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The role of nitric oxide on contractile impairment during endotoxemia in rat diaphragm muscle

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Abstract

We examined the contribution of nitric oxide (NO) on the contractile impairment in diaphragm muscles of endotoxemic rats. Force–frequency relationship was depressed 24 h after lipopolysaccharide administration. 7-Nitroindazole, aminoguanidine and 1H-[1,2,4]Oxadiazole (4,3-a)quinoxalin-1-one (ODQ) partially restored the contractile impairment, Nω-Nitro-L-Arginine (L-NNA) was ineffective. K^+ contractions were reduced by 50% in endotoxemic muscles, 7-nitroindazole partially recovered, while aminoguanidine and L-NNA were ineffective. Verapamil reduced contractility to a greater extent in endotoxemic muscles. Caffeine and ryanodine contractions were augmented during endotoxemia without NOS contribution. L-NNA, 7-nitroindazole, ODQ and hemoglobin did not affect, but aminoguanidine completely restored partially inhibited neurotransmission by d-tubocurarine. Endotoxemia did not change membrane potentials and neurotransmitter release but slightly increased excitability. At this stage of endotoxemia, (1) constitutive NOS appears to be the dominant isoform, (2) NO does not have a major role on contractile dysfunction and (3) impairment could be explained by altered sensitivity of the voltage sensor. (4) NO does not substantially modulate neuromuscular transmission in normal and endotoxemic rats.

Keywords: NO (Nitric oxide); Lipopolysaccharide; Contractility; Excitation–contraction coupling; Neuromuscular junction; Skeletal muscle

1. Introduction

It has been well documented that respiratory failure is one of the major factors leading to mortality in septic shock (Montgomery et al., 1985) that can be due to lipopolysaccharides. Consecutively, immune reactions following the bacterial endotoxin exposure were shown to induce formation or increase of cytokines and other mediators such as nitric oxide (NO), reactive oxygen species, tumor necrosis factor alpha and interleukins, which were supposed to play a role in impaired ventilatory muscle contractility (Stamler and Meissner, 2001).

In the recent years, several investigators have suggested that NO has a crucial role in the vascular collapse and respiratory failure in sepsis. This suggestion is based on the studies with in vivo lipopolysaccharide injection to various species that lead to increased NO production as a result of inducible NO synthase (iNOS) expression in diaphragm and other skeletal muscles (Boczkowski et al., 1996; el-Dwairi et al., 1998). The functional link between the contractile impairment and elevated NO production during sepsis was suggested by previous studies. Lipopolysaccharide-induced diaphragmatic contractile dysfunction was partially recovered upon pretreatment with NOS inhibitors or iNOS induction blockers by systemic injection prior to lipopolysaccharide. On the other hand, administration of nonselective NOS inhibitors raised the question whether other constitutive NOS isoforms also take part in enhanced NO production. Hussain et al. (1997) showed that bacterial endotoxin inoculation up-regulated neuronal NOS (nNOS) and endothelial NOS (eNOS) (el-Dwairi et al., 1998). However, contradictory results about the contributions of iNOS and nNOS to the

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endotoxin-induced contractile failure obscured the NO hypothesis. Anderson et al. (2001) reported that lipopolysaccharide-induced muscle contractility failure did not recover even with the repeated injections of iNOS inhibitor during sepsis. Moreover, disruption of iNOS gene in mice resulted in worsening of contractile force decline induced by endotoxin (Comtois et al., 1999), and nNOS expression levels were found to be significantly higher than that of the wild-type animals. Surprisingly, in diaphragm muscles of nNOS knock-out mice, lipopolysaccharide-induced contractile impairment was greater than that of the wild-type (Comtois et al., 2001). Activity of iNOS or nNOS has a protective role during sepsis rather than being deleterious, according to these observations. Deletion of one of the isoforms increased severity of contractility failure. These findings imply that NO may have both protective and deleterious effects, depending on the differences in temporal NOS expression. However, contractile status of the diaphragm muscle during different phases of endotoxemia, and the contribution of different NOS isoforms were not fully elucidated.

Diaphragmatic contractile impairment during sepsis was also explained by neuromuscular transmission failure. Leon et al. (1992) suggested that impaired contractility during endotoxemia resulted from dysfunction in neuromuscular transmission due to decreased resting membrane potential. Increased spontaneous neurotransmitter release and depolarization of the muscle cells were attributed to the increased NOS activity following lipopolysaccharide administration in mice (Liu et al., 1995). Whether neuromuscular transmission failure also contributes to the pathology and the role of nitrergic system during endotoxemia remains yet to be evaluated.

Based on these facts, we attempted to investigate the role of NO on diaphragmatic failure during endotoxemia. Firstly, we examined the influence of NO on contractile failure during the stage where the impairment was already established. Secondly, we tried to find which isoform of NOS is involved at this stage. Thirdly, we tried to locate the level of impairment in excitation–contraction coupling. Finally, we assessed whether NO overproduction during endotoxemia has a blocking action on neuromuscular transmission as a contributing factor in this contractile failure.

2. Materials and methods

2.1. Dissection and tissue preparation

Experiments were performed on male Wistar rats weighing 220–310 g. Written approval was obtained from the Animal Ethics Committee of Hacettepe University. Muscles were dissected under diethyl ether anesthesia. Control and lipopolysaccharide-treated animals were randomly allocated into two groups to receive either the intraperitoneal administration of sterile saline (1 ml/kg) or *E. coli* lipopolysaccharide (15 mg/kg dissolved in sterile saline). This dose of lipopolysaccharide was chosen from previous studies (Anderson et al., 2001) and resulted in hypotension, piloerection, tachypnea, diarrhea, nasal discharge, lethargy, hepatomegaly and ~15% mortality in 24 h. Diaphragm muscles were isolated 6, 12 and 24 h later.

2.2. Solutions

The diaphragm muscles were kept in mammalian Ringer's solution with the following composition (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 $NaHCO_3$, 1 Na_2HPO_4, 11 glucose and at pH 7.40. All the solutions were bubbled with a gas mixture of 5% CO_2 and 95% O_2, and temperature was kept constant at 37±0.5 °C for contractility measurements and 20±0.5 °C for electrophysiology.

2.3. Measurement of contractility

Muscles were mounted vertically into the isolated organ baths between an FT-03 force transducer (Grass Instrument, Quincy, MA) and a holder attached to a micrometer that allowed adjustment of the preparation to optimal length. The optimal length of the muscle was defined as the muscle length at which twitch amplitude was maximal. At this optimal length, resting tensions were measured about 2 g and maintained throughout the experiments. Following 1 h of equilibration period, isometric contractions were induced by rectangular pulses generated with a Grass stimulator (S48, Grass Instruments) and delivered through a stimulus isolation unit (Grass SIU 5). Stimulus intensity was increased until maximal twitch responses were obtained, and they were 110–120 V for direct and 10–12 V for indirect stimulations. Then, they were set at 1.2× of the maximal to ensure supramaximal stimulation (150 and 15 V for direct and indirect stimulations, respectively). Pulse durations were 1 ms for direct responses, whereas 0.1 ms were adequate during indirect stimulation. Tetanic force–frequency relationship was evaluated by applying 500-ms long trains of 1, 10, 20, 40, 60, 80, 100 and 150 Hz for every 5 min. Isometric contractions were recorded on a polygraph (7B Grass Instruments) and also fed to a data acquisition unit (Maclab 4/s and Apple Macintosh Quadra 650). The force was expressed in Newtons (N) and normalized for muscle cross-sectional area. Following the removal of non-muscle tissue, muscle strips were blotted and weighed, and force per cross-sectional area were calculated according to the following equation: force/cm^2=[(force (N)×specific density of skeletal muscle (1.06 g/cm3)×length of strip (cm)]/[mass of strip (g)] (Close, 1972). Caffeine and high K^+ contractions were induced by rapid introduction of Ringer's solutions containing 10 mM caffeine or equimolar substitution of KCl (170 mM) for NaCl.

