Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle

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Submitted 1 November 2004; accepted in final form 23 February 2005

Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle. Am J Physiol Heart Circ Physiol 289: H336–H343, 2005. First published February 25, 2005; doi:10.1152/ajpheart.01105.2004.—Vascular endothelial growth factor (VEGF) is considered to be important in promotion of capillary growth in skeletal muscles exposed to increased activity. We studied its interactions with nitric oxide (NO) by examining the expression of endothelial NO synthase (NOS), VEGF, and VEGF receptor-2 (VEGFR-2) proteins in relation to capillary growth in rat extensor digitorum longus muscles electrically stimulated for 2, 4, or 7 days with and without NOS inhibition by Nω-nitro-L-arginine (L-NNA, 3 mg/day). Stimulation increased all proteins from 2 days onward, concomitantly with capillary proliferation (labeling for proliferation fractionation) and increased capillary-to-fiber ratio (C/F) (33). Stimuli proposed to trigger angiogenesis in stimulated muscles, where inhibition of NOS activity abolished the increase in capillary proliferation and the increase in C/F at all time points. We conclude that, in stimulated muscles, NO, generated by activation of neuronal NOS by muscle activity or endothelial NOS by increased blood flow and capillary shear stress, may increase capillary proliferation in the early stages of stimulation through upregulation of VEGFR-2 and VEGF. With longer stimulation, capillary growth appears to require NO, and high levels of VEGF and VEGFR-2 may be contributing to maintenance of the increased capillary bed.

vascular endothelial growth factor; capillary proliferation; chronic muscle stimulation; nitric oxide synthase inhibition

VEGF promotes endothelial cell division and migration (71) and has been extensively studied in muscles exposed to increased activity. Work by Wagner and colleagues has described elevated levels of VEGF mRNA in rat muscles after one bout of exercise (9), a response that was eliminated by repeated exercise during training (58). Furthermore, the increased density of microvessels that was associated with higher levels of VEGF protein in the rat hindlimb after several days of training could be blocked by a VEGF-neutralizing antibody (2), and increased C/F in trained muscles appeared concomitantly with a higher density of VEGF positively stained capillaries (68). When muscle activity was increased by electrical stimulation for several days, VEGF mRNA (26, 63) and protein (3, 5) were upregulated in association with increased capillary supply. In contrast, our own experiments have shown an increase in the proportion of capillaries undergoing proliferation in muscles stimulated for 2 days, but only after 4 days were there more capillaries positively expressing VEGF protein, and C/F increased even later (50). These studies confirm a role for VEGF in activity-induced angiogenesis but do not elucidate the exact temporal link between VEGF expression and capillary growth.

VEGF exerts its angiogenic effect by coupling to the tyrosine kinase receptors VEGF receptor (VEGFR)-1 and VEGFR-2, expressed predominantly on endothelial cells. VEGF-2 is involved in mitogenesis in cultured endothelial cells (23), whereas VEGFR-1 is implicated in endothelial cell differentiation (13). Reports of changes in VEGFR expression in muscles subjected to increased activity are inconclusive. VEGF and VEGFR-1 mRNA were elevated after a single bout of exercise in rat muscles, but VEGFR-2 mRNA was not changed (20), although it was increased 4 h after exercise in human muscles (22). By contrast, there was no change in VEGFR-1 protein in muscles stimulated for 2 days (51), whereas during training of ischemic muscles, increases in mRNA for VEGFR-1 and VEGFR-2 were reported to occur later than for VEGF (48). The relation between expression of VEGF receptors and expression of ligand in active muscles therefore remains unclear.

