Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta

MICHELA ZANETTI,1 JUN'ICHI SATO,1 ZVONIMIR S. KATUSIC,2 AND TIMOTHY O'BRIEN1

Departments of 1Endocrinology and 2Anesthesiology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received 6 June 2000; accepted in final form 4 January 2001

Zanetti, Michela; Satoko, Jun'ichi; Katusic, Zvonimir S.; O'Brien, Timothy

Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. Am J Physiol Heart Circ Physiol 280: H2516–H2523, 2001.—Increased production of oxygen free radicals is an important mechanism of endothelial dysfunction in diabetes mellitus. Our goal was to test whether adenovirus (Ad)-mediated gene transfer of copper/zinc (CuZn) or manganese superoxide dismutase (Mn SOD) improves relaxation of diabetic vessels. The aortas from 9 alloxan-induced diabetic mellitus (DM) and 16 control rabbits were used. Control and DM rings were transduced ex vivo with Ad vectors encoding Mn SOD (AdMn SOD), CuZn SOD (AdCuZn SOD), β-galactosidase (Adβgal), or diluent. In the absence of gene transfer, SOD activity was significantly increased in DM aortas. Transgene expression in DM AdCuZn SOD and DM AdMn SOD-transduced vessels was confirmed by Western blot analysis and by increased SOD activity (DM AdCuZn SOD, 76.2 ± 9.3; DM AdMn SOD, 65.2 ± 4.8; P < 0.05 vs. DM Adβgal; 50.9 ± 4.4 U/mg protein). Superoxide production was increased in DM Adβgal-transduced aorta and relaxations to acetylcholine were impaired in these vessels. Gene transfer of CuZn SOD and Mn SOD corrected both of these defects. Thus Ad-mediated gene transfer CuZn and Mn SOD to the diabetic aorta improves endothelium-dependent relaxation.

Address for reprint requests and other correspondence: T. O’Brien, Mayo Clinic, 200 First St. SW, 5-194 Joseph, Rochester, MN 55905 (E-mail: obrien.timothy@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ENDOTHELIAL DYSFUNCTION is one of the earliest events that occurs in the development of diabetic cardiovascular disease. Abnormal endothelium-dependent relaxation can be detected before the onset of overt atherosclerosis in diabetic patients. In vitro evidence (7) suggests that hyperglycemia as a consequence of diabetes is associated with increased intracellular oxidative stress, which may be involved in the pathogenesis of vascular complications. Exposure of endothelial cells to high glucose results in superoxide generation (9), enhanced cell-mediated low-density lipoprotein peroxidation (16), increased expression of antioxidant enzymes (6), and activation of the redox-sensitive nuclear factor NF-κB (28). In vivo animal and clinical studies (13, 22, 26, 36, 39) showed abnormal endothelium-dependent relaxation in the setting of hyperglycemia. A causal relationship between oxidative stress and diabetic vascular dysfunction is further supported by recent reports (8, 20, 25, 37) that show protection against endothelial oxidative damage in diabetes with administration of exogenous free-radical scavengers, including superoxide dismutase (SOD), glutathione, and vitamins C and E.

However, the use of exogenous antioxidants to investigate the role of superoxide as a mediator of diabetic endothelial-dependent dysfunction may be biased by the limitation that systemic delivery of antioxidants may not provide therapeutic concentrations in the target tissue. Moreover, whether and to what extent exogenous antioxidant enzymes are actually taken up by endothelial cells and expressed within their physiological location remain to be ascertained.

This potential limitation could be addressed by gene transfer techniques, which allow high levels of transgene expression. In the present study, we investigated the role of superoxide anion in endothelial dysfunction by transducing aortic rings from alloxan-induced diabetic rabbits with replication-deficient adenoviral (Ad) vectors containing genes for human copper/zinc SOD (CuZn SOD) and manganese SOD (Mn SOD).

