Regulation of nitric oxide production in response to skeletal muscle activation

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Fujii, Yoshitaka, Yang Guo, and Sabah N. A. Hussain. Regulation of nitric oxide production in response to skeletal muscle activation. J. Appl. Physiol. 85(6): 2330–2336, 1998.—Nitric oxide (NO) is synthesized in normal muscle fibers by the neuronal (nNOS) and the endothelial (ecNOS) isoforms of nitric oxide synthase (NOS). NO contributes to the regulation of several processes such as excitation-contraction coupling and mitochondrial respiration. We assessed in this study whether NO production is regulated in response to an acute increase in muscle activation. Three groups of anesthetized, tracheostomized, spontaneously breathing rats were examined after an experimental period of 3 h. Group 1 served as a control (no loading), whereas groups 2 and 3 were exposed to moderate and severe inspiratory resistive loads, respectively, which elicited peak pressures of 50 and 70% of maximum, respectively. Ventilatory (diaphragm, intercostal, and transverse abdominis) and limb (gastrocnemius) muscles were excised at the end of the experimental period and examined for NOS activity and NOS protein expression. Neither sub-maximal nor maximum tracheal pressures were altered after 3 h of resistive loading. Diaphragmatic and intercostal muscle NOS activities declined significantly in response to moderate and severe loading, whereas those of transverse abdominis and gastrocnemius muscles remained unchanged. On the other hand, resistive loading had no significant effect on ventilatory and limb muscle NOS isoform expression. We propose that a contraction-induced decline in muscle NOS activity represents a compensatory mechanism through which muscle contractility and mitochondrial function are protected from the inhibitory influence of NO.

nitric oxide; diaphragm; fatigue; contraction

Nitric oxide (NO), a multifunctional second messenger with numerous biological functions, is synthesized from L-arginine by a group of hemoproteins known as nitric oxide synthases (NOS). So far, only three genes are known to code for three NOS isoforms. The neuronal (nNOS) and endothelial (ecNOS) isoforms, which were first identified in neuronal and endothelial cells, respectively, have widespread tissue distribution (20). The third NOS isoform, also known as the inducible isoform (iNOS), is expressed in numerous cell types in response to bacterial endotoxin and inflammatory cytokines such as tumor necrosis factor, interleukins, and interferon-γ (20).

Recent studies suggest that normal skeletal muscle fibers express nNOS, which is localized beneath the sarcolemma of fast-twitch fibers (21) and associates with the dystrophin complex through a direct interaction with α-syntrophin (7). In addition to the sarcolemma, nNOS expression is enriched at the muscular endplate as well (23). Muscle fibers are also reported to express the ecNOS isoform, which has a cytoplasmic distribution and is localized mainly in muscle fibers rich in succinate dehydrogenase (22). There has been recent evidence indicating abundant iNOS expression in skeletal muscle fibers and cultured myocytes in response to bacterial endotoxin and inflammatory cytokines (4, 13, 31, 35).

Although the exact functional significance of any of the NOS isoforms in regulating skeletal muscle function remains under investigation, recent evidence points to an important role for NO in regulating muscle contractile force. This regulation is believed to be mediated through cGMP-dependent and cGMP-independent processes that involve Ca2+ release by ryanodine receptors and inhibition of contractile proteins (24, 26). In addition, NO contributes to the regulation of mitochondrial respiration in skeletal muscles by inhibiting cytochrome-c oxidase, a process that is likely to decrease cellular ATP and increase ADP, AMP, GDP, and Pi. Furthermore, NO also modulates skeletal muscle glucose uptake (2) and creatine kinase activity (36).

Initial studies suggested that the rate of NO production by constitutive NOS isoforms (nNOS and ecNOS) is regulated through changes in intracellular Ca2+ (20). More recent reports indicate, however, that NO production may also be influenced by alterations in ecNOS and nNOS gene expression, such as those noticed during development and in sex hormones (11, 34).