VEGF mRNA expression in response to acute exercise could be attenuated by inhibition of nitric oxide (NO) synthase (NOS) activity (21), indicating a relation between these two factors. NO was also shown to be involved in the early stages of angiogenesis in stimulated muscles, where inhibition of NOS activity abolished the increase in capillary proliferation (34). Increased muscle activity is known to upregulate neuro-
nal NOS (nNOS) in rat muscle (60) and endothelial NOS (eNOS) in skeletal muscle arterioles (67). NO is released in vitro from contracting muscles (7) and from endothelium by increased shear stress (43) and in vivo in response to increased blood flow (17). When shear stress was increased in skeletal muscle capillaries by chronic electrical stimulation or long-term vasodilator treatment, a higher proportion of VEGF-positive capillaries was found before the increase in C/F (50). NO and VEGF are known to be synergistic and/or complementary in other angiogenic situations. For example, VEGF stimulated synthesis of eNOS mRNA and production of NO in vitro (28, 46) via VEGFR-2 (44, 61), implying that the action of NO lies downstream of VEGF. Furthermore, VEGF-induced angiogenesis in the rabbit cornea could be blocked by NOS inhibition (74), and the angiogenic response to hindlimb ischemia in eNOS−/− mice was impaired and could not be reversed by VEGF (52). The nature of any interaction between NO and VEGF in the course of activity-induced angiogenesis is as yet undefined, and even less is known about the interaction between NO and VEGF receptors.

Most studies of capillary growth and factors involved in it have been performed in animals or humans undergoing many weeks of endurance exercise training, which involves recruitment of predominantly oxidative fibers. In recent experiments, training by exhaustive exercise that recruits glycolytic fibers demonstrated a rapid increase in the size of the capillary bed preceded by an increase in VEGF protein (68, 69). This type of training also resulted in an increase in capillaries, first, around glycolytic fibers (69), which showed a higher level of VEGF mRNA after exhaustive exercise (8). A similar pattern of fiber recruitment with rapid capillary growth initially in the vicinity of glycolytic fibers was observed in chronically stimulated muscles (6, 32). Defined periods of stimulated contractions allow the effects of muscle activity to be studied without possible complications from exhaustion associated with whole body exercise. We have therefore investigated the relation between eNOS, VEGF, and VEGFR-2 with respect to capillary growth by following changes in their expression in chronically stimulated rat skeletal muscles without and with inhibition of NO formation by a nonspecific NOS inhibitor, Nω-nitro-L-arginine (L-NNA). We hypothesized that, in chronically stimulated muscles, increased activity and increased blood flow (14) may generate release of NO, which not only affects capillary proliferation directly but may also contribute to the upregulation of VEGF and VEGFR-2 protein.

MATERIALS AND METHODS

Surgical and pharmacological treatment. The following groups of adult male Sprague-Dawley rats (292 ± 9 g final body wt) were studied: 1) control rats, 2) rats with ankle flexor muscles stimulated for 2, 4, or 7 days, 3) rats treated with the NOS inhibitor L-NNA for 2, 4, or 7 days, and 4) stimulated rats treated concurrently with L-NNA. L-NNA was used as a nonspecific blocker of NOS, because nNOS and eNOS both appear necessary for arteriolar dilation in contracting fast-twitch muscles (45). Separate animals from these groups were used for experiments where intravital observations of muscle microcirculation were made to assess the effects of NO inhibition and for experiments where muscles were removed for biochemical and immunohistochemical analysis. Rats were given L-NNA (Sigma Aldrich Chemicals, Steinhausen, Germany; 100 mg/l in drinking water. On the basis of daily measured fluid consumption, each rat received ~3 mg L-NNA per day. Control rats drank water only. Chronic muscle stimulation was performed using electrodes placed close to the right peroneal nerve, with parameters of subcutaneously implanted stimulators programmed to 10-Hz frequency, 0.1-ms pulse width, and 3 V for 8 h/day (47). Electrodes and stimulators were implanted under isoflurane anesthesia and aseptic conditions. All rats were anesthetized with pentobarbital sodium (50 mg/kg) 16 h after the last 8-h stimulation period. Muscles were removed for analysis, and rats were then killed by pentobarbital sodium overdose. All procedures conformed to the United Kingdom Animals (Scientific Procedures) Act of 1986. The protocol for this study was approved by the Birmingham Ethical Review Subcommittee.

