Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases

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1Critical Care and Respiratory Divisions, Royal Victoria Hospital and Meakins-Christie Laboratories, McGill University, Montreal, Quebec H3A 1A1; and 2Respiratory Division, Notre-Dame Hospital, Université de Montréal, Montreal, Quebec, Canada H3C 3J7

El-Dwairi, Q., A. Comtois, Y. Guo, and S. N. A. Hussain. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. Am. J. Physiol. 274 (Cell Physiol. 43): C770–C779, 1998.—The aims of this study were to assess the role of nitric oxide (NO) and the contribution of different NO synthase (NOS) isoforms in skeletal muscle contractile dysfunction in septic shock. Four groups of conscious rats were examined. Group 1 served as control; groups 2, 3, and 4 were injected with Escherichia coli endotoxin [lipopolysaccharide (LPS), 20 mg/kg ip] and killed after 6, 12, and 24 h, respectively. Protein expression was assessed by immunoblotting and immunostaining. LPS injection elicited a transient expression of the inducible NOS isoform, which peaked 12 h after LPS injection and disappeared within 24 h. This expression coincided with a significant increase in nitrotyrosine formation (peroxynitrite footprint). Muscle expression of the endothelial and neuronal NOS isoforms, by comparison, rose significantly and remained higher than control levels 24 h after LPS injection. In vitro measurement of muscle contractility 24 h after LPS injection showed that incubation with NOS inhibitor (N-methylisothiourea) restored the decline in submaximal force generation, whereas maximal muscle force remained unaffected. We conclude that NO plays a significant role in muscle contractile dysfunction in septic animals and that increased NO production is due to induction of the inducible NOS isoform and upregulation of constitutive NOS isoforms.

endotoxin; sepsis; shock

**SEPTIC SHOCK SYNDROME** represents a major cause of death in intensive care units and is usually associated with high cardiac output, severely reduced arterial pressure, and intense peripheral vasodilation (28). Bacterial lipopolysaccharide (LPS), the outer membrane of gram-negative bacteria, is known to play a central role in the pathogenesis of septic shock by activating the release of mediators and cytokines from various cells (36). In vitro and in vivo measurements of ventilatory muscle contractility and fatigue resistance have confirmed that endotoxemia or septic shock leads to a significant decline in contractility and fatigue resistance (4, 31). Many mediators such as prostaglandins, thromboxanes, reactive oxygen species, tumor necrosis factor, and lately nitric oxide (NO) have been proposed to cause muscle contractile dysfunction in septic shock.

NO, a highly reactive second messenger with numerous biological functions, is synthesized from l-arginine by a group of hemoproteins known as NO synthases (NOS). Three different genes that code for three NOS isoforms have been recognized. The neuronal (nNOS) and endothelial (eNOS) isoforms, which were first identified in neuronal and endothelial cells, respectively, are known to have widespread tissue distribution in the brain, skeletal muscles, and endothelial cells (18). The third NOS isoform, also known as the inducible (iNOS) isoform, is expressed in numerous cell types in response to bacterial endotoxin and inflammatory cytokines such as tumor necrosis factor, interleukins, and interferon-γ (18).

There has been increasing interest in the past few years in the role of NO in LPS-mediated contractile dysfunction. We have documented that enhanced NO release contributes to diaphragmatic vascular dysfunction in endotoxemic dogs (14). Others have reported that in vivo and in vitro exposure to LPS or inflammatory cytokines leads to enhanced NO production and iNOS induction in muscle fibers and cultured myocytes (27, 34, 37). The functional significance of iNOS induction in muscle fibers was not addressed in these studies. Two recent reports in rats and guinea pigs suggest that muscle contractile dysfunction in septic shock coincides with iNOS induction and that inhibitors of NOS activity restore muscle contractility (5, 11). Both groups of investigators, however, have ignored the possibility that eNOS and nNOS, which are constitutively expressed in normal muscle fibers (19, 20), could contribute to enhanced NO formation in septic muscles. Heightened NO production by these constitutive isoforms is likely to have a negative influence on muscle contractility (19). Whether constitutively expressed NOS isoforms contribute to enhanced NO production and muscle contractile dysfunction in septic shock remains unknown.

