Expressional downregulation of neuronal-type NO synthase I in guinea pig skeletal muscle in response to bacterial lipopolysaccharide

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Abstract We have investigated the expression of neuronal-type NO synthase I (NOS I) and inducible-type NOS II in guinea pig skeletal muscle (diaphragm). Expression of NOS I mRNA and protein was highest in muscle of specific pathogen-free animals, lower in normally bred animals, and lowest in lipopolysaccharide (LPS)-treated animals. NOS II mRNA and protein levels were highest in muscle of LPS-treated animals. Elevated NOS activity in muscle from LPS-treated animals was less susceptible to the NOS I-selective inhibitor N^G-nitro-L-arginine. Expressional downregulation of NOS I in sepsis may have implications for contractile function of skeletal muscle.

Key words: NO synthase mRNA; Anti-NO synthase antibodies; NO synthase activity; N^G-nitro-L-arginine

1. Introduction

Neuronal-type NO-synthase (NOS I) was first identified in rat and porcine brain [1–3], and has subsequently been found in spinal cord, peripheral nerves, epithelial cells, and skeletal muscle (for review see [4]). In brain, NOS I is mainly a soluble enzyme with a molecular mass of 160 kDa [1–3,5]. Expression of NOS I in skeletal muscle has been first reported by Nakane et al. [6]. Most of the NOS I protein found in skeletal muscle is membrane-bound [7,8]. In several reports, the sarcolemmal endplate region has been described as the major site of expression of NOS I in skeletal muscle [8–11]. The molecular basis for membrane association of NOS I has recently been investigated. The N-terminal region of NOS I contains a sequence motif, referred to as GLGF- or PDZ-domain, which mediates association of this enzyme with cytoskeletal proteins [9,12,13].

NOS I is considered a constitutive enzyme, but there are some reports of expressional regulation. However, many of these stimuli reported to modulate expression are rather non-specific. Occlusion of the middle cerebral artery of the rat produced an upregulation of NOS I mRNA and immunoreactive protein [14]. Chronic salt loading of rats increased expression of NOS I mRNA in the supraoptic and paraventricular nuclei of the hypothalamus, and elevated NOS activity in the posterior pituitary [15]. Upregulation of NOS I mRNA was detected in rat sympathetic neurons and central motor neurons following axotomy [16]. One report suggested that estrogens can upregulate NOS I mRNA in skeletal muscle [17]. Developmental regulation of NOS I expression has been reported in rat lung and murine skeletal muscle [18,19], and Ogura et al. [20] have described induction of NOS I expression in the development of neural precursor cells isolated from embryonic murine neural tubes.

In the present study we demonstrate that NOS I mRNA and protein are downregulated in guinea pig skeletal muscle when the animal is treated with bacterial lipopolysaccharide (LPS), a stimulus which has been reported to enhance expression of inducible-type NOS II in this tissue [8].

2. Materials and methods

2.1. Animals

Male Dunkin Hartley guinea pigs (200–250 g; Charles River, Sulzfeld, Germany) were kept in our animal facilities on regular chow and water until the day of the experiment. For treatment of guinea pigs with bacterial lipopolysaccharide (LPS), LPS was dissolved in phosphate-buffered saline at a concentration of 7.5 mg/ml by thoroughly mixing and briefly sonicating the solution. A few hours after treatment with an i.p. injection of LPS (7.5 mg/kg), guinea pigs behaved apathetic and started shivering. Animals were sacrificed 6 hours after injection of LPS, a time at which high expression of NOS II protein has been reported in different tissues of LPS-treated animals [21]. 'Specific pathogen-free' (SPF) guinea pigs were obtained from Charles River, delivered in microorganism-tight containers and sacrificed immediately upon arrival.

