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Potentiation of Endogenous Nitric Oxide With Superoxide Dismutase Inhibits Platelet-Mediated Thrombosis in Injured and Stenotic Arteries

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Objectives. **We tested the hypothesis that dismutation of superoxide anion increases endogenous levels of nitric oxide, resulting in inhibition of cyclic variations in blood flow in arteries that are injured and stenotic.**

Background. **Platelet adhesion and aggregation leading to cyclic flow variations might result, in part, from generation of superoxide anion that can deplete endogenously produced nitric oxide.**

Methods. **Spontaneous cyclic flow variations, monitored with a proximal Doppler probe, were induced in the carotid artery of anesthetized rabbits by clamping the vessel with forceps and placing a high grade stenosis at the site of injury. Bovine copper/zinc superoxide dismutase (12 mg/kg body weight, n = 5), a synthetic low molecular weight mimetic (12 mg/kg, n = 8) or buffer vehicle (n = 8) was administered intravenously as divided boluses over 45 min, and the frequency of cyclic flow variations was monitored for 4 h.**

Results. **Cyclic flow variations remained stable for 4 h in vehicle-treated animals (15 ± 1 [mean ± SEM]/30 min at baseline and 16 ± 1/30 min after 4 h, n = 8) but exhibited a marked and persistent reduction in animals given copper/zinc superoxide dismutase (from 14 ± 1/30 min at baseline to 4 ± 1/30 min after 4 h) or the mimetic (from 15 ± 1/30 min at baseline to 3 ± 1/30 min after 4 h, $p < 0.005$). They were restored in three of four mimetic-treated animals during infusion of N^G-monomethyl- L-arginine (100 mg/kg), an inhibitor of nitric oxide production. In addition, levels of cyclic guanosine 5′-monophosphate in platelets were elevated after administration of the mimetic (from 2.4 ± 0.5 fmol/10^6 platelets at baseline to 4.9 ± 0.6 fmol/10^6 platelets 45 min after the mimetic, $p < 0.03$, n = 6), whereas mean arterial blood pressure was decreased and flow velocity in the carotid artery was increased consistent with mediation of the effect on cyclic flow variations by increased endogenous nitric oxide.**

Conclusions. **Dismutation of superoxide anion appears to attenuate platelet thrombus formation at a site of vessel injury by potentiation of endogenously produced nitric oxide. This approach may have utility to inhibit platelet-rich thrombosis in injured and stenotic arteries where production of superoxide anion is increased.**

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Activation of platelets has been shown to play a major role in the pathogenesis of unstable angina (1–3). In experimental animals, transient accumulation and dislogment of platelets in injured and stenotic coronary arteries result in cyclic variations in blood flow (4,5). Analogous cyclic flow variations have been observed in the peripheral arteries of patients with vascular disease and intermittent claudication (6) and in the coronary arteries of patients with unstable angina undergoing angioplasty (7,8). However, administration of aspirin in these patients frequently fails to abolish cyclic flow variations, possibly because it does not prevent intense and sustained activation of platelets in the injured vessel.

Oxygen-derived radicals have been implicated in the generation of cyclic flow variations after vessel injury in experimental animals (9). Superoxide anion elaborated by damaged endothelial cells (10), activated leucocytes (11), and smooth muscle cells rapidly inactivate endothelium-derived relaxing factor, also identified as nitric oxide (12–15), which inhibits platelet adhesion and aggregation under physiologic conditions (16–19). Previous studies (20) have shown that inhibition of nitric oxide production with N^G-monomethyl-L-arginine induces cyclic flow variations in injured and stenotic coronary arteries in dogs. In the same study, administration of L-arginine, a precursor for nitric oxide synthesis, abolished cyclic flow variations.

Superoxide dismutase may protect endogenous nitric oxide from inactivation by scavenging superoxide anion. In vitro, the inhibitory action of nitric oxide on aggregation of platelets as well as their adhesion to endothelium induced by thrombin is

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potentiated by superoxide dismutase consistent with its preventing inactivation of endothelium-derived nitric oxide (12,17,21). This study was designed to test in vivo the hypothesis that superoxide dismutase, or a low molecular weight macrocyclic mimetic of superoxide dismutase, prevents inactivation of endogenously produced nitric oxide, thereby attenuating cyclic flow variations induced by vessel injury and stenosis.

