Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide

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Lloyd, Pamela G., Hsiao T. Yang, and Ronald L. Terjung. Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. Am J Physiol Heart Circ Physiol 281: H2528–H2538, 2001.—Nitric oxide (NO) has been implicated in both collateral expansion (arteriogenesis) and capillary growth (angiogenesis). Exercise training increases collateral-dependent blood flow to tissues at risk of ischemia and enhances capillarity in active skeletal muscle. Exercise also acutely elevates NO. Thus we assessed the role of NO in training-induced arteriogenesis and angiogenesis. These studies utilized a rat model of peripheral vascular disease (bilateral femoral artery ligation). Untreated rats (control) and rats treated with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 65–70 mg/kg drinking water) were divided into sedentary or exercise-trained subgroups. After ~3 wk, L-NAME treatment had elevated mean arterial pressure ~39–58%, confirming NO synthesis inhibition. The training program (treadmill exercise twice per day, 20–25 m/min, 15% grade, ~18 days) increased collateral-dependent blood flow to the distal hindlimb, with the greatest increase (~59%) in the calf (P < 0.001). This increase was inhibited by L-NAME. In contrast, the training-induced increase in muscle capillarity was not blocked by L-NAME. Thus arteriogenesis and angiogenesis appear to differ in their requirement for NO.

Nω-nitro-L-arginine methyl ester; exercise training; capillaries; arterioles; nitric oxide synthase

THE ADULT VASCULATURE possesses the ability to remodel itself in response to a variety of stimuli, including hemodynamic forces (shear) and tissue metabolic demands (ischemia). This remodeling attempts to increase blood supply to better serve tissue metabolic demand. In humans, a number of clinical conditions exist in which blood supply is insufficient to meet tissue demands (e.g., cardiac ischemia resulting from coronary artery disease and intermittent claudication resulting from peripheral arterial disease). Better understanding of the mechanisms of vascular remodeling could lead to more effective treatments for these conditions.

Animal models of peripheral arterial disease (hindlimb ischemia produced by femoral artery ligation) have been used extensively to study vascular remodeling. Recent studies in these models have demonstrated that at least two distinct processes are involved in vascular remodeling in adults. These processes have been termed “arteriogenesis” and “angiogenesis” (42).

Arteriogenesis and angiogenesis occur in different types of vessels and are regulated by separate stimuli. Arteriogenesis refers to the enlargement of preexisting collateral arteries, allowing increased blood flow to downstream tissue. Thus it serves to restore tissue perfusion when the primary pathway for blood flow has been obstructed. For example, arteriogenesis in the upper thighs of animals with experimental peripheral vascular disease increases blood flow to the distal limb (14, 21, 34). Increased shear stress through collateral vessels (as would occur after occlusion of a major artery) is thought to be a primary stimulus for arteriogenesis (42). In animal models of peripheral vascular disease, tissue ischemia does not appear to be an important controller of arteriogenesis, because the collateral vessels that are undergoing remodeling are typically not surrounded by ischemic tissue (14, 21).

Angiogenesis refers to the proliferation of capillaries within a tissue. Angiogenesis can improve oxygen delivery to individual cells of the tissue. However, because blood flow is mainly controlled by upstream arterial resistance, angiogenesis does not significantly enhance tissue blood flow. Unlike arteriogenesis, angiogenesis is thought to be initiated by tissue ischemia (14, 21).

Because the initiating stimuli for arteriogenesis and angiogenesis differ, it is of interest to determine whether downstream signaling events also vary between these two processes. Nitric oxide (NO), a potent vasodilator and signal mediator produced by the vascular endothelium, has recently been implicated in both arteriogenesis and angiogenesis. Transgenic mice lacking the endothelial NO synthase (NOS) isoform show reduced angiogenic capability in a variety of situations (26, 30). Similarly, inhibition of NOS has been shown to inhibit various types of vascular remodeling in vivo (23, 38, 57) and migration and prolifer-
tion of cultured endothelial cells in vitro (7, 16, 31, 36). Thus NO appears likely to play a significant role in vascular remodeling. However, its involvement may vary depending on the situation studied. For example, pathological angiogenesis, angiogenesis in avascular implants, or embryonic vascular development may involve control processes distinct from those involved in physiological angiogenesis in adult tissue or in arteriogenesis. Thus in vivo studies of arteriogenesis and angiogenesis in a physiological context are necessary to define how “normal” vascular remodeling may be regulated.

