Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise

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Gavin, Timothy P., David A. Spector, Harrieth Wagner, Ellen C. Breen, and Peter D. Wagner. Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. J Appl Physiol 88: 1192–1198, 2000.—Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1) mRNA increase in rat skeletal muscle in response to a single acute exercise bout. Nitric oxide (NO) is released locally by muscle vascular endothelium and muscle fibers during exercise, contributes to the blood flow response to exercise, and regulates mitochondrial respiration. We hypothesized that a reduction in NO production, via NO synthase inhibition, would demonstrate a link between NO and the VEGF, bFGF, and TGF-β1 gene responses to exercise. To investigate this hypothesis, 9-wk-old female Wistar rats were divided into eight treatment groups (n = 6 each): 1) saline + rest, 2) saline + exercise, 3) 30 mg/kg Nω-nitro-L-arginine methyl ester (L-NAME, a known NOS inhibitor) + rest, 4) 30 mg/kg L-NAME + exercise, 5) 300 mg/kg L-NAME + rest, 6) 300 mg/kg L-NAME + exercise, 7) 300 mg/kg Nω-nitro-o-arginine methyl ester (o-NAME, inactive enantiomer of L-NAME) + rest, and 8) 300 mg/kg o-NAME + exercise. Exercise consisted of 1 h of running at 20 m/min on a 10° incline. VEGF, TGF-β1, and bFGF mRNA from left gastrocnemius were analyzed by quantitative Northern blot. Submaximal exercise for 1 h increased VEGF mRNA 4.2-fold and TGF-β1 mRNA 1.5-fold in untreated rats but did not increase bFGF mRNA. The exercise-induced increase in VEGF mRNA was attenuated ~50% by 30 and 300 mg/kg L-NAME; the TGF-β1 mRNA increase was unaffected by 300 mg/kg L-NAME. In addition, 300 mg/kg o-NAME had no effect on the exercise-induced increase in VEGF mRNA. Administration of 300 mg/kg L-NAME had no effect on bFGF mRNA. These findings suggest that NO is important in the regulation of the VEGF gene response to exercise through increases in VEGF transcription or by increases in the VEGF mRNA half-life.

VEGF transcription or by increases in the VEGF gene response to exercise through increases in VEGF mRNA levels after acute exercise was attenuated (~50%) by NOS inhibition, suggesting that NO regulates VEGF mRNA through increased VEGF tran-
sion or increased VEGF mRNA half-life. This attenuation was not observed after the administration of 300 mg/kg N-nitro-o-arginine methyl ester (o-NAME), the inactive enantiomer of L-NAME. The exercise-induced increase in TGF-β1 mRNA was unaffected by NOS inhibition. Neither exercise nor L-NAME affected bFGF mRNA levels. These results suggest that NO is an important signaling mechanism in the regulation of the exercise-induced increase in VEGF mRNA.

METHODS

This study was approved by the University of California, San Diego, Animal Subjects Committee. Female Wistar rats [68 ± 7 (SD) days old, 220 ± 11 (SD) g body wt] were first familiarized with a rodent treadmill (Omnipacer model LC-4, Omnitech, Columbus, OH) and taught to run at 20 m/min on an incline of 10° for 5 min, 24–48 h before the experimental bout. Animals were housed in their cages and allowed standard rat food and water ad libitum before undertaking the study. The exercise bout consisted of 1 h of treadmill running at 20 m/min on a 10° incline. This speed represents 55% of the speed required to attain maximal oxygen consumption (6). Initially, four treatment groups were defined with six rats in each group: 1) saline + rest, 2) saline + exercise, 3) 300 mg/kg L-NAME (Sigma Chemical, St. Louis, MO) + rest, and 4) 300 mg/kg L-NAME + exercise. After the initial results of 300 mg/kg L-NAME on the exercise-induced increase in VEGF mRNA were obtained and to eliminate the potential for nonspecific drug interactions, four additional groups of animals were defined with six rats in each group: 1) 30 mg/kg L-NAME + rest, 2) 30 mg/kg L-NAME + exercise, 3) 300 mg/kg d-NAME, the inactive enantiomer of L-NAME (Sigma Chemical) + rest, and 4) 300 mg/kg d-NAME + exercise. Animals were injected intraperitoneally with saline, L-NAME, or d-NAME 20 min before the start of rest or exercise. After completing the 1 h of rest or exercise, animals were anesthetized with pentobarbital sodium (50 mg/kg ip) while breathing 100% O2 to avoid hypoxemia, which has been shown to stimulate skeletal muscle VEGF mRNA (6). Within 20 min of the completion of exercise and after topical administration of lidocaine, the left carotid artery was catheterized for the measurement of mean arterial pressure (MAP) to determine the effect of L-NAME on the vasculature. After the measurement of MAP and within 40 min of the completion of exercise, the left gastrocnemius muscles (both heads combined) were removed for RNA isolation, frozen in liquid nitrogen, and stored at −80°C until further RNA analysis.