Efficacy of L-NNA treatment. Efficacy of L-NNA treatment was assessed by measurement of mean arterial blood pressure from a catheter in the carotid artery via a pressure transducer (type PDT75-1166, Elcomatic) in all animals and by estimation of the diameter of the terminal precapillary arterioles on the surface of the right extensor digitorum (EDL) muscle. In four or five animals from each group, the jugular vein was cannulated for maintenance of anesthesia (5 mg/kg bolus when required). The EDL was exposed for intravital observation, superfused by deoxygenated buffered physiological salt solution (pH 7.35–7.45, 32–34°C), epi-illuminated by green-filtered fiber-optic light (Schott, Oberkochen, Germany), and viewed with an immersion objective (×25, 0.6 numerical aperture; Leitz) (4). Images of precapillary arterioles were displayed via a video camera (Cohu 1472) on a monitor at a final magnification of ×1,000, and vessel diameters were tracked and measured offline using a calibrated video caliper (Dynatek Laboratories, Chantilly, VA) connected to a chart recorder (Lectromel, Welwyn Garden City, UK) (38).

Muscle analysis. In three or four rats from each group, EDL muscles were removed after measurement of arterial blood pressure under pentobarbital sodium anesthesia. A midportion of each muscle was frozen in precooled isopentane for cryostat sectioning and immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and capillaries (alkaline phosphatase) (50). The remainder of the muscle was frozen in liquid nitrogen for analysis of eNOS, VEGF, and VEGFR-2 protein by Western blot analysis (12, 51). Briefly, 50 μg of whole muscle extract per lane were resolved using SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia) in blotting buffer (15% methanol, 25 mM Tris, and 120 mM glycerine). Membranes were blocked with 10% nonfat dry milk in TTBS (20 mM Tris, 137 mM NaCl, and 0.01% Tween 20, pH 7.6). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG was used as secondary antibody at a dilution of 1:2,500 in TTBS (Amersham Pharmacia). Antibody detection was carried out by a chemiluminescence-based detection system (ECL, Amersham Pharmacia). Equal protein loading was normalized to β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The following antibodies were used: mouse monoclonal anti-eNOS (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-Flk-1/VEGFR-2 (sc-504, Santa Cruz Biotechnology), and anti-VEGF 1 (sc-507, Santa Cruz Biotechnology). The anti-VEGF antibody recognizes the 165-, 189-, and 121-amino acid splice variants of VEGF-A, which were clearly seen after separation, and densitometric analysis was performed on all three protein bands.

Data and statistical analysis. Mean arterial blood pressure and arteriolar diameters and dilator response values are means ± SE, and n is the number of animals or number of vessels, respectively. For protein analysis, expression of the relevant band with respect to β-actin for each experimental muscle was normalized to the value for the first control muscle on the same gel, which was designated 1.0. Time-dependent effects were analyzed, and comparisons between groups at a given time were made by ANOVA with repeated measures where appropriate, with Fisher’s protected least-significant difference post hoc test. Results are considered statistically significant at the 5% level.
inhibition alone had no effect on VEGFR-2 protein at any time point (Table 2).

**Mean blood pressure and arteriolar diameters.** The efficacy of L-NNA in reducing basal systemic NO release was shown by a significant rise in mean blood pressure at all time points in unstimulated rats compared with control animals (Fig. 3; \( P < 0.05 \)). Rats that were stimulated and received L-NNA showed similar hypertension after 2, 4, or 7 days, although by 7 days, blood pressures were no longer significantly higher than in controls (Fig. 3). Stimulation alone had no effect on mean blood pressure after 7 days (89 ± 13 vs. 102 ± 8 mmHg in controls, not significant) or at any earlier time point (14).

