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## Research report

## Rapid development of nitric oxide-induced hyperalgesia depends on an alternate to the cGMP-mediated pathway in the rat neuropathic pain model

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**Abstract**

Intrathecal injection of a nitric oxide releasing compound, NOC-18, was used to define the role of nitric oxide (NO) in the spinal mechanism of neuropathic pain caused by unilateral chronic constriction injury to rat sciatic nerves. Paw withdrawal latency was used to evaluate nociception induced by thermal stimuli before surgery and afterwards at 1, 3, and 6 h, and on days 1, 2, 3, 4, 5, 8, and 12 after the nerve ligation. In the sham-surgery control groups, intrathecal injection of 10 or 100  $\mu\text{g}$  of NOC-18 did not produce any change in withdrawal latencies. In rats with unilateral nerve ligation, however, administration of 1 or 10  $\mu\text{g}$ , but not 0.1  $\mu\text{g}$ , of NOC-18 significantly shortened the time in which thermal hyperalgesia developed after nerve injury. Injection of 1  $\mu\text{g}$  of NOC-18 decreased the onset time of thermal hyperalgesia from 2 days to 3 h and with 10  $\mu\text{g}$  hyperalgesia developed within 1 h after the nerve injury. The effects of intrathecal injection of MK-801, a *N*-methyl-D-aspartate (NMDA) receptor antagonist, *N*-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, methylene blue (MB), a soluble guanylate cyclase inhibitor, and hemoglobin (Hb), a NO scavenger, on the development of thermal hyperalgesia after the sciatic nerve ligation were examined in the presence and absence of 1 and 10  $\mu\text{g}$  of NOC-18. Acceleration of the development of thermal hyperalgesia induced by 1 and 10  $\mu\text{g}$  NOC-18 was completely inhibited by Hb, but was not affected by either MK-801, L-NAME or MB. These findings indicate that NO plays an important role in the rapid development of thermal hyperalgesia after the nerve injury, but that facilitation of nociceptive processing in the spinal cord may entail an alternate to the NO–cyclic guanosine 3',5'-monophosphate (cGMP) pathway. © 1998 Elsevier Science B.V.

**Keywords:** Nitric oxide; NO-releasing compound; Hyperalgesia; Nerve injury; Spinal cord; Cyclic GMP

**1. Introduction**

Traumatic nerve injury can lead to the development of hyperalgesia or/and allodynia. An increase in the afferent impulse rate from the damaged nerve area enhances the activity of spinal dorsal horn neurons, producing pain. Activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn neurons is critical to the development and maintenance of the hyperalgesia induced by nerve injury [1,10,40]. Bennett and Xie reported an animal model of thermal hyperalgesia induced by chronic constriction injury to the sciatic nerve in rats [5]. Chronic nerve constriction leads to a marked demyelination and degeneration of both myelinated A-fibers and unmyelinated C-fibers [3], but the damage of C-fibers is later and to a lesser extent compared with A-fibers after nerve liga-

tures. This model mimics some aspects of neuropathic pain in humans.

The NMDA receptor is one of the receptors for excitatory amino acids, and plays an important role in changes such as wind-up, facilitation, central sensitization, and hyperalgesia, all of which may be manifestations of the same mechanisms [19,27]. In support of this, recent evidence has demonstrated that intraperitoneal or intrathecal administration of MK-801, a NMDA receptor antagonist, resulted in complete abolition of the thermal hyperalgesia in the neuropathic pain model [12,47,56]. These results suggest that NMDA induces thermal hyperalgesia through the activation of NMDA receptors and subsequent production of nitric oxide (NO).

