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### Research Article

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# Nitric Oxide Synthase Modulates Angiogenesis in Response to Tissue Ischemia

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## Abstract

We tested the hypothesis that endothelial nitric oxide synthase (eNOS) modulates angiogenesis in two animal models in which therapeutic angiogenesis has been characterized as a compensatory response to tissue ischemia. We first administered L-arginine, previously shown to augment endogenous production of NO, to normal rabbits with operatively induced hindlimb ischemia. Angiogenesis in the ischemic hindlimb was significantly improved by dietary supplementation with L-arginine, compared to placebo-treated controls; angiographically evident vascularity in the ischemic limb, hemodynamic indices of limb perfusion, capillary density, and vasomotor reactivity in the collateral vessel-dependent ischemic limb were all improved by oral L-arginine supplementation. A murine model of operatively induced hindlimb ischemia was used to investigate the impact of targeted disruption of the gene encoding for eNOS on angiogenesis. Angiogenesis in the ischemic hindlimb was significantly impaired in eNOS<sup>-/-</sup> mice versus wild-type controls evaluated by either laser Doppler flow analysis or capillary density measurement. Impaired angiogenesis in eNOS<sup>-/-</sup> mice was not improved by administration of vascular endothelial growth factor (VEGF), suggesting that eNOS acts downstream from VEGF. Thus, (a) eNOS is a downstream mediator for in vivo angiogenesis, and (b) promoting eNOS activity by L-arginine supplementation accelerates in vivo angiogenesis. These findings suggest that defective endothelial NO synthesis may limit angiogenesis in patients with endothelial dysfunction related to atherosclerosis, and that oral L-arginine supplementation constitutes a potential therapeutic strategy for accelerating angiogenesis in patients with advanced vascular obstruction. (*J. Clin. Invest.* 1998; 101:2567–2578.) Key words: angiogenesis • nitric oxide (NO) • eNOS (NOS-3) • vascular endothelial growth factor (VEGF) • L-arginine

## Introduction

Nitric oxide (NO)<sup>1</sup> was initially identified by Furchgott and Zawadzki as endothelium-derived relaxing factor (EDRF), a

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molecule with profound vasomotor regulatory effects (1). Subsequent investigations established a plethora of diverse regulatory activities for NO, including inhibition of platelet aggregation, leukocyte adherence, and smooth muscle proliferation (2). Synthesis of NO has been shown to be tightly regulated by a family of isoenzymes (3), which share in common the property of converting arginine to citrulline, yielding free NO (4, 5). These fundamental observations have been extrapolated to in vitro and in vivo studies, in which administration of L-arginine supplements has been shown to reduce vasomotor tone (6), whereas deletion of the gene encoding for endothelial cell NO synthase (NOS 3) has been shown to result in systemic hypertension (7) and pulmonary vasoconstriction (8).

Previous investigations have provided inferential evidence that biological processes modulated by NO might extend to include angiogenesis. Brock et al. (9), for example, found that vascular endothelial growth factor (VEGF) increased cytosolic Ca<sup>2+</sup> in human umbilical vein endothelial cells (HUVECs), and Ku et al. (10) documented dose-dependent relaxation of isolated canine coronary arteries in response to VEGF that could be abolished by prior endothelial disruption and/or N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) pretreatment. In vitro studies performed in our laboratory demonstrated that VEGF stimulates the release of NO from the normal arterial wall (11), promotes recovery of disturbed endothelium-dependent flow in the rabbit ischemic hindlimb (12), causes NO-dependent hypotension in animals (13) as well as patients (14), and inhibits neointimal thickening in two different animal models of arterial injury (15, 16). Direct in vitro evidence that NO may induce angiogenesis was demonstrated recently by Papatropoulos et al. (17, 18). Moreover, Ziche and co-workers established the first line of evidence that NO can induce angiogenesis in vivo (19, 20).

Accordingly, we tested the hypothesis that endothelial nitric oxide synthase (eNOS) modulates angiogenesis, i.e., neovascularization developing in vivo in response to ischemia. For this purpose, we used two animal models, in which therapeutic angiogenesis has been characterized as a compensatory response to tissue ischemia. We first administered L-arginine, previously shown to augment endogenous production of NO, to normal rabbits with operatively induced hindlimb ischemia (21). We next used a mouse model of operatively induced hindlimb ischemia (22) to investigate the impact of targeted disruption of the gene encoding for eNOS on angiogenesis. The results of these experiments demonstrate that eNOS is

1. *Abbreviations used in this paper:* Ach, acetylcholine; APV, average peak velocity; cGMP, cyclic guanylate; EDRF, endothelium-derived relaxing factor; eNOS, endothelial NO synthase; 5-HT, serotonin; LDPI, laser Doppler perfusion imaging; NO, nitric oxide; PECAM-1, platelet endothelial cell adhesion molecule-1; SNP, sodium nitropruside; VEGF, vascular endothelial growth factor.

critical for angiogenesis, which occurs in response to tissue ischemia *in vivo*.

## Methods

### *Animal models: rabbit ischemic hindlimb model*

**Rabbits.** We used a previously described rabbit model of unilateral hindlimb ischemia (21). In brief, male New Zealand White rabbits (3.8–4.2 kg) (Pine Acre Rabbitry, Norton, MA) were anesthetized with xylazine (2 mg/kg), followed by ketamine (50 mg/kg) and acepromazine (0.8 mg/kg). After the skin incision, the entire femoral artery and all its major branches were dissected free. The external iliac artery and all of the above arteries were ligated with 4-0 silk (Ethicon, Sommerville, NJ). Finally, the femoral artery was excised from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. As a consequence, blood flow to the ischemic limb becomes completely dependent upon collateral vessels issuing from the internal iliac artery.

**Study design.** Animals were randomly divided into three groups. The L-arginine-treated rabbits ( $n = 18$ ) received 2.25% (wt/vol) L-arginine in drinking water starting either (a) immediately after surgery (L-arg group;  $n = 10$ ) or (b) 10 d after surgery (L-arg/10 group;  $n = 8$ ). Dietary L-arginine supplementation was continued until 37 d postoperatively. These rabbits then received drinking water without L-arginine for the next 3 d (i.e., until 40 d after operation), and were finally killed. The control ischemic group (c) consisted of 10 rabbits receiving no dietary L-arginine.

At 10 and 40 d after initial surgery, lower limb blood pressure, vasomotor reactivity, and angiographic score were examined (*vide infra*) in nontreated, ischemic (control) rabbits. The L-arginine treatment group was similarly investigated at 10, 37, and 40 d after initial surgery. The L-arginine 10-d group was evaluated only at day 40 after initial surgery. On day 40 after surgery, all animals were killed and tissue sections from the ischemic limb were submitted for histologic examination.

**Lower limb blood pressure ratio.** Each rabbit was anesthetized, the hindlimb skin shaved and cleaned, and the systolic blood pressure in each limb was measured from the posterior tibial artery using standard Doppler probe techniques (21). The blood pressure ratio was expressed as the ratio of systolic pressure in the ischemic versus normal limb (ischemic/normal blood pressure ratio); this ratio should be 1.0 in normal animals, and typically falls to  $< 0.3$  after surgery (21).

**Measurement of vasomotor activity.** After measurement of blood pressure ratio, a 3-Fr. infusion catheter (Tracker-18<sup>®</sup>; Target Therapeutics, San Jose, CA) was inserted into the abdominal aorta via the left common carotid artery. A 0.018-in Doppler guide wire (Cardiometrics, Mountain View, CA) was advanced through the 3-Fr. infusion catheter into the proximal segment of the internal iliac artery supplying the ischemic limb. The Doppler wire detects a real time spectral Doppler signal, from which the average peak velocity (APV, temporal average of the instantaneous peak velocity wave form) was calculated and displayed (23).

A second catheter (Tracker-18<sup>®</sup>) was introduced into the left common carotid artery through the same cutdown and placed at the origin of the common iliac artery of the ischemic limb using a separate 0.018-in guide wire (Hi-Torque Floppy II; Advanced Cardiovascular Systems, Temecula, CA) under fluoroscopic guidance. This catheter was used for infusion of vasoactive drugs, for measurement of intraarterial blood pressure via a pressure transducer, and for selective angiography (*vide infra*). The use of this catheter for drug infusion precluded graphic display of the blood pressure during drug infusion; intraarterial blood pressure was therefore determined immediately before and immediately after drug infusion.

The endothelium-dependent vasodilators, serotonin (5-HT), and acetylcholine (ACh) were administered intraarterially over 2 min via an infusion pump (1 ml/min). Each was administered at a dose of 1.5

$\mu\text{g}/\text{min}/\text{kg}$  on day 10 in L-arg, L-arg/10, and control groups, and again on day 37 in L-arg groups. In addition, on day 40, each group received 5-HT and ACh in increasing doses of 0.15, 1.5, and 15  $\mu\text{g}/\text{kg}/\text{min}$ ; 5 min elapsed between each dose to reestablish basal blood flow. After completing the 5-HT and ACh infusion, a 2-min intraarterial infusion of sodium nitroprusside (SNP, 1.5  $\mu\text{g}/\text{kg}/\text{min}$ ) was performed to evaluate endothelium-independent vasomotor reactivity.

