

## Activation of Peripheral NMDA–Nitric Oxide Cascade in Formalin Test

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**Background:** It has been suggested that peripheral glutamate and nitric oxide (NO) released by tissue-damaging stimuli play an important role in peripheral nociceptive transmission. This study was conducted to determine whether NO was released in the periphery after subcutaneous injection of formalin and whether the peripheral N-methyl-D-aspartate (NMDA)–NO cascade was activated.

**Methods:** During pentobarbital anesthesia, a microdialysis probe was implanted subcutaneously into the glabrous skin of both hind paws of Sprague-Dawley rats. After sample collection to obtain the basal level of NO metabolites (total amount of nitrite [ $\text{NO}_2^-$ ] and nitrate [ $\text{NO}_3^-$ ] [ $\text{NO}_2^-$ – $\text{NO}_3^-$ ]), 5% formalin was injected into the plantar surface of the right hind paw during perfusion of the dialysis catheters with artificial cerebrospinal fluid (ACSF), the NO synthase inhibitor *N*<sup>G</sup>-monomethyl-L-arginine acetate, or the NMDA antagonist *D,L*-2-amino-5-phosphonovaleric acid through a microdialysis probe. Formalin also was injected in the animals that underwent sciatic nerve sectioning. In another series of experiments, NMDA was perfused through one probe. Samples for measurement of  $\text{NO}_2^-$ – $\text{NO}_3^-$  were collected and immediately analyzed using high-performance liquid chromatography.

**Results:** Subcutaneous formalin significantly increased the dialysate concentrations of  $\text{NO}_2^-$ – $\text{NO}_3^-$  (maximum increase  $144 \pm 12\%$  of baseline value 30 min after formalin administration;  $P < 0.05$ ) on the side ipsilateral to the injection. Both *N*<sup>G</sup>-monomethyl-L-arginine acetate and *D,L*-2-amino-5-phosphonovaleric acid significantly ( $P < 0.05$ ) suppressed the formalin-induced increases in  $\text{NO}_2^-$ – $\text{NO}_3^-$  concentration. In the rats with denervation of the sensory nerves, formalin did not change the

$\text{NO}_2^-$ – $\text{NO}_3^-$  concentration. In addition, NMDA perfusion significantly ( $P < 0.05$ ) increased the concentrations of  $\text{NO}_2^-$ – $\text{NO}_3^-$ .

**Conclusion:** The results of the current study show that subcutaneous formalin injection induces peripheral release of NO, the production of which is mediated by activation of NMDA receptors in the peripheral nervous system. (Key words: Microdialysis; nociception; pain.)

NITRIC oxide (NO) acts as an intra- and intercellular messenger and has been implicated in many physiologic pathways.<sup>1,2</sup> There is abundant evidence that NO plays a role in nociceptive signaling because NO has been located in sensory afferent neurons and in spinal neurons within the dorsal horn.<sup>3,4</sup> Increases in NO synthase (NOS) have been observed in dorsal root ganglia and the spinal dorsal horn after sensory nerve axotomy, capsaicin administration, or sensory nerve ligation.<sup>5-7</sup> Although a facilitatory role of NO in nociceptive transmission has been reasonably well-established in the spinal cord, quantitative analysis and examination of its action at peripheral site of the nociceptive pathway has not been undertaken. In the spinal dorsal horn, *N*-methyl-D-aspartate (NMDA) receptor activation and the associated  $\text{Ca}^{2+}$  influx result in the generation of NO by activation of NOS. These changes in the central nervous system induce the prolonged nociceptive transmission. We previously reported that subcutaneous injection of formalin induced the release of excitatory amino acid glutamate in the plantar surface of the rat hind paws, which would contribute to the nociception and inflammation.<sup>8</sup> The relation between glutamate receptor activation and NO release in peripheral sites, however, is unknown. The purpose of the current study was to determine whether NO would be released peripherally after subcutaneous injection of formalin. We also evaluated whether the peripheral NMDA–NO system would be activated after formalin injection, as observed in the central nervous system.

### Materials and Methods

Experiments were conducted according to a protocol approved by the Sapporo Medical University Animal

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Care and Use Committee. The animals used in this study were male Sprague-Dawley rats (weighing 300–350 g; Japan SLC, Hamamatsu, Japan), which were housed individually in a temperature-controlled room (at  $21 \pm 1^\circ\text{C}$ ) with a 12-h light-dark cycle and given free access to food and water.

