Gene Transfer of Endothelial Nitric Oxide Synthase Improves Relaxation of Carotid Arteries From Diabetic Rabbits

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Background—Diabetes mellitus is associated with impairment of NO-mediated vascular relaxation. The purpose of this study was to determine whether adenovirus-mediated gene transfer of endothelial NO synthase (eNOS) or Cu/Zn superoxide dismutase (SOD1) improves responsiveness to acetylcholine in alloxan-induced diabetic rabbits.

Methods and Results—After 8 weeks, plasma glucose was greater in diabetic rabbits (418±35 mg/dL) (mean±SEM) than in normal rabbits (105±4 mg/dL). Carotid arteries were removed and cut into ring segments. Arteries were incubated for 2 hours with adenoviral vectors driven by a CMV promoter expressing β-galactosidase (β-gal), eNOS, SOD1, or vehicle. After incubation with virus, arteries were incubated for an additional 24 hours to allow transgene expression. Vascular reactivity was examined by recording isometric tension. After precontraction with phenylephrine, responses to the endothelium-independent vasodilator sodium nitroprusside were similar in diabetic and normal arteries. Endothelium-dependent relaxation to acetylcholine (3×10−6 mol/L) was significantly less in arteries from diabetic animals (68±5%) than in normal vessels (90±3%). Adenoviral transfection of arteries with eNOS improved relaxation in response to acetylcholine in diabetic (EC50 eNOS 5.06±0.12×10−7 mol/L versus vehicle 5.17±0.43×10−7 mol/L) but not normal arteries. Vasorelaxation in response to acetylcholine was inhibited by Nω-nitro-L-arginine (100 μmol/L) in all groups. Responses to acetylcholine were unchanged after gene transfection of SOD1 or β-gal in arteries from diabetic or normal rabbits.

Conclusions—Adenovirus-mediated gene transfer of eNOS, but not SOD, improves impaired NO-mediated relaxation in vessels from diabetic rabbits. (Circulation. 2000;101:1027-1033.)

Key Words: diabetes mellitus ■ acetylcholine ■ viruses ■ gene therapy ■ nitric oxide

adenoviral vectors are a useful method for introducing genetic material into blood vessels to alter vascular function. This approach has been used to improve function in blood vessels with functional impairment.

Diabetes mellitus is often associated with impairment of endothelium-dependent relaxation in response to acetylcholine in humans and experimental animals. This observation suggests that an increase in vascular levels of endothelial NO synthase (eNOS), a major mediator of endothelium-dependent relaxation, might improve vascular dysfunction in diabetes. Thus, the first goal of this study was to determine whether gene transfer of eNOS improves vascular function in diabetic vessels.

Other studies indicate that endothelial dysfunction in diabetic vessels may be produced by excess production of reactive oxygen species. Furthermore, administration of superoxide dismutase (SOD), which dismutes superoxide anion to H2O2, to diabetic animals improves endothelium-dependent relaxation. This finding suggests that the dilator response to acetylcholine might be improved by an increase in levels of SOD in vessels from diabetic animals. Thus, the second goal of this study was to determine whether gene transfer of SOD1 to carotid arteries from diabetic rabbits improves vascular function.

We have shown previously that after gene transfer, expression is greater in atherosclerotic than normal vessels when a cytomegalovirus (CMV) promoter is used. Because the CMV promoter may be activated by reactive oxygen species, it seemed likely that expression of the transgene would be augmented in diabetic as well as atherosclerotic vessels. The third goal of this study was to determine whether efficiency of gene transfer is augmented in diabetic vessels.

Methods

Adenovirus Vector

Three replication-deficient adenoviruses were used: (1) AdCMVeNOS (eNOS) constructed with cDNA for bovine eNOS, (2) AdCMVSOD1 (SOD1) containing cDNA for human Cu/Zn-SOD, and (3) AdCMVβ-galactosidase (β-gal) containing the reporter gene for β-gal, used as a control.
control virus.$^{34}$ A 3% sucrose PBS solution (vehicle) served as a virus-free control. Adenovirus was obtained from the Vector Core Laboratory of University of Iowa and stored at $-80^\circ$C until used.

