Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia

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Objective: **Although nitric oxide (NO) has a critical role in angiogenesis, the therapeutic potential of NO synthase overexpression in severe ischemia remains undefined. We tested the hypothesis that overexpression of endothelial NO synthase (eNOS) would improve tissue perfusion in severe hind limb ischemia.**

Methods: **Severe hind limb ischemia was induced in 122 adult male Sprague-Dawley rats. Ten days after the induction of hind limb ischemia, vascular isolation and intraarterial delivery of an adenoviral vector encoding eNOS (AdeNOS), a control adenoviral vector (AdE1), or phosphate-buffered saline solution (PBS) was performed. Skeletal muscle blood flow, muscle oxygen tension, angiography, and immunohistochemistry for capillary counts were measured.**

Results: **Gene transfer of AdeNOS increased eNOS protein expression and enzyme activity. Two weeks after gene transfer, skeletal muscle blood flow was fourfold higher in eNOS-transduced than in AdE1-transduced or PBS treated rats and was similar to exercise-induced maximal flow in nonischemic muscle. eNOS overexpression increased muscle oxygen tension in a titer-dependent fashion. This increase persisted 1 month after transduction, even though eNOS enzyme activity had declined to normal levels. Angiography and capillary counts showed that eNOS overexpression increased the size and number of collateral arteries, but did not significantly increase the capillary–muscle fiber ratio.**

Conclusions: **eNOS overexpression in an ischemic rat hind limb significantly increased skeletal muscle blood flow, muscle oxygen tension, and collateral arteries (arteriogenesis). Furthermore, eNOS overexpression did not result in capillary angiogenesis above control levels. These studies demonstrate the potential for eNOS overexpression as treatment for severe limb ischemia in human beings. (J Vasc Surg 2003;38:820-6.)**

Critical limb ischemia due to atherosclerotic occlusive disease is a significant clinical problem that can lead to disability and limb loss. Despite advances in surgical and catheter-based limb revascularization, critical limb ischemia still results in 150,000 major amputations annually.[1] Consequently there is intense interest in the development of therapeutic angiogenesis to reverse the effects of critical limb ischemia. This disease is a logical target for application of therapeutic angiogenesis, because of easy accessibility of the target tissue, ease in monitoring limb blood flow, and lack of treatment options if surgical or percutaneous revascularization is not feasible. A variety of peptide growth factors induce therapeutic angiogenesis in experimental models of hind limb ischemia[2-4]; vascular endothelial growth factor (VEGF) has been the most widely studied.[4-6]

However, nitric oxide (NO), produced by endothelial NO synthase (eNOS), has a critical role in angiogenesis. For example, autocrine production of NO is essential for endothelial cell proliferation and migration in vitro.[7] Furthermore, angiogenesis is impaired in eNOS knockout mice.[8] In addition, these mice are also resistant to VEGF-induced angiogenesis,[8] which suggests that the angiogenic effect of VEGF depends on production of NO.[9] Finally, dietary supplementation with L-arginine, the NO precursor substrate for eNOS, increases blood flow and improves vasomotor tone in rabbit hind limb ischemia[8] and corrects the impaired angiogenesis induced by hypercholesterolemia in rat hind limb ischemia.[10]

NO is important for other reasons. Critical limb ischemia often occurs in patients with other disorders that impair endothelial NO function, such as hypertension, diabetes, smoking, and hyperlipidemia. Decreased NO synthesis or activity in these disorders may be responsible for impairment of collateral artery development and resultant persistent tissue ischemia. These patients also may not receive the full therapeutic benefit of angiogenic therapy, which requires downstream activation of eNOS, such as administration of VEGF, as evidenced by lack of effect of VEGF on angiogenesis in eNOS knockout mice.[8] For these reasons, direct augmentation of eNOS activity is an attractive therapeutic target in patients with critical limb ischemia. We hypothesized that adenoviral overexpression of

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Competition of interest: none.

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eNOS would induce arteriogenesis and increase skeletal muscle blood flow and oxygen tension (PO_2) in a rat model of severe hind limb ischemia.

