Endogenous vascular remodeling in ischemic skeletal muscle: a role for nitric oxide

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Buckwalter, John B., Valerie C. Curtis, Zoran Valic, Stephen B. Ruble, and Philip S. Clifford. Endogenous vascular remodeling in ischemic skeletal muscle: a role for nitric oxide. J Appl Physiol 94: 935–940, 2003. First published October 18, 2002; 10.1152/japplphysiol.00378.2002.—To test the hypothesis that nitric oxide (NO) production is essential for endogenous vascular remodeling in ischemic skeletal muscle, 22 New Zealand White rabbits were chronically instrumented with transit-time flow probes on the common iliac arteries and underwent femoral ligation to produce unilateral hindlimb ischemia. Iliac blood flow and arterial pressure were recorded at rest and during a graded exercise test. An osmotic pump connected to a femoral arterial catheter continuously delivered N-nitro-L-arginine methyl ester (a NO synthase inhibitor) or a control solution (N-nitro-L-arginine methyl ester or phenylephrine) to the ischemic limb over a 2-wk period. At 1, 3, and 6 wk after femoral ligation, maximal treadmill exercise blood flow in the ischemic limb was reduced compared with baseline in each group. However, maximal exercise blood flow was significantly (P < 0.05) lower in the L-NAME-treated group than in controls for the duration of the study: 48 ± 4 vs. 60 ± 5 ml/min at 6 wk. Consistent with the reduction in maximal blood flow response, the duration of voluntary exercise was also substantially (P < 0.05) shorter in the L-NAME-treated group: 539 ± 67 vs. 889 ± 87 s. Resting blood flow was unaffected by femoral ligation in either group. The results of this study show that endogenous vascular remodeling, which partially alleviated the initial deficit in blood flow, was interrupted by NO synthase inhibition. Therefore, we conclude that NO is essential for endogenous collateral development and angiogenesis in ischemic skeletal muscle in the rabbit.

A BASIC UNDERSTANDING of the physiological mechanisms that govern the growth of blood vessels tantalizes researchers with the potential clinical applications. It is widely believed that the ability to prevent angiogenesis could be effective in the treatment of tumors and ocular neovascularization (11). In contrast, the promotion of new blood vessel growth has been touted as a potential treatment for myocardial ischemia and peripheral vascular disease. Peripheral vascular disease occurs in 12% of people between the ages of 65 and 70 yr. Although inadequate peripheral circulation can compromise daily living activities and possibly result in limb amputation, treatment options are limited. Invasive treatments such as peripheral angioplasty and reconstructive surgery are associated with considerable risks and high likelihood of restenosis (12). Exercise training appears to be the most effective treatment to alleviate intermittent claudication and increase exercise tolerance in humans suffering from peripheral vascular disease (13); however, the physiological mechanism by which these improvements occur is not fully understood.

Humans suffering from peripheral vascular disease have a limited ability to increase skeletal muscle blood flow in the affected limbs during exercise. This limited blood flow reserve leads to muscle ischemia during exercise and claudication. To further investigate peripheral vascular disease, animal models that replicate the human disease state have been developed. A particularly good model of peripheral vascular disease can be achieved in the rabbit by femoral ligation, which produces an immediate, sustained reduction in hindlimb blood flow reserve (29). This persistent hindlimb ischemia model allows serial examination of hindlimb perfusion and function. Interestingly, although blood flow reserve remains compromised to the hindlimb, endogenous angiogenesis and vascularization lessen the initial blood flow deficit to the ischemic hindlimb. This endogenous vascular remodeling appears to stabilize ~10 days after femoral ligation (31, 32).

