Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex

JIANHUA LI AND JERE H. MITCHELL
Moss Heart Center and Department of Internal Medicine, University of Texas Southwest Medical Center, Dallas, Texas 75390-9174

Received 6 March 2002; accepted in final form 15 May 2002

Li, Jianhua, and Jere H. Mitchell. Role of NO in modulating neuronal activity in superficial dorsal horn of spinal cord during exercise pressor reflex. Am J Physiol Heart Circ Physiol 283: H1012–H1018, 2002. First published May 23, 2002; 10.1152/ajpheart.00174.2002.—Static contraction of hindlimb skeletal muscle in cats induces a reflex pressor response. The superficial dorsal horn of the spinal cord is the major site of the first synapse of this reflex. In this study, static contraction of the triceps surae muscle was evoked by electrical stimulation of the tibial nerve for 2 min in anesthetized cats (stimulus parameters: two times motor threshold at 30 Hz, 0.025-ms duration). Ten stimulations were performed and 1-min rest was allowed between stimulations. Muscle contraction caused a maximal increase of 32 ± 5 mmHg in mean arterial pressure (MAP), which was obtained from the first three contractions. Activated neurons in the superficial dorsal horn were identified by c-Fos protein. Distinct c-Fos expression was present in the L6-S1 level of the superficial dorsal horn ipsilateral to the contracting leg (88 ± 14 labeled cells per section at L7), whereas only scattered c-Fos expression was observed in the contralateral superficial dorsal horn (9 ± 2 labeled cells per section, P < 0.05 compared with ipsilateral section). A few c-Fos-labeled cells were found in control animals (12 ± 5 labeled cells per section, P < 0.05 compared with stimulated cats). Furthermore, double-labeling methods demonstrated that c-Fos protein coexisted with nitric oxide (NO) synthase (NOS) positive staining in the superficial dorsal horn. Finally, an intrathecal injection of an inhibitor of NOS, N-nitro-l-arginine methyl ester (l-NAME), an inhibitor of NOS, on c-Fos expression and the pressor reflex evoked by muscle contraction.

METHODS

General surgical preparation. Experiments were performed on 12 cats (3.8–5.8 kg body wt) that were anesthetized by inhalation of 3–5% halothane in oxygen. An endotracheal tube was inserted into the trachea via a trachotomy to maintain an open airway, and a jugular vein and carotid artery were catheterized for drug administration and measurement of arterial blood pressure, respectively. Anesthesia was then maintained with α-chloralose (80 mg/kg) injected intravenously. Throughout the experiment, supplemental α-chloralose (15 mg/kg iv) was given if the cats exhibited a corneal reflex or they withdrew a limb in response to a noxious stimulus. Arterial blood gases and pH were periodically determined (ABL-3, Radiometer; Copenhagen, Denmark) and were maintained within normal limits (pH 7.30–7.40; PaO2 32–36 mmHg; PaCO2 >80 mmHg) by adjusting the ventilator (model 661, Harvard Apparatus; South Natick,
MA) or injecting a 1 M solution of sodium bicarbonate intravenously. Body temperature was continuously monitored with the use of a rectal probe and was maintained between 37.0 and 38.5°C with a water-perfused heating pad and an external heat lamp.

The tibial nerves in both legs were carefully exposed and separated. A pool was formed around the exposed muscular tissue by suturing the skin flaps to steel posts around each leg. Each exposed region was immersed in a pool of warm mineral oil (37°C). The nerves were then placed on platinum bipolar stimulating electrodes. The calcanean bone of each hindlimb was cut, allowing the Achilles tendons to be connected to force transducers (model FT10, Grass Instruments) for measurement of induced tension by the triceps surae muscle. The pelvis was stabilized in a spinal unit (Kopf Instruments; Tujunga, CA) and the knee joints were secured by attaching the patellar tendon to a steel post. In four cats that received intrathecal injection, the spinal cord at L1 was exposed and a polyethylene-10 catheter was placed under the dura. The tip of the catheter was advanced carefully 6–7 cm so that it was located near the L7 dorsal root. The cannula was then secured by tightening a suture placed in the dura. Arterial blood pressure (ABP) was measured with a pressure transducer (model P23 ID, Statham; Oxnard, CA) connected to an arterial catheter. Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. Heart rate was derived from the arterial pressure pulse by a Biotach (Gould Instruments; Cleveland, OH). All measured variables were continuously recorded on an eight-channel chart recorder (model 2800s, Gould Instruments).