**Measurement of blood flow and vascular resistance.** Doppler-derived flow was calculated as follows (23):

$$Q_D = (\pi d^2 / 4) (0.5 \times APV);$$

where  $Q_D$  = Doppler-derived time average flow,  $d$  = vessel diameter, and  $APV$  = time average of the spectral peak velocity.

The mean velocity was estimated as  $0.5 \times APV$  by assuming a time-averaged parabolic velocity profile across the vessel. The Doppler-derived flow calculated in this fashion has been shown to correlate with flow measurements determined by electromagnetic flowmeters both *in vitro* and *in vivo* (23). The vascular resistance was calculated by the following equation:

$$R_i = (P_c / Q_D);$$

where  $R_i$  = vascular resistance at internal iliac artery and  $P_c$  = blood pressure at common iliac artery.

The angiographic diameter of main collateral artery was measured at the site of the Doppler sample volume at 5 mm distal to its origin from the internal iliac artery as described above.

**Quantitative angiography.** Angiographic luminal diameter of the internal iliac artery in the ischemic limb at baseline and after drug (5-HT, ACh, or SNP) infusion was determined using an automated edge-detection system (23). The film selected for analysis was scanned with a high resolution video camera and the signal displayed on a digitized monitor. Center lines were traced manually for a 10-mm-long segment, beginning immediately distal to the tip of the Doppler wire. The contours were subsequently detected automatically on the basis of the weighted sum of first and second derivative functions applied to the digitized brightness information. The vascular diameter was then measured at the site of the Doppler sample volume (5 mm distal to the wire tip) (23). Cross-sectional area was calculated assuming a circular lumen. Angiography was performed immediately after drug administration and intraarterial blood pressure recording, using 1 ml of contrast media (Isovue-370; Squibb Diagnostics, New Brunswick, NJ). Serial images of the ischemic limb were recorded on 105-mm spot film at a rate of 2/s for 4 s.

At day 40, angiographic films recorded at the level of the medial thigh to evaluate morphological development of collateral vessels were performed at a rate of 1/s for 10 s, using 5 ml of contrast media (Isovue-370; Squibb Diagnostics) injected at 1 ml/s from an automated angiographic injector (Medrad, Pittsburgh, PA). The 4-s angiogram recorded at the thigh level of the ischemic limb was used to perform quantitative analysis of collateral vessel development (21). A composite of 5-mm<sup>2</sup> grids was placed over the medial thigh area. An angiographic score was calculated for each film by a single observer blinded to the treatment regimen as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the medial thigh.

**Analysis of capillary density.** The impact of L-arginine administration on microscopic angiogenesis was assessed by measuring the number of capillaries in light microscopic histologic sections taken from the normal and ischemic hindlimbs. Tissue specimens were obtained as transverse sections from the adductor muscle and the semi-membranous muscle of both limbs of each animal at the time of death (day 40). These two muscles were chosen for light microscopic analysis because they are the two principal muscles of the medial thigh, and each was originally perfused by that artery (deep femoral artery) ligated when the common/superficial femoral artery was excised. Muscle samples were embedded in optimal cutting temperature compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Multi-

ple frozen sections 5  $\mu\text{m}$  in thickness were cut from each specimen on a cryostat (Miles) so that the muscle fibers were oriented in a transverse fashion, after which the two sections were placed on glass slides. The tissue sections were stained for alkaline phosphatase with an indoxyl-tetrazolium method to detect capillary endothelial cells as previously described (24–26), and counterstained with eosin. Capillaries were counted under a 20 $\times$  objective to determine the capillary density (mean number of capillaries/ $\text{mm}^2$ ). 20 different fields from the two muscles were randomly selected for capillary counts. To ensure that analysis of capillary density was not overestimated as a result of muscle atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined for each histologic section. The counting scheme used to compute the capillary/muscle fiber ratio was otherwise identical to that used to compute capillary density.

**Serum levels of L-arginine.** The serum concentrations of L-arginine in the blood samples obtained from ear vein at days 37 and 40 were analyzed using ion-exchange liquid chromatography.

**Cyclic guanylate (cGMP) assay.** Five muscle samples each were harvested from the ischemic and nonischemic hindlimbs, respectively, of six rabbits whose drinking water had been supplemented with L- or D-arginine (2.25%) for 1 wk. The tissue samples were weighed (wet weight), frozen in liquid nitrogen, and stocked at  $-80^\circ\text{C}$  for cGMP measurement.

The assay for cGMP was performed as previously described (27) with slight modification. Briefly, at the time of assay, tissues were homogenized in 10-fold volume (wt/vol) of 6% trichloroacetic acid in polypropylene tubes at  $4^\circ\text{C}$ , and centrifuged at 2,000 g for 15 min. The supernatant was removed from each sample, placed in a polypropylene test tube, and washed four times with fivefold volume (vol/vol) of water-saturated diethyl ether. The liquid samples were then frozen in liquid nitrogen and lyophilized overnight. The lyophilate was resuspended in 1 ml of 0.05 M sodium acetate buffer (pH 5.8). Measurement of cGMP was performed using a cGMP enzyme-immunoassay system (Biotrak<sup>®</sup>; Amersham Life Science, Arlington Heights, IL), according to the manufacturer's protocol.

The weighed tissues remaining from cGMP measurement were digested using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Values for cGMP were standardized for protein content.

#### *Animal models: murine ischemic hindlimb model*

**Mice.** Mice with targeted disruption of the eNOS gene (eNOS<sup>-/-</sup> mice) and age-matched (8 wk) C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, ME) were used for these experiments. The eNOS<sup>-/-</sup> mice are deficient in eNOS protein, confirmed previously by Western blot analysis (7). Ringed segments of aorta from these mice when tested *ex vivo* lack endothelium-dependent relaxation in response to acetylcholine, although endothelium-independent relaxation to sodium nitroprusside is preserved intact (7). The eNOS<sup>-/-</sup> mice were obtained by breeding heterozygous eNOS mutant mice to obtain both homozygotes and wild-type littermates. Littermates (as well as SV129 wild-type mice) were tested and disclosed no differences versus C57BL/6. The fact that each of the three groups (C57BL/6, wild-type littermates, and SV129) behaved the same suggests that any differences seen in the eNOS<sup>-/-</sup> mice are due to eNOS gene deletion, although we cannot rule out with certainty an effect of other genes that segregate with the eNOS locus. For this to occur, one would have to postulate that a unique interaction occurs with these other genes so that their effects are not visible in each of the three wild-type groups.

**Surgery.** All animals were anesthetized by intraperitoneal pentobarbital injection (160 mg/kg) for subsequent surgical procedures and laser Doppler analysis (*vide infra*) of hindlimb blood flow. Skin incision was performed at the mid-portion of the left hindlimb overlying the femoral artery. The femoral artery was then gently isolated and the proximal portion of the femoral artery was ligated with 3-0 silk ligatures. The distal portion of the saphenous artery was ligated. The remaining arterial branches as well as veins were all dissected free, then excised. The overlying skin was closed using two surgical staples.

After surgery, mice were kept on a heating plate at  $37^\circ\text{C}$ , and special care was taken to monitor the animals until they had recovered completely from anesthesia.

**Monitoring hindlimb blood flow.** We measured ischemic (left)/normal (right) limb blood flow ratio using a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System [LDPI], Lisa Inc., North Brunswick, NJ). The LDPI uses a 12-mW helium–neon laser beam that sequentially scans a  $12 \times 12$  cm surface area. As the scanning is performed, moving blood cells shift the frequency of incident light according to the Doppler principle. A photodiode collects the backscattered light, and the original light intensity variations are transformed into voltage variations in the range of 0–10 V. A perfusion output value of 0 V was calibrated as 0% perfusion, whereas 10 V was calibrated as 100%. Upon termination of scanning, a color-coded image representing blood flow distribution is displayed on a monitor. The perfusion signal is subdivided into six different intervals, each displayed as a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as red. The stored perfusion values behind the color-coded pixels remain available for data analysis. Previous studies (28) established that laser Doppler flow velocity correlates with capillary density in the ischemic limb, including animals (C57 wild type), in which angiogenesis was retarded by administration of neutralizing antibodies prepared against VEGF, as well as the angiogenesis inhibitor platelet factor 4 (PF-4). Double immunolabeling for BrdU and CD-31 in ischemic versus normal murine hindlimbs established that endothelial cell proliferation peaks at 7 d ( $1,235 \pm 254$  versus  $8 \pm 14$  BrdU-positive cells/ $\text{mm}^2$  for the ischemic versus normal limbs, respectively,  $P < 0.001$ ); proliferative activity is then subsequently reduced at days 14 and 21. In mice treated with PF-4 and killed 14 d after surgery, capillary density ( $268 \pm 195$  versus  $1,053 \pm 371$  capillaries/ $\text{mm}^2$ ,  $P < 0.01$ ) and endothelial cell proliferation ( $16 \pm 29$  versus  $935 \pm 239$  BrdU-positive cells/ $\text{mm}^2$ ,  $P < 0.01$ ) were significantly reduced in PF-4 versus PBS-injected mice, respectively.