2.2. Ribonuclease protection analyses with guinea pig-specific cRNA probes

Plasmids containing guinea pig specific NOS I, NOS II and β-actin cDNA fragments were generated as previously described [8]. Ribonuclease protection analyses were performed according to standard procedures [22]. Briefly, radiolabeled antisense RNA (cRNA) probes were generated by in vitro transcription of 0.5 mg plasmid DNA using T3 or T7 RNA polymerase (Stratagene) and 90 μCi [α-^{32}P]-UTP per reaction. Thereafter, template DNA was degraded by incubation with DNase I; labeled cRNA probes were purified on NucTrap probe purification columns (Stratagene). For each hybridization 4 μg poly(A)$^+$-enriched RNA [22] from skeletal muscle and control tissues (cerebellum of normal animals, and small intestine of LPS-treated animals) were incubated overnight at 50°C with 100000 cpm NOS cRNA probe and 30000 cpm β-actin cRNA probe. Unhybridized probe was digested by treatment with a mixture of RNase A and RNase T1 (Boehringer-Mannheim). RNase activity was stopped by the addition of proteinase K in a buffer containing 10% sodium dodecyl sulfate. Protected fragments were extracted with phenol/chloroform, precipitated with ethanol and electrophoretic separated in a denaturing 6% polyacrylamide gel. DNA fragments derived from pGl2-Basic restricted with *Hin*fI were labeled with [γ-^{32}P]ATP and served as size markers. Densitometric analyses of gels were performed using a Molecular Imager (Bio-Rad) and results were quantified by comparison with the hybridization signal of a β-actin cRNA probe.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; LPS, bacterial lipopolysaccharide; NBT/X-phosphate, 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NOS I, neuronal-type NOS; NOS II, inducible-type NOS; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate

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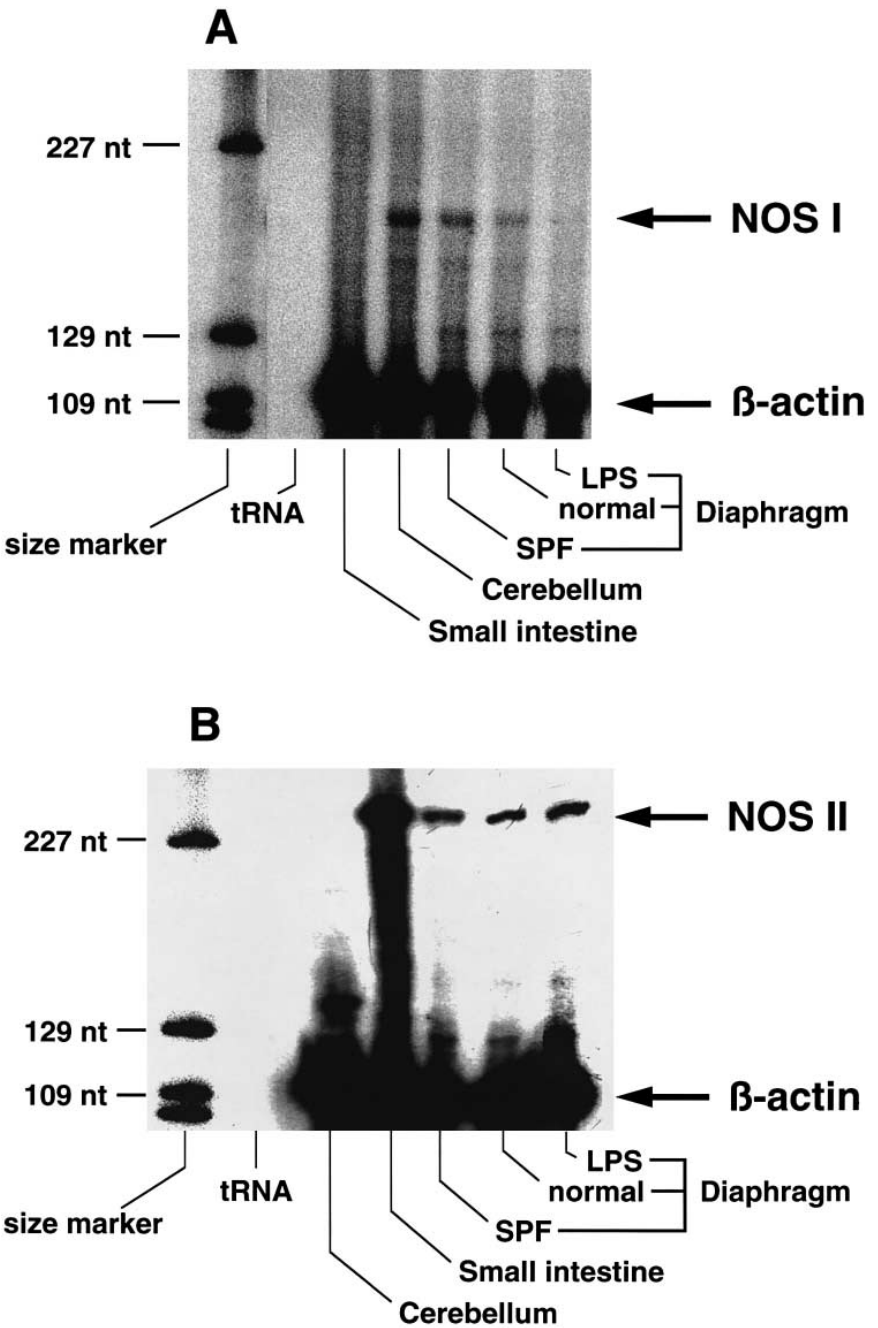

