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Stimulation of nitric oxide release from rat spinal cord by prostaglandin E_2

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1 We recently demonstrated that intrathecal administration of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ induced allodynia through a pathway that includes the glutamate receptor and nitric oxide (NO)-generating systems from pharmacological studies. In order to clarify the involvement of NO in prostaglandin-induced allodynia, we measured NO released from rat spinal cord slices by a chemiluminescence method.

2 PGE_2 stimulated NO release from both dorsal and ventral regions all along the spinal cord. PGE_2 stimulated the release within 10 min and increased it in a time-dependent manner.

3 The PGE_2-induced NO release was observed at 100 nM – 10 μM. $PGF_{2\alpha}$ stimulated the release at concentrations higher than 1 μM, but PGD_2 (up to 10 μM) did not enhance it.

4 17-Phenyl-ω-trinor PGE_2 ($EP_1 > EP_3$) and sulprostone ($EP_1 < EP_3$) were as potent as PGE_2, but PGE_1 was less potent, in stimulating NO release. While M&B 28767 (EP_3) did not enhance the release, butaprost (EP_2) stimulated it at 1 μM. The PGE_2-evoked release was blocked by ONO-NT-012, a bifunctional EP_1 antagonist/EP_3 agonist.

5 The PGE_2-evoked release was Ca^{2+}-dependent and blocked by MK-801 (NMDA receptor antagonist) and L-NAME (NO synthase inhibitor). The release was also inhibited by PGD_2 and dibutyryl-cyclic AMP.

6 The present study demonstrated that PGE_2 stimulates NO release in the rat spinal cord by activation of NMDA receptors through the EP_1 receptor, and supports our previous findings that the NO-generating system is involved in the PGE_2-induced allodynia.

Keywords: Prostaglandins; EP_1 receptor; nitric oxide; measurement; NMDA; spinal cord

Introduction

Nitric oxide (NO) plays important roles in many physiological functions such as neurotransmission, the regulation of vascular tone, and the mediation of immune responses (Moncada & Higgs, 1993). Neuronal type NO synthase (nNOS) is a calcium/calmodulin-dependent enzyme that is stimulated by activation of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor. NO activates soluble guanylate cyclase to increase the intracellular content of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Bredt & Snyder, 1992). A role of NO in sensory signalling was initially suggested based on the localization of nNOS in sensory pathways (Bredt *et al*., 1990). Immunohistochemical studies have demonstrated that nNOS is discretely localized in the superficial dorsal horn and the intermediolateral cell column (Dun *et al*., 1992; 1993; Saito *et al*., 1994). In accordance with these locations, NO is thought to regulate autonomic tone and sensory transduction at the spinal cord level. Many pharmacological studies have indirectly demonstrated that NO released in the spinal cord may be involved in the mediation of hyperalgesia by use of inhibitors for NOS and guanylate cyclase (Moore *et al*., 1991; Haley *et al*., 1992; Kitto *et al*., 1992; Meller *et al*., 1992; Malmberg & Yaksh, 1993). The mechanisms of the production and roles of NO during nociceptive transmission have been reviewed; it may act as a retrograde messenger between neurones, as a modulator of postsynaptic function, or as a paracrine mediator in adjacent neurones or glia (Meller & Gehart, 1993). One or more modes of action may underline the involvement of NO in spinal nociceptive processing.