Experimental protocol. The cats were allowed to stabilize for 4 h after surgery. Three groups of animals were studied. In the first group [stimulated group (n = 5)], the cats received electrical stimulation of the tibial nerve of one hindlimb (left side). The tibial nerve of the other hindlimb (right side) was placed on platinum bipolar stimulating electrode without delivery of electrical stimulation. In the second group [control group (n = 3)], the cats received the same surgical procedures as in stimulated animals, and electrical stimulation of the tibial nerve was delivered after paralysis of muscle with intravenous injection of pancuronium bromide (200 μg/kg). In the third group [L-NAME (n = 4)], the cats received intrathecal injection of 5 mM L-NAME prepared in artificial cerebrospinal fluid (100 μl; Sigma) 10 min before electrical stimulation of the tibial nerve. The duration of the injection was 2 min. Before the animals were terminally perfused, 100 μl of 2% Evans blue dye was injected intrathecally to confirm whether the dye had spread within the L1-S1 spinal cord.

ABP was monitored during static muscular contraction of the triceps surae muscle. The contraction was induced by electrical stimulation of the tibial nerve for 2 min. Stimulus parameters were two times motor threshold at 30 Hz, 0.025-ms duration. It has been reported (34) that direct activation of group III and group IV afferent fibers within the tibial nerve does not occur with these stimulus parameters. Ten stimulations were performed and a 1-min rest period was allowed between stimulations. These 2-min contractions and 1-min rest periods were performed for a total of 30 min. The motor threshold was readjusted over the 30-min period of muscle contraction to attempt to maintain a consistent increase in muscle tension during stimulation of the tibial nerve.

Ninety minutes after the end of the electrical stimulation, the cats were perfused transcardially with 1 liter of saline, followed by 1.5 liter of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Robust c-Fos expression in the medulla induced by static muscle contraction has been found at 90 min after the end of the electrical stimulation of the ventral roots (22). Therefore, 90 min was used as the time point to perfuse the cat in the present experiments. After being perfused, the spinal cord was removed and stored in the same fixative solution for 2 h. The spinal cord was then transferred to a 30% sucrose solution overnight to prevent ice crystal formation. Coronal sections (25 μm) were cut on a cryostat (model 2800 Frigocut-E, Cambridge Instruments), placed serially into four wells containing cryoprotectant, and then kept in a −20°C freezer.

Immunocytochemistry. Tissue was removed from cryoprotectant and then rinsed in PBS for 30 min. The sections were washed in PBS for 15 min, followed by 0.5% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Sections were placed in PBS containing 1% normal goat serum and 0.1% Triton X-100 for 15 min. They were then incubated in a primary antibody to c-Fos (Santa Cruz Biotechnology, catalog no. sc-52, 1:10,000 dilution) for 48 h at 4°C. At the end of this incubation period, sections were rinsed in PBS and then in the PBS-normal goat serum-Triton X-100 mixture for 15 min. The sections were incubated in biotinylated goat anti-rabbit IgG (Vector Kit, 1:200) for 30 min, washed in PBS for 30 min and incubated with ABC solution (Vector Kit, 1:50) for 30 min. After a serial rinse in PBS and Tris buffer, the c-Fos reaction product was made visible by incubation of sections with hydrogen peroxide and 3,3′-diaminobenzidine (DAB). The sections were then washed in distilled water, mounted in PBS, and air-dried overnight. The sections were subsequently cleared in ascending alcohol and xylene baths. Permount medium was used for a coverslip. Sections were examined under a light microscope. c-Fos reaction product appeared as dark brown staining in the cell nucleus. The sections were stained for NADPH-diaphorase (NADPH-d, a NOS) according to the histochemical method described by Vincent and Kimura (39) after they were previously stained for c-Fos product and washed in PBS for 1 h. Sections were incubated for 60 min at 37°C in a PBS solution containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium (Sigma), and 1 mg/ml β-NADPH (Sigma). The sections were then rinsed in PBS, mounted on slides, air-dried overnight, dehydrated, and coverslipped.

Cell counts and statistical analysis. Tissue sections were examined under a standard light microscope. The cellular nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB as a c-Fos label. Four to five sections of the spinal cord at the L6, L7, and S1 levels were selected for each level of each animal. The total number of c-Fos-labeled cells was counted in each spinal level for each animal. The number of labeled cells was then divided by the total number of sections counted to provide a mean cell count per slice for each level. The cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB as the c-Fos label. NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites, and axons. This offered us the opportunity to examine the codistribution of c-Fos label and NADPH-d-positive staining.