An excellent example of physiological angiogenesis is found in adult skeletal muscle, where remodeling of the capillary bed occurs in response to muscle use. A characteristic increase in muscle capillarity is observed when a muscle of relatively low capillary density is recruited by daily exercise training (19). In rats with experimentally induced peripheral vascular disease, exercise training also enhances collateral-dependent blood flow, indicating that arteriogenesis is occurring as well (47, 49, 52–54). These adaptations may occur in response to a unique combination of stimuli produced by exercise, including increased flow, relative ischemia, and/or metabolic events associated with muscle contractions. Thus the exercise-trained femorally ligated rat is an excellent model for studying both arteriogenesis and angiogenesis.

NO may mediate vascular remodeling in response to exercise training, because exercise training upregulates angiogenic growth factors (3, 12) that act via NO (7, 31, 36, 57). Indeed, increased activity of the NO pathway has been detected in response to training (24, 11, 44, 25). Shear stress, which is thought to be the primary signal for arteriogenesis, also stimulates NO pathway activity (8), suggesting a link between NO and arteriogenesis. Thus we hypothesized that exercise training stimulates both angiogenesis and arteriogenesis by upregulating growth factors that act via NO.

In agreement with this hypothesis, Hudlicka et al. (20) recently found that angiogenesis in rat skeletal muscle in response to chronic electrical stimulation is blocked by NOS inhibition. Furthermore, we (56) previously found that NOS inhibition eliminates the increase in collateral blood flow established by arteriogenesis in response to exogenous delivery of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). However, neither of these studies utilized the physiological angiogenic stimulus of physical activity. Therefore, in the present study, we examined the role of NO in exercise training-stimulated arteriogenesis and angiogenesis. We found that NOS inhibition blocked arteriogenesis in response to exercise but not angiogenesis. Thus, in this model of peripheral arterial insufficiency, arteriogenesis and angiogenesis in response to chronic physical activity appear to differ in their requirement for NO.

**MATERIALS AND METHODS**

**Experimental design.** All rats had experimentally induced hindlimb ischemia, produced by bilateral occlusion of the femoral arteries. Rats were randomly divided into N-\(\text{\textit{N}}\)-nitro-L-arginine methyl ester-treated (\(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME}; \(n = 25\)) or untreated (CONT; \(n = 26\)) groups. \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} animals received \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} daily in drinking water.

Within each treatment group, rats were further divided and randomly assigned to either sedentary (SED) or exercise-trained (EX) subgroups (\(n = 13\) animals in the CONT-SED group, 13 animals in the CONT-EX group, 9 animals in the \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME}-SED group, and 16 animals in the \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME}-EX group). Animals in the EX groups exercised daily on a treadmill. This experimental design permitted 1) direct evaluation of the main treatment effects of NOS inhibition and/or physical training and 2) evaluation of the interaction between NOS inhibition and physical training on expansion of collateral blood flow and angiogenesis.

**Animal care.** Fifty-one male Sprague-Dawley rats (~325 g, Taconic Farms; Germantown, NY) were housed two per cage in a temperature (21°C)- and light-controlled (12:12-h dark-light cycle) room. Purina Rat Chow and water were provided ad libitum. On arrival, all rats were accustomed to handling and to daily treadmill walking (5–10 min at 20 m/min, 15% grade) for ~5 days. Rats were conditioned to run at the front of the treadmill by briefly turning it off and on. Our previous experiments (46, 48, 54) showed that this protocol does not induce peripheral adaptations in rats.

This study was approved by the Animal Care and Use Committee of the University of Missouri. The care and treatment of the animals and all experimental procedures were carried out in accordance with National Institutes of Health (NIH) guidelines.

**Femoral artery ligation.** Rats were anesthetized with 100 mg/kg ketamine-0.5 mg/kg acepromazine for bilateral femoral artery ligation. The surgical sites were shaved and treated with topical antiseptic. Through small incisions on the medial aspect of both thighs, the right and left femoral arteries were isolated and completely occluded ~5–6 mm distal to the inguinal ligament by ligation with 3-0 surgical silk (46, 52, 53). The wounds were closed with skin clips, and the animals were allowed to recover in their cages. All animals appeared fully recovered within 24 h, with no signs of tissue necrosis.

