Relationship between neuronal nitric oxide synthase and NADPH-diaphorase in the dorsal root ganglia during neuropathic pain

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Background: Changes in nitric oxide (NO) production in the dorsal root ganglia (DRG) may contribute to allodynia after nerve injury. It is known that the histochemistry of NADPH-diaphorase (NADPH-d) is known to be not always coincident with NOS. This study was conducted to investigate the relationship between nNOS and NADPH-d expression in the DRG in a spinal nerve injury model of neuropathic pain, and to elucidate role that NO plays in neuropathic pain.

Methods: nNOS immunohistochemistry and/or NADPHP-d histochemistry were conducted in the DRG of a spinal nerve transection model of neuropathic pain, and the pain behavior was then measured by a von Frey filament test of the hindpaws of wild type and nNOS knock-out mice.

Results: nNOS immunoreactive neurons and NADPH-d stained neurons were not always identical. Additionally NADPH-d increased, but nNOS did not increase significantly in the DRG after spinal nerve transection. Neuropathic pain behavior increased in the hindpaw of nNOS(−/−) mice after spinal nerve transection, but was lower than that of wild type mice after spinal nerve transection.

Conclusions: nNOS immunoreactive neurons and NADPH-d stained neurons were not always identical in the DRG, and a novel NADPH-d positive source may be involved in neuropathic pain after spinal nerve transection. Changes in nNOS expression in the DRG were not the primary cause of neuropathic pain behavior in a spinal nerve transection model of neuropathic pain. (Korean J Anesthesiol 2009; 57: 342~9)

Key Words: Dorsal root ganglion, NADPH-diaphorase, Neuropathic pain, Nitric oxide synthase.

INTRODUCTION

Changes in nitric oxide (NO) production in spinal cord or DRG may contribute to allodynia after nerve injury [1]. NO release in spinal cord or DRG is evoked by N-methyl-D-aspartate (NMDA) receptors activation [2,3]. NO has been shown to enhance the release of excitatory amino acids [4]. Pharmacological evidence regarding the role of spinal NO in the development of nerve injury-evoked allodynia has been conflicting. Some investigators observed allodynia inhibition in nerve-injured rats after treatment L-NG-nitro-arginine methyl ester (L-NAME), a nonspecific nitric oxide synthase (NOS) inhibitor [4,5]. However, other investigators reported that nNOS regulation in DRG neurons may play an important role in neuroplasticity, not in neuropathic pain, after nerve injury [6,7].

Previous studies have indicated that NOS-labeled cells increase in the ipsilateral DRG following sciatic nerve transections of L5 and L6 spinal nerves [8,9]. In addition, peripheral nerve section results in increase in NOS mRNA in the corresponding DRG [10]. A model of nerve-injury-induced pain demonstrates that ipsilateral decrease in paw withdrawal threshold is associated with an increase in total NOS activity in the DRG, which show that NOS activity are relevant to the genesis and/or maintenance of altered pain behavior [11].
However, Luo et al. [6] reported that systemic treatment with a specific pharmacological inhibitor of nNOS failed to prevent or reverse alldynia in nerve-injured rats. They insisted that upregulation of nNOS is not responsible for the development and/or maintenance of alldynia after nerve injury.

The activity of all known subtype of NOS (nNOS, eNOS, iNOS) is dependent on NADPH, flavin mononucleotide (FMN), and flavin-adenin dinucleotide (FAD), therefore, the activity of NOS has often measured cytochemically as NADPH-diaphorase (NADPH-d) activity [12,13]. Neuronal NADPH-d is a nitric oxide synthase, and NADPH-d histochemistry provides a specific histochemical marker for neurons producing NO.

However, a study demonstrates that NADPH-d histochemistry is not always coincident with NOS and provides evidence for an as yet uncharacterized subtype of NOS in the rat anterior pituitary [14]. nNOS-immunoreactive neurons and NADPH-d stained neurons are not identical and that nNOS does not increase as a result of hindpaw inflammation, leaving the source of NO involved in thermal hyperalgesia following injury in question [15].

The aim of this study was to investigate relationship between nNOS and NADPH-d expressions in DRG in a spinal nerve transection model of neuropathic pain, and elucidate role of NO in neuropathic pain using nNOS knock-out mice.

