial dysfunction in cholesterol-fed rabbits and suggests a potential role for antioxidant therapy in atherosclerosis.

As shown in previous studies, fatty streak formation and intimal thickening, with a marked impairment of endothelium-dependent relaxation in vitro. The beneficial effect of PEG-SOD treatment on endothelial dysfunction in hypercholesterolemic rabbits is unlikely to be due to anatomic improvement after PEG-SOD. The duration of treatment was short, and the extent of lesions did not appear grossly different. Furthermore, scanning electron microscopy revealed no obvious difference in the percent area covered with endothelium in the groups. This finding excludes the potential artifact that the preparation technique for organ bath studies preferentially damaged endothelium from untreated rabbits, resulting in exaggeration of endothelial dysfunction in this group. Regions of endothelial denudation were present when the vessels were examined by electron microscopy. However, scanning electron microscopy was performed after the vessels had been removed from the organ chambers, and inadvertent endothelial damage could not be avoided.

SOD does not penetrate endothelial cells. Therefore, PEG-SOD was used to increase tissue levels of SOD. PEG-SOD has been shown to be readily assimilated by cultured porcine endothelial cells in sufficient concentration to afford the cells protection.
against damage induced by xanthine oxidase. It is not clear whether the increase in total SOD activity after PEG-SOD treatment in the present study is due to uptake of PEG-SOD or induction of SOD biosynthesis. Chronic therapy with PEG-SOD was chosen based on preliminary experiments in our laboratory. In these studies, neither 1-day treatment with PEG-SOD (n=2) nor acute administration of SOD in the organ chamber (n=4) improved endothelium-dependent relaxation of atherosclerotic vessels.

EDRF is an unstable factor in aqueous solutions with a short half-life. The degradation rate of EDRF in aerated Krebs' buffer depends on the oxygen tension, and it is decreased by SOD. These findings suggest that superoxide radicals contribute to the instability of EDRF in aqueous solu-

![Superoxide Dismutase and EDRF]

**FIGURE 2.** Representative anti-bovine copper-zinc superoxide dismutase (SOD) immunoblot (out of three experiments). Standard lane (S) contains 0.1 μg bovine liver copper-zinc SOD. Lanes 1-4 contain 50 μl vascular cell homogenate (2 mg protein/ml) from normal rabbits, normal rabbits treated with polyethylene-glycolated SOD (PEG-SOD), cholesterol-fed rabbits treated with PEG-SOD, and cholesterol-fed rabbits, respectively. Two immunoreactive bands are visible. The lower band comigrates with the copper-zinc SOD standard monomer band; the upper band is increased in intensity in cholesterol-fed rabbits. The function of the upper band is unknown.

**FIGURE 3.** Total superoxide dismutase activity in homogenates of aortas obtained from normal and cholesterol-fed rabbits. Open bars represent untreated animals, cross-hatched bars animals treated with polyethylene-glycolated superoxide dismutase (PEG-SOD). n=8 for each group. *p<0.05.

**FIGURE 4.** Effect of treatment with polyethylene-glycolated superoxide dismutase (PEG-SOD) on relaxation induced by acetylcholine in aortas of rabbits fed either normal diet (N) or cholesterol-enriched diet (AS). Vascular rings were precontracted with 1 μM l-phenylephrine. Phenylephrine contractions were 5.5±0.4 g in normal group (n=6), 5.3±0.4 g in normal group treated with PEG-SOD (n=6), 4.7±0.4 g in cholesterol-fed group (n=8), and 5.5±0.4 g in cholesterol-fed group treated with PEG-SOD (n=6). Acetylcholine was given cumulatively. Values are mean±SEM. *p<0.05 vs. PEG-SOD untreated animals.

**FIGURE 5.** Effect of polyethylene-glycolated superoxide dismutase (PEG-SOD) on relaxation induced by calcium ionophore A23187 in aorta in vitro obtained from rabbits fed either normal diet (N) or cholesterol-enriched diet (AS). Vascular rings were precontracted with 1 μM l-phenylephrine. Phenylephrine contractions were 5.6±0.5 g in normal group (n=6), 5.6±0.5 g in normal group treated with PEG-SOD (n=6), 4.8±0.3 g in cholesterol-fed group (n=8), and 5.5±0.4 g in cholesterol-fed group treated with PEG-SOD (n=6). A23187 was given cumulatively. Values are mean±SEM. *p<0.05 vs. PEG-SOD untreated animals.
Results. Recent data from our laboratory suggest that the release of biologically active EDRF can be markedly impaired by inhibition of endothelial SOD activity but not by inhibition of other antioxidant defense mechanisms such as catalase or the glutathione redox cycle. This finding suggests that inactivation of EDRF by intracellularly produced superoxide radicals is prevented by intrinsic SOD activity under normal conditions. Previous studies\(^{10,11}\) and the present experiments, however, strongly suggest that there is an excess generation of oxygen-derived free radicals within atherosclerotic vessels. The source of radicals could be either the endothelial cell itself or resident monocyte-macrophages present within the intima of these vessels. Imbalance between intrinsic SOD activity and excess generation of superoxide radicals could be responsible for augmented inactivation of EDRF, resulting in impaired endothelium-dependent relaxation in atherosclerotic arteries. The finding of the present study that a twofold increase in vascular SOD activity partially restored endothelium-dependent vascular relaxations toward normal in hypercholesterolemic rabbits suggests that this imbalance may be present in cholesterol-fed rabbits. The finding that PEG-SOD treatment did not augment endothelium-dependent relaxations in normal aorta suggests that intrinsic SOD within the endothelium of normal vessels is sufficient to prevent the degradation of EDRF by the comparatively small amount of superoxide anion present. Thus, in contrast to the atherosclerotic aorta, increases in SOD in normal rabbit aorta do not enhance endothelium-dependent vascular relaxations.