2.4. Electrophysiology

Electrophysiological recordings were performed by using conventional microelectrode techniques. Cells were impaled by glass microelectrodes with tip resistances of 10–30 MΩ. Electrical signals were fed through an amplifier (Axoclamp 2A, Axon Instrument, CA), and digitized data were recorded on a computer. Direct action potentials were induced by electrical stimulation of the muscle fibers locally with 15 V and 0.1 ms long pulses. To measure quantal content, end-plate potentials were isolated and recorded by partially blocking presynaptic release to a level below the action potential threshold with a low Ca^{2+} (0.5–1.5 mM) and high Mg^{2+} (10–15 mM) solution and stimulating the nerve at 0.7 Hz (0.1 ms, 10–15 V). The mean end-plate potential amplitudes and their quantal contents were calculated according to formula derived by Boyd and Martin (1956).

2.5. Reagents

E. coli endotoxin (Serotype O55:B5), aminoguanidine, verapamil, Nω-Nitro-L-Arginine (L-NNA), caffeine, ryanodine, sodium nitroprusside, hemoglobin, d-tubocurarine and 1H-[1,2,4]Oxadiazole (4,3-a)quinoxalin-1-one (ODQ), were obtained from Sigma, USA. 7-Nitroindazole was supplied by RBI, UK.

2.6. Statistical analysis

Values are expressed as mean±S.E.M. The data were analyzed and tested for statistical significance by two-tailed unpaired Student's *t* tests or two-way analysis of variance followed by Bonferroni's method for multiple comparisons. *P* values less than 5% were considered significant. The effective frequency of 50% maximum response (EF_{50}) was estimated from the plots computed using Graphpad Prism (Graphpad Software).

3. Results

3.1. Effects of lipopolysaccharide on diaphragmatic contractility

We evaluated the time course of the depressant effect of endotoxemia on diaphragmatic muscle contractility. Following lipopolysaccharide injection, direct twitch amplitudes were not altered after 6 h, but 28% inhibition was observed after 12 h ($P<0.05$). This inhibitory effect reached 45% following 24 h of endotoxemia (Table 1, $P<0.01$). Lipopolysaccharide administration did not change the time course of direct twitch contractions. Bath administration of NOS inhibitors, 7-nitroindazole (100 μM), aminoguanidine (1 mM) and L-NNA (1 mM) did not affect the twitch responses either in the control ($n=8$) and endotoxemic animals ($n=5$ for each group).

Table 1
Twitch amplitudes and their time courses in different treatment groups

	Amplitude (N/cm^2)	1/2 Rise time (ms)	1/2 Relaxation time (ms)	*n*
Control	2.2±0.1	8.7±0.7	18.3±1.6	8
LPS 6	2.1±0.2	8.8±0.9	17.8±2.0	5
LPS 12	1.6±0.2[a]	8.9±0.7	18.7±1.1	5
LPS 24	1.2±0.2[b]	8.6±0.3	19.1±0.7	5

Values are mean±S.E.M. For the control group.
[a] $P<0.05$, compared to the control.
[b] $P<0.01$, compared to the control.

Force–frequency relationship of diaphragmatic muscle strips from endotoxemic animals displayed significant reduction above 40 Hz after 12 h ($P<0.01$). Maximal tetanic responses elicited in lipopolysaccharide-treated muscles were 37% lower after 12 h and 66% lower after 24 h with respect to controls (Fig. 1A). To evaluate the contribution of NOS isoforms on lipopolysaccharide-induced contractile dysfunction, we investigated the effects of different NOS inhibitors on endotoxemic rat muscles. In the control muscle strips, bath application of 1 mM aminoguanidine and 100 μM 7-nitroindazole increased submaximal tetanic contractions at 40 and 60 Hz and shifted the force–frequency curves to the left without altering maximal tetanic contraction amplitudes ($P<0.05$, $n=5$). L-NNA (1 mM) was ineffective (Fig. 1B). Among the different NOS inhibitors tested, 7-nitroindazole increased contractility in the 24 h lipopolysaccharide-treated group by 20% between 40 and 80 Hz, while the others were found to be ineffective (Fig. 1C, $P<0.05$, $n=5$). To further evaluate the contribution of different NOS isoforms in lipopolysaccharide-induced skeletal muscle dysfunction, we compared the submaximal tetanic contraction amplitudes before and after L-NNA, 7-nitroindazole and aminoguanidine on the same muscle strips. Following stabilization of 40-Hz subtetanic responses evoked every 5 min, we applied NOS inhibitors and measured the percentage change at the plateau of drug effect. 7-Nitroindazole (100 μM) and aminoguanidine (1 mM) significantly elevated subtetanic responses in both control and 24-h lipopolysaccharide-treated muscle strips (Fig. 1D, $P<0.01$, $n=5$). However, this increase following NOS inhibition in the control muscles was more pronounced when compared with lipopolysaccharide-treated group ($P<0.05$). In these experiments, 7-nitroindazole was more effective than aminoguanidine, but L-NNA did not appreciably alter tetanic contractions. To determine the changes in cGMP pathway which is the principal downstream signal transduction system for NO, we compared 40 Hz subtetanic contractions of the control and lipopolysaccharide group of muscles after the inhibition of soluble guanylyl cyclase by its selective inhibitor, ODQ. In the presence of ODQ, tetanic responses of control muscle strips slightly increased, while 24-h lipopolysaccharide group of muscles displayed significantly higher augmentation during soluble guanylyl cyclase blockade ($P<0.01$, $n=5$).

