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Rho-kinase mediates spinal nitric oxide formation by prostaglandin E_2 via EP3 subtype

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Abstract

Prostaglandin E_2 (PGE_2), the principal pro-inflammatory prostanoid, is known to play versatile roles in pain transmission via four PGE receptor subtypes, EP1–EP4. We recently demonstrated that continuous production of nitric oxide (NO) by neuronal NO synthase (nNOS) following phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) and NMDA receptor NR2B subunits is essential for neuropathic pain. These phosphorylation and nNOS activity visualized by NADPH-diaphorase histochemistry were blocked by indomethacin, a PG synthesis inhibitor. To clarify the interaction between cyclooxygenase and nNOS pathways in the spinal cord, we examined the effect of EP subtype-selective agonists on NO production. NO formation was stimulated in the spinal superficial layer by EP1, EP3, and EP4 agonists. While the EP1- and the EP4-stimulated NO formation was markedly blocked by MK-801, an NMDA receptor antagonist, the EP3-stimulated one was completely inhibited by H-1152, a Rho-kinase inhibitor. Phosphorylation of MARCKS and NADPH-diaphorase activity stimulated by the EP3 agonist were also blocked by H-1152. These results suggest that PGE_2 stimulates NO formation by Rho-kinase via EP3, a mechanism(s) different from EP1 and EP4.

Keywords: Prostaglandin E_2; Nitric oxide; EP1; EP3; Myristoylated alanine-rich C-kinase substrate; Spinal cord; Rho-kinase; Neuropathic pain

The dorsal horn of the spinal cord is an important site for pain transmission and many substances are involved in the transmission and modulation of incoming pain information from the periphery [1,2]. Glutamate is the main excitatory neurotransmitter in the central nervous system and mediates fast neurotransmission at the vast majority of excitatory synapses via *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors [3,4]. The NMDA receptor activation results in the production of a number of intracellular signaling molecules including prostanoids [1,2] and nitric oxide (NO) [5]. Since Vane [6] first reported that aspirin-like drugs prevented the development of inflammation by blocking the synthesis of prostanoids, it is widely accepted that prostanoids are involved in pain, fever, edema, and various aspects of inflammation. Among them, prostaglandin E_2 (PGE_2) is considered to be the principal pro-inflammatory prostanoid and play an important role in nociceptive processing and sensitization in the spinal cord as well as in the periphery through four PGE receptor subtypes, EP1–EP4 [7]. Prostanoids are produced from arachidonic acid by two isoforms of cyclooxygenase COX-1 and COX-2, which are both constitutively expressed in the spinal cord [1,8]. NO is produced from L-ar-

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ginine by three isoforms of NO synthase (NOS) and NMDA receptor stimulation is linked to the activation of neuronal NOS (nNOS) [9,10]. In the spinal cord, nNOS is discretely localized in the superficial dorsal horn, lamina X, and intermediolateral cell column of the spinal cord [11,12]. In accordance with these localizations, it has been considered that NO from neurogenic origin is involved in nociceptive processing of nociceptive input and persistent pain as intracellular and intercellular messengers in the spinal cord [1,5]. Because of the short lifetime and highly reactive nature of NO, however, the role of NO in nociception and pathological pain was less clear as compared with that of PGE_2.

nNOS exhibits a bidomain structure, in which an N-terminal oxygenase domain containing L-arginine-binding site is linked to a C-terminal reductase domain containing cofactors NADPH, FAD, and FMN [13]. In addition to NO formation, the C-terminal sequence of NOS can also transfer electrons from NADPH to other substrates including nitroblue tetrazolium. In the presence of a relatively high concentration of NADPH, NOS reduces nitroblue tetrazolium to the water-insoluble dye nitroblue tetrazolium formazan. While the histochemical reaction known as NADPH diaphorase has long been used to map the distribution of cells containing NADPH diaphorase in the spinal cord as well as in the brain [14], the discovery that paraformaldehyde-resistant NADPH-diaphorase activity in the central nervous system is identical to nNOS activity [15,16] has provided a specific histochemical marker for cellular analysis of neurons producing NO at the light microscopic level. The advent of the fluorescent NO indicator diaminofluorescein-FM (DAF-FM) diacetate with high sensitivity and broad concentration–response range allows us to visualize NO formation in situ [17]. By use of these histochemical and fluorometric methods, we have recently shown that the increase in nNOS activity in the superficial dorsal horn reflects a neuropathic pain state even 1 week after nerve injury [18] and that this activation of nNOS is linked to phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCK) at Ser159 [19] and NMDA receptor NR2B subunit at Tyr1472 [20]. We also suggested that the NR2B phosphorylation was inhibited by an EP1 subtype-selective antagonist [20] and that PGE_2 was essential for the induction of neuropathic pain by use of membrane-associated PGE synthase-$1^{-/-}$ mice [21]. PGE_2 was previously shown to stimulate NO release from spinal slices [22]. To elucidate a molecular mechanism(s) of NO production by PGE_2 in relation to neuropathic pain, we examined the effect of EP subtype-selective agonists on NO formation in the spinal cord.