MATERIAL AND METHODS

Adenoviral vectors. All adenoviral vectors were first generation (E1 and E3 region deleted) replication-incompetent vectors. The adenoviral vector encoding eNOS (Ad-eNOS) contains the bovine endothelial NO synthase complementary DNA driven by the Rous sarcoma virus promoter,[11] and was a generous gift of Dr Beverly L. Davidson of the University of Iowa College of Medicine. AdE1 contains the identical viral backbone without a transgene. Propagation, purification, and storage of these vectors were performed as described,[12] and concentration of adenoviral vectors was determined with standard plaque assay techniques.[12] Frozen vector stocks were thawed immediately before use and diluted in sterile phosphate-buffered saline solution (PBS) to achieve the indicated titers.

Rat hind limb ischemia model and adenoviral gene transfer. One hundred twenty-two male Sprague-Dawley rats (300-350 g; Simonsen, Gilroy, Calif) were used. Animals were housed in an environmentally controlled room and were given food and water as desired. The care of animals complied with the Guide for the Care and Use of Laboratory Animals.

Rats were anesthetized by inhalation of 1% to 2% isoflurane, and hind limb ischemia was created with ligation of the left common iliac artery, external iliac artery, and femoral artery, as described.[12,13] Ten days after induction of ischemia, gene transfer to the vasculature and skeletal myocytes was accomplished with intraarterial injection of Ad-eNOS or appropriate control solution during vascular isolation of the hind limb, as described.[14] In brief, tourniquet isolation of the proximal thigh was used to prevent collateral inflow or outflow. The left saphenous artery was cannulated, and blood in the vasculature was flushed out with saline solution through a venotomy in the saphenous vein. The venotomy was clamped temporarily, and 0.7 mL of PBS or adenoviral vector was infused via the saphenous artery. After a 30-minute dwell time the vein clamp was removed, and vector was flushed out of the limb with 5 mL of PBS, followed by ligation of the saphenous artery and vein. This technique results in efficient adenoviral gene transfer to hind limb capillary endothelium and skeletal muscle, with minimal gene transfer in distant organs.[14]

eNOS immunoblotting and activity assay. Western blotting and eNOS enzyme activity assays were performed on homogenates of gastrocnemius muscle as previously.[12] In brief, gastrocnemius muscle homogenates were prepared, fractionated with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nylon membranes for Western blotting. Rabbit polyclonal antibody to eNOS (Transduction Laboratories, Lexington, Ky) was used to probe membranes, and secondary detection was done with horseradish peroxidase–linked donkey anti-rabbit antibody with chemiluminescent detection (ECL Plus; Amersham Bioscience, Piscatawny, NJ). Densitometry was determined with Scion Image (NIH Imaging, Bethesda, Md). Nitric oxide synthase activity was determined in gastrocnemius muscle homogenates on days 3, 14, and 30 after gene transfer, with a commercial assay that measures conversion of radiolabeled arginine to citrulline (Calbiochem-Novabiochem Corp, San Diego, Calif) according to the manufacturer's instructions.[12] Calcium-dependent NO synthase activity (NOS-1, NOS-3) was calculated by subtracting NOS activity measured without calcium from total NOS activity, and is expressed as counts per minute per milligram of protein.

Measurement of skeletal muscle blood flow and (Po_2). Rats were anesthetized 14 days after gene transfer, and regional blood flow to the gastrocnemius muscle was determined with fluorescent microspheres, as described.[13] In brief, 4×10^5 polystyrene blue or yellow-green fluorescent 15-μm-diameter microspheres (Triton Technology, San Diego, Calif) were injected into the left ventricle. Fluorescence in the gastrocnemius muscle was quantified with spectrophotometry after processing tissue with potassium hydroxide and 2-ethoxyethyl acetate. Blood flow rate was calculated as milligrams per liter per minute per gram of tissue. For measurement of skeletal muscle (Po_2), rats were anesthetized 14 and 30 days after gene transfer and their gastrocnemius muscles were exposed. Licox Clark-type oxygen and temperature probes (GMS, Keil-Mielkendorf, Germany) were inserted into the muscle through 18-gauge needles, and the sensors were positioned at the midportion of the muscle. (PO_2) and gastrocnemius muscle temperature were recorded every 1 to 3 minutes with the rats breathing 100% oxygen until a plateau was reached (approximately 45 minutes) and then room air (21% oxygen) until a plateau was noted (approximately 45 minutes). Measurements were made on 100% oxygen to unmask perfusion deficits in chronic ischemia, as described.[15,16] Control values were determined in the right (nonischemic) limbs of animals.

