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# Nitric oxide (NO) serves as a retrograde messenger to activate neuronal NO synthase in the spinal cord via NMDA receptors

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**Abstract**

We have recently demonstrated that nitric oxide (NO) produced by neuronal NO synthase (nNOS) in the spinal cord is involved in the maintenance of neuropathic pain. To clarify whether NO itself affected nNOS activity in the spinal cord as a retrograde messenger, we examined the involvement of the NO/cGMP signaling pathway in the regulation of nNOS activity by NADPH-diaphorase histochemistry. NO-generating agents NOR3 ($t_{1/2} = 30$ min) and SNAP ($t_{1/2} = 5$ h), but not NOR1 ($t_{1/2} = 1.8$ min), significantly enhanced NADPH-diaphorase staining in the spinal cord. 8-Br-cGMP also enhanced it similar to that by NOR3, and 8-Br-cAMP and forskolin, an activator of adenylate cyclase, enhanced it moderately. NOR1 and NOR3 markedly increased the cGMP level in the spinal cord. The enhancement of NADPH-diaphorase staining by NOR3 was significantly inhibited by CPTIO, an NO scavenger, ODQ, a soluble guanylate cyclase inhibitor, and KT5823, an inhibitor of cGMP-dependent protein kinase. Additionally, the NOR3-enhanced nNOS activity was completely inhibited by NMDA antagonists MK-801 and D-AP5, partially by the GluRε2-selective antagonist CP-101,606, and was attenuated in GluRε1$^{-/-}$ and GluRε1$^{-/-}$/ε4$^{-/-}$ mice. These results suggest that NO may regulate nNOS activity as a retrograde messenger in the spinal cord via activation of NMDA receptor containing GluRε1 and GluRε2 subunits.


*Keywords:* Nitric oxide (NO); Neuronal nitric oxide synthase (nNOS); NO donor; NADPH diaphorase; NMDA receptors; Spinal cord

Nitric oxide (NO)[1] is produced from L-arginine by three isoforms of NO synthase (NOS); neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) and plays important roles in a wide variety of physiological and pathophysiological processes such as neurotransmission, regulation of vascular tone, and mediation of immune responses [1–3]. Considerable evidence has demonstrated that activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors and subsequent NO production are key events in neurotransmission and synaptic plasticity in the nervous system including pain perception and central sensitization in the spinal cord [4,5]. NO activates soluble guanylate cylcase and increases the generation of guanosine 3′,5′-cyclic monophosphate (cGMP) [6]. In fact, noxious stimulation increased NOS expression [7–9] and cGMP content [10] in the spinal cord. Although cGMP modifies several intracellular processes including activation of protein kinases, ion channels and phosphodiesterases, a primary action of elevated cGMP levels is the stimulation of cGMP-dependent protein kinase I (PKGI) in

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[1] *Abbreviations used:* ACSF, artificial cerebrospinal fluid; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; CPTIO, 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide; eNOS, endothelial NOS; IBMX, 3-isobutyl-1-methylxanthine; iNOS, inducible NOS; NADPH-d, NADPH-diaphorase; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; nNOS, neuronal NOS; NOR1, (±)-(E)-methyl-2[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexemide; NOR3, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide; NOS, NO synthase; ODQ, 1*H*-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; PKGI, cGMP-dependent protein kinase I; PKGIα, α isoform of PKGI; PBS, phosphate-buffered saline; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside.

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the nervous system as well as the vascular system [11,12]. PKGI exists in two isoforms designated α and β [11]. Recently, the α isoform of PKGI (PKG-Iα) has been shown to be expressed in a subpopulation of small- and medium-sized neurons in dorsal root ganglia and superficial laminae of the dorsal horn of the spinal cord [13,14]. Thus many studies have demonstrated that the NO/cGMP signaling pathway is present in neurons of the spinal cord and contributes to the development of hyperalgesia in models of acute and chronic pain [15–19] and to spinal motor neuron death, which was prevented by NO-stimulated cGMP synthesis [20,21]. Although the NO-cGMP-PKG pathway was thus considered to mediate NO-induced hyperalgesia and motor neuron death in the spinal cord, PKG was shown to mediate a depression of synaptic transmission in the cerebellum and amygdala [22,23], and long-term potentiation and long-term depression in the hippocampus [24–26]. Therefore, the mechanisms through which NO mediates its hyperalgesic effects are not completely understood.