**Animals**

Hyperglycemia was produced in adult male New Zealand White rabbits (2.2 to 2.5 kg) by administration of alloxan (150 mg/kg IV) via the lateral ear vein. Rabbits that did not develop diabetes served as controls. At the end of 8 weeks, body weight increased in normal rabbits ($n=6$) from 2.75±0.06 to 3.73±0.04 kg and in diabetic rabbits ($n=10$) from 2.68±0.07 to 2.76±0.15 at the end of the study ($P<0.05$ versus normal). Blood glucose concentrations at the end of the study were 105±4 mg/dL in normal rabbits and 418±35 mg/dL in diabetic animals ($P<0.05$, normal versus diabetic).

Eight weeks after induction of diabetes, rabbits were euthanized by injection of sodium pentobarbital (50 mg/kg) followed by exsanguination. The carotid arteries were quickly removed and placed in cold (4°C) oxygenated Krebs solution (mmol/L: NaCl 133, KCl 4.7, NaH$_2$PO$_4$ 1.35, NaHCO$_3$ 16.3, MgSO$_4$ 0.61, glucose 7.8, and CaCl$_2$ 2.52). The carotid arteries were then cut into segments 4 mm long for ex vivo incubation with adenovirus. All procedures and handling of animals were reviewed and approved by the Animal Care and Use Committee of the University of Iowa.

**Ex Vivo Infection**

Rings from the carotid arteries were placed in a 96-well culture plate and incubated with either Ad5CMV$\beta$-gal (1×10$^9$ and 3×10$^9$ pfu/mL), Ad5CMVeNOS (3×10$^9$ pfu/mL), Ad5CMVSOD (3×10$^9$ pfu/mL), or vehicle (PBS with 3% sucrose) for 2 hours at 37°C. The rings were placed in Eagle's minimal essential media (Boehringer Mannheim) containing 100 U/mL penicillin and 100 µg/mL streptomycin for an additional 24 hours at 37°C in a chamber aerated with 95% O$_2$ and 5% CO$_2$.

**Detection of $\beta$-Galactosidase**

After ex vivo incubation, some vessels were rinsed with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for detection of $\beta$-gal as previously described.$^3$ Vessel segments were incubated in 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal, Sigma) solution for 2 hours at room temperature, embedded in paraffin, sectioned, and counterstained with nuclear fast red.

In other vessels, after incubation in adenovirus, the vessel segments were rinsed with PBS, frozen in liquid nitrogen, and stored at $-70^\circ$C until use. $\beta$-Gal activity was measured with a chemiluminescent reporter assay (Galacto-Light Plus, Tropix) as previously described.$^3$ Tissue was minced with a scalpel blade and placed in 150 µL of Galacto-Light Lysis Solution (100 mmol/L, potassium phosphate [pH 7.8], 0.2% Triton X-100). The homogenate was centrifuged at 10 000g for 10 minutes, and the supernatant was removed. The assay was performed with 10 µL of supernatant in 200 µL Galacto-Plus substrate:reaction buffer diluent (1:100 dilution). This reaction was carried out at room temperature, and light emissions were measured with a Moonlight 2010 luminometer (Analytical Luminescence Laboratory). A standard calibration curve was generated with purified Escherichia coli $\beta$-galactosidase (Boehringer Mannheim). Protein concentrations were determined with a Bio-Rad DC Protein Assay. $\beta$-Gal activity was expressed as mU $\beta$-gal/mg protein. Values for each group were calculated as an average of 2 rings from each animal.

**Detection of Superoxide**

Hydroethidine, an oxidative fluorescent dye, was used to evaluate levels of superoxide in situ as described previously.$^{21}$ Cells are permeable to hydroethidine, and in the presence of O$_2^-$, hydroethidine is oxidized to fluorescent ethidium bromide, in which form it is trapped by intercalation with DNA. This method provides sensitive detection of O$_2^-$ levels in situ. Unfixed frozen ring segments were cut into sections 30 µm thick and placed on glass slides. Hydroethidine (2×10$^{-6}$ mol/L) was applied to each tissue section and coveredslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. Normal and diabetic tissues were processed and