We recently showed that intrathecal (i.t.) administration of prostaglandin E_2 (PGE_2) to conscious mice induced hyperalgesia in the hot plate test and that i.t. PGE_2 and $PGF_{2\alpha}$ induced allodynia, a state of discomfort and pain evoked by innocuous tactile stimuli (Uda *et al*., 1990; Minami *et al*., 1992; 1994c). The diversity of PGE_2 actions is ascribed to PGE receptor subtypes EP_1, EP_2, EP_3 and EP_4 coupled to different signal transduction pathways (Coleman *et al*., 1994). We suggested with EP receptor agonists that PGE_2 may exert allodynia through EP_1 receptors and hyperalgesia through EP_2 and/or EP_3 receptors in the mouse spinal cord (Minami *et al*., 1994a). Furthermore, the bifunctional EP_1 antagonist/EP_3 agonist ONO-NT-012, (5(Z)-7-[(1**S**,2**S**,3**S**,5**R**)-3-(*trans*-β-styrene) sulphonamido-6,6-dimethylbicyclo (3.1.1.)hept-2-yl]-5-heptenoic acid), is found to be a highly potent, simple competitive antagonist for the PGE_2-induced allodynia (Minami *et al*., 1995b). Recent studies from our laboratory further demonstrated, with glutamate receptor antagonists and inhibitors for NOS and guanylate cyclase (Minami *et al*., 1994b; 1995a; 1996b; Nishihara *et al*., 1995a) that prostaglandin-induced hyperalgesia and allodynia are mediated by the glutamate receptor-NO generating pathways. However, whether PGE_2 and $PGF_{2\alpha}$ stimulate NO synthesis in the spinal cord has not been examined directly. Kitade *et al*. (1996) recently established a sensitive system for NO measurement. In order to elucidate the involvement of NO in prostaglandin-induced pain responses, in the present study we measured NO production by prostaglandins in rat spinal cord slices.

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Methods

Release of NO from rat spinal cord slices

Adult male Wistar rats (230±10 g) were killed by decapitation and spinal cords were removed quickly. After careful removal of the pia-arachnoid membranes on ice and washing the tissue in Krebs-Ringer buffer (KRB in mM: NaCl 135, KCl 4.8, $CaCl_2$ 2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10 and *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane-sulphonic acid 12.5; pH 7.4), the dorsal halves of whole spinal cords were separated from the ventral ones and sliced into small pieces (cubic-like block; 0.5 mm thick). If necessary, the tissue was first cut transversely into three regions-cervical, thoracic and lumbar, and each selected region was then divided into the dorsal and ventral parts by a horizontal cut. The slices (0.84±0.2 mg protein) were washed with KRB for 10 min, transferred to 12-well culture dishes and incubated at 37°C with test agents in 2 ml of KRB in a CO_2 incubator for indicated times. In Ca^{2+}-free experiments, the slices were incubated with a Ca^{2+}-free KRB to which 0.5 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid was added.

Measurement of NO content in the incubation medium

The concentrations of NO_2^- and NO_3^- in the medium were determined with a redox chemiluminescence NO analyser (FES-450, Scholar Tec., Osaka, Japan) essentially as described previously (Kitade *et al*., 1996). Briefly, 200 μl of the medium was mixed with 2 ml of saturated ascorbic acid solution in a sealed tube for 10 to 15 s at room temperature to reduce NO_2^- to NO or with 2 ml of 0.1 M vanadium trichloride in 1 N HCl solution in a sealed tube for 60 s at 70°C to reduce NO_2^- and NO_3^- to NO (Hendrix & Braman, 1995). Measurement of NO was based on the observation of chemiluminescence (660–900 nm) produced by the reaction of NO with ozone. Sodium nitrite or sodium nitrate was used as standard. The sensitivity threshold for detection of NO was around 50 pmol with either sodium nitrite or sodium nitrate as standard and the NO-chemiluminescence relationship was linear between 50 and 1000 pmol in a full-scale condition by the above-mentioned reduction methods. NO_3^- is not detected by incubation with saturated ascorbic acid, because it cannot be reduced to NO. The background of NO in the medium was determined with dishes without slices and the background levels of NO_2^- and NO_2^- plus NO_3^- were 0.235±0.03 and 1.27±0.29 nmol/dish, respectively. If not specified, accumulation of NO_2^- and NO_3^- in the medium was used as a measure of NO formation. NO release was calculated by subtracting the background from measured NO and normalized by protein concentration. Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

Chemicals

The following chemicals were generous gifts: PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$ and ONO-NT-012, from Ono Research Institute (Osaka, Japan); sulprostone from Dr K.-H. Thierauch, Schering AG, Berlin; M&B 28767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost 13E-enoic acid) from Rhône-Poulenc Rorer, U.K.; butaprost from Dr P.J. Gardiner of Bayer, U.K.; and MK-801 ((+)-5-methyl-10,11-dihydro-5H-diabenzyl[a,d]cyclohepten-5,10-imine hydrogen maleate) from Merck Research Laboratories (Rathway, NJ, U.S.A.). 17-Phenyl-ω-trinor PGE_2 was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). N^{ω}-nitro-L-arginine methyl ester (L-NAME) and dibutyryl-cyclic AMP were purchased from Sigma (St. Louis, MO, U.S.A.). Prostanoids were stored in an ethanol solution at −20°C. An aliquot of the desired stock solution was put into a borosilicate tube and the ethanol was removed by evaporation to dryness under nitrogen gas. Prostanoids and all other drugs were dissolved in sterile saline on the day of experiments and kept on ice until used.