#### Animal Preparation

Animals were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally, and additional sodium pentobarbital was administered throughout the experiment to maintain areflexia. A microdialysis probe (A-I-4-03; Eicom, Kyoto, Japan) was inserted subcutaneously into the bilateral glabrous skin of the hind paw, as described previously.<sup>8</sup> The probe was perfused with artificial cerebrospinal fluid (ACSF) (140 mM NaCl, 4.0 mM KCl, 1.26 mM  $\text{CaCl}_2$ , 1.15 mM  $\text{MgCl}_2$ , 2.0 mM  $\text{Na}_2\text{HPO}_4$ , and 0.5 mM  $\text{NaH}_2\text{PO}_4$ ; pH 7.4), at a constant flow rate of 3  $\mu\text{l}/\text{min}$  for 120 min to establish a diffusion equilibrium, followed by sample collection to obtain basal release levels of  $\text{NO}_2^-$  and  $\text{NO}_3^-$ . In another series, during general anesthesia (isoflurane in oxygen), the right sciatic nerve was exposed in the upper thigh. A transection of the nerve was made just distal to the greater trochanter, with a 2-cm distal stump excision. Seven days after surgery, a microdialysis probe was inserted subcutaneously according to the method described previously.

#### Drugs

The drugs and chemicals used in the experiment were  $N^G$ -monomethyl-L-arginine acetate (L-NMMA; Research Biochemicals, Natick, MA), *D,L*-2-amino-5-phosphonovaleic acid (AP-5; Sigma Chemical, St Louis, MO) and NMDA (Sigma Chemical). L-NMMA, AP-5, and NMDA were dissolved in physiologic saline. The pH of each solution was adjusted to 7.4.

#### Microdialysis Study

After sample collection to obtain the basal level, the probe was perfused with ACSF, L-NMMA (40 mM), or AP-5 (10 mM) at a constant flow rate of 3  $\mu\text{l}/\text{min}$ . Thirty minutes after perfusion, the sample was collected to obtain the basal level of NO metabolites (total amount of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  [ $\text{NO}_2^-$ - $\text{NO}_3^-$ ]) and a 50  $\mu\text{l}$  volume of 5% formalin was injected into the plantar surface of the right hind paw using a 29-gauge needle. Perfusate collection was started with a 4-min delay because of the dead space of the outflow catheter to sample subcutaneous perfusate from the time of formalin injection. In another series of rats, NMDA (5 mM) was perfused through a microdi-

alysis probe for 20 min. Samples were collected in polypropylene tubes for 5 min at each sampling time point, and the samples were immediately analyzed for  $\text{NO}_2^-$ - $\text{NO}_3^-$ . After the microdialysis study, the animals were killed with a overdose of sodium pentobarbital.

#### Analysis of Nitrite-Nitrate

The concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  in the dialysate was analyzed using an automated NO-detecting high-performance liquid chromatography system (ENO-10; Eicom).  $\text{NO}_2^-$ - $\text{NO}_3^-$  in the dialysate was separated by a reverse-phase separation column packed with polystyrene polymer ( $4.6 \times 50$  mm; NO-PAK; Eicom), and  $\text{NO}_3^-$  was reduced to  $\text{NO}_2^-$  in a reduction column packed with copper-plated cadmium filling (NO-RED; Eicom).  $\text{NO}_2^-$  was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven that was set at  $35^\circ\text{C}$ . The absorbance of the color of the product dye at 540 nm was measured using a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl- $\text{NH}_4\text{Cl}$  and 0.5 g/l 4Na-EDTA. The Griess reagent, which was 1.25% HCl that contained 5 g/l sulfanilamide with 0.25 g/l *N*-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min. The contamination of  $\text{NO}_2^-$ - $\text{NO}_3^-$  in ACSF and the reliability of the reduction column were evaluated in each experiment. To determine the *in vitro* recovery of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  across the dialysis probe, a microdialysis probe was put into aliquots at a 10-mm concentration of  $\text{NaNO}_2$  and  $\text{NaNO}_3$  and perfused with Ringer's lactate solution at a constant flow rate of 3  $\mu\text{l}/\text{min}$  at room temperature. The *in vitro* recovery was estimated based on the levels of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in the 5-min dialysate sample.