NO is a free radical which is synthesized from L-arginine and molecular oxygen by NO synthase. It produces an increase in intracellular cyclic guanosine 3',5'-monophosphate (cGMP) through activation of soluble guanylate cyclase. In 1977, NO was first shown to stimulate the

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soluble guanylate cyclase in homogenates of animals' brain [13,35]. There is now considerable evidence that NO plays an important role in neural transmission to act as a second messenger in the central [17,18,43] and peripheral nervous systems [8,20,51], and other part of the body [7,22,36,41,48,49]. NO is deeply involved in the nociceptive processing in the central nervous system, at both spinal and supraspinal levels. Intrathecal injection of L-arginine and NO-releasing compounds produced hyperalgesia in tail flick test and formalin pain model [23,28,32]. In contrast with these reports, others showed that intracerebroventricular administration of L-arginine or dibutyl cGMP produced antinociception via the NO–cGMP pathway in tail flick procedure [26,50,52]. That is, NO exerts a dual role in acute nociceptive processing: intrathecal NO plays a role in thermal hyperalgesia while intracerebroventricular NO plays a role in thermal antinociception.

However, the functional role of NO at spinal or supraspinal level in the mechanism of chronic neuropathic pain remains to be clarified. Yamamoto and Shimoyama [57] demonstrated that pretreatment with a NO synthase inhibitor, such as *N*-nitro-L-arginine methyl ester (L-NAME) or a NO scavenger, hemoglobin (Hb), delayed the development of thermal hyperalgesia following the chronic nerve constriction injury in the rat. Meller et al. [33] also reported that three days after nerve ligation, methylene blue (MB) blocked the thermal hyperalgesia for a period of 2 h, and L-NAME blocked it for 4 h.

NOCs are newly developed NO-releasing compounds which need no cofactors (Fig. 1). They are a convenient and useful source of NO for studies of the many effects of NO [23,43,44,46,58], including nociception. In the solid form, NOCs are stable for months at room temperature but release NO rapidly when dissolved in acidic solutions and more slowly in near neutral solutions. Among of NOCs, NOC-18 releases NO most slowly, with a half life of 78 min at pH 7.4 and 37°C [21]. One mole of NOC can release 1 to 1.5 mole of NO [25]. The form of NO releasing from NOC-18 is NO' (gaseous form).

The functional aspect of NO in the spinal mechanism of chronic neuropathic pain remains to be clarified. The purpose of the present study is to determine whether the NMDA-produced thermal hyperalgesia in a model of neuropathic pain is mediated through the production of NO and activation of soluble guanylate cyclase by examining the effects of NOC-18 administered intrathecally on the

development of thermal hyperalgesia following sciatic nerve ligation in the rat.

## 2. Materials and methods

The study protocol was reviewed and approved by the Animal Care Committee at Osaka University Medical School. One hundred and one male Sprague–Dawley rats (250–280 g; Nihon, Hamamatsu, Japan) were used in this study. Animals were housed five per cage in a room maintained at  $22 \pm 0.5^\circ\text{C}$  with an alternating 12-h light–dark cycle. Food and water were allowed ad libitum until they were transported to the laboratory approximately an hour before the experiments. We performed all experiments under normal room light and temperature.

### 2.1. Animal preparation

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal, Abbott, North Chicago) (45 mg/kg). Then, chronic intrathecal catheters were inserted by passing a polyethylene-10 catheter through an incision in the atlanto–occipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar subarachnoid space, according to the methods of Yaksh and Rudy [55]. The animals were allowed to recover for a week before experiments.

### 2.2. Sciatic nerve constriction injury

The hyperesthetic state was induced by chronic constriction of a sciatic nerve with four loose ligatures. Rats were anesthetized by inhalation of halothane, maintained at a concentration of 2–3%. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Four chronic gut ligatures (4-0) were tied loosely around the right sciatic nerve about 1 mm apart above the level at which the sciatic nerve trifurcates. Ligatures were tied such that they were able to slide freely along the sciatic nerve thus producing no indentation of the sciatic nerve and no apparent impedance of epineural blood flow. The left sciatic nerve was exposed but not ligated and served as a sham surgery. The incision was closed in layers [5].