Materials and methods

Chemicals. EP subtype-selective agonists for EP1 (ONO-DI-004), EP2 (ONO-AE1-259-01), EP3 (ONO-AE-248), and EP4 (ONO-AE1-329) were kindly donated by Ono Pharmaceutical (Osaka, Japan). H-1152, a Rho-kinase specific inhibitor, was synthesized as reported previously [23]. Ro31-8220 (bisindoylmaleimidine IX, methanesulfonate), a protein kinase C inhibitor, and MK-801, an NMDA receptor antagonist, were obtained from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan).

Animals, neuropathic-pain model, and drug administration. Adult male C57BL/6 mice weighing 22 ± 2 g and 2- to 3-week-old C57BL/6 mice were used for histochemistry and NO measurement, respectively. Mice were housed under conditions of a 12 h light–dark cycle, a constant temperature of 22 ± 2 °C, and 60 ± 10% humidity. They were received food and water ad libitum. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Animal Experimentation Committee of Kansai Medical University.

For intrathecal administration, a 27-gauge stainless-steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae and drugs (5 μl) were injected into the subarachnoid space of conscious mice. ONO-AE-248 (10 pg/mouse) was intrathecally injected to naive mice 30 min before sacrifice. If necessary, H-1152 (500 ng/mouse) or Ro31-8220 (0.5 nmol/mouse) was intrathecally injected 10 min before injection of ONO-AE-248.

A neuropathic-pain model was made by selective transection of L5 spinal nerve (L5-SNT) as reported previously [18]. Briefly, mice were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg) and the left L5 spinal nerve was isolated, tightly ligated with 4-0 silk threads at two locations (5 mm apart), and transected between ligatures. During recovery from surgery, mice were individually housed and alert without signs of debilitating pain. Indomethacin (0.1 or 1.5 mg/mouse) was orally administered 2 or 3 h before sacrifice on day 7 after L5-SNT for histochemical studies.

Immunohistochemistry. Monoclonal antibody against phospho-Ser159 of MARCKS (pS159-MARCKS) was raised in mice as described previously [19]. Rabbit polyclonal antibody against phospho-Tyr1472 of NR2B (pY1472-NR2B) was obtained from Calbiochem (La Jolla, CA).

Mice were anesthetized as described above and perfused through the left cardiac ventricle with 50 ml of physiological saline followed by a fixative containing 4% paraformaldehyde in 0.12 M sodium phosphate (pH 7.4). Spinal cords were removed, immersed in the fixative for 4 h, and then cryoprotected overnight in 20% (w/v) sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The lumbar enlargement was dissected out, rapidly frozen in hexane at −50 °C, and stored at −80 °C until required for sectioning. Transverse frozen sections (20 μm thick) were cut on a cryostat and thaw-mounted on slides. Slices were washed in PBS, blocked with 2% bovine serum albumin in PBS containing 0.2% Triton X-100 for 30 min at room temperature, and incubated with cultured medium of murine hybridoma producing pS159-MARCKS monoclonal antibody (1:5) overnight at 4 °C or rabbit polyclonal pY1472-NR2B antibody (1:2000) for 2 days at 4 °C, followed by 1-h incubation with Cy3-conjugated donkey anti-mouse IgG or goat anti-rabbit IgG (1:300; Jackson ImmunoResearch, West Grove, PA) as the secondary antibody at room temperature. After washing three times with PBS, the slides were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA). Control and experimental sections were concurrently immunostained under the same conditions. The specificity of immunostaining of the spinal cord by pS159-MARCKS and pY1472-NR2B antibodies was confirmed by the cognate peptide used for immunogen. Fluorescence images were captured with a cooled charge-coupled device camera mounted on a fluorescence microscope (BX-50, Olympus, Tokyo, Japan).

NADPH-diaphorase histochemistry. NADPH-diaphorase activity is a reliable marker of nNOS activity in the spinal cord [24]. NADPH-diaphorase histochemistry was carried out on free-floating sections (30–40 μm thick) of the spinal cord as described previously [18]. The incubation was carried out for 3 h at 37 °C in a reaction mixture containing 0.5 mg/ml β-NADPH, 0.2 mg/ml nitroblue tetrazolium, and 0.25% Triton X-100 in 0.1 M PBS.