Statistics

Data are presented as mean±s.e.mean. The statistical analyses were carried out by analysis of variance (ANOVA) followed by Dunnett's *t* test for multiple comparisons or by Student's *t* test.

Results

PGE_2-induced NO release from rat spinal cords

In order to study whether PGE_2 could stimulate NO release from the spinal cord, we examined its effect on the dorsal and ventral parts of rat spinal cords at the three different levels: cervical, thoracic and lumbar. After reduction of NO_2^- and NO_3^- to NO with acidic vanadium (III), the NO level was determined by the chemiluminescence method. As shown in Figure 1, 60 min incubation of the dorsal part of lumbar with 100 nM PGE_2 stimulated NO release (7.67±1.89 nmol mg^{-1}) when compared to that (0.53±0.14 nmol mg^{-1}) incubated with KRB. NO_2^- formation was 2.19±0.37 nmol/dish when determined by reduction with saturated ascorbic acid. Therefore, the ratio of NO_3^- and NO_2^- was 2.5. Similar stimulation of NO release by PGE_2 was observed with spinal cords at cervical and thoracic levels. Although PGE_2 stimulated NO release from the ventral parts as well as the dorsal parts at the three levels examined (Figure 1), we used the dorsal half of whole spinal cords and measured the concentrations of NO_2^- and NO_3^- in the medium for the following experiments.

Figure 2 shows the time course of NO release induced by 100 nM PGE_2. The NO level in KRB was 0.70±0.44 nmol mg^{-1} at 10 min which was not significantly changed in KRB during the 120 min experimental period. PGE_2 increased the NO level in the medium in a time-

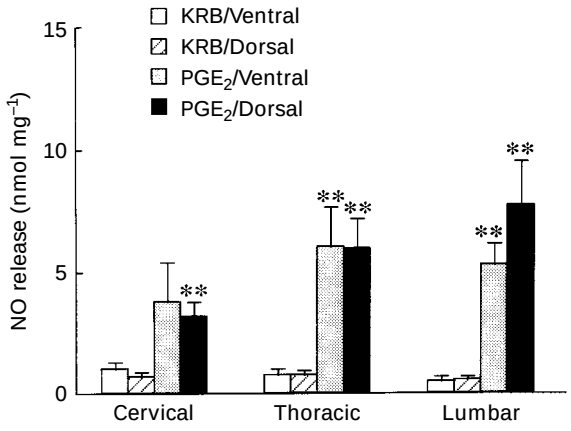

Figure 1 The effect of PGE_2 on NO release (nmol mg^{-1} protein) at three different levels of rat spinal cord. Slices in 12-well culture dishes were incubated in 2 ml of KRB in the absence or presence of 100 nM PGE_2 for 60 min in a CO_2 incubator. NO content in the medium was measured as described under Methods. Each column represents the mean±s.e.mean of fifteen independent experiments. Statistical analyses were carried out by Student's *t* test. $^{**}P<0.01$, as compared with the control (KRB).

dependent manner; it increased to 1.46 ± 0.44, 3.26 ± 1.14, 6.34 ± 0.96, and 9.54 ± 2.32 nmol mg^{-1} at 10, 30, 60 and 120 min, respectively.

Specificity of prostaglandin and EP agonists for NO release

Figure 3a shows the dose-dependent effect of PGD_2, PGE_1, PGE_2 and $PGF_{2\alpha}$ on NO release from rat dorsal spinal cord slices over 60 min. PGE_2 increased the NO release at 100 nM – 10 μM. PGE_1 showed a similar dose-dependent pattern, but to a lesser extent. $PGF_{2\alpha}$ only increased NO release at concentrations higher than 1 μM. On the other hand, PGD_2 did not enhance NO release.