### 2.3. Thermal nociceptive test

The rats were placed in a clear plastic cage ( $23 \times 13 \times 13 \text{ cm}^3$ ) upon an elevated floor of clear glass (2 mm thick) and were allowed 5–10 min to acclimatize. A radiant heat source (Planter Test No. 7370; Ugo Basile; Italy) was focused on the planter surface of the hindpaw. Withdrawal latency was measured with a stopwatch to the nearest 0.1 s as the time from onset of the heat to withdrawal of the hindpaw from the beam. A cut off latency of 20 s was used

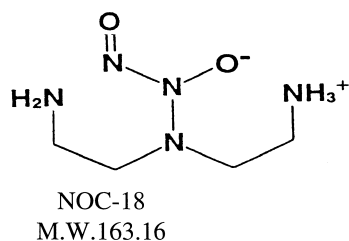


Fig. 1. Chemical structure of a NO-releasing compound, NOC-18.

to avoid tissue damage. Paw withdrawal latency (PWL) was measured 5 times and the basal latency defined as the mean of the last four stable latencies. PWLs were tested alternately with 5 min intervals between consecutive tests. To analyze the magnitude of the hyperesthesia, the difference score (DS) was calculated by subtracting the PWL of the control side (left side) from the PWL of the nerve injury side (right side).

#### 2.4. Behavior analysis

The general behavior of rats was carefully observed at 1, 2, and 3 h after intrathecal administration of drugs. Righting and ambulation were assessed by placing the rat horizontally with its back on the table which normally gives rise to an immediate coordinated twisting of the body to an upright position.

#### 2.5. Drugs

The animals were randomly assigned into 11 groups as follows: no drug (control) ( $n = 10$ ); 0.1  $\mu\text{g}$  of NOC-18 ( $n = 8$ ); 1  $\mu\text{g}$  of NOC-18 ( $n = 9$ ); 10  $\mu\text{g}$  of NOC-18 ( $n = 7$ ); 10  $\mu\text{g}$  of MB ( $n = 7$ ); 50  $\mu\text{g}$  of Hb ( $n = 10$ ); 10  $\mu\text{g}$  of MB and 1  $\mu\text{g}$  of NOC-18 ( $n = 8$ ); 10  $\mu\text{g}$  of MB and 10  $\mu\text{g}$  of NOC-18 ( $n = 9$ ); 50  $\mu\text{g}$  of Hb and 10  $\mu\text{g}$  of NOC-18 ( $n = 7$ ); 100  $\mu\text{g}$  of L-NAME and 10  $\mu\text{g}$  of NOC-18 ( $n = 7$ ); 10  $\mu\text{g}$  of MK-801 and 10  $\mu\text{g}$  of NOC-18 ( $n = 7$ ). All drugs were dissolved in 0.9% saline shortly before administration and the injection volume was adjusted to 5  $\mu\text{l}$ . Each drug solution was administered intrathecally through a catheter connected to a motor-driven Hamilton syringe 15 min before the sciatic nerve ligature. Thermal hyperalgesia was measured before surgery, and at 1, 3, 6 h and 1, 2, 3, 4, 5, 8, and 12 days after the nerve injury. Two additional groups were used to verify the effect of NOC-18 on rats with bilateral sham surgery. These groups received 10  $\mu\text{g}$  of NOC-18 ( $n = 6$ ) or 100  $\mu\text{g}$  of NOC-18 ( $n = 6$ ) intrathecally and were tested at hours 1, 3, and 6 after administration. The agents used in this study were L-NAME, MB, Hb (Sigma, St Louis, MO), MK-801 (Research Biochemicals, Natick, MA) and NOC-18 (Dojindo, Kumamoto, Japan).

#### 2.6. Data analysis

Data were expressed as mean  $\pm$  S.E.M. Student's *t*-test (comparison of two groups) or ANOVA, followed by the Newman-Keuls test (comparison among multiple groups), were used for the statistical evaluation.  $P < 0.05$  was considered as statistically significant.

### 3. Results

Preoperatively, there were no differences observed between right and left PWLs in each group. And at no time were there differences among left PWLs after the sham surgery in each group.

Subjective observation of rats injected with NOC-18 and/or L-NAME, MB, Hb or MK-801 revealed no obvious changes in animal behavior during a period of 3 h when compared with control animals. Neither sedative nor toxic effect was observed after intrathecal administration of any of the drugs used in this study.

#### 3.1. Effects of NOC-18 in the rat with bilateral sham surgery

In the rats with bilateral sham surgery, intrathecal administration of NOC-18 (10 or 100  $\mu\text{g}$ ) did not alter the PWLs to thermal stimuli at hours 1, 3, and 6 after the treatment.

