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UPREGULATION OF NEURONAL NITRIC OXIDE SYNTHASE IN THE PERIPHERY PROMOTES PAIN HYPERSENSITIVITY AFTER PERIPHERAL NERVE INJURY

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Abstract—Peripheral nerve injury often results in neuropathic pain that is manifested as hyperalgesia, and allodynia. Several studies suggest a functional role for neuronal nitric oxide synthase (nNOS) in the development or maintenance of neuropathic pain, but such a contribution remains unclear. In our current study, we found that intraplantar injection of the NOS substrate L-arginine or NO donor 3-morpholino-synonimine (SIN-1) produced mechanical hypersensitivity that lasted more than 24 h. Following L5 spinal nerve ligation (L5 SNL), immunoreactivity for nNOS in the ipsilateral L5 but not L4 dorsal root ganglion (DRG) was dramatically increased in mainly small- and medium-sized neurons and non-neuronal cells. L5 SNL caused increased nNOS immunoreactivity in the ipsilateral sciatic nerve, mainly in Schwann cells and the ipsilateral glabrous hind paw skin, mainly on the basement membrane. Furthermore, total nNOS protein and mRNA in the ipsilateral sciatic nerve and hind paw skin were markedly upregulated following nerve injury. Intraplantar injection of the NOS inhibitor 7-nitroindazole (7-NI) or the non-specific NOS inhibitor L-N^G-nitro-arginine methyl ester (L-NAME) effectively suppressed SNL-induced mechanical allodynia. Collectively, these data suggest that in the periphery nNOS upregulation induced by peripheral nerve injury contributes to mechanical hypersensitivity during the maintenance phase of neuropathic pain. Blocking nNOS signaling in the periphery may thus be a novel therapeutic strategy for the treatment of neuropathic pain.

Key words: peripheral nerve injury, mechanical allodynia, periphery, neuronal nitric oxide synthase, nitric oxide.

Peripheral nerve injury often results in hyperalgesia and allodynia, which are associated with neuropathic pain. Although many studies have made considerable progress toward understanding neuropathic pain, the mechanisms underlying neuropathic pain are poorly understood. In the past, most reports have focused on only the neurons that drive the establishment and/or maintenance of neuropathic pain. However, recent investigations have demonstrated the involvement of non-neuronal cells, such as immune cells and glial cells, in the pathogenesis of neuropathic pain (Oh et al., 2001; Ma and Eisenach, 2003; Zelenka et al., 2005; Thacker et al., 2009; Shibasaki et al., 2010). Nerve injury-induced events in non-neuronal cells can stimulate or recruit other cells or neurons, release a variety of factors that are crucial for pain, and induce peripheral sensitization, thus directly increasing the excitability of nociceptors. These immune and glial cell responses to peripheral nerve injury occur at several locations such as dorsal root ganglia (DRG), spinal cord, sciatic nerve, and peripheral sensory terminals (Takahashi et al., 2004; Zhuang et al., 2006; Scholz and Woolf, 2007; Shibasaki et al., 2010). However, much less is known about the interaction between neuronal and non-neuronal cells in the periphery under neuropathic pain conditions.

Nitric oxide (NO) is a diffusible molecule that acts as an important modulator in the central and peripheral nervous systems and that functions in various physiologic and pathophysiologic processes (Snyder, 1992; Meller and Gebhart, 1993; Prast and Philippu, 2001). As NO activity is tightly regulated by nitric oxide synthase (NOS), changes in expression on NOS may regulate the pathophysiologic functions on NO in the nervous system. Neuronal NOS (nNOS) is expressed in the neurons of the central and peripheral nervous system and predominantly produces NO in neuronal tissues. The contribution of nNOS to pain hypersensitivity has been characterized in neuropathic pain models (Luo and Cizkova, 2000; Guan et al., 2007). First, nerve injury upregulates nNOS expression in DRG neurons (Choi et al., 1996; Luo et al., 1999; Cizkova et al., 2002; Shortland et al., 2006; Guan et al., 2007), changes nNOS immunoreactivity in the spinal cord (Fiallos-Estrada et al., 1993; Steel et al., 1994; Cizkova et al., 2002; Ma and Eisenach, 2007; Chacur et al., 2010), and