Immunohistochemistry. Immunohistochemical staining for eNOS protein was performed, as described,[12] on 10-μm frozen sections of gastrocnemius muscle, with a mouse monoclonal antibody to human eNOS (Transduction Laboratories; cross-reacts with human, rat, and bovine eNOS) at 1:250 dilution. Biotinylated goat anti-mouse immunoglobulin G (IgG) was applied, followed by avidin-biotin horseradish peroxidase complex, and colorimetric detection was performed with 0.05% 3,3′-diaminobenzidine (DAB) tetrahydrochloride and 0.01% hydrogen peroxide. Capillary and arteriole counts were determined by staining for the endothelial-specific antigen CD31 with a monoclonal antibody to rat CD31 (Pharmigen, San Diego, Calif). Sections were incubated overnight in 1:250 dilution at 4°C, washed, and incubated for 60 minutes in biotinylated horse anti-mouse IgG, and colorimetric detection was performed as described previously. Capillary and arteriole density and capillary–muscle fiber ratio were determined by a blinded observer examining the middle portion of sections at 400× magnification. To determine capillary and arteriole densities, six random fields were counted in a box

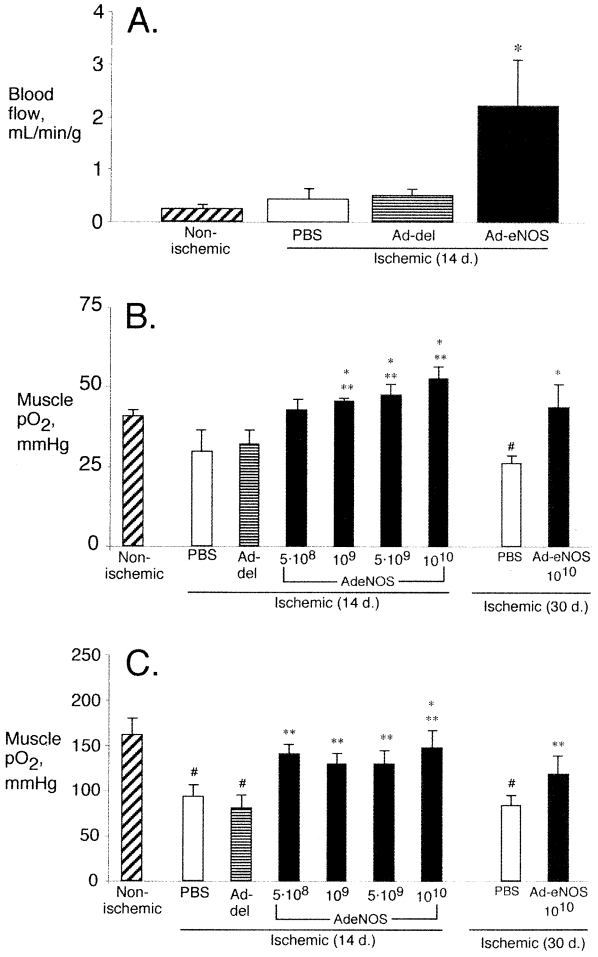

Fig 1. Effect of endothelial nitric oxide synthase *(eNOS)* overexpression on muscle blood flow and oxygen tension (Po_2). **A,** Skeletal muscle blood flow determined by microspheres 14 days after gene transfer. Significant increase in encoding eNOS *(AdeNOS)*–treated rats. $*P < .05$ vs other groups. **B,** Muscle Po_2 (in mm Hg) of normal and ischemic gastrocnemius muscles 14 and 30 days after gene transfer with rats breathing room air. Animals received the indicated titers of AdeNOS or control solutions as indicated. Note dose-dependent increase in Po_2 14 days after gene transfer with AdeNOS treatment, which persisted at 30 days. **C,** Rats breathing 100% oxygen to amplify perfusion differences between normal and ischemic muscles. Statistically significant decrease in Po_2 in phosphate-buffered saline solution *(PBS)*–treated and AdE1-treated ischemic animals; dose-dependent correction with AdeNOS treatment. $*P < .05$ vs PBS; $**P < .05$ vs AdE1; $\#P < .05$ vs normal.