Measurement of nitric oxide. Slices were prepared from the lumbar spinal cord of 2- to 3-week-old mice and nitric oxide (NO) measurement was carried out by use of the fluorescent NO indicator DAF-FM as reported previously [18]. Lumbosacral segments were cut using a vibrating

blade microtome (Leica VT-1000S) and slices (350 μm thick) obtained from lumbar segments L4–L6 were incubated for 2 h in the artificial cerebrospinal fluid bubbled with 95% O_2/5% CO_2 at 37 °C. After loading with DAF-FM by incubation of spinal slices in the artificial cerebrospinal fluid containing 20 μM DAF-FM diacetate for 2 h at room temperature, they were placed in the recording chamber and mechanically fixed using an overlaying grid of nylon threads attached to a platinum ring. The slice was superfused in a Krebs solution containing one of EP subtype-selective agonists (10 μM) or glutamate (1 mM) equilibrated with 95% O_2/5% CO_2 at 3 ml/min. The recording chamber was mounted on an inverted fluorescence microscope (IX-70, Olympus) equipped with a dichroic mirror (505 nm) and an emission filter (515–550 nm). Intracellular NO was detected as fluorescence intensity obtained with excitation at 480 ± 10 nm using a high-speed excitation wavelength switcher (Hamamatsu Photonics, Hamamatsu, Japan). Optical signals were recorded with the AQUACOSMOS imaging system (Hamamatsu Photonics) with a cooled charge-coupled device camera.

Results

Effect of indomethacin on nNOS activity in neuropathic pain-model mice

We have shown that the increase in nNOS activity in the superficial dorsal horn of the spinal cord reflects a neuropathic pain state even 1 week after nerve injury [18] and that this nNOS activation may be reversibly regulated by phosphorylation of MARCKS at Ser159 [19] and NMDA receptor NR2B subunit at Tyr1472 [20]. To clarify the role of PGE_2 in maintenance of neuropathic pain in relation to nNOS activation, we examined the effect of indomethacin, a PG synthesis inhibitor, on phosphorylation of MARCKS and NR2B subunit in neuropathic pain model (L5-SNT) mice on day 7 after operation (Fig. 1). The nerve injury-associated increase of Ser159-phosphorylated MARCKS (Fig. 1B) and Tyr1472 phosphorylation of NR2B subunit (Fig. 1E) in the superficial layer of the spinal cord were significantly reduced 2 h after oral administration (1.5 mg/mouse) of indomethacin (Figs. 1C and F).

To confirm the involvement of PGE_2 in nNOS activation in a neuropathic-pain state, we measured NADPH-diaphorase reactivity, a reliable marker of spinal nNOS activity after fixation [24], in the lumbar spinal cord prepared from L5-SNT mice. Consistent with pS159-MARCKS and pY1472-NR2B immunostaining, although NADPH diaphorase-reactive neurons were sparsely distributed and neuropiles were weakly stained in the superficial dorsal horn of naive mice, the number of NADPH diaphorase-reactive neurons and the intensity of NADPH-diaphorase staining were obviously increased in L5-SNT mice; and these increases were reversibly reduced to the basal level of naive mice 3 h after oral administration (0.1 mg/mouse) of indomethacin (Figs. 1G–I).