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Abbreviations: ATF3, activating transcription factor 3; CCI, chronic constriction injury; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene related peptide; D-NAME, D-N^G-nitro-arginine methyl ester; DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein; IB4, isolectin 4; IL, interleukin; IR, immunoreactive; L-NAME, L-N^G-nitro-arginine methyl ester; L5 SNL, L5 spinal nerve ligation; MPE, maximal possible effect; NF200, neurofilament 200; NMDA, N-methyl-D-aspartate, nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffer saline; PGP9.5, protein gene peptide 9.5; PWTs, paw withdrawal thresholds; SIN-1, 3-morpholino-synonimine; TRPV1, transient receptor potential cation channel subfamily V member 1; 7-NI, 7-nitroindazole.

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alters the catalytic activity of nNOS in the DRG and spinal cord (Choi et al., 1996; Cizkova et al., 2002). In addition, genetic knockout of nNOS in mice attenuates pain hypersensitivity induced by nerve injury (Guan et al., 2007). It is suggested that NO synthesized by nNOS in the DRG or spinal cord following nerve injury activates protein kinase and/or ion channels and thus results in neuronal excitability to cause pain hypersensitivity (Qian et al., 1996; Kim et al., 2000; Yoshimura et al., 2001). However, pharmacological evidence of specific and non-specific nNOS inhibitors administrated systemically or spinally to alleviate nerve-injury induced pain hypersensitivity remains controversial (Meller et al., 1992; Yoon et al., 1998; Luo et al., 1999; Pan et al., 1998; Lee et al., 2005; Guan et al., 2007; Chacur et al., 2010). This discrepancy can be explained by the differences in the delivery method, dose, drug potency or administration time after nerve injury. However, non-systemic or non-spinal administration can attenuate pain hypersensitivity induced by nerve injury, but the analgesic effects of peripherally administrated specific and non-specific nNOS inhibitor on neuropathic pain have not been reported.

In our current study, we hypothesized that upregulation of nNOS in the periphery of nerve-injured rats promotes pain hypersensitivity. To test this hypothesis, we (1) asked whether injection of an exogenous NOS substrate or NO donor injection into the peripheral hind paw can induce mechanical hypersensitivity, (2) investigated nNOS expression in DRGs, sciatic nerve, and hind paw skin after peripheral nerve injury, and (3) examined the effects of injected nNOS or NOS inhibitors into the periphery on spinal nerve ligation (SNL)-induced mechanical allodynia.

EXPERIMENTAL PROCEDURES

These experiments conformed to the ethics guidelines of the International Association for the Study of Pain (IASP, 1983) and the National Institutes of Health (USA). All procedures in this study were accorded with the National Institute of Health Guide for the Care and Use of Laboratory Animals (USA) and approved by the Seoul National University Hospital Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Animals

Male Sprague–Dawley rats (230–250 g, Harlan) were used in all experiments. The animals were housed in groups of two to four per case, with food and water available *ad libitum*. All animals were acclimated on 12-h light/dark cycle under standardized environmental conditions.

Surgery

L5 SNL was performed as described previously (Jang et al., 2007). Briefly, all experimental procedures were performed under enflurane anesthesia (3% for induction and 2% for maintenance). A skin incision was made above the middle lumbar spine and the left transverse process of L6 vertebra was identified. After carefully removing the process, the L5 spinal nerve was isolated. The nerve was tightly ligated with 6-0 silk thread and transected about 1 mm distal to the ligation. The wound was aseptically sutured and maintained with proper postoperative care. In sham operation group, these procedures were done in the same manner, except for ligation and cut of the L5 spinal nerve.