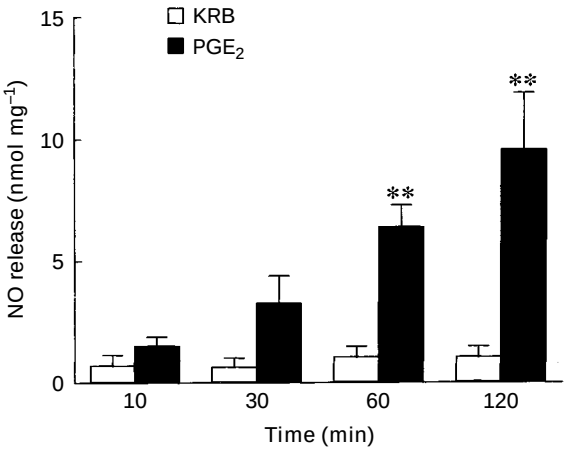

Figure 2 Time courses for the effect of PGE_2 on NO release (nmol mg^{-1} protein) from rat spinal cord. Slices in 12-well culture dishes were incubated in 2 ml of KRB in the absence or presence of 100 nM PGE_2 for the indicated times in a CO_2 incubator. NO content in the medium was measured as described under Methods. Each column represents the mean $\pm$ s.e.mean of five independent experiments. Statistical analyses were carried out by Student's t test. $^{**}P<0.01$, as compared with the control (KRB).

In order to specify the EP receptor subtype(s) involved in the PGE_2-induced NO release, we examined the effect of various EP agonists on the 60 min release from the spinal cord. As shown in Figure 3b, 17-phenyl-ω-trinor PGE_2 (an EP_1 and EP_3 agonist, $EP_1 > EP_3$) and sulprostone (an EP_1 and EP_3 agonist, $EP_1 < EP_3$) significantly increased the NO level, in a dose-dependent manner similar to that of PGE_2. NO release was also increased at the concentration of 1 μM of butaprost (an EP_2 agonist). On the other hand, M&B 28767 (an EP_3 agonist) did not stimulate NO release over the basal level.

Effect of various agents on the PGE_2-induced NO release from the rat spinal cord

We previously demonstrated that PGE_2 induced allodynia through activation of NMDA receptors mediated by EP_1 receptors and that PGD_2 blocked the PGE_2-induced allodynia (Minami *et al*., 1994b; 1995b; 1996a). In order to clarify the correlation between the PGE_2-induced allodynia and NO release, we next examined the effect of various agents on the release of NO from the spinal cord. As expected, ONO-NT-012 and the NMDA receptor antagonist MK-801 as well as the NOS inhibitor L-NAME inhibited the PGE_2-evoked NO release from spinal cord slices (Figure 4). The PGD_2 receptor is coupled to stimulation of adenylate cyclase. Both PGD_2 and dibutyryl-cyclic AMP, a membrane-permeable cyclic AMP analogue, blocked the PGE_2-induced NO release. Consistent with the notion that nNOS is a calcium/calmodulin-dependent enzyme, the PGE_2-induced NO release was not observed in Ca^{2+}-free KRB.

Discussion

NO has recently been recognized as an important neuromodulator and neurotransmitter in the central nervous system (Bredt & Snyder, 1992; Snyder, 1992). A variety of methods have been used to measure NO synthesis in biological models, each with its own advantages and disadvantages (Archer, 1993). In the spinal cord, direct