Methods

Animal preparations. Experiments involving animals were performed according to the "Position of the American Heart Association on Research Animal Use" adopted by the Association in November 1984 and were approved by the Animal Studies Committee at Washington University. Forty-two New Zealand white rabbits weighing 2.3 to 2.5 kg were premedicated with intravenous ketamine (20 to 30 mg/kg body weight) and ventilated with oxygen-enriched room air containing 2% isoflurane. A femoral artery was cannulated for measurement of mean arterial blood pressure and withdrawal of blood samples. An ear vein was cannulated for drug administration. Mean arterial blood pressure and the electrocardiogram were monitored continuously with a four-channel recorder (model 2000, Gould Inc.).

A segment of the right common carotid artery was exposed and instrumented with a proximal Doppler probe for measuring flow velocity and frequency of cyclic flow variations. In 39 animals, the carotid artery was gently clamped six to eight times with cushioned forceps at a site 1 cm distal to the Doppler probe. A 2.5-mm long band of polypropylene tubing was placed around the injured segment and constricted until the mean flow velocity just began to decline. This degree of constriction has been shown to reduce the diameter of the carotid artery by ~75% (5). Immediately after placing the stenosis around the vessel, a pattern of cyclic variations in blood flow velocity was induced, characterized by intermittent, spontaneous decreases followed by abrupt increases of flow velocity. By carefully controlling the amount of intimal damage induced initially, cyclic flow variations occurred spontaneously and persistently for at least 4 h without additional manipulation of the stenosis. The remaining three rabbits were not subjected to vessel injury as a control.

Experimental protocol. Thirty minutes after the frequency of cyclic flow variations had stabilized, rabbits with vessel injury were randomly assigned to receive either 8 mg/kg (n = 5) or 12 mg/kg (n = 8) of a novel, synthetic manganese complex of a 15-membered macrocylic ligand that mimics superoxide dismutase (SC 52608, molecular weight 341.2, catalytic rate constant [k_{cat}] = 4.13×10^7 m/s at pH 7.4 [22]) (Monsanto Company); 12 mg/kg (n = 5) of bovine copper/zinc superoxide dismutase (molecular weight 32,500, $k_{cat} = 2.3 \times 10^9$ m/s [23] (Sigma Corp.); 5 mg/kg (n = 5) of a water-soluble form of aspirin (Aspégic, Laboratoires Synthelabo, Paris, France) or 10 μg/kg per min (n = 4) of nitroglycerin over 4 h as positive controls; or buffer (n = 8) as a negative control. Superoxide dismutase and the mimetic were dissolved in 1 mmol/liter of *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) buffer (Sigma Corp.) at pH 7.4 and administered intravenously over 1 min in divided bolus doses of 4 mg/kg at intervals of 15 min. In four additional animals given 12 mg/kg of the mimetic, N^G-monomethyl-L-arginine (100 mg/kg) (Sigma Corp.) was infused over 45 min beginning 1 h after the end of the administration of mimetic. The remaining three animals in whom vessel injury was not induced received 12 mg/kg of the mimetic as a control.

The frequency of cyclic flow variations and the peak and nadir of the flow velocity profiles were observed for 4 h after the onset of administration of agents. Serial blood samples were collected before and 1 and 3 h after the onset of administration of agents for measurement of platelet count and aggregation assessed ex vivo in platelet-rich plasma, activated partial thromboplastin time and prothrombin time. Samples were also obtained before and 45 min after administration of the mimetic for analysis of platelet cyclic guanosine 5′-monophosphate levels in platelet-rich plasma.

In some rabbits, the site of carotid arterial injury was examined by scanning electron microscopy 4 h after the onset of administration of agents. Vessels were perfused in situ through the aorta with 0.2 mol/liter of phosphate-buffered saline followed by a solution of 1% paraformaldehyde and 1.5% glutaraldehyde fixative in phosphate buffer (pH 7.4) at a pressure of 100 mm Hg. Vessels from animals exhibiting cyclic flow variations were perfused consistently at the time of peak flow velocity (i.e., just after a cyclic flow variation was registered). The injured arterial segment was excised and immersed in fixative for an additional 12 h, transferred into phosphate buffer and stored at 4°C before processing.