Immunohistochemistry

Rats were anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.4. The L4 and L5 DRGs, sciatic nerve, and surface of hind paw skin were dissected. For sciatic nerve staining, about 50 mm of the sciatic nerve was transected at the proximal portions of 50 mm apart from the region where the distal sciatic nerve splits. The sciatic nerve was then cut longitudinally or transversely. All tissues were post-fixed for 12 h at 4 °C in the same fixative and cryoprotected in 0.1 M PBS containing 20% sucrose overnight at 4 °C. Samples were mounted in OCT and cryosectioned at 10 μm (DRGs and sciatic nerve) or 30 μm (hind paw skin). For a single nNOS staining, standard biotin-streptavidin techniques were used. Frozen sections were washed three times with 0.3% Triton X-100 in 0.1 M PBS (T-PBS), incubated with 0.3% hydrogen peroxide for 15 min, and then blocked with 3% normal goat serum in T-PBS for 1 h. Samples were incubated with mouse anti-nNOS (1:1000; BD Biosciences, San Jose, CA, USA) overnight at 4 °C. After additional wash, sections were incubated with biotinylated secondary antibody (Zymed, San Francisco, CA, USA) for 1 h at room temperature and then applied with streptavidin-conjugated horseradish peroxidase (Zymed) for 15 min at room temperature. The specific nNOS binding was visualized with 3, 3-diaminobenzidine (DAB) (Zymed) and the sections were lightly counterstained with Hematoxylin (Sigma, St. Louis, MO, USA).

For double immunofluorescent staining, frozen sections were washed and blocked as mentioned above. Rabbit anti-calcitonin gene related peptide (CGRP) (1:2000; Millipore, Billerica, MA, USA), transient receptor potential cation channel subfamily V member 1 (TRPV1) (1:1000; Millipore), activating transcription factor 3 (ATF3) (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glial fibrillary acidic protein (GFAP) (1:200; Sigma), S100 (1:400; Santa Cruz), or protein gene product 9.5 (PGP9.5) (1:1000; Abcam, Cambridge, UK) was incubated with mouse anti-nNOS (1:1000; BD) overnight at 4 °C. After an additional rise with T-PBS, the sections were incubated with anti-rabbit Alexa-Fluoro594 (1:500; Invitrogen, Carlsbad, CA, USA) and anti-mouse Alexa-Fluoro488 (1:500; Invitrogen) for 2 h at room temperature. For IB4 colocalization with nNOS, biotinylated isolectin 4 (IB4) (1:300; Sigma) and mouse anti-nNOS (1:1000; BD) were incubated overnight at 4 °C. After washing, the sections were incubated with anti-mouse Alexa-Fluoro488 (1:500; Invitrogen) and CY3-conjugated streptavidin (1:500; Sigma). For analysis of double-staining with neurofilament 200 (NF200) and nNOS, mouse monoclonal anti-NF200 antibody N52 (1:1000; Sigma) or mouse monoclonal anti-nNOS (1:1000; BD) were labeled with Alexa-Fluoro594 monoclonal antibody labeling kit (Invitrogen) or Alexa-Fluro488 monoclonal antibody labeling kit (Invitrogen), respectively. For glabrous hind paw and sciatic nerve samples, DAPI staining was examined as control staining. The specificity of the staining was confirmed by omitting of primary or secondary antibodies. Stained sections were visualized under LSM510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

Quantitative analysis

Visualized DRG neurons with DAB were identified by the typical morphology in the presence of a nucleus. In each rat, four to seven sections of the L4/L5 DRG on day 7 post-SNL were randomly selected. Splitting of neuronal nuclei sections between sections can overestimate true cell profiles. We, therefore, corrected split nuclei and calculated the neuronal number for each ganglion according to Konigsmark's formula (Konigsmark, 1970). We divided the DRG neurons into small ($<$30 μm), medium (30–50 μm), and large ($>$50 μm) neurons according to the mean of long- and short-axes of the neuronal soma (Harper and Lawson, 1985). Nuclear diameters were measured as described previously (Jang et al., 2007). At least 300