#### 3.2. Effects of NOC-18 after chronic nerve constriction

In the control group, the DS levels on days 2, 3, 4, 5, 8, and 12 after the sciatic nerve ligature were significantly more negative than the presurgical values. In the groups treated with NOC-18 (1  $\mu\text{g}$  or 10  $\mu\text{g}$ ), the DS levels were significantly more negative at all testing times after the nerve injury compared to the presurgical (0 day) values, and the DS levels at hours 1, 3, 6 and on day 1 after the nerve injury were significant more negative than the con-

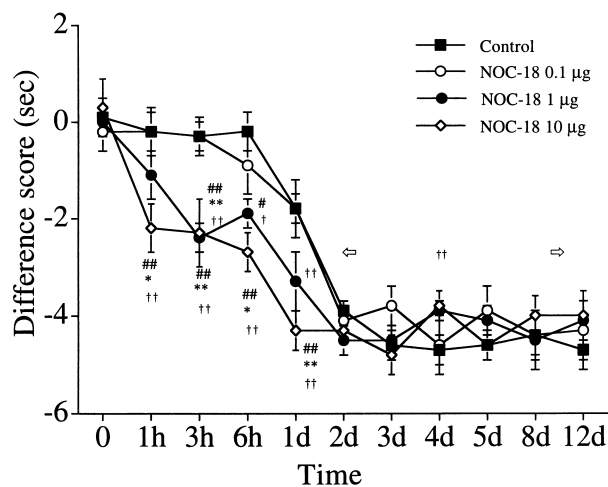


Fig. 2. Time course of the effects of intrathecally administered NOC-18 on the development of thermal hyperalgesia following application of the unilateral sciatic nerve constriction injury in the rat. The DS was calculated by subtracting the paw withdrawal latency of the control side from that of the nerve injury side. Saline control (■) ( $n = 10$ ); 0.1  $\mu\text{g}$  of NOC-18 (○) ( $n = 8$ ); 1  $\mu\text{g}$  of NOC-18 (●) ( $n = 9$ ); 10  $\mu\text{g}$  of NOC-18 (◇) ( $n = 7$ ). All drugs were dissolved in 0.9% saline shortly before administration. The injection volume was always 5  $\mu\text{l}$ . Administration of 1 or 10  $\mu\text{g}$ , but not 0.1  $\mu\text{g}$  of NOC-18 accelerated the development of thermal hyperalgesia in the rat. Data represent the mean  $\pm$  S.E.M. # $P < 0.05$ , ## $P < 0.01$  vs. control, \* $P < 0.05$ , \*\* $P < 0.01$  vs. 0.1  $\mu\text{g}$  of NOC-18. † $P < 0.05$ , †† $P < 0.01$  compared with presurgery (0 day) value in the same group.