On first glance, our findings in normal rabbit aorta seem to be in conflict with prior studies in which SOD prolonged the half-life of EDRF in bioassay.\(^{12,13}\) The differences between the present findings and previous studies likely relate to differences in the experimental preparation. The previous experiments used superfusion bioassay techniques, in which the EDRF was exposed for several seconds, while in transit from the donor vessel to the detector vessel, to an oxygen-enriched buffer solution and superoxide anions generated by the endothelium of the donor tissue. In the present organ chamber studies of normal vessels, the transit of the EDRF from the endothelium to the underlying vascular smooth muscle likely was more rapid and degradation by the superoxide anion less favored.

The present findings may explain in part our previous observations regarding the production of nitrogen oxides by endothelium of atherosclerotic rabbits.\(^9\) In that study, release of nitrogen oxides (NO and one-electron oxidation products of NO, including NO\(_2^-\)), measured by chemiluminescence in the effluent of superfused aorta from hypercholesterolemic and atherosclerotic rabbits, was paradoxically increased, although the vasorelaxant activity (measured by bioassay) was markedly depressed. The augmented release of nitrogen oxides by endothelium of cholesterol-fed animals clearly demonstrated that the activity of the endothelial NO synthetase enzyme was not impaired and that the enzyme had sufficient substrate (L-arginine) and cofactors to function normally. The reduced biological activity strongly suggests that the nitrogen oxides were released in an inactivated, nonvasoactive form such as nitrite. In light of our present findings, it is likely that this process involved degradation of EDRF to nitrite by the superoxide anion.

Another role of PEG-SOD may be to preserve EDRF substrate. It has been suggested that EDRF...
may not be identical with NO² and that one of the nitrosothiols, S-nitroscysteine, shares many of the biological and chemical properties of EDRF.²⁵⁻²⁷ It is conceivable that excess generation of superoxide radicals within the endothelium of atherosclerotic vessels may oxidize sulfhydryl-containing compounds to their disulfide form, thereby reducing the availability of substrate for the formation of such a compound.

PEG-SOD also may have prevented lipid peroxidation within the vascular wall of cholesterol-fed animals. This may reduce the oxidative environment within the vessel wall and thus prevent intracellular degradation of EDRF. Additionally, macrophages within the intima of atherosclerotic vessels may serve as a ready source of superoxide anion. PEG-SOD may have been taken up by these cells and subsequently prevented the degradation of EDRF by decreasing release of superoxide anions into the intimal space.

Endothelial cells in culture increase the biosynthesis of SOD in response to increased oxygen concentration or in the presence of generators of superoxide radical.²⁸ In the present study, we found no difference in total SOD activity in vessels from normal and cholesterol-fed rabbits. A previous study reported an increase in vascular SOD activity by approximately 70% in rabbits fed with 1% cholesterol for 6.5 months.²⁹ The reason for this discrepancy is not clear, but it could be related to the different duration of cholesterol feeding (4 versus 6.5 months). Immunoblotting of vascular tissue from normal and cholesterol-fed animals indicated the presence of two immunoreactive bands. The lower, or fast-moving, band comigrated with the bovine copper-zinc SOD monomer band (Figure 2). The identity of the upper band is not known, but the intensity of this band was greater in cholesterol-fed animals than in controls and was unaffected by treatment with PEG-SOD.

As shown previously, the vasoconstrictor response to α-adrenoceptor agonists was reduced in hypercholesterolemic rabbits.³,³⁰ In the present study, the potency of phenylephrine was reduced slightly in cholesterol-fed rabbits. Of interest was the finding that vasoconstrictor responses to phenylephrine were restored by PEG-SOD treatment in cholesterol-fed rabbits. The mechanism of impaired responsiveness to α-adrenoceptor agonists in atherosclerosis is not known. Several processes may be involved, including altered receptor affinity, changes in uptake or metabolism, or degradation of phenylephrine.

The present studies may have important clinical implications. It has been suggested that endothelial dysfunction contributes to vasospasm in atherosclerosis and may play a role in the development of hypertension in individuals with atherosclerosis.³¹,³² SOD has been used successfully in a variety of other diseases, including bronchopulmonary dysplasia in neonates, Crohn's disorder, chronic cystitis, and cardiac ischemia-reperfusion injury.³³ The present study suggests that therapy with PEG-SOD or other antioxidants may be beneficial in clinical syndromes in which endothelial dysfunction may contribute.

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References


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