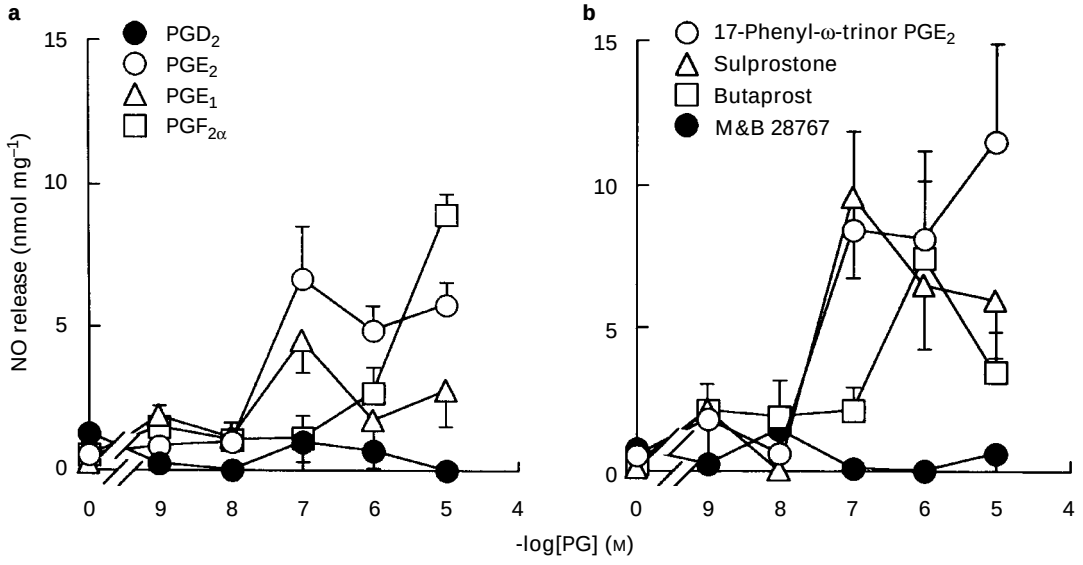

Figure 3 Dose-dependence for NO release (nmol mg^{-1} protein) from rat spinal cord induced by prostaglandins and EP agonists. (a) Slices in 12-well culture dishes were incubated with various doses of PGD_2, PGE_1, PGE_2 and $PGF_{2\alpha}$ for 60 min in a CO_2 incubator. (b) Slices in 12-well culture dishes were incubated with various doses of 17-phenyl-ω-trinor PGE_2, sulprostone, butaprost and M&B 28767 for 60 min in a CO_2 incubator. NO contents in the medium are expressed as the mean of five experiments; vertical lines show s.e.mean.

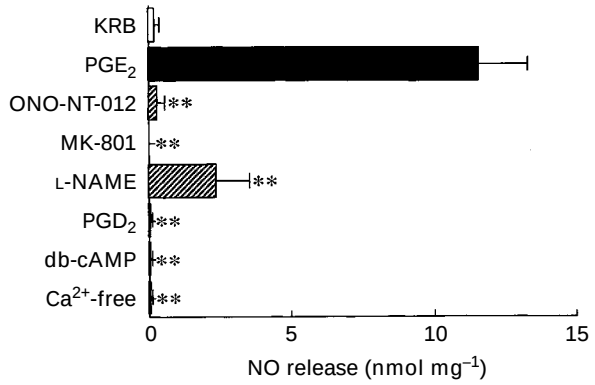

Figure 4 Effect of various agents on the PGE_2-evoked NO release (nmol mg^{-1} protein) from rat spinal cord. Slices in 12-well culture dishes were incubated with 100 nM PGE_2 for 60 min in KRB containing PGD_2 (1 μM) or dibutyryl-cyclic AMP (db-cAMP, 1 mM). After 10 min incubation with L-NAME (10 μM), MK-801 (10 μM) or ONO-NT-012 (10 μM), slices in 12-well culture dishes were incubated with 100 nM PGE_2 for 60 min. Slices in 12-well culture dishes were also incubated with 100 nM of PGE_2 for 60 min in Ca^{2+}-free KRB. Each column represents the mean + s.e.mean of five experiments. Statistical analyses were carried out by Dunnett's t test. $^{**}P<0.01$, as compared with PGE_2-induced NO release.