Another possible mediator of muscle contractile dysfunction in septic shock is peroxynitrite, a highly reactive compound formed by the reaction of NO with superoxide anions (2). Peroxynitrite exerts an adverse effect on protein and lipid functions as a result of oxidative modifications and nitration of tyrosine residues (2). In septic humans and animals, significant nitrotyrosine formation has been found in lung and cardiac tissue samples, suggesting that peroxynitrite formation plays a significant role in the pathogenesis of tissue injury (3, 21). The possibility that peroxynitrite forms in skeletal muscles during septic shock has not been investigated previously.

The main objectives of this study were to investigate, in a rat model of septic shock, 1) the contribution of NO to muscle contractile dysfunction, 2) which NOS isoform participates in enhanced muscle NO production, and 3) whether enhanced NO production is associated with peroxynitrite formation.
METHODS

Reagents. Materials for L-citrulline assay were purchased from Sigma Chemical (St. Louis, MO). L-[2,3-3H]arginine was purchased from Dupont. The Western blotting apparatus, buffers, precasted gels, membranes, and protein markers were obtained from Novex (San Diego, CA). Specific monoclonal antibodies for the three NOS isoforms were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antinitrotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham and the protein measurement kit from Bio-Rad (Hercules, CA).

Animal preparation. The procedures for the care and use of animals were approved by the Animal Care Committee of the Royal Victorian Hospital. Four groups (n = 6 in each group) of pathogen-free male Sprague-Dawley rats (250–300 g) were housed in the animal facility of the Royal Victorian Hospital and were studied 1 wk after arrival. Group 1 was injected with vehicle alone (normal saline, control group). Groups 2, 3, and 4 were injected with Escherichia coli LPS (serotype 055:B5, Sigma Chemical; 20 mg/kg ip) and killed by cervical dislocation 6, 12, and 24 h after injection, respectively. We determined in our preliminary experiments that this dose of E. coli LPS elicits a moderate degree of shock, which is associated with a significant decline in arterial pressure over a 24-h period and an ~30% mortality rate. Ventral and limb muscles were dissected and frozen quickly in liquid nitrogen. The tissues collected included the diaphragm, intercostal, gastrocnemius (red and white portions), and soleus limb muscles were dissected and frozen quickly in liquid nitrogen for the presence of L-[3H]citrulline by liquid scintillation counting. 

NOS measurement. The presence of 1 mM N8-nitro-L-arginine methyl ester (NOS inhibitor). Ca2+/calmodulin-dependent NOS activity was calculated as the difference between activity measured in the presence of CaCl2 and that measured in EDTA/EGTA buffer. Ca2+/calmodulin-independent NOS activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and in the presence of N8-nitro-L-arginine methyl ester.

Immunoblotting. Crude muscle homogenate proteins (100 µg; see above) from three animals in each group were heated for 15 min at 90°C and then loaded on a gradient (4–12%) sodium dodecyl sulfate-tris(hydroxymethyl)aminomethane glyceine polyacrylamide gel. The proteins were transferred electrophoretically to polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and incubated overnight at 4°C with primary monoclonal anti-iNOS, anti-eNOS, and anti-nNOS antibodies (1:500 for each antibody). Our previous studies indicated that NOS antibodies specifically detect NOS isoforms in rat tissue samples. Lysates of cytokine-activated murine macrophages, human endothelial cells, and pituitary cells were used as positive controls for iNOS, eNOS, and nNOS proteins, respectively (provided by Transduction Laboratories). We also evaluated the influence of LPS injection on nitrotyrosine formation (footprint of peroxynitrite) using primary murine macrophages and nitrotyrosine antibody (Upstate Biotechnology; 1 µg/ml) (2). Nitrotyrosine molecular weight standard (Upstate Biotechnology) was used as positive control. Specific proteins were detected using horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies and ECL 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). Predeetermined molecular weight standards (Novex) were used as markers.