Hematologic measurements. Arterial blood was drawn into plastic syringes containing 3.8% sodium citrate solution (9 vol of blood/1 vol of sodium citrate). Platelet-rich plasma was obtained by centrifugation of the blood sample at 160 *g* for 10 min at room temperature. Platelet count was measured and adjusted to 3×10^8/ml by addition of platelet-poor plasma obtained by recentrifugation of the remaining plasma at 1,500 *g* for 10 min. Aggregation of the count-adjusted platelet-rich plasma was measured turbidometrically with an aggregometer (Payton Associates). Collagen (4 to 8 μg/ml) (Chronolog) and thrombin (0.5 to 0.75 U/ml) (Chronolog) were used as agonists. The minimal (threshold) concentration of each agonist required to induce ≥50% change of light transmission was determined in baseline samples and then used in serial samples to assess changes in aggregation after treatment. Activated partial thromboplastin and prothrombin times were measured in citrated plasma samples with use of an automated coagulation timer (Coag-a-mate, Organon Teknika), as described by the manufacturer.

Measurement of platelet cyclic guanosine 5′-monophosphate. Cyclic guanosine 5′-monophosphate levels in platelets were measured in platelet-rich plasma to which 1 mmol/liter of 3-isobutyl-L-methyl-xanthine (Sigma Corp.) was added to inhibit phosphodiesterase activity. Samples were frozen in liquid

nitrogen and stored at −70°C before assay. Each was thawed and 250 μl incubated with an equal volume of 20% trichloroacetic acid on ice for 5 min. The mixture was centrifuged at 800 *g* for 5 min and the supernatant removed. Trichloroacetic acid in the supernatant was extracted by four washes with water-saturated ether and the sample evaporated to dryness in a Speed-vac. The sample was reconstituted in buffer and assayed with use of an enzyme immunoassay kit (Amersham). Results were expressed as fmol of cyclic guanosine 5′-monophosphate/10^6 platelets.

Scanning electron microscopy. Vessel segments were post-fixed with 1.0% osmium tetroxide at room temperature, followed by several washes in phosphate buffer. The segments were pinned onto sheets of silicon rubber, dehydrated in an ascending series of ethanol and dried by the critical point method in a Polaron G-3000 critical point dryer. The dried specimens were mounted on aluminum stubs with sticky tabs, rimmed with silver colloidal paint and coated with gold in a Polaron G-5000 sputter coater. They were observed using a Hitachi S-450 scanning electron microscope, operated at 20 kV.

Statistics. All results are expressed as mean value ± SEM. Differences within and between groups over time were evaluated with use of a two-way analysis of variance for repeated measurements incorporating a Duncan's post hoc test to define the significance of observed differences. A p value ≤0.05 was considered significant.

Results

Frequency of cyclic flow variations. Spontaneous cyclic flow variations were induced in all 39 rabbits with vessel injury and stenosis. They were unchanged in frequency and magnitude for 4 h in rabbits given buffer vehicle (Fig. 1), and their incidence was not associated with changes in blood pressure (Fig. 2). Administration of 12 mg/kg of the mimetic of superoxide dismutase inhibited cyclic flow variations significantly from 15 ± 1/30 min at baseline to 3 ± 1/30 min at 4 h. Similar results were obtained with administration of the same dose (on a weight basis) of copper/zinc superoxide dismutase (Fig. 1). A lower dose of mimetic (8 mg/kg) also inhibited cyclic flow variations markedly, but the frequency was partially restored 1 h after drug administration in three of five rabbits. Aspirin inhibited cyclic flow variations comparable to that observed with the high dose of superoxide dismutase (Fig. 1) as did