#### Statistical Analysis

The  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration was represented as the mean  $\pm$  SD of percentage basal values. The statistical significance of differences between groups was assessed by a two-way analysis of variance for repeated measures, followed by the Scheffé F test. Changes in  $\text{NO}_2^-$ - $\text{NO}_3^-$  during NMDA perfusion were compared with baseline values using one-way analysis of variance, followed by the Scheffé F test.  $P < 0.05$  was considered to be statistically significant.

## Results

### Basal Value Condition of Nitrite-Nitrate in Dialysates

A preliminary study showed that dialysis equilibrium was obtained within 120 min after the start of ACSF perfusion at a constant flow rate of 3  $\mu\text{l}/\text{min}$ , and basal values were stable for at least 240 min (data not shown). The basal value of  $\text{NO}_2^-$ - $\text{NO}_3^-$  was  $13.19 \pm 1.42 \text{ pmol}/10 \mu\text{l}$ . The *in vitro* recovery of  $\text{NO}_2^-$ - $\text{NO}_3^-$  was estimated to be  $68.97 \pm 0.02\%$  ( $n = 6$ ) across the dialysis probe.

### Effects of Formalin Injection on Nitrite-Nitrate Concentration

Subcutaneous injection of formalin significantly ( $P < 0.05$ ) increased the concentrations of  $\text{NO}_2^-$ - $\text{NO}_3^-$  on the side ipsilateral to the injection, but not on the contralateral side (fig. 1). Ipsilateral  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration significantly increased immediately after formalin injection ( $P < 0.05$ ), and the increases were observed for 100 min. The increase in the  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration after formalin injection was suppressed significantly ( $P < 0.05$ ) during L-NMMA perfusion (fig. 2). L-NMMA alone did not affect the basal value of  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration (data not shown). In the animals with denervated sensory nerves in the hind paws, the concentrations of  $\text{NO}_2^-$ - $\text{NO}_3^-$  did not show significant changes after formalin injection (fig. 3).

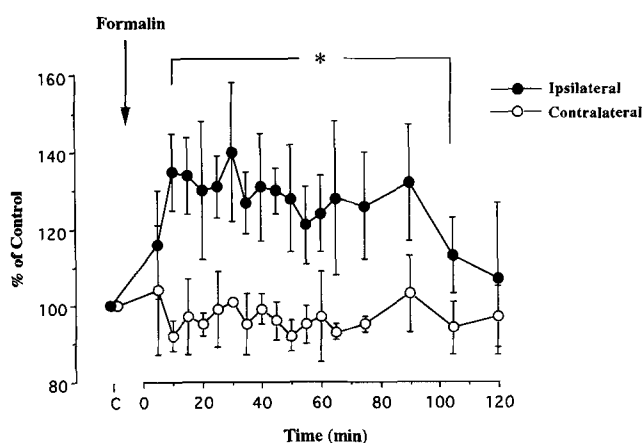


Fig. 1. Time courses of the subcutaneous concentrations of  $\text{NO}_2^-$ - $\text{NO}_3^-$  on the ipsilateral and contralateral sides of the hind paw after subcutaneous injection of formalin. Data are represented as the mean  $\pm$  SD from six rats. \* $P < 0.05$ , showing statistical significance of the difference between ipsilateral and contralateral sides.

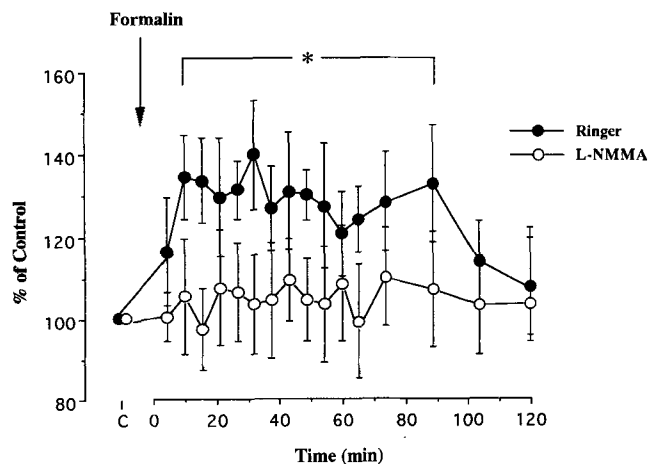


Fig. 2. Effect of  $N^G$ -monomethyl-L-arginine acetate (L-NMMA) perfusion on the formalin-induced increase in the subcutaneous concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . L-NMMA perfusion suppressed the increase in the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  that was observed with perfusion with Ringer's solution. Data are represented as the mean  $\pm$  SD from six rats in each group. \* $P < 0.05$ , showing statistical significance of the difference between Ringer's solution and L-NMMA.