**MATERIALS AND METHODS**

Twelve male wild type C57BL/6 (3–4 months old, 25–30 g) and 12 male nNOS(−/−) (3–4 months old, 25–30 g), NOS(−/−) mice were used in this experiment. The nNOS(−/−) mice were obtained from Dr. Dae-Yeul Yu (Induced Mutant Resources Program, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). They were maintained under the standard laboratory conditions on a 12-hour light/dark cycle, with free access to food and water. All animal-related procedures and care were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology.

A unilateral peripheral neuropathy was induced by spinal nerve transection previously described by Wolf et al. [16]. Briefly, under gaseous anesthesia with a mixture of halothane and 1 : 2 flow ratio of N2O/O2, the left 5th lumbar (L5) spinal nerve was isolated and cut, and the distal stump was excised forming a gap of a few millimeters between the stumps to avoid nerve regeneration. Hemostasis was confirmed and the wound was then sutured. Neuropathic pain behavior was measured in all animals (24 mice) before surgery and 1 week after surgery. nNOS immunohistochemistry and NADPH-d histochemistry were performed in 6 mice of each (wild and nNOS knock-out) group after perfusion fixation. Neuropathic pain behavior was measured in remaining 6 mice of each group 2 weeks after surgery.

Seven days after neuropathic surgery, neuropathic pain behavior was measured in all animals, and then 6 mice in each group were selected randomly. The selected animals were subjected to thiopental sodium anesthesia (50 mg/kg, intraperitoneal) and transcardiac perfusion fixation with saline followed by 4% phosphated-buffered paraformaldehyde. The L5 DRG of ipsilateral and contralateral side of the mouse were obtained, post-fixed in cold 4% paraformaldehyde solution, and then placed in a 30% sucrose solution at 4°C overnight. Frozen sections (30 μm thick) parallel to the spinal nerve were prepared in each tissue and collected in phosphated buffered solution (PBS) in 24-well plates.

To perform immunohistochemistry for nNOS, the 3 tissue sections of each DRG were immersed for 30 min in 3% H2O2 to inactivate endogenous peroxidases. Sections were incubated 1 hour at room temperature (RT) in the polyclonal nNOS antibody (1 : 250, Santa Cruz, USA) in 0.1 M PBS, pH 7.4, containing 0.1% Triton X-100, 1.5% bovine serum albumin (BSA), and 1 : 200 normal goat serum (NGS), followed by incubation for 1hour at RT in 1 : 200 biotinylated goat anti-rabbit IgG (Vector, USA) and 1 : 200 NGS in PBS. Immunoreactions were visualized by incubation for 1 hour at RT in avidin-biotin-peroxidase complex (1 : 100, ABC kit, Vector, USA) in PBS and 5–10 min in 0.05% 3,3′-diaminobenzidine (DAB) and 0.01% H2O2 in 0.1 M PBS.

The three sections of each DRG were processed in multiwell for NADPH-d activity according to the procedure of Vincent and Kimura [17]. The sections were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.1 mg/ml β-NADPH (Sigma Chemical, USA) at 37°C for 45–60 min. Following the reaction, the sections were rinsed in phosphate buffer (pH 7.4), air-dried overnight, and coverslip with Canada Balsam (Kanto, Japan).

To do double labelling of NADPH-d and nNOS, NADPH-d histochemistry was performed in the sections of DRG, and then followed by nNOS immunohistochemistry in the same tissue sections.
One and two weeks after surgery, frequency of foot withdrawal in response to normally innocuous mechanical stimuli was measured. Mechanical stimuli were applied with a set of von Frey filaments ranging from 8.4 to 186.7 mN (8.4, 13.5, 24.5, 54.4, 100.5, and 186.7 mN). The mice were placed on a metal mesh floor covered by a transparent plastic dome, adapted 30 min in the test cage, and von Frey filaments were applied from underneath the metal mesh floor to the plantar surface of the foot. The von Frey filament was applied 10 times (once every 3−4 s) to each paw. The occurrence of foot withdrawal was measured as a percentage (number of trials accompanied by foot withdrawal/10 × 100 = % frequency of foot withdrawal). Average of foot withdrawal frequencies to 6 von Frey filaments was used for final foot withdrawal frequency of each foot.

All area of the tissue section was photographed with a light microscope (magnification, ×200). The total number labeled and unlabeled cell profiles was counted manually in the photographs of three tissue sections in each DRG, and the percentage of nNOS immunoreactive or NADPH-d positive profiles per section was calculated. The percentage of nNOS labeled profiles per section and foot withdrawal frequencies were expressed as mean ± SD. The differences were analyzed by Student t-test. P < 0.05 was considered to be statistically significant.