MATERIALS AND METHODS

Construction, propagation, and purification of Ad vector. Recombinant Ad vectors containing the cDNA encoding Mn SOD and CuZn SOD genes driven by a cytomegalovirus promoter were gifts from Dr. J. F. Engelhardt (University of Iowa; Iowa City, IA). The vectors were generated, propagated, and purified as described previously (17, 31).

An Ad vector encoding the Escherichia coli β-galactosidase gene (Adβgal) driven by a cytomegalovirus promoter was obtained from Dr. J. M. Wilson (University of Pennsylvania; Philadelphia, PA) and used in all experiments as a control. It was propagated, isolated, and quantified as described above. Viral stocks were stored at −70° C.

Diabetic animal model. Male New Zealand White rabbits weighing 3.0–3.5 kg were randomly assigned to control (n = 16) and alloxan (n = 9) groups. The animals were housed individually in stainless steel wire-bottomed cages in a room with a 12:12-h light-dark cycle. All of the experimental protocols were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the

Mayo Clinic, 200 First St. SW, 5-194 Joseph, Rochester, MN 55905
recommendations of the American Association for the Accreditation of Laboratory Animal Care. Diabetes mellitus was induced in the alloxan group by intravenous injection in the marginal ear vein of alloxan monohydrate (150 mg/kg body wt) freshly dissolved in 30 ml of saline. All of the animals were provided with food and water ad libitum, and, in the alloxan group, blood glucose levels were monitored on a weekly basis. The alloxan-injected animals with blood glucose ≥22.2 mmol/l were included in the protocol. Ten weeks after diabetes mellitus was induced, the thoracic aortas were harvested. Sedation and induction of anesthesia were obtained with intramuscular injections of ketamine (65 mg/kg), xylazine (13 mg/kg), and acepromazine (22 mg/kg). After the animals were sedated, blood was collected for glucose, glycated hemoglobin, and lipid analysis. Thoracic aortas were harvested, and the animals were then euthanized. Dissected vessels were immersed in cold modified Krebs-Ringer bicarbonate solution (pH 7.4) supplemented with streptomycin and penicillin. The adhering perivascular tissue was carefully removed. Special care was taken to avoid damage to the endothelium. Aortas, 5 mm in length, from both diabetic and control rabbits, were excised. Each artery was divided into 6–8 rings, which were used for ex vivo gene transfer as described below.

**Ex vivo thoracic aorta gene transfer.** At the time of death, aortic rings from control and alloxan-induced diabetic rabbit aortas were randomly exposed either to AdCuZn SOD, AdMn SOD, or AdΔgal [100 μl of 1 × 10^10 plaque-forming units (PFU)/ml] diluted in Dulbecco’s modified Eagle’s medium for 1 h at 37°C in a CO₂ incubator. Additional rings from control and diabetic animals were exposed to diluted alone. Vessel rings were then placed in tissue culture dishes and incubated in medium 199 with 10% fetal calf serum and antibiotics for 24 h. Of these rings, one was used for 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside X-Gal staining, one for West-24 h. Of these rings, one was used for 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside X-Gal staining, one for Western blot analysis, one for SOD activity, one for the determination of in situ production of superoxide anion, and the rest for vascular reactivity studies.

**Determination of plasma glucose, lipids, and total glycated hemoglobin.** For glucose and lipid assays, blood samples were centrifuged at 2,000 rpm for 10 min at 4°C and plasma was stored at −70°C until measurements were performed. Plasma glucose was determined by the glucose oxidase method on a glucose analyzer (model 2700, Select Biochemistry Analyzer, Yellow Springs Instruments; Yellow Springs, OH). Total plasma cholesterol and triglycerides were measured with the use of standard techniques (1, 5). Total glycated hemoglobin was measured by high-performance liquid chromatography (38).

**Histochemical analysis of gene expression.** The fresh rings were frozen in optimum cutting tissue compound (Miles Scientific) and were then cut into serial 5-μm-thick sections. For histochemical staining of β-galactosidase, the sections were fixed in 2% paraformaldehyde and 0.4% glutaraldehyde for 15 min at 4°C and then rinsed twice with phosphate-buffered saline (PBS). The sections were stained in a 500 μg/ml solution composed of X-Gal (Boehringer-Mannheim; Indianapolis, IN) for 4 h at 37°C and then rinsed in PBS and counterstained with eosin.

