Inhibition of Coronary Artery Superoxide Dismutase Attenuates Endothelium-Dependent and -Independent Nitrovasodilator Relaxation

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Isolated bovine coronary arteries were treated with 10 mM diethylidithiocarbamate (DETCA) for 30 minutes to deplete the cytosolic ZnCu form of superoxide dismutase (SOD). This treatment completely inhibited the endothelium- and cGMP-dependent relaxation to acetylcholine (mediated via the endothelium-derived relaxing factor, which is thought to be nitric oxide) without significantly inhibiting endothelium-dependent relaxation to arachidonic acid (mediated by prostaglandins). DETCA treatment of endothelial cells cultured from the coronary arteries inhibited bradykinin-elicted release of endothelium-derived relaxing factor, which was detected by bioassay on an isolated rabbit aorta in the presence of extracellular SOD. DETCA also inhibited cGMP-associated relaxations to nitric oxide and to vasodilators thought to function via the generation of this mediator (nitroglycerin and nitroprusside), but cAMP-associated relaxations to isoproterenol and papavarine were not altered. The inhibitory effects of DETCA against the relaxation to nitroprusside and nitroglycerin were attenuated by severe hypoxia. DETCA treatment of isolated coronary arterial smooth muscle or cultured endothelial cells produced an increase of chemiluminescence elicited in the presence of lucigenin, a detector of superoxide anion generation. The addition of SOD markedly attenuated the effects of DETCA treatment on arterial relaxation and chemiluminescence. Therefore, control of cellular superoxide anion levels by endogenous SOD appears needed for the release of endothelium-derived relaxing factor and relaxation of vascular smooth muscle to nitrovasodilators mediated via cGMP in the bovine coronary artery, but SOD is not critical for other endothelium-dependent or cAMP-associated relaxant mechanisms. (Circulation Research 1991;69:601–608)

The characterization of endothelium-derived relaxing factor (EDRF), now thought to be a species related to nitric oxide,1−3 has uncovered a potential physiologically important interaction of this mediator with superoxide anion. It was first reported in the cerebral microcirculation that superoxide anion (and derived O2 species) could attenuate the vasodilation to acetylcholine (ACh) in vivo,4 a response that was subsequently shown to be dependent on an endothelium-derived mediator.5 It was then demonstrated that superoxide anion inactivated EDRF in bioassay experiments in vitro.1−3,6 Many of the pharmacological probes used in the study of EDRF have been observed to result in the extracellular generation of superoxide anion (and derived O2 species) both in vitro7 and in vivo.8,9 It is now well documented that both nitric oxide and EDRF show similar profiles of instability in the presence of superoxide anion,1−3 which may originate from a direct chemical reaction between nitric oxide and superoxide anion.10

Reactive O2 species have been known to be associated with the modulation of the activity of the soluble form of guanylate cyclase in broken-cell and purified preparations since 1976.11 Superoxide dismutase (SOD) was initially shown to participate in expression of the activation of guanylate cyclase.12,13 More recent studies14−17 have perhaps provided an explanation for these early observations. SOD, by
removing endogenously produced superoxide anion, can prevent inhibition of the activation of guanylate cyclase by agents that generate nitric oxide. Guanylate cyclase is also activated during the metabolism of H2O2 by catalase, through a process that does not appear to involve the production of nitric oxide (authors’ unpublished observations), and this activation mechanism is likewise inhibited by superoxide anion.14,15 In addition, we have recently reported evidence that inhibitors of the activation of soluble guanylate cyclase in intact tissues, methylene blue and LY88583, increase the intracellular production of superoxide anion in vascular smooth muscle.16,17 Therefore, superoxide anion may function as an inhibitory modulator of the activation of the soluble form of guanylate cyclase and physiological regulatory mechanisms, such as vascular smooth muscle relaxation mediated by cGMP.