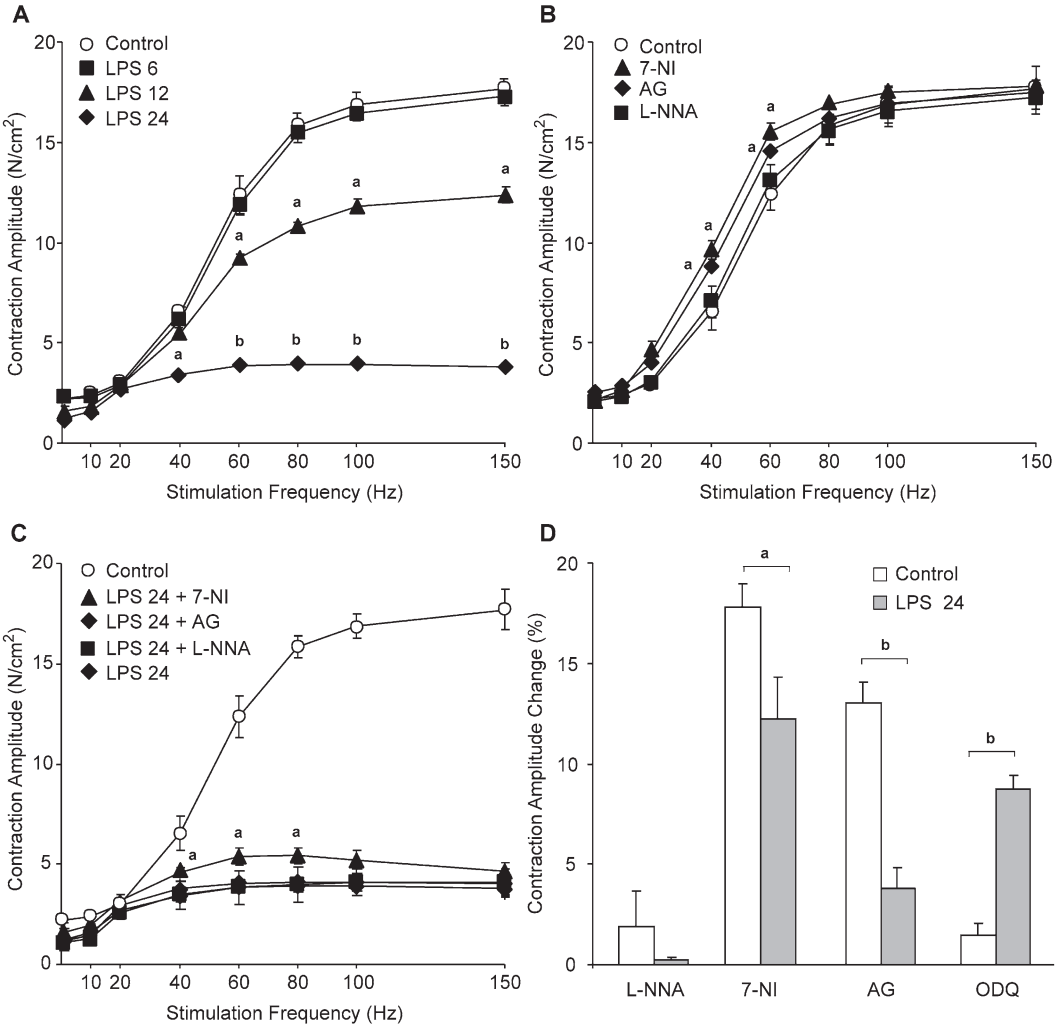

Fig. 1. The effects of lipopolysaccharide-induced endotoxemia on contractility of rat diaphragm muscles. (A) Lipopolysaccharide (LPS) depressed force–frequency curves in diaphragm muscle strips at 12 and 24 h, while no inhibition was observed at 6 h. (B) In the control muscles, 7-nitroindazole (7-NI, 100 μM) and aminoguanidine (AG, 1 mM) shifted force–frequency curves to the left. No change was detected after L-NNA (1 mM). (C) In endotoxemia group, 7-nitroindazole slightly restored contractile impairment. No significant change was observed after L-NNA and aminoguanidine. (D) Effects of NOS inhibitors were compared on the same muscle strips stimulated with trains of 40 Hz in the control and 24-h lipopolysaccharide groups. 7-Nitroindazole, aminoguanidine and ODQ (10 μM) increased contractility in both groups, L-NNA was ineffective. Values are expressed as mean±S.E.M. [a]$P<0.05$, [b]$P<0.01$, with respect to control.

To investigate the depolarization–contraction coupling in the skeletal muscle during endotoxemia, we compared K^+ contractions in the controls and after lipopolysaccharide injection (Table 2). In diaphragm muscles, the rapid introduction of 170 mM KCl solution induced a fast and transient contractile response that reached to a peak within 0.2 min. K^+-induced contraction amplitudes of the muscles obtained from lipopolysaccharide-treated animals after 12 h were found to be 30% lower than controls ($P<0.05$, $n=6$). This decline was more pronounced after 24 h ($P<0.01$, $n=6$). No change was observed in the time course of contractions during endotoxemia. Furthermore, to determine the contribution of NO on the decreased K^+ contractions in endotoxemia, NOS inhibitors were applied on muscle strips obtained from 24-h lipopolysaccharide-treated rats. In these muscles,

Table 2
Amplitudes and time courses of K^+ contractions in different treatment groups

	Amplitude (N/cm^2)	1/2 Rise time (min)	1/2 Relaxation time (min)
Control	2.6±0.3	0.1±0.03	0.5±0.06
Control+7-NI	2.6±0.4	0.1±0.01	0.5±0.03
LPS 6	2.4±0.1	0.1±0.01	0.4±0.01
LPS 12	1.8±0.2[a]	0.1±0.01	0.5±0.03
LPS 24	1.3±0.1[b]	0.1±0.01	0.6±0.05
LPS 24+ AG	1.5±0.3[b]	0.1±0.01	0.5±0.04
LPS 24+ 7-NI	1.6±0.1[a,c]	0.1±0.01	0.5±0.03
LPS 24+ L-NNA	1.4±0.1[b]	0.1±0.01	0.5±0.04

Values are mean±S.E.M. For all groups, $n=6$.
[a] $P<0.05$, compared to the control.
[b] $P<0.01$, compared to the control.
[c] $P<0.01$, compared to the LPS 24.

K^+ contractions partially recovered in the presence of 7-nitroindazole (100 μM, $P<0.05$, $n=6$). On the other hand, K^+ contraction characteristics were unchanged after 7-nitroindazole (100 μM) in the control muscle strips. L-NNA (1 mM) and AG-induced (1 mM) increase in contraction amplitudes did not reach statistical significance. Time courses of K^+ contractions remained unchanged after L-NNA and aminoguanidine treatment ($n=6$ for each group).