LDPI was used to evaluate perfusion of both left (ischemic) and right (nonischemic) murine hindlimbs. Excess hair was removed from the hindlimbs using a depilatory cream. Before initiating scanning, mice were placed on a heating plate at  $37^\circ\text{C}$  to minimize variations in temperature. For each time point described, we used LDPI to perform two consecutive measurements over the same region of interest (legs and feet), and found essentially little or no difference between the two scans. Accordingly, after twice recording laser Doppler color images, the average perfusions of the ischemic and nonischemic foot were calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, calculated perfusion was expressed as the ratio of left (ischemic) to right (nonischemic) hindlimb perfusion. Perfusion analyses were performed sequentially (a) before surgery, (b) immediately after surgery, and at (c) 3 d, (d) 7 d, (e) 14 d, (f) 21 d, (g) 28 d, and (h) 35 d after surgery under pentobarbital anesthesia.

**Tissue preparation.** Animals were killed at predetermined arbitrary time points after surgery with an overdose of sodium pentobarbital. For immunohistochemistry, whole ischemic and nonischemic limbs were immediately fixed in methanol overnight. After bones had been carefully removed, 3- $\mu\text{m}$ -thick tissue sections were cut and paraffin-embedded. For total RNA extraction, isolated tissue samples were rinsed in PBS to remove excess blood, flash-frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until use.

**Immunohistochemistry.** Histologic sections, 5- $\mu\text{m}$ -thick, prepared from paraffin-embedded tissue samples of the lower limbs were used for immunohistochemical analysis. Identification of endothelial cells was performed by immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) using a rat monoclonal antibody directed against mouse CD31 (PharMingen, San Diego, CA). Immunohistochemical localization of VEGF was performed using a rabbit polyclonal antibody directed against human VEGF amino-terminal peptides 1–20 (Santa Cruz Biotechnology, Santa Cruz, CA). Previous experiments revealed that this antibody

precipitates a 46-kD protein (nonreducing condition) or a 23-kD protein (reducing condition) from mouse uterus protein extract corresponding to the size of recombinant human VEGF<sub>165</sub> used as a control.

Immunoperoxidase staining was performed as previously described. In brief, sections were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. To prevent nonspecific antibody binding, sections were preincubated for 20 min in PBS containing 10% horse serum. Next, sections were incubated with the primary antibodies directed against either CD31 or VEGF at appropriate dilutions overnight at 4°C. Sections were then rinsed for 15 min with PBS, followed by incubation with biotinylated secondary antibody for 30 min at room temperature. After a 15-min wash, sections were treated with streptavidin-horseradish-peroxidase complex (Biogenex, San Ramon, CA) at room temperature for 30 min. Sections were then rinsed with PBS, and incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride dihydrate (for VEGF staining) or 3-amino-9-ethylcarbazole (for CD31 staining). Sections were finally counterstained with 20% Gill's hematoxylin, and subsequently covered. Negative control slides were prepared by substituting preimmune rat serum for CD31 and preimmune rabbit serum for VEGF antibody staining.

**Analysis of capillary density.** Capillary densities in both ischemic and nonischemic limbs were analyzed for specific evidence of neovascularity. Endothelial cells positively stained with CD31 were counted under light microscopy. Five different microscopic fields on two different light microscopic sections from each animal (three animals at each time point) were counted, and capillary density was expressed as number of capillaries/mm<sup>2</sup>.

**Analysis of VEGF mRNA expression.** Total tissue RNA was isolated from ischemic hindlimb by phenol/chloroform extraction. 15 µg of total RNA per lane was separated by electrophoresis on 1% agarose gels containing formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham). The membranes were hybridized with <sup>32</sup>P-labeled probe specific for VEGF, a 675-bp EcoRI/BglIII fragment of plasmid pSV1.VEGF.21 (29). Hybridization conditions were carried out as previously described (29).

### Drugs

Recombinant human VEGF protein (rhVEGF<sub>165</sub>) was the generous gift of Bruce Keyt (Genentech, South San Francisco, CA). Replication-defective adenovirus encoding VEGF<sub>165</sub> was the generous gift of Brian Annex and Kevin Peters. L-arginine hydrochloride, acetylcholine, serotonin, and SNP were all purchased from Sigma Chemical Co. (St. Louis, MO). L-arginine was prepared in normal water for rabbits as a 2.25% concentrated solution. Fresh stock solutions of acetylcholine, serotonin, and SNP were prepared daily.

### Statistical analysis

All results are expressed as mean ± standard error (m ± SEM). Statistical significance was evaluated using unpaired Student's *t* test for

comparisons between two means. Multiple comparison between three groups was performed using ANOVA. When a significant difference was detected, multiple-comparison analysis was performed using Student-Newman-Keuls test. A value of *P* < 0.05 was interpreted to denote statistical significance.

## Results

### Evidence of enhanced angiogenesis in rabbits receiving dietary L-arginine supplementation

**Effects of dietary L-arginine in rabbits.** Body weight and systolic blood pressure did not differ between control and L-arginine-treated animals. Blood flow and vascular resistance measured from the internal iliac artery before L-arginine or saline administration did not differ between treatment and control groups at any time points. L-arginine administration significantly increased serum L-arginine concentrations by twofold, compared to control, nontreated rabbits (Table I).

To evaluate the direct effects of L-arginine on hemodynamic findings at day 40, dietary L-arginine was discontinued from day 37 to day 40 in L-arginine groups, and this was sufficient to lower serum L-arginine concentration to levels similar to those measured in control, nontreated animals (Table I).

**Calf blood pressure ratio.** L-arginine administration promoted greater recovery of ischemic/normal calf blood pressure ratio than was observed in the control group. The ischemic/normal calf blood pressure ratio measured at day 40 was 0.84 ± 0.04 for L-arginine group compared to 0.61 ± 0.02 for control rabbits (*P* < 0.05; Fig. 1). Likewise, blood pressure ratio of L-arg/10 group at day 40 (0.74 ± 0.02) was also significantly greater than that measured in the control group (*P* < 0.05). Thus, dietary L-arginine supplementation significantly improved ischemic/normal calf blood pressure ratio, suggesting augmented perfusion in both L-arginine-treated groups compared to control rabbits.

**Iliac arterial blood flow.** Blood flow measured at baseline was similar for both L-arginine and control groups. At day 10, we evaluated iliac blood flow response to a single dose of each of three vasoactive substances, 5-HT, ACh, and SNP (Fig. 2). In controls, 5-HT infusion (1.5 µg/ml/kg) markedly reduced iliac arterial blood flow. In L-arginine-treated animals, the reduction in blood flow induced by 5-HT was significantly less than in control animals (*P* < 0.01). Similarly, ACh increased iliac arterial blood flow in L-arginine-treated animals to a markedly greater extent than was seen in control animals. The increased iliac blood flow in response to endothelium-indepen-

Table I. Basal Data of Rabbits

	<i>n</i>	Serum L-arg concentration (µmol/dl)	Body weight (kg)	Systolic blood pressure at healthy limb (mmHg)	Rest blood flow (ml/min)	Rest vascular resistance
Control group (day 10)	7		2.97 ± 0.20	96.6 ± 8.5	14.8 ± 1.3	3.8 ± 0.5
Control group (day 40)	8	11.0 ± 1.5	3.19 ± 0.23	108.9 ± 13.2	16.9 ± 2.6	4.2 ± 0.9
L-arg group (day 10)	9		2.98 ± 0.17	100.4 ± 9.7	12.2 ± 1.5	4.7 ± 0.4
L-arg group (day 37)	6	24.7 ± 3.3*	3.27 ± 0.15	119.3 ± 8.7	15.1 ± 2.0	4.3 ± 0.3
L-arg group (day 40)	6	9.0 ± 1.0	3.34 ± 0.11	106.3 ± 8.2	13.2 ± 1.0	4.3 ± 0.3

The body weight, basal hemodynamic data, and serum L-arginine concentration are indicated in control group (at day 10 and 40) and L-arginine treatment group (at day 10, 37, and 40). Serum L-arginine level significantly increased after dietary L-arginine supplementation (\**P* < 0.01; control group day 40 versus L-arginine group day 37). There were no significant differences in body weight, systolic blood pressure at healthy hindlimb, rest blood flow, and rest vascular resistance between control and L-arginine group at day 10 and at day 40. There were also no differences in these values between days 37 and 40 within L-arginine treatment group.