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**Figure 1.** In situ detection of O$_2^-$ in carotid artery. Confocal fluorescent photomicrographs of normal and diabetic rabbit carotid artery incubated with hydroethidine. Carotid artery from normal rabbit shows minimal fluorescence in endothelium (e) and adventitia. In contrast, carotid artery from diabetic rabbit shows increased ethidium bromide fluorescence reflecting increased O$_2^-$ levels throughout vessel wall, which was greatest in endothelium. Similar findings were observed in 4 of 5 diabetic rabbits.
Figure 2. Histochemical staining of a carotid artery from a diabetic rabbit transfected with AdCMV β-gal. A, Carotid artery at ×100; B, carotid artery at ×400. Blue color after X-gal staining represents expression of β-gal. Sections were counterstained with nuclear fast red. β-Gal is observed in endothelium and adventitia.
Superoxide levels were also measured by lucigenin-enhanced chemiluminescence for 5 minutes. Background counts were determined and subtracted, and RLUs were normalized to surface areas.

Measurement of Vascular Function
Isometric tension was recorded to assess function of transfected carotid vessels. Vascular rings were mounted on stainless steel hooks at optimal resting tension (3 g) in individual organ baths bathed in Krebs bicarbonate solution at 37°C and aerated with 95% O2/5% CO2. Tension was periodically adjusted to the desired level during a 45-minute equilibration period. The vascular rings were then contracted with 100 mmol/L KCl and rinsed 3 times after each contraction. Responses to phenylephrine (10−7 to 10−5 mol/L) were then examined. Concentration-response curves for the endothelium-dependent dilators acetylcholine (10−9 to 10−7 mol/L) and A23187 (10−7 to 10−5 mol/L) or the endothelium-independent dilator sodium nitroprusside (10−8 to 10−5 mol/L) were then generated after precontraction of vessels with an EC50 dose of phenylephrine. In a separate set of vessels, responses to acetylcholine were examined to determine the presence of N^o-nitro-L-arginine (L-NNA, 100 μmol/L) to inhibit eNOS.

Statistical Analysis
Contractile responses were expressed as percent contraction of response to 100 mmol/L KCl, and relaxation was expressed as percent relaxation to contraction produced by an EC50 dose of phenylephrine. All data are expressed as mean±SEM. Intergroup comparisons were performed with an independent 1-way ANOVA to test for difference among treatment groups, followed by Bonferroni’s corrected t test. Comparisons between diabetic and normal groups were made with Student’s paired t test. Differences were considered to be significant at a value of P<0.05.

Results

Superoxide Production
Vessels from diabetic rabbits had increased O2− levels measured by hydroethidine fluorescence compared with nor-
mals (Figure 1). The increase in basal $O_2^{-}$ levels was observed in endothelial cells, media, and adventitia.

With lucigenin chemiluminescence, there was no detectable basal production of $O_2^{-}$ in segments of carotid artery. Because previous studies suggest that NADH/NADPH oxidase is a major source of $O_2^{-}$ production in vessels, we assessed $O_2^{-}$ production in vessels from normal or diabetic rabbits that were stimulated by NADH oxidase. Superoxide production in response to NADH (0.1 mmol) was >2-fold greater in carotid arteries from diabetic rabbits than in normal carotids ($277 \pm 54$ versus $117 \pm 15$ RLU·min$^{-1}$·mm$^{-2}$, $P<0.05$). These data suggest increased propensity to generate $O_2^{-}$ levels in carotid arteries from diabetic animals during treatment with NADH.

Expression of β-Gal

Twenty-four hours after incubation with AdCMVβ-gal, rings from the carotid artery were analyzed histochemically for transgene expression. Positive staining for β-gal was noted in adventitial and endothelial cells but not in vascular muscle (Figure 2). There was no staining in vehicle-treated vessels. The activity of β-gal was similar in normal and diabetic rabbits when carotid arteries were incubated with β-gal, which indicates similar expression of transgene product (Figure 3).

Vasomotor Responses

In normal vessels after gene transfer of β-gal, SOD1, or eNOS, vasomotor responses to phenylephrine (Table), sodium nitroprusside (Figure 4A, Table), and A23187 (Table) were not different from those of vehicle-treated vessels. Relaxation to acetylcholine was also similar in vessels transfected with β-gal, eNOS, or SOD1 and in vehicle-treated vessels (Figure 5A). Relaxation to acetylcholine was inhibited after pretreatment with L-NNA (100 μmol/L) in all vessels (Figure 5A).