Membrane depolarization opens the voltage-dependent Ca^{2+} channels located in the t-tubule membrane. These channels have been shown to be dihydropyridine-sensitive and have a major role in excitation–contraction coupling by triggering Ca^{2+} release from the sarcoplasmic reticulum. To examine the dihydropyridine receptor sensitivity, we partially blocked the channels by using 10 μM verapamil, which slightly depressed the tetanic responses in control muscles, and then compared the force–frequency relationship in the muscles obtained from lipopolysaccharide-treated animals (Fig. 2). In control muscles, verapamil application did not affect the amplitudes of twitch and low frequency tetanic contractile responses and produced a minor depression in tetanic contraction amplitudes at higher frequencies (~11%, at 150 Hz). The control EF_{50} (43.09±0.83 Hz) displayed no significant difference from the EF_{50} (43.58±0.80 Hz) after addition of verapamil. On the other hand, in the lipopolysaccharide group, verapamil-induced depression started at lower frequencies, and the maximal depression obtained at 150 Hz (~%70, $P<0.01$) was more pronounced (Fig. 2). The EF_{50} was 58.24±1.07 Hz after 24 h of endotoxemia, and, in the presence of verapamil, EF_{50} significantly increased to 65.27±1.33 Hz ($P<0.01$). To further examine the steps in excitation–contraction coupling during lipopolysaccharide-induced contractility depression, we investigated the caffeine-induced contractions. It was previously shown that caffeine administration releases Ca^{2+}

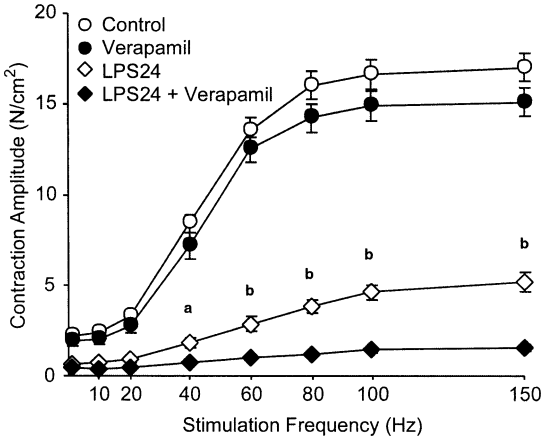

Fig. 2. Inhibitory effect of verapamil on force–frequency relationship in diaphragm muscles obtained from 24-h lipopolysaccharide-treated rats. Verapamil (10 μM) did not change the twitch amplitudes, but a slight depression was observed in tetanic contractile responses of the control muscles. Verapamil inhibited force–frequency relationship in the endotoxemic animals to a greater extent than that observed in the control muscles. [a]$P<0.05$ and [b]$P<0.01$, $n=7$ for each group.

from the sarcoplasmic reticulum, which results in muscle contraction. In our experiments, application of 10 mM caffeine induced a biphasic contraction that reached maximum in 5.4±0.7 min (Fig. 3A). After 24 h of endotoxemia, caffeine-induced contraction amplitudes were significantly augmented (Fig. 3A, $n=6$, $P<0.01$). Incubation with the NOS inhibitors did not reverse this amplitude increase in caffeine contractions. We also investigated the effects of ryanodine after 24 h of lipopolysaccharide administration. Ryanodine is a sarcoplasmic reticulum ryanodine receptor channel opener at low concentrations (<10 μM) (Franzini-Armstrong and Protasi, 1997). In control muscles, ryanodine (3 μM) application induced a slowly progressing monophasic contraction that reached maximum in 85±1.4 min (Fig. 3B, $n=6$). In the lipopolysaccharide group, ryanodine contraction amplitudes were increased by almost twofold, and these contractions attained maximum after 47±4.1 min ($P<0.01$, $n=6$). However, NOS inhibitors did not alter ryanodine contractions ($n=6$ for each group).

3.2. Effects of lipopolysaccharide and NO on neuromuscular transmission

As the neuromuscular impairment due to NO overproduction is proposed to be one of the mechanisms responsible from respiratory muscle failure during endotoxemia, we compared the neuromuscular transmission in the presence of inhibitors of different NOS isoforms on muscles obtained from lipopolysaccharide-treated and control rats. Indirect twitch amplitudes were reduced to ~50% by d-tubocurarine. These partially blocked preparations have enabled us to examine the effects of minute alterations in neurotransmitter release indirectly. Concentration of d-tubocurarine to achieve 50% neuromuscular blockade was 0.7±0.03 μM for the control muscles and 0.7±0.04 μM for the muscles from 24-h lipopolysaccharide-treated rats ($n=6$ for each group). L-NNA (0.01–1 mM, $n=4$) and 7-nitroindazole (100 μM, $n=4$) did not alter amplitudes and time courses of twitch contractions in the control and lipopolysaccharide-treated control muscles (Fig. 4A and B). To further investigate the involvement of NO on neuromuscular transmission, we used agents interacting with the nitrergic system. The NO donor (sodium nitroprusside, 0.1–1 mM, $n=3$), NO scavenger (hemoglobin, 10 μM, $n=3$) and soluble guanylyl cyclase inhibitors, ODQ (10 μM, $n=4$) and methylene blue (30 μM, $n=3$), did not affect indirect twitch contractions in this experimental setting (results not shown). On the other hand, aminoguanidine increased twitch amplitudes in both groups of muscles in a concentration-dependent manner (Fig. 4C, $P<0.01$, $n=6$).

3.3. Effects of endotoxemia and NOS inhibitors on electrical properties of neuromuscular junction

Effects of endotoxemia and NOS activity on neurotransmitter release and muscle cell membrane excitability

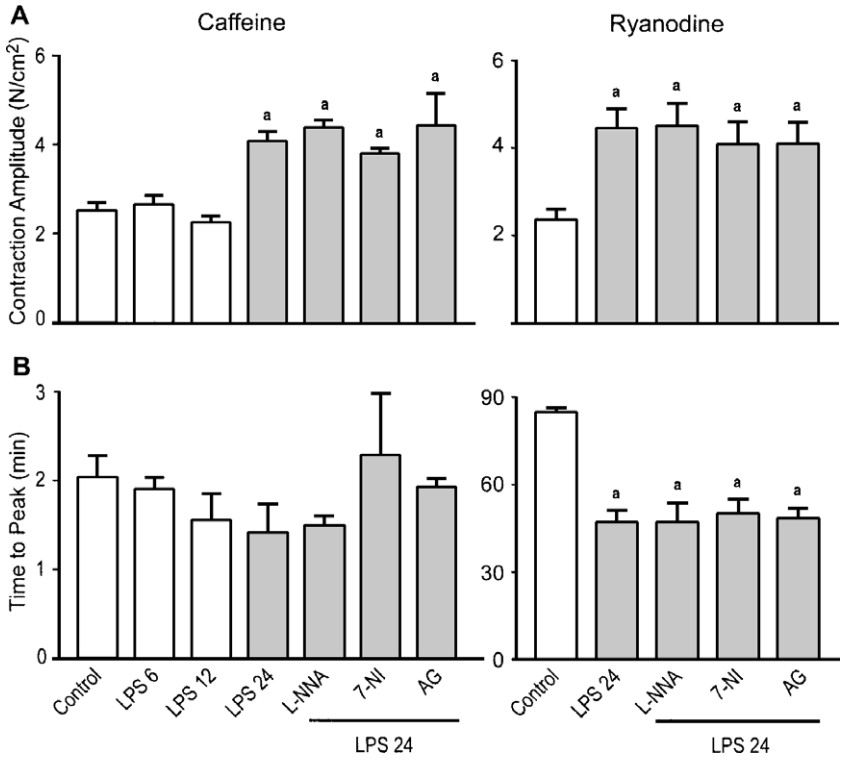

Fig. 3. Caffeine and ryanodine-induced contractions during endotoxemia. (A) Caffeine contraction amplitudes were similar in muscles from 6- and 12-h lipopolysaccharide-treated rats. After 24 h of endotoxemia, caffeine (10 mM) and ryanodine (3 μM) contraction amplitudes were increased. (B) Time to peak values of caffeine contractions were not changed after lipopolysaccharide, whereas ryanodine contractions were faster after 24 h. Alterations in caffeine and ryanodine contractions were not affected by NOS inhibition. n=6 for each treatment group, ^{a}P<0.01 when compared to the controls.