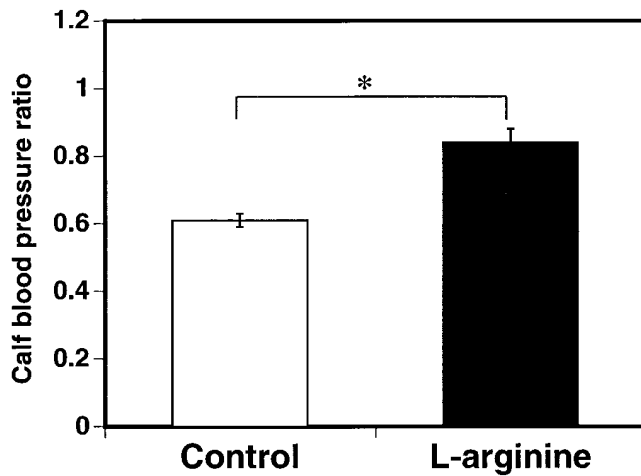


Figure 1. The ratio of systolic blood pressures measured in the ischemic/normal hindlimb at calf level at day 40. L-arginine supplementation significantly ( $* = P < 0.05$ ) improved ischemic/normal calf blood pressure ratio, suggesting augmented perfusion in L-arginine-treated versus control rabbits. ( $* = P < 0.01$ ; control group versus L-arginine group).

dent vasodilator SNP was also significantly higher in the L-arginine group than control group ( $P < 0.01$ ; Fig. 2).

At day 40 (3 d after discontinuing L-arginine supplements), iliac blood flow response to these same vasoactive agents was

restudied (Fig. 2). Lower doses (0.15–1.5  $\mu\text{g/ml/kg}$ ) of 5-HT increased iliac blood flow in L-arginine group, but caused dose-dependent reduction in blood flow in control rabbits ( $P < 0.01$  versus control). Although higher doses (15  $\mu\text{g/ml/kg}$ ) of 5-HT reduced blood flow in both groups, flow reduction was significantly less in L-arginine than in control rabbits ( $P < 0.05$ ; Fig. 2). ACh increased blood flow in a dose-dependent manner in both groups; however, the magnitude of increase in blood flow was significantly higher in L-arginine group than in control group ( $P < 0.01$ ; Fig. 2). The response to SNP also disclosed significantly increased blood flow in L-arginine versus control animals ( $P < 0.01$ ; Fig. 2). Thus, dietary L-arginine increased iliac blood flow in response to both endothelium-dependent and -independent vasodilators.

**Arterial resistance.** At day 10, we evaluated arterial resistance after administration of 5-HT, ACh, and SNP (Fig. 3). In control rabbits, we observed a markedly greater increase in arterial resistance in response to 5-HT than in the L-arginine rabbits. ACh induced a markedly greater reduction in vascular resistance in L-arginine than in control rabbits, as did SNP (Fig. 3). Similar findings were recorded at day 40 (Fig. 3).

**L-arginine concentrations versus hemodynamic measurements.** After 37 d of L-arginine therapy, serum L-arginine equaled  $24.7 \pm 3.3 \mu\text{mol/dl}$ . 3 d after discontinuing L-arginine, serum concentration of L-arginine returned to normal ( $9.0 \pm 1.0 \mu\text{mol/dl}$ ). Vasomotor responses to 5-HT, ACh, and SNP (1.5  $\mu\text{g/ml/kg}$ ) were comparable ( $P = \text{NS}$ ) at these two time points. Calf blood pressure ratio was also comparable at days 37 and 40 despite the above-noted differences in serum L-arginine

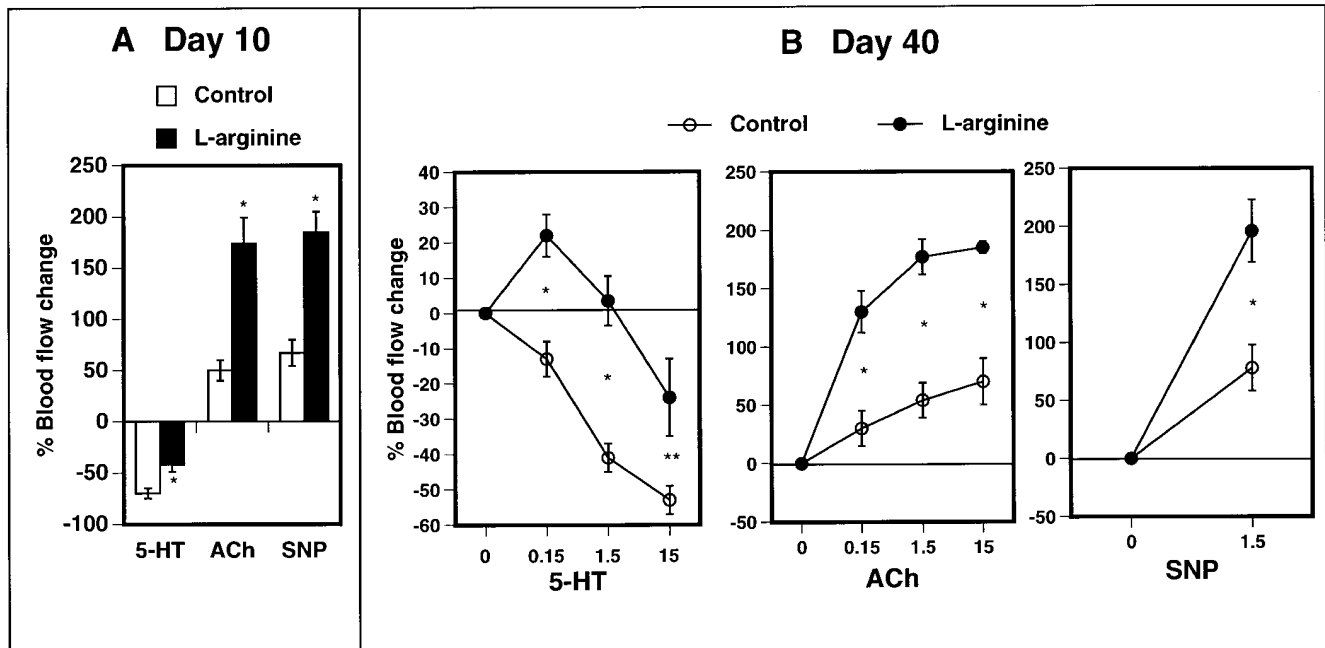


Figure 2. (A) Day 10: administration of 5-HT reduced hindlimb blood flow in L-arginine-treated as well as control rabbits, but the decrease in blood flow was significantly attenuated by treatment with L-arginine. ACh and SNP moderately increased blood flow in both groups, but the increase in blood flow was significantly enhanced only in L-arginine-treated animals. (B) Day 40: low to medium doses (0.15–1.5  $\mu\text{g/ml/kg}$ ) of 5-HT increased blood flow in L-arginine group, whereas these doses decreased blood flow in a dose-dependent manner in control group. High dose (15  $\mu\text{g/ml/kg}$ ) of 5-HT decreased blood flow in both groups; the decrease observed for the L-arginine group, however, was significantly less than that seen in the control group. ACh increased blood flow in both groups in adose-dependent manner; however, the increase was significantly higher in L-arginine-treated rabbits. A single dose of SNP increased blood flow in both groups, but the increase was greater for the L-arginine than for control rabbits. ( $* = P < 0.01$ ,  $** = P < 0.05$ ; control group versus L-arginine group).