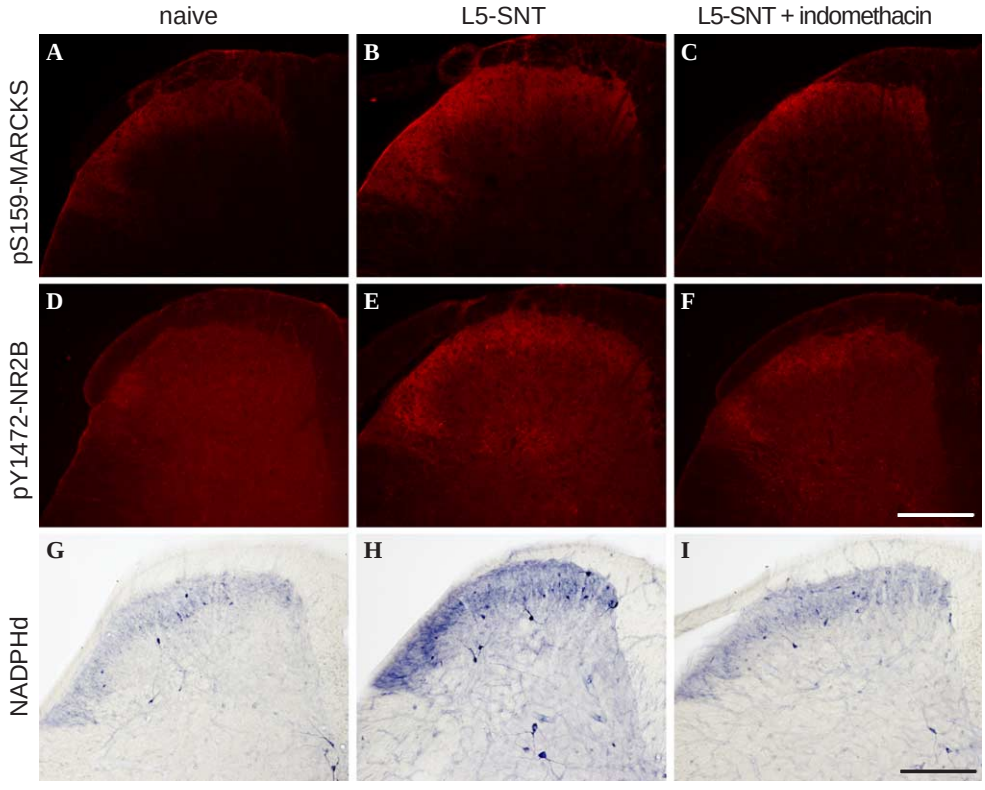

Fig. 1. Effects of indomethacin on phosphorylation of MARCKS and NMDA receptor NR2B subunit, and nNOS activity in the spinal cord prepared from L5-SNT mice. Transverse sections (20 μm thick) of lumbar spinal cord were immunostained with pS159-MARCKS (A–C) or pY1472-NR2B (D–F) antibody. Transverse sections (40 μm thick) of lumbar spinal cord were subjected to NADPH-diaphorase histochemistry (G–I). The sources of the spinal cord were from naive mice (A, D, and G) or L5-SNT mice without (B, E, and H) or with pretreatment with oral indomethacin for 2 h (C,F) or 3 h (I). Scale bars, 200 μm.

Formation of NO by EP subtype-selective agonists in the spinal cord

EP subtype-selective agonists have been recently developed on the basis of the binding affinity to cloned EP subtypes [25]. To further clarify the involvement of PGE_2 in nNOS activation in the spinal cord, we examined the effect of EP subtype-selective agonists on NO formation in spinal slices prepared from naive mice using the fluorescent NO indicator DAF-FM. Figs. 2A–D illustrate fluorescence and ratio images of the dorsal horn of the spinal cord before and after the addition of 10 μM ONO-AE-248, an EP3 agonist, and a representative of time courses of NO formation. The EP3 agonist markedly increased NO formation immediately after stimulation in the superficial layer, but weakly in the deeper layer of most spinal slices (54 slices from 17 mice). In addition to the EP3 agonist, the EP1 agonist and the EP4 agonist also stimulated NO formation, whereas the EP2 agonist had little or no effect (Fig. 2E). In contrast, glutamate added exogenously increased NO formation in all layers of the dorsal horn (Fig. 2E).

To study whether NMDA receptors were involved in NO formation by EP subtype-selective agonists, we examined the effect of MK-801, an NMDA receptor antagonist, on NO formation. When spinal slices were pretreated with MK-801 (200 μM) for 3 min, it markedly inhibited NO formation by the EP1 agonist (4 slices from 3 mice), the EP4 agonist (6 slices from 4 mice), and glutamate (4 slices from 3 mice), but not by the EP3 agonist (5 slices from 5 mice) (Fig. 3A).

Involvement of Rho-kinase in NO formation and MARCKS phosphorylation by the EP3 subtype

Since PGE_2 was reported to activate Rho-kinase through the EP3 subtype [26], we next examined the effect of H-1152, a Rho-kinase specific inhibitor [27], on NO formation by EP subtype-selective agonists. As shown in Fig. 3B, NO formation by the EP3 agonist was completely inhibited by H-1152 and it resumed after the washout in 15 out of 18 slices from 12 mice. On the other hand, NO formation by the EP1 agonist, the EP4 agonist, or glutamate was negligibly affected by H-1152 (Fig. 3B). To clarify how

