Gene transfer of endothelial nitric oxide synthase (eNOS) in eNOS-deficient mice

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Lake-Bruse, Kristy D., Frank M. Faraci, Edward G. Shestely, Nobuyo Maeda, Curt D. Sigmund, and Donald D. Heistad. Gene transfer of endothelial nitric oxide synthase (eNOS) in eNOS-deficient mice. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H770–H776, 1999.—Relaxation to acetylcholine (ACH) and calcium ionophore (A-23187) is absent in aortas from endothelial nitric oxide synthase (eNOS)-deficient (eNOS -/-) mice. We hypothesized that gene transfer of eNOS would restore relaxation to ACh and A-23187 in eNOS -/- mice. Aortic rings from eNOS +/- and eNOS +/+ mice were exposed in vitro to vehicle or adenoviral vectors encoding β-galactosidase (lacZ) or eNOS. Histochemical staining for β-galactosidase and eNOS demonstrated transduction of endothelial cells and adventitia. Vehicle-treated vessels from eNOS -/- mice did not relax to ACh or A-23187 compared with eNOS +/- mice. In contrast, relaxation to nitroprusside (NP) was significantly greater in eNOS -/- mice than in eNOS +/- mice. Gene transfer of eNOS, but not lacZ, to vascular rings of eNOS -/- mice restored relaxation to ACh and A-23187. In vessels from eNOS -/- mice that were transduced with eNOS, Nω-nitro-L-arginine (10-4 M) inhibited relaxation to ACh and A-23187 but not NP. Thus vascular function can be significantly improved by gene transfer in vessels where a major relaxation mechanism is genetically absent.

The role of the endothelial isoform of nitric oxide synthase (eNOS) in vascular pathophysiology is difficult to evaluate with pharmacological approaches because most NOS inhibitors affect all three isoforms (endothelium derived, neuronal, and inducible; Refs. 17, 26). Mice with targeted disruption of the gene provide a new tool to study the role of eNOS in regulation of vasomotor tone (10). Aorta and carotid artery from eNOS-deficient (eNOS -/-) mice exhibit impaired endothelium-dependent vasodilation to acetylcholine (6, 10). It is not known, however, whether this abnormal vascular phenotype is the result of eNOS gene disruption per se or embryological and developmental abnormalities that result from life-long eNOS deficiency. We therefore performed an ex vivo complementation study (replacement of a disrupted gene) with a replication-deficient recombinant adenovirus containing the eNOS transgene to distinguish between gene deficiency vs. embryological and developmental anomalies. We hypothesized that vascular abnormalities caused by simple loss of eNOS would be complemented by ex vivo gene transfer, whereas abnormalities caused by life-long deficiency and compensatory changes in other vasoactive systems would not be corrected.

Gene complementation has been used previously in gene-targeted mice. For example, gene transfer of apolipoprotein E or low-density lipoprotein receptor to the liver of mice deficient in those proteins reduces plasma cholesterol (12, 27, 29). Several studies have demonstrated gene transfer to blood vessels (2, 13, 21, 22, 24, 31), but gene transfer to blood vessels of gene-targeted mice has not been reported.

In the present study, we modified the ex vivo gene transfer method that we have used in other species for use in mice (19). We evaluated effects of overexpression of the eNOS transgene in control mice (C57BL/6 and eNOS +/- mice) and eNOS -/- mice, to answer the question, Does overexpression of recombinant eNOS in aorta of eNOS -/- mice improve relaxation to acetylcholine and A-23187?

MATERIALS AND METHODS

Four-month-old male and female C57BL/6 mice (Harlan, 18-30 g) were used to establish a method for gene transfer to aortas of mice. Four-month-old littermate male and female eNOS -/- and eNOS +/- mice (18-30 g), originally generated at the University of North Carolina, were used for gene complementation studies. Generation of eNOS -/- mice has been described previously (25). Animals were maintained in the Animal Care Facility at the University of Iowa, which is American Association for Accreditation of Laboratory Care approved. Experiments were conducted in accordance with guiding principles of the American Physiological Society and the University of Iowa Institutional Animal Care and Use Committee.