measuring 0.0625 mm^2. Arterioles were differentiated from capillaries by vessel wall thickness (greater than 1 cell layer was consistent with an arteriole). To determine capillary–muscle fiber ratio, capillaries and muscle fibers were counted in five random muscle fiber bundles.

A bromodeoxyuridine (BrdU)–labeling and monoclonal antibody detection kit (Roche Diagnostics Corp, Indianapolis, Ind) was used to determine cellular proliferation. Rats were injected intraperitoneally with BrdU (31 mg/kg) 24 hours and 12 hours before they were killed. Frozen sections (10 μm) were prepared from the gastrocnemius and tibialis anterior muscles and the small intestine. In brief, after fixation, peroxidase blocking with 0.3% hydrogen peroxide, and rinsing in tris-buffered saline solution (TBS), the slides were incubated in 2N hydrocholoride for 30 minutes at room temperature. Slides were then rinsed with TBS and blocked with avidin and biotin. The slides were incubated with anti-BrdU mouse monoclonal antibody for 30 minutes at room temperature, and rinsed with TBS. The slides were treated with a biotinylated secondary antibody (horse anti-mouse [rat adsorbed]; Vector Laboratories, Burlingame, Calif) followed by a streptavidin peroxidase conjugate, and developed with DAB chromogen. The slides were counterstained with hematoxylin, dehydrated, and mounted. Slides from all groups were run in parallel on the same day. Negative controls consisted of similar sections treated in an identical manner but excluding the primary antibody. Positive controls consisted of the intestinal mucosa of the same rats. Cellular proliferation was quantified by counting BrdU-stained cells in six fields per slide at 400× magnification.

Hindlimb angiography. Inasmuch as no significant differences were noted in blood flow (Fig 1, *A*) or muscle oxygenation (Fig 1, *B*) between rats receiving PBS or AdE1, angiography was carried out on PBS-treated and AdeNOS-treated animals 30 days after gene transfer. Rats were anesthetized with 1% to 2% inhaled isoflurane. The infrarenal abdominal aorta was ligated proximally and cannulated distally with a 20-gauge polyethylene catheter. The hind limbs were exanguinated through a venotomy in the inferior vena cava, with heparinized saline solution (10 U/mL). Barium sulfate (0.6 g/mL, 1.5 mL) was then injected into the aortic catheter, and the vena cava was ligated. The skin was removed from the rat hind limbs to avert imaging the dermal vasculature. Images were acquired with a single enveloped Kodak X-OMAT TL film (Eastman Kodak, Dallas, Tex), with 500 mA, 50 kV, and 0.5 s exposure. Rats were killed before imaging. Images were scored independently by three raters blinded to treatment, who counted the number of vessels that crossed a standardized 1 × 1-cm grid overlying the image within an area of interest defined as the greater trochanter to the distal femur. The number of vessels was divided by the lines of the grid in the area of interest to produce an angioscore.

Statistical analysis. Mean and SEM values are reported. Statistical analysis was performed with the Student *t* test for two independent samples, and analysis of variance for multiple group comparisons was performed with the Fisher exact test for post-hoc analysis.

RESULTS

Gene transfer and expression of eNOS. Successful adenoviral gene transfer and eNOS overexpression were demonstrated with Western blotting, immunohistochemistry, and measurement of eNOS activity (Fig 2, *A-C*).

NOS activity was significantly higher in AdeNOS-treated rats than in AdE1-treated and PBS-treated rats at 3 and 14 days after gene transfer. By 30 days after gene transfer, eNOS activity in AdeNOS-treated rats decreased to normal levels (Fig 2, *C*).