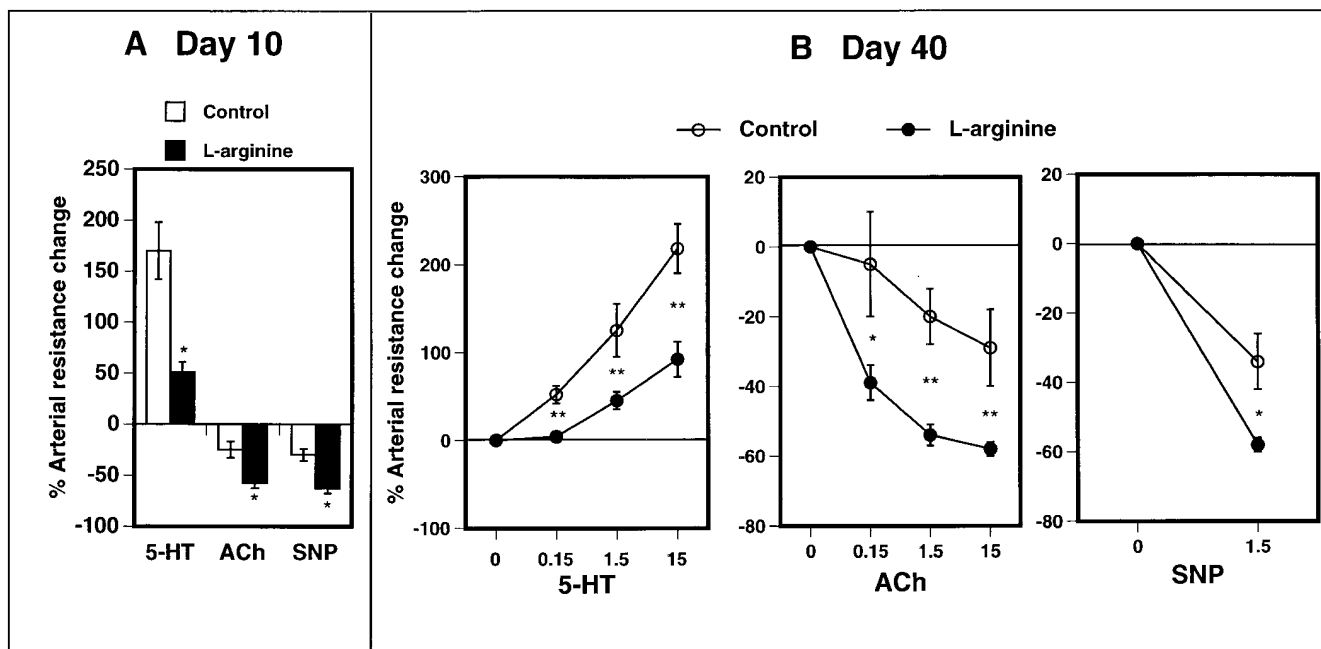


Figure 3. (A) Day 10: increase in vascular resistance after 5-HT was significantly higher in control than in the L-arginine group. ACh and SNP decreased vascular resistance in both groups, but to a significantly greater extent in L-arginine group. (B) Day 40: 5-HT increased vascular resistance in both groups in a dose-dependent manner, but the increase was significantly higher in control than in L-arginine-treated rabbits. ACh decreased vascular resistance in both groups, also in dose-dependent manner, with significantly greater reduction in L-arginine group. Single dose of SNP decreased vascular resistance in both groups, but reduction was greater in L-arginine group. (\* =  $P < 0.01$ , \*\* =  $P < 0.05$ ; control group versus L-arginine group).

concentrations (Table II). Thus, continuing administration of L-arginine did not itself alter hemodynamic parameters in L-arginine-treated rabbits, suggesting that improved hemodynamics in the L-arginine group were due to the consequences rather than the concentration of serum L-arginine.

**Angiographic analysis.** The mean diameter of conduit collateral arteries was significantly greater in L-arginine-treated rabbits (baseline =  $1.16 \pm 0.06$  mm, SNP =  $1.36 \pm 0.08$  mm) than in controls (baseline =  $0.97 \pm 0.05$  mm, SNP =  $1.01 \pm 0.03$  mm;  $P < 0.05$ ).

Table II. Comparison of Hemodynamics between Day 37 and 40 in L-arginine-treated Rabbits

	Day 37	Day 40
<i>n</i>	6	6
Serum L-arginine concentration ( $\mu\text{mol/dl}$ )	$24.7 \pm 3.3^*$	$9.0 \pm 1.0$
Blood pressure ratio (calf)	$0.85 \pm 0.02$	$0.84 \pm 0.04$
Blood flow change (%) (iliac artery)		
5-HT ( $1.5 \mu\text{g/ml/kg}$ )	$3.0 \pm 5.5$	$2.9 \pm 7.4$
ACh ( $1.5 \mu\text{g/ml/kg}$ )	$138 \pm 21$	$179 \pm 16$
SNP ( $1.5 \mu\text{g/ml/kg}$ )	$154 \pm 16$	$197 \pm 32$
Vascular resistance change (%) (iliac artery)		
5-HT ( $1.5 \mu\text{g/ml/kg}$ )	$15.3 \pm 11.2$	$35.9 \pm 17.6$
ACh ( $1.5 \mu\text{g/ml/kg}$ )	$-56.7 \pm 3.3$	$-53.3 \pm 3.5$
SNP ( $1.5 \mu\text{g/ml/kg}$ )	$-62.3 \pm 2.2$	$-58.6 \pm 3.0$

The blood pressure ratio (at calf), iliac arterial blood flow change, and iliac arterial vascular resistance were all comparable between day 37 and 40 in L-arginine-treated animals, despite marked difference in serum L-arginine levels. (\* =  $P < 0.01$ ; day 37 versus day 40).

Collateral vessel development in the medial thigh was assessed by calculating the angiographic score as described above. The angiographic score at day 40 in the L-arginine group was significantly higher than that of control group (control =  $0.21 \pm 0.03$ , L-arginine group =  $0.30 \pm 0.02$ ;  $P < 0.05$ ; Fig. 4).

**Measurement of capillary density.** Histologic evaluation of tissue sections retrieved from the medial thigh muscles of the ischemic hindlimb on day 40 showed a marked increase in capillary density in L-arginine-treated animals. Capillary densities in the L-arginine group and L-arginine 10-d group ( $210.6 \pm 9.7/\text{mm}^2$ ) were significantly higher than those of control group ( $159.1 \pm 9.6/\text{mm}^2$ ;  $P < 0.05$ ; Fig. 5). Capillary/muscle fiber ratio was also higher in the L-arginine group ( $0.70 \pm 0.04$ ) than controls ( $0.41 \pm 0.03$ ;  $P < 0.01$ ; Fig. 5).

Capillary density in the nonischemic limb of L-arginine rabbits ( $178.3 \pm 1.4/\text{mm}^2$ ) was similar to that in the nonischemic limb of controls ( $181.3 \pm 5.8 \text{ mm}^2$ ;  $P = \text{NS}$ ; Fig. 5). The capillary/muscle fiber ratio was also equivalent in the nonischemic limb of each group (L-arginine group =  $0.59 \pm 0.05$ ; control group =  $0.57 \pm 0.03$ ;  $P = \text{NS}$ ; Fig. 5). Thus, L-arginine did not promote neovascularization in nonischemic healthy hindlimbs.

**Assay for cGMP.** Administration of L- but not D-arginine resulted in increased tissue levels of cGMP (Fig. 6). After D-arginine, cGMP (pmol/mg protein) level was  $0.168 \pm 0.005$  in nonischemic, and  $0.202 \pm 0.073$  in ischemic tissues. After L-arginine, cGMP was  $0.264 \pm 0.011$  in nonischemic tissues, and  $0.627 \pm 0.022$  in ischemic tissues. The level of cGMP for the L-arginine group was markedly increased compared to the D-arginine group ( $P < 0.01$ ). In nonischemic hindlimbs, cGMP levels in the L-arginine group were significantly higher than in the D-arginine group ( $P < 0.01$ ). For D-arginine groups, there was

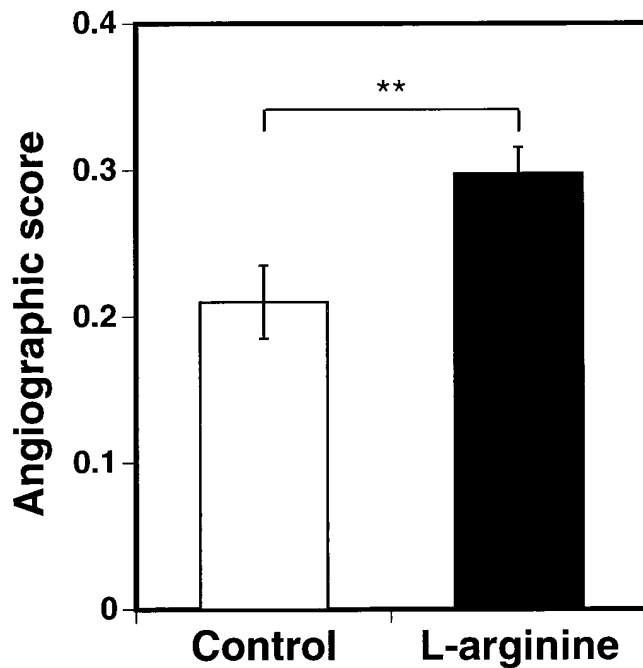


Figure 4. Quantitative analysis of collateral vessel development judged from angiograms recorded systematically at mid-calf level. The angiographic score (see Methods) at day 40 was significantly greater in rabbits receiving L-arginine than in control animals. (\*\* =  $P < 0.05$ ; control group versus L-arginine group).

no significant difference in cGMP between ischemic and nonischemic muscles; in contrast, after L-arginine, cGMP levels in ischemic muscle were higher than in nonischemic muscles ( $P < 0.01$ ).