In the present study, we used diethyldithiocarbamate (DETCA), a copper chelator that inhibits the cytosolic or ZnCu form of SOD,18,19 to probe for the role of this enzyme; we also used superoxide anion that was produced from endogenous metabolic sources in the modulation of coronary arterial tone in vascular tissue obtained from calf hearts.

Materials and Methods

Materials

ACH, arachidonic acid (sodium salt), bradykinin triacetate, DETCA, lucigenin (bis-N-methylacridinium nitrate), phenylephrine hydrochloride, sodium nitroprusside, papavarine hydrochloride, bovine erythrocyte SOD, HEPES, and isoproterenol were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of arachidonic acid (10 mM) were prepared in equimolar Na2CO3 and handled under nitrogen atmosphere until use. Solutions of nitric oxide were prepared, diluted, and handled under an argon atmosphere, as previously described.9 Nitroglycerin solutions were prepared dissolving 0.4 mg sublingual tablets (Parke-Davis, Morris Plains, N.J.) in distilled water. Other chemicals were analyzed reagent grade from Baker Chemical Co.

Determination of Changes in Force in the Bovine Coronary Artery

Isolated endothelium-intact or rubbed arterial rings from the left anterior descending coronary artery of calf hearts obtained immediately after slaughter were prepared and studied by adaptation of previously described methods.14,20 Briefly, arterial rings were mounted on wire hooks attached to force displacement transducers (model FT-03, Grass Instrument Co., Quincy, Mass.) for measurement of changes in isometric force on a polygraph (model 7, Grass Instrument). Arteries were incubated in individually thermostated (37°C) 10-ml baths (Metro Scientific) for 2 hours at an optimal passive tension of 5 g in Krebs’ bicarbonate buffer (pH 7.4) containing the following (mM): NaCl 118, KCl 4.7, CaCl2 1.5, NaHCO3 25, MgSO4 1.1, KH2PO4 1.2, and glucose 5.6, initially gassed with 95% O2−5% CO2. After a 2-hour equilibration, the vessels were depolarized with Krebs’ bicarbonate containing KCl in place of NaCl. This treatment produces maximal contraction and enhances the reproducibility of subsequent contractions. The arteries were then reequilibrated with Krebs’ bicarbonate for 30 minutes before conducting experiments.

In all experiments, the functional retention or removal of endothelium by gentle rubbing of the lumen of the vessel with the wooden handle of a cotton applicator was confirmed by examining the effect of 10⁻⁸–10⁻⁶ M ACh on arteries precontracted with 0.1 μM serotonin. Endothelium-intact arteries relaxed 70–100%, whereas endothelium-denuded arteries used in this study usually contracted at the largest dose of ACh, and relaxations to ACh were not observed. Arterial rings were subjected to a two-cycle experimental protocol in which they were typically precontracted to 50–60% of maximal tone (tone, ~6 g) with ~20 mM KCl, and a 30-minute equilibration was used between responses. Drugs dissolved in distilled/deionized water were added to the 10-ml baths in 10-μl aliquots in a cumulative concentration-dependent fashion. When indicated, coronary arterial rings were incubated for 30 minutes with 10 mM DETCA, followed by washout of the chelating agent. Conditions for the DETCA treatment were optimized by maximization of the detection of superoxide anion by chemiluminescence (described below). Control or DETCA-treated arteries were then recontracted to a tone of ~6 g by carefully adjusting the concentration of KCl; typically, the procedures of the first experimental cycle were then repeated. Since the DETCA treatment usually caused a detectable inhibition (~35%) of tone generation, vessels treated with DETCA generally required a slightly greater concentration of KCl. For example, the concentration of KCl used for endothelium-intact arteries in the study of ACh required an increase from 20±1 to 25±1 mM (n=11, p<0.05) to produce a force of 7.0±0.6 and 6.0±0.8 g, respectively. Endothelium-denuded arteries studied with nitroglycerin required an increase in KCl from 20±1 to 26±1 mM (n=11, p<0.05). Since the inhibition of tone generation by DETCA treatment was not prevented by the addition of 0.3 μM SOD or by contracting arteries under N2 atmosphere (data not shown), the origin of this effect remains to be established. Studies under severe hypoxia (P0₂=8–10 mm Hg) were conducted using a gas mixture of 95% N2−5% CO2 and required a significantly greater concentration of KCl (26±1 mM) for the generation of tone.