Nitrotyrosine formation was also quantified by slot blotting. Diaphragmatic crude homogenates (100 µg) were mixed with an equal volume of sample buffer, boiled for 5 min, and loaded on nitrocellulose membrane using slot-blotting apparatus (Life Technologies, Gaithersburg, MD). Membranes were then blocked with 5% nonfat dry milk and probed overnight with primary polyclonal antinitrotyrosine antibody (Upstate Biotechnology; 1 µg/ml) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Negative controls were produced by replacing antinitrotyrosine antibody with a rabbit immunoglobulin G (IgG). The specificity of antinitrotyrosine antibody was assessed by preincubation with 10 mM authentic nitrotyrosine (Alexis, San Diego, CA). Specific proteins were detected with the ECL kit, and the intensity of the slots was evaluated by densitometry, as mentioned above.

Immunohistochemistry. Muscle tissues were flash frozen in cold isopentane (20 s) and then immersed in liquid nitrogen and stored at −80°C. Air-dried cryostat sections (10 µm) were rehydrated with phosphate-buffered saline (PBS, pH 7.4, 3–5 min) and blocked for 1 h with normal donkey or horse serum, then washed with PBS. For accurate detection of iNOS and nitrotyrosine expressions, we used monoclonal anti-iNOS (Transduction Laboratories; 20 µg/ml in PBS containing 1% bovine serum albumin) and polyclonal antinitrotyrosine (Upstate Biotechnology; 2 µg/ml) antibodies. Sections were incubated with primary antibodies overnight at 4°C. For iNOS expression, the sections were incubated with biotinylated goat anti-mouse secondary antibody and then treated with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Sites of immunoreaction were visualized by immersing the sections in a solution of diaminobenzidine and hydrogen peroxide. Counterstaining was performed with
hematoxylin (Sigma Chemical). A similar protocol was used for negative control sections, except anti-NOS antibody was replaced with mouse or rabbit IgG. For nitrotyrosine expression, we used Cy3-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Sites of immunoreactivity were visualized with a Nikon fluorescence microscope equipped with a Cy3 filter.

Reverse transcriptase-polymerase chain reaction. Total RNA was extracted from frozen diaphragm samples following the method described by Chomczynski and Sacchi (8). Total RNA (1 μg) was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reverse transcription (RT)-generated cDNA encoding ecNOS, nNOS, and β-actin (as an internal standard and positive controls) were amplified by polymerase chain reaction (PCR). RNA with no clear β-actin band in the RT-PCR products (35 cycles) was discarded from further studies. ecNOS oligonucleotide primers (synthesized in the McGill University DNA Synthesis Facility) were 5’-TACGGACGACAACTCCAC-3’ (forward) and 5’-CAGGCTGCAGTCTCTTGGATC-3’ (reverse; 240 bp) (30). The experimental conditions for all PCRs were initial denaturation at 95°C for 5 min, 35 cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min), and a final 10-min 72°C extension. Ethidium bromide-stained 2% agarose gels were used to separate PCR products, which were visualized under ultraviolet light. Optical densities of the DNA bands were scanned with a densitometer (see above) and quantified with SigmaGel software. To verify the accuracy of the amplified sequence, PCR products were cloned in PCRII cloning vector (Invitrogen, San Diego, CA) and sequenced in the McGill University DNA Sequencing Facility. To use RT-PCR semi-quantitatively, we assessed the relationship between total RNA concentration per sample and the optical density of PCR products by varying RNA concentrations and fixing the number of PCR cycles at 35, as indicated previously (13). We chose to study total RNA concentrations of 50 and 20 ng for ecNOS and nNOS amplification, respectively, on the basis of a previous study we found that, at this concentration, exposure to S-methylisothiourea completely inhibited muscle NOS activity (15). Tetanic contractions were digitized at a frequency of 1 kHz with a personal computer and stored on the hard disk for later analysis. At the end of the experiment, the strip was blotted dry and weighed. Muscle length (cm) and weight (g) were measured and used to calculate the cross-sectional area.

Isometric forces were normalized for muscle cross-sectional area estimated by using the value of 1.056 g/cm³ for muscle density (9). The peak force (N/cm²) was measured for each contraction within the force-frequency curve.

Data analysis. NO activity and muscle force values are means ± SE. Differences in NO activity between the control and LPS groups for a given muscle and between muscles were compared by two-way analysis of variance for repeated measures. Any different detection was evaluated post hoc by the Student-Newman-Keuls test. P < 0.05 was considered significant.