The average diameter of precapillary arterioles in a total of 34 vessels in 8 control rats was 9.2 ± 0.4 μm. We previously reported that stimulation for 2 days caused precapillary arteriole diameters to increase (55), and, similarly, in the present study, arterioles were 16% wider in stimulated muscles than in controls (10.7 ± 0.6 μm, \( P < 0.05 \)). This effect was transient, because after 7 days, diameters had reverted to control values (8.6 ± 0.2 μm, not significant). L-NNA treatment alone for 2 or 7 days significantly decreased arteriolar diameters (Fig. 4; \( P < 0.05 \)). The combination of stimulation and L-NNA had different effects depending on the treatment duration. It prevented the stimulation-induced diameter increase after 2 days, and arterioles were constricted compared with stimulated or control muscles. After 7 days, vessels were paradoxically 63% wider than in control muscles (Fig. 4).

**DISCUSSION**

This study has shown that chronic electrical stimulation of skeletal muscle leads to increases in eNOS, VEGF, and VEGFR-2 proteins and early capillary proliferation. The proliferation declines over time as new capillaries are formed in stimulated muscles by a combination of proliferation and endothelial cell migration (15, 25), so that C/F is increased later on. The gradual decrease in proliferation can be partly accounted for by the fact that, as shown by electron-microscopic studies, the population of labeled cells at capillary sites in muscles stimulated for 2 days includes endothelial cells and fibroblasts, whereas after 7 days, only endothelial cells are labeled (15). The main aim of this study was to investigate whether NO produced in contracting muscles is involved in the upregulation of VEGF or VEGFR-2. Treatment with the NOS inhibitor L-NNA did not alter the level of eNOS protein, although it blocked NOS activity on the basis of the increase in blood pressure and constriction of arterioles. It suppressed the elevation of VEGF and VEGFR-2 and abolished capillary proliferation during the first few days of chronic stimulation, with the result that there was no increase in C/F after 7 days.
The presence of NO therefore appears to be a key requirement for capillary proliferation in the first few days of muscle stimulation, possibly by upregulating VEGF and VEGFR-2 proteins. The importance of NO in the upregulation of VEGF mRNA has been previously shown in muscles exposed to an acute bout of exercise (21).

Before further consideration of the role of NO, VEGF, and VEGFR-2 in the regulation of capillary growth in stimulated muscles, the relation between the estimation of these proteins in whole muscle extracts and specific sites of capillary proliferation needs to be addressed. For any of these factors to play a role in signaling the start of endothelial cell proliferation, it is evident that they should be localized close to the capillaries themselves. eNOS is confined chiefly in endothelial cells in large and small blood vessels (18). In EDL muscles, it has also been colocalized with mitochondrial markers (42), where its activity increases during contractions (64), with the potential for release of NO into capillaries. VEGF receptors are also expressed predominantly on endothelial cells in all vessel types (16). There was good correlation between the increase in protein level of VEGFR-2 and the number of proliferating capillaries during angiogenesis in ischemic skeletal muscles (51), and, on the basis that the time course of changes in VEGFR-2 expression matched that of cell proliferation in the present experiments, a similar relation can be inferred.

The elevation of whole muscle VEGF protein, on the other hand, represents changes in all muscle compartments, because its expression has been reported within individual muscle fibers (8, 37), in interstitially located cells such as macrophages (70), in vascular smooth muscle (39), and in the extracellular matrix (5). Although VEGF was increased in whole muscle extracts after 2 days of stimulation, its immunolocalization to capillary sites, taken to indicate spatially appropriate ligand binding to the endothelial receptor, occurred only after the peak of capillary proliferation (Fig. 5) (50). Inasmuch as it is clear that factors initiating endothelial cell proliferation must act at the site of these cells, it seems unlikely that VEGF protein in capillaries after the peak of proliferation would be such a factor. VEGF may be important for directing the migration of endothelial cells to potentially hypoxic sites within stimulated muscles, but our previous electron microscopic studies did not

![Image](http://ajpheart.physiology.org/)