**Western blot analysis for SOD proteins.** Aortic segments were isolated and immediately stored in liquid nitrogen. The frozen segments were pulverized and resuspended in lysis buffer composed of 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% sodium dodecyl sulfate, 0.1% deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.2% leupeptin, 0.2% aprotinin, and 0.1% peptatin. Aortic debris was homogenized on ice and then centrifuged at 4,000 rpm for 10 min to remove the insoluble pellet, and protein concentration was determined by the bicinchoninic acid assay. Protein (50 μg) was loaded on 4% stacking/12.5% separating sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Ponceau S staining was used to assess an equal protein load. The resolved proteins were transferred to a 0.2-μm nitrocellulose membrane on a semidry electrophoretic transfer system (Bio-Rad) for Western blot analysis. Blots were blocked and incubated either with an anti-human CuZn SOD or Mn SOD antibody (1:5,000; Stressgene) overnight at 4°C. After the blots were washed extensively, horseradish peroxidase-linked secondary antibody was added (1:5,000; Amersham Life Science) and visualized with the use of the enhanced chemiluminescence Western blotting detection system (Amersham Life Science). In control experiments, we tested a specific binding of the secondary antibody by omitting the primary antibody and incubating the blot with the secondary antibody. After the blots were exposed to enhanced chemiluminescence, no bands were detectable on the membrane.

**Measurement of SOD activity.** The aortas were carefully cleaned of fat and connective tissue and were then cut into pieces in ice-cold RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton-X 100, 10% glycerol, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 50 mM NaF, and 10 mM Na₃PO₄). The minced tissue-buffer mixture was homogenized with 50 passes of a ground glass-on-glass homogenizing system at 4°C and sonicated on ice with one 10-s burst. The homogenates were then centrifuged at 4°C at 4,000 rpm for 15 min to remove cellular debris. The supernatant was collected and assayed for SOD activity. Protein content of the tissues was assessed with the use of a bicinchoninic acid assay. All of the assays were performed at 25°C. SOD activity was measured by the reduction of cytochrome c method (29). Briefly, xanthine/xanthine oxidase was used to generate O₂⁻, which was detected by the reduction of cytochrome c. Spectrophotometric measurement of the rate of reduction of cytochrome c in the presence of increasing amounts of SOD protein was performed. Total SOD activity was determined from the amount of inhibition of cytochrome c reduction.

**In situ detection of superoxide anion.** The oxidative fluorescent dye hydroethidine was used to evaluate in situ production of superoxide, as described previously (19). Unfixed frozen rings of aortic segments were cut into 30-μm-thick sections and placed on a glass slide. Slides were incubated with hydroethidine (2 × 10⁻⁶ mol/l) in a light-protected, humidified chamber at 37°C for 30 min and coverslipped. Tissue sections were imaged with the use of a Olympus Fluoview laser scanning confocal microscope mounted on an upright microscope (model BW50WI; Olympus) equipped with Ar and Kr lasers. Fluorescence was detected with a 585-nm long-pass filter. Unlabeled sections were used to obtain background images of aortas from alloxan-induced diabetic rabbits. Identical photomultiplier settings were then used for the image acquisition from all samples. The images were analyzed by using an image processing software (Meta-Morph, version 3.7) running on a Pentium III computer.