### Effects of AP-5 and NMDA on Nitrite-Nitrate Concentration

The perfusion of AP-5 through the microdialysis probe suppressed significantly ( $P < 0.05$ ) the formalin-induced increase in  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration, as shown in figure 4. Figure 5 shows the effects of the perfusion of NMDA through the microdialysis probe on the concentration of

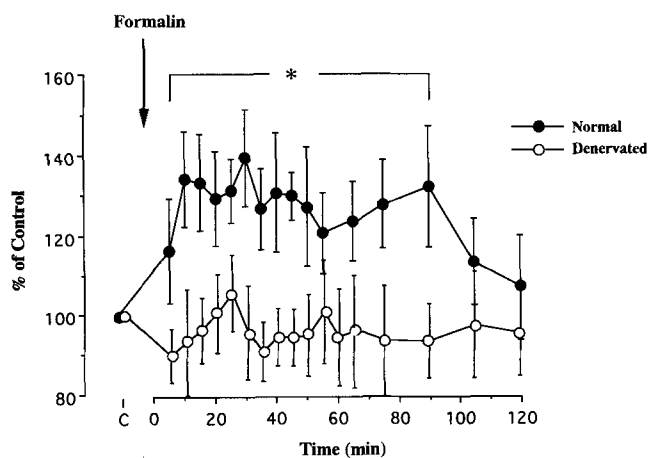


Fig. 3. Time courses of the subcutaneous concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  after subcutaneous injection of formalin in the normal and denervated rats. Data are represented as the mean  $\pm$  SD from five or six rats in each group. \* $P < 0.05$ , showing statistical significance of the difference between normal and denervated rats.

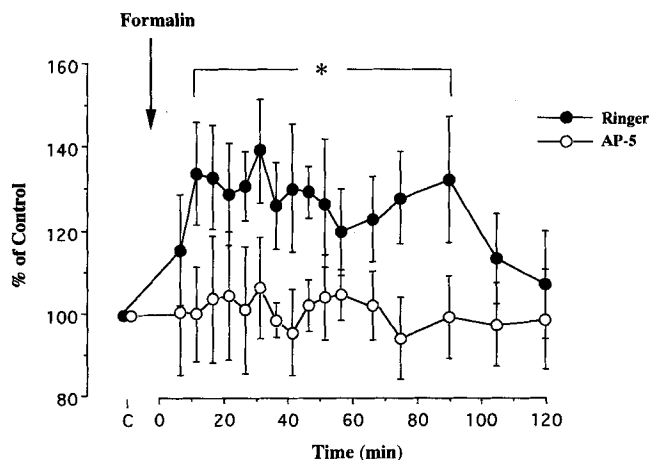


Fig. 4. Effect of *D,L*-2-amino-5-phosphonovaleric acid (AP-5) perfusion on the formalin-induced increase in the subcutaneous concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . AP-5 perfusion suppressed the increase in the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  that was observed with Ringer's solution. Data are represented as the mean  $\pm$  SD from six rats in each group. \* $P < 0.05$ , showing statistical significance of the difference between Ringer's lactate solution and AP-5.

the  $\text{NO}_2^-$ - $\text{NO}_3^-$ . NMDA perfusion increased the concentrations of  $\text{NO}_2^-$ - $\text{NO}_3^-$  significantly ( $P < 0.05$ ).

## Discussion

In the current study, injection of formalin into the plantar surface of the rat hind paw significantly increased the subcutaneous concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  on the side ipsilateral, but not the side contralateral, to the injection. This increase was suppressed by the perfusion of NOS inhibitor L-NMMA. The perfusion of competitive NMDA antagonist AP-5 also suppressed the formalin-induced increase in the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . In addition, the subcutaneous perfusion of NMDA alone increased the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . In the animals that had undergone sciatic nerve sectioning, the increase in the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  was not observed. These data show that subcutaneous formalin induced the peripheral release of NO through activation of an NMDA-NO cascade in the peripheral nervous system.