With the use of this rabbit model, the administration of exogenous basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) has been shown to ameliorate peripheral blood flow insufficiencies and promote angiogenesis (2–5, 31). The angiogenic growth factors bFGF and VEGF are endogenous heparin-binding proteins. Tissue hypoxia has been shown to increase endogenous production of VEGF, which may provide the driving force that stimulates in vivo angiogenesis and vascularization (25, 30). Interestingly, bFGF and VEGF have been shown to produce dose-dependent release of nitric oxide (NO) in vivo and in vitro (6, 8, 34). The role of NO in the vascular...
remodeling of ischemic tissue is unclear. Recent studies provide contradictory conclusions about the role of NO in angiogenesis and the restoration of blood flow to ischemic tissue. Murohara and colleagues (24) showed that dietary L-arginine supplementation to augment endogenous production of NO improved angiogenesis in an ischemic rabbit hindlimb. In contrast, Hopkins and colleagues (14) reported that continuous exogenous infusions of the NO donor nitroglycerin into the ischemic rabbit hindlimb did not enhance vascular growth. Thus the role of NO in endogenous vascular remodeling and angiogenesis remains somewhat unclear.

In the present study, we hypothesized that NO plays a role in the endogenous vascular remodeling that occurs in the rabbit hindlimb after femoral ligation. We hypothesized that chronic inhibition of NO production in experimental rabbits would reduce endogenous vascular remodeling. We further postulated that the reduction in endogenous vascular remodeling would be manifested physiologically as a lower maximal blood flow response during voluntary exercise and functionally as a reduced tolerance for voluntary exercise.

METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with American Physiological Society guidelines for the care and use of animals. Twenty-two New Zealand White rabbits (3.0–3.5 kg) were selected for their willingness to run on a motorized treadmill. The animals were chronically instrumented in a series of sterile surgical procedures. During the first surgical procedure, a midline abdominal incision was made, 1.5-mm flow probes (ultrasonic transit-time flow probes, Transonic Systems, Ithaca, NY) were placed on right and left common iliac arteries, and the cables were tunneled to the back. A subcutaneous pocket was fashioned, and the flow probe connectors remained subcutaneous to be retrieved for experimental measurements. Sufficient recovery (>7 days) time from the first surgical procedure was allowed for the flow probes to “grow in” so that adequate signal could be recorded. Baseline data were gathered at rest and during exercise (see below) before the second surgical procedure. During the second surgical procedure, the femoral artery was ligated and an osmotic pump was implanted for chronic drug infusion. The right or left femoral artery was dissected from the inguinal ligament to the bifurcation into the saphenous and popliteal arteries. After implantation of a catheter, the femoral artery was ligated proximally at the inguinal ligament and distally just above the bifurcation into the saphenous and popliteal arteries to produce unilateral ischemia in the hindlimb of the rabbit. All side branches of the femoral artery between these two points were also ligated. A heparin-impregnated catheter (0.027 in. ID, 0.047 in. OD; Solomon Scientific, Plymouth Meeting, PA) was placed into the ligated femoral artery for chronic intra-arterial infusion of experimental drugs. The tip of the catheter in the femoral artery was placed just proximal to the inguinal ligament. Thus all drugs were infused upstream from the site of ligation. The other end of the catheter was attached to an osmotic infusion pump (model 2ML2, Alzet, Palo Alto, CA). These osmotic pumps are designed to hold a volume of 2 ml and deliver the loaded solution at 5 μl/h for 2 wk. The pump was tunneled under the skin and left subcutaneously on the back of the rabbit. The rabbits were randomly divided into three groups: group 1 (n = 9) received osmotic pumps loaded with N-nitro-L-arginine methyl ester (L-NAME; 400 mg/ml; Sigma Chemical, St. Louis, MO) in heparinized (100 U/ml) saline, group 2 (n = 7) received osmotic pumps loaded with N-nitro-D-arginine methyl ester (D-NAME; 400 mg/ml; Sigma Chemical) in heparinized (100 U/ml) saline, and group 3 (n = 6) received osmotic pumps loaded with phenylephrine (PE; 10 mg/ml; Baxter, Deerfield, IL) in heparinized (100 U/ml) saline. In one rabbit, the contralateral flow probe malfunctioned, and thus only six nonischemic limbs are represented in the D-NAME-treated group. L-NAME was given to inhibit NO production in the ischemic hindlimb and reduce endogenous vascular remodeling. The inactive isomer D-NAME was given as a control infusion. PE was given as a control for the vasoconstrictor effect produced when L-NAME blocks NO-mediated vasodilation.