**Measurement of vascular reactivity.** Rings were connected to isometric force-displacement transducers (Grass Instrument) and suspended in organ chambers filled with 25 ml of gassed (94% O₂/6% CO₂) Krebs-Ringer bicarbonate control solution (pH 7.4, 37°C) composed of (in mmol/l) 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, 0.0026 EDTA, and 11.1 glucose. Isometric tension was recorded continuously. Rings were allowed to equilibrate for 30 min and then gradually stretched to the optimal point on the length-tension curve (g), as determined by the contraction to repeated exposure to 30 mmol/l KCl. After three washouts,
maximal contraction with phenylephrine (10^{-5} \text{ mol/l}) was obtained. Concentration responses to acetylcholine and diethylaminodiazene-1,ium-1,2.-dioate (DEA-NONOate) were then examined during a submaximal contraction obtained with 3 \times 10^{-7} to 10^{-6} \text{ mol/l} phenylephrine. Care was taken to match the contractions in different experimental groups. Only one concentration-response curve per drug was obtained in each tissue preparation. All of the drugs were obtained from Sigma and were freshly made immediately before the study.

**RESULTS**

**Animal data.** Plasma glucose and glycated hemoglobin were significantly increased in the diabetic compared with the control rabbits (Table 1). In contrast, plasma levels of cholesterol and triglycerides were similar in both groups.

**Histochemical localization of β-galactosidase expression.** Aortic rings transduced with Adβgal at a concentration of 1 \times 10^{10} \text{ PFU/ml} and stained 24 h later showed transgene expression in the endothelium and adventitia as confirmed by X-Gal staining (Fig. 1B). In contrast, no X-Gal-positive cells could be detected in nontransduced vessels (Fig. 1A).

**Western blot analysis for SOD proteins.** To demonstrate antioxidant transgene expression, diabetic aortas were analyzed by Western blotting for CuZn and Mn SOD. Diabetic aortas transduced with Adβgal, AdCuZn SOD, or AdMn SOD were evaluated. Representative immunoblot images revealed elevated CuZn and Mn SOD signals in diabetic rings transduced with AdCuZn SOD and AdMn SOD compared with diabetic aortas transduced with Adβgal (Fig. 2, top).

**Measurement of SOD activity.** Ten weeks after the induction of diabetes, total SOD activity was significantly (P < 0.05) increased in aortic rings from diabetic animals (45.0 ± 4.6 \text{ U/mg protein}) compared with control vessels (35.4 ± 1.3 \text{ U/mg protein}).

To confirm that elevated levels of transgene expression observed by Western blot analysis represented functionally increased antioxidant activity, total aortic SOD activity from nine animals was assayed. Diabetic aortic rings transduced with Adβgal, DM vessels exposed to AdCuZn SOD and DM vessels transduced with AdMn SOD were evaluated. Quantitative determination of SOD activity showed a statistically significant (P < 0.002) increase, by 50% in AdCuZn SOD-transduced diabetic rings (76.24 ± 9.3 \text{ U/mg protein}) and a 30% increase (P < 0.05) in AdMn SOD-transduced vessels (65.2 ± 4.8 \text{ U/mg protein}) compared with DM Adβgal-transduced aortas (50.9 ± 4.4 \text{ U/mg of protein}; see Fig. 2, bottom).

**In situ detection of superoxide anion.** With the use of confocal microscopy and hydroethidine staining, aortic sections from alloxan-induced diabetic rabbits showed a moderate ethidium bromide (EtBr) fluorescence (red, Fig. 3A), reflecting O_2^- production mainly in the endothelium (Fig. 3A). After ex vivo gene transfer of either CuZn SOD or MnSOD, EtBr fluorescence was reduced.

---

**Table 1. Characteristics of control and alloxan-induced diabetic rabbits**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>3.8 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>8.8 ± 0.5</td>
<td>25.7 ± 1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>HDL-Ch, mmol/l</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.04</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Total glycated hemoglobin, %</td>
<td>2 ± 0.1</td>
<td>5.5 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SE of the number of rabbits in each experiment. HDL-Ch, high-density lipoprotein cholesterol.
compared with aortic rings from diabetic animals transduced with Adβgal (Fig. 3, B and C).