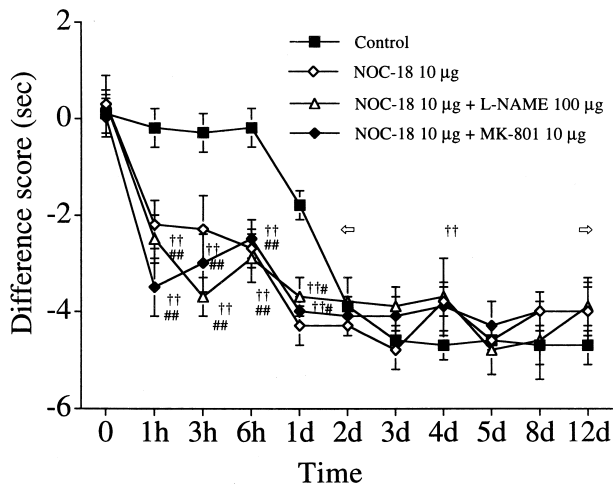


Fig. 3. Time course of the effects of intrathecally co-administered NOC-18 with MK-801 or L-NAME on the development of thermal hyperalgesia induced by a nerve constriction injury. Saline control (■) ( $n=10$ ); 10  $\mu\text{g}$  of NOC-18 ( $\diamond$ ) ( $n=7$ ); 10  $\mu\text{g}$  of NOC-18+100  $\mu\text{g}$  of L-NAME ( $\triangle$ ) ( $n=7$ ); 10  $\mu\text{g}$  of NOC-18+100  $\mu\text{g}$  of MK-801 ( $\blacklozenge$ ) ( $n=7$ ). Both MK-801 and L-NAME did not block the rapid development of hyperalgesia caused by NOC-18. Data represent the mean  $\pm$  S.E.M.  $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$  vs. control.  $^{\dagger}P < 0.05$ ,  $^{\dagger\dagger}P < 0.01$  compared with presurgery (0 day) value in the same group.

control values. The DSs in the 0.1  $\mu\text{g}$  of NOC-18 group were not significantly different from those of the control group (Fig. 2).

### 3.3. Effects of NOC-18 and MK-801 or L-NAME after chronic nerve constriction

In the group treated with NOC-18 (10  $\mu\text{g}$ ) and MK-801 (10  $\mu\text{g}$ ), the DS levels at hours 1, 3, 6 and on day 1 after the nerve ligature were significantly more negative than those of the control group. And in the group treated with NOC-18 (10  $\mu\text{g}$ ) and MK-801 (10  $\mu\text{g}$ ), the DS levels were not significantly different from those of the NOC-18 (10  $\mu\text{g}$ ) group (Fig. 3). In the groups treated with NOC-18 (10  $\mu\text{g}$ ) and L-NAME (100  $\mu\text{g}$ ), the DS levels at hours 1, 3, 6 and on day 1 after the nerve ligature were significantly more negative than those of the control group. And in the group treated with NOC-18 (10  $\mu\text{g}$ ) and L-NAME (100  $\mu\text{g}$ ), the DS levels were not significantly different from those of the NOC-18 (10  $\mu\text{g}$ ) group (Fig. 3).

### 3.4. Effects of Hb alone or NOC-18 and Hb after chronic nerve constriction

On days 2, 3, 4, 5 and 8 after the nerve ligature, the DS levels of Hb (50  $\mu\text{g}$ ) group were less negative than those of the control group (Fig. 4). In the group treated with NOC-18 (10  $\mu\text{g}$ ) and Hb (50  $\mu\text{g}$ ), the DS levels were more negative than those of Hb (50  $\mu\text{g}$ ) group on days 2 after nerve ligature (Fig. 5). At hours 1, 3, 6 and on days 1, 2, 3 and 5 after the nerve injury, the DS levels of NOC-18 (10  $\mu\text{g}$ ) group were significantly more negative than those of the groups treated with NOC-18 (10  $\mu\text{g}$ ) and Hb (50  $\mu\text{g}$ ) (Fig. 5).

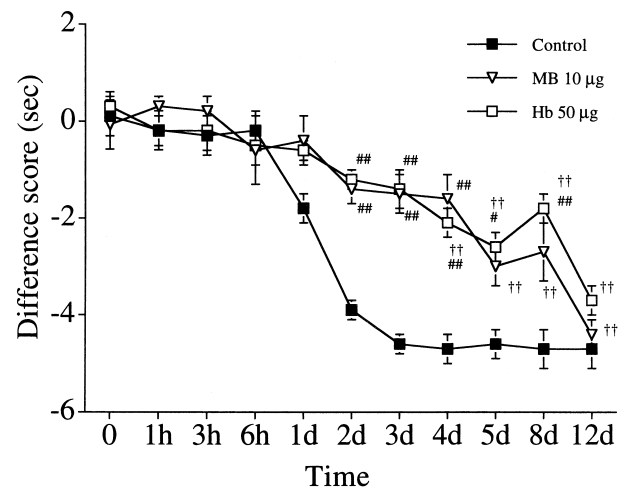


Fig. 4. Time course of the effects of intrathecally administered rat Hb or MB on the development of thermal hyperalgesia induced by a nerve constriction injury. Saline control (■) ( $n=10$ ); 50  $\mu\text{g}$  of Hb ( $\square$ ) ( $n=10$ ); 10  $\mu\text{g}$  of MB ( $\nabla$ ) ( $n=7$ ). The vehicle group is presented for comparison. Data represent the mean  $\pm$  S.E.M.  $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$  vs. control.  $^{\dagger}P < 0.05$ ,  $^{\dagger\dagger}P < 0.01$  compared with presurgery (0 day) value in the same group.