### Peripheral Nitric Oxide-in-Formalin Test

Several lines of observations suggest that peripheral NO acts as a pronociceptive mediator.<sup>9-11</sup> Human and animal studies support a role of NO in mediating peripheral nociceptive transmission.<sup>12-15</sup> Intracutaneous injection of NO precursors in humans evoke pain in a dose-

dependent manner,<sup>12,14</sup> suggesting that NO may activate cutaneous nociceptors directly or indirectly. In the mouse, the NOS inhibitor *N*<sup>G</sup>-nitro-L-arginine methylester hydrochloride (L-NAME), if coadministered with intraplantar formalin, exhibited antinociceptive activity in a dose-dependent manner.<sup>13</sup> Intraarticular injection of L-NAME resulted in a complete reversal of heat hyperalgesia in arthritic model of rats.<sup>15</sup> It is suggested that NO is released during the inflammation and that it acts to increase vascular permeability to different chemical substances activating specific receptors involved in pain signaling.<sup>16</sup> It is conceivable that endogenous NO is released at the site of acute inflammation.<sup>17</sup> In the formalin test, it has been hypothesized that the first phase of formalin-induced behavior reflects formalin-induced activation of C-fiber primary afferent nociceptors, and that the second phase is an associated phase of inflammation in which various chemical mediators are able to alter the functions of peripheral afferent fibers.<sup>18-20</sup> As shown in the current study, formalin induces the elevation of the subcutaneous concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  on the ipsilateral side of the hind paw; however, the increase does not show biphasic change. Peripheral NO was released in acute nociception and the inflammatory stage.

### Source of Nitric Oxide-in-Formalin Test

Nitric oxide is synthesized from the terminal guanidino nitrogen atoms of the amino acid L-arginine by an enzyme, NOS.<sup>21</sup> Because NO has a short half-life, it does

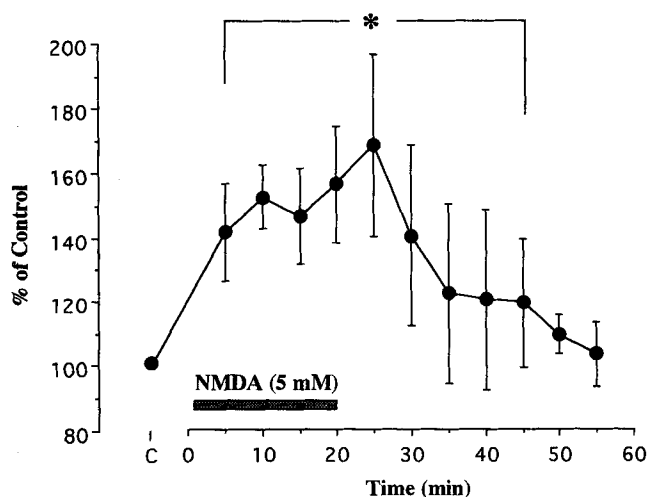


Fig. 5. Effect of *N*-methyl-D-aspartate (NMDA) perfusion on the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . Note that 5 mM NMDA perfusion for 20 min evoked the release of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . Data are represented as the mean  $\pm$  SD from six rats. \* $P < 0.05$  compared with baseline.