Endothelial Cell Culture and Superfusion

Bioassay Methods

Arterial endothelial cells were obtained from the left anterior descending coronary artery of bovine hearts by mechanical removal with a plastic cell scraper. Cells were suspended and grown in Dulbec-
co’s modified Eagle’s medium containing 15% fetal calf serum. Each line of cells was cloned from individual colonies, and purity was demonstrated using uptake of diacytlated low density lipoprotein. Cells were grown to confluence on Cytodex III microcarrier beads. Cell densities were estimated by microscopic cytometry after trypsinization of cells and were found to be \( \sim 2 \times 10^5 \) cells/ml beads. The present study used cells from passages 3–7.

For superfusion–bioassay experiments, 1 ml beads was placed in a plastic column (Evergreen Scientific) supported in a water jacket maintained at 37°C. The column was pump-perfused at 4 ml/min with oxygenated Krebs’ bicarbonate buffer. The column effluent was directed over an endothelium-denuded rabbit aortic ring. The aortic ring was contracted with phenylephrine (0.1–1.0 \( \mu \)M) to elicit \( \sim 3 \) g active force. In each experiment, a preliminary test of the direct effect of ACh (1 \( \mu \)M) was conducted to confirm the loss of functional endothelium. Release of EDRF was stimulated by bradykinin (10^{-6} M) initially in the absence of SOD (phenylephrine tone=3.3±0.3 g, \( n=6 \)) and then in the presence of 0.3 \( \mu \)M SOD perfused through the endothelial cell column (phenylephrine tone=2.3±0.4 g, \( p<0.05 \) versus tone in the absence of SOD). Treatment of the endothelial cells with DETCA (10 mM) was conducted by incubating the cells for 30 minutes with the compound, without exposing the rabbit aortic ring to DETCA. After treatment of the cells with DETCA, the release of EDRF was again stimulated by bradykinin in the absence of SOD (phenylephrine tone=2.9±0.4 g) and then in the presence of SOD (phenylephrine tone=1.7±0.1 g, \( p<0.05 \) versus tone in the absence of SOD). Responses are described in “Results” as percent relaxation of the level of tone present under the conditions examined, in the absence of the relaxant agent.

Detection of Superoxide Anion

Ring segments of endothelium-denuded bovine coronary arteries were prepared as described above for organ bath tone studies but were placed in plastic scintillation minivials containing 0.25 mM lucigenin and other additions in a final volume of 1 ml Krebs’ solution buffered with 10 mM HEPES-NaOH (pH 7.4). Aliquots of endothelial cells on microcarrier beads (50 \( \mu l \)) in a final volume of 1 ml, were used for the determination of superoxide anion–elicited chemiluminescence in the presence of 0.1 mM lucigenin. The chemiluminescence elicited by superoxide anion in the presence of lucigenin was measured using a scintillation counter (Mark 5303, TM Analytic, Inc., Elk Grove Village, Ill.) in out-of-coincidence mode with a single active photomultiplier tube. After 3 minutes of dark adaptation, vials containing all components with the exception of arterial rings (blanks) were counted three times for 0.1 minute over the next 20 minutes. This procedure was then repeated after placing an arterial ring of \( \sim 25 \) mg in each vial. Blanks were subtracted from the average of the relatively constant levels of chemiluminescence produced under each condition by the arteries to obtain the data reported as counts per minute (cpm) in “Results.” The chemical specificity of the light-yielding reaction for superoxide anion has been reported previously and was checked with \( \mathrm{H}_2\mathrm{O}_2 \) and with xanthine/xanthine oxidase as a superoxide anion generating system. Superoxide anion increases lucigenin-elicited chemiluminescence over the nanomolar concentration range, which is eliminated by SOD (3 \( \mu \)M) and is not affected by scavengers of \( \mathrm{H}_2\mathrm{O}_2 \) (0.1 \( \mu \)M catalase) or hydroxyl radical (100 mM mannitol). \( \mathrm{H}_2\mathrm{O}_2 \) was found to produce chemiluminescence in the millimolar concentration range.