### 3.5. Effects of MB alone or NOC-18 and MB after chronic nerve constriction

The DS levels of MB (10  $\mu\text{g}$ ) treated group were significantly less negative than those of the control group on days 2, 3, 4 and 5 after the nerve ligature (Fig. 4). In the group treated with NOC-18 (10  $\mu\text{g}$ ) and MB (10  $\mu\text{g}$ ),

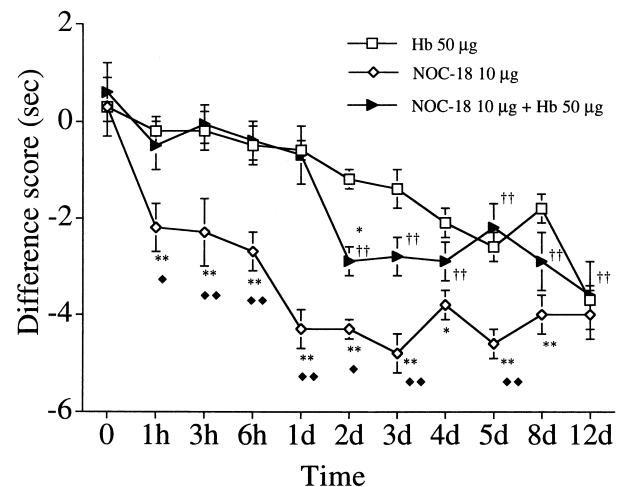


Fig. 5. Time course of the effects of intrathecally co-administered NOC-18 with Hb on the development of thermal hyperalgesia induced by a nerve constriction injury. Ten micrograms of NOC-18 ( $\diamond$ ) ( $n=7$ ); 10  $\mu\text{g}$  of NOC-18 and 50  $\mu\text{g}$  of Hb ( $\blacktriangleright$ ) ( $n=7$ ); 50  $\mu\text{g}$  of Hb ( $\square$ ) ( $n=10$ ). Hb blocked the development of hyperalgesia after the nerve injury in the presence or absence of NOC-18. Data represent the mean  $\pm$  S.E.M.  $^{\blacklozenge}P < 0.05$ ,  $^{\blacklozenge\blacklozenge}P < 0.05$  vs. 10  $\mu\text{g}$  of NOC-18 and 50  $\mu\text{g}$  of Hb,  $^{\ast}P < 0.05$ ,  $^{\ast\ast}P < 0.05$  vs. 50  $\mu\text{g}$  of Hb.  $^{\dagger}P < 0.05$ ,  $^{\dagger\dagger}P < 0.01$  compared with presurgery (0 day) value in the same group.