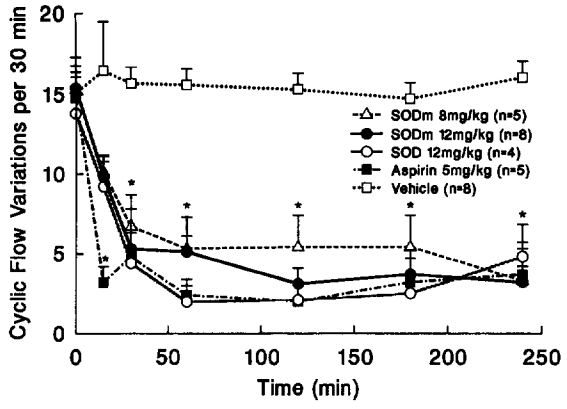
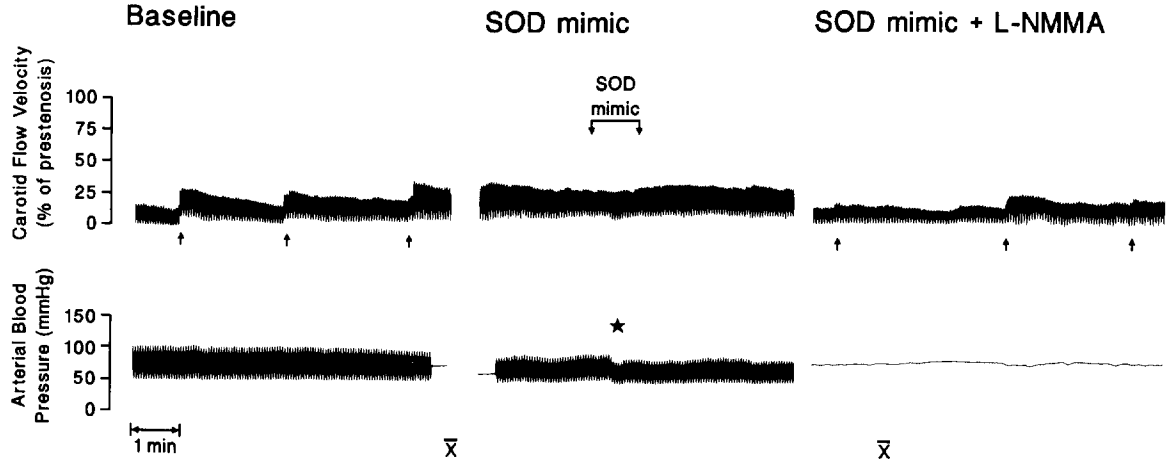


Figure 1. Inhibition of cyclic flow variations after administration of different doses of a mimetic of superoxide dismutase (SODm, boluses of 4 mg/kg at 0 and 15 min for 8 mg/kg and at 0, 15 and 30 min for 12 mg/kg), bovine copper/zinc superoxide dismutase (SOD, boluses of 4 mg at 0, 15 and 30 min), aspirin (bolus at 0 min) or buffer used as the vehicle for superoxide dismutase. **Data points** are values calculated from cyclic flow variations recorded over 30-min intervals. *p < 0.005 compared with baseline and vehicle-treated animals.

Figure 2. Recordings of phasic carotid blood flow velocity and both pulsatile and mean ($\bar{x}$) arterial blood pressure measured simultaneously in an animal after induction of vessel injury and stenosis (Baseline), which resulted in spontaneous cyclic flow variations (**arrows**) during administration of 4 mg/kg of the mimetic of superoxide dismutase (SOD) (total dose of 12 mg/kg) and 30 min after the start of infusion of N^G-monomethyl-L-arginine (L-NMMA). Cyclic flow variations were not associated with changes in blood pressure. Administration of the mimetic induced a transient decrease in blood pressure (**star**) and abolished cyclic flow variations, whereas concomitant administration of the inhibitor of nitric oxide production resulted in the return of cyclic flow variations and increased mean blood pressure.



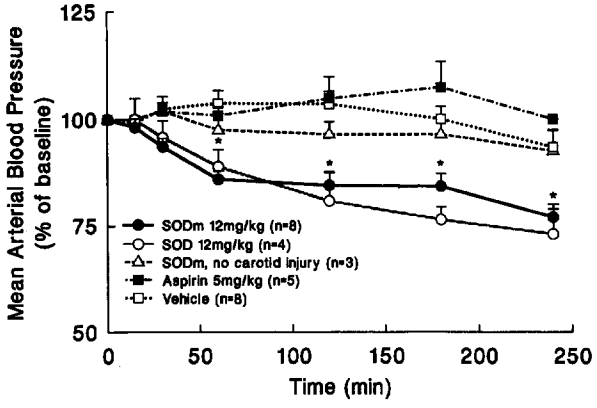


Figure 3. Changes in aortic mean arterial blood pressure expressed as percentages of baseline values after intravenous administration of the mimetic of superoxide dismutase (SODm), either with or without injury and stenosis induced in a carotid artery, or administration of copper/zinc superoxide dismutase, aspirin or vehicle in the presence of vessel injury and stenosis. Values at baseline were 71 ± 3 mm Hg for the mimetic with vessel injury, 65 ± 4 mm Hg for the mimetic without vessel injury, 69 ± 8 mm Hg for superoxide dismutase, 57 ± 5 mm Hg for aspirin and 67 ± 4 mm Hg for vehicle. In the absence of injury, blood pressure changed minimally after administration of mimetic. *$p < 0.001$ compared with baseline values.