**\(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} administration.** Rats began receiving the NOS inhibitor \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} (Sigma; St. Louis, MO) in their drinking water 2 days before femoral artery ligation. \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} was dissolved in distilled water (1 mg/ml), and the water was supplied fresh daily to the animals (56). \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} administration was continued until the day of blood flow measurement (total treatment time ~20 days). \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} consumption per rat was calculated from the amount of water consumed per day per cage (2 rats/cage). \(\text{\textit{N}}\)-\(\text{\textit{N}}\)-nitro-\(\text{\textit{d}}\)-arginine methyl ester (\(\text{\textit{d}}\)-\(\text{\textit{N}}\)\textit{-NAME}) is often assumed to be an “inactive” enantiomer of \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} and is thus commonly used as a control for \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME} administration. However, a recent study (2) suggested that 4-wk \(\text{\textit{d}}\)-\(\text{\textit{N}}\)\textit{-NAME} treatment produces hemodynamic and structural adaptations similar to those caused by \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME}, with \(\text{\textit{d}}\)-\(\text{\textit{N}}\)\textit{-NAME} being approximately one-half as active as \(\text{\textit{L}}\)-\(\text{\textit{N}}\)\textit{-NAME}. Thus we chose not to administer \(\text{\textit{d}}\)-\(\text{\textit{N}}\)\textit{-NAME} to the CONT rats to avoid potentially confounding results.

**Exercise training.** Rats in the EX subgroups began walking on a motor-driven rodent treadmill (model 42-15, Quinton) 24 h after femoral artery ligation, as we have previously described (53, 54). Rats exercised twice daily, 7 days/wk. Each day, the rats exercised both morning and afternoon, with at least 4 h between the two exercise bouts. The exercise program was progressive in nature. Rats began by walking on the treadmill (20 m/min, 15% grade). Rats exercised until showing signs of fatigue (a change in gait from walking to hopping). If the rats were able to walk at 20 m/min for >15
min without becoming fatigued, the treadmill speed was increased to 25 m/min. If the rats were able to run at 25 m/min for a further 10 min without becoming fatigued, the speed was increased to 30 m/min until fatigue occurred. The total length of time each animal was able to exercise before becoming fatigued was recorded. Total daily exercise time increased from ~10 min at the beginning of the training program to ~30 min by the end. Exercise time of the CONT rats was matched to the performance of the L-NAME rats. This was an attempt to ensure that the training stimulus was similar, on the average, across groups.

**Blood flow determination.** Eighteen days after femoral artery ligation, rats were surgically prepared for blood flow measurement, as previously described (46, 48, 54). After anesthetization with ketamine, each rat was instrumented with a polyethylene-50 catheter in the left carotid artery. The catheter was advanced to the arch of the aorta, where it served to monitor mean arterial pressure and heart rate. In addition, the aortic catheter was used to infuse radioactive microspheres. After placement of the aortic catheter, a second catheter was placed in the caudal artery for monitoring caudal blood pressure and collecting reference blood samples. Rats were instrumented in the morning and allowed to recover for >4 h before further experimentation.

After the rats recovered from surgery, blood flow to the hindlimbs and other tissues was measured (46, 48, 54). Blood flow was measured twice by infusing differentially radioisotopically labeled microspheres ($^{85}$Sr and $^{141}$Ce, 15 ± 0.1-μm diameter, New England Nuclear; Boston, MA) while the rats first walked on the treadmill at low speed (15 m/min, 15% grade) and then ran at high speed (25 m/min, 15% grade) (see below). The upstream resistance of the collateral circuit controls maximal blood flow to the collateral-dependent muscle when downstream resistance in the active muscle is minimal. Thus, if blood flow to the collateral-dependent muscle is no higher at 25 m/min than at 15 m/min, the measured flow is expected to represent maximal collateral-dependent blood flow.

After 1 min of walking at low speed (15 m/min), a well-mixed suspension of microspheres was administered to each animal via the aortic catheter. The catheter was then flushed with saline for ~20 s. As the microspheres were infused, a reference blood sample was collected from the caudal catheter (flow rate, 0.5 ml/min). Blood collection began 10 s before microsphere infusion and continued throughout microsphere actions. Repeated-measures ANOVA was applied to data collection for each muscle using a Spot digital charge-coupled device camera (Diagnostic Instruments; Sterling Heights, MI) connected to a Nikon Eclipse E600 microscope (Nikon; Torrance, CA). Images were acquired and processed using Adobe Photoshop software. A 20-square grid was overlaid on each composite image. With the use of the grid as a guide, 20 nonoverlapping fields were selected for analysis. A new image was acquired in each field (image area, ~0.06 mm$^2$). The number of myocytes and the number of capillaries surrounding each myocyte were counted using NIH Image software and recorded for calculation of capillary contacts per fiber.