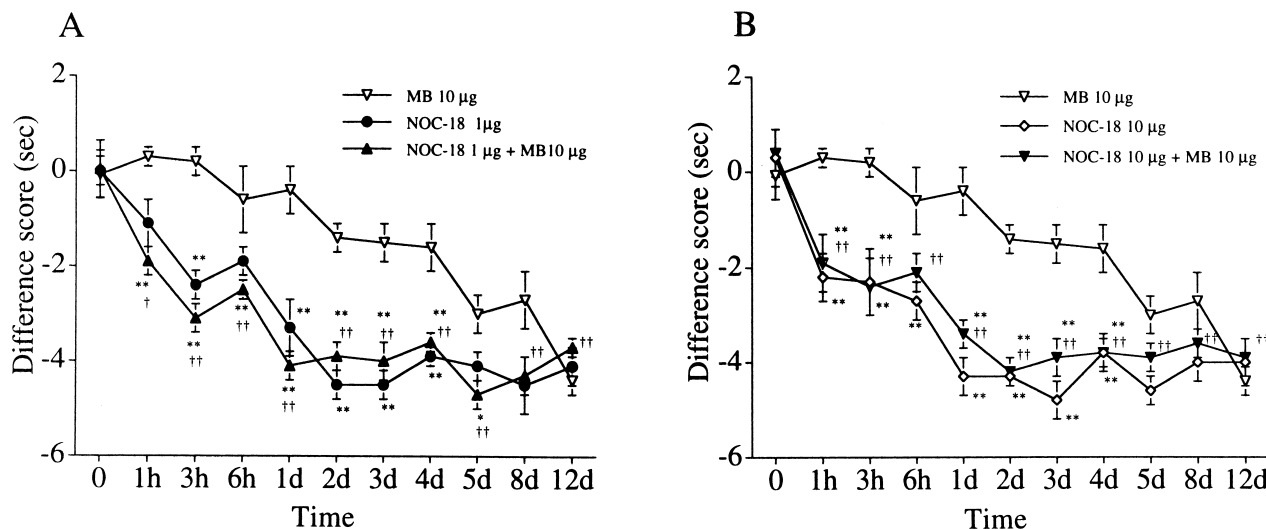


Fig. 6. Time course of the effects of intrathecal co-administration of 1  $\mu$ g (A) or 10  $\mu$ g (B) of NOC-18 with MB on the development of thermal hyperalgesia induced by a nerve constriction injury. Ten micrograms of NOC-18 ( $\diamond$ ) ( $n=7$ ); 10  $\mu$ g of NOC-18 and 10  $\mu$ g of MB ( $\blacktriangledown$ ) ( $n=7$ ); 1  $\mu$ g of NOC-18 and 10  $\mu$ g of MB ( $\blacktriangle$ ) ( $n=8$ ); 10  $\mu$ g of MB ( $\nabla$ ) ( $n=7$ ). MB delayed the development of hyperalgesia after the nerve injury in the absence of NOC-18, but it did not block the rapid development of hyperalgesia caused by NOC-18. Data represent the mean  $\pm$  S.E.M. \*  $P < 0.05$ , \*\*  $P < 0.05$  vs. 10  $\mu$ g of MB.  $\dagger P < 0.05$ ,  $\dagger\dagger P < 0.01$  compared with presurgery (0 day) value in the same group.

the DS levels on hours 1, 3, and 6 and on day 1 after the nerve injury were significantly more negative than those of the control group. The DS levels of the group treated with NOC-18 (1  $\mu$ g) and MB (10  $\mu$ g) were not significantly different from those treated with NOC-18 (1  $\mu$ g) alone. In the group treated with NOC-18 (1  $\mu$ g) and MB (10  $\mu$ g), the DS levels at hours 1, 3, and 6 and on days 1, 2, 3, 4 and 5 after the nerve injury were significantly more negative than those of the MB (10  $\mu$ g) group (Fig. 6A).

The DS levels of the group treated with NOC-18 (10  $\mu$ g) and MB (10  $\mu$ g) group were significantly more negative than those of the control group at hours 1, 3, and 6 and on 1 day after the nerve injury. The DS levels of the group treated with NOC-18 (10  $\mu$ g) and MB (10  $\mu$ g) were not significantly different from those treated with NOC-18 (10  $\mu$ g) alone. In the group treated with NOC-18 (10  $\mu$ g) and MB (10  $\mu$ g), the DS levels at hours 1, 3, and 6 and on days 1, 2, 3 and 4 after the nerve injury were significantly more negative than those treated with MB (10  $\mu$ g) group (Fig. 6B).

#### 4. Discussion

The current study shows that intrathecal administration of NOC-18 has no effect on the PWLs to thermal stimuli in rats with bilateral sham surgery. Thus we have clear evidence that NO does not produce thermal hyperalgesia in animals without nerve injury as evaluated by the paw withdrawal behavior. However, our previous study [23] showed that intrathecal NOC-18 produced a dose-dependent curtailment of the radiant heat tail-flick latency in rats