RESULTS

Figure 1 illustrates the changes in total and Ca²⁺/calmodulin-independent NOS activities of various muscles in the four groups of rats. Total muscle NOS activity in the control group was dependent on the

![Graph showing changes in NOS activity](image-url)
presence of Ca\textsuperscript{2+} and calmodulin and varied significantly among different muscles, with the gastrocnemius showing the highest activity and the soleus the lowest. The diaphragm exhibited a significant rise in total NOS activity 6 h after LPS injection (P < 0.05 compared with control values), whereas intercostal and gastrocnemius NOS activities increased significantly only after 12 and 24 h, respectively (P < 0.01 compared with control values; Fig. 1). These elevations in diaphragmatic, intercostal, and gastrocnemius NOS activity were due mainly to augmented Ca\textsuperscript{2+}/calmodulin-dependent activity (Fig. 1). Ca\textsuperscript{2+}/calmodulin-independent activity in the diaphragm, intercostal, and gastrocnemius muscles rose significantly only 12 h after LPS injection (P < 0.01 compared with control values). LPS injection had no significant effect on soleus NOS activity.

Figure 2 depicts iNOS protein expression in the diaphragm (Fig. 2A) and intercostal (Fig. 2B) muscles of the four groups of animals. iNOS expression in both muscles peaked 12 h after LPS injection and disappeared completely within 24 h. In both muscles, extensive staining with anti-iNOS antibody was detected in muscle fibers 12 h after LPS injection (Fig. 3, A and B). No iNOS staining was found in control muscles or muscles obtained 6 h after LPS injection (data not shown).

Changes in diaphragmatic eNOS and nNOS protein expression in the four groups are shown in Fig. 4. Diaphragmatic eNOS protein expression rose twofold 24 h after LPS injection (Fig. 4A). Similarly, diaphragmatic nNOS expression peaked (5-fold increase) 12 h after LPS injection and remained elevated after 24 h (Fig. 4B). These changes in constitutive NOS isoform expression were due to increased mRNA levels (Fig. 5). Optical densities of nNOS PCR product rose by >28-
and 22-fold after 12 and 24 h of LPS injection, respectively (Fig. 5). Similarly, optical densities of diaphragmatic ecNOS mRNA increased to 140 and 190% of control values after 12 and 24 h of LPS injection, respectively. No significant changes in β-actin expression were detected (Fig. 5).

Figure 6 shows the mean densitometry of various NOS isoforms in the diaphragm. iNOS expression rose by 100-fold 12 h after LPS injection and then declined to control levels by 24 h. In comparison, nNOS and ecNOS increased by more than five- and twofold, respectively, after 24 h.

Immunoblotting of diaphragmatic muscle samples from control animals with antinitrotyrosine antibody revealed two faint bands equivalent to molecular masses of 50 and 42 kDa (Fig. 7A). In addition to these bands, two new prominent bands with apparent molecular masses of 196 and 86 kDa were detected in muscles of LPS-injected animals (Fig. 7A). Figure 7B illustrates the densitometric values of diaphragmatic and intercostal nitrotyrosine expression assessed by slot blotting. In both muscles a significant increase in nitrotyrosine intensity was detected only 12 h after LPS injection, with a later decline to values similar to those of the controls. Preincubation of antinitrotyrosine antibody with 10 mM authentic nitrotyrosine eliminated the positive bands mentioned above, confirming the specificity of the antinitrotyrosine antibody. Immunostaining with antinitrotyrosine antibody of diaphragmatic
samples obtained from control animals showed weak positive staining close to the sarcolemma of several muscle fibers (Fig. 8A). Similar localization but more intense staining was detected in muscle fibers 12 h after LPS injection (Fig. 8B). In addition, intense nitrotyrosine staining of diaphragmatic muscle vessels was also noted after LPS injection (Fig. 8D). Replacement of antinitrotyrosine with rabbit IgG completely eliminated the staining (Fig. 8C). Similarly, sites of reactivity were eliminated when antinitrotyrosine antibody was preincubated with 10 mM authentic nitrotyrosine (not shown).