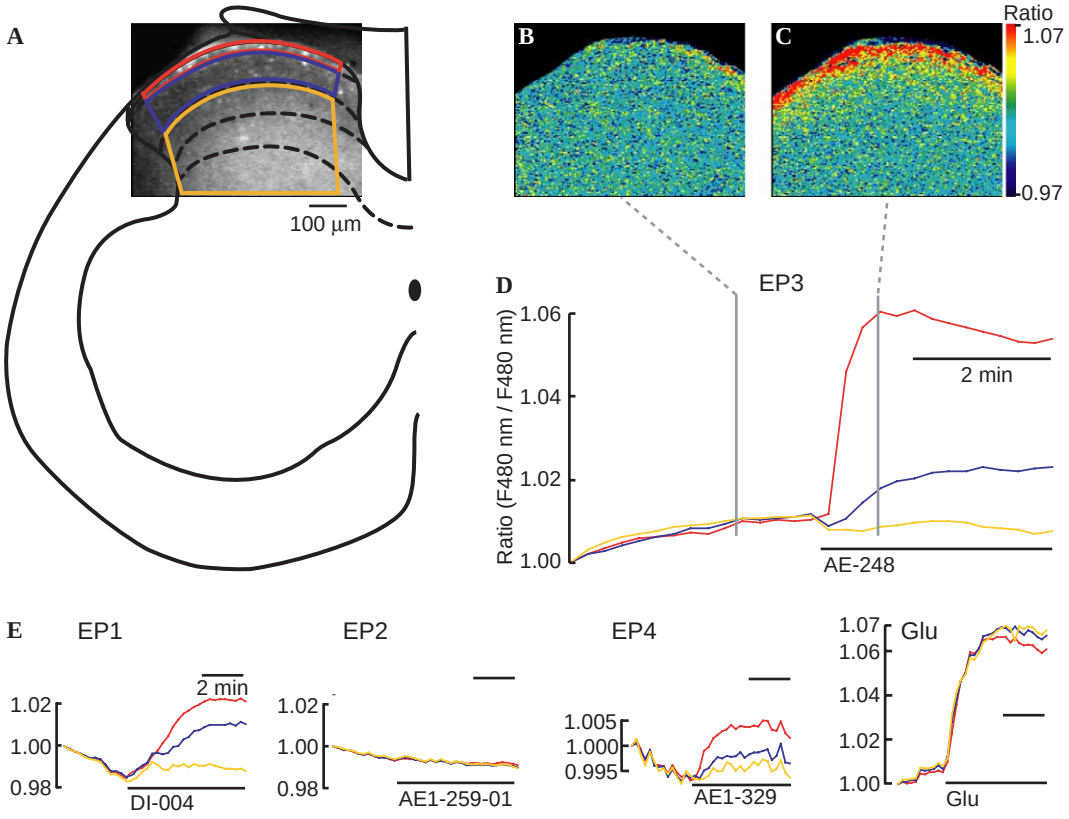

Fig. 2. NO production by EP subtype-selective agonists in spinal slices in vitro. (A–D) NO production in the superficial dorsal horn of the spinal cord by the EP3 agonist. Fluorescence image of DAF-FM-loaded transverse spinal section at L5 level prepared from 2- to 3-week-old mice (A) and NO formation in pseudocolor as ratio images before (B) and 30 s after (C) the addition of 10 μM ONO-AE-248. Time courses of NO formation in DAF-FM-loaded spinal slices (D) were measured at 15-s intervals. Broken lines indicate laminae in DAF-FM-loaded dorsal horn and boxes depict the regions to monitor fluorescence images excited at 480 nm. (E) Time courses of NO formation by EP subtype-selective agonists. DAF-FM-loaded transverse spinal slices were stimulated by 10 μM EP subtype-selective agonist for EP1, EP2 or EP4 and 1 mM glutamate. The numbers of slices and mice examined were as follows: EP1, 26 slices from 17 mice; EP2, 7 slices from 4 mice; EP3, 54 slices from 17 mice; and EP4, 50 slices from 19 mice.