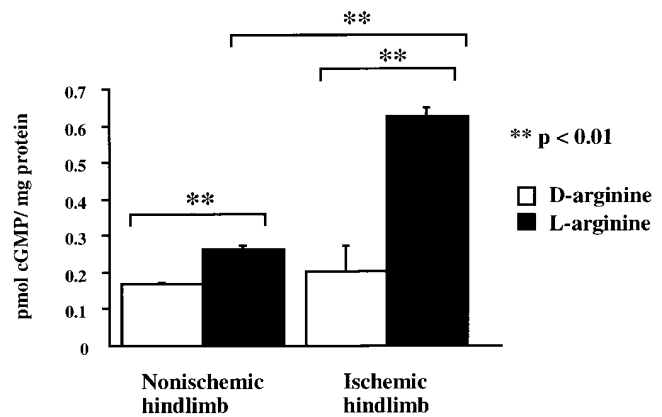


Figure 6. Levels of cGMP in muscle samples retrieved from ischemic and nonischemic rabbit hindlimbs after 1 wk of D- or L-arginine dietary supplementation. White and black bars respectively show groups fed D- and L-arginine. Values of cGMP represent mean  $\pm$  SEM (pmol/mg protein) of five samples each from six rabbits.

#### Evidence of retarded angiogenesis in $eNOS^{-/-}$ mice

**Induction of unilateral limb ischemia in mice.** Immediately after the femoral artery was resected from the left hindlimb, the ratio of ischemic (left)/normal (right) blood flow was reduced to  $0.34 \pm 0.07$  in wild-type C57, and  $0.43 \pm 0.05$  in  $eNOS^{-/-}$  mice, respectively ( $P = NS$ ). Thus, a comparable reduction in limb perfusion was generated in both groups.

**Serial perfusion measurements.** Serial LDPI examinations performed at days 3, 7, 14, 21, 28, and 35 disclosed progressive recovery of limb perfusion within 28 d after induction of limb ischemia in C57 wild-type mice (Fig. 7). By day 28, the ra-

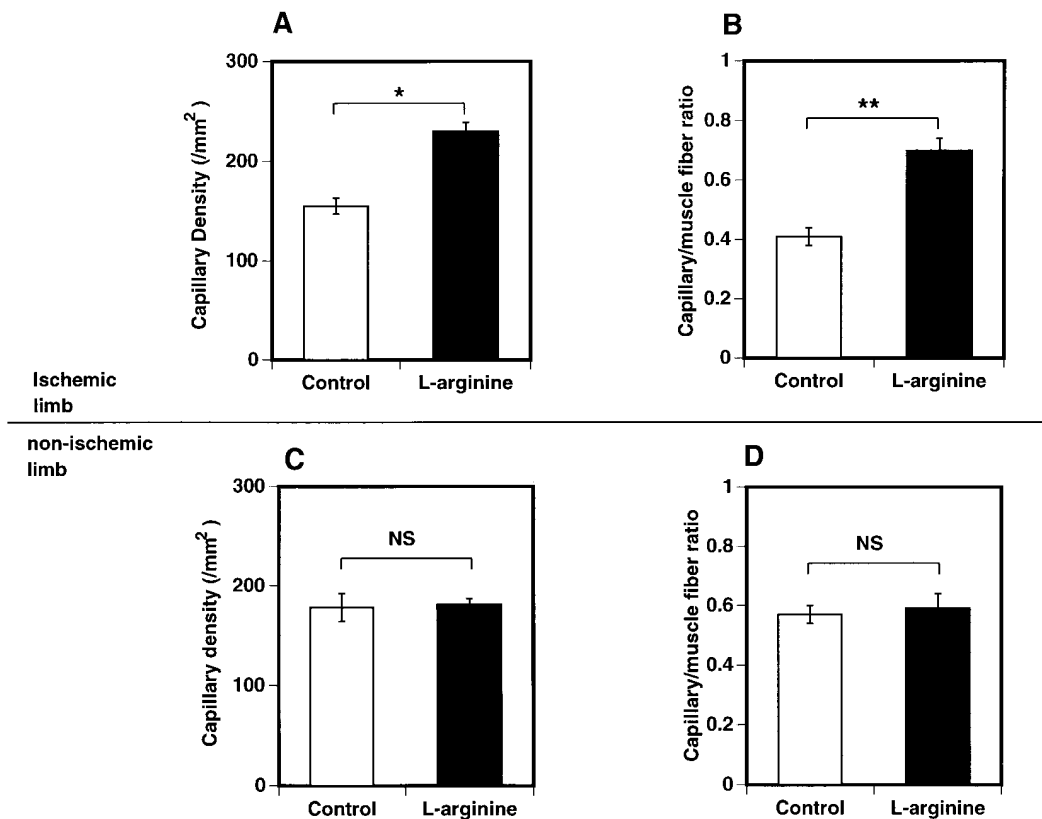
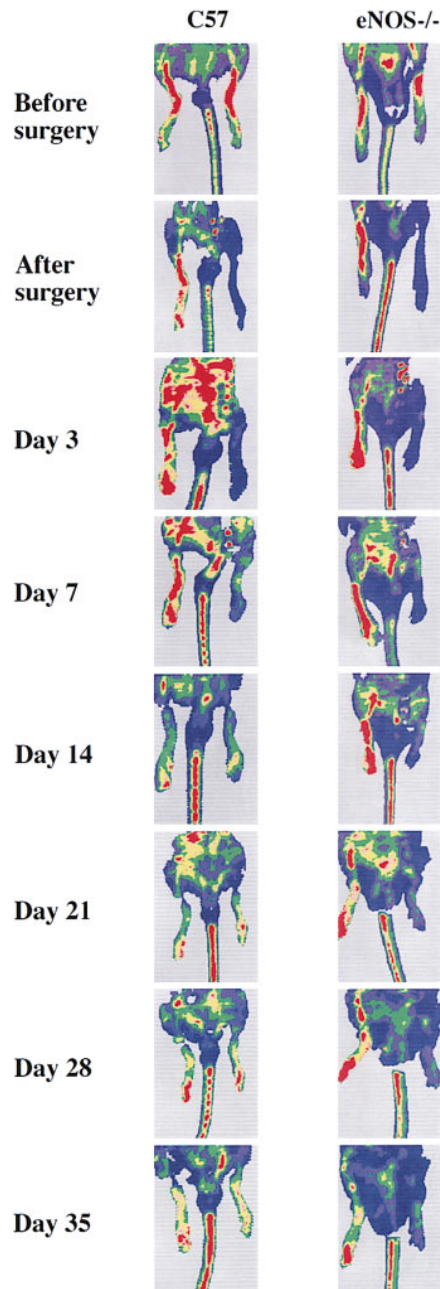


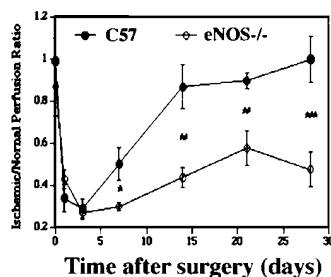
Figure 5. Histologic evaluation of angiogenesis. (A and B) In ischemic hindlimb, both capillary density and capillary/muscle fiber ratio were significantly increased in L-arginine versus control animals at day 40. (C and D) In nonischemic, healthy hindlimb, both capillary density and capillary/muscle fiber ratio were comparable ( $P = NS$ ) for L-arginine and control animals at day 40. (\* =  $P < 0.01$ , \*\* =  $P < 0.05$ ; control group versus L-arginine group).



## A Serial laser Doppler color image



## B Time course of ischemic/normal blood perfusion ratio



tion of ischemic/normal blood flow had improved to  $1.0 \pm 0.1$  in C57 mice, respectively. In contrast, limb perfusion among  $eNOS^{-/-}$  mice remained impaired throughout the 28-d follow-up period, so that by day 28, the ratio of ischemic/normal flow ( $0.48 \pm 0.08$ ) was persistently reduced in comparison with wild-type mice ( $P < 0.001$ ). Representative digital color-coded representations of hindlimb perfusion assessed by LDPI at each time point after induction of limb ischemia are demonstrated for C57 and  $eNOS^{-/-}$  mice in Fig. 7.

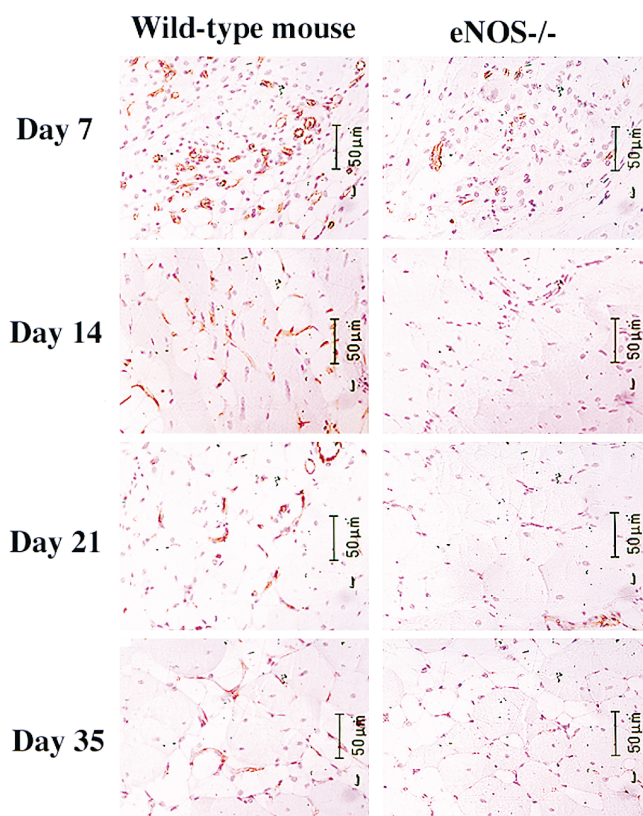
**Analysis of capillary density.** To confirm that impaired recovery in hindlimb perfusion assessed by LDPI reflected retarded angiogenesis, we performed measurements of capillary density on histologic sections harvested from the ischemic hindlimbs of C57 and  $eNOS^{-/-}$  mice. As is shown in Fig. 8, capillary density after induction of and recovery from limb ischemia was significantly reduced in  $eNOS^{-/-}$  mice compared to wild-type controls. Representative photomicrographs illustrating capillary density for each group are shown in Fig. 8.