**RESULTS**

nNOS immunoreactivity was found in the cytoplasm of the small and medium-sized neurons in the DRG, not in the large neurons in the contralateral (not operated) and ipsilateral (spinal nerve transecting) DRGs of C57/B6 mice. Number of nNOS positive cells tended to increase in the ipsilateral DRG, compared with contralateral DRG, but failed to reach statistical significance (P = 0.08, Fig. 1).

NADPH-d histochemical reactivity was found in the cyto-
plasm of the small and medium-sized neurons in the contralateral DRG, as similar to expression pattern of nNOS immunoreactivity (Fig. 2A) in the C57/BL6 mice. However, number of NADPH-d positive cells increased significantly (P < 0.001) in the ipsilateral DRG 1 week after spinal nerve transection, compared with contralateral DRG (Fig. 2B,C). Especially, NADPH-d histochemical reactivity increased in large neurons in the ipsilateral DRG of C57/BL6 mice (Fig. 2B).

Double labeling with nNOS and NADPH-d was found in the small and medium-sized neurons in the contralateral DRG of C57/BL6 mouse (white asterisk in Fig. 3A). However, nNOS positive and NADPH-d negative labeling was seen in the small-sized neurons (black asterisks in Fig. 3B) in the DRG. Double labeling with nNOS and NADPH-d was also found in the small and medium-sized neurons in the ipsilateral DRG of C57/BL6 mouse 1 week after spinal nerve transection (white asterisk in Fig. 3B). In contrast to the contralateral DRG, NADPH-d positive, not nNOS positive, large neurons (vacant arrow in Fig. 3B) was found in the ipsilateral DRG 1 week after spinal nerve transection. In addition, NADPH-d positive fibers showing beaded pattern were found among the neurons in the DRG (arrows in Fig. 3B).

nNOS immunoreactivity was found in the DRG of wild type (C57/BL6) mouse, but was not detected in the DRG of nNOS(−/−) mouse (Fig. 4A,B). However, NADPH-d positive cells were found in the contralateral DRG of nNOS(−/−) mouse, similar to the contralateral DRG of wild type mouse (Fig. 5A,C) after spinal nerve transection. Furthermore, number of the NADPH-d positive cells increased in the ipsilateral DRG of nNOS(−/−) mouse 1 week after spinal nerve transection compared with the contralateral DRG of the nNOS(−/−) mouse (Fig. 5B,D). Especially, localization of NADPH-d histochemical reactivity was eccentric in the small neurons in the ipsilateral DRG of nNOS(−/−) mouse (Fig. 5D).

Fig. 2. (A) NADPH-d histochemical reactivity is found in the small and medium sized neurons in the contralateral DRG of C57/BL6 mice 1 week after spinal nerve transection. (B) NADPH-d histochemical reactivity is found in the small, medium, and large sized-neurons in the ipsilateral DRG of C57/BL6 mice 1 week after spinal nerve transection. (C) Percentage of NADPH-d positive profiles is higher significantly in the ipsilateral DRG compared with the contralateral DRG of C57/BL6 mouse 1 week after spinal nerve transection (*P < 0.01). Values are expressed as mean ± SD. Scale bar, 100 μm.
Fig. 3. (A) nNOS positive and NADPH-d positive neurons (white asterisk) and nNOS positive and NADPH-d negative neurons (black asterisk) were found in the contralateral DRG of C57/BL6 mice 1 week after spinal nerve transection. (B) nNOS positive and NADPH-d positive neurons (white asterisk), nNOS positive and NADPH-d negative neurons (black asterisk), nNOS negative and NADPH-d positive neurons (vacant arrow), and NADPHD-d positive fibers (arrows) were found in the ipsilateral DRG of C57/BL6 mice 1 week after spinal nerve transection. Scale bar, 50 μm.

Fig. 4. (A) nNOS immunoreactive neurons are found in the DRG of wild type (C57/BL6) mice. (B) nNOS immunoreactive neurons are not found in the DRG of nNOS(−/−) mice. Scale bar, 50 μm.

Foot withdrawal responses to von Frey filament increased in the ipsilateral hindpaws in both wild type and nNOS(−/−) mice 1 week after spinal nerve transection. However, foot withdrawal frequencies in nNOS(−/−) mice were lower than that of wild type mice 1 week after spinal nerve transection ($P < 0.01$), and tend to be lower than that of wild type mice 2 weeks after surgery, but failed to reach statistical significance ($P = 0.09$, Fig. 6).