In carotid arteries from diabetic rabbits, phenylephrine produced dose-dependent contraction, which was not altered by transfection with either eNOS or SOD1 compared with β-gal or vehicle-treated animals (Table). Maximal contraction in vessels from diabetic rabbits (151 ± 16%) tended to be greater than that in normal rabbits (117 ± 8%), but it did not achieve statistical significance. In vehicle-treated vessels, responses to sodium nitroprusside, an endothelium-independent vasodilator, were similar in diabetic and normal rabbits. Transfection with β-gal, eNOS, or SOD1 did not alter the response to nitroprusside in vessels from diabetic rabbits (Figure 4A). In addition, vascular responses to the calcium ionophore A23187, an endothelium-dependent vasodilator, were similar in vehicle-treated rings in diabetic and normal rabbits. Transfection with β-gal, eNOS, or SOD1 did not alter the response to A23187 in vessels from diabetic rabbits (Table).

In vehicle-treated rings, maximal relaxation to acetylcholine was significantly less in diabetic (68 ± 5%; Figure 5B) than in normal (90 ± 3%; Figure 5A) rabbits ($P<0.05$). Transfection of β-gal or SOD1 did not alter responses to acetylcholine. Relaxation to acetylcholine was augmented in vessels from diabetic rabbits transfected with eNOS compared with incubation with vehicle or β-gal (Figure 5B). The EC50 for eNOS was significantly different from that for vehicle or β-gal (Table) in diabetic rabbits. Relaxation to acetylcholine was inhibited in all vessels after pretreatment of rings with L-NNA (Figure 5B).

Discussion

The major finding of this study is that adenovirus-mediated gene transfer of eNOS to carotid arteries improves impaired NO-mediated responses to acetylcholine in diabetic rabbits. This is the first demonstration, to the best of our knowledge, of the use of gene transfer to improve impaired vascular function in vessels during diabetes.

Endothelium-dependent relaxation is impaired in humans with both type I, insulin-dependent diabetes mellitus and type II, non–insulin-dependent diabetes mellitus. Endothelium-dependent relaxation is also a hallmark of impaired vascular responses in genetic models of diabetes or in animals in which diabetes is induced with either alloxan or streptozotocin. In this study, we observed less relaxation of carotid artery in response to acetylcholine in diabetic than in normal rabbits. Relaxation to sodium nitroprusside, an endothelium-independent vasodilator, was similar in normal and diabetic animals, which suggests that impaired relaxation to acetylcholine in diabetic rabbits is not due to dysfunction of vascular smooth muscle. These studies instead suggest impaired endothelium-dependent relaxation.

There was a tendency (not statistically significant) for greater maximal contraction to KCl in vessels from diabetic animals, which might make it difficult to compare relaxation in these vessels. Sodium nitroprusside was given to compare with responses to acetylcholine and to address differences in baseline. The key finding in this study is that gene transfer of eNOS improves relaxation to acetylcholine but not to sodium nitroprusside. These results cannot be explained by a tendency for augmented contraction to KCl in vessels from diabetic animals.

We did not measure vascular reactivity in fresh vessels to determine whether there is a change when vessels are maintained in culture, but we have previously reported that...
vascular function in normal arteries is not impaired by incubation in tissue culture for 24 hours. It was important for us to demonstrate, however, that vascular reactivity is still abnormal in arteries from diabetic animals after maintenance in tissue culture for 24 hours. We found that impairment of responses in arteries from diabetic animals was comparable to that described previously.

We and others have been successful in transferring eNOS cDNA to blood vessels. Although these studies have shown alteration in function after overexpression of eNOS in normal and diseased blood vessels, the functional effects of gene transfer of eNOS to vessels from diabetic animals have not been examined. It seemed important to determine whether overexpression of eNOS in arteries from diabetic rabbits might produce functional changes.

In this study, mechanisms responsible for endothelial dysfunction during diabetes appeared to be specific for a receptor-dependent stimulus of NO release, because the response to the receptor-independent endothelium-dependent vasomotor relaxing agent calcium ionophore A23187 was not impaired. Other investigators have also noted that vascular responses to A23187 may not be impaired in diabetic animals.