**Impact of VEGF protein administration and VEGF gene transfer on angiogenesis in  $eNOS^{-/-}$  mice.** We next examined whether impaired angiogenesis in  $eNOS^{-/-}$  mice could be restored by treatment with the angiogenic growth factor VEGF, administered as recombinant human protein (rhVEGF) or gene transfer. Previous studies have shown that rhVEGF or phVEGF<sub>165</sub> augments angiogenesis in human (30), porcine (31, 32), rabbit (21, 33), and murine (22) species. The replication-defective adenoviral vector, which we used for VEGF gene transfer, encodes the murine VEGF sequence, and has been shown previously to augment angiogenesis in vivo in apolipoprotein E-deficient mice (22). Both the protein and gene were administered via an intraperitoneal route. Neither of these strategies, however, successfully restored perfusion to the ischemic limb of  $eNOS^{-/-}$  mice (Fig. 9). Failure of VEGF administration to improve angiogenesis in  $eNOS^{-/-}$  deficient mice is consistent with the notion that NO is an essential downstream element regulating VEGF-induced angiogenesis.

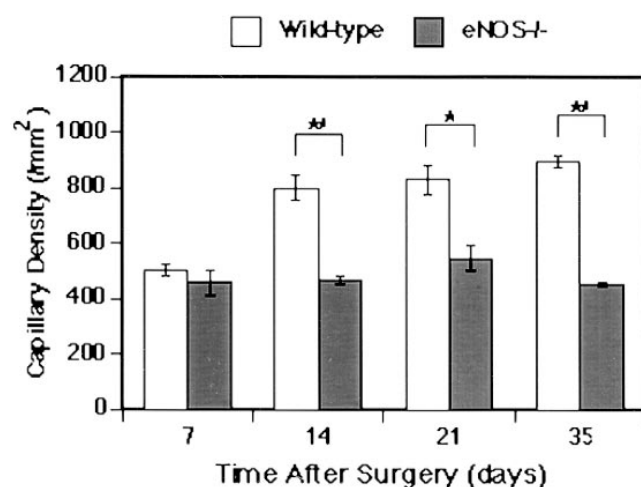
**Endogenous expression of VEGF.** To determine whether impaired angiogenesis in  $eNOS^{-/-}$  mice is associated with a reduction in endogenous VEGF expression, we performed Northern blot analyses to detect VEGF mRNA levels in tissues retrieved from the ischemic limb. However, no apparent difference in VEGF gene expression at the mRNA level was observed between  $eNOS^{-/-}$  and control mice (Fig. 10). Furthermore, immunohistochemical staining to detect VEGF protein in ischemic tissues at several time points after surgery revealed comparable VEGF staining in both groups (Fig. 10). These findings thus indicate that endogenous VEGF expression is preserved in  $eNOS^{-/-}$  mice.

**Figure 7.** Angiogenesis was impaired in the ischemic hindlimb of  $eNOS^{-/-}$  mice. (A) Serial laser Doppler analyses of hindlimb perfusion revealed impaired perfusion after induction of hindlimb ischemia in  $eNOS^{-/-}$  mice. Very low perfusion signal (dark blue) was observed in ischemic limb of an  $eNOS^{-/-}$  mouse, whereas high perfusion pattern (red to orange) was detected in a control C57 mouse. (B) Computer-assisted quantitative analyses of hindlimb perfusion demonstrates significant reduction in ischemic/normal hindlimb perfusion ratio in  $eNOS^{-/-}$  mice ( $n = 6$ ) compared to wild-type animals ( $n = 8$ ). (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ ; C57 versus  $eNOS^{-/-}$ ).

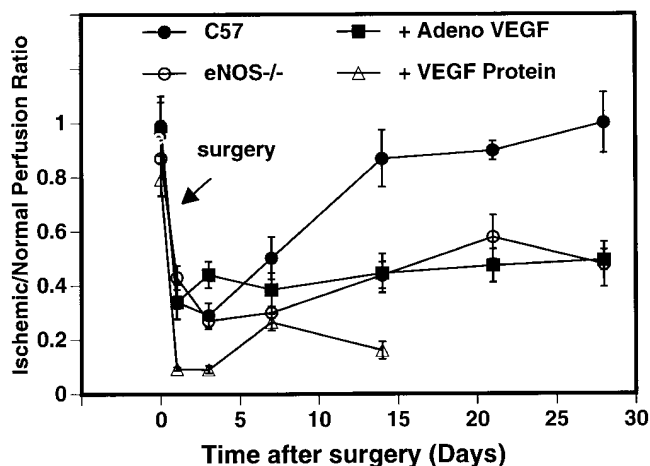
## A CD31 Immunostaining in ischemic limbs



## B Time Course of Capillary Density



**Figure 8.** Capillary density in ischemic hindlimb tissue evaluated by light microscopy. (A) Immunostaining of ischemic hindlimb tissue with anti-PECAM-1 antibody (bright red reaction product) showed markedly reduced capillary density in eNOS<sup>-/-</sup> mice compared to control C57 mice. (B) Quantitative analyses revealed reduced capillary density in eNOS<sup>-/-</sup> mice compared to control C57 mice examined 7, 14, 21, and 35 d after induction of ischemia ( $n = 3$  each time point). (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ ; C57 versus eNOS<sup>-/-</sup>).



**Figure 9.** Serial laser Doppler blood perfusion analyses disclosed no improvement in ischemic/normal hindlimb perfusion ratio in response to either rhVEGF protein or adenovirus-VEGF gene transfer.

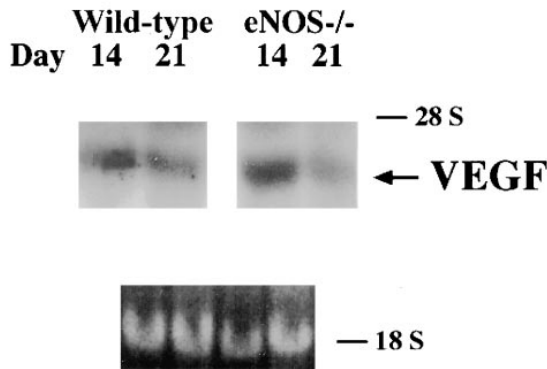
*Effect of NO donor in eNOS<sup>-/-</sup> mice.* To determine whether exogenous administration of an NO donor could restore spontaneous angiogenesis in the ischemic limb of eNOS<sup>-/-</sup> mice, these animals were treated with the NO donor DETA-NONOate (half-life = 20 h at 37°C) (34). The concentration of NONOate used in the present study was based on in vivo work by Liebmann et al. (35) who used the related NO donor DEA-NO. However, use of DETA-NONOate (50 mg/kg/d) did not improve angiogenesis in the ischemic hindlimb of eNOS<sup>-/-</sup> mice as judged by Doppler flow analysis and/or capillary density (data not shown).