RNA isolation and Northern analysis. Rat gastrocnemius muscles were removed, and total cellular RNA was isolated from each sample by the method of Chomczynski and Sacchi (9). These RNA preparations were quantitated by absorbance at 260 nm, and RNA intactness and integrity were assessed by ethidium bromide staining after separation by electrophoresis in a 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to Zeta-probe membrane (Bio-Rad, Hercules, CA). After transfer, RNA was cross-linked to the membrane by ultraviolet irradiation for 1 min and stored at 4°C. The blots were then probed with oligolabeled [α-32P]dCTP cDNA probes, which had a specific activity >1 × 106 disintegrations·min⁻¹·μg DNA⁻¹ (13). The rat VEGF probe is a 0.9-kb cDNA Pst I/Sma I insert cloned into pBluescript II KS (+) vector (25). The rat TGF-β1 cDNA probe is a 0.985-kb Hind III/Xba I insert cloned into pBluescript II KS (+) vector (31). The bFGF probe is a 1-kb Xho I fragment of human bFGF cDNA (23). Prehybridization and hybridization were performed in 50% formamide, 5× saline-sodium citrate (SSC; 20× SSC is 0.3 M sodium chloride, 0.3 M sodium citrate), 10× Denhardt's solution (100× Denhardt's solution is 2% Ficoll, 2% polyvinylpyrrolidone, 2% BSA factor V), 50 mM sodium phosphate (pH 7.0), 1% SDS, and 250 μg/ml salmon sperm DNA at 42°C. Blots were washed at 2× SSC and 0.1% SDS at room temperature and 0.1× SSC and 0.1% SDS at 55°C (bFGF and TGF-β1) or 65°C (VEGF). Blots were exposed to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) for 2–3 (VEGF and bFGF) or 7 days (TGF-β1) with use of a Cronex Lightning Plus screen at −80°C. Autoradiographs were quantitated by densitometry within the linear range of signals and normalized to 18S rRNA levels.

Statistical treatment. Quantitative densitometry was used to measure the mRNA levels for all three growth factors. Normalization of growth factor densitometric signals with the 18S RNA was used to control for lane loading variation. One sample was lost from the o-NAME + rest group and was not replaced. A two-way ANOVA (3 × 2, drug × exercise level) was used to determine changes in MAP. A two-way ANOVA (2 × 2, drug × exercise level) was used to determine changes in mRNA. Bonferroni's test was used to determine significance between conditions. For VEGF mRNA, 30 mg/kg L-NAME, 300 mg/kg L-NAME, and 300 mg/kg D-NAME samples were compared with saline data, respectively. Significance was established at P ≤ 0.05 for all statistical sets, and values are means ± SE.

RESULTS

Injection of 30 mg/kg L-NAME, 300 mg/kg L-NAME, and 300 mg/kg D-NAME resulted in significant systemic hypertension as measured under anesthesia (133 ± 2, 151 ± 4, 155 ± 3, and 154 ± 4 mmHg for saline, 30 mg/kg L-NAME, 300 mg/kg L-NAME, and D-NAME, respectively) −1 h and 40 min after the injection of L-NAME or D-NAME (Fig. 1). There was no significant difference in MAP between rest and exercise for any treatment group; thus these values were combined. Figure 2 shows representative Northern blots in which VEGF mRNA levels were examined after the single 1-h submaximal exercise run. It is clear that VEGF mRNA increased after exercise and that this increase was attenuated by 30 and 300 mg/kg L-NAME (Fig. 2, A and B), whereas 300 mg/kg D-NAME did not alter the exercise-induced increase in VEGF mRNA (Fig. 2C).
VEGF mRNA. L-NNAME attenuated the exercise-induced increase in VEGF mRNA (A and B); D-NNAME had no effect on the exercise-induced increase (C).