Adenoviral vectors. Two replication-deficient recombinant adenovirus vectors were used. Ad-CMVlacZ (AdlacZ; generously provided by Dr. Zvonimir Katusic) was used as the control virus. Bovine eNOS [Ad-CMVeNOS (AdeNOS)] (kindly provided by Dr. Zvonimir Katusic) was used to overexpress eNOS. These viral vectors were constructed with methods similar to those described previously (3, 4, 5). Viral titer was determined by plaque assay on human embryonic kidney 293 cells that complement the E1 early replication-deficient recombinant adenovirus containing the eNOS transgene to distinguish between gene deficiency vs. embryological and developmental anomalies. We hypothesized that vascular abnormalities caused by simple loss of eNOS would be complemented by ex vivo gene transfer, whereas abnormalities caused by life-long deficiency and compensatory changes in other vasoactive systems would not be corrected.

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viral promoters. Wild-type virus concentration was $< 3 \times 10^4$ plaque-forming units (PFU)/ml as determined by plaque assay on human airway carcinoma A549 cells. After purification, the virus was suspended in phosphate-buffered saline with 3% sucrose added for stabilization of virus particles and stored at $-80°C$. 

Gene transfer to aorta. The descending thoracic aorta was removed from the mice and placed in a dish containing cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 7.1H₂O, 11.1 glucose, 25 NaHCO₃, and 2.54 CaCl₂H₂O. Loose fat and connective tissue were gently dissected away without disruption of the adventitia, and the vessel was cut into four rings 3 mm in length. With the use of a 96-well cell culture dish, each ring was incubated in a 200-µl volume of vehicle (PBS with 3% sucrose) or virus ($3 \times 10^8$ PFU/200 µl of AdlacZ or Ad-eNOS) for 3 h. Stock virus titters ($10^{10}$ PFU/ml) were diluted with Eagle’s minimal essential medium (MEM) containing 100 µg/ml of penicillin per 100 U/ml of streptomycin.

We selected the final viral titer on the basis of preliminary studies with viral titters ranging from $10^7$ to $10^8$ PFU/200 µl and exposure times to virus ranging from 2 to 5 h. After incubation with virus, the vessels were transferred into MEM to remove nonadherent virus particles. Vessels were then placed in 1 ml of MEM and incubated at 37°C with 95% O₂-5% CO₂ for 24 h. Vessel segments were then evaluated for vasomotor function or histochemical and biochemical analysis of transgene expression.

Histochemical and biochemical analysis of expression of reporter transgene. We examined expression of β-galactosidase in blood vessels that were transduced by AdlacZ, washed with PBS, lightly fixed on 10 min in 2% paraformaldehyde and 0.25% gluteraldehyde, and incubated with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) solution for 2 h at room temperature. After incubation with X-gal, vessels were rinsed in PBS and fixed in 7% Formalin. Tissues were embedded in paraffin, sectioned, and counterstained with hematoxylin. Transgene expression in the vessel was examined (but not quantified) by identifying blue nuclei in each cell layer (intima, media, and adventitia).

Expression of β-galactosidase was quantitated with a chemiluminescent assay (Galacto-Light Plus, Tropix, Bedford, MA). Vessels were minced and soaked in 90 µl of lysis buffer containing 0.2% Triton X-100 and 100 mmol/l potassium phosphate, pH 7.8. After 60 min, the tissue suspension was centrifuged at 10,000 g for 10 min and the supernatant was assayed for β-galactosidase. Light emission was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) and calibrated to a standard curve that was generated with purified Escherichia coli β-galactosidase. Enzyme activity was normalized to tissue protein concentration with the Bradford assay (Hercules, CA). Light emission was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) and calibrated to a standard curve that was generated with bovine albumin. Data are presented as β-galactosidase (mU/mg protein).