Although the contribution of ecNOS activity to the regulation of skeletal muscle blood flow has been extensively addressed, little is known about changes in skeletal muscle NO production in response to alterations in muscle activity. Reiser and colleagues (28) reported recently that chronic stimulation of limb muscles in rats was accompanied by a significant upregulation of muscle NOS activity and nNOS gene expression. These results indicate that NO production undergoes significant changes in response to increased muscle activity. Whether muscle NO production undergoes similar regulation in response to acute exercise remains unknown. The main purpose of this study was to evaluate the influence of increased muscle activity, of a relatively short duration (3 h), on ventilatory muscle NOS activity and on expression of nNOS and ecNOS isoforms.

METHODS

Animal preparation. The procedures used were approved by the Animal Research Committee of McGill University.
Twenty-four pathogen-free, male Sprague-Dawley rats (250–300 g) were studied 1 wk after arrival. All animals were lightly anesthetized with pentobarbital sodium (30 mg/kg) and were given supplemental doses (10 mg/kg) as needed (on the basis of the absence or presence of corneal reflexes). The animals were tracheostomized with polyethylene tubing (ID 2.2 mm) (Intamedic; Clay Adams), which was sutured firmly in place with a silk tie. The cannula was connected to a two-way nonrebreathing valve (model 2300, Hans Rudolph, Kansas City, MO). Tracheal pressure was measured with a differential transducer (Validyne, Northridge, CA), which was connected to the tracheal cannula via a side port. The inspiratory line was connected to a 5-liter bag, which was continuously filled with 70% O2-balance N2. Preliminary experiments indicated that this gas composition prevented the development of severe hypoxemia during moderate and severe resistive loading. An arterial catheter (22 gauge) was placed into the internal carotid artery and used for measuring blood pressure (Trantec model 60–800, American Edwards Laboratories, Santa Ana, CA) and for the sampling of arterial blood. Arterial blood gases were analyzed by means of an automatic blood-gas analyzer (AVL model 995, Instrumentation Laboratories, Lexington, MA). A catheter, placed into the jugular vein, was used to access the venous circulation. All animals were monitored for 3 h during the experimental protocol and then euthanized with an overdose of pentobarbital sodium. The chest was then opened, and the diaphragm and intercostal and transverse abdominis muscles were quickly excised and frozen in liquid nitrogen. In addition, the gastrocnemius muscle was also excised and frozen.

L-citrulline assay. Frozen muscle tissues from each animal were homogenized in six volumes (wt/vol) of homogenization buffer (10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 0.32 mM sucrose, 1 mg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 µg/ml pepstatin A, pH 7.4). The crude homogenates were centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant (50 µl) was added to 10 ml prewarmed (37°C) tubes, each containing 100 µl of reaction buffer of the following composition: (in mM) 50 KH2PO4, 60 valine, 1.5 NADPH, 1.2 MgCl2, and 2 CaCl2, as well as 1 mg/ml bovine serum albumin, 1 µg/ml calmodulin, 10 µM tetrahydrobiopterin (BH4), and 25 µl of 120 µM stock L-[2,3-3H]arginine (150–200 counts·min−1·pmol−1). The samples were incubated for 30 min at 37°C, and the reaction was terminated by the addition of 500 µl of cold (4°C) stop buffer (100 mM HEPES, 12 mM EDTA, pH 5.5). To obtain free L-[3H]citrulline for the determination of enzyme activity, 2 ml of Dowex 50w resin (8% cross-linked, Na+) form) were added to eliminate excess L-[2,3-3H]arginine. The supernatant was removed and examined for the presence of L-[3H]citrulline by liquid scintillation counting. Enzyme activity was expressed in picomoles of L-citrulline produced per milligram protein per minute. Protein was measured by the Bradford technique with bovine serum albumin as the standard. NOS activity was also measured in the presence of 1 mM of Nω-nitro-l-arginine methyl ester (NOS inhibitor). Total specific muscle NOS activity was calculated as the difference between that measured in the presence and absence of Nω-nitro-l-arginine methyl ester.