Skeletal muscle blood flow and (Po_2). Blood flow in the gastrocnemius muscle, measured 14 days after gene transfer, was significantly higher in AdeNOS-treated rats (Fig 1, *A*). The flow rate of 2 mL/min/g of tissue achieved in ischemic rats treated with AdeNOS is similar to the maximal blood flow seen in nonischemic muscle when stimulated with exercise.[13] Resting blood flow was similar in nonischemic muscle and ischemic muscle treated with PBS or the control vector AdE1. We have demonstrated previously that resting skeletal muscle blood flow is low and requires exercise-induced hyperemia to demonstrate lack of flow reserve in experimental chronic ischemia.[13]

While the rats were breathing room air, (Po_2) of the left gastrocnemius muscle was significantly higher in AdeNOS-treated rats compared with PBS-treated or AdE1-treated rats at all titers of AdeNOS except 5×10^8 plaque-forming units (pfu)/mL (Fig 1, *B*). This increase persisted up to 30 days after gene transfer in rats receiving the highest dose of AdeNOS (1×10^{10} pfu/mL). To unmask perfusion deficits, rats were exposed to 100% inhaled oxygen.[15,16] Tissue PO_2 in the ischemic left gastrocnemius muscle was significantly higher in AdeNOS-treated rats than in AdE1-treated rats at all titers (Fig 1, *C*). However, PO_2 in AdeNOS-treated rats was significantly higher than in PBS-treated rats only at the highest titer of AdeNOS (147.3 ± 19.6 mm Hg; n = 6; $P < .05$).

Cellular proliferation. To determine cellular proliferation in AdeNOS-treated rats, sections of muscle were stained with BrdU. Despite the increase in blood flow and tissue Po_2 in AdeNOS-treated rats 14 days after gene transfer, no difference in BrdU staining was found in the calf muscles of experimental and control rats 5 days after gene transfer (data not shown).

Histology and angiography. Gross and microscopic examination of muscle did not reveal any evidence of angioma or other arteriovenous malformation, as has been described with VEGF overexpression.[17] Thirty days after gene transfer, the persistent increase in tissue oxygenation after eNOS overexpression correlated with increased collateral artery development and a trend toward increased capillary–muscle fiber ratio. Capillary density was not significantly higher in AdeNOS-treated rats compared with control rats (data not shown). However, when capillary counts were normalized per muscle fiber (capillary-fiber ratios) to compensate for tissue edema and muscle atrophy,[8] control rats had 1.8 ± 0.3 capillaries per fiber and AdeNOS-treated rats had 2.8 ± 0.7 capillaries per fiber ($P = .08$; Fig 3, *A*). Histologically, there was no difference in number of arterioles in AdeNOS-treated rats compared with control rats (data not shown). Contrast angiography revealed significantly more collateral artery development in AdeNOS-treated rats than in PBS-treated rats (Fig 3, *B*).