Immunohistochemical analysis of eNOS expression. Arterial rings were fresh-frozen in Tissue-Tek embedding medium (Miles). Serial 11-µm thick sections were cut, adhered to poly-L-lysine-coated slides, and stored in a cryostat at 4°C overnight. Before being stained, slides were allowed to dry in room air for 1 h. Horse serum (5%) was applied for 60 min to block nonspecific binding of protein. Mouse anti-eNOS antibody (1:50; kit no. 30020, Transduction Laboratories, Lexington, KY) was applied for 1 h. After sections of vessels were washed for 15 min in PBS, biotinylated goat anti-mouse immunoglobulin G (kit no. 4002, Vector Laboratories, Burling- ton, CA) was applied for 30 min. Sections were rinsed for 15 min in PBS, and then a complex of avidin and biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA) for peroxidase was further diluted fourfold and applied for 2 min and then washed with water for 5 min. Vessel sections were counterstained with hematoxylin and examined for positive staining of eNOS (dark purple color) by light microscopy.

Vasomotor function. Rings of aorta were suspended in an organ bath containing 25 ml of oxygenated Krebs buffer maintained at 37°C. The rings were connected to a force transducer to measure isometric tension (contraction and relaxation). Resting tension was increased stepwise to reach a final optimal tension of 0.5 g, and rings were allowed to equilibrate for at least 30 min. Krebs solution was changed before and twice after each curve (approximately every 30 min).

We measured vascular responses to acetylcholine (receptor-mediated agonist), calcium ionophore A-23187 (non-receptor-mediated activation of eNOS), and nitroprusside (a NO donor, endothelium-independent agonist). Cumulative concentration-response curves ($10^{-10}$–$10^{-5}$ M for nitroprusside and $10^{-8}$–$10^{-5}$ M for acetylcholine and A-23187) were generated after precontraction of vessels with 9,11-dideoxy-11β,9α-epoxymethanoprostaglandin F₂α (U-46619). Vessels were precontracted to 30–50% of maximal contraction. Preliminary studies were performed to determine the average maximal contraction elicited in aortic rings.

In each experiment, acetylcholine and nitroprusside were examined first. The order was alternated between acetylcholine and nitroprusside. The final intervention was A-23187 or maximal contraction to U-46619. Because A-23187 has prolonged effects and interfered with maximal contraction, vessels could not be evaluated for both relaxation to A-23187 and to U-46619 maximal contraction.

In studies of vasomotor function in ex vivo transduced aorta, vessels were incubated with a relatively low concentration of nifedipine ($3 \times 10^{-7}$ M) for 25 min before examination of vascular responses (see discussion). Nifedipine was rinsed from the organ bath after the vessels were stretched to the resting tension and maintained in the organ bath for the duration of the assay.

Drugs. Acetylcholine, nitroprusside, N-nitro-L-arginine, calcium ionophore A-23187, U-46619, and X-Gal were obtained from Sigma (St. Louis, MO). Nifedipine was obtained from Research Biochemicals International (Natick, MA). U-46619 was obtained from Cayman Chemical (Ann Arbor, MI). A-23187 was dissolved in dimethyl sulfoxide and diluted with distilled water. Nifedipine was dissolved in absolute ethanol and diluted with isotonic saline. Dimethyl sulfoxide and ethanol were diluted so that the final bath concentration was $\leq 0.1%$. Vehicles for nifedipine and A-23187 did not alter vasomotor tone. N-nitro-L-arginine was warmed and dissolved in Krebs solution. Acetylcholine and sodium nitroprusside were dissolved in isotonic saline. All concentrations are expressed as the final concentration of each drug in the tissue bath.