**Table 2. Protein expression of eNOS, VEGF-R2, and VEGF ligand in EDL from rats treated for 2, 4, or 7 days with t-NNA**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>2 Days (n = 3)</th>
<th>4 Days (n = 3)</th>
<th>7 Days (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>1.00 ± 0.01</td>
<td>1.01 ± 0.02</td>
<td>0.99 ± 0.04</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>1.00 ± 0.01</td>
<td>1.04 ± 0.06</td>
<td>0.94 ± 0.05</td>
<td>1.11 ± 0.08</td>
</tr>
<tr>
<td>VEGF</td>
<td>1.00 ± 0.01</td>
<td>1.18 ± 0.10</td>
<td>1.01 ± 0.03</td>
<td>1.05 ± 0.04</td>
</tr>
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Values are means ± SE, expressed as protein-to-actin ratio normalized to control muscles in each gel; n, number of muscles. eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2. *P* = not significant vs. control for all t-NNA groups.

Fig. 2. Effect of t-NNA on endothelial nitric oxide synthase (eNOS, A), vascular endothelial growth factor (VEGF, B), and VEGF receptor-2 (VEGFR-2, C) protein expression in control and stimulated EDL muscles. *Top*: two representative immunoblots from each experiment. *Bottom*: densitometric analyses of Western blots. The same blots were stripped and reprobed with β-actin antibody to check equal loading of samples. Values are means ± SE from 3–4 muscles in each experimental group. *P* < 0.05 vs. control. †P < 0.05 vs. stimulation alone.
detect sprouting capillaries after 2 days of stimulation, although they appeared by 7 days (15).

NO can be generated by increased activity of eNOS or nNOS. Although it is known that chronic electrical stimulation upregulates nNOS in muscle fibers (57), eNOS upregulation by training has been reported only in skeletal muscle arterioles (65). The activity of both enzymes can be increased by acute exercise: nNOS due to muscle fiber contractions and eNOS due to changes in blood flow and shear stress (17). It is also possible that chronic stimulation could increase eNOS in muscle fibers, because the expression of eNOS was found to be correlated with mitochondrial content in normal rat muscles (42), and stimulation is known to increase the proportions of mitochondria (29) and highly oxidative fibers (36).

During acute contractions, a specific blocker of nNOS inhibited NOS activity (measured by conversion of [3H]arginine to [3H]citrulline) by ~30%, whereas nonspecific NOS inhibition by N-nitro-L-arginine methyl ester almost completely suppressed it (60), implying that a considerable amount of NO during exercise must be generated via eNOS. L-NNA used in the present experiments acts similarly to N-nitro-L-arginine methyl ester as a competitive inhibitor, preventing the conversion of L-arginine to citrulline and, hence, the generation of NO. Although the quantity of eNOS protein in muscles was increased by stimulation, regardless of NOS inhibition, the activity of the enzyme was depressed, as demonstrated by an increase in mean arterial pressure. Furthermore, the fact that NOS inhibition prevented the stimulation-induced increase in arteriolar diameter in the early stages would imply limitation of capillary flow and shear stress and a further curb to generation of NO. This is also supported by findings of decreased blood flow in rat hindlimb muscles under conditions of NOS inhibition at rest and during exercise (27, 53) and by our preliminary data on the suppression of stimulation-induced increases in capillary shear stress by L-NNA (unpublished observations).

The regulatory role of NO in capillary growth differed at the early and later stages of stimulation. The presence of NO during the first few days was necessary for upregulation of VEGF and VEGFR-2. NO donors are known to increase expression of VEGF in many types of cells in vitro, including keratinocytes (19), macrophages (70), and vascular smooth muscle cells (39), but similar effects on endothelial cells are not established, although VEGF produced by other cell types in stimulated muscles could travel to the capillaries and influence their growth in vivo. NO donors increased the levels of VEGF and the number of proliferating endothelial cells in the brain (72), and the absence of eNOS in knockout mice resulted in lower VEGF mRNA and capillary density in the heart (73). After 7 days of stimulation, levels of VEGF and VEGFR-2 were unaffected by L-NNA, yet capillary growth was suppressed, suggesting that NO is modulating capillary growth independently of VEGF or its receptor. It could elicit proliferation of endothelial cells directly by increasing the activity of extracellular regulated kinases, ERK-1/2 (54, 59), by an NO-cGMP pathway (72), or by activation of protein kinase C, ERK-2, and c-Jun (38).