## Discussion

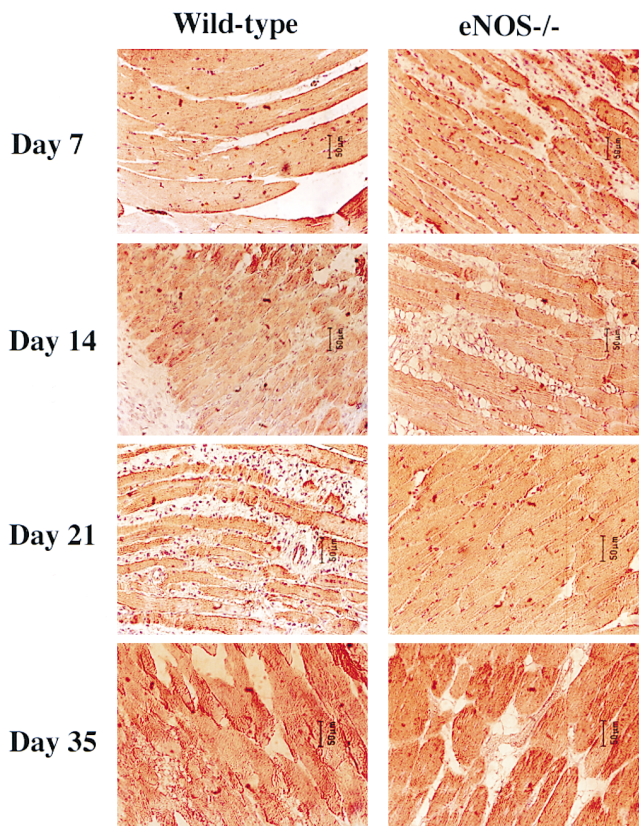
The role of NO in angiogenesis has been controversial. Lau and Ma (36), for example, reported that NO inhibits migration of cultured endothelial cells, an essential step for angiogenesis (37). Pipili-Synetos et al. (38) reported that NO donors inhibit angiogenesis in the chick chorioallantoic membrane and tube formation in the matrigel tube formation assay (39). In contrast, Guo et al. (40) showed that exogenous administration of a novel NO donor stimulated proliferation of cultured rat aortic endothelial cells. Ziche et al. suggested that NO may play a role in angiogenesis elicited by substance P (19) and VEGF (20, 41) but not bFGF (20). Furthermore, the extent to which endogenous NO synthesis is critical for angiogenesis, which develops in vivo in ischemic tissues, has not been determined previously.

The experiments performed in this study establish that NO is a critical regulatory molecule for physiologic angiogenesis that constitutes a naturally occurring, compensatory response to ischemia. Experiments performed in the rabbit model documented that oral administration of L-arginine is alone sufficient to augment angiogenesis in the setting of limb ischemia. Evidence for this included favorable and statistically significant increased blood pressure, reduced arterial resistance, increased flow at rest, and increased flow reserve in the ischemic limb of L-arginine-treated animals, compared to untreated controls. That improvement in these functional indexes per-

## A Northern Analysis for VEGF Expression in Mouse Ischemic Hindlimb



## B VEGF immunostaining



**Figure 10.** (A) Northern analysis showed no difference between VEGF mRNA expression in ischemic hindlimb tissue of  $eNOS^{-/-}$  versus control mice. Each time point comprises ischemic hindlimb tissues obtained from three different animals. (B) Immunostaining for VEGF showed comparable intensity of staining for control and  $eNOS^{-/-}$  mice.

sisted 3 d after discontinuation of L-arginine suggests that these findings were not simply the result of transient, pharmacologically mediated improvement in endothelium-dependent blood flow. Furthermore, angiography performed *in vivo* and

histologic examination performed at necropsy provided anatomic evidence of neovascularity: angiographically evident collateral blood vessels and capillary density were significantly more abundant in the ischemic limbs of L-arginine-treated than control rabbits. These hemodynamic and histologic data collectively indicate that enhancement of endogenous NO production by means of dietary L-arginine supplementation significantly augmented collateral formation in ischemic rabbit hindlimbs.

Experiments performed in the mouse model indicate that eNOS (NOS 3) is essential for angiogenesis in ischemic tissues *in vivo*. Spontaneous angiogenesis developing in response to limb ischemia was severely attenuated in mice lacking the eNOS gene ( $eNOS^{-/-}$ ). Evidence that angiogenesis was significantly impaired was documented by a combination of LDPI analysis and capillary density measurement. Whereas  $eNOS^{-/-}$  mice may spontaneously develop systemic hypertension (42), it is unlikely that this was responsible for impaired angiogenesis in these mice. The possibility that systemic hypertension *per se* may impair angiogenesis *in vivo* has not to our knowledge been documented previously. In fact, coronary capillary angiogenesis has been shown to develop uninhibited in at least two hypertensive rat models (43, 44).

Because previous studies have demonstrated that the endothelial cell mitogen VEGF promotes neovascularization *in vitro* (45) and *in vivo* (21, 32, 46), we examined whether VEGF could compensate for impaired angiogenesis in  $eNOS^{-/-}$  mice. Administration of the same murine  $adVEGF_{165}$  vector has been shown previously to achieve marked restoration of angiogenesis in mice lacking apolipoprotein E (22). Angiogenesis in  $eNOS^{-/-}$  mice, however, was not improved by either recombinant VEGF protein administration or adenovirus-mediated VEGF gene transfer.

These results are consistent with the work of Papatropoulos et al., who demonstrated that autocrine production of NO appeared to be necessary for *in vitro* capillary tube formation (17), including vascular networks developing in response to VEGF (18). Our findings are similarly consistent with *in vivo* studies performed by Ziche et al. that were the first *in vivo* experiments to establish a role for NO in angiogenesis in general (19), and VEGF-induced angiogenesis in particular (20). Using a rabbit corneal pocket assay, Ziche et al. observed that VEGF-induced angiogenesis was blocked by systemic administration of L-NAME. In the current investigation, Northern analysis and immunohistochemistry documented comparable expression of VEGF gene and protein in ischemic limb tissues of  $eNOS^{-/-}$  mice versus wild-type animals; in spite of this, angiogenesis was retarded in the former. Our findings thus extend the work of Ziche et al. to show that VEGF expression in the absence of eNOS is insufficient for angiogenesis, which develops naturally in response to tissue ischemia *in vivo*. Moreover, whereas L-NAME used in the study of Ziche et al. inhibits all NOS isoforms, the current findings establish that VEGF-mediated angiogenesis is mediated specifically by eNOS.

The precise mechanism responsible for defective angiogenesis in  $eNOS^{-/-}$  mice remains enigmatic. Previous workers have shown that endogenous NO may be required for endothelial cell proliferation and migration (19, 47, 48). Because inhibition of NO synthesis has multiple other effects, however, alternative mechanisms, including increased leukocyte-endothelial interaction (49), increased perimicrovascular fibrosis,

and increased activity of tissue angiotensin-converting enzyme (50–52) cannot be excluded.

Our results suggest that endogenous eNOS activity is critical for angiogenesis *in vivo*, but exogenously administered NO failed to restore angiogenesis *in vivo*. In this regard, eNOS<sup>-/-</sup> mice have been shown recently to develop increased total pulmonary resistance, and the resulting pulmonary hypertension is also not reversed by inhaled NO (8). The explanation for this discrepancy remains enigmatic. It is possible, however, that the amount of NO delivered, local levels of NO achieved, and/or temporal requirements may be different from endogenous synthesis when NO donors are used to compensate for a deficiency of authentic NO (2).

Previous studies from our laboratory indicated that NO may function as an endogenous (negative) regulator of VEGF expression in the vascular wall (53). Although we considered the possibility that a similar paradigm might lead to augmented expression of VEGF in skeletal muscle of eNOS<sup>-/-</sup> mice, we were unable to detect any such difference between control and eNOS<sup>-/-</sup> mice. The reason for this discrepancy remains enigmatic, but it must be recognized that the two paradigms differ in several important respects. The arterial wall contains smooth muscle which is (normally) in direct contact with a continuous layer of endothelial cells, and the regulatory activity of NO was demonstrated after balloon-induced stretch of the artery wall. In contrast, the ischemic hindlimb is comprised of skeletal muscle lacking such an adjacent organized endothelial monolayer, and the injury (arterial excision) in this case results in profound ischemia, including necrosis, with a corresponding inflammatory cell infiltrate. Precisely how these salient differences may directly contribute to modulated NO–VEGF interaction remains to be elucidated.

The findings observed in these two animal models of hindlimb ischemia may have clinical implications for analogous patients with vascular insufficiency, including peripheral artery disease limiting blood flow to the lower extremities. Findings in the rabbit ischemic hindlimb model suggest that clinical administration of L-arginine to promote eNOS activity deserves consideration as a therapeutic strategy for individuals with claudication and/or critical limb ischemia. Previous *in vivo* studies have established a precedent for favorable alteration of vascular dysfunction in response to L-arginine administration, independent of the presence or absence of global L-arginine deficiency (54–59). Weyrich et al. (60), in particular, showed that L-arginine could ameliorate myocardial ischemia–reperfusion injury, and that this effect was associated with the preservation of endothelial function. The finding that L-arginine may elicit beneficial effects in the setting of ischemia unassociated with a global deficiency of L-arginine is directly relevant to our ischemic hindlimb model. Previous studies from our laboratory (12) have documented that endothelium in the ischemic hindlimb is dysfunctional; it is possible that the handling of L-arginine by such dysfunctional endothelium in the ischemic hindlimb is disturbed. Alternatively, others (54) have established that natural antagonists of NOS such as asymmetrical dimethylarginine may accumulate in dysfunctional endothelium. Thus, administration of L-arginine in such cases may favorably alter the enzymatic kinetics of eNOS, including competing out the effects of antagonists such as asymmetrical dimethylarginine, and thereby lead to augmented production of NO. Whereas we cannot rule out the possible contribution of L-arginine–induced insulin release (61), previous investiga-

tors (6) have found that D-arginine and L-arginine both increase insulin levels, although only L-arginine favorably impacted endothelium-dependent forearm blood flow.

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