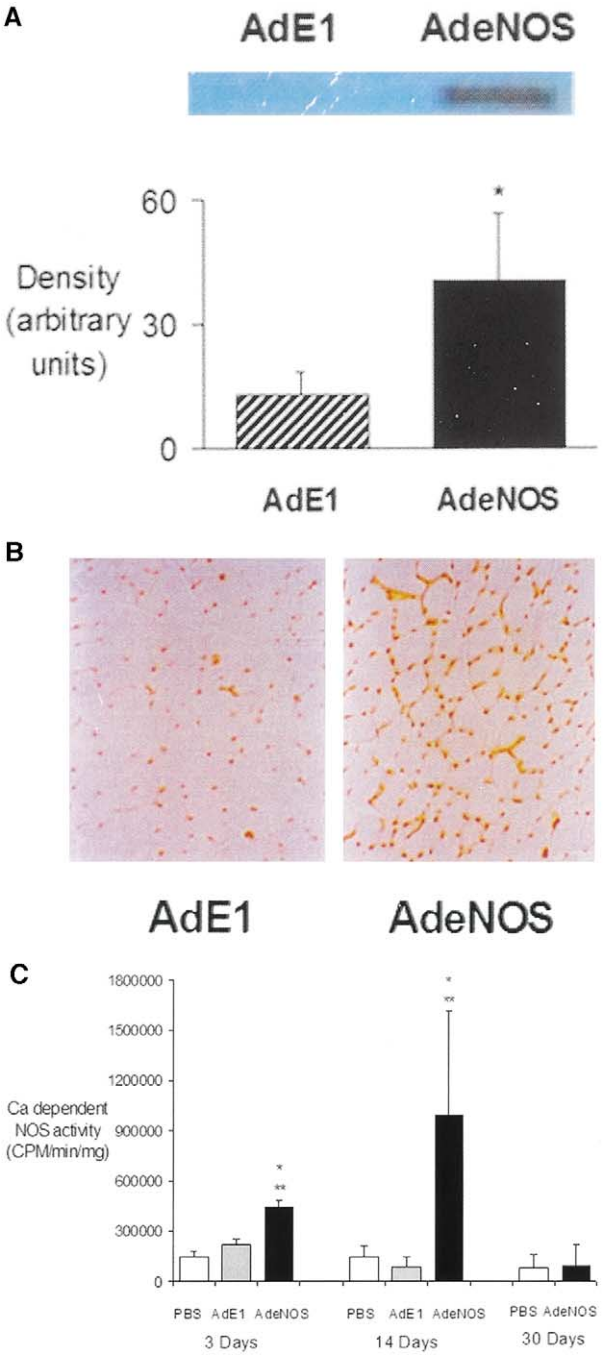

Fig 2. Transgene expression of endothelial nitric oxide synthase *(eNOS)* after adenoviral-mediated gene transfer in ischemic hind limbs. **A,** Western blot for eNOS protein on gastrocnemius muscle homogenates 3 days after gene transfer of either AdE1 (control vector) or adenoviral vector encoding eNOS *(AdeNOS)*; graph of densitometric analysis with Western blot (n = 3 animals each). **B,** Immunohistochemistry of cross-sectioned gastrocnemius muscle with anti-eNOS antibody 3 days after gene transfer of either AdE1 or AdeNOS (×80). **C,** Calcium-dependent NOS activity of muscle homogenates at 3, 14, and 30 days after gene transfer. *$P < .05$ vs phosphate-buffered saline solution *(PBS)*; **$P < .05$ vs AdE1.