Figure 3 portrays the quantitative densitometry for VEGF mRNA for 30 and 300 mg/kg L-NNAME and 300 mg/kg D-NNAME normalized to 18S rRNA. Figure 3, A and B, demonstrates that exercise induced a 4.2-fold increase in VEGF mRNA; whereas L-NNAME did not completely inhibit this response, it did attenuate the response ~50%. There was no difference between the VEGF mRNA values for saline + rest and L-NNAME + rest. With the same saline samples (rest and exercise) used in Fig. 3, A and B, Fig. 3C shows similar exercise-induced increases in VEGF mRNA between saline and D-NNAME groups. There was no difference between the VEGF mRNA values for saline + rest and D-NNAME + rest. For the drug × exercise interaction effect, P = 0.009 for 30 mg/kg L-NNAME, P = 0.002 for 300 mg/kg L-NNAME, and P = 0.79 for 300 mg/kg D-NNAME. Figure 4 demonstrates close correlation between the saline + rest and saline + exercise samples used for the 30 mg/kg L-NNAME blot with the 300 mg/kg D-NNAME blot (Fig. 4A) and for the 300 mg/kg L-NNAME blot with the 300 mg/kg D-NNAME blot (Fig. 4B).

Representative Northern blots for the TGF-β1 and bFGF responses to exercise and 300 mg/kg L-NNAME are presented in Fig. 5. Exercise increased TGF-β1 but did not increase bFGF mRNA. Administration of 300 mg/kg L-NNAME had no effect on TGF-β1 or bFGF mRNA levels. Figure 6 portrays the quantitative densitometry for TGF-β1 and bFGF mRNA normalized to 18S rRNA. Figure 6A demonstrates that exercise increased TGF-β1 ~1.5-fold and that L-NNAME did not affect this exercise-induced increase. Figure 6B shows that neither exercise nor L-NNAME affected bFGF mRNA levels.

**DISCUSSION**

The principal findings of the present study are as follows: 1) VEGF and TGF-β1 mRNA were increased after 1 h of submaximal exercise; 2) L-NNAME attenuated the VEGF mRNA increase ~50%; and 3) L-NNAME did not affect rest or exercise TGF-β1 or bFGF mRNA levels. Previously, we demonstrated that skeletal muscle VEGF mRNA levels were increased after 1 h of acute exercise, with this increase being intensity and oxygen concentration dependent (6). This report is the first to demonstrate that NO plays a significant role in the exercise-induced increase in VEGF mRNA.

Exercise and VEGF. VEGF is a potent endothelial mitogen for arterial and venular endothelial cells (14). It has been shown that VEGF mRNA increases in skeletal muscle after 1 and 3 days of chronic nerve stimulation and immediately after a single exercise bout (6, 21, 39). Changes in mRNA are not necessarily followed by increases in protein, but VEGF protein is increased almost threefold after 3 days of chronic electrical nerve stimulation (3). Recent work has shown that VEGF mRNA is increased in humans in response to exercise (20, 33). Our data (Figs. 2 and 3) provide additional evidence supporting the hypothesis that VEGF plays an important role in exercise-induced skeletal muscle angiogenesis.

NO, exercise, TGF-β1, and bFGF. As is evident from Figs. 5 and 6, exercise produced at best only modest increases in TGF-β1 and bFGF mRNA, which were unaltered by NOS inhibition. Although NO has been shown to modify bFGF (12) and TGF-β1 (44) gene expression, we believe that the relatively modest changes in bFGF and TGF-β1 mRNA with exercise and NOS inhibition suggest that these growth factors do not play a significant role in the initial stages of exercise-induced angiogenesis. In a previous report our group demonstrated a small, but significant, increase in bFGF mRNA with exercise; in this report we do not (6). One possible explanation for this apparent discrepancy may be related to the delay required for the measurement of MAP before the gastrocnemius samples were removed. Breen et al. (6) demonstrated that the exercise-induced increase in bFGF mRNA returns to baseline within 2 h after the completion of exercise. Perhaps the 40 min required for the measurement of MAP and gastrocnemius removal in this report represented a sufficient amount of time for the bFGF mRNA to return to baseline.