Effects of diabetes mellitus on vascular reactivity. In DM Adβgal aortas, endothelium-dependent relaxation to acetylcholine was significantly impaired relative to responses in nondiabetic control animals (see Figs. 5A and 6A). EC25 was significantly different in control compared with DM Adβgal-transduced vessels (6.9 ± 0.1 vs. 6.6 ± 0.1, P < 0.05). In contrast, relaxations to nitric oxide (NO) donor were similar in control and DM Adβgal-transduced aortas (see Figs. 5B and 6B).

Effects of ex vivo gene transfer of SOD isoforms on vascular reactivity in normal and diabetic aorta. In nondiabetic aortas, after gene transfer of Adβgal, AdCuZn SOD, or AdMn SOD, relaxations to acetylcholine were not different from those of nontransduced vessels (Fig. 4A). Relaxations to diethylaminodiazen-1-ium-1,2-dioate were also similar in control aortas transduced with Adβgal, AdCuZn SOD, or AdMn SOD and in nontransduced vessels (Fig. 4B).

The functional effect of increased aortic antioxidant activity in diabetic aortas was evaluated next. Diabetic rings transduced with AdCuZn SOD showed significantly improved relaxations to acetylcholine (10−7 to 3 × 10−6 mol/l) (Fig. 5A). EC25 was significantly (P < 0.05) different in AdCuZn SOD-transduced aortas (7 ± 0.1) compared with Adβgal-transduced vessels (6.6 ± 0.1). After Ad-mediated gene transfer of Mn SOD to the diabetic aorta, endothelium-dependent relaxations to acetylcholine were also significantly improved (Fig. 6A). EC25 was 7 ± 0.1 in AdMn SOD-transduced aortas (P < 0.05 vs. Adβgal-transduced vessels). Relaxations to NO donor were not different among control, Adβgal, AdCuZn SOD, and AdMn SOD-transduced vessels (Fig. 5B and 6B).

DISCUSSION

Vascular function has been extensively evaluated in human and animal models of diabetes mellitus. Impaired endothelium-dependent relaxation has been found in arteries from diabetic animals (26) as well as in vessels from normal animals exposed to hyperglycemia (36). Finally, studies (13, 22, 30) in humans with insulin- and non-insulin-dependent diabetes have reported endothelial dysfunction in different vascular beds. In agreement with previous reports (34–36), we showed that endothelium-dependent relaxation is impaired in the aorta of the alloxan-induced diabetic rabbit. In addition, we found increased activity of SOD in the diabetic aorta, which may possibly be interpreted as a response to enhanced superoxide production in the setting of high glucose concentrations. Superoxide generation was increased in the endothelium of vessels from diabetic animals. Ad-mediated gene transfer of Mn and CuZn SOD augmented enzyme
activity and expression, decreased superoxide levels, and enhanced endothelium-dependent relaxation of this vascular bed. Therefore, scavenging excess superoxide via SOD gene transfer ameliorates endothelial dysfunction in experimental diabetes mellitus.

Fig. 3. In situ detection of superoxide in rabbit aorta. Fluorescent photomicrographs of confocal microscopic sections of aorta labeled with the oxidative dye hydroethidine (HE), a red fluorescence when oxidized to ethidium bromide (EtBr) by O$_2^\cdot$. At identical laser and photomultiplier settings, moderate fluorescence was found in the alloxan-induced diabetic aorta transduced with Adβgal (A). Fluorescence reflecting O$_2^\cdot$ was especially seen in the endothelium of DM Adβgal-transduced aorta. After ex vivo transduction with either AdCuZn SOD or AdMnSOD, O$_2^\cdot$ levels are decreased in the aorta from diabetic animals (B and C, respectively). Similar findings were observed in 4 diabetic rabbits. Arrows point to endothelium.