We observed that cells were stained for β-galactosidase in the endothelium and adventitia of vessels from normal and diabetic animals. Fibroblasts in culture have muscarinic receptors and it is possible that adventitial fibroblasts in vivo also contain muscarinic receptors and thus could release NO in response to acetylcholine after transduction with eNOS. Other studies indicate that recombinant eNOS in adventitial fibroblasts in the dog basilar artery can be activated by bradykinin. We have observed, however, that in the carotid artery of rabbits, after transduction of adventitia with eNOS and after denudation of endothelium, A23187 but not acetylcholine produces vascular relaxation. Thus, it appears that endothelium is necessary to produce relaxation in response to acetylcholine, even after gene transfer of eNOS, in the rabbit carotid artery.

Numerous studies suggest that the mechanism of impaired endothelium-dependent relaxation in diabetes and atherosclerosis may involve inactivation of NO by oxygen-derived free radicals. Production of superoxide anion inactivates NO, and dismutation of free radicals has general significance oxidizes hydroethidine. Polyethylene-glycolated SOD abolished endothium time in blood vessels, which confirms the specificity of the fluorescent signal for superoxide anion. Although we found increased O₂·⁻ levels in vessels from diabetic animals, gene transfer of SOD1 failed to improve vasomotor response to acetylcholine. The finding may result from several factors. First, in other studies in which exogenous SOD improves endothelium-dependent relaxation, the enzyme has access throughout the vessel wall. Adenovirus-mediated gene transfer increases SOD in the endothelium and adventitia not the media. Thus, enhanced production of free radicals in the media, which we demonstrated with hydroethidine (Figure 1), may not be corrected by gene transfer to endothelium and adventitia. Second, SOD1, which is present in cytosol, may not be able to protect NO from O₂·⁻ if the reaction of these radicals occurs in the extracellular space. Third, it is possible that abnormal relaxation to acetylcholine may not involve reaction with O₂·⁻. Our findings and those of others that responses to A23187 are not impaired in vessels from the diabetic animals suggest that there may be a selective effect on receptor-mediated endothelium-dependent relaxation, which may not be mediated by reactive oxygen species.

In previous studies, we observed greater expression after gene transfer to arteries in atherosclerotic than normal rabbits when an adenovirus with a CMV promoter was used. In the present study, we observed no difference in activity of β-galactosidase in carotid arteries from normal and diabetic rabbits. Although both atherosclerosis and diabetes may have elevated concentrations of oxygen-derived free radicals and thus would be expected to increase expression when the CMV promoter is used, we speculate that this mechanism may not be as active in diabetic vessels as in atherosclerotic vessels and thus might fail to enhance gene expression.

The goal of this study was to use gene transfer as a tool for vascular biology. The major factor for studying the effects of gene transfer ex vivo instead of in vivo is that multiple mechanisms can be studied in the same rabbits; eg, in this study, we compared vehicle with gene transfer of eNOS, CuZn-SOD, and 2 concentrations of β-gal in the carotid arteries of each rabbit. Using in vivo techniques, we would need to use vehicle in 1 carotid artery versus an intervention in the other carotid artery. Because the design is less efficient, many more rabbits must be studied. With a similar design, to accomplish the same goals, we would need ~3 times as many rabbits, and the approach would be less sensitive because comparison would be between multiple animals. Thus, this ex vivo gene transfer approach seems appropriate for mechanistic studies. In addition, the absence of an immune response in vitro also makes this approach attractive for mechanistic studies of vascular biology. Nevertheless, because of the immune response to adenovirus, it will be necessary to study gene transfer to vessels in vivo, especially if the approach were to move toward gene therapy.
In summary, this study demonstrates that adenoviral gene transfer of eNOS can improve impaired vascular function in diabetic vessels. Although several mechanisms may contribute to this response to eNOS, gene transfer provides a novel approach to study diabetic arteries. Gene transfer of SOD failed to improve vascular function. The finding of increased production of superoxide anion in the media of the artery, to which gene transfer of SOD does not have access, suggests that increased generation of superoxide throughout the arterial wall may play an important role in vascular dysfunction associated with diabetes mellitus.

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