were also studied by using electrophysiological techniques. Lipopolysaccharide treatment did not alter the resting membrane potentials (Table 3), amplitude, frequency and time course of spontaneous miniature end-plate potentials. Control miniature end-plate potential frequency was 1.6±0.2 Hz, and miniature end-plate potential frequencies measured at 6, 12 and 24 h of endotoxemia were 1.6±0.2, 1.7±0.1 and 1.6±0.2 Hz, respectively (Fig. 5, n=5 muscle, 40–60 cells). Control miniature end-plate potential amplitude was 1.6±0.1 mV. After lipopolysaccharide injection, miniature end-plate potential amplitudes were 1.6±0.1, 1.5±0.1 and 1.6±0.1 mV, 6, 12 and 24 h, respectively. L-NNA (1 mM), 7-nitroindazole (100 μM) and aminoguanidine (1 mM) did not change resting membrane potential in both control and lipopolysaccharide-treated muscles. Among the inhibitors tested on spontaneous miniature

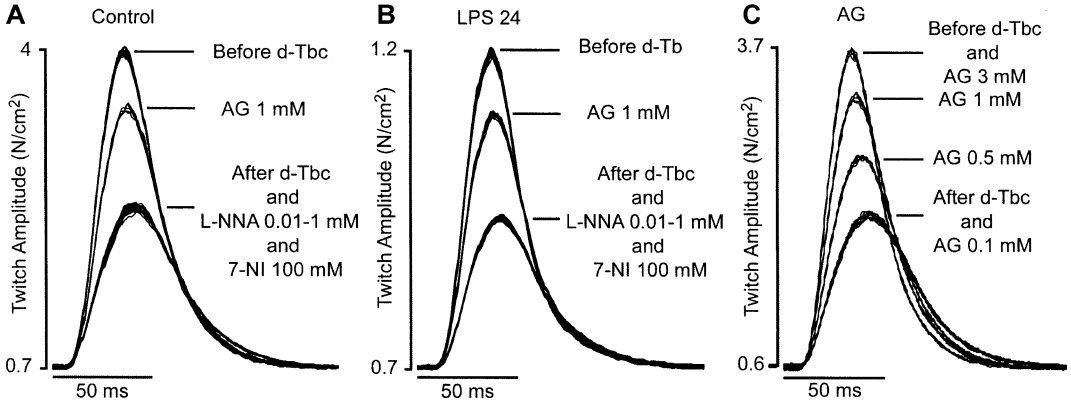

Fig. 4. The effects of NOS inhibitors on neuromuscular transmission in muscles from control and 24-h endotoxemia groups. Indirect twitches were recorded during 50% inhibition of neurotransmission by d-tubocurarine (~0.7 μM). Indirect twitches obtained from the same muscle are superimposed (10 traces for each group) with different treatments to display both amplitude and time course changes. (A) Control and (B) lipopolysaccharide groups. L-NNA (0.01–1 mM) and 7-nitroindazole (7-NI, 100 μM) did not change indirect twitches. Aminoguanidine (AG, 1 mM) recovered indirect twitch amplitudes to the same extent in both control and lipopolysaccharide groups. (C) Aminoguanidine (0.1–3 mM) increased indirect twitch amplitudes in a concentration-dependent manner in the control muscles. n=6, P<0.01.

Table 3
Effects of LPS and aminoguanidine on action potential parameters in rat diaphragm muscles

	Control	LPS 6	LPS 12	LPS 24	AG	LPS 24+AG
RMP	−73.6±0.20	−74.1±0.50	−73.8±0.30	−74.2±0.50	−74.1±0.60	−73.9±0.60
Amplitude (mV)	103.1±0.50	104.4±0.20	104.3±0.50	110.6±0.10[a]	102.2±0.70	109.8±0.50[a]
Overshoot (mV)	29.5±0.50	30.3±0.30	30.5±0.60	36.4±0.10[a]	28.7±0.60	35.7±0.30[a]
1/2 Rise time (ms)	0.4±0.02	0.4±0.03	0.4±0.05	0.4±0.03	0.4±0.03	0.4±0.02
1/2 Decay time (ms)	0.8±0.05	0.8±0.01	0.9±0.07	1.0±0.01[a]	0.7±0.01	1.0±0.03[a]
Rate of rise (V/s)	309.8±2.30	310.4±3.10	315.4±5.40	383.2±9.40[a]	310.4±4.40	385.1±6.80[a]
Rate of decay (V/s)	96.1±0.70	98.2±2.10	94.1±3.20	85±1.70[a]	97.2±0.50	85.4±2.10[a]

RMP—resting membrane potential. Values are mean±S.E.M. For all groups, n=5 muscles, 70–120 fibers.
[a] P<0.01, compared to the control.

activity, only bath application of aminoguanidine for 1 h increased miniature end-plate potential frequency by threefold in the controls (4.5±0.2 Hz, P<0.01) and lipopolysaccharide-treated muscles (4.3±0.1 Hz, P<0.01) without altering their time courses (Fig. 5, n=4 muscle, 40–50 cells, data not shown for the other NOS inhibitors). The effects of aminoguanidine on neuromuscular transmission were further studied by measuring the quantal content of the end-plate potentials. Baseline end-plate potential amplitude in the control muscles was 2.75±0.4 mV, and its quantal content was 2.54±0.3 (n=4 muscles, 50–70 end-plates). In the 24-h lipopolysaccharide group, end-plate potential amplitude and their quantal contents were 2.33±0.3 and 2.15±0.6 mV, respectively (n=3 muscles, 30–45 end-plates). Following 100 min of aminoguanidine (1 mM) perfusion, end-plate potential amplitudes and their quantal contents in both groups increased gradually and reached a plateau level. After aminoguanidine, end-plate potential amplitudes and their quantal contents increased up to 278.2% (7.64±0.6 mV) and 313.9% (7.97±0.5) in the control muscles and 273.3% (6.37±0.7 mV) and 302.6% (6.51±0.9) in the 24-h lipopolysaccharide group, respectively (P<0.01 for both groups).

To identify the changes induced by lipopolysaccharide on muscle cell excitability, we analyzed the action potential configuration. No appreciable change was observed in the 6- and 12-h lipopolysaccharide groups. However, following 24 h of lipopolysaccharide treatment, action potential amplitudes, overshoots and half decay times were increased, and there was no apparent change in the rise times. Lipopolysaccharide treatment also altered rate of action potential development, namely, this treatment increased the rate of rise and reduced the rate of decay of the muscle action potentials. However, perfusion of the muscles with aminoguanidine (1 mM) for 1 h did not reverse this effect (P<0.01, n=5 muscles, 70–120 cells, Table 3).