measurement of NO is limited by the instability of the molecule itself and by sensitivity of the assay. Indirect measures such as change in citrulline or cyclic GMP levels have been used as marker for NO synthesis (Meller & Gebhart, 1993; Malmberg & Yaksh, 1995). NMDA and acetylcholine have recently been shown to stimulate NO release from rat spinal cord with a bioassay system using a ring of endothelium-denuded rat aorta (Li *et al*., 1994; Xu *et al*., 1996). The drawback of this technique is that vasodilating substances such as prostaglandins cannot be used in perfusates in the bioassay system. Compared with the increase in NO content (1–70 nmol/dish) produced by iNOS in primary cultured hepatocytes (Kitade *et al*., 1996), the enhancement of NO release from spinal cord slices by PGE_2 was rather small, at most 10 nmol/dish. Therefore, we measured accumulation of NO_2^- and NO_3^- in the medium, instead of NO_2^- alone, as a measure of NO formation by a sensitive redox chemiluminescence method. After reduction of NO_2^- and NO_3^- to NO with acidic vanadium (III), NO was detected by the interaction of ozone with NO, which generates chemiluminescence (Archer, 1993). Because aeration of incubation buffers with 5% CO_2/95% O_2 increased the background NO level in the medium and this increase could not be neglected in the present study, the slices were incubated in a 5% CO_2 incubator without aeration. NO_3^-, but not NO_2^-, increased around 0.5 nmol/dish in the tissue-free medium during the 60 min incubation period (M. Sakai, unpublished observations), suggesting that the contamination of air may account for a considerable portion for the measured NO in KRB. On the other hand, increase in NO_2^- as well as NO_3^- was observed in the medium after stimulation by PGE_2. Similar problems of contamination from the air were noticed for the measurement of NO/nitrite in cultured cells (Weikert *et al*., 1997). Furthermore to minimize contamination from outside, incubation media were freshly made up with Mill-Q water.

PGE receptors are classified into four subtypes EP_1–EP_4. While the EP_1 receptor is coupled to Ca^{2+} mobilization, EP_2 and EP_4 receptors stimulate adenylate cyclase and the EP_3 receptor inhibits adenylate cyclase (Coleman *et al*., 1994). Recently, we showed that i.t. administration of PGE_2 in conscious mice induced allodynia through EP_1 receptor stimulation and hyperalgesia through EP_2/EP_3 receptors (Minami *et al*., 1994a). The present study demonstrated that PGE_2 stimulated NO release via EP_1 receptors for the following reasons: (1) NO release was significantly stimulated by 17-phenyl-ω-trinor PGE_2 ($EP_1 > EP_3$) and sulprostone ($EP_1 < EP_3$), followed by PGE_1, but not by M&B 28767 (EP_3) (Figure 3). The order of potency of PG agonists to stimulate NO release is in good agreement with that required to displace [^{3}H]-PGE_2 binding to the EP_1 receptor (Watabe *et al*., 1993). (2) PGE_2-evoked NO release was blocked by the EP_1-receptor antagonist ONO-NT-012 (Figure 4). We further demonstrated that PGE_2-stimulated NO release was blocked by MK-801 and L-NAME and that the release was abolished in the Ca^{2+}-free medium (Figure 4). These results suggest that nNOS may be responsible for the PGE_2-evoked NO production. Minami *et al*. (1996a) recently demonstrated that PGD_2 inhibits the PGE_2-induced allodynia in conscious mice. The blockade of PGE_2-evoked NO release by PGD_2 and dibutyryl-cyclic AMP (Figure 4) supports the above-mentioned notion that NO released by PGE_2 is involved in the induction of allodynia. We previously showed that PGE_2 stimulated the glutamate release from synaptosomes of rat spinal cord (Nishihara *et al*., 1995b). Taken together, the present results support the notion that PGE_2 may stimulate NO production following NMDA receptor activation via EP_1 receptors and evoke allodynic responses.

In the present study, we also found that $PGF_{2\alpha}$ and butaprost could stimulate NO release from the spinal cord (Figure 3). While $PGF_{2\alpha}$ is thought to cause Ca^{2+} mobilization, butaprost is coupled to stimulation of adenylate cyclase through the EP_2 receptor. Therefore, the butaprost-induced NO release must be mediated by a substance which raises intracellular Ca^{2+}. We previously showed in pharmacological studies that butaprost evoked hyperalgesia through the NMDA-NO cascade (Nishihara *et al*., 1995a; Minami *et al*., 1996b). The involvement of NMDA receptors in PGE_2-induced hyperalgesia was confirmed by the use of NMDA receptor ε subunit knockout mice (Minami *et al*., 1997). Although it is possible that glutamate may mediate the butaprost-induced NO release, the mechanism of NO production by butaprost remains to be clarified.

In summary, the present study is the first in which NO production is measured directly in the spinal cord and confirms that NO production is involved in pain responses induced by prostaglandins, especially the PGE_2-induced allodynia. The good correlation of *in vitro* NO production in rat spinal cord slices with *in vivo* behavioural studies confirms that the redox chemiluminescence method for NO measurement, employed here, is valuable for studies on the involvement of NO in nociception at the spinal level.

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