The paradoxical dilation of arterioles in muscles stimulated for 7 days in L-NNA-treated animals could be explained by incomplete inhibition of eNOS activity, because higher doses of L-NNA were found to be necessary for inhibition of flow-mediated dilator responses of rat skeletal muscle arterioles after exercise training (67). We previously reported that the same dose of L-NNA for 7 days changed ACh-induced dilation of EDL arterioles to constriction, and this could be prevented by chronic stimulation applied intermittently, suggesting restoration of endothelial function (41). It is not possible to say whether a higher dose of L-NNA would have suppressed the increase in VEGF and VEGFR-2 after 7 days of stimulation, and we did not wish to use a higher dose of L-NNA, insomuch as this may be toxic for the whole animal (53). Moreover, at the dose used, L-NNA was effective at inhibiting endothelial cell proliferation and the increase in C/F on stimulation. Another
more likely explanation for the persistence of arteriolar dilation and, to a certain degree, for high levels of VEGF and VEGF-R2 in stimulated l-NNA-treated animals is an increased role for other factors substituting for NO. In eNOS−/− mice, release of endothelium-derived hyperpolarizing factor (30) or prostaglandins (66) can compensate for the lack of NO in the maintenance of arterial dilation, and prostaglandins can themselves upregulate VEGF expression (62).

NOS inhibition may also result in an imbalance between muscle perfusion and oxygen consumption. Rat muscles stimulated for 7 days have slightly higher blood flow during contractions and considerably lower maximal twitch tension than control muscles (14). NOS inhibitors are known to increase muscle oxygen consumption and increase the force of contraction and considerably lower maximal twitch tension (9). Rat muscles stimulated for 7 days have slightly higher blood flow during muscle perfusion and oxygen consumption. Rat muscles stimulated for 7 days have slightly higher blood flow during muscle perfusion and oxygen consumption. Rat muscles stimulated for 7 days have slightly higher blood flow during muscle perfusion and oxygen consumption.

It is considered an important factor for the upregulation of VEGF, and it may be involved in the very early stages of stimulation, when muscle PO2 did not increase after acute contractions to the same extent as in normal muscles and the percentage of VEGF-positive capillaries was transiently higher (35).

Exercise results in a number of changes on a molecular level that could contribute to capillary growth in skeletal muscles (for review see Ref. 56), but unfortunately experiments defining a clear relation between the type and duration of exercise, modulation of the expression of VEGF and its receptors, and NO production and angiogenesis have not been performed (56). In view of the fact that chronic electrical stimulation resembles changes in skeletal muscles subjected to exhaustive exercise, at least with respect to the speed and location of capillary growth, this present study may help elucidate the relation between the three most important factors involved.

To summarize, increased capillary proliferation induced by chronic electrical stimulation occurs concomitantly with increased levels of eNOS, VEGF-R2, and VEGF proteins in whole muscle extracts, whereas VEGF protein localized to capillary sites increases slightly later. Although it is not possible to deduce which of these, or any other factors, actually initiate capillary proliferation, the present experiments demonstrate a significant role for NO, in that inhibition of NO activity by l-NNA suppressed capillary proliferation and, ultimately, capillary growth at all time points, whereas raised VEGF-R2 and VEGF protein expression were attenuated only during the first few days of stimulation. NO therefore contributes to capillary proliferation in the first few days, possibly via upregulation of VEGF and VEGF-R2 proteins but, later, may contribute to capillary growth in other ways. Whether the release of NO is due to increased muscle activity per se or to increased blood flow and shear stress remains to be elucidated.

ACKNOWLEDGMENTS

We thank Julia Verhaag for immunohistochemical estimation of proliferating capillaries and Dr. Stuart Egginton for use of the facilities where this work was performed. We also thank Michael Kloehn for advice and practical help with replacement of stimulators (47). Present address of M. Milkiewicz: Dept. of Kinesiology, York University, Toronto, Ontario, Canada M3J 1P3.

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