**Biochemical analysis.** The white quadriceps muscle samples were analyzed for citrate synthase activity. Because citrate synthase is a component of the tricarboxylic acid cycle and thus an index of tissue oxidative capacity, increased citrate synthase activity is indicative of the efficacy of the training program in inducing peripheral adaptations in the active muscle (50, 51).

**RESULTS**

**NOS inhibition.** The majority of animals tolerated L-NAME treatment well, with acceptable running performance. However, three animals in the L-NAME-EX group were only able to exercise for <15 days and were thus excluded from the study.

Daily L-NAME intake averaged 69.1 ± 2.8 mg·kg$^{-1}$·day$^{-1}$ in the L-NAME-SED group and 64.9 ± 2.5 mg·kg$^{-1}$·day$^{-1}$ in the L-NAME-EX group. Body weights were ~8% less in L-NAME-SED rats and ~12% less in L-NAME-EX rats relative to the corresponding CONT animals ($P < 0.001$; Table 1). Hindlimb tissue weights were modestly but significantly lower (~4%) in the L-NAME groups than in the CONT groups ($P < 0.001$; Table 1). However, when the hindlimb weights were normalized to body weight, it became apparent that this decrease was due to the overall decrease in body weight in L-NAME-treated animals rather than to a specific effect of L-NAME on the musculature of the hindlimb (data not shown).
Mean arterial pressure was significantly increased by L-NAME administration (P < 0.001; Table 2), demonstrating that L-NAME effectively inhibited NO synthesis. After ~3 wk of L-NAME treatment, preexercise blood pressures were ~58% higher in L-NAME-SED rats than in CONT-SED rats and ~39% higher in L-NAME-EX rats than in CONT-EX rats. Blood pressure remained elevated in the L-NAME group even during treadmill exercise (P < 0.001). L-NAME administration did not affect heart rate either before or during exercise.

L-NAME exhibited a significant main treatment effect to reduce blood flow to the total, proximal, and distal hindlimb segments (Table 3; P < 0.001 and P < 0.025). This effect was primarily due to a difference between the L-NAME-SED group and the CONT-EX group. This was also true for many of the individual muscles that comprise the entire hindlimb (see Table 4). L-NAME treatment did not alter collateral-dependent blood flow to the calf muscles in sedentary animals. In contrast, calf muscle blood flow was decreased by L-NAME in the L-NAME-EX group (i.e., a significant interaction effect; Table 3).

**Exercise training.** Running time of the CONT-EX rats was matched with that of the L-NAME-EX rats. On the first day of the training program, CONT-EX rats ran 12.7 ± 2.8 min, whereas L-NAME-EX rats ran 9.3 ± 1.3 min. After 2 wk of training, daily running time had increased to 33.6 ± 2.7 min for CONT-EX rats and 35.9 ± 3.3 min for L-NAME-EX rats. Little further change in daily running time occurred during the final week of training. The effectiveness of the ~3-wk treadmill exercise program at inducing muscle adaptation was demonstrated by increased citrate synthase activity in superficial quadriceps muscles. In CONT rats, training increased citrate synthase activity from 12.4 ± 0.8 to 19.2 ± 1.6 μmol·g⁻¹·min⁻¹. Training increased citrate synthase activity in L-NAME rats from 13.2 ± 1.8 to 19.4 ± 1.6 μmol·g⁻¹·min⁻¹. The increases were significant (P < 0.01) and similar for both groups (~55% in CONT-EX and ~47% in L-NAME-EX rats relative to the corresponding SED rats). Thus L-NAME treatment did not prevent a typical biochemical adaptation in response to exercise training. Body weights were decreased in both CONT-EX rats (~8%) and L-NAME-EX rats (~12%) relative to the corresponding SED rats (Table 1), another well-known response of male rats to exercise training.