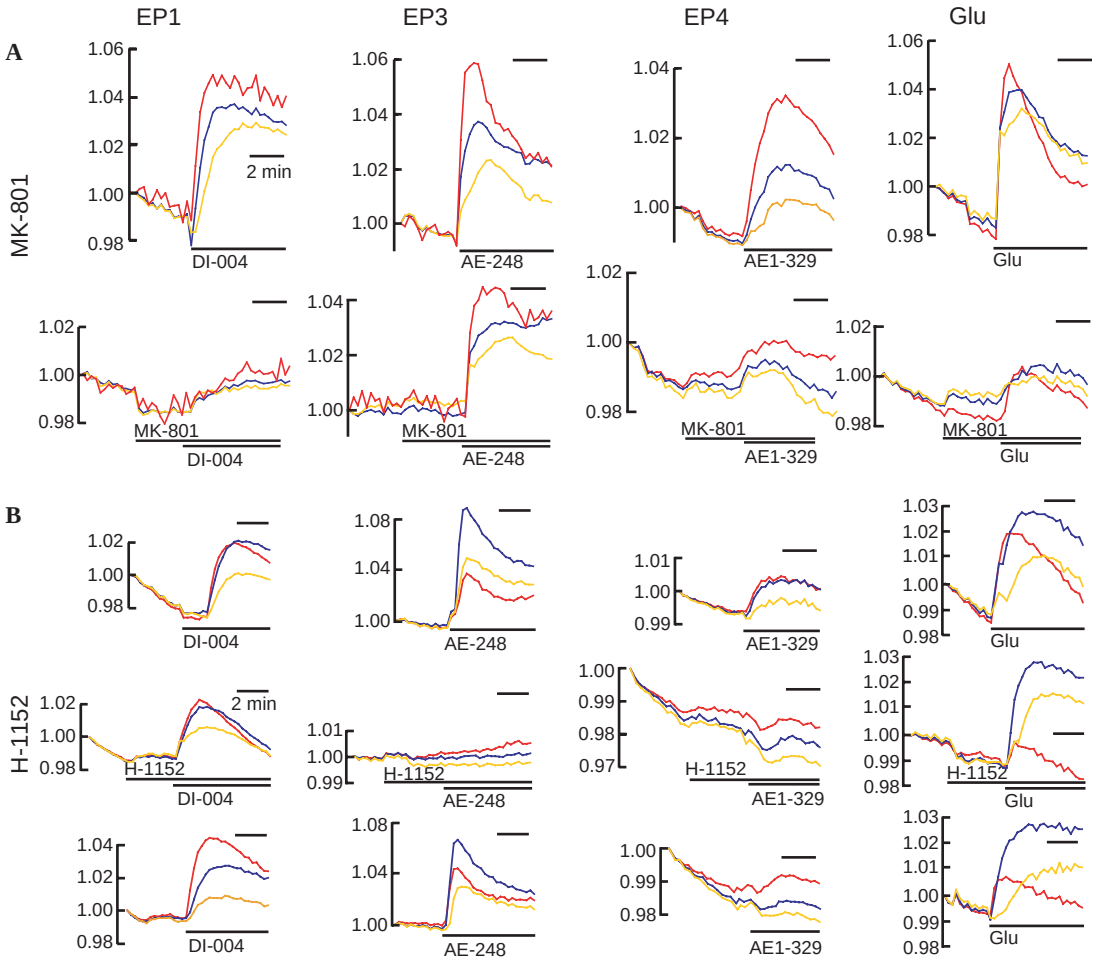

Fig. 3. Effects of MK-801 and H-1152 on NO formation by EP subtype-selective agonists and glutamate. Spinal slices were sequentially stimulated twice or three times by EP1, EP3, or EP4 agonist or glutamate at more than 30-min intervals for washing. MK-801 (A, 200 μM) or H-1152 (B, 10 μM) was added to a perfusion solution 3 min before the second stimulation.