On the day of an experiment, the rabbit was brought to the laboratory, and the flow probe leads were connected to the flowmeter. Blood pressure was measured at the central ear artery through percutaneous insertion of a 24-gauge intra-arterial catheter (Becton-Dickinson, Sandy, UT) attached to a solid-state pressure transducer (Abbott, North Chicago, IL). The rabbit sat quietly in a restraining cage in the laboratory as resting blood flow and blood pressure were measured continuously for 20 min. The rabbit was then moved to a motorized treadmill and performed a graded exercise test that consisted of 2-min workloads at 7 m/min and 0% grade, 12 m/min and 0% grade, and 15 m/min and 10% grade until exhaustion. Exhaustion was defined as the time at which the rabbit no longer voluntarily ran on the treadmill. Common iliac blood flow and blood pressure were measured continuously. The duration of voluntary exercise was recorded for each rabbit. The data were collected before and 1, 3, and 6 wk after femoral ligation.

Blood pressure and blood flows were continuously displayed on a computer (Macintosh Power PC G3) using a MacLab system at 100 Hz (ADInstruments, Castle Hill, Australia) for later playback and data analysis. Hemodynamic measurements were averaged over 15 s at rest and during the last 15 s of exercise. Each rabbit served as its own control for all experiments to examine the effect of femoral artery ligation on resting and exercise blood flow. The effect of chronic infusion of the various drugs was examined between the groups of rabbits. This experimental design necessitated a two-way (drug × time) repeated-measures analysis of variance. Significant differences are reported at P < 0.05.

RESULTS

Before femoral ligation, common iliac blood flow at rest was similar in the leg to undergo femoral ligation for each group of rabbits [23 ± 2, 21 ± 2, and 20 ± 2 (SE) ml/min for L-NAME, D-NAME, and PE, respectively]. Furthermore, before femoral ligation, maximal blood flow during exercise in the limb to undergo femoral ligation was not different among the groups of rabbits (Table 1). Voluntary exercise times (seconds) were also similar between the groups (1,179 ± 100, 1,151 ± 72, and 1,141 ± 139 s for L-NAME, D-NAME, and PE, respectively) before femoral ligation. Because there were no significant differences in any hemodynamic data at any point in the study between the two control groups (D-NAME and PE), these groups were combined for ease of comparison with the L-NAME group. Table 1 gives the heart rate and blood pressure...
ARGinine methyl ester; PE, phenylephrine; ischemic limb blood flow was no signifi-
cant difference in resting iliac blood flow between animals treated with N-nitro-
l-arginine methyl ester (L-NAME) and control group, which received N-nitro-d-
arginine methyl ester (d-NAME) or phenylephrine (PE). Values are means ± SE.

![Blood Flow vs. Time](image)

Fig. 1. Resting iliac blood flow over duration of experiment. There was no significant difference in resting iliac blood flow at any time between animals treated with N-nitro-l-arginine methyl ester (L-NAME) and control group, which received N-nitro-d-arginine methyl ester (d-NAME) or phenylephrine (PE). Values are means ± SE.

![Blood Flow vs. Exercise](image)

Fig. 2. Iliac blood flow during exercise. Drug infusions started after femoral ligation and continued for 2 wk (ending between weeks 1 and 3). Femoral ligation significantly (P < 0.05) reduced iliac blood flow at weeks 1, 3, and 6 compared with preligation in both groups. In addition, hindlimb blood flow was significantly (P < 0.05) lower at weeks 1, 3, and 6 in L-NAME-treated rabbits than in controls, which received d-NAME or PE. Values are means ± SE.