Fig. 1. Ribonuclease protection analyses for NOS I mRNA (A) and NOS II mRNA (B); β-actin mRNA was used for standardization. Poly $(A)^+$RNA was prepared from the diaphragm of specific pathogen-free, normal and LPS-treated guinea pigs, and from control tissues (cerebellum of normal animals and small intestine of LPS-treated animals). Protected RNA fragments were separated on a 6% denaturing polyacrylamide gel. Panel (A) shows a hybridization with a cRNA probe specific for guinea pig NOS I mRNA. The protected fragment is 175 nucleotides. Panel (B) shows a hybridization with a cRNA probe specific for guinea pig NOS II mRNA. Here the protected fragment is 239 nucleotides. Molecular size markers and t-RNA controls (20 μg RNA/lane) are also shown. NOS I mRNA was detected in the cerebellum (positive control) and in diaphragm. NOS II mRNA was found in small intestine (positive control) and in diaphragm. Results are representative of three independent experiments yielding the same results.

2.3. Western blotting

Particulate protein fractions from diaphragm were solubilized with the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, 20 mM final concentration) and enriched for NOS by 2′,5′-ADP Sepharose chromatography [23]. A CHAPS-solubilized homogenate from guinea pig cerebellum and a partially purified soluble fraction from RAW 264.7 macrophages, induced for 18 h with LPS (1 μg/ml) and interferon-γ (100 U/ml), were used as control protein preparations. Proteins were separated by denaturing discontinuous polyacrylamide gel electrophoresis (Disc-PAGE) using 7.5% resolving gels. Western blotting was performed as described [8]. Briefly, blots were incubated with a polyclonal anti-NOS I antibody ([24]; 1:2000) and a monoclonal anti-NOS II antibody (Transduction Laboratories, Lexington, KY; 1:500). After incubation with the secondary antibody conjugated to alkaline phosphatase, immunoreactive proteins were visualized with NBT/X-phosphate (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate).

2.4. Assay of NOS activity

The NO-forming capacity of affinity-purified fractions from diaphragm muscle was measured by bioassay using RFL-6 rat lung fibroblasts, as previously described [25,26]. This assay measures NO-stimulated cGMP accumulation in these reporter cells. The enzymatic reaction was performed on the reporter cells in a volume of 1 ml. The reaction mixture contained 100 μM arginine, 100 μM NADPH, 3 μM tetrahydrobiopterin, 300 nM FAD, 250 μM glutathione and 500 U/ml calmodulin. The reaction was started by the addition of the respective protein fraction and was allowed to proceed for 3 min. In NOS inhibitor studies, protein preparations (10–25 μg) were preincubated with NG-nitro-L-arginine (L-NNA) in Locke's solution [26] at 37°C for 5 min.

3. Results

3.1. Regulation of NOS I and NOS II mRNAs

Ribonuclease protection assays with a NOS I cRNA probe and mRNA from guinea pig diaphragm revealed a protected RNA fragment of the expected molecular size (Fig. 1A). The intensity of the signal was strongest in RNA from SPF guinea pigs (100%), lower in RNA from normal animals (68% of SPF), and lowest in RNA from LPS-treated animals (36% of SPF). Small intestine of LPS-treated animals and cerebellum of normal animals were utilized as control tissues. A strong signal was seen in cerebellum of normal animals, but none in small intestine from LPS-treated animals (Fig. 1A). Using a NOS II cRNA probe, a protected RNA fragment of the expected molecular size was detected in skeletal muscle, with the highest intensity in muscle from LPS-treated animals (Fig. 1B). Small intestine from LPS-treated animals (used as a positive control) gave a strong NOS II signal, whereas no significant signal was found in cerebellum from normal animals (negative control) (Fig. 1B).

3.2. Regulation of NOS I and NOS II protein

In Western blots with particulate protein fractions from guinea pig diaphragm, significant NOS I immunoreactivity was detected in protein preparations from SPF animals (Fig. 2A). This signal was weaker in muscle from normal animals and still less in muscle from LPS-treated guinea pigs (Fig. 2A). Muscle NOS I migrated with the expected molecular mass of about 160 kDa which was not different from the NOS I in protein preparations from guinea pig cerebellum (positive control) (Fig. 2A). Also, NOS II immunoreactivity was detected in the particulate fractions of muscle from the different types of animals (Fig. 2B). Marked staining was present in muscle from SPF- and normal animals; the signal was significantly increased in muscle from LPS-treated animals. Interestingly, muscle NOS II migrated slightly above the immunoreactive band obtained with the soluble fraction from induced RAW 264.7 macrophages (Fig. 2B). No relevant signals of NOS I or NOS II immunoreactivities were found in the soluble protein fractions of skeletal muscle (n = 3, not shown).