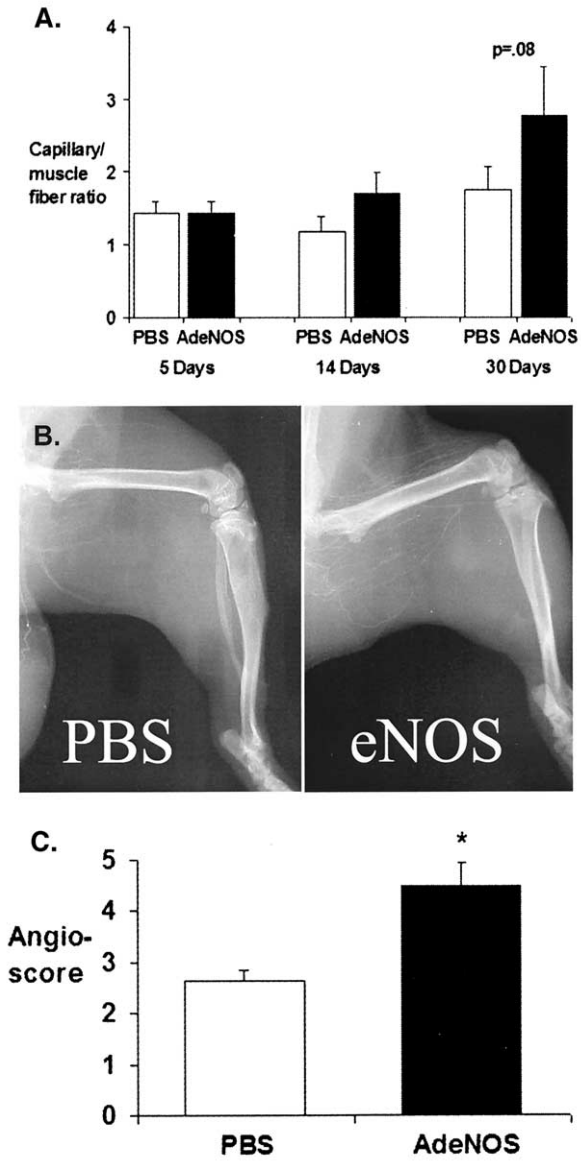

Fig 3. Capillary–muscle fiber ratio and angiography after endothelial nitric oxide *(eNOS)* overexpression. **A,** Trend toward increased capillary–muscle fiber ratio 30 days after gene transfer ($P = .08$). **B,** More large collateral arteries were observed in adenoviral eNOS (AdeNOS)–treated rats 30 days after gene transfer. Fewer large collateral arteries were observed in phosphate-buffered saline solution *(PBS)*–treated rats. **C,** Significantly higher angioscores in AdeNOS-treated rats (n = 7) vs PBS-treated rats (n = 6). $*P < .001$).

Mean angioscore was 4.5 ± 0.5 in AdeNOS-treated rats and 2.6 ± 0.2 in control rats ($P < .01$; Fig 3, *C*).

DISCUSSION

We examined the effects of eNOS overexpression on hind limb blood flow, muscle (Po_2), and neovascularization after induction of severe hind limb ischemia. Our major findings were that intraarterial administration of AdeNOS during vascular isolation results in increased NOS activity up to 2 weeks after gene transfer; and that overexpression of eNOS increases resting skeletal muscle blood flow to near maximal levels, increases (Po_2) in the gastrocnemius muscle 1 month after gene transfer, and increases collateral artery development, with a trend toward increased capillary-fiber ratios.

Overexpression of eNOS in this study increased blood flow fourfold and led to supranormal tissue (Po_2), substantially more than the 37% increase observed by Smith et al.[18] The two studies were both performed with rat hind limb ischemia models. However, they differ most notably in the delivery technique used for adenovirus, intraarterial to the calf in our study and intramuscular to the thigh in the study by Smith et al[18]; by the different vectors, bovine eNOS driven by the Rous sarcoma virus promoter and human eNOS driven by the cytomegalovirus promoter; and possibly by the level of eNOS overexpression induced.

Intra-arterial gene transfer results in greater adenoviral transduction of the endothelium and adventitia of arteries, arterioles, and capillaries than muscle fibers, whereas intramuscular delivery primarily transduces muscle fibers and capillaries.[14] This difference may account for the arteriogenesis and lack of significant capillary angiogenesis seen in our study. Our finding of increased blood flow and tissue oxygenation without a significant change in capillary counts indicates that the ability of NO to accelerate capillary angiogenesis[8,10] is not critical to its beneficial effects in hind limb ischemia. Hershey et al[19] also found that VEGF-induced increases in blood flow in hind limb ischemia correlated temporally with growth of large collateral vessels rather than increases in capillary density. In addition, we documented a threefold increase in eNOS activity, but Smith et al[18] did not measure in vivo increases in eNOS activity. A more direct comparison of the levels of induced eNOS activity might shed light on the differences between the results of that study and our own. Both studies taken together suggest that eNOS overexpression can drive both capillary angiogenesis and arteriogenesis, but that intraarterial delivery may be better to drive arteriogenesis. This interpretation is also suggested by our recent finding of increased blood flow without significant capillary angiogenesis after intraarterial delivery of an adeno-associated virus, using the same model of rat hind limb ischemia.[20]

As opposed to the fourfold increase in blood flow we observed after eNOS overexpression, either angiogenic peptides or oral supplementation with L-arginine[8,10] increases ischemic-normal blood pressure ratio by 30% to 50% in the rabbit femoral artery excision model of hind limb ischemia. These studies include intraarterial administration of VEGF protein,[4] intramuscular injection of VEGF protein,[5] intraarterial gene transfer of VEGF,[6] gene transfer of fibroblast growth factor,[21] and hepatocyte growth factor.[22] We measured regional blood flow rate and direct muscle (Po_2), and thus direct comparison with studies that used indirect indices such as calf ischemic-normal blood pressure ratio may be limited. Nonetheless, studies in which

both regional blood flow and calf blood pressure ratio were measured can serve as a basis for this comparison. The greater increases in blood flow after eNOS overexpression compared with other molecules have important clinical implications in reversing the effects of critical limb ischemia. Substantial and immediate increase in blood flow is necessary to prevent limb loss.