**Table 1. Hemodynamic variables at rest and during exercise for L-NAME-treated and control groups**

<table>
<thead>
<tr>
<th></th>
<th>HR, beats/min</th>
<th>MAP, mmHg</th>
<th>Limb Blood Flow, ml/min</th>
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<tr>
<td></td>
<td>Rest</td>
<td>Exercise</td>
<td>Rest</td>
</tr>
<tr>
<td>L-NAME</td>
<td>182 ± 10</td>
<td>383 ± 6</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>d-NAME</td>
<td>185 ± 9</td>
<td>406 ± 6</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>PE</td>
<td>194 ± 4</td>
<td>404 ± 11</td>
<td>67 ± 3</td>
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**Before ligation**

<table>
<thead>
<tr>
<th></th>
<th>Nonischemic</th>
<th>Ischemic</th>
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<tbody>
<tr>
<td>L-NAME</td>
<td>180 ± 13</td>
<td>340 ± 14</td>
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<tr>
<td>d-NAME</td>
<td>168 ± 6</td>
<td>395 ± 4</td>
</tr>
<tr>
<td>PE</td>
<td>217 ± 13</td>
<td>396 ± 11</td>
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</table>

**After ligation**

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NAME</td>
<td>184 ± 9</td>
<td>174 ± 9</td>
<td>186 ± 11</td>
</tr>
<tr>
<td>d-NAME</td>
<td>182 ± 5</td>
<td>199 ± 6</td>
<td>186 ± 15</td>
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Values are means ± SE. HR, heart rate; MAP, mean arterial pressure; L-NAME, N-nitro-l-arginine methyl ester; d-NAME, N-nitro-d-arginine methyl ester; PE, phenylephrine; ischemic limb blood flow, blood flow in limb that underwent femoral ligation and drug infusion; nonischemic blood flow, blood flow to contralateral limb.
rabbits than in controls, which received D-NAME or PE. Values are means ± SE.

DISCUSSION

The salient finding in this study is that NO plays a role in the endogenous vascular remodeling in the rabbit hindlimb after femoral ligation. Chronic inhibition of NO production reduced endogenous vascular remodeling compared with the control. This reduction was manifested physiologically as a lower maximal blood flow response during voluntary exercise and functionally as a reduced tolerance for voluntary exercise.

Numerous experimental models have been used to study angiogenesis in vivo and in vitro. However, we believe there are a number of advantages to using the ischemic rabbit hindlimb model (surgically ligated femoral artery) to examine in vivo arteriogenesis and angiogenesis. The ischemic rabbit hindlimb model is a particularly good representation of human peripheral vascular disease, because resting hindlimb blood flow is normal while blood flow reserve during exercise remains compromised. In addition, there is a well-established body of literature demonstrating the feasibility of using pharmacological agents to alter vascular growth in the ischemic hindlimb of the rabbit (3–5, 14, 31). Moreover, previous studies have described a well-defined, short period of endogenous vascular remodeling (~10 days) after femoral ligation (31, 32). In the present study, the continuous infusion of L-NAME over this period attenuated endogenous vascular remodeling that spontaneously occurred after femoral ligation. Even after the L-NAME infusion ceased (14 days after femoral ligation), endogenous vascular remodeling over the next 28 days could not remedy the stunted growth during the first 2 wk immediately after femoral ligation. It is unknown whether, given enough time, a slow continuous vascular remodeling would have occurred in the L-NAME-treated group to equal the revascularization in the control group. Nevertheless, exercise blood flow and exercise tolerance remained compromised throughout the duration of the experiment in the rabbits that received L-NAME compared with control infusions. These results suggest that there is a critical window shortly after femoral ligation in which the availability of NO is essential for vascular remodeling. Once this time period has passed, NO production is not as effective in producing vascular remodeling to alleviate vascular insufficiencies.