Different from many conventional neurotransmitters that are stored in synaptic vesicles and released by exocytosis, a labile, free radical mediator NO simply diffuses from the nerve terminal into adjacent cells and acts as anterograde and retrograde messengers at nociceptive synapses in the spinal cord [4]. Since paraformaldehyde-resistant NADPH-diaphorase (NADPH-d) activity is identical to nNOS in the central nervous system, NADPH-d histochemistry has long been used to detect nNOS activity at the microscopic level [27,28]. To elucidate biochemical and molecular mechanisms for the NO/cGMP signaling pathway in the spinal cord, we have recently established a unique *ex vivo* system in combination of NADPH-d histochemistry with isolated intact spinal cord preparations [29]. We have demonstrated that NO produced by nNOS, but not by eNOS or iNOS, in the spinal cord was mainly involved in the maintenance of neuropathic pain [30–32]. NO has been supposed to act as a retrograde messenger [4], i.e. to diffuse back to the presynaptic terminals of primary afferent fibers where it stimulates soluble guanylate cyclase resulting in the formation of cGMP. It may, in turn, activate PKG resulting in further glutamate release and nNOS activation. However, whether NO itself affects nNOS activity positively or negatively as retrograde messenger in the spinal cord remains unknown. To gain more insight into the retrograde messenger of NO in the spinal cord, we took an advantage of our unique *ex vivo* system and studied whether NO generated by NO donors served as retrograde messenger in the dorsal horn of the spinal cord. The present study demonstrated that NO itself enhanced nNOS activity mediated by NMDA receptors.

## Materials and methods

### *Materials*

NMDA antagonists MK 801 and D-AP5, forskolin, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and 8-Br-cGMP were obtained from Sigma-Aldrich (St. Louis, MO, USA). NO donors including sodium nitroprusside (SNP), (±)-(E)-methyl-2[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexemide (NOR1), (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR3), and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), and 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide (CPTIO, an NO scavenger) were purchased from Dojindo (Kumamoto, Japan). KT5823 (an inhibitor of PKG) and 1*H*-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, an inhibitor of soluble guanylate cyclase) were supplied by Wako Pure Chemical (Osaka, Japan) and Cayman Chemical (Ann Arbor, MI, USA), respectively.

### *Animals and preparation of intact spinal cords*

Male C57BL/6 mice were obtained from Shizuoka Laboratory Center (Hamamatsu, Japan). Mice lacking either the GluRε1 (GluRε1$^{-/-}$) [33] or GluRε4 (GluRε4$^{-/-}$) [34] subunit of the NMDA receptor were obtained by the gene targeting technique. The double knockout (GluRε1$^{-/-}$/GluRε4$^{-/-}$) mice lacking both GluRε1 and GluRε4 were generated by crossing of GluRε1$^{-/-}$ and GluRε4$^{-/-}$mice. The animals were housed under conditions of a 12-h light/12-h darkness cycle, a constant temperature of 22 ± 2 °C and 60 ± 10% humidity. They were allowed free access to food and water during experiments. All animals conformed to the regulations of the Animal Care Committee of Kansai Medical University and received humane care in accordance with the National Institutes of Health guide. Spinal cords were prepared from 20-day-old mice according to the method described previously [29]. Briefly, under ether anesthesia, spinal cords below thoracic segments were quickly dissected and were immediately placed in ice-cold artificial cerebrospinal fluid (ACSF) equilibrated with 95% $O_2$/5% $CO_2$. ACSF consists of 124 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 22 mM $NaHCO_3$, 1.25 mM $NaH_2PO_4$ and 10 mM glucose and was aerated by 95% $O_2$/5% $CO_2$ throughout the experiments. The isolated spinal cord preparations were hemisected and the left half was incubated at 34 °C for 30 min with test chemicals in ACSF and the cognate right half was incubated in ACSF alone as control.

### *NADPH-diaphorase (NADPH-d) histochemistry*

NADPH-d histochemistry was carried out according to the method described previously [29]. After 30-min incubation with various chemicals, the spinal halves were fixed overnight with 4% paraformaldehyde and then cryoprotected with 30% sucrose in phosphate-buffered saline (PBS, pH 7.4) for 1 day at 4 °C. The hemisected spinal cords were cut on a cryostat and transverse frozen sections (40-μm thick) from lumbar segments at L3–L6 levels were thaw-mounted on silane-coated glass slides. The reaction of NADPH-d was carried out at 37 °C for 110 min in the standard reaction mixture containing 0.5 mg/ml β-NADPH, 0.2 mg/ml nitroblue tetrazolium and 0.3% Triton X-100 in 0.1 M PBS (pH 7.4). The reaction was stopped by rinsing the slides in PBS three times for 10 min. Digital images were captured using a charged-coupled device camera mounted on an optic microscope (E-1000, Nikon, Tokyo, Japan). Areas of laminae I and II (superficial layer) were analyzed by a Windows-computerized image processing system (Scion Image).

### *Determination of cGMP content*

Isolated spinal cord preparations were preincubated for 10 min in ACSF containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) equilibrated with 95% $O_2$/5% $CO_2$ at 34 °C. Subsequently, the spinal cord preparations were incubated for the indicated times with 100 μM NO donors. To extract cGMP, the spinal cord preparations were snap frozen in liquid nitrogen, homogenated for 30 sec in 6% trichloroacetic acid solution using a Polytron homogenizer at the maximum speed, and then centrifuged at 20,000*g* for 15 min at 4 °C. The supernatants were washed 4 times with water-saturated diethyl ether, and the upper ether layer was discarded after each wash. The aqueous extracts were incubated for 30 min at 37 °C. Aliquot of the supernatant was measured using cGMP Enzymeimmunoassay Biotrak (EIA) system (Amersham Biosciences, Little Chalfont,