Immunoblotting. Crude muscle homogenate proteins (100 µg) from four animals in each group were heated for 15 min at 90°C and then loaded on gradient (4–12%) sodium dodecyl sulfate-Tris glycine polyacrylamide gels. The proteins were separated, transferred electrophoretically onto polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and subsequently incubated overnight at 4°C with primary monoclonal anti-eNOS and anti-nNOS antibodies (1:500 for each antibody, Transduction Laboratories, Lexington, KY). Lysates of human endothelial cells and pituitary cells were used as positive controls for eNOS and nNOS proteins, respectively (provided by Transduction Laboratories). Specific proteins were detected by using horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies and enhanced chemiluminescence reagents (Amersham). The blots were scanned with an imaging densitometer (model GS700, Bio-Rad, 12-bit precision and 42-µm resolution), and optical densities of the protein bands were quantified with SigmaGel software (Jandel Scientific, San Rafael, CA). Predetermined molecular weight standards (Novex) were used as markers.

Experimental protocol. At the end of the surgical procedure, a 30-min stabilization period was allowed. The animals were then randomly assigned to one of three groups. Group 1 (n = 8) was designated as control (no loading), and animals in that group were allowed to breath spontaneously for a period of 3 h. Group 2 (moderate load, n = 8) and group 3 (severe load, n = 8) animals were exposed to moderate and severe inspiratory resistance loading. In each animal, maximum tracheal pressure was measured before loading by occluding the inspiratory port of the Hans Rudolph valve for a period of 30 s. The degree of resistance in groups 2 and 3 was set initially to result in the generation of tracheal pressures of ~30 and 70% of maximum tracheal pressure, respectively. At the end of 3 h of loading, the inspiratory resistance was removed and maximum tracheal pressure was remeasured. The animals were then euthanized, and muscles were quickly excised and frozen in liquid nitrogen.

Statistical analysis. All values are expressed as means ± SE. Comparisons between groups and within a group (initial vs. end) were carried out by using a two-way ANOVA and Scheffé’s test for multiple comparison. Student’s t-test was used for comparison between two groups. P < 0.05 was regarded as a statistically significant difference.

RESULTS

Table 1 lists the changes in arterial pressure and arterial blood gases in the three groups of animals. Mean arterial pressure values measured initially were not different among the three groups. Moreover, load-

![Table 1. Changes in mean arterial pressure and arterial blood gases in the 3 groups of animals](http://jap.physiology.org/content/33/4/A2731.full.pdf)
ing elicited no significant decline in mean arterial pressure (Table 1). Similarly, initial arterial Po2, Pco2, and pH were not different among the three groups. In groups 1 and 2, arterial blood gases at the end of a 3-h experimental period remained similar to the initial values. Severe resistive loading, on the other hand, elicited a significant decline in arterial pH and a rise in arterial Pco2 (Table 1).

Figure 1 shows the changes in tracheal pressure generated in response to moderate and severe inspiratory loads. Through the resistive loading period (3 h), the tracheal pressure generated in response to moderate and severe loads remained similar to the initial values (measured within a few minutes of the load application). Thus there was no significant difference between the initial and the end values of tracheal pressure measured in groups 2 and 3 (Fig. 1). Moreover, 3 h of resistive loading in these two groups of animals elicited no significant decline in maximum tracheal pressure (measured in response to complete occlusion of the airway) (Fig. 2).

Figure 3 illustrates the changes in muscle NOS activity measured at the end of a 3-h experimental period in the three groups of animals. Moderate resistive loading elicited an ~50% decline in diaphragmatic and intercostal NOS activity (P < 0.05 compared with control values). A similar decline in diaphragmatic and intercostal muscle NOS activities was observed in response to severe loading (Fig. 3). By comparison, NOS activities of the transverse abdominis and gastrocnemius muscles in groups 2 and 3 remained similar to those measured in the control animals.