**Effect of exercise training on hindlimb blood flow.** In CONT rats, training did not significantly increase total hindlimb blood flow or proximal hindlimb blood flow (Table 3). However, blood flow to the collateral-dependent calf muscles was increased by exercise training.

### Table 1. Body and hindlimb tissue weights

<table>
<thead>
<tr>
<th></th>
<th>CONT SED</th>
<th>CONT EX</th>
<th>L-NAME SED</th>
<th>L-NAME EX</th>
<th>P Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>405 ± 7.4</td>
<td>373 ± 8.0 †</td>
<td>373 ± 12.8 †</td>
<td>327 ± 8.9 † §</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hindlimb weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22.5 ± 0.69</td>
<td>21.9 ± 0.45</td>
<td>20.3 ± 0.54 †</td>
<td>19.2 ± 0.46 † §</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Proximal</td>
<td>14.1 ± 0.51</td>
<td>13.8 ± 0.37</td>
<td>12.8 ± 0.47</td>
<td>12.0 ± 0.30 † §</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Distal</td>
<td>8.1 ± 0.23</td>
<td>7.9 ± 0.18</td>
<td>7.3 ± 0.23 †</td>
<td>7.1 ± 0.13 † §</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Calf muscles</td>
<td>2.7 ± 0.08</td>
<td>2.6 ± 0.07</td>
<td>2.4 ± 0.07 †</td>
<td>2.3 ± 0.07 † §</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = no. of animals. Calf muscles, gastrocnemius-plantaris-soleus muscle group; CONT, control; L-NAME, N⁵-nitro-L-arginine methyl ester; SED, sedentary; EX, exercise trained; NS, not significant. Differences were considered significant when P < 0.05. *These significant L-NAME effects were due to the significant effect of L-NAME on total body weight, because the statistical difference is lost when the analyses are performed on hindlimb weights corrected to animal body weight (e.g., g/100 g body wt). †Significantly different from the CONT-SED group (Tukey’s test); ‡significantly different from the CONT-EX group (Tukey’s test); §significantly different from the L-NAME-SED group (Tukey’s test).

### Table 2. Mean arterial pressure and heart rate at preexercise and during treadmill exercise at 15 and 25 m/min

<table>
<thead>
<tr>
<th></th>
<th>CONT SED</th>
<th>CONT EX</th>
<th>L-NAME SED</th>
<th>L-NAME EX</th>
<th>P Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Preexercise</td>
<td>141 ± 4.1</td>
<td>143 ± 3.9</td>
<td>223 ± 8.2 †‡</td>
<td>199 ± 6.3 †‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low speed</td>
<td>152 ± 4.4</td>
<td>144 ± 3.4</td>
<td>221 ± 10.1 †‡</td>
<td>202 ± 6.9 †‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High speed</td>
<td>154 ± 5.2</td>
<td>152 ± 3.7</td>
<td>214 ± 11.1 †‡</td>
<td>187 ± 11.3 †‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preexercise</td>
<td>499 ± 10.5</td>
<td>508 ± 16.1</td>
<td>467 ± 21.9</td>
<td>508 ± 18.8</td>
<td>NS</td>
</tr>
<tr>
<td>Low speed</td>
<td>577 ± 10.8</td>
<td>568 ± 12.9</td>
<td>553 ± 8.8</td>
<td>549 ± 13.3</td>
<td>NS</td>
</tr>
<tr>
<td>High speed</td>
<td>572 ± 12.9</td>
<td>568 ± 16.1</td>
<td>574 ± 12.1</td>
<td>560 ± 14.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = no. of animals. P values <0.05 were considered significant. *Significantly different from preexercise value (P < 0.001); †significantly different from the CONT-SED group (Tukey’s test); ‡significantly different from the CONT-EX group (Tukey’s test). See Table 1 for abbreviations.
Blood flow to the calf muscles (gastrocnemius-planta-ris-soleus muscle group) was much higher in CONT-EX rats (~50%) than in CONT-SED rats (Table 3; \( P < 0.001 \)). Thus exercise training specifically enhanced blood flow to the collateral-dependent tissue in CONT animals.