In the present study, conscious rabbits performed treadmill exercise before and after femoral ligation. Treadmill exercise provides a natural physiological stimulus to induce skeletal muscle hyperemia in the ischemic hindlimb. In addition, treadmill exercise also gives an indication of exercise tolerance and, thus, skeletal muscle function under normal conditions. There is evidence of increases in exercise tolerance with exercise training in animals and humans with peripheral vascular insufficiency. Interestingly, an increase in exercise tolerance is possible without an improvement in limb blood flow (9, 10, 20, 21, 37) and may reflect a redistribution of blood flow within the ischemic limb to areas most affected by the stenosis. Therefore, in the present study, we believed it was imperative to make in vivo measurements of skeletal muscle performance and blood flow, because vascular remodeling within the ischemic muscle could have improved exercise tolerance without an increase in total hindlimb blood flow. However, we found no dissociation between exercise tolerance and total hindlimb blood flow. NO inhibition resulted in a decrease in total hindlimb blood flow and exercise tolerance.

It is well known that inadequate blood flow to ischemic tissues provides a stimulus for the development of collateral vascular networks. In the ischemic hindlimb of a rabbit, endogenous vascular remodeling can be accomplished through angiogenesis or arteriogenesis and is likely a combination of both of these events (1, 4, 5, 14, 17, 18, 31). Angiogenesis refers to the process by which new capillary blood vessels sprout and proliferate within a tissue. Angiogenesis is associated with endothelial cell proliferation and the formation of endothelial cell tubes (26). Angiogenesis in the ischemic rabbit hindlimb model results in an increase in the number of capillaries per skeletal muscle fiber. In contrast, arteriogenesis is the rapid proliferation of preexisting collateral arteries that dramatically increases the vessel lumen through growth to enhance perfusion of an ischemic area (7). These collateral vessels develop from preexisting arteriolar anastomoses.

L-NAME-treated and control groups. For both groups, these reductions were most pronounced 1 wk after femoral ligation. Exercise tolerance improved at 3 and 6 wk after femoral ligation compared with 1 wk after femoral ligation for each group. However, at each time point after femoral ligation, exercise tolerance was lower (P < 0.05) in the rabbits that received L-NAME than in the control group.

Fig. 3. Exercise tolerance (duration of voluntary exercise). Drug infusions started after femoral ligation and continued for 2 wk (ending between weeks 1 and 3). Femoral ligation significantly (P < 0.05) reduced exercise tolerance at weeks 1, 3, and 6 compared with baseline (preligation) in both groups. Tolerance for exercise was significantly (P < 0.05) less at weeks 1, 3, and 6 in L-NAME-treated rabbits than in controls, which received d-NAME or PE. Values are means ± SE.

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and have a “corkscrew” appearance in angiography (17). An increase in capillary density within ischemic tissue through angiogenesis is of minimal value without the large increase in perfusion of the ischemic tissue produced through arteriogenesis. Capillary networks are not designed for conductance of large volumes of blood but, rather, for local delivery of nutrients and oxygen (35). Thus arteriogenesis is thought to be more important than angiogenesis is restoring flow to alleviate occlusive artery disease (35).

A limitation to the present study is the inability to distinguish the contributions of arteriogenesis and angiogenesis to the endogenous vascular remodeling that occurred after femoral ligation. Neither angiographic nor histological documentation of new vascular growth in the ischemic hindlimb was provided in the present study. Although this study provides evidence that NO plays an important role in the revascularization of the ischemic rabbit hindlimb, it is unclear whether NO does so primarily through angiogenesis or arteriogenesis. However, it seems reasonable to speculate that the alterations in hindlimb blood flow and function in the present study are likely a result of a combination of inhibition of NO-mediated angiogenesis and arteriogenesis.