Immunoblotting of muscle protein with selective anti-nNOS and anti-ecNOS antibodies revealed that the expression of nNOS and ecNOS in various ventilatory and limb muscles was not altered in response to moderate and severe resistive loading. Figure 4 shows immunoblotting of diaphragmatic tissues samples obtained from the three groups of animals. In control diaphragm samples, anti-nNOS and anti-ecNOS antibodies detected 165- and 130-kDa protein bands, respectively. The intensity of these bands was not altered in response to moderate and severe loading (Fig. 4). Similar findings were observed when intercostal, transverse abdominis, and gastrocnemius muscle samples were probed with these antibodies (Table 2).
on the rate of NO production. To achieve this goal, we applied moderate and severe inspiratory loads that significantly elevated the pressure generation by the inspiratory muscles without eliciting fatigue in these muscles (as indicated by the preservation of maximum tracheal pressure). A similar protocol has recently been used to assess the influence of resistive loading on the generation of oxygen-reactive species in the diaphragm (5). Unlike artificial nerve or muscle stimulation, in which nonphysiological paradigms are delivered via electrodes implanted around the nerve fibers or muscle tissues, resistive loading provides a physiological means for increasing ventilatory muscle metabolic demands. The major drawback of using resistive loading is the difficulty in assessing the contribution of various inspiratory muscles to the generation of negative intrathoracic pressure. It is safe to assume, however, that the diaphragm and intercostal muscles were the main ventilatory muscles recruited during resistive breathing.

It should be emphasized that maximum tracheal pressure values measured in our experiments are lower than those reported by other investigators (1, 5, 8, 25). In addition to inspiratory muscle strength, maximum tracheal pressure measurements in anesthetized, supine animals are influenced by several factors, among which are the type and depth of anesthesia, arterial blood gases, lung volume, age, and body weight. We speculate that deeper levels of anesthesia, but most importantly, the use of relatively younger and lighter rats, in our study compared with other studies (1, 5, 8, 25) may have contributed to the generation of lower maximum tracheal pressure values.

It has been established in the past few years that NO generation in normal skeletal muscle fibers originates mainly from the activity of the nNOS isoform. Indeed, several studies have documented that the nNOS isoform is expressed at the junctional and extrajunctional sarcolemma and associates with the dystrophin complex by interacting directly with α-syntrophin (6). The close association of nNOS with the dystrophin complex is believed to be the main mechanism behind the absence of nNOS expression in muscle fibers of human patients with Duchenne muscular dystrophy (6). In certain species, such as rats, there appears to be fiber-type-specific distribution of nNOS expression, with abundant nNOS expression being localized in type II fibers (21). On the other hand, more homogenous nNOS expression has been identified in human skeletal muscles fibers (12). In addition to the nNOS isoform, Kobzik et al. (22) reported the presence of the ecNOS isoform in rat skeletal muscle fibers that are rich in succinate dehydrogenase. Our group (16) and others (12), however, failed to detect ecNOS expression in human, canine, or rabbit skeletal muscles, suggesting that ecNOS expression may be limited to rat and murine skeletal muscle fibers.

In the endothelial cells, constitutive NO production in response to agonist stimulation is regulated through enhanced intracellular Ca2+ concentration (20). A similar mechanism has been shown to exist in cardiac

DISCUSSION

The main finding of this study is that, after 3 h of moderate and severe inspiratory resistive loading, diaphragmatic and intercostal muscle NOS activity declined significantly, whereas NOS activities of transverse abdominis and gastrocnemius muscles were not changed. The decline in diaphragmatic and intercostal NOS activities was not due to changes in muscle NOS protein expression.