A two-way ANOVA was used for statistical comparison of changes in MAP and tension (across time and among groups) with a Student-Newman-Keuls post hoc analysis. The ipsilateral vs. contralateral data for cell counts labeled with c-Fos protein per section were analyzed by a paired t-test. The data (stimulated group, control group, and L-NAME group) for cell count labeled with c-Fos protein per section were analyzed by a one-way ANOVA. Linear regression analysis was used to characterize the relationship between the change of blood pressure and number of c-Fos-labeled cells.
RESULTS

Changes in muscle tension and MAP. The changes in peak muscle tension and MAP after electrical stimulation of the tibial nerve to induce static muscle contraction in cats are shown in Fig. 1, A and B, respectively. There was no difference for peak tension over 30 min between stimulated animals and animals with L-NAME injection. The basal MAP before induced contraction was $102 \pm 9$ mmHg in stimulated animals. Because maximal response in MAP was observed during the first three contractions, we also analyzed this response.

The maximal increase in MAP attained during the first three muscle contractions (maximal peak tension: $3.9 \pm 0.2$ kg) was $32 \pm 5$ mmHg ($P < 0.05$). The peak increases in MAP for each of the 10 contractions are shown in Fig. 1B. The MAP responses to 10 induced muscle contractions were significantly increased above baseline over 30 min of protocol in stimulated animals. In control animals, the basal MAP was $105 \pm 12$ mmHg and there was no significant difference for the MAP during the 30-min electrical stimulation period. Intrathecal injection of 5 mM L-NAME significantly reduced the maximal increases ($20 \pm 3$ mmHg) in MAP from the basal MAP of $98 \pm 10$ mmHg during the first three contractions (maximal peak tension: $3.9 \pm 0.3$ kg). There was no difference for the basal levels of MAP before stimulation among the three groups of animals.

Distribution of c-Fos label in superficial dorsal horn of spinal cord. Photomicrographs of c-Fos-positive staining in the superficial dorsal horn of the spinal cord in animal with muscle contraction and in control animal are shown in Fig. 2, A–C. Distinct c-Fos expression was observed in the superficial dorsal horn at the L6, L7, and S1 levels on the side ipsilateral to the contracting muscle after electrical stimulation of the tibial nerve. The number of c-Fos-labeled cells on the stimulated side was higher than that on the contralateral side. At the L6, L7, and S1 levels, there were $68 \pm 15$, $88 \pm 14$, and $55 \pm 12$ labeled cells per section on the ipsilateral side, respectively, and $10 \pm 4, 9 \pm 2$, and $8 \pm 2$ labeled cells per section on the contralateral side ($P < 0.05$), respectively. A few c-Fos-labeled cells were observed in control animals. For example, there were $12 \pm 5$ labeled cells per section at the L7 level in control ($P < 0.05$ compared with stimulated cats). Also, c-Fos expression was observed in the deep dorsal horn at the L6, L7, and S1 levels on the side ipsilateral to the contracting muscle after electrical stimulation of the tibial nerve. The number of c-Fos-labeled cells on the stimulated side was $15 \pm 4$ at L7. In contrast, c-Fos expression was not observed in the deep dorsal horn on the contralateral side.

In addition, a photomicrograph of c-Fos staining in the superficial dorsal horn in an animal with the prior intrathecal injection of 5 mM L-NAME is shown in Fig. 2D. At the L7 level, the number of c-Fos-labeled neurons ($58 \pm 12$ labeled cells per section) on the side ipsilateral to the contracting muscle was higher than that in control animals ($12 \pm 5$ labeled cells per section, $P < 0.05$). However, this number was reduced compared with that in stimulated animals ($88 \pm 14$ labeled cells per section, $P < 0.05$). A relationship between the reflex MAP response and number of c-Fos-labeled cells in the dorsal horn is shown in Fig. 3. The results show that there is a correlation between the maximal MAP response to muscle contraction and the number of c-Fos-labeled neurons in the superficial dorsal horn at L6, L7, and S1. In this study, it was confirmed that the dye had spread within the L6-S1 spinal cord when the spinal cord was exposed after perfusion.

Furthermore, utilizing a double-label method, it was observed that distinct c-Fos expression was in close...
proximity to neuronal processes with NADPH-d-positive staining in the superficial dorsal horn of the L6, L7, and S1 levels. For example, 48% of c-Fos-positive cells were observed to have close connections (codistributions) with NADPH-d in the superficial dorsal horn at the L7 level. It was not observed that c-Fos-positive cells had colocalization with NADPH-d in this region. The photomicrograph in Fig. 4 shows that c-Fos-positive neurons codistribute with NADPH-d staining in the superficial horn of the spinal cord at the L7 level.