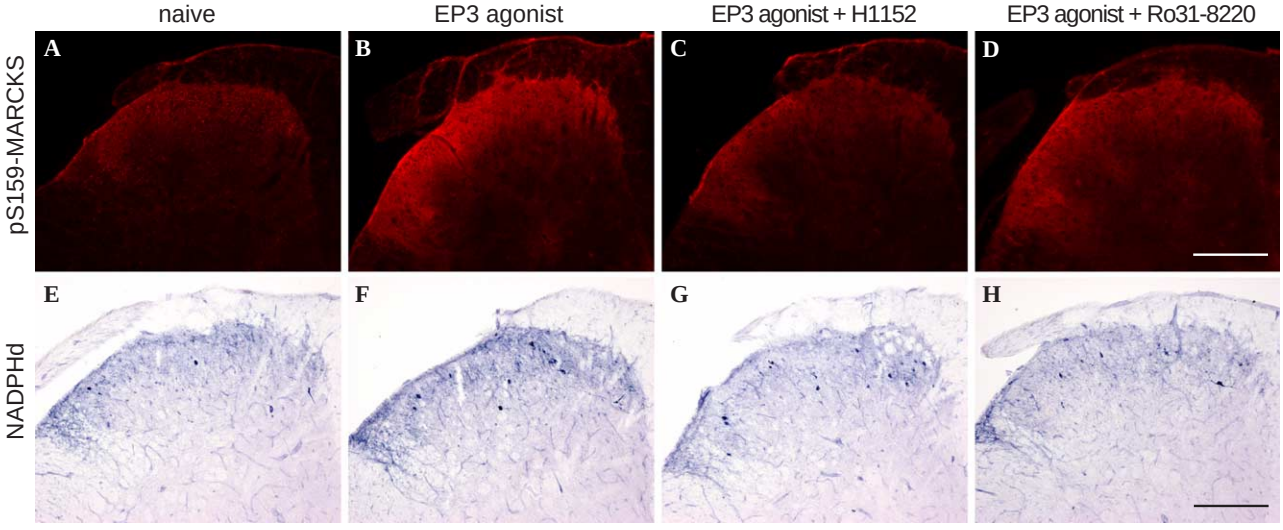

Fig. 4. Effects of EP3 agonist on phosphorylation of MARCKS at Ser159 and NADPH-diaphorase activity in the spinal cord of naive mice. The EP3 agonist ONO-AE-248 (10 pg/mouse) was intrathecally injected into mice 30 min before sacrifice without (B,F) or with 10-min pretreatment of H-1152 (500 pg/mouse, C,G) or Ro31-8220 (0.5 nmol/mouse, D,H). Transverse sections of lumbar spinal dorsal horn prepared from naive mice (A,E) or mice treated with the EP3 agonist were immunostained with pS159-MARCKS antibody (A–D) or subjected to NADPH-diaphorase histochemistry (E–H). Scale bars, 200 μm.

the EP3 agonist stimulated NO formation in the spinal cord, we also examined whether the EP3 agonist enhanced Ser159 phosphorylation of MARCKS. As shown in Fig. 4, the EP3 agonist markedly enhanced MARCKS phosphorylation in the superficial layer of the spinal cord (Fig. 4B), which was strongly inhibited by H-1152 (Fig. 4C) and weakly inhibited by the protein kinase C inhibitor Ro31-8220 (Fig. 4D). The distribution and intensity of NADPH-diaphorase activity in the spinal cord (Figs. 4E–H) were well correlated with those of Ser159 phosphorylation of MARCKS, suggesting that NO formation by the EP3 agonist in the spinal cord is mainly mediated by phosphorylation of MARCK by Rho-kinase.

Discussion

As many of the effects of NMDA receptor activation are mediated through NO production in the central nervous system, NO has been considered to play versatile roles in nociceptive processing in the spinal cord [5]. Immunocytochemical studies showed that COX-2 was colocalized with nNOS immunoreactivity in neurons of the superficial laminae of the dorsal horn 8. Because NO as well as PGE_2 cannot be stored in vesicles, its signaling specificity must be controlled at the level of synthesis and the NOS/NO pathway was suggested to interact with the COX/PGE_2 pathway in nociceptive processing in the spinal cord [8,20,22,28,29]. We propose that PGE_2 may stimulate NO formation in a neuropathic-pain state by two different pathways: phosphorylation of NMDA receptor NR2B subunits at Tyr1472 via the EP1 subtype and phosphorylation of MARCKS at Ser159 via the EP3 subtype (Fig. 5).

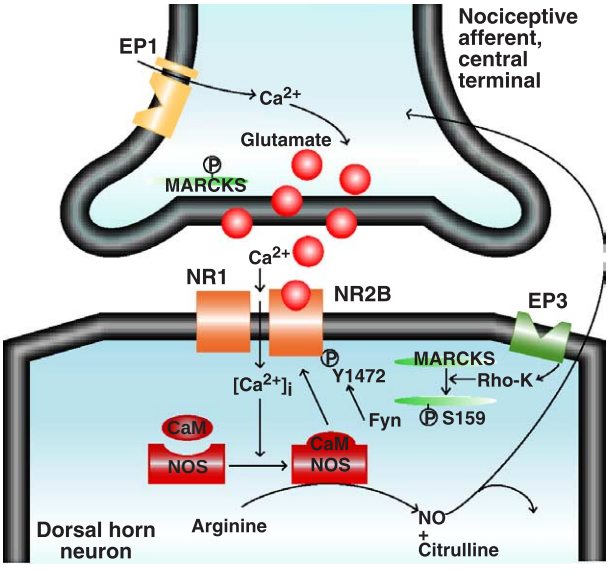

Fig. 5. Two possible mechanisms of nNOS activation in the spinal cord by PGE_2. PGE_2 stimulates Fyn-kinase-mediated phosphorylation of NR2B subunit at Tyr1472 via EP1 [20] and Rho-kinase-mediated phosphorylation of MARCKS at Ser159 via EP3, which in turn activate nNOS in postsynaptic neurons in the superficial layer of the dorsal horn.