not diffuse for a long distance. Therefore, NO must be synthesized locally in the formalin-injected site. Different types of NOS exist in different cells.<sup>2</sup> Neuronal NOS is expressed constitutively in neurons. Inducible NOS (iNOS) originally was described in macrophages and hepatocytes but also is found in central nervous system glial cells. Endothelial NOS is expressed constitutively in endothelial cells. Possible peripheral sources of NO include the peripheral afferent fibers, neutrophils, infiltrating leukocytes, and the endothelial cells lining the capillaries. In the current study, L-NMMA perfusion suppressed the increase in concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  after formalin injection. Because L-NMMA is a nonselective NOS inhibitor, it is unclear which type of NOS would be associated with production of NO. Peripheral NO may be generated through neuronal NOS within nociceptors because neuronal NOS-like immunoreactivity is expressed in small- and medium-diameter dorsal root ganglion neurons<sup>22,23</sup>; neuronal NOS-like immunoreactivity is colocalized in some dorsal root ganglia neurons with substance P-like and calcitonin gene-related-peptide-like immunoreactivity<sup>22,24</sup>; and NOS-like immunoreactivity is coexpressed in some dorsal root ganglia neurons with substance P-like or calcitonin gene-related-peptide-like immunoreactivity and is reduced greatly in neonatal rats treated with capsaicin.<sup>22,25</sup> Selective neuronal NOS inhibitor 7-nitro-indazole (7-NINA) exhibits antinociceptive activity if injected intraperitoneally into mice, by producing dose-related inhibition of the second phase of hind paw licking after subplantar injection of formalin.<sup>26</sup> Salvemini *et al.*<sup>27</sup> reported that NO produced by constitutive NOS (neuronal or endothelial NOS) was involved in the development of inflammation at early time points (< 4 h) after subcutaneous administration of carrageenan, and NO produced by inducible NOS was involved in the maintenance of the inflammatory response at later time points (> 5 h). It is possible that the NO released after formalin injection may be generated by the neuronal NOS. The increase in  $\text{NO}_2^-$ - $\text{NO}_3^-$  concentration after formalin injection was suppressed by NMDA receptor antagonist AP-5, and NMDA perfusion caused the release of  $\text{NO}_2^-$ - $\text{NO}_3^-$ . These facts suggest that NO is produced by neuronal NOS because NMDA receptors exist in the peripheral nervous system.<sup>28</sup> The current results, in which the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  did not increase in the rats with denervated sensory nerves, imply that an important source of NO is peripheral afferent fibers.

#### NMDA-Nitric Oxide Cascade

Several studies have shown that peripheral glutamate has nociceptive and inflammatory roles. Application of glutamate to skin evokes pain-related behaviors,<sup>28,29</sup> and peripherally administered glutamate antagonists can prevent the nociception produced by inflammation.<sup>29,30</sup> Thus, glutamate receptor activation should play an important role in nociceptive signaling at the level of the peripheral nerve terminal in the skin. In our previous report, the increase in glutamate concentration in the plantar surface of the rat hind paw was observed on the side ipsilateral, but not the side contralateral, to the subcutaneous injection of formalin.<sup>8</sup> We demonstrated that perfusion with the NMDA antagonist AP-5 suppressed the increase in NO after formalin injection, and that NMDA perfusion induced the increase in NO concentration. These facts suggest that formalin evokes the release of glutamate, which may induce the activation of a peripheral NMDA-NO cascade. This cascade was activated throughout the current experiment; the concentration of  $\text{NO}_2^-$ - $\text{NO}_3^-$  did not show the biphasic changes seen in the behavioral study. A possible explanation for this discrepancy is that the central inhibitory systems (descending inhibitory system and intraspinal inhibitory system) are activated in the period between first and second phases in the formalin test.<sup>31-33</sup> We previously reported that intrathecal pretreatment with yohimbine and methysergide produced greater increases in the number of flinches in phase 1, the interphase period, and phase 2 after formalin injection than without pretreatment.<sup>31</sup> Formalin injection induced increases in noradrenaline, 4-hydroxy-3-methoxyphenylglycol (MHPG), serotonin, and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in both the ipsi- and contralateral dorsal halves.<sup>31</sup> Henry *et al.*<sup>32</sup> compared the nociceptive behaviors produced by a single hind paw injection of formalin with those produced by two formalin injections administered 20 min apart. They showed that there was a second decrease in nociceptive responses after the second injection, suggesting that the interphase period in the formalin test results from active inhibition. Matthies and Franklin<sup>33</sup> reported that decerebrate rats (those having undergone transection of the brain at the rostral metencephalon or mesencephalon) did not show the abatement in the interphase period in the formalin test, which suggests that the cessation of the nociceptive behavior at the interphase period may be caused by a central inhibitory modulation of nociceptive behaviors. In summary, subcutaneous formalin injection induces peripheral release of NO, the production of which is

mediated by activation of NMDA receptors in the peripheral nervous system. This peripheral NMDA-NO cascade could contribute to formalin-induced nociception.

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