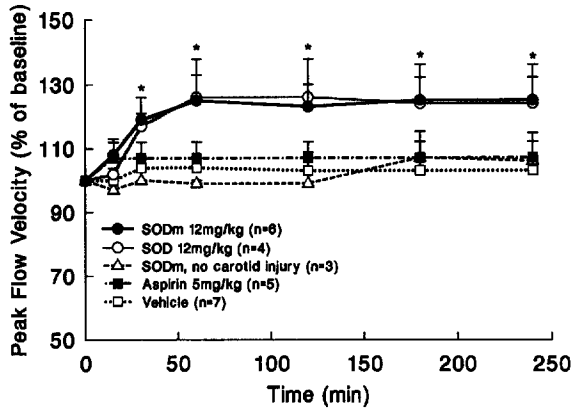


Figure 4. Changes in peak flow velocity in the carotid artery assessed with a proximal Doppler probe after administration of the mimetic of superoxide dismutase (SODm), either with or without distal injury and stenosis of the vessel, or after administration of copper/zinc superoxide dismutase, aspirin or vehicle in the presence of injury and stenosis of the carotid artery. In the absence of vessel injury, peak flow velocity was changed minimally after administration of the mimetic. *$p < 0.05$ compared with baseline values.

continuous infusion of nitroglycerin (from 15 ± 1/30 min at baseline to 1 ± 1/30 min after 4 h, n = 4).

Hemodynamic variables and carotid flow velocity. In the presence of vessel injury and stenosis, bolus injections of the mimetic caused a transient decrease in mean arterial blood pressure of between 10 and 15 mm Hg that recovered within 2 min (Fig. 2). In animals given either superoxide dismutase or the mimetic, a more modest but sustained decrease in blood pressure was observed compared with vehicle- and aspirin-treated animals (Fig. 3). A constant infusion of nitroglycerin also decreased blood pressure from 53 ± 3 to 40 ± 5 mm Hg after 4 h. However, in the absence of vessel injury, blood pressure was decreased minimally after administration of the mimetic (Fig. 3). Heart rate was not changed significantly in any group (data not shown).

Administration of superoxide dismutase or the mimetic induced a sustained increase of peak flow velocity in the injured carotid artery (Fig. 4). Changes were minimal in animals given vehicle or aspirin and in those given the mimetic in the absence of vessel injury (Fig. 4). Nadir flow velocity increased also in injured vessels beginning 30 min after the administration of superoxide dismutase (139 ± 13% of baseline velocity after 12 mg/kg) or the mimetic (145 ± 13% of baseline after 8 mg/kg and 149 ± 8% after 12 mg/kg). Similar effects were observed in animals given nitroglycerin (data not shown).

Hematologic findings. Platelet count was unchanged during experiments except in vehicle-treated rabbits in which the count decreased from $3.0 \pm 0.2 \times 10^8$ platelets/ml at baseline to $2.2 \pm 0.5 \times 10^8$ platelets/ml after 4 h ($p < 0.05$, n = 5). Platelet aggregation in platelet-rich plasma assessed ex vivo in response to added thrombin or collagen was not changed after administration of superoxide dismutase or the mimetic. However, in animals given aspirin, platelet aggregation was decreased from 60 ± 3% transmittance at baseline to 21 ± 9% transmittance 1 h after aspirin and 17 ± 10% after 4 h ($p < 0.05$, n = 5). Activated partial thromboplastin and prothrombin times were not changed significantly in any of the groups of animals (data not shown).

Cyclic guanosine 5′-monophosphate levels in platelets. Levels increased markedly from 2.4 ± 0.5 fmol/10^6 platelets at baseline to 4.9 ± 0.6 fmol/10^6 platelets 45 min after administration of 12 mg/kg of the mimetic of superoxide dismutase ($p < 0.03$, n = 6).