Statistical Analysis

For studies of smooth muscle relaxation to vasodilator agents, Student’s t test (paired analysis) was used to compare the percent relaxation in an experimental cycle before and after treatment with DETCA. The time-control preparations experienced no significant change in sensitivity across the two experimental cycles in which all significant drug effects were observed. For multiple comparisons in chemiluminescence, an analysis of variance was performed, followed by a post hoc Duncan’s test. The accepted level of significance was \( p<0.05 \). The number of experimental determinations \( (n) \) in all cases is the number of treated or control animals from which an arterial ring was obtained or is the number of experiments conducted on cultured endothelial cells. Data in the figures are mean±SEM.

Results

Effects of DETCA Treatment on Endothelium-Dependent Relaxation

Isolated bovine coronary arteries are known to show a cGMP-associated relaxation to nanomolar concentrations of ACh that is endothelium dependent and inhibited by nitro-L-arginine (30 \( \mu \)M) (authors’ unpublished observations) or by methylene blue (10 \( \mu \)M), antagonists of the biosynthesis or action of EDRF, respectively, implicating mediation of the response by this mediator. Higher concentrations of ACh are known to produce contraction of endothelium-denuded bovine coronary smooth muscle. Potassium-precontracted endothelium-intact bovine coronary arteries show a relaxation to micromolar concentrations of arachidonic acid (Figure 1), which is eliminated by indomethacin (10 \( \mu \)M) and not observed in endothelium-denuded arteries (not shown), indicating mediation of the relaxation by prostaglandins produced by the endothelium. As shown in Figure 1, the EDRC mediated relaxation of potassium-precontracted bovine coronary arteries to \( 10^{-8}-10^{-6} \) M ACh is eliminated by pretreatment with DETCA, and the direct smooth muscle contractile effect of ACh is observed. However, DETCA pretreatment does not alter the endothelium-derived prostaglandin-mediated relaxation to micromolar concentrations of arachidonic acid.
caused the cultured endothelial cells to release a relaxing factor (48±9% relaxation, n=6), which was slightly enhanced (62±14%, p=NS) in the presence of SOD in the perfusate. DETCA treatment of the endothelial cells, without treatment of the detector arterial ring, eliminated the relaxation, and the direct contractile effects (−26±12% relaxation, p<0.05 versus the absence of DETCA treatment) of bradykinin were observed. After inclusion of SOD in the perfusate, exposure of DETCA-treated endothelial cells to bradykinin produced an initial transient relaxation of the arterial ring (28±6% relaxation, p<0.05 versus DETCA treatment or SOD perfusion), which was immediately followed by reversal to a contraction (−60±14% relaxation, p<0.05 versus SOD perfusion). The relaxation to nitric oxide injected through the endothelial cell column was not eliminated by the DETCA treatment (Figure 2). In preliminary bioassay experiments using endothelium-intact bovine coronary arteries, DETCA treatment also eliminated the detection of EDRF released by ACh (not shown).

Effects of DETCA Treatment on Non-Endothelium-Dependent Vascular Smooth Muscle Relaxation

The contribution of superoxide anion production by the endothelium to the attenuation of EDRF-mediated relaxation was investigated by selective treatment of cultured bovine coronary endothelial cells with DETCA, using an untreated endothelium-denuded artery for bioassay detection of EDRF. In these experiments, conducted in the presence of indomethacin, bradykinin was used as a stimulus for the release of EDRF; since ACh was ineffective in eliciting EDRF release from cultured cells. Endothelium-denuded rabbit aortic rings precontracted with phenylephrine were used to detect EDRF, since they were less sensitive to the direct smooth muscle contractile effects of bradykinin, as compared with the bovine coronary artery. As shown in the typical experiment in Figure 2, bradykinin