Fig. 4. Relaxation responses to acetylcholine (A) and diethylamin-odiazene-1,2-dione (DEA-NONOate) (B) in control aortas exposed to 1) Adβgal, 2) AdCuZn SOD, 3) AdMnSOD, or 4) the control solution, phosphate-buffered saline (PBS), during submaximal contraction to phenylephrine. Submaximal contractions obtained with $3 \times 10^{-6}$ to $10^{-5}$ mol/l phenylephrine were not significantly different among the four control groups. Acetylcholine: PBS, 5 ± 0.4 g; Adβgal, 5.1 ± 0.5 g; AdCuZn SOD, 4.5 ± 0.4 g; AdMn SOD, 4.5 ± 0.2 g. DEA-NONOate: PBS, 4.6 ± 0.6 g; Adβgal, 4.4 ± 0.4 g; AdCuZn SOD, 3.5 ± 0.5 g; AdMn SOD, 4.4 ± 0.4 g. n = 7 rabbits.
SOD is an important antioxidant in the endothelium, capable of scavenging superoxide anion \(^{(21)}\). Three isoforms of SOD are known in mammals. The intracellular CuZn-containing SOD is located predominantly in the cytoplasm and nucleus of cells. The Mn-containing SOD is found predominantly in the mitochondria. The third isoenzyme of SOD is the extracellular SOD. There is evidence \(^{(11, 40)}\) suggesting that generation of free radicals is increased in diabetes. Both the cytosol and mitochondria are responsible for increased superoxide production in diabetes mellitus \(^{(12)}\). However, the relative contribution of the two isoforms of SOD to the scavenging process is not known. Accordingly, we sought to examine whether a selective overexpression of Mn or CuZn of SOD may differentially affect endothelium-dependent relaxation in diabetic vessels.

After 10 wk of hyperglycemia, SOD activity was significantly increased in the diabetic rabbit aorta. This finding is in keeping with in vitro data, showing an upregulation of antioxidant enzymes, including SOD, catalase, and glutathione peroxidase, in vascular endothelial cells cultured in high glucose \(^{(6)}\). In vascular beds from diabetic animals, SOD activity has been reported to be unaltered \(^{(27)}\), increased \(^{(14)}\), or decreased \(^{(15, 32)}\). These discrepancies may depend on variations of enzyme activity over time as well as by the type of tissue under examination. It is known that glycosylation of CuZn SOD, which occurs in the presence of high glucose, is associated with impaired enzyme activity \(^{(2)}\).

Despite increased SOD activity and expression, impaired endothelium-dependent relaxation was still
present in the diabetic aorta. Gene transfer of CuZn SOD and Mn SOD significantly increased SOD activity in this vessel (by 50 and 30%, respectively, vs. Adβgal-transduced diabetic aorta). SOD activity almost doubled in diabetic aortas transduced with Mn and CuZn SOD compared with control vessels. Responses to acetylcholine were not affected in vessels from control animals after SOD gene transfer but were greatly improved in diabetic animals. This is in contrast to the recent findings by Lund et al. (19), who did not find any improvement in endothelium-dependent relaxation to acetylcholine after ex vivo Ad-mediated gene transfer of CuZn SOD to the diabetic carotid artery. However, disease duration (8 vs. 10 wk in our study) as well as vascular bed studied (carotid artery vs. aorta) may be relevant to whether CuZn SOD overexpression improves relaxation in diabetic vessels.

Moderate EtBr fluorescence, reflecting superoxide anion production, was found in the endothelium of vessels from diabetic rabbits. The specificity of EtBr for superoxide in the vessels has been previously demonstrated (19). SOD gene transfer reduced EtBr fluorescence in diabetic aortas, which is consistent with decreased endothelial levels of superoxide in these vessels. Thus enhanced production of oxygen free radicals is associated with endothelial dysfunction in diabetes mellitus. These abnormalities may be corrected by ex vivo SOD gene transfer, which results in endothelial and adventitial transgene expression.

Overexpression of CuZn SOD and Mn SOD under free-radical generating conditions such as hyperglycemia might have deleterious effects, because increased scavenging of O$_2^-$ might result in enhanced production of hydrogen peroxide, which is known to be a powerful oxidant. In addition, H$_2$O$_2$ is known to produce vasodilation via the cGMP pathway (23). However, Teixeira et al. (33) reported that O$_2^-$ dismutation in fibroblasts overexpressing CuZn SOD prevents the formation of increased H$_2$O$_2$ by alternative pathways. Therefore, improved scavenging of superoxide anion via SOD overexpression may not necessarily result in increased formation of H$_2$O$_2$ in diabetic vessels. This is consistent with our finding that CuZn SOD and Mn SOD gene transfer to the diabetic aorta was associated with decreased superoxide levels and improved vascular function.