**DISCUSSION**

The purpose of this study was to determine whether neurons in the superficial dorsal horn were activated during the exercise pressor reflex. Also, the role of NO in modulating the activity of these neurons in the dorsal horn and in determining the pressor response induced by muscle contraction was examined. The results of this study show that the number of c-Fos-labeled neurons was increased in the superficial dorsal horn.
from the region shown at top. Activated neurons mediate the exercise pressor response and the number of c-Fos-labeled neurons in the dorsal horn strongly suggest that these neurons in the dorsal horn were activated by muscle afferents from contracting muscle. In addition, our data showing a correlation between paralysis. Muscle afferents were not directly activated by c-Fos expression. The c-Fos-labeled neurons were distributed from the L6 to the S1 levels of the dorsal horn activated by muscle contraction. Furthermore, it has recently been shown that the lamina I neurons of the dorsal horn that project to the caudal ventrolateral medulla are activated by static muscle contraction (40). In addition, the codistribution of c-Fos neurons and NOS-positive staining in this region of the superficial horn was determined by a double-label method and provided a unique opportunity to determine the neurochemical characteristics of activated neurons. The results show that c-Fos-labeled neurons and NOS coexisted in the dorsal horn. Also, intrathecal injection of L-NAME reduced c-Fos expression and attenuated the increase in blood pressure to static muscle contraction. This suggests that NO plays a role in the activation of neurons in the dorsal horn that are involved in the exercise pressor reflex.

This finding also supports the idea that multiple spinal segments are involved in producing the exercise pressor reflex, which has been demonstrated previously (43). Furthermore, the results of this study showed that activated neurons and NOS in the dorsal horn coexisted in the superficial dorsal horn and that intrathecal injection of L-NAME to inhibit NOS activity reduced both c-Fos expression in the dorsal horn and the pressor reflex evoked by muscle contraction.

The reflex cardiovascular responses to static muscle contraction are mediated by both group III (thinely myelinated Aδ) and group IV (unmyelinated C) fibers (25, 26). The majority of group III and group IV skeletal muscle afferent fibers make their first synapse in the dorsal horn of the spinal cord (24, 29). Previous studies (12, 13, 42, 44) have shown the modulatory role of neurotransmitters and neuromodulators in the dorsal horn in the exercise pressor reflex. For example, static muscle contraction increased the extracellular concentration of substance P and glutamate in the dorsal horn (12, 42). It was also found that microdialysis of antagonists to substance P and to N-methyl-D-aspartic acid (NMDA) and non-NMDA glutamate receptors into the dorsal horn significantly attenuated the reflex cardiovascular responses to static muscle contraction (13, 14, 44). These findings show that the reflex pressor response to muscle contraction is mediated by release of neurotransmitters and/or neuromodulators, and by activation of their receptors at the level ofafferent fiber entry into the dorsal horn. Furthermore, it has been recently reported that NO production in the dorsal horn had a modulatory role in pressor reflex evoked by muscle contraction (41). The study suggests that an increase of NO in the dorsal horn enhances the excitability of neurons to muscle afferent input (41).

In the present study, neurons in the superficial dorsal horn activated by muscle contraction were identified by c-Fos expression. The c-Fos-labeled neurons were distributed from the L6 to the S1 levels of the dorsal horn, in which skeletal muscle afferents were believed to form the first synaptic sites for expression of the exercise pressor reflex (43). Previous studies (4, 5) using electrophysiological methods have also shown that neurons in the superficial and deep dorsal horn at the L7 level are excited during muscle contraction. Furthermore, it has recently been shown that the lamina I neurons of the dorsal horn that project to the caudal ventrolateral medulla are activated by static muscle contraction (40). In addition, the codistribution of c-Fos neurons and NOS-positive staining in this region of the superficial horn was determined by a double-label method and provided a unique opportunity to determine the neurochemical characteristics of activated neurons. The results show that c-Fos-labeled neurons and NOS coexisted in the dorsal horn. Also, intrathecal injection of L-NAME reduced c-Fos expression and attenuated the increase in blood pressure to static muscle contraction. This suggests that NO plays a role in the activation of neurons in the dorsal horn that are involved in the exercise pressor reflex.
Excitatory amino acid (EAA) in the superficial dorsal horn of the spinal cord have been shown to participate in the neurotransmission of signals from the primary afferent fibers to dorsal horn neurons (6, 7). EAA excites NMDA and non-NMDA glutamate receptors located in the dorsal horn (10). EAA binds to NMDA receptors to cause calcium channel opening, and influx of calcium ions into the cell then activates a variety of cellular events, including activation of NOS (9, 20, 46), which is also located in the dorsal horn (1). It has been demonstrated that activation of NMDA receptors produced NO in neural tissue (9, 23, 27, 28). At present, it is believed that diffusible NO then activates a cGMP-dependent mechanism as an intercellular messenger via guanylate cyclase (2, 8, 9, 20). Inhibition of guanylate cyclase activation by methylene blue has been reported to block the effect of a NO donor in the central nervous system (21).