NO and exercise-induced angiogenesis. One theory regarding exercise-induced angiogenesis in skeletal muscle suggests that prolonged imbalances between the perfusion capabilities of the blood vessels and the metabolic requirements of the tissue cells lead to modification of the vasculature to satisfy the tissue needs (see Ref. 1 for review). This theory suggests that decreased oxygenation resulting from this imbalance causes the tissues to become hypoxic and produces a variety of metabolites implicated in vessel growth, including adenosine, ADP, lactate acid, nicotinamide derivatives, and prostaglandins of the E series. The resulting increase in vascularity promotes oxygen deliv-
Fig. 3. Effect of 30 mg/kg L-NAME (A), 300 mg/kg L-NAME (B), or 300 mg/kg d-NAME (C) on the exercise-induced VEGF mRNA response. Saline + rest data were normalized to 1.0. All other data were normalized to saline + rest to allow for comparisons. Results from quantitative densitometry of Northern blots are shown as ratio of VEGF mRNA to 18S rRNA. VEGF was increased 4.2-fold, with this increase attenuated 50% by L-NAME (A and B). d-NAME had no effect on exercise-induced increase in VEGF mRNA (C). Error bars, SE. # Significantly greater than saline + rest, L-NAME + rest, and L-NAME + exercise; *significantly greater than saline + rest and L-NAME + rest (P = 0.05).

Fig. 4. Correlation between saline + rest ratio of VEGF mRNA to 18S rRNA for 30 mg/kg L-NAME and 300 mg/kg D-NAME (A) and for 300 mg/kg L-NAME and 300 mg/kg D-NAME (B), which shows close agreement between these values.

Fig. 5. Representative Northern blots showing mRNA signals for transforming growth factor-β1 (TGF-β1, A) and basic fibroblast growth factor (bFGF, B) with saline or 300 mg/kg L-NAME. Exercise consisted of 1 h at 20 m/min on 10° incline. Normalization with 18S rRNA was used to control for lane loading. Exercise increased TGF-β1 mRNA. L-NAME had no effect on TGF-β1 or bFGF mRNA.
physiological oxygen gradient, regulating mitochondrial respiration, and within the regulation during exercise and is a cellular signal.

Fig. 6. Effect of 300 mg/kg L-NAME on TGF-β1 (A) or bFGF (B) mRNA. Saline + rest data were normalized to 1.0. All other data were normalized to saline + rest to allow for comparisons. Values are from quantitative densitometry of Northern blots for ratio of TGF-β1 or bFGF mRNA to 18S rRNA. TGF-β1 was increased ~1.5-fold by exercise; bFGF was not increased by exercise. L-NAME did not affect TGF-β1 or bFGF mRNA levels. Error bars, SE.*Significantly greater than saline + rest. (P = 0.05).

Fig. 6. Effect of 300 mg/kg L-NAME on TGF-β1 (A) or bFGF (B) mRNA. Saline + rest data were normalized to 1.0. All other data were normalized to saline + rest to allow for comparisons. Values are from quantitative densitometry of Northern blots for ratio of TGF-β1 or bFGF mRNA to 18S rRNA. TGF-β1 was increased ~1.5-fold by exercise; bFGF was not increased by exercise. L-NAME did not affect TGF-β1 or bFGF mRNA levels. Error bars, SE.*Significantly greater than saline + rest. (P = 0.05).

ery to the tissue cells by increasing the capillary-to-fiber surface area interface and increasing maximal blood flow. NO is known to be important in blood flow regulation during exercise and is a cellular signal regulating mitochondrial respiration, and within the physiological oxygen gradient, S-nitrosohemoglobin brings local blood flow into line with oxygen requirements in the brain (22, 37, 40). Given this influence of NO on blood flow and mitochondrial respiration, it appears reasonable that NO may be important for exercise-induced angiogenesis.

NO and VEGF. NO appears to be an important regulator of endothelial cell growth and angiogenesis. One such role for NO is to function in VEGF-induced angiogenesis as a cellular signal stimulated by VEGF mediating the mitogenic effect of VEGF in the coronary venular endothelium (29). In addition, NOS has been shown to lie downstream from VEGF during VEGF-induced angiogenesis (44).

It is now known that hypoxia increases VEGF transcription through hypoxia-inducible factor 1 (HIF-1) (16, 28) and stabilizes VEGF mRNA via the RNA-binding protein HuR (26). We have shown that hypoxic exercise produces a greater increase in VEGF mRNA levels than does exercise alone (6). In addition to HIF-1 regulation, the murine VEGF gene promoter region contains consensus binding sites for transcription factors special protein-1 (SP-1), activator protein-1 (AP-1), and activator protein-2 (AP-2), as well as nuclear factor-κB (NF-κB) (38). NO has been shown to inhibit VEGF upregulation through inhibition of HIF-1 in aortic smooth muscle and pulmonary artery cells or AP-1 in aortic smooth muscle cells (27, 41). In contrast, NO increases VEGF mRNA via guanylate cyclase activity in human A-172 glioblastoma cells and human Hep G2 hepatocellular carcinoma cells and increases VEGF mRNA stability in these same cells (8). The mechanism of action for NO includes stimulation of soluble guanylate cyclase, which converts GTP to the intracellular second-messenger cGMP, consistent with the reported mechanism for AP-1 (30).