Figure 9 shows the influence of LPS injection on diaphragmatic contractility measured in vitro. Although 6 h of endotoxemia had no effect on the force-frequency relationship, diaphragmatic force generated in response to 50 Hz decreased significantly ($P < 0.05$ compared with control animals) 12 h after LPS injection (Fig. 9, middle). Submaximal diaphragmatic force declined further after 24 h and was associated with a reduction in maximal force ($P < 0.05$ compared with control values; Fig. 9, right). Incubation of diaphragmatic strips with $S$-methylisothiourea restored the decrease in submaximal diaphragmatic force (10- and 50-Hz stimulation) to values similar to those recorded in control animals, whereas the LPS-induced diminution of maximal diaphragmatic force was not altered by this NOS inhibitor (Fig. 10).
DISCUSSION

The novel findings of this study are as follows. 1) Endotoxemia elicits a significant rise in ventilatory and limb muscle NOS activity, which is due to iNOS induction as well as nNOS and eNOS upregulation. 2) Significant nitrotyrosine formation in the ventilatory muscles is detected 12 h after LPS injection and coincides with iNOS expression. 3) NOS inhibition completely restores the LPS-induced decline in submaximal force generation, whereas maximal muscle force remained unchanged.

iNOS expression in normal skeletal muscles. iNOS expression was not detected in different skeletal muscles of pathogen-free rats (10, 12). The current study confirms the absence of iNOS protein expression in normal rat muscles. No significant iNOS expression was detected in the ventilatory and limb muscles of dogs and rabbits (15). Similarly, cultured C2C12 cells and skeletal muscles from pathogen-free C3H/HeN mouse skeletal muscles do not express iNOS protein under normal conditions (34, 37). Gath et al. (11), on the other hand, noted detectable iNOS protein expression in skeletal muscles of normal guinea pigs. These results suggest that constitutive iNOS expression in normal muscle fibers is species dependent. The functional significance of constitutive iNOS expression in guinea pig muscles remains to be assessed.

Using L-citrulline assay as an indicator of iNOS activity, Salter et al. (29) reported for the first time that diaphragmatic iNOS activity rose significantly 6 h after inoculation of rats with Salmonella typhimurium endotoxin. In a more recent study, Boczkowski et al. (5) described iNOS expression in the diaphragm of rats injected with E. coli LPS. Interestingly, early iNOS expression (within 6 h of LPS injection) localized mainly in inflammatory cells infiltrating the diaphragm, whereas myofibrillar iNOS expression was evident after 12 and 24 h (5). iNOS expression was not detected in limb or ventilatory muscles other than the diaphragm. We observed transient iNOS expression in the diaphragm, which peaked 12 h after LPS injection and disappeared within 24 h. The expression of iNOS

Fig. 9. Changes in diaphragmatic contractility in response to 10-, 50-, and 120-Hz stimulation frequencies. *P < 0.05, **P < 0.01 compared with control values. A significant decline in diaphragmatic force was detected at submaximal and maximal stimulation frequencies 24 h after LPS injection.

Fig. 10. Influence of NOS inhibition on diaphragmatic contractility. Diaphragmatic strips isolated 24 h after LPS injection were stimulated at different frequencies before (cross-hatched bars) and after (filled bars) exposure to 1 mM S-methylisothiourea (SMT). **P < 0.01 compared with control values. SMT exposure restored submaximal force with no influence on maximal diaphragmatic force generation.

![Graph showing changes in diaphragmatic contractility](attachment:image.png)
protein on Western blots was associated with a significant rise in Ca\textsuperscript{2+}/calmodulin-independent NOS activity (Fig. 1). Not only was the time course of iNOS expression in the diaphragm in our study different from that in the study of Boczkowski et al., but we detected significant iNOS induction in the intercostal and limb muscles, whereas only diaphragmatic iNOS expression was observed by Boczkowski et al. We speculate that these differences between the two studies are related to variations in the dose and serotype of E. coli endotoxin, which could elicit different time courses of inflammatory cytokine production. The two studies, however, confirm that myofibrillar iNOS expression lags behind that of inflammatory cells infiltrating muscle fibers. Similarly, Thompson et al. (34) failed to detect iNOS expression in skeletal muscle fibers, despite abundant iNOS protein in the endothelium and macrophages of mice injected with E. coli LPS. The molecular mechanisms responsible for iNOS induction in muscle fibers have not been addressed in our study. We speculate, however, that activation of the iNOS promoter in skeletal muscle fibers is mediated through mobilization of the transcription factor nuclear factor-κB through pathways involving tyrosine kinases and protein kinase C. The importance of these pathways in iNOS induction in L6 cultured myoblasts was confirmed recently (27).