It has been shown that muscle contraction increased EAA concentrations, glutamate, and aspartate in the dorsal horn (12). Furthermore, blockade of NMDA and non-NMDA receptors in this region attenuated the pressor response to muscle contraction (13, 14). Therefore, activation of skeletal muscle afferents evokes release of EAA into the dorsal horn of the spinal cord. EAA acts on NMDA receptors to activate NOS, which catalyzes the formation of NO from L-arginine (20). Microdialysis of L-arginine into the L7 dorsal horn increased the peak response to static muscle contraction (41). In the present study, we have found that intrathecal administration of L-NAME to inhibit NOS activity attenuated the pressor response to muscle contraction in addition to a reduction in c-Fos expression.

C-Fos protein has been used as an anatomic marker of neuronal activation by a variety of physiological and pharmacological stimulations (35). However, it has been shown that c-fos is an early response gene and its transcription products control the expression of the late response genes (31). This may be an important link between cell stimulation and subsequent alteration in gene expression. It has been reported that c-fos antisense oligodeoxynucleotide applied to the spinal cord alters formalin-induced nociception (17, 18). Furthermore, NMDA glutamate receptors have been shown to couple to c-fosactivation (15, 36, 37). NMDA receptors have also been implicated in long-term excitability of dorsal horn cells (27, 28). For example, hyperexcitability of the dorsal horn neurons induced by peripheral inflammation was reduced by blockade of NMDA receptors (32). Furthermore, NO has also been implicated in causing hyperexcitability of the dorsal horn in hyperalgesic animals (27, 28). It has been shown that NO release in the dorsal horn was caused by an intradermal injection of capsaicin, an intervention that produces hyperexcitability (45). It seems that the NMDA-NO cascade appears to be involved in producing a hyperexcitable state in the dorsal horn neurons. Activation of the c-fos early response gene is linked to a cGMP mechanism, which is activated by diffusible NO via guanylate cyclase (11). It has been shown that intrathecal injection of the selective inhibitor cGMP-dependent protein kinase Iα produced a significant antinociception, and this was accompanied by a marked reduction in formalin-induced c-fos expression in the spinal cord (38). In addition, c-Fos expression induced by noxious stimulation in the dorsal horn was reduced by L-NAME to inhibit NOS (16, 33). In this study, L-NAME reduced the number of activated neurons with c-Fos expression and attenuated the pressor response to muscle contraction. A cGMP-dependent mechanism via guanylate cyclase mediated by NMDA-NO cascade is likely to be involved in the c-Fos expression in activated neurons during the exercise pressor reflex.

In summary, our study has shown that static contraction of skeletal muscle activates neurons in the dorsal horn of the spinal cord, the major site of the first synaptic involved in skeletal muscle afferents eliciting the exercise pressor reflex. The neurons activated by muscle contraction coexist with NOS-positive staining in the dorsal horn, suggesting that NO is one of the neurotransmitters or neuromodulators involved in modulating this reflex. This concept is supported by our data showing that decreases in NO formation by the intrathecal injection of L-NAME reduces c-Fos expression and attenuates the blood pressure response to muscle contraction. Thus the results suggest that the pressor reflex induced by static muscle contraction is mediated by the activation of neurons in the superficial dorsal horn and that the formation of NO modulates the activation of these neurons and the blood pressure response to muscle contraction.

The authors express gratitude to James Jones, Julius Lamar, Jr., and Margaret Robledo for technical assistance.

This study was supported by American Heart Association, Texas Affiliate, Grant 9960088Y (to J. Li), by National Heart, Lung, and Blood Institute Grant HL-06296, and by the Lawson and Rogers Lacy Research Fund in Cardiovascular Diseases (to J. H. Mitchell).

REFERENCES