Possible mechanism of NO formation by the EP1 subtype

We previously showed that PGE_2 stimulated NO release from spinal cord slices by activation of NMDA receptors through the EP1 subtype [22] and that PGE_2-induced allodynia was blocked by the NOS inhibitor L-NAME [30]. In agreement with the previous reports, the EP1 subtype-selective agonist ONO-DI-004 stimulated NO formation in the superficial layer of the spinal cord, which was blocked by MK-801 (Figs. 2E and 3A). Since PGE_2 was known to stimulate the release of glutamate in the spinal cord via EP1 [28], it is likely that the released glutamate by EP1 activates NMDA receptors and increases intracellular Ca^{2+} concentration in postsynaptic neurons, which leads to activation of nNOS and subsequent NO production.

Possible mechanism of NO formation by the EP3 subtype

We have recently shown that Rho-kinase is in part involved in the maintenance of neuropathic pain through phosphorylation of MARCKS at Ser159 by use of H-1152 and pSer159-MARCKS antibody [19]. In agreement with the previous report that PGE_2 activates Rho-kinase through the EP3 subtype [26], the EP3 agonist ONO-AE-248 stimulated NO formation (Figs. 2C and D) and phosphorylation of MARCKS at Ser159 (Fig. 4B) in the superficial layer of the spinal dorsal horn, which were strongly blocked by H-1152 (Figs. 3B and 4C). Similar to the restricted localization of nNOS and MARCKS in the superficial dorsal horn of the spinal cord, EP3 immunoreactivity was mainly detected in the neuropiles of lamina II and weakly in lamina I of the spinal cord and in small-sized neurons of dorsal root ganglia [31]. MARCKS is an actin-binding protein and has been implicated in cellular processes associated with cytoskeletal restructuring such as synaptic trafficking and neurotransmitter release [32]. We showed that nNOS activity might be reversibly regulated by the translocation of nNOS from the cytosol to the membrane in the presence of NMDA [33]. These results together suggest that activation of the EP3 subtype on the postsynaptic neurons may stimulate NO production through translocation of nNOS from the cytosol to the membrane by MARCKS phosphorylation.

Involvement of PGE_2 and NO in neuropathic pain

Recent studies from our laboratory have demonstrated that NO produced by nNOS is involved in the maintenance of neuropathic pain and that Tyr1472 phosphorylation of NR2B by Fyn-kinase is linked to nNOS activation, which may lead to the development and maintenance of neuropathic pain in the pain-model of mice [20]. Pretreatment of indomethacin reduced NADPH-diaphorase activity as well as Tyr1472 phosphorylation of NR2B and Ser159 phosphorylation of MARCKS in L5-SNT mice in vivo (Fig. 1). The present and recent findings that (1) neuropathic pain was not produced in $Fyn^{-/-}$mice, (2) Tyr1472 phos-

phorylation of NR2B subunit did not occur in $Fyn^{-/-}$mice and inhibited by the EP1 antagonist, and (3) NO formation by the EP1 agonist did not occur in $Fyn^{-/-}$mice and inhibited by the NMDA receptor antagonist MK-801 (Fig. 3A) strongly suggest that PGE_2 is involved in the maintenance of neuropathic pain in the spinal cord via EP1 through phosphorylation of NR2B subunit by Fyn-kinase. Disappearance of mechanical allodynia and thermal hyperalgesia in membrane-associated PGE synthase-1 knockout mice over a week supports the involvement of PGE_2 in the induction and maintenance of neuropathic pain [21].

Whereas the EP3 agonist phosphorylated MARCKS at Ser159 in the superficial dorsal horn and the phosphorylation was blocked by H-1152 in naive mice (Fig. 4), but not by the EP3 antagonist in L5-SNT mice (data not shown). MARCKS is phosphorylated at multiple sites by protein kinase C in vivo and in vitro [34], and at Ser159 by protein kinase A, protein kinase C as well as Rho-kinase in vitro [35]. It is likely that PGE_2 may modulate functions of MARCKS by phosphorylation at multiple sites in a neuropathic-pain state (Fig. 5).

The present and recent studies from our laboratory have demonstrated that PGE_2 and NO produced by nNOS are crucial for the maintenance of neuropathic pain in the pain-model of mice. Although neuropathic pain is often poorly relieved by conventional analgesics including nonsteroidal anti-inflammatory drugs (NSAIDs) [36], NSAIDs are worthy to be considered for pain management in the early stage of neuropathic pain.

Acknowledgments

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