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\[ \text{Graphs showing relaxation of potassium-precontracted bovine coronary arterial rings. DETCA, diethyldithiocarbamate; ACH, acetylcholine (n=11); AA, arachidonic acid (n=10). Effects of pretreatment with DETCA on ACH- and AA-induced relaxation of endothelium-intact bovine coronary arterial rings precontracted with potassium are shown. Experimental conditions are described in "Materials and Methods." Standard error symbols are not shown when they are obscured by the symbols.} \]
Evidence That Superoxide Anion Mediates the Actions of DETCA Treatment

The inhibitory actions of DETCA treatment were found to be attenuated when characterized in a hypoxic environment. As shown in Figure 5, when examined in the presence of an O2 atmosphere, coronary arterial relaxation elicited by nitroprusside (1 μM) is also markedly inhibited by DETCA pretreatment. However, in the presence of severe hypoxia (Po2=8–10 mm Hg) produced by gassing the tissue bath with 95% N2 (containing 5% CO2), DETCA treatment caused only a minor attenuation of the relaxation to nitroprusside. Severe hypoxia also inhibited the inhibitory effect of DETCA on relaxation to nitroglycerin (0.1 μM).

Table 1. Reversal of Diethyldithiocarbamate-Elicited Inhibition of Coronary Relaxation to Nitroglycerin by Superoxide Dismutase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relaxation to 0.1 μM nitroglycerin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
</tr>
<tr>
<td>Time control</td>
<td>79±13%</td>
</tr>
<tr>
<td>0.3 μM SOD</td>
<td>78±10%</td>
</tr>
<tr>
<td>DETCA</td>
<td>91±5%</td>
</tr>
<tr>
<td>DETCA+SOD</td>
<td>90±4%</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=5. SOD, superoxide dismutase; DETCA, diethyldithiocarbamate. Experimental conditions described in "Materials and Methods" were used to determine the percent relaxation of KCl-induced tone.

*p<0.05 vs. before treatment.
with DETCA also increased the level of detectable chemiluminescence from 5,755 ± 2,861 to 10,863 ± 4,930 cpm (p < 0.05, n = 4).

Discussion

The results of this study suggest that the inhibition of ZnCu SOD increased the levels of endogenously generated superoxide anion, which produced both inhibition of the release of EDRF from coronary arterial endothelial cells and antagonism of vascular smooth muscle relaxation by agents thought to act via nitric oxide.

Effects of Superoxide Dismutase Inhibition on Endothelium-Dependent Relaxation

The inactivation of the EDRF by superoxide anion during its transfer from the endothelial cell to vascular smooth muscle is a well-documented interaction. Data in the present study suggest that increased levels of superoxide anion within the endothelial cell appear to inactivate EDRF at an intracellular site before its release, since the removal of extracellular superoxide anion with added SOD only partially restored detection of EDRF by bioassay. An alternative possibility that DETCA treatment inhibits the biosynthesis of EDRF appears unlikely, according to the results of a recent study. In this study, it was found that DETCA treatment inhibited the detection of EDRF but did not reduce the quantity of the nitrogen oxide decomposition products of EDRF. The observed actions of DETCA treatment on endothelial cell mediator release did not appear to originate from a nonspecific toxic effect, because endothelium-dependent prostaglandin-mediated relaxation to arachidonic acid was not altered (see Figure 1).