3.3. Regulation of NOS activity

Specific NOS activity was lowest in muscle from SPF animals and increased more than 2-fold in muscle from LPS-treated animals (Table 1). In further experiments, the inhibition of muscle NOS activities by L-NNA and aminoguanidine was investigated. Based on previous reports, L-NNA is about ten times more potent in inhibiting NOS I activity compared with NOS II activity [27,28]. L-NNA produced significantly less NOS inhibition in muscle protein preparations from LPS-treated animals than normal or SPF animals (Table 1). Aminoguanidine is usually considered a selective inhibitor of NOS II [28]. However, as previously reported [8], guinea pig muscle NOS was only marginally inhibited by aminoguanidine (up to 300 μM, not shown). This is in agreement with a report by Nakane et al. [27] demonstrating that aminoguanidine has very low inhibitory potency and lacks isoform selectivity with human NOS.

4. Discussion

The major finding of the current study is that exposure of animals to bacterial LPS not only upregulates NOS II expression, but also downregulates NOS I expression in skeletal muscle (diaphragm) of guinea pigs. This finding is surprising because NOS I is usually considered a constitutively expressed gene. Upregulation of NOS I gene expression has previously been reported in nerves in response to traumatic stimuli [14,16] and in lung, nerve cells and skeletal muscle cells during maturation [18–20]. To our knowledge, downregulation of NOS I in response to a specific agent has not yet been described; but a recent report suggested that there is a defect in NOS I expression in dystrophin-deficient muscular dystrophy [19].

Evidence has been presented for an alternatively spliced NOS I in rat skeletal muscle. This muscle NOS I was found to be slightly larger than brain NOS I owing to a 34-amino acid (102-base pair) insert between amino acids 839 and 840 of the brain form [29]. The muscle isoform had similar catalytic activity as brain NOS I [29]. In our study, we did not specifically search for a muscle isoform. However, in Western blots, guinea pig muscle NOS I migrated with a molecular mass which was not different from NOS I in preparations

Table 1
Specific NOS activities in particulate fractions of guinea pig skeletal muscle, and their inhibition by the NOS I-selective inhibitor NG-nitro-L-arginine

Inhibitor treatment	NOS activities (cGMP/g tissue)[a]		
	SPF animals	Normal animals	LPS-treated animals
None (basal activity)	10.8 ± 0.7	17.9 ± 0.5	24.1 ± 0.9
	% residual activity following inhibitor treatment		
3 μM L-NNA	7.9 ± 0.6	7.2 ± 0.8	16.3 ± 1.2
30 μM L-NNA	0.3 ± 0.2	1.7 ± 0.2	2.9 ± 0.3

Activity was measured by the stimulation of soluble guanylyl cyclase in RFL-6 reporter cells as described in Section 2. Results represent ± SEM of four determinations.
[a]These activities were set 100% in subsequent studies with L-NNA.