**Angiogenesis and NO.** Many angiogenic molecules appear to be associated with NO production (6, 8, 23, 27, 34). The potent angiogenic growth factors bFGF and VEGF have been shown to produce dose-dependent release of NO (6, 8, 34). The release of NO produces profound vasodilation in vivo (6, 8). In addition, NO generation may participate in the angiogenic properties of platelet-activating factor and tumor necrosis factor-α (23). The generation of NO is consistent with the observation that administration of platelet-activating factor can produce substantial vasodilation and hypotension (27). Sodium nitroprusside, which releases NO spontaneously, has also been shown to promote growth and mobilization of the endothelium in vitro in a dose-dependent fashion (39). Endothelial cell proliferation and the formation of endothelial cell tubes are hallmarks of angiogenesis. In skeletal muscle, angiogenesis can be documented by an increase in capillary-to-fiber ratio. Recently, Murohara and colleagues (24) showed that dietary L-arginine supplementation (to augment endogenous production of NO) produced an increase in capillary density and capillary-to-muscle fiber ratio in the ischemic hindlimb of a rabbit. It has long been known that exercise training results in an increase in capillary-to-fiber ratio (15). It has been hypothesized that the angiogenesis in skeletal muscle associated with exercise training is dependent on the release of NO by skeletal muscle vasculature via shear stress (16). Indeed, chronic elevations in skeletal muscle blood flow, which should increase shear stress, produced an increase in capillarity (38). Two recent studies have elucidated a role for NO in the angiogenic effects produced by chronic electrical stimulation (16) and exercise training (19). Hudlicka and colleagues (16) reported that skeletal muscle angiogenesis produced by chronic electrical stimulation of a rat hindlimb was abolished by the NO synthase inhibitor N-nitro-L-arginine. Lloyd and colleagues (19) showed that exercise training improved collateral-dependent blood flow to the calf muscle in a rat model of peripheral vascular disease. The training induced vascular remodeling was inhibited by L-NAME (19), although they found that the increase in capillarity (angiogenesis) of the calf with exercise training was not altered by NO inhibition. Thus, Lloyd and colleagues concluded that arteriogenesis and angiogenesis appear to differ in their requirement for NO. In the present study, the stimulus for arteriogenesis and angiogenesis was not exercise training. We examined the effect of NO inhibition on the endogenous vascular remodeling in the rabbit hindlimb that occurs shortly after femoral ligation. Just as NO appears to be important for exercise training-induced arteriogenesis and/or angiogenesis in skeletal muscle, the present results demonstrate that NO plays a similar role in the endogenous vascular remodeling of the ischemic rabbit hindlimb.

**Arteriogenesis and NO.** NO production could be particularly important in arteriogenesis. Continuously synthesized NO controls basal tone of arterioles and small arteries and does so most effectively in large (>50-μm) arterioles (28). Shear stress, which is already highest at the arteriolar level, rises acutely after femoral artery occlusion in preexisting arteriolar connections bypassing the occlusion. Because shear stress can increase the release of endothelium-dependent relaxing factors, including NO (22), this could provide a local stimulus other than ischemia that might induce vascular development. Bauters et al. (4) and Takeshita et al. (31) reported a direct relationship between improvements in skeletal muscle blood flow and angiographic score produced by VEGF administration and the baseline vascular deficit produced by femoral ligation. This implies that severe ischemia is required for optimal effectiveness of growth factor-induced angiogenesis. However, there is evidence of arteriogenesis in tissue that is unlikely to be ischemic. Large collateral arteries grow in the quadriceps region of the rabbit after femoral ligation, even though this portion of the hindlimb remains well perfused (17). Thus, Ito et al. (17) argue that a local factor other than ischemia is necessary to explain collateral vascular growth, since it is unlikely that surrounding tissue is ischemic. An increase in blood flow through collateral circulation produces an increase in wall shear stress, which could provide an adequate stimulus for NO release and arteriogenesis. Recent data from Tronc et al. (33) support this hypothesis. In conscious rabbits, chronic elevation of blood flow through the carotid artery resulted in increases in vessel caliber that were inhibited by L-NAME (33).

**Conclusion.** The data presented in this study are consistent with the hypothesis that NO plays an essential role in the endogenous vascular remodeling that occurs in the rabbit hindlimb after femoral ligation. The results of the present study indicate that chronic inhibition of NO production in experimental rabbits reduced endogenous vascular remodeling compared with control rabbits. This reduction was manifested physiologically as a lower maximal blood flow response.
during voluntary exercise and functionally as a reduced tolerance for voluntary exercise.

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REFERENCES