Calculation and statistical analysis. All data are presented as means ± SE; n indicates the number of animals. Relaxation to acetylcholine, A-23187, and nitroprusside was expressed as percent relaxation from the amount of precontra-
concentrations of virus used above). Incubation with duplicate segments after incubation with the same mU/mg protein (5 nM) for a total of 24 h. After gene transfer of 200 µl for 2, 3, or 5 h followed by incubation in media (Krebs, medium 199, and DMEM), polymyxin B, or addition of indomethacin (10 -5 M) did not prevent these spontaneous contractions or relaxations. However, incubation of the vessels with nifedipine (3 x 10 -7 M) for 25 min before precontraction with U-46619 (in the absence of nifedipine) greatly reduced or prevented spontaneous changes in vascular tone. This approach was used previously in human epicardial coronary artery rings to inhibit phasic activity and allow quantification of vasodilator responses in vitro (26). To determine if vasomotor function was altered by pretreatment with nifedipine, freshly harvested vessels from C57BL/6 mice were studied. Relaxation and contraction were examined after pretreatment with vehicle or nifedipine (3 x 10 -7 M). Contraction to U-46619 (3 x 10 -7 M) tended to be less in nifedipine-pretreated vessels than in untreated vessels (1.29 ± 0.14 vs. 1.58 ± 0.10 g of tension; n = 9, P < 0.05). Nevertheless, pretreatment with nifedipine did not alter relaxation to acetylcholine or nitroprusside and only slightly reduced relaxation to low (10 -7 M) concentrations of A-23187. On the basis of these findings, all vessels in subsequent gene transfer studies were pretreated for 25 min with nifedipine, and then the nifedipine was washed out of the organ bath before vascular responses were examined.

RESULTS

Preliminary studies. We evaluated activity of β-galactosidase in C57BL/6 mice aortic segments that were exposed to vehicle or three concentrations of AdlacZ (final viral titer concentration: 10 7, 3 x 10 8, 10 9 PFU/200 µl) for 2, 3, or 5 h followed by incubation in media for a total of 24 h. After gene transfer of β-galactosidase, enzyme activity was related to both the viral titer and duration of incubation with virus. After incubation with AdlacZ for 2 h, enzyme activity in vessels increased from 0 ± 2 (control) to 4 ± 1, 10 ± 5, and 50 ± 7 mU/mg protein (n = 4–6 duplicate segments) after incubation with 10 7, 3 x 10 8, 10 9 PFU/200 µl, respectively. After incubation with AdlacZ for 3 h, enzyme activity in vessels increased from 0 ± 2 (control) to 6 ± 1, 20 ± 5, and 130 ± 7 mU/mg protein (n = 4–6 duplicate segments after incubation with the same concentrations of virus used above). Incubation with 3 x 10 8 PFU/200 µl AdlacZ for 5 h increased enzyme activity in vessels to 408 ± 106 mU/mg protein (n = 4). In preliminary studies of vasomotor function, we found that relaxation to acetylcholine and nitroprusside was impaired after incubation with 3 x 10 9 and 10 9 PFU/200 µl AdlacZ for 3 h (data not shown). On the basis of these data, we exposed vessel segments to a viral titer of 3 x 10 8 PFU/200 µl for 3 h. This protocol provided moderate transduction of AdlacZ but retained intact vasomotor function.

Immunohistochemistry. Immunostaining of aorta from C57BL/6 and eNOS +/- mice confirmed the presence of endogenous eNOS only in endothelial cells (Fig. 1A). After transduction by AdeNOS, staining for eNOS was evident in both endothelium and many cells in adventitia (Fig. 1B). Staining of endothelial cells for eNOS was absent in aorta from eNOS -/- mice (Fig. 1C). In eNOS -/- mice after transduction with eNOS, staining was observed in some endothelial cells and many cells in adventitia (Fig. 1D). No eNOS staining was observed in aorta from eNOS -/- mice transduced with AdlacZ (data not shown).

Vasomotor function after incubation with virus. After incubation in media, vehicle, 3 x 10 8 PFU/200 µl, or 10 9 PFU/200 µl of AdlacZ or AdeNOS for 2 or 3 h, vessels often developed irregular oscillations in vascular tone, which were not observed in freshly harvested vessels. Incubation of vessels with other types of culture media (Krebs, medium 199, and DMEM), polymyxin B, or addition of indomethacin (10 -5 M) did not prevent these spontaneous are not present.
vessels from eNOS−/− mice without inhibition of relaxation to sodium nitroprusside (Fig. 4). These data provide evidence that restoration of relaxation was a result of eNOS transgene expression.