Peroxynitrite formation. It has been established that a diffusion-limited reaction of NO with superoxide anions produces peroxynitrite, which is a powerful oxidant capable of oxidizing many proteins, leading to the addition of a nitro group to the ortho position of tyrosine to form nitrotyrosine (2). Although this reaction occurs spontaneously, low-molecular-mass transition metals and superoxide dismutase are known to catalyze it (2). More recent studies have documented extensive nitrotyrosine formation in the lungs and aorta of endotoxemic animals and in patients with acute lung injury (21, 32, 38).

To the best of our knowledge, our results provide the first evidence of nitrotyrosine formation in normal and septic skeletal muscles. It is interesting that the time course of increased nitrotyrosine formation in the diaphragm and intercostal muscles was similar to that of iNOS expression, suggesting that iNOS was the isoform responsible for peroxynitrite formation in our experiments. On the other hand, we speculate that the low level of nitration in the controls was due to the activity of constitutively expressed NOS isoforms. Our results also indicate that enhanced nitration of tyrosine residues in septic muscles is a selective process and involves the nitration of two major proteins with apparent molecular masses of ~196 and 86 kDa. The nature of these proteins remains to be elucidated.

nNOS and ecNOS expression. Recent studies suggest that normal skeletal muscle fibers express nNOS, which is localized beneath the sarcolemma of fast-twitch fibers (19, 26). The association of nNOS with the sarcolemma is mediated through the interaction of nNOS with the dystrophin complex (7). In addition to the sarcolemma, nNOS expression is also enriched at the muscular end plate (22). Muscle fibers are reported to express the ecNOS isoform, which has a cytoplasmic distribution and is localized mainly in muscle fibers rich in succinate dehydrogenase (20).

Our data indicate that LPS injection elicited a substantial rise in muscle NOS activity, which was due mainly to augmented Ca\textsuperscript{2+}/calmodulin-dependent NOS activity (Fig. 1). We attribute this finding to increased protein expression of the ecNOS and nNOS isoforms as detected by immunoblotting with isoform-selective antibodies. The heightened expression of these isoforms was not limited to the diaphragm, since similar changes in nNOS and ecNOS expression were also seen in other muscles such as the intercostals, soleus, and gastrocnemius. Little is known about the influence of LPS and inflammatory cytokines on the regulation of skeletal muscle nNOS and ecNOS isoforms. In endothelial cells, cytokines and LPS increase NO production, presumably via enhanced activation and/or expression of the ecNOS isoform (17, 23). Upregulation of ecNOS transcription rate in response to LPS and cytokines has been confirmed recently in bovine aortic endothelial cells (16). Similarly, there is evidence that in vivo exposure to LPS (24) or in vitro administration of interferon-γ (1) leads to upregulation of nNOS expression in the brain. Several mechanisms are likely to be involved in the upregulation of muscle nNOS and ecNOS protein expression in septic animals. Our study indicates that one of these mechanisms is increased mRNA concentration of these isoforms (Fig. 5). More research is needed to elucidate whether the increase in mRNA expression of these isoforms is due to improved mRNA stability and/or increased transcription rate. In addition, we need to investigate the exact transcription factors involved in the regulation of nNOS and ecNOS gene expression in response to endotoxemia or inflammatory cytokines.