Effects of N^G-monomethyl-L-arginine. Inhibition of nitric oxide production with N^G-monomethyl-L-arginine infused after administration of the mimetic of superoxide dismutase restored cyclic flow variations within 7 to 9 min in three of four rabbits (Fig. 2) and resulted in a decrease in carotid flow velocity in all four (from 130 ± 11% of baseline to 105 ± 6% of baseline after inhibitor, $p < 0.05$). Mean arterial blood pressure was increased from 66 ± 3 to 87 ± 7 mm Hg ($p = 0.05$) during inhibitor infusion. Within 10 min after the end of the infusion of inhibitor, cyclic flow variations were again attenuated, carotid flow velocity increased, and blood pressure decreased as with the mimetic alone. Lower doses of N^G-monomethyl-L-arginine (5 or 40 mg/kg) did not renew cyclic flow variations and did not change carotid flow velocity (data not shown).

Scanning electron microscopy. In nine rabbits, the injured carotid arteries were examined by scanning electron microscopy. Accumulation of platelet-rich thrombus at the site of injury was diminished in animals given the mimetic of super-

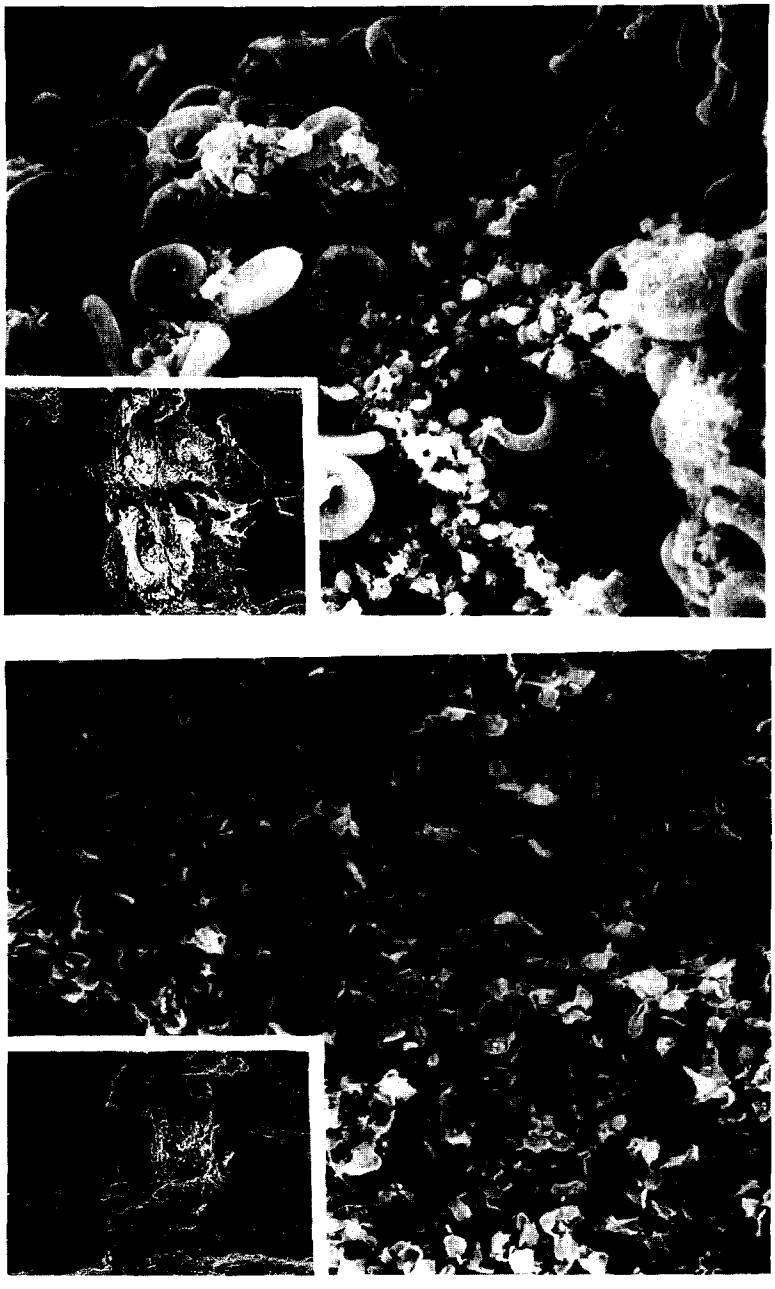


Figure 5. Scanning electron micrographs of injured carotid arteries and associated thrombi from two animals 4 h after the induction of injury. **Top**, Artery from an animal given buffer vehicle. **Inset** is a low magnification of the artery incised longitudinally showing the clamp-injured segment as a transverse band devoid of endothelium and with considerable thrombus attached to the subendothelium. The thrombus (high magnification) comprises platelets, red cells and fibrin. **Bottom**, Artery from an animal given the mimetic of superoxide dismutase. Thrombus is diminished compared with the vehicle-treated animal and comprises primarily platelets.