**DISCUSSION**

Two novel tools, targeted disruption of a selected gene and gene transfer, offer unique opportunities to study the role of a gene in vascular biology. These approaches can be combined to assess effects of replacement of genes in genetically altered mice. In normal mouse aorta, relaxation to acetylcholine is mediated by NO. However, in eNOS−/− mice, the aorta does not relax to acetylcholine (10). The present study confirms that relaxation of aorta in response to acetylcholine and A-23187 is mediated by eNOS. The novel finding in this study is that gene replacement (complementation) restores vascular function toward normal. This study...
Fig. 3. Relaxation to acetylcholine (A), calcium ionophore A-23187 (B), and nitroprusside (C) in aorta from wild-type control mice. Vessels were treated with vehicle (○) or were incubated with AdCMVlacZ (AdlacZ; ■) or AdCMVeNOS (AdeNOS; △). Data are means ± SE; n = 5–13. *P < 0.05 for comparison between groups (2-way ANOVA with adjusted Bonferroni’s post hoc).

Fig. 4. Relaxation of eNOS-transduced aorta from eNOS-deficient mice in response to acetylcholine (A), calcium ionophore A-23187 (B), and nitroprusside (C). Vessels were evaluated in the absence (○) or presence (■) of a NOS inhibitor, Nω-nitro-L-arginine (10^{-4} M; n = 4–13). Data are means ± SE; n = 5–13. *P < 0.05 without vs. with NOS inhibitor (2-way ANOVA with adjusted Bonferroni’s post hoc).

The tissue damage was avoided by reducing the concentration of virus in the infusion solution. In our ex vivo studies, we found attenuation of relaxation of mice aorta in response to acetylcholine and A-23187 (but not nitroprusside) after a high viral titer (10^9 PFU/200 µl) or a long exposure time to virus. Thus we used a submaximal titer that did not cause detectable vascular dysfunction but nevertheless improved vasculature responses.

Spontaneous oscillation in tone was observed in vessels incubated for 24 h in either the absence or the presence of virus. Spontaneous phasic contractile activity in isolated coronary arteries from humans has been reported by many researchers over the past 20 years (7, 8, 11, 23). In human coronary arteries, the mechanisms controlling tone are complex; however, the phasic contractions have been shown to be dihydropyridine-sensitive, voltage-operated Ca^{2+} channels (7, 28). Pretreatment with nifedipine inhibited spontaneous phasic activity in human coronary arteries and allowed quantitative analysis of vasoconstrictor and vasodilator agents (28). We also found that nifedipine was useful in preventing phasic activity of isolated segments of mice aorta. Pretreatment with nifedipine and removal of nifedipine before examination of vascular response did not prevent contraction with U-46619, nor did it alter maximal relaxation to acetylcholine, A-23187, and nitroprusside in vessels. Thus quantitative analysis of responses to vasodilator agents is feasible in mice aorta after ex vivo gene transfer.

Relaxation of eNOS-transduced aorta in eNOS--/ mice. Overexpression of eNOS in aorta of eNOS−/− mice produced histochemical evidence of eNOS staining of endothelial cells and adventitia and significantly improved relaxation to acetylcholine and A-23187. In contrast, overexpression of eNOS in aorta of control mice produced similar eNOS staining but without altering relaxation to acetylcholine and A-23187. We cannot explain the lack of enhanced relaxation in eNOS transduced vessels from eNOS+ /+ mice. It is possible that insufficient concentrations of cofactors or substrate in the in vitro vasomotor assay could account for these results. Inhibition of relaxation to acetylcholine and A-23187 with a NOS inhibitor provided evidence...
that restoration of relaxation in eNOS-/- mice was
mediated by NO via recombinant eNOS.

In eNOS-/- mice, relaxation of the aorta in response to
low concentrations of nitroprusside was augmented,
perhaps as a compensatory response to the absence of
eNOS (6). Overexpression of eNOS in these eNOS-/-
vessels did not significantly alter the hypersensitivity
to nitroprusside but restored maximal relaxation to
acetylcholine and A-23187, which was minimal in aor-
tic segments treated with vehicle or Adlacz.