4. Discussion

The major findings of this study are the following: (1) lipopolysaccharide administration decreased contractility of the rat diaphragm muscles after 24 h, 7-nitroindazole, aminoguanidine and ODQ, but not L-NNA, partially improved this contractile impairment; (2) lipopolysaccharide treatment slightly modified action potential characteristics, and NOS inhibition did not influence this effect; (3) endotoxemia decreased high K^+-induced contractions, and they were partially recovered by 7-nitroindazole; (4) endotoxemia increased inhibitory effect of verapamil on the voltage sensor; (5) endotoxemia increased caffeine and ryanodine contractions, and they were not altered by NOS

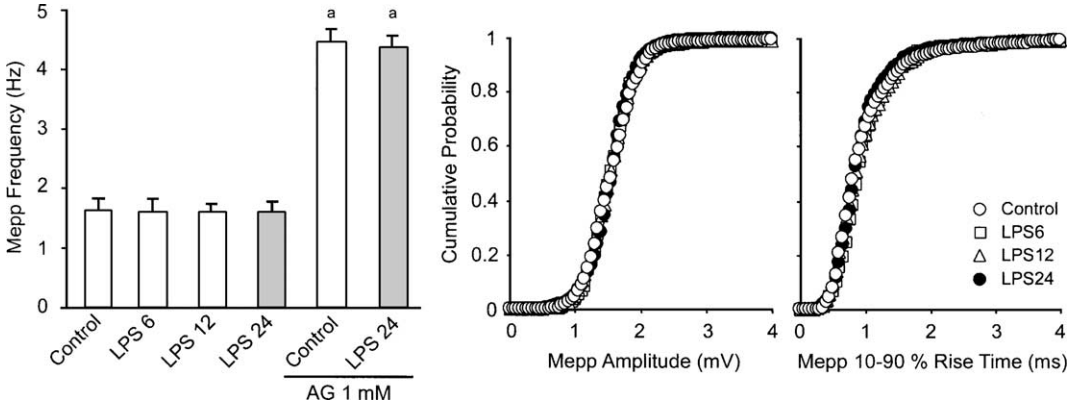

Fig. 5. The effects of 24-h endotoxemia and aminoguanidine on miniature end-plate potentials in rat diaphragm muscles. Miniature end-plate potential (mepp) frequencies were similar in the control and in 6-, 12- and 24-h endotoxemia groups (n=5 muscle, 40–60 cells). Aminoguanidine (AG, 1 mM) treatment for 1 h increased miniature end-plate potential frequencies similarly (~threefold) in both control and 24-h endotoxemia groups ([a]P<0.01, n=4 muscles, n=40–50 cells for each group). Endotoxemia did not change amplitudes and time courses of miniature end-plate potentials, as illustrated in the cumulative amplitude and 10–90% rise time histograms.

inhibition; (6) pharmacological manipulation of the nitrergic system in the rat diaphragm muscle did not alter neurotransmission during physiological and endotoxemic conditions; (7) aminoguanidine increased presynaptic transmitter release at the neuromuscular junction with a mechanism not related to NOS blockade.

In accordance with the previous studies, we found that the contractile impairment became manifest after 12 h and was maximum at 24 h of endotoxemia. Therefore, we evaluated the effects of NOS inhibition on muscle strips at this late stage of endotoxemia. Bath application of NOS inhibitor-induced contractile improvement that we observed in this study was not as pronounced as that achieved by their systemic administration before lipopolysaccharide injection (Sambe et al., 1998; Boczkowski et al., 1996). Additionally, in the experiments where we compared the force output induced by 40 Hz subtetanic stimulation between the control and lipopolysaccharide 24-h groups, potentiation of the contractile responses by 7-nitroindazole and aminoguanidine were significantly lower in the lipopolysaccharide group, while L-NNA was ineffective under both circumstances. Furthermore, soluble guanylyl cyclase inhibition by ODQ yielded higher potentiation of subtetanic contractions in endotoxemia when compared with controls. Yet, this increase did not reach to the level of 7-nitroindazole, showing that this effect appears to be mediated through cGMP-dependent and as well as cGMP-independent mechanisms. Besides, it was shown that cGMP levels were not correlated with NOS activity at the late stage of endotoxemia (Boczkowski et al., 1996). These results imply that, in our experimental setting, NOS activity "at this stage of endotoxemia" is not the major contributor to the contractile failure.

Another aspect investigated in this study was to find which NOS isoform was dominant at this stage of endotoxemia. NOS inhibitors with differing specificities can be used to reveal the contribution of different NOS isoforms to a particular response. L-NNA inhibits all three isoforms of the NOS (Southan and Szabo, 1996). 7-Nitroindazole displays selectivity for the nNOS isozyme and is reported to be approximately 50 times more potent at inhibiting nNOS than the inducible form (Kalisch et al., 1996). Aminoguanidine shows partial selectivity for the iNOS (Griffiths et al., 1993). Among the NOS inhibitors used, only 7-nitroindazole partially reversed lipopolysaccharide-induced decrease in tetanic contractions and high K^+-induced contractions. Similarly, aminoguanidine recovered 40-Hz tetanic contractions but to a lesser extent than 7-nitroindazole, and it was ineffective on high K^+-induced contractions. Interestingly, L-NNA did not influence lipopolysaccharide effects on contractility at all. According to the NOS inhibitor IC_{50} values obtained from isolated enzyme studies, concentration of L-NNA in this study should be enough to block almost all isoforms (Southan and Szabo, 1996). However, it was shown in the previous studies that L-NNA was only effective at 10 mM to block skeletal muscle NOS actions on tetanic contractions (Reid et al., 1998; Marechal Beckers-Bleukx, 1998). At these high concentrations, L-NNA was found to be less effective than 7-nitroindazole and aminoguanidine, indicating a lower potency for the skeletal muscle NOS enzymes. The reason for these contradictory results between these studies, including our study and isolated enzyme IC_{50} studies, may be explained by the tissue-related factors. For instance, in the pig gastric fundus, Dick and Lefebvre (1997) found that aminoguanidine up to 10 mM and 7-nitroindazole 300 μM did not inhibit peripheral neuronal NOS. On the other hand, L-NNA blocked the enzyme with an IC_{50} value of 45 μM. Therefore, our results as well as the previous studies showed that, in rat diaphragm muscles, NOS inhibitors can block NOS activity at higher concentrations than they are normally used in other tissues.

The iNOS isoform is not expressed in skeletal muscles of normal rats (Hussain et al., 1997). Therefore, under normal conditions, the effects of 7-nitroindazole and aminoguanidine on contractility should be solely related with their inhibitory action on constitutive NOS isoforms, namely, nNOS and eNOS. After lipopolysaccharide administration, if iNOS activity is still high at this late stage, it is expected that the recovery of tetanic contractions will be more pronounced when NOS isoforms are inhibited. However, tetanic contractile output increased by 7-nitroindazole and aminoguanidine was significantly lower in endotoxemic muscles when compared with controls, and aminoguanidine was less effective than 7-nitroindazole. Unlike the results of Boczkowski et al. (1996), our findings do not indicate an increased iNOS activity at this stage. Rather, the effects of 7-nitroindazole and aminoguanidine appear to be mediated by nNOS and/or eNOS. Similarly, Thompson et al. (1996), el-Dwairi et al. (1998) and Gocan et al. (2000) reported that there was no induction of iNOS after 24 h of endotoxemia in skeletal muscles. Thus, according to our results, constitutive NOS isoforms seem to be the major producers of NO. Inasmuch as 7-nitroindazole is selective for nNOS, at this late stage of endotoxemia, nNOS appears to be the prominent isoenzyme for NO synthesis.