Differences in degree of ischemia and local concentration of NO may account for the differing effects of eNOS overexpression in our study and those that used L-arginine supplementation or pharmacologic vasodilators. Tissue hypoxia impairs endogenous eNOS enzyme activity,[23] and thus L-arginine supplementation may not be so effective as overexpression of eNOS in improving collateral artery growth and tissue perfusion.[24] In chronic hypoxia, rat arteries are dilated, VEGF is up-regulated, and production of NO is stimulated. Increased permeability and angiogenesis help rats maintain tissue oxygen delivery and consumption.[25] The failure of sufficient collateral blood flow development in patients with critical limb ischemia may be related to eNOS dysfunction in these patients; this enzyme dysfunction may also explain the lack of response to some pharmacologic vasodilators.

The mechanism by which eNOS overexpression increases blood flow and oxygenation may be multifactorial. Increased blood flow with only a trend toward increased capillary–muscle fiber ratio could be due to NO-induced vasodilation, as noted in eNOS gene transfer studies, to nonischemic hind limbs[26] and in hind limbs with attenuated vasodilatation after myocardial infarction.[24] However, we find this unlikely to be the sole mechanism, because there was persistent increase in muscle oxygenation and collateral artery development at 30 days (Fig 1) after NOS activity had subsided to basal levels (Fig 2). Rather, we suggest that eNOS overexpression leads to structural changes in the hind limb vasculature and that the sustained increase in tissue perfusion is not just from increased tonic vasodilation by NO. Of note, NO induces both endothelial cell migration and proliferation through the mitogen-activated protein kinase pathway in vitro,[27] and increase VEGF secretion through the hypoxia response element.[28] These nonvasodilatory functions of NO also support the idea that eNOS overexpression could lead to structural changes in the hind limb vasculature. Finally, release of NO from the endothelium inhibits adhesion of platelets and inflammatory cells; these anti-inflammatory and antithrombotic effects of NO may also contribute to the beneficial effect of eNOS overexpression in hind limb ischemia.

We hypothesize that eNOS overexpression may increase arteriogenesis via the following mechanism. Arteriolar vasodilation due to eNOS overexpression results in decreased vascular resistance in the calf. Blood flow increases through preformed collateral arteries in the thigh, thereby inducing enlargement of these vessels via shear stress–responsive arterial remodeling.[19] The early phase of the shear stress response consists of stimulation of endogenous eNOS to release increased NO, which in turn may stimulate collateral artery development. This proposed mechanism is consistent with our major findings, as well as the lack of cellular proliferation in the calf at 5 days.

Blood flow rate of the gastrocnemius muscle in eNOS-treated animals (>2 mL/min/100 g of tissue) approached flow rate induced by maximal exercise in nonischemic limbs, not resting flow rate.[13] Of no surprise, these rates of blood flow induce Po_2 greater than normal tissue Po_2 of 40 mm Hg (Fig 1, *A*). Sustained supranormal tissue oxygenation could lead to oxidant-mediated tissue injury; thus optimal treatment of limb ischemia may use lower titers of AdeNOS (ie, 5×10^8 pfu/mL; Fig 1, *A*). Lower adenoviral titers are less likely to produce side effects, such as inflammation caused by the adenoviral capsid protein, which can cause dose-dependent gangrene.[12]

The finding that eNOS overexpression results in substantial, dose-dependent increases in blood flow and tissue oxygenation without significant induction of capillary angiogenesis is novel and stands in contrast to the effects of angiogenic peptides and dietary L-arginine supplementation. The rapid and substantial increases in skeletal muscle blood flow and tissue oxygenation suggest that eNOS gene transfer is a molecular therapy of potential value in treating critical limb ischemia. Additional work on the exact mechanism or mechanisms by which eNOS overexpression increases collateral artery development also remains to be done. Impairment of eNOS function in many vascular diseases, the critical role of NO in mediating VEGF-induced angiogenesis, and the multiple beneficial effects of NO in the vasculature make overexpression of this enzyme a logical approach for treating critical limb ischemia.

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