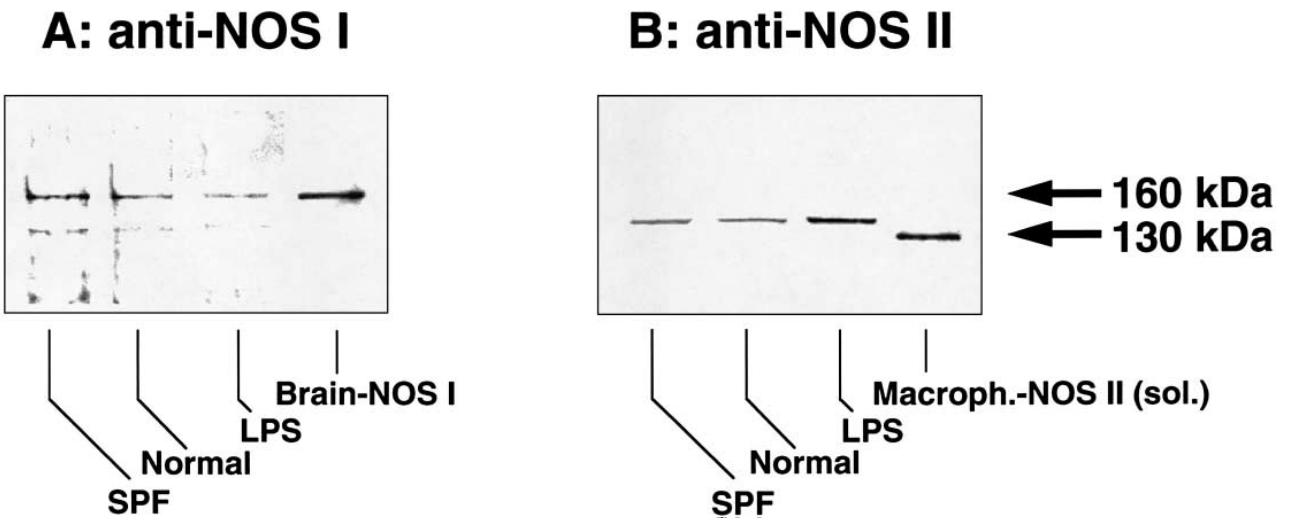

Fig. 2. Western blots of particulate protein fractions from diaphragm muscle of specific pathogen-free, normal and LPS-treated guinea pigs. Electrophoreses were performed in 7.5% polyacrylamide resolving gels and blots were immunostained with anti-NOS I antibody (A) or anti-NOS II antibody (B). Positive control proteins were included (CHAPS-solubilized homogenate of guinea pig cerebellum for NOS I, and partially purified soluble fraction from LPS-induced RAW 264.7 macrophages for NOS II; 100 μg protein/lane and 10 μg protein/lane, respectively). Diaphragm muscle samples were applied at 10 μg protein/lane. Results are representative of four independent experiments with identical results.

from guinea pig cerebellum (Fig. 2A), murine or porcine cerebellum (not shown). Thus, either a separate muscle form does not occur in guinea pig diaphragm, or its difference in molecular weight is too small to be detected in our Western blots.

On the other hand, the NOS II immunoreactivity found in guinea pig skeletal muscle was slightly more massive than the control NOS II obtained from induced RAW 264.7 macrophages (Fig. 2B). In RAW 264.7 cells, NOS II is primarily soluble, whereas in striated muscle most of the enzyme is particulate. The increase in molecular weight could reflect posttranslational modifications responsible for the membrane association of the NOS II in muscle. Thoenes et al. [30] found a similar shift in the molecular weight of NOS II in homogenates of heart muscle samples from patients with sepsis, relative to affinity-purified NOS II from RAW 264.7 macrophages. Furthermore, Vodovotz et al. [31] reported a 4.5 kDa increase in the molecular mass of the membrane-associated NOS II in primary macrophages, compared to NOS II in the soluble fraction of these cells. However, the posttranslational modifications responsible for the membrane association of NOS II (and the increase in apparent molecular mass) have not yet been identified. NOS II contains five pairs of di-lysines, a motif that may undergo ε-NH_2 myristylation [31]. Moreover, NOS II can be phosphorylated on tyrosine residues [32] and probably serine/threonine residues [31].

The functions of NO in different areas of skeletal muscle are still largely unclear. Skeletal muscle represents an important source of NOS I expression [6–9], and the highest enzyme levels are found at the neuromuscular endplate [8,10,11,13]. In the CNS, NO has been shown to regulate synaptic connectivity during development [33], and synaptic plasticity in the adult [34]. Also at developing neuromuscular synapses, NO can serve as a retrograde messenger mediating presynaptic suppression [35]. If similar mechanisms were active in mature striated muscle, a downregulation of membrane-associated NOS I by LPS could result in an improved synaptic transmission. However, the NOS I downregulation in response to LPS was accompanied by an upregulation of the NOS II isoform. Even though this is likely to occur in different cell compartments, the NOS II-derived NO production may still mask a reduced NO production by NOS I. Indeed, recent findings in our laboratory indicate that contractile function is depressed in striated muscles from LPS-treated animals [8]. Furthermore, we and others found NO donor compounds to reduce, and NOS inhibitors to improve contractile force [7,8]. The molecular mechanisms of this negative regulation of contraction by NO are unknown. In mitochondria isolated from rat brain or heart, NO has been reported to reduce respiration rate by reversibly inhibiting the activity of cytochrome c oxidase [36,37]. Similarly, in primary astrocyte cultures induced with LPS/interferon-γ, NOS II-generated NO has been shown to significantly decrease the activity of cytochrome c oxidase [38]. Other evidence suggests that NO could reduce contractile force of skeletal muscle by inhibiting the ryanodine receptor calcium release channel [39].

In conclusion, LPS treatment of guinea pigs leads to an overexpression of NOS II in skeletal muscle. Interestingly, this tissue has been found to express this isoform already under basal conditions [8]. On the other hand, the NOS I isoform which is also present in muscle tissue, is significantly downregulated following treatment of guinea pigs with LPS. Additional studies are needed to characterize the functional consequences of this expressional regulation.

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