To locate the level of lipopolysaccharide-induced contractile impairment, we used a stepwise approach and looked for the possible alterations in membrane excitability and different levels of excitation–contraction coupling. Our evaluation on muscle excitability showed that resting membrane potential was unchanged, and alterations in action potential configuration favored a slight increase in excitability during endotoxemia. The finding that NOS inhibition did not alter lipopolysaccharide-induced increase in excitability implies that NO is not involved in this endotoxin action. It has been reported that lipopolysaccharide promotes hyperexcitability by releasing a variety of proinflammatory cytokines, including interleukin-1 and tumor necrosis factor in applysia sensory neurons (Clatworthy and Grose, 1999). In septic rat models, Na^+/K^+ pump activity and its concentration in the plasma membrane were found to be increased (McCarter et al., 2002). It is well known that catecholamines

stimulate skeletal muscle Na^+/K^+ pump activity (Overgaard et al., 1999). Therefore, sustained sympathetic discharge and consequently elevated plasma cathecolamine levels (Jones and Romano, 1984) in the circulation may contribute to stimulation of sarcolemmal Na^+/K^+ ATPase which in turn can induce sarcolemmal hyperexcitability.

Inasmuch as sarcolemmal excitability is not depressed, the mechanism(s) of contractile impairment in endotoxemia should be at the level of excitation–contraction coupling or beyond. Potassium contractions induced by prolonged depolarizations have been widely used to evaluate the relationship between t-tubule depolarization and contractile response or alterations in intracellular Ca^{2+} handling in the skeletal muscle (Dulhunty, 1992). In our study, lipopolysaccharide treatment decreased the amplitudes of high-K^+ contractions without significantly altering their time course. It is generally assumed that the amplitude and the time course of K^+ contractions depend on the activation and inactivation of the processes regulating the release of Ca^{2+} from the sarcoplasmic reticulum. Although time course of K^+ contractions was not changed in our study after lipopolysaccharide, it has been reported that amplitude alterations without time course modifications were related to the changes in the voltage sensor sensitivity (Joumaa et al., 2002). Therefore, we further examined the possible lipopolysaccharide effects at the voltage-sensing dihydropyridine receptor L-type Ca^{2+} channels in the t-tubular membrane. We observed significantly greater depression in the tetanic contractile force in the lipopolysaccharide group with respect to the controls after the partial blockade of dihydropyridine receptors by verapamil. In the 24-h endotoxemia group, the force–frequency relationship of the diaphragm muscle tended to be shifted to the right after the addition of the same concentration of verapamil, which became evident by the increased EF_{50} value, meaning that a higher stimulation frequency is necessary to produce the same relative force. These findings imply that lipopolysaccharide induced robust changes in the voltage sensor. Although we did not measure directly, it may be speculated that the sensitivity or the density of dihydropyridine receptors is reduced due to lipopolysaccharide action.

Experiments were conducted to investigate the contribution of excitation–contraction coupling below the level of voltage sensors by means of caffeine- and ryanodine-induced contractions. Interestingly, caffeine and ryanodine contractions were potentiated by about 60% when the contractility was depressed by 70%, implying that lipopolysaccharide does not hamper sarcoplasmic reticulum Ca^{2+} release during endotoxemia. Thus, decreased contractile power of muscles after lipopolysaccharide cannot be explained by these results; instead, these changes could be related to compensatory mechanisms. Moreover, lipopolysaccharide effect on sarcoplasmic reticulum Ca^{2+} kinetics was not related to the nitrergic activity because augmented responses to caffeine and ryanodine prevailed after NOS inhibition. Data from caffeine experiments do not implicate a deficit in the releasable stores of Ca^{2+} as a mechanism for contractile impairment in endotoxemia. Twitch amplitudes were depressed without alteration in their time courses. It has been shown that sarcoplasmic reticulum Ca^{2+} pump is the rate limiting step in the twitch relaxation of skeletal muscles (Chua and Dulhunty, 1988); therefore, our observations indirectly suggest that sarcoplasmic reticulum Ca^{2+} pump activity is not affected during lipopolysaccharide-induced contractile impairment. Taken together, these experiments showed that lipopolysaccharide-induced contractile failure resulted from a defect in the excitation–contraction coupling above the level of the sarcoplasmic reticulum Ca^{2+} channel. The voltage sensor and dihydropyridine receptors appear to be the target of this inhibitory action.

NO was proposed as a modulator of transmission in neuromuscular junctions in certain species (Ribera et al., 1998). NO donors inhibit acetylcholine release, and, in opposition, NOS inhibitors augmented the neurotransmitter output in neuromuscular junctions of frog sartorious muscle, xenopus and torpedo electric organ (Richmonds and Kaminski, 2001). However, in our study, pharmacological manipulation of nitrergic activity by bath application of NO donors, NO scavengers, NOS inhibitors (7-nitroindazole and L-NNA) and inhibition of cGMP synthesis did not change indirect twitch amplitudes, which were used as an indirect measure of neurotransmitter release. These findings demonstrate that nitrergic system does not exert an appreciable influence on neuromuscular transmission in the rat skeletal muscle during physiological conditions. Our experiments also showed that, during endotoxemia, NO does not play a role on the evoked neurotransmitter release as evidenced by two sets of experiments. First, during the indirect twitch measurements, the same concentrations of d-tubocurarine inhibited neurotransmission to the same degree in the control and lipopolysaccharide-treated groups. Second, aminoguanidine increased end-plate potentials and their quantal contents to the same extent in both groups. In addition, spontaneous neurotransmitter release was also not altered during endotoxemia inasmuch as miniature end-plate potential frequency and amplitudes were the same, and aminoguanidine-induced miniature end-plate potential frequency elevation was identical in both groups. These experiments demonstrated that, under these circumstances, there was no apparent defect in neuromuscular transmission.

Among the NOS inhibitors tested, only aminoguanidine increased the evoked and spontaneous neurotransmitter release. The mechanism of aminoguanidine-induced augmentation in neurotransmitter release is not likely to be due to NOS inhibition. Inasmuch as aminoguanidine did not change miniature end-plate potential amplitudes and their time course, this effect cannot be attributed to altered acetylcholinesterase activity and nicotinic receptor sensitivity. Our findings indicate that aminoguanidine increases neuromuscular transmission by augmenting presynaptic neurotransmitter release. Aminoguanidine could be exerting its effects by blocking K^+ channels and consequently prolonging the

action potential duration and depolarizing the presynaptic membrane. However, lack of aminoguanidine effects on resting membrane potential and decay phase of the action potentials that are closely related to K^+ channel activity excludes this possibility. The exact mechanism of aminoguanidine action still needs to be further investigated.