One possibility for the beneficial effect of SOD overexpression in our study is that a further increase in SOD levels by Ad-mediated gene transfer over and above that induced by diabetes mellitus per se might completely scavenge the excessive amounts of superoxide generated in the setting of high glucose. Scavenging superoxide may reverse the known effect of this free radical on activation of the prostaglandin H$_2$/thromboxane A$_2$ receptor, which directly generates a vasoconstrictor effect (3). Another possibility for the improvement of diabetes-induced vasomotor dysfunction by gene transfer of SOD relates to its protective properties on basal release of NO (18). Thus we hypothesize that increased expression of SOD after Ad-mediated gene transfer may reduce diabetes-induced endothelial dysfunction by augmenting NO availability, preventing the formation of potent oxidants such as peroxynitrite and attenuating superoxide-mediated constrictor effect.

In this study, the aorta was harvested after 10 wk of hyperglycemia and exposed to viral fluid or control solution for 1 h. The aortic rings were subsequently incubated for 24 h. We showed that after 24 h in organ culture, endothelial function is still intact in control vessels (24) and that abnormal vascular reactivity is present in the diabetic aorta (41). These findings were confirmed in the present study. In our model, we chose to compare vascular reactivity in diabetic vessel segments transduced with Adβgal to control rings exposed to diluent alone after 24 h in culture. Zanetti et al. (41) showed that at the vector dose used in this study, vascular function was not different in the diabetic aorta transduced with Adβgal or exposed to diluent. Also, use of Adβgal as control vector allows the examination of transgene expression.

These results may be compared with those recently obtained in the same experimental model using gene transfer of endothelial NO synthase (eNOS) (41). It has been hypothesized that reduced NO generation from eNOS and/or inactivation of NO by superoxide anions is responsible for endothelial dysfunction in diabetes mellitus (10). Thus the defect in endothelial function observed in diabetes mellitus could potentially be corrected by overexpression either of eNOS or SOD. Because production of oxygen-derived free radicals in increased in diabetes mellitus, and because they may interact with NO, resulting in increased peroxynitrite levels (4), it is difficult to predict whether overexpression of eNOS would favorably affect NO-dependent relaxation in diabetic vessels. In contrast, overexpression of SOD resulting in increased scavenging of superoxide anions might prevent the formation of peroxynitrite and therefore protect the endothelium. In the diabetic aorta, whereas eNOS overexpression improved vascular responses to low acetylcholine concentrations, it did not reverse contractions observed in the diabetic vessels to higher doses of acetylcholine (41). In contrast, in the current study we found that vascular reactivity to acetylcholine is completely normalized by gene transfer of SOD to the dysfunctional diabetic aorta.

In conclusion, endothelial dysfunction was observed in the aorta of the alloxan-induced diabetic rabbit despite increased activity of SOD. However, further augmentation of vascular SOD levels obtained after gene transfer of Mn SOD or CuZn SOD completely normalized endothelial dysfunction. This suggests that gene therapy approaches to diabetic vascular dysfunction aimed at overexpression of SOD may have beneficial therapeutic effects.

The authors thank S. Stephan for invaluable technical assistance. This work was supported by National Heart, Lung, and Blood Institute Grants HL-44116, HL-53542 (to Z. S. Katusic), and HL-58080 (to T. O’Brien). M. Zanetti is the recipient of an American Heart Association Northland Affiliate Fellowship, and T. O’Brien is the recipient of a Career Development Award from the Juvenile Diabetes Foundation.
REFERENCES


4. Beckman JS, Beckman TW, Chen J, Marshall PA, and Libby P. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624, 1990.