\( \text{L-NAME-treated rats also showed no improvement in blood flow to the total or proximal hindlimb with training (Table 3). Blood flow to the collateral-dependent calf muscles was greater in L-NAME-EX animals than in L-NAME-SED animals (at 25 min/min only). However, this apparent increase was well below that observed with exercise training in CONT animals (Fig. 1 and Table 3), and blood flow was significantly lower in several of the individual calf muscles of L-NAME-EX rats relative to CONT-EX rats (Table 4). Furthermore, the presence of a greater perfusion pressure at the head of the collateral in L-NAME-treated animals confounds interpretation of this apparent training effect. Because increased arterial pressure can increase collateral-dependent blood flow, we calculated vascular conductance to account for this pressure difference. Exercise training significantly increased the conductance of the collateral circulation in the absence of L-NAME (Fig. 2; \( P < 0.01 \)). L-NAME treatment eliminated this training-induced increase in conductance, because conductance in L-NAME-EX rats was not significantly different than conductance in CONT-SED rats. However, it still remained greater than in the L-NAME-SED group (\( P < 0.01 \)).}
NO, ARTERIOGENESIS, AND ANGIOGENESIS

Effect of training and L-NAME on nonhindlimb blood flow. Blood flow to nonhindlimb muscles and the kidneys was measured to verify proper distribution of the microspheres throughout the circulation (Table 5). In CONT rats, training spared the reduction in renal blood flow typically seen during acute exercise ($P < 0.05$). This effect of training was not seen in the L-NAME-treated animals. Neither training nor L-NAME had any effect on blood flow to nonischemic muscle tissue (abdominal muscles, psoas muscle, and diaphragm; Table 5).

Effect of L-NAME and physical training on muscle capillarity. The number of capillary contacts per fiber was $\sim 24\%$ higher in CONT-EX animals than in CONT-SED animals ($P < 0.01$; Fig. 3), demonstrating that exercise training stimulated angiogenesis within the active muscle. Images of capillarity in all four groups are shown in Fig. 4. L-NAME had no influence on muscle capillarity in SED animals, because there was no difference in capillary contacts per fiber between L-NAME-SED animals and CONT-SED animals. Surprisingly, L-NAME treatment had no effect on the increase in capillary contacts per fiber observed after exercise training ($\sim 27\%$ increase). This is in contrast with the marked inhibitory effect of L-NAME on the enhancement of collateral-dependent blood flow described above. Thus NOS inhibition affects arteriogenesis and angiogenesis differently.

DISCUSSION

In this study, we assessed the effects of exercise training and NOS inhibition on the processes of collateral vessel enlargement (arteriogenesis) and capillary proliferation (angiogenesis). We employed a rat model of acute-onset peripheral arterial insufficiency established by bilateral occlusion of the femoral arteries. Proximal occlusion of the femoral artery eliminates $\sim 85\%$–$90\%$ of the flow reserve to the distal hindlimb tissue of the rat (49); however, small conduits exist that circumvent the femoral obstruction. These collateral vessels are able to deliver limited flow downstream, which suffices to avoid tissue complications associated with rest ischemia (21, 34, 49). Maximal blood flow to the calf muscles after acute occlusion of the femoral arteries is $20$–$25\, \text{ml}\cdot\text{min}^{-1}\cdot\text{100 g}^{-1}$ (49), approximately two to three times the flow measured in resting quiescent muscle (27). This collateral-derived flow arises from the collateral vessels of the thigh and reenters the normal vascular tree of the distal hindlimb tissues, which is not modified by the surgical obstruction. Approximately 80% of the resistance in the vascular circuit for calf blood flow resides in the upstream collateral vessels that circumvent the femoral artery obstruction (40, 55). While these vessels are subject to vasomotor control (39), the possible increase in blood flow to the distal tissue at risk of ischemia is...
Significantly different from SED groups (P, L-NAME treatment had no effect on exercise-induced angiogenesis.

The increases in diameter of these collateral casts of animals with femoral artery occlusion (15, 21, 46). Expansion of the collateral tree is demonstrated by enlargement of the collateral tree (vessels in the thigh) dependent on structural enlargement of the collateral tree within the thigh.

Data are expressed as means ± SE; n = no. of animals. Blood flow was measured in milliliters per minute per 100 g. P values < 0.05 were considered significant. †Significantly different from low speed (Tukey’s test); ‡significantly different from the CONT-SED group (Tukey’s test); §significantly different from the CONT-EX group (Tukey’s test). See Table 1 for abbreviations.