In conclusion, our results indicate instantaneous NO production during lipopolysaccharide-induced contractile failure is not the major contributor to the pathology in rat diaphragm muscles. The level of excitation–contraction coupling affected by endotoxemia appears to be the voltage sensor step, and this inhibition is not related to ongoing NOS activity. The nitrergic system is not primarily involved in the neuromuscular transmission in the rat diaphragm during both normal and endotoxemic conditions. Aminoguanidine selectively increases presynaptic release with a mechanism not related to NOS inhibition. Thus, these results suggest that lipopolysaccharide reduces muscle contractility with the mechanisms unrelated to nitrergic system and does not influence the neuromuscular transmission.

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References

Anderson, J.L., Williams, G., Head, S.I., 2001. The role of nitric oxide in diaphragmatic dysfunction in endotoxemic rats. Muscle Nerve 24, 30–36.

Boczkowski, J., Lanone, S., Ungureanu-Longrois, D., Danialou, G., Fournier, T., Aubier, M., 1996. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats: role on diaphragmatic contractile dysfunction. J. Clin. Invest. 98, 1550–1559.

Boyd, I.A., Martin, A.R., 1956. The end-plate potential in mammalian muscle. J. Physiol. 132, 74–91.

Chua, M., Dulhunty, A.F., 1988. Inactivation of excitation–contraction coupling in rat extensor digitorum longus and soleus muscles. J. Gen. Physiol. 91, 737–757.

Clatworthy, A.L., Grose, E., 1999. Immune-mediated alterations in nociceptive sensory function in *Aplysia californica*. J. Exp. Biol. 202 (Pt 5), 623–630.

Close, R.I., 1972. Dynamic properties of mammalian skeletal muscles. Physiol. Rev. 52, 129–197.

Comtois, A.S., El-Dwairi, Q., Laubach, V.E., Hussain, S.N., 1999. Lipopolysaccharide-induced diaphragmatic contractile dysfunction in mice lacking the inducible nitric oxide synthase. Am. J. Respir. Crit. Care Med. 159, 1975–1980.

Comtois, A.S., Barreiro, E., Huang, P.L., Marette, A., Perrault, M., Hussain, S.N., 2001. Lipopolysaccharide-induced diaphragmatic contractile dysfunction and sarcolemmal injury in mice lacking the neuronal nitric oxide synthase. Am. J. Respir. Crit. Care Med. 163, 977–982.

Dick, J.M., Lefebvre, R.A., 1997. Influence of different classes of NO synthase inhibitors in the pig gastric fundus. Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 488–494.

Dulhunty, A.F., 1992. The voltage-activation of contraction in skeletal muscle. Prog. Biophys. Mol. Biol. 57, 181–223.

el-Dwairi, Q., Comtois, A., Guo, Y., Hussain, S.N., 1998. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. Am. J. Physiol. 274, C770–C779.

Franzini-Armstrong, C., Protasi, F., 1997. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. Physiol. Rev. 77, 699–729.

Gocan, N.C., Scott, J.A., Tyml, K., 2000. Nitric oxide produced via neuronal NOS may impair vasodilatation in septic rat skeletal muscle. Am. J. Physiol. Heart Circ. Physiol. 278, H1480–H1489.

Griffiths, M.J., Messent, M., MacAllister, R.J., Evans, T.W., 1993. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br. J. Pharmacol. 110, 963–968.

Hussain, S.N., Giaid, A., El Dawiri, Q., Sakkal, D., Hattori, R., Guo, Y., 1997. Expression of nitric oxide synthases and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. Am. J. Respir. Cell Mol. Biol. 17, 173–180.

Jones, S.B., Romano, F.D., 1984. Plasma catecholamines in the conscious rat during endotoxicosis. Circ. Shock 14, 189–201.

Joumaa, W.H., Serrurier, B., Bigard, X., Leoty, C., 2002. Nandrolone decanoate treatment induces changes in contractile responses of rat untrained fast-twitch skeletal muscle. Acta Physiol. Scand. 175, 189–199.

Kalisch, B.E., Connop, B.P., Jhamandas, K., Beninger, R.J., Boegman, R.J., 1996. Differential action of 7-nitro indazole on rat brain nitric oxide synthase. Neurosci. Lett. 219, 75–78.

Leon, A., Boczkowski, J., Dureuil, B., Desmonts, J.M., Aubier, M., 1992. Effects of endotoxic shock on diaphragmatic function in mechanically ventilated rats. J. Appl. Physiol. 72, 1466–1472.

Liu, S.H., Sheu, T.J., Lin, R.H., Lin-Shiau, S.Y., 1995. The in vivo effect of lipopolysaccharide on the spontaneous release of transmitter from motor nerve terminals. Br. J. Pharmacol. 116, 1757–1760.

Marechal, G., Beckers-Bleukx, G., 1998. Effect of nitric oxide on the maximal velocity of shortening of a mouse skeletal muscle. Pflugers Arch. 436, 906–913.

McCarter, F.D., Nierman, S.R., James, J.H., Wang, L., King, J.K., Friend, L.A., Fischer, J.E., 2002. Role of skeletal muscle Na+–K+ ATPase activity in increased lactate production in sub-acute sepsis. Life Sci. 70, 1875–1888.

Montgomery, A.B., Stager, M.A., Carrico, C.J., Hudson, L.D., 1985. Causes of mortality in patients with the adult respiratory distress syndrome. Am. Rev. Respir. Dis. 132, 485–489.

Overgaard, K., Nielsen, O.B., Flatman, J.A., Clausen, T., 1999. Relations between excitability and contractility in rat soleus muscle: role of the Na+–K+ pump and Na+/K+ gradients.J.Physiol.518(Pt1),215–225.

Reid, M.B., Kobzik, L., Bredt, D.S., Stamler, J.S., 1998. Nitric oxide modulates excitation–contraction coupling in the diaphragm. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 119, 211–218.

Ribera, J., Marsal, J., Casanovas, A., Hukkanen, M., Tarabal, O., Esquerda, J.E., 1998. Nitric oxide synthase in rat neuromuscular junctions and in nerve terminals of Torpedo electric organ: its role as regulator of acetylcholine release. J. Neurosci. Res. 51, 90–102.

Richmonds, C.R., Kaminski, H.J., 2001. Nitric oxide synthase expression and effects of nitric oxide modulation on contractility of rat extraocular muscle. FASEB J. 15, 1764–1770.

Sambe, A., Ungureanu-Longrois, D., Danialou, G., Lanone, S., Benessiano, J., Aubier, M., Boczkowski, J., 1998. Role of nitric oxide on diaphragmatic contractile failure in *Escherichia coli* endotoxemic rats. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 119, 167–175.

Southan, G.J., Szabo, C., 1996. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem. Pharmacol. 51, 383–394.

Stamler, J.S., Meissner, G., 2001. Physiology of nitric oxide in skeletal muscle. Physiol. Rev. 81, 209–237.

Thompson, M., Becker, L., Bryant, D., Williams, G., Levin, D., Margraf, L., Giroir, B.P., 1996. Expression of the inducible nitric oxide synthase gene in diaphragm and skeletal muscle. J. Appl. Physiol. 81 (6), 2415–2420.