**DISCUSSION**

This study shows that nNOS immunoreactive neurons and NADPH-d stained neurons were not always identical, and NADPH-d increased, nNOS did not increase significantly, in the DRG after spinal nerve transection. Neuropathic pain behaviors in ipsilateral hindpaws in nNOS(−/−) mice, even lower than wild type mice, increased after spinal nerve transection com-
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Fig. 5. (A) NADPH-d positive cells are found in the contralateral DRG of wild type mice 1 week after spinal nerve transection. (B) Number of the NADPH-d positive cells increase in the ipsilateral DRG of wild type mice 1 week after spinal nerve transection compared with the contralateral DRG. (C) NADPH-d positive cells are found in the contralateral DRG of nNOS(−/−) mice, similar to the contralateral DRG of wild type mice, 1 week after spinal nerve transection. (D) Number of the NADPH-d positive cells increase in the ipsilateral DRG of nNOS(−/−) mouse 1 week after spinal nerve transection compared with the contralateral DRG. Scale bar, 100 μm.

Immunoreactivity for nitric oxide synthase (NOS) was investigated in lumbar (L1, L4, L5 and L6) dorsal root ganglia from naive controls and from rats surviving for 3, 7, and 14 days after unilateral ligation of the L5 and L6 spinal nerves [12]. Quantitative analysis revealed significant increases in the percentage of NOS immunoreactive cells profiles in L5 and L6 ganglia on the operated side at all time points, with the number of labeled profiles increasing with time following ligation. These findings suggested that nitric oxide may have a role in the generation and/or maintenance of neuropathic pain [6]. Increase in DRG nNOS protein was observed and localized principally to small and occasionally medium-size sensory neurons. However, upregulation of nNOS is not responsible for the development and/or maintenance of allodynia after nerve injury [6].

In this study, number of nNOS positive cells tended to increase in the DRG after spinal nerve transection, but failed to reach statistical significance. Quantitative analysis of the immunostaining data revealed that L5/6 DRGs ipsilateral to the nerve injury contained (P < 0.001) more nNOS positive cells (11.3 ± 1.4%) than do L5/6 DRGs from sham-operated rats (2.2 ± 0.3%) [6]. Quantitative study in this experiment showed that percentage of nNOS positive neurons were 30.5 ± 4.3% and 35.0 ± 3.7% respectively in the contralateral and ipsilateral DRGs of C57/BL6 mice 1 week after spinal nerve
Fig. 6. Foot withdrawal frequencies to von Frey filaments are higher in the ipsilateral hindpaw of wild type mice 1 week after spinal nerve transection compared with the ipsilateral hindpaw of nNOS(-/-) mice (P < 0.01), and tend to be lower than that of wild type mice 2 weeks after spinal nerve transection, but fail to reach statistical significance (n = 12 in each group 1 week after surgery, n = 6 in each group 2 weeks after surgery).

In this study, nNOS immunoreactive neurons and NADPH-d stained neurons were not identical, and NADPH-d positive cells increased, nNOS immunoreactive cells did not increase significantly, in the DRG after spinal nerve transection. There were 3 types of cells in the DRG in double labeling of nNOS and NADPH-d. First type of cells was both nNOS and NADPH-d positive. Second type was nNOS positive and NADPH-d negative. Third type was nNOS negative and NADPH-d positive. First type (both nNOS and NADPH-d positive) was definitely nNOS containing neurons producing NO. nNOS is primary NO regulator in neurons. The activity of the enzyme is inhibited by a protein inhibitor called protein inhibitor of neuronal nitric oxide synthase (PIN) [20]. Immunohistochemical analysis revealed that spinal cord injury (SCI) causes an increase in both NP847 and p-Thr286-CaM-KIIα in the nucleus intermediolateralis. These findings suggest that SCI induces p-Thr286-CaM-KIIα, which phosphorylates the nNOS at Ser847 [21]. Second type of cells in this study (nNOS positive and NADPH-d negative) may contain inactive nNOS (inhibited by PIN or not phosphorylated) in the DRG. Third type of cells (nNOS negative and NADPH-d positive) may be an uncharacterized subtype of NOS or a novel substance which is not related to NO production.

In conclusion, change of nNOS expression in the DRG was not main cause of neuropathic pain behavior, and novel NADPH-d positive source may involved in neuropathic pain in a spinal nerve transection model of neuropathic pain.
REFERENCES