Fig. 3. Capillarity (assessed as capillary contacts per fiber) in white gastrocnemius muscle (means ± SE). Numbers in parentheses are the numbers of animals in each group. Training significantly affected capillarity (P < 0.01). In both CONT and L-NAME rats, capillarity was increased similarly by exercise training, demonstrating that L-NAME treatment had no effect on exercise-induced angiogenesis. *Significantly different from SED groups (P < 0.01).
els. Recovery of limb blood flow after surgical induction of hindlimb ischemia in endothelial NOS knockout mice is impaired compared with wild-type mice, suggesting that NO is required for collateral expansion (30). Similarly, L-NAME inhibits enlargement of the rabbit common carotid artery in response to a chronic increase in flow (38). Finally, our laboratory (56) recently found that NOS inhibition prevents collateral vessel enlargement in response to exogenous delivery of the angiogenic growth factors bFGF and VEGF. Taken together, these findings imply that NO is an integral component of the signaling pathway for remodeling of existing arterial vessels.

Shear stress and angiogenic growth factors may mediate NO-dependent collateral growth. Although the precise signal prompting vascular remodeling in response to exercise training is not known, several candidates have been suggested (19). The importance of metabolic influences located within the active ischemic muscle has been depreciated as providing upstream signals for collateral vessel remodeling, because of the remote distance and the likelihood that collateral vessels in the periphery are not within frankly ischemic tissue (14, 21, 34, 42). Rather, hemodynamic factors related to increased blood flow (e.g., shear stress) through the collateral vessels during exercise have been suggested (18, 19). The observation that daily exercise enhances the bFGF-induced increase in collateral blood flow (54) is consistent with this hypothesis and suggests that the ability of the endothelium to sense and respond to shear stress as well as to angiogenic growth factors is important in vessel enlargement.

Interestingly, both VEGF and bFGF have been found to act via NO (see below), and the mRNA levels of both growth factors are reportedly increased in skeletal muscle by exercise (3, 10). Thus both bFGF and VEGF are potential mediators of NO-dependent arteriogenesis in response to exercise training. VEGF is a particularly promising candidate for such a role. Numerous studies have shown that VEGF acutely increases NO production in vascular cells. In vivo, exogenous VEGF increases NO release from the rabbit thoracic aorta, inferior vena cava, and pulmonary artery (41), whereas VEGF gene transfer enhances endothelium-dependent dilation in the rat ischemic hindlimb (37). In vitro, VEGF increases NO (9, 13, 17) and NOS levels (8, 17, 57) in a variety of cultured vascular cell types. Consistent with these data, NO appears to be an important mediator of vascular remodeling in response to VEGF. L-NAME inhibits angiogenesis into VEGF-treated pellets in vivo (57), and NOS inhibitors decrease proliferation (16, 31, 32, 57) and migration (7, 36, 57) of cultured endothelial cells in response to VEGF. Thus VEGF activates the NO pathway, and VEGF-induced vascular remodeling appears to require normal NO production. The NO-dependent arteriogenesis that we observed in this study is therefore consistent with a VEGF-mediated response to training. However, specific inhibition of endogenous VEGF and/or its receptors will be needed to provide definitive evidence that VEGF affects this physiological response.

Although there is evidence that bFGF can also activate the NO pathway, it is unclear whether NO is an important downstream effector of bFGF. bFGF acutely enhances endothelium-dependent dilation in isolated...
rat cremaster arterioles (45) and increases NO production in cultured endothelial cells grown on fibrin (1). It is also unclear whether exercise enhances bFGF production, because some data indicate that bFGF mRNA is increased (3), whereas other work does not (10). Certainly, capillarity can be increased with no measurable change in bFGF protein in the muscle (6). Furthermore, it is unclear whether NO is an obligatory mediator of the actions of bFGF. Although studies (57, 58) in vitro suggest that vascular remodeling in response to bFGF is not NO mediated, NO does appear to be an important downstream mediator of bFGF in vivo. For example, we (56) have previously shown that L-NAME treatment inhibits bFGF-induced increases in collateral blood flow in the same animal model of peripheral arterial insufficiency used in this study. Thus involvement of bFGF in vascular remodeling in response to training remains a possibility, although it appears less likely than VEGF involvement.