oxide dismutase (n = 5) as well as in those given aspirin (n = 2) compared with those given vehicle (n = 2) (Fig. 5). Indeed, the injured surface in animals given the mimetic appeared to be covered only with a thin layer of platelets (Fig. 5).

Discussion

The results show that administration of superoxide dismutase or a synthetic low molecular weight mimetic inhibit cyclic flow variations in damaged and stenotic carotid arteries (Fig. 1 and 2) and decrease accumulation of platelet-rich thrombus at the site of vascular injury (Fig. 5). This is accompanied by reduced mean arterial blood pressure (Fig. 3) and increased blood flow velocity in the injured vessel (Fig. 4), similar to the effects observed with nitroglycerin, which is metabolized to nitric oxide in vivo (24). The effects of superoxide dismutase are reversed by infusion of N^G-monomethyl-L-arginine (Fig. 2), an inhibitor of nitric oxide production (25). Thus, inhibition of cyclic flow variations and vasodilation induced by superoxide dismutase appear to reflect increased endogenous nitric oxide, possibly derived from endothelium located proximal to the site of vessel injury and stenosis where high shear could induce its synthesis (26).

Cyclic variations in blood flow analogous to those induced in this study have been observed in both peripheral and coronary arteries of patients (6–8). By carefully controlling the

extent of vessel injury induced, we modified the experimental preparation reported initially by Folts et al. (4) to one exhibiting totally spontaneous cyclic flow variations with a stable frequency for at least 4 h.

Antiplatelet agents, including aspirin (4), antagonists of thromboxane (27), serotonin (28) and platelet-activating factor (29,30), all abolish cyclic flow variations, as does L-arginine (20), the precursor of nitric oxide, nitroglycerin (31) and sodium nitroprusside (32), consistent with a mechanism for their induction involving platelet activation. Our data confirm previous results (9) showing attenuation of cyclic flow variations by administration of superoxide dismutase consistent with oxygen free radicals elaborated by damaged endothelial cells (10), leucocytes (11) and smooth muscle cells contributing to local activation of platelets and inactivation of endogenous nitric oxide.

Increased endogenous levels of nitric oxide resulting from dismutation of superoxide anion by superoxide dismutase or the mimetic could account for the observed vasodilation and the twofold increase in the levels of platelet cyclic guanosine 5′-monophosphate. Both of these responses could contribute to the observed decrease in cyclic flow variations. Changes in flow velocity and blood pressure were minimal in animals given the mimetic in the absence of vascular injury and stenosis indicating the dependence of nitric oxide release on vessel damage or stenosis, or both. Previous studies have documented that flow-induced shear stress and thrombin both increase nitric oxide released from endothelium (21,26,33,34). Thus, by removing superoxide anion, superoxide dismutase may have unmasked the nitric oxide released in response to shear forces and thrombin generation associated with high grade stenosis. Inhibition of endothelium-derived nitric oxide release during infusion of N^G-monomethyl-L-arginine may explain the return of cyclic flow variations (Fig. 2).

Despite bolus administration, superoxide dismutase and the mimetic produced prolonged inhibition of cyclic flow variations, increased flow velocity and lowered blood pressure. This may have resulted from prolonged association of superoxide dismutase with heparin binding sites on endothelium documented previously (35). Thus, as generation of nitric oxide as well as superoxide anion persisted at the site of stenosis, prolonged availability of superoxide dismutase could have protected the endogenous nitric oxide from inactivation.

Clinical implications. Unstable angina is characterized by intermittent accumulation of platelets at sites of coronary stenosis (3). The prolonged inhibitory effects of superoxide dismutase or its mimetic on local platelet adhesion and activation in an injured artery may be useful to attenuate unstable angina and warrants further investigation.

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