Recently, Benoit et al. (5) demonstrated that nitroprusside, a direct NO donor, and ACh, an NO donor via endothelial NOS, increased VEGF mRNA in resting skeletal muscle. Our findings from NOS inhibition during exercise are consistent with these findings. Given that NO is important for vasodilation during exercise (22), it would appear that NO should function to increase VEGF levels, rather than inhibit VEGF, as had been demonstrated in aortic smooth muscle and pulmonary artery cells (27, 41). It is well recognized that different tissues can respond differently to the same stimulus, as evidenced by the response to hypoxia: in the lung, hypoxia causes vasoconstriction of the vasculature, whereas in skeletal muscle, hypoxia vasodilates the vasculature. It appears, therefore, that the regulation of VEGF gene expression is different depending on the specific tissue under investigation.

Use of L-NAME and D-NAME. We used D-NAME, the inactive enantiomer of L-NAME, to control for nonspecific effects of L-NAME. The results from the Northern blots demonstrate that the attenuation of VEGF mRNA by L-NAME is not a result of nonspecific effects, as evidenced by no difference in the exercise-induced increase in VEGF mRNA levels between saline and D-NAME (Figs. 2 and 3). However, the increase in blood pressure with D-NAME is not consistent with previous reports in the literature (32). Although it has been shown previously that acute administration of 100 mg/kg iv D-NAME does not affect blood pressure (32), there are no known reports on the effects of acute administration of 300 mg/kg D-NAME. A recent report (4) demonstrated that chronic administration of D-NAME may increase resting blood pressure, suggesting that D-NAME may exhibit some activity similar to L-NAME.

In this report we have demonstrated that two different dosages of L-NAME produce similar results on VEGF gene expression. Figure 4 demonstrates that although the values for the increase in VEGF mRNA with exercise in the saline group are not exactly the same for the L-NAME and D-NAME blots, these values are in close agreement and that differences in these values result from technical issues inherent in compari-
sons of this type. Among the various NOS inhibitors, we selected L-NAME because of its nonspecific inhibition of all the NOS isoforms and because of its universal use as an NOS inhibitor. The majority of other NOS inhibitors, such as aminoguanidine and nitroindazole, which are chemically unrelated to L-NAME, demonstrate only isoform-specific inhibition. Although their use is unwarranted here, these isoform-specific inhibitors will be useful in the future to determine the NOS isoform responsible for the exercise-induced skeletal muscle VEGF gene expression.

Our exercise model provides a unique opportunity to study growth factor regulation resulting from systemic, in vivo exercise. In this model it would be impossible to locally administer NO donors to the exercising skeletal muscle, inasmuch as this would require catheterizing the animal hindquarters, which would significantly impair running performance. It is also not possible to administer an NO donor systemically, inasmuch as this would result in undesirable side effects on blood pressure regulation. In addition, it would not be possible to measure local skeletal muscle NO release via a central catheter because of the short half-life of NO.

On the basis of the work by Breen et al. (6), which demonstrated the largest increase in VEGF, TGF-β1, and bFGF mRNA in animals killed immediately after exercise, we harvested our samples as soon as possible after the measurement of MAP (6). Breen et al. also demonstrated that the increase in VEGF mRNA with exercise, we harvested our samples as soon as possible and bFGF mRNA in animals killed immediately after exercise in the rat gastrocnemius is localized to the subsarcolemmal region of the muscle.

In summary, we have demonstrated that 1 h of acute exercise increases VEGF and TGF-β1 mRNA. We have also demonstrated that NOS inhibition by L-NAME attenuates the exercise-induced increase in VEGF mRNA ~50%, consistent with previous findings that NO donors increase VEGF mRNA in resting skeletal muscle and that NO increases VEGF mRNA half-life. Our data suggest that NO plays an important role in exercise-induced VEGF gene regulation in skeletal muscle.

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REFERENCES