Training-induced angiogenesis does not require intact NO synthesis capability. The most intriguing finding of the present study is that training-induced angiogenesis in the active ischemic calf muscle is completely unaffected by L-NAME. The increased number of capillary contacts per fiber in the L-NAME-EX animals suggests that capillary growth in response to training does not require normal NO production. While we do not know that NO production within the active muscle was completely eliminated, it is clearly apparent that NO production was not normal. The dose of L-NAME used was sufficient to elevate arterial blood pressure and to eliminate the increase in collateral blood flow (in contrast with its lack of effect on the increase in capillarity). Thus we believe that the absence of any inhibition of angiogenesis in response to the training-induced stimulus warrants careful consideration. In agreement with our findings, Morris and Hansen-Smith (29) have recently shown that the NOS inhibitor N-nitro-L-arginine (L-NNA) does not inhibit angiogenesis induced by muscle stretch over a 28-day period. These data suggest that the requirement for NO is strongly dependent on the nature of the angiogenic stimulus, with hemodynamic factors (e.g., shear) initiating a NO-dependent angiogenic pathway, whereas other stimuli (e.g., exercise training and muscle stretch) activate an NO-independent pathway (29).

Our data and those of Morris and Hansen-Smith (29) are in contrast with the results in some other in vivo models of angiogenesis. For example, angiogenesis into Matrigel implants is inhibited in endothelial NOS knockout mice relative to wild-type controls (26). Likewise, L-NAME inhibits angiogenesis into tumor cell-containing Matrigel implants in mice (22, 23) and VEGF-containing pellets in the rabbit cornea (57). Fundamental differences between our model and these models may explain the disparity in results. The rat model of hindlimb ischemia allows us to examine angiogenesis in vascularized adult tissue (muscle) induced by a physiological stimulus (exercise training), whereas these models prompt angiogenesis in avascular material (implants and pellets) in response to externally supplied stimuli (tumor cells and exogenous growth factors). As discussed above, the nature of the stimulus may be critically important in determining the requirement for NO in the angiogenic process.

A requirement for NO in angiogenesis has also been demonstrated in ischemic hindlimbs of endothelial NOS knockout mice, in which angiogenesis is reduced relative to ischemic hindlimbs of wild-type animals (30). Again, however, there are considerable differences between this model and ours that could be responsible for the disparity in results. The mouse model of hindlimb ischemia results in a more severe impairment than the rat model, with the mice unable to use the ischemic limbs for up to a week after surgery and occurrence of toe necrosis in ~10% of the animals (4). Thus severe ischemia and low flow are constantly present in this model, even at rest. In contrast, in the rat model of hindlimb ischemia, blood flow to the limb is sufficient for resting needs. Thus tissue ischemia is intermittent, because it occurs only during exercise, when blood flow becomes limiting. Recent studies suggest that intermittent ischemia may be a more effective stimulus for induction of genes regulated by hypoxia-regulated transcription factors (e.g., activator protein-1 and hypoxia-inducible factor 1) than constant ischemia (33). Thus it is likely that different mechanisms may be operating to produce angiogenesis in the constantly ischemic mouse hindlimb and the intermittently ischemic rat hindlimb. These mechanisms may differ in their requirement for NO.

In another recent study, Hudlicka et al. (20) found that L-NNA blocked capillary growth in response to chronic electrical stimulation in the rat extensor digitorum longus muscle. The use of electrical stimulation rather than endurance exercise as an angiogenic stimulus is a fundamental difference between this study and the present study and may account for the disparity in results. For instance, it may be that load bearing and muscle mechanics (force and stretch) associated with treadmill running are critical signals for angiogenesis in active muscle. The chronically stimulated extensor digitorum longus muscle is relatively unloaded. It is also possible that the sustained high-frequency contractions produced by electrical stimulation may result in some degree of tissue damage (28), with angiogenesis occurring as part of a recovery process (although this is less likely to occur in the rat than in other species) (5). Finally, the hindlimb muscles of ligated rats are likely to become ischemic during exercise, whereas the muscles of chronically stimulated rats undergo angiogenesis even though O2 tension does not change (38), suggesting another fundamental difference in the nature of the angiogenic stimulus in exercising versus stimulated muscle.

In conclusion, this study provides evidence that angiogenesis and arteriogenesis in response to exercise training are regulated differently and are not equivalent in their requirement for NO. The role of NO in angiogenesis may vary depending on the nature of the angiogenic stimulus and the tissue involved. These
novel results provide new insights into the regulation of vascular remodeling in skeletal muscle.

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