Effects of Superoxide Dismutase Inhibition on Vascular Smooth Muscle Relaxation

In the present study, the inhibitory actions of DETCA appear to be selective for vascular smooth muscle relaxants thought to function via the activation of soluble guanylate cyclase. Although it is not unambiguously established, there is substantial evidence to support a role for nitric oxide as a mediator of guanylate cyclase activation and vascular relaxation elicited by the EDRF released by ACh,1–3 by nitroglycerin,2,28 and by nitroprusside.2,28 It is well documented that superoxide anion inactivates EDRF or nitric oxide with a high level of selectivity,1,3,6,7,9,10 suggesting that this interaction could be involved in physiological regulatory processes. Thus, the chemical reaction between superoxide anion and nitric oxide may explain the selective antagonism of relaxation to ACh and to nitrovasodilators by DETCA treatment. Our recent studies in the isolated bovine pulmonary artery16 suggest that DETCA treatment appears to inhibit cGMP-associated relaxation to increased oxygen tension and hydrogen peroxide, in addition to the inhibition of nitrovasodilator mechanisms. Since guanylate cyclase activity in the absence of stimuli appears to be inhibited by superoxide anion,29 a direct interaction with this enzyme in vascular smooth muscle could also be a site of action of superoxide anion.

Relaxation to endothelium-derived prostaglandins produced on exposure to arachidonic acid, to isoproterenol, and to papavaren were not antagonized by DETCA treatment, presumably because these agents may function via mechanisms not involving nitric oxide or the activation of soluble guanylate cyclase. It has been demonstrated that isoproterenol and papawarne elicit relaxation of bovine coronary arteries associated with increases in cAMP. Therefore, the results do not provide evidence supporting an interaction of superoxide anion with the generation of a relaxant prostaglandin from arachidonic acid, with adenylyl cyclase, or with cAMP-mediated relaxation mechanisms.

Superoxide Anion as a Mediator of the Actions of DETCA Treatment

Several observations suggest that treatment of bovine coronary arterial endothelial cells and arteries with DETCA results in an increase in the levels of superoxide anion and that this oxygen species mediates the actions of this inhibitor on nitrovasodilator-related vascular responses. It should be noted that DETCA treatment may result in effects other than inhibition of SOD, which may account for the depression of tone produced by treatment of arteries by this copper chelator (see "Materials and Methods"). However, the apparent absence of inhibition of EDRF biosynthesis27 and the restoration of relaxation to nitroglycerin by the addition of SOD (Table 1) are consistent with a role for inhibition of SOD in the antagonism of nitrovasodilator-related vascular responses. Since the inhibitory actions of DETCA treatment on arterial relaxation to nitroglycerin and nitroprusside were attenuated when examined under a nitrogen atmosphere (P02 = 8–10 mm Hg), the actions of DETCA are O2 dependent, consistent with a requirement for the reduction of O2 in the endogenous production of superoxide anion. The relatively weak inhibitory effect of DETCA under hypoxic conditions suggests that the effects of this probe are not a consequence of a direct action of DETCA on the events that mediate the cGMP-associated relaxation to these nitrovasodilators. Treatment of bovine coronary endothelial cells or arterial smooth
muscle with DETCA caused an increase in lucigenin-elicited chemiluminescence, suggesting the detection of increased levels of superoxide anion.\textsuperscript{22,23} Since exogenous membranes that lucigenin-elicited chemiluminescence, it appears that superoxide anion may readily permeate cell membranes to produce its effects. It is currently thought that superoxide anion is transported across membranes via anion channels.\textsuperscript{30} The results of the present study are also in accord with our recent observations that intracellular superoxide anion production may contribute to the inhibitory effects of methylene blue\textsuperscript{17} and LY83583\textsuperscript{16} on vascular relaxation associated with the activation of soluble guanylate cyclase.

The results of this study suggest that increased superoxide anion levels derived from endogenous sources within the endothelial cell and the vascular smooth muscle cell can attenuate the release of EDRF and the actions of vasodilators thought to function via the generation of nitric oxide, respectively. Thus, endogenous SOD appears to permit the release of EDRF and cGMP-mediated relaxation of vascular smooth muscle to nitrovasodilators, but SOD does not appear to be critical for other endothelium-dependent or cAMP-associated relaxant mechanisms. The concepts examined in the present study suggest that physiological stimuli or pathophysiological processes that increase intracellular levels of superoxide anion will antagonize the stimulation of soluble guanylate cyclase resulting in the attenuation of phenomena mediated via cGMP.

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