Adventitial transduction of eNOS alters vascular
function. Gene transfer of eNOS and lacZ to adventitia
by adenoviral vectors has been demonstrated by our lab
(20–22) and others (1–3, 14, 15). Intracisternal admin-
istration of AdeNOS resulted in transduction of adventi-
titial fibroblasts in cerebral arteries (2). Electron micros-
copy with immunogold labeling demonstrated recombina-
tional eNOS cellular localization (caveoli) in adventitial fibroblasts. In the present study, both immu-
nohistochemistry for eNOS and X-Gal staining for
β-galactosidase showed transduction of both endothe-

cial cells (eNOS-/- mice transduced with eNOS) and
many fibroblast-like cells in the adventitia (control and
eNOS-/- mice).

It was not surprising to find that endothelial cells
from eNOS-/- mice could be transduced with eNOS, but
it is of interest that restoration of relaxation to acetyl-
choline resulted from transduction of what appears to
be only a relatively limited number of endothelial cells
in eNOS-/- mice. We do not know why such an
apparently low level of transduction resulted in signifi-
cant improvement of acetylcholine relaxation in aorta
from eNOS-/- mice. One possible explanation for this
restored relaxation to acetylcholine relates to increased
sensitivity of vessels to NO. We observed a significant
increase in sensitivity to the NO donor nitroprusside
in aorta from eNOS-/- mice. A second possibility is that
eNOS-transduced fibroblasts in the adventitia contrib-
uted to acetylcholine-induced relaxation. There is evi-
dence that fibroblasts, in culture, contain muscarinic
receptors (9, 13), and thus it is possible that adventitial
fibroblasts express muscarinic receptors that could be
coupled to recombinant eNOS. Previous studies in
other species have shown that recombinant eNOS in
fibroblasts can be activated after stimulation of fibro-
blast receptors (18, 30). Thus we cannot exclude the
possibility that activation of eNOS in adventitia contrib-
uted to relaxation in response to acetylcholine in eNOS
-/- mice after gene transfer with eNOS. Immunohisto-
chemistry suggested that adventitia was transduced
with eNOS in both control (eNOS +/- and C57BL/6)
and eNOS -/- mice. In common carotid arteries of
rabbits, with adventitia transduced with eNOS and
eNOS-/- mice. In contrast to denuded vessels, many
fibroblast-like cells in the adventitia (control and
eNOS-/- mice).

Because of the small size of the mouse aorta, it is
difficult to completely remove endothelium without
damage to smooth muscle and adventitia. For example,
rolling of vascular rings, which is a common approach
to denuding vessels, may damage transduced adventi-
titia. Previous studies (30) have shown that endothelium-
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In summary, we have demonstrated, first, that ex
vivo gene transfer to mice aorta is feasible and that
functional consequences can be evaluated. Second,
eNOS transduction results in restoration of relaxation
to acetylcholine and A-23187 in aorta of eNOS-/- mice.
This restoration is mediated by NOS, as demonstrated
with a NOS inhibitor. Finally, this is the first study to
our knowledge that demonstrates improvement of vas-
cular function in gene-targeted mice with gene transfer
to blood vessels. This study indicates that, even in
vessels in which a major relaxation mechanism is
genetically absent, vascular function can be signifi-
cantly improved by gene transfer.

We thank Zvonimir Katusic for providing AdCMVeNOS; Beverly
L. Davidson for providing AdCMVlacz; Pamela K. Tompkins
for technical assistance, and Arlinda LaRose for secretarial assistance.
We also thank the University of Iowa Gene Transfer Vector Core and
Richard D. Anderson for preparation of the viruses and Lisa Hancock
from the Transgenic Animal Facility for genotyping the mice. Geneti-
cally deficient mice generated at the University of North Carolina
were supported in part by the College of Medicine and the Diabetes
Endocrinology Research Center.

This work was supported by National Institute of Neurological
Disorders and Stroke Grant NS-24621 and National Heart, Lung,
and Blood Institute Grants HL-16066, HL-14388, and HL-38901. K. D.
Lake-Bruse is supported by Institutional Training Grant DK-
07690. F. M. Faraci and C. D. Sigmund are Established Investigators
of the American Heart Association.

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Received 12 November 1998; accepted in final form 13 April 1999.

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