The principal purpose of our study was to evaluate the influence of an acute increase in muscle activation on the rate of NO production. To achieve this goal, we applied moderate and severe inspiratory loads that significantly elevated the pressure generation by the inspiratory muscles without eliciting fatigue in these muscles (as indicated by the preservation of maximum tracheal pressure). A similar protocol has recently been used to assess the influence of resistive loading on the generation of oxygen-reactive species in the diaphragm (5). Unlike artificial nerve or muscle stimulation, in which nonphysiological paradigms are delivered via electrodes implanted around the nerve fibers or muscle tissues, resistive loading provides a physiological means for increasing ventilatory muscle metabolic demands. The major drawback of using resistive loading is the difficulty in assessing the contribution of various inspiratory muscles to the generation of negative intrathoracic pressure. It is safe to assume, however, that the diaphragm and intercostal muscles were the main ventilatory muscles recruited during resistive breathing.

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Table 2. Changes in optical densities of nNOS and ecNOS protein expressions in various muscles

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Moderate Load</th>
<th>Severe Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS isoform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>1.206±4</td>
<td>1.230±35</td>
<td>1.243±37</td>
</tr>
<tr>
<td>Intercostals</td>
<td>1.948±4</td>
<td>2.015±56</td>
<td>2.355±62</td>
</tr>
<tr>
<td>Transverse abdominis</td>
<td>1.352±29</td>
<td>1.361±36</td>
<td>1.374±39</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>2.496±106</td>
<td>2.655±110</td>
<td>2.661±123</td>
</tr>
<tr>
<td>ecNOS isoform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>138±12</td>
<td>134±14</td>
<td>142±9</td>
</tr>
<tr>
<td>Intercostals</td>
<td>123±8</td>
<td>134±11</td>
<td>136±14</td>
</tr>
<tr>
<td>Transverse abdominis</td>
<td>145±15</td>
<td>139±19</td>
<td>155±21</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>160±21</td>
<td>148±17</td>
<td>151±13</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 different animals, expressed in arbitrary units. nNOS and ecNOS, neuronal and endothelial nitric oxide synthase (NOS), respectively. Moderate and severe loading resulted in no significant changes in ecNOS and nNOS protein expression of various muscles.
myocytes, where ecNOS activity rises in parallel with the increase in Ca^{2+} levels elicited by pacing with a uniform electric field (18). In addition to regulation of NOS activity by intracellular concentration of Ca^{2+}, there is evidence that both ecNOS and nNOS expressions are transcriptionally regulated under certain conditions. For instance, we have reported recently that subacute hypoxia in conscious rats elicited significant upregulation of cerebellar nNOS expression (15). Interestingly, hypoxia provoked the opposite effect on cerebellar ecNOS gene expression (15). More importantly, we found that diaphragmatic NOS activity during postnatal development is strongly influenced by alterations in muscle ecNOS and nNOS gene expression (11). Transcriptional control of skeletal muscle constitutive NOS expression has also been described in response to systemic endotoxemia in rats (10). These findings strongly support the notion that skeletal muscle NOS activity is controlled at multiple levels, including gene transcription.