It should be emphasized that, unlike the diaphragm, intercostal, and gastrocnemius muscles, total NOS activity of the soleus muscle did not increase after LPS injection (Fig. 1). The reasons behind this observation are unclear. We speculate that we were unable to detect a significant rise in soleus total NOS activity after LPS injection because this muscle expresses very low levels of constitutive NOS isoforms, especially nNOS. Indeed, in the control group of animals, NOS activity was lower in the soleus than in the muscles (Fig. 1). Similar findings have been reported by Kobzik et al. (19). Moreover, we recently compared NOS expression in rat muscles and observed very low nNOS expression in the soleus compared with the intercostal, diaphragm, and gastrocnemius muscles (15). Thus it is possible that LPS-induced changes in soleus constitutive NOS expression might not have been large enough to be detected with the L-citrulline assay. It is also likely that variations in local cytokine levels, the degree of inflammatory cell infiltration, and other unknown factors may have contributed to the lack of a significant rise in soleus NOS activity after LPS injection.
NO and muscle contractility. Despite recent publications dealing with muscle NOS isoform expression and iNOS induction in response to LPS injection (27, 34, 37), to the best of our knowledge, only two studies have addressed the contribution of NO to LPS-induced contractile dysfunction. Boczkowski et al. (5) were the first to report that administration of NOS inhibitor 90 min after LPS infusion significantly improved twitch and tetanic diaphragmatic force to ~75% of control values. In another study, Gath et al. (11) assessed the fatigability of guinea pig diaphragmatic strips by repeated stimulation of the phrenic nerve. These authors reported that strips isolated from endotoxemic animals fatigued faster than normal strips and that preincubation with NOS inhibitor restored muscle fatigability to normal levels. Our findings of decreased diaphragmatic contractility 24 h after LPS injection and of restored submaximal diaphragmatic force after incubation with NOS inhibitor support the notion that enhanced NO production participates in inducing contractile dysfunction in septic animals. However, unlike the results of Boczkowski et al., we noted that inhibition of muscle NOS activity by 5-methylisothiourea did not restore the LPS-induced decline in maximal diaphragmatic force (Fig. 10). This observation is consistent with the hypothesis of Kozlik et al. (19) that NO influences excitation-contraction coupling at submaximal stimulation frequencies, whereas force production at maximal stimulation frequencies is insensitive to NO.

The exact sites through which NO influences muscle force remains to be elucidated. There is evidence, however, that NO regulates muscle contractile force through guanosine 3',5'-cyclic monophosphate (cGMP)-dependent and cGMP-independent processes, which involve Ca\(^{2+}\) release by ryanodine receptors (25). This notion is supported by the finding that agents that increase intracellular cGMP, such as 8-Br-cGMP, reverse the influence of NOS inhibitors on muscle force (19). It has also been proposed that NO depresses 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 P\(_i\). These metabolites regulate diverse cellular processes, such as ion transport, protein synthesis, and muscle contraction. NO could also modulate muscle contractility by inhibiting creatine kinase (39). Finally, it has been proposed that peroxynitrite, formed by NO and superoxide anions, is involved in sepsis-induced skeletal muscle dysfunction through increased lipid peroxidation and oxygen free radical damage (5, 31). Our results confirm that endotoxemia leads to enhanced tyrosine nitration of the ventilatory muscles of septic rats, which coincided with iNOS expression in these muscles.

The source of enhanced NO production in endotoxin muscles has not been identified. Boczkowski et al. (5) and Gath et al. (11) attributed LPS-induced muscle contractile dysfunction to iNOS induction in muscle fibers. Although our results confirm iNOS induction in skeletal muscles, upregulation of nNOS and ecNOS protein expressions and the significant rise in Ca\(^{2+}/\)calmodulin-dependent NOS activity in these muscles suggest that iNOS may not be the only source of enhanced NO production in septic animals. This is particularly true in the late phase of septic shock (24 h), where iNOS expression could no longer be detected and ecNOS and nNOS expression peaked. The functional significance of this rise in ecNOS and nNOS expression remains to be assessed by using selective gene knockouts of NOS isoforms or potent isoform-selective NOS inhibitors.

In summary, our study indicates that LPS-induced muscle contractile dysfunction is due to enhanced NO release, which, in turn, could be attributed to iNOS induction and upregulation of ecNOS and nNOS expressions. We also found that iNOS induction in muscle tissues was associated with nitrotyrosine formation, suggesting that iNOS induction is involved in muscle peroxynitrite production.

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REFERENCES


33. Szabo, C., G. J. Southan, and C. Thiemermann. Beneficial effects and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibi-