Acute exercise increases nitric oxide synthase activity in skeletal muscle

CHRISTIAN K. ROBERTS, R. JAMES BARNARD, ARNIE JASMAN, AND THOMAS W. BALON

1Department of Physiological Science, University of California, Los Angeles 90024; and 2Department of Diabetes, Gonda Research Center and Beckman Research Institute, City of Hope National Medical Center, Duarte, California 91010-3000

Roberts, Christian K., R. James Barnard, Arnie Jasman, and Thomas W. Balon. Acute exercise increases nitric oxide synthase activity in skeletal muscle. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E390–E394, 1999.—This study examined the effects of acute exercise on skeletal muscle nitric oxide synthase (NOS) activity. Female Sprague-Dawley rats were divided into three groups: control, exercise, and exercise + N\textsuperscript{G}-nitro-L-arginine methyl ester (\textit{L}-NAME). In the exercise + \textit{L}-NAME group, \textit{L}-NAME was administered in the drinking water (1 mg/ml) for 2 days and subsequently the exercise and exercise + \textit{L}-NAME groups underwent a 45-min bout of exhaustive treadmill running after which NOS activity and muscle glycogen were measured. In the control and exercise groups, 1-amino-S-methylisothiourea (AMITU), a selective neuronal NOS inhibitor, with and without additional nonselective NOS blockade (with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA)), was used in vitro to assess the contribution of nNOS to total NOS activity. The exercise bout increased NOS activity by 37% in exercise compared with control groups, and both groups had significantly greater NOS activity compared with exercise + \textit{L}-NAME. AMITU decreased total NOS activity in the control and exercise groups by 31.8 and 30.2%, respectively, and these activities were significantly greater than AMITU + L-NMMA in both control and exercise groups. We conclude that 1) there is basal neuronal NOS and endothelial NOS activity in skeletal muscle, 2) an acute exercise bout increases NOS activity in skeletal muscle, and 3) glycogen depletion during exercise occurs irrespective of NOS activity.

1-amino-S-methylisothiourea; glycogen; muscle contraction

NITRIC OXIDE (NO) is a signaling molecule formed from \textit{L}-arginine by a number of different isoforms of nitric oxide synthase (NOS; Ref. 19). Both the neuronal NOS (nNOS) and the endothelial NOS (eNOS) isoforms are expressed in skeletal muscle (12, 13). nNOS is thought to be localized in the subsarcolemmal region of skeletal muscle and at the neuromuscular junction (12, 14), whereas eNOS is uniformly distributed in muscle fibers (13) as well as being located in the vessel wall. In addition, it has been demonstrated that both fast- and slow-twitch skeletal muscle can express both nNOS and eNOS protein (12, 13, 26). The quantity of the nNOS isoform appears to be related to the fiber-type distribution of a particular muscle, i.e., higher nNOS levels are associated with fast-twitch-glycolytic muscles (12). On the other hand, eNOS levels are associated with more oxidative muscles (13).

Balon and Nadler (3) were the first to demonstrate an increase in NO release from muscles exposed to prior electrical stimulation. Prior exercise increases NO metabolite production (11), and NO concentrations in expired air increase during exercise (17); however, the sources of the NO production have not been investigated. Additionally, it has been demonstrated that chronic electrical stimulation and in vitro muscle loading increase nNOS protein in skeletal muscle (21, 26). Furthermore, we recently (22) demonstrated that exercise-stimulated glucose transport is NO dependent in vivo, and this has been shown in vitro as well, as reviewed by Balon (1).

Consequently, we hypothesized that an acute exercise session would increase total skeletal muscle NOS activity. Furthermore, we attempted to determine which isoforms of NOS are activated in skeletal muscle during exercise, utilizing a specific nNOS inhibitor, 1-amino-S-methylisothiourea (AMITU), in vitro. Finally, it has been suggested that the glycogen status of skeletal muscle may also be a signal for induction of glucose transport during exercise. Consequently, we hypothesized that glycogen depletion would be associated with increased NOS activity.

METHODS

Animals and design. All protocols were conducted in accordance with the University of California, Los Angeles, Animal Research Committee. Female Sprague-Dawley rats weighing 175–200 g were purchased from Harlan Sprague Dawley (San Diego, CA). Standard rat chow (Purina) and water were provided ad libitum to the rats with the animals on a 12:12-h light-dark cycle starting at 0700. To determine the role of exercise on NOS activity, animals were assigned to one of three groups, consisting of 8 rats/group: control, exercise, exercise + N\textsuperscript{G}-nitro-L-arginine methyl ester (\textit{L}-NAME). The exercise + \textit{L}-NAME treated group was administered \textit{L}-NAME...
Exercise and Nitric Oxide Synthase Activity

Acute exercise session. The exercise protocol used was the same as in our previous study (22). After acclimatization to the motor-driven treadmill (Quinton Instruments, Seattle, WA) for 2 days, consisting of 15 min at 1.3 km/h, the exercise and exercise + L-NAME groups underwent a 45-min exhaustive bout of acute exercise. The protocol consisted of a 15-min run at 1.3 km/h up a 15% grade, followed by 15 min at 1.6 km/h up a 20% grade, followed by 15 min at 1.9 km/h up a 25% grade, or until exhaustion, whichever came first. On completion of the run, the animals were immediately killed by cervical dislocation, and the gastrocnemius muscles were removed, immediately placed in liquid nitrogen, and stored at −70°C until analyses were performed.

Muscle glycogen determination. Muscle glycogen content in gastrocnemius muscles was determined by the anthrone method as previously described (16). Muscle samples were cut into pieces weighing 90–200 mg. Solution (0.5 ml of 30% KOH) was added to each sample and placed into a boiling water bath for 15–20 min. Once the tissue was in solution, samples were cooled on ice for 30 min followed by addition of 1.1–1.2 vol of 95% ethanol to precipitate the glycogen and were centrifuged at 800 g for 30 min. The supernatants were aspirated, and glycogen precipitates were redissolved in 3.0 ml of distilled water. One milliliter of the above solution was added to 1 ml of 5% phenol, and 5 ml of 96–98% sulfuric acid were added rapidly to the surface of the liquid. Samples were incubated at 30°C for 15–20 min, and then the absorbance was read on a Beckman DU-40 spectrophotometer at 490 nm.

NOS activity. Total NOS activity of all groups was assessed by measuring the conversion of [3H]arginine to [3H]citrulline, similar to the protocol previously described by Kobzik et al. (12). Frozen gastrocnemius muscles were homogenized in 10 vol of homogenization buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The homogenate was centrifuged at 4°C for 15 min at 20,000 g. The pellet was resuspended in one-half of the original volume of homogenization buffer. Supernatant and pellet homogenates (25 µl) were added to 25 µl of 100 nM [3H]arginine (50 Ci mmol−1) and 100 µl of reaction buffer containing 50 mM HEPES (pH 7.4), 1 mM NADPH, 1 mM EDTA, and 1.25 mM CaCl₂. After incubation for 15 min at 22°C, assays were stopped with 4 ml of termination buffer containing 20 mM HEPES (pH 5.5) and 2 mM EDTA and were placed on ice. Samples were applied to 10-ml columns of Dowex AG50WX-8 (Na+ form) that were eluted with an additional 4 ml of termination buffer. [3H]citrulline was quantified by liquid scintillation spectroscopy of the 8-ml flow through.

AMITU studies. AMITU (Calbiochem, San Diego CA) is a selective, noncompetitive, irreversible inhibitor of nNOS at low concentration (28). The dose required to inhibit purified nNOS 50% (IC50) is 3 µM and eNOS is 103 µM. A dose-response curve was formulated for the homogenized rat brain to assess the IC50 in biological suspension. In the control and the exercise groups, AMITU was used at a 50% effective dose (AMITU50) in vitro, with and without the addition of nonspecific NOS blocker with Nω-N-monomethyl-L-arginine (L-NMMA) (AMITU50 + L-NMMA) at a concentration of 100 µM to assess the contribution of eNOS and nNOS to the total NOS activity in the particulate fraction.

Statistics. Muscle glycogen and NOS activity of all groups were compared with a one-way ANOVA. When significant F values were noted, post hoc analyses were performed with a Tukey-Kramer multiple comparison test. Differences were considered statistically significant at P < 0.05. Values reported are means ± SE.

RESULTS

Running times. As in our previous study (22), L-NAME had no apparent adverse effects on the rats at a dose effective at inhibiting NO synthesis except that in the present study, animals in the exercise group ran longer than the exercise + L-NAME group (47.7 ± 1.3 vs. 40.6 ± 1.2 min, respectively, P < 0.05).

NOS activity. The total muscle NOS activity (supernatant + particulate fractions) was significantly elevated in the exercise group compared with control values, increasing from 0.286 ± 0.01 to 0.391 ± 0.01 pmol/mg protein (P < 0.01) with exercise. L-NAME administration decreased NOS activity to 0.008 ± 0.01 pmol/mg protein (P < 0.0001 vs. control and exercise groups; Fig. 1). In agreement with Reiser et al. (21), the majority of the NOS activity (∼90%) was in the particulate fraction for both the control and exercise groups. Thus only the particulate fraction was assayed in the subsequent experiments. A dose-response curve was formulated with AMITU in brain homogenate (Fig. 2). The results showed 50% inhibition at 20 µM AMITU. AMITU50 decreased total NOS activity in the control and exercise groups by 31.8% (P < 0.05) and 30.2% (P < 0.004), respectively, and these remaining activities...
were significantly greater than AMITU\textsubscript{50} + l-NMMA in both control (90.9% inhibition, $P < 0.001$ vs. AMITU\textsubscript{50}) and exercise groups (93.5% inhibition, $P < 0.001$, Fig. 3).

Muscle glycogen. The muscle glycogen concentration of the control group was $4.79 \pm 0.46$ mg glycogen/g wet wt of tissue. The acute exercise bout significantly reduced muscle glycogen to $2.36 \pm 0.22$ mg glycogen/g wet wt, $P < 0.0003$. The glycogen level of the exercise + l-NAME group was also significantly reduced postexercise compared with control ($2.86 \pm 0.24$ mg glycogen/g wet wt; $P < 0.003$). Although the exercise group exercised longer than the exercise + l-NAME group, there was no significant difference between the exercise and exercise + l-NAME groups in postexercise glycogen content (Fig. 4).

DISCUSSION

In a previous study (22), we found that blocking NOS with l-NAME prevented the translocation of GLUT-4 to the sarcolemma of skeletal muscle and the increase in glucose transport normally seen with acute exercise. The main hypothesis of this study was that total skeletal muscle NOS activity would increase as a result of an acute exercise bout. The data reported here are the first evidence of increased NOS activity in skeletal muscle during acute exercise. In agreement with our hypothesis, total NOS activity increased by $\sim 40\%$ with exercise. Balon and Nadler (3) reported that electrical stimulation increased total nitrosylated compound efflux from incubated extensor digitorum longus muscle. More recently, it has been demonstrated that in vivo electrical stimulation and in vitro muscle loading increase NOS activity in skeletal muscle (21, 26). However, electrical stimulation is not identical to true physiological muscle activity (5). Others have shown that exercise may increase NOS activity as demonstrated by the indirect indexes, urinary nitrate and cGMP production (4, 11), and increased NO concentrations in expired air during physical activity (17). The present data also demonstrate that resting skeletal muscle has significant NOS activity, which agrees with previous studies (12). Furthermore, the NOS activity both at rest and during exercise was nearly completely eliminated by NOS inhibition with l-NAME.

Studies have previously investigated the regulation of constitutive NOS isoforms in skeletal muscle. Reiser et al. (21) reported that chronic electrical stimulation (3 wk) increased total muscle NOS activity and that this correlated with an increase in nNOS protein. Tidball et al. (26) have demonstrated increases in NOS activity after 2 min of stimulation at a physiological frequency of 2 Hz and also reported that passive stretch of C3C12 cultured myotubes for 2 min increased NO release.

In an attempt to determine which NOS isoform(s) activity was increased with treadmill exercise, we used the specific nNOS inhibitor AMITU while assaying NOS activity. This isothiourea compound has been reported to give 50% inhibition of purified nNOS at 3 $\mu$M and 50% inhibition of eNOS at 103 $\mu$M (28). Because the inhibitor had not been used in tissue homogenates, we did a pilot dose-response curve with rat brain homogenate, a tissue in which most all of the constitutive NOS is nNOS, and found that 50% inhibition was achieved with 20 mM AMITU (Fig. 2). When we used this AMITU\textsubscript{50} with the control muscle samples, basal NOS activity was reduced by 32%. If we extrapolate and assume that $\sim 65\%$ of the activity is attributable to nNOS, the remainder is attributable to the eNOS isoform. It is possible that the activity of each of the isoforms is different in brain and muscle; however, we cannot test this because no purely specific inhibitors are available at the present. These results indicate that there is a significant contribution from both calcium-calmodulin-dependent isoforms of NOS, nNOS and eNOS, in fast-twitch skeletal muscle and that nNOS appears to be the more active isoform at rest, which agrees with the histochemical data of Kobzik et al. (12, 13). Furthermore, the results indicate that nNOS and eNOS appear to increase with exercise and that these isoforms are, in fact, not only constitutively expressed in skeletal muscle, but their activity is inducible when the metabolic demand of the muscle increases. However, from our data, we cannot deduce whether the
inducible activity was due to more protein or an increase in the activity of the existing protein. After treadmill exercise, total NOS activity was increased by ~40%. When AMITU was added to the assay medium, NOS activity was reduced by 30%. Although NOS activity was reduced by AMITU in the exercise samples, it is not possible to definitively state that the increase in measured total activity was due primarily to an increase in nNOS activity. However, we speculate this is the case based on the reports of Reiser et al. (21) and Tidball et al. (26) showing increased nNOS protein with chronic electrical stimulation and in vivo reloading, respectively. Furthermore, increasing the AMITU concentration in our assay would have resulted in some inhibition of eNOS.

Although vascular eNOS activity may be elevated during exercise due to shear stress (24), the vascular contribution to the total muscle homogenate is very small. Muscle itself expresses a significant amount of eNOS protein (13). In addition, from our homogenate measurements, we cannot assess the contribution of neuromuscular junction nNOS to the total NOS activity (14). Macrophage NOS (or inducible NOS) did not contribute to the total NOS activity, as this isoform is not expressed in skeletal muscle under normal conditions (15, 25). Furthermore, if macrophage NOS were present in the blood cells in the muscle vasculature, it would be lost in the muscle homogenization preparations and therefore would not contribute to the total NOS activity.

One possible explanation for the increase in muscle NOS activity with exercise is the result of increased calcium content in the muscle fibers during contraction. As the muscle is activated, calcium is released from the sarcoplasmic reticulum into the cytosol of the muscle fibers, which could activate NOS. Consistent with this is the observation that both constitutively expressed isoforms of NOS (eNOS and nNOS) require calcium as a cofactor for activation (19), and there is also evidence that extracellular calcium may also enhance NOS activation during increased loading (26). Thus, as calcium enters the sarcoplasm, not only does it induce muscle contraction, but it also activates calcium-dependent, constitutively expressed isoforms of NOS.

Potential functions of increased NO production during physical activity include increased glucose transport (2, 22), optimal force production, shortening velocity and power production (20), alteration of oxidative metabolism (13), and increase in blood flow to skeletal muscle (10, 18). With regard to the latter function, because skeletal muscle has a relatively high NOS activity in comparison with other tissues (23), and when tissue mass is taken into consideration, skeletal muscle produces the greatest amount of NO. As a result, NO may be an autacoid that elicits vascular smooth muscle relaxation during exercise to increase perfusion via a paracrine mechanism.

Another goal of this study was to assess if an increase in NOS activity with acute exercise is associated in any way with glycogen depletion. It has been previously reported that NO plays a role in contraction-stimulated glucose transport in skeletal muscle (2, 22). In addition, it has been noted that the glycogen status of skeletal muscle may be a signal for the increase in glucose transport seen during and immediately after exercise (6). The exercise bout reduced muscle glycogen in both exercise and exercise + L-NAME groups to the same extent, suggesting that there is no requirement for NOS activity in order for glycogen to be depleted. Our results further indicate that glycogen depletion is not the only factor that regulates glucose transport during exercise, as our previous study demonstrated complete inhibition of GLUT-4 recruitment to the plasma membrane in the presence of L-NAME (22). Furthermore, if increases in glucose transport during exercise are associated with both muscle glycogen reduction and NO, NOS is activated downstream of the breakdown of glycogen in the signaling mechanism. This NO production, occurring either downstream or independent of glycogen depletion, subsequently causes GLUT-4 translocation. Interestingly, Balon and Nadler (3) also demonstrated that electrical stimulation, which elicited ~50% glycogen depletion, was associated with an ~200% increase in NO release.

Prior studies indicate that glycogen depletion with high intensity exercise (of similar intensity to that employed in the present study) occurs in all gastrocnemius fiber types (29, 30). The ability to vasodilate the skeletal muscle vasculature is reduced in the presence of a NOS inhibitor (10, 18). However, the decrease in blood flow with NO-inhibitor administration during exercise is similar to the decrease seen in resting flow (8, 9, 27). Nevertheless, with vasoconstriction, this would suggest an increase in the hypoxic state of the muscle and therefore further depletion of muscle glycogen. However, this did not occur, as the muscle glycogen concentration was not lower in the exercise + L-NAME group. Other mechanisms are also thought to be important for exercise-induced vasodilation of the muscle vasculature (7), and furthermore oxygen extraction would increase in the presence of vasoconstriction. Although the exercise group ran slightly longer, there was no difference in glycogen depletion between the exercise and the exercise + L-NAME groups.

Overall, NO appears to be a positive regulator of muscle function. NO production increases during exercise and may be involved in the increases in exercise-induced glucose transport and skeletal muscle blood flow and force production, all of which suggest that NO is a key mediator of exercise metabolism. Confirmation of which isoforms of NOS are involved in skeletal muscle exercise metabolism requires further study.

We thank Steven S. Dilauro for help with the glycogen determination.

This study was supported by National Institute on Aging Grant AG-07592 (R. J. Barnard), a grant from the L-B Research/Education Foundation (R. J. Barnard) and the American Diabetes Association (T. W. Balon).

Address for reprint requests and other correspondence: R. J. Barnard, Dept. of Physiological Science, UCLA, PO Box 951527, Los Angeles, CA 90095-1527 (E-mail: jbarnard@physc.ucla.edu).

Received 11 March 1999; accepted in final form 29 April 1999.
REFERENCES