Many investigators have studied the influence of exercise on NO synthesis and release in the vascular system. Chronic exercise in dogs (running for 1 h, twice a day, for 10 days) enhances NO release from isolated coronary arteries and upregulates aortic ecNOS mRNA levels (30). Similarly, 4 wk of cycle training in humans elicited a significant rise in forearm NO release (19). Little is known regarding the influence of exercise on muscle NO production. Reiser et al. (28) reported recently that chronic electrical stimulation (3 wk) of rat tibialis anterior and extensor digitorium longus muscles elicits a significant induction in nNOS expression and doubles NOS activity in these muscles. These data provide the first evidence that muscle nNOS expression can be modulated by muscle activity. Our results, on the other hand, indicate that a relatively short period of increased activity (3 h of resistive loading) had no significant effect on the expression of constitutive NOS isoforms. We found, however, that NOS activity of the diaphragm and intercostal muscles declined significantly after 3 h of moderate or severe inspiratory resistive loading. The precise mechanisms involved in this response remain unclear. We propose the following pathways through which muscle NOS activity may be reduced during acute exercise. First, a significant reduction in cofactor availability, especially that of BH_{4}, in response to increased muscle activity is likely to result in reduced muscle NOS activity. Availability of BH_{4} has been demonstrated to be a major factor in determining NOS activity under normal conditions (20) and in response to inflammatory cytokine exposure (29). Little is known about the synthesis of BH_{4} in skeletal muscle cells. We recently reported, however, that GTP cyclohydrolase I (the rate-limiting enzyme in BH_{4} synthesis) is upregulated, along with iNOS induction, in ventilatory and limb muscles of endotoxemic rats (16). Whether increased muscle activity alters the expression or the activity of GTP cyclohydrolase I remains to be investigated. Second, alterations in the levels of endogenous NOS inhibitors in exercising muscles may lead to a reduction in muscle NOS activity. These inhibitors include N^{G},N^{G}-dimethylarginine and N^{G}-N^{G}-dimethyl-arginine, which are known to be synthesized and released by the endothelial cells (32). Another endogenous inhibitor is the newly discovered PIN (protein inhibitor of nNOS) (17). This small protein (89 amino acids) is known to be widely expressed and to inhibit, selectively, NOS activity by preventing the dimerization of nNOS monomers (17). We recently identified abundant PIN expression in various skeletal muscles, including the diaphragm (14). Our results also indicate that PIN is localized at the sarcolemma of skeletal muscle fibers and is transcriptionally controlled during skeletal muscle development (14). Little is known about the changes in PIN expression in various pathophysiological conditions. It is possible, however, that an increase in muscle activity may result in increased PIN expression, leading to reduction in muscle NOS activity without any significant changes in nNOS or ecNOS protein expression. Finally, there is recent evidence indicating that in normal skeletal muscles, caveolin-3 (a membrane-bound protein) interacts selectively with nNOS and potently inhibits nNOS activity (33). The functional significance of this interaction between nNOS and caveolin-3 in determining muscle NOS activity under resting conditions and in response to increased muscle activity remains to be assessed. Third, we speculate that the most important cause of decreased muscle NOS activity in exercising muscles is the formation of peroxynitrite. This potent oxidant, which is formed by the diffusion-controlled reaction of superoxide anions and NO, is produced in various cells, such as macrophages, endothelial, and smooth muscle cells (3). We recently reported the formation of peroxynitrite in ventilatory muscle fibers in response to endotoxin injection (10). Although no published data are available regarding the formation of peroxynitrite during increased muscle activity, the presence of abundant NOS expression and the well-documented rise in reactive oxygen species levels (5, 27) are likely to augment peroxynitrite production during strong muscle contractions. Elevated peroxynitrite concentration is likely to exert a strong inhibitory influence on muscle NOS activity through oxidation of sulfhydryl groups and tyrosine nitration.

Although our results do not provide a direct answer as to why augmentation of ventilatory muscle activity leads to the reduction in muscle NOS activity, one can predict several advantages gained by this response. These advantages include the removal of a significant inhibitory influence exerted by NO on muscle contractility. It was shown by Kobzik et al. (21) and by our group (11) that endogenous NO synthesis inhibits muscle contractility, especially in response to low-frequency stimulation. This effect appears to be mediated through cGMP-dependent and cGMP-independent mechanisms involved in excitation-contraction coupling and sarcoplasmic reticulum Ca^{2+} flux (21). NO also interferes with muscle contractility through the direct inhibition of myosin ATPase activity and creatine kinase activity (26, 36). Finally, it has been well established that endogenous NO synthesis in a variety of cells, including
skeletal muscles, attenuates mitochondrial respiration by reversibly inhibiting mitochondrial enzymes such as cytochrome-c oxidase (9). Thus reduction in NO synthesis during acute exercise is likely to augment mitochondrial respiration and to protect sarcoplasmic reticulum calcium release in response to muscle membrane depolarization.

In summary, our results indicate that increased skeletal muscle activation is associated with a significant decline in muscle NOS activity, which is not mediated by reduction in muscle NOS isoform expression. This reduction in muscle NOS activity represents a compensatory mechanism by which muscle contractility and metabolism are protected from the negative effects of endogenous NO synthesis.

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