without nerve injury. We have no distinct explanation for the discrepancy in findings with the tail flick response and the paw withdrawal response. The interpretation of these reflexive measures has not been clearly defined in pain research. The inconsistency in these results may originate from the different methods of pain measurement, that is, the tail flick response and the paw withdrawal behavior may be derived from different reflexive processings. The present and previous [23] studies indicate that paw withdrawal response exhibits a longer latency (10–12 s) compared with the tail flick response (4–5 s). The tail flick is a monosynaptically mediated sensorimotor reflex test, assessed by timing the response to radiant heat on the skin of the tail, which does not measure the critical higher central nervous system functions involved in the experience of pain [24]. But the paw withdrawal behavior, which is more complex and appears more purposive, is derived from complex organized behavior that may be either normally elicited or unlearned pain reaction [38]. The recent study [30] demonstrated that blockade of thalamic NMDA receptors produced by intrathalamic injection of D,2-amino-5-phosphonovaleric acid antagonized carrageenan-induced decrease in the rat PWLs. Our recent study [42] also demonstrated that brain electroconvulsive treatment blocked the curtailment of PWLs produced by unilateral chronic constriction of sciatic nerve in the rat. These findings suggest that the paw withdrawal behavior may be integrated and modulated not only at spinal but also at supraspinal synaptic junctions in neural pathways. There may be a descending inhibitory pathway from the brain involved.

It has been demonstrated that the application of loose unilateral sciatic nerve ligations induces a time-dependent

thermal [5] and mechanical hyperalgesia [2]. Our present data showed that the DS levels on days 2, 3, 4, 5, 8, and 12 after right sciatic nerve ligation were significantly more negative than the presurgical values, whereas the PWL of the left hind paw after unilateral sham surgery did not change during the study period. The average time change observed (difference score) in the present study was approximately  $-4.0$  s at 2–12 days after the unilateral sciatic nerve constriction injury. These data clearly indicate that thermal hyperalgesia developed in the control saline group 2 days after the nerve injury and continued for at least 12 days. The onset time and duration of hyperalgesia are comparable with published data [5,57], demonstrating that thermal hyperalgesia was evident on the second postoperative day and lasted for 2 months.

While the precise mechanisms by which hyperalgesia or hyperesthesia are produced after the application of loose sciatic nerve ligatures are not clear, it is possible that the peripheral nerve lesion may cause facilitation of spinal nociceptive processing. Chronic nerve constriction leads to a marked demyelination and degeneration of large and small myelinated fibers; A $\beta$ - and A $\delta$ -fibers, and the unmyelinated C-fiber [3]. However, the damage of C-fibers is later and to a lesser extent compared with A-fibers after loose nerve ligatures. An increase in the impulse rate mediated by C-fibers from the damaged nerve area enhances the activity of spinal dorsal horn neurons. It has been proposed that high-frequency barrages of C-fiber discharges after nerve injury result in the release of glutamate from primary afferent terminals, and a subsequent induction of an influx of  $\text{Ca}^{2+}$  through the NMDA receptor ion channel [14,53,54]. This leads to a sequence of events including the production of NO which causes the long-term changes observed in hyperalgesia.

In the present study, we used a newly developed NO-donor, NOC-18 to examine the effects of exogenous NO on the spinal processing. From the published data on the half-life of NO release [21], we estimated that the duration of the effect of NOC-18 would be about 2 h since the pH of the cerebrospinal fluid was 7.53 to 7.60. Kitto et al. [28] used sodium nitroprusside as a NO-donor, injecting it intrathecally to examine the nociceptive effect of NO. Although nitroprusside spontaneously releases NO, seemingly independent of the pH [15], the mechanism of NO release from sodium nitroprusside remains obscure. Aqueous solutions of sodium nitroprusside are temperature- and light-sensitive [4], and during decomposition cyanide is released [57]. Compared with sodium nitroprusside, NOC-18 releases a large amount of NO spontaneously, and its mechanism of NO release is very simple; hence, no effects of other metabolites need to be taken into account.

The current study demonstrates that a single intrathecal injection with NOC-18 (1 and 10  $\mu\text{g}$ ) 15 min prior to surgery to place the ligatures decreased the time before development of thermal hyperalgesia, while no effects were observed with 0.1  $\mu\text{g}$  of NOC-18 pretreatment (Fig.

2). Administration of 1  $\mu\text{g}$  of NOC-18 shortened the onset time of the development of thermal hyperalgesia from 2 days to 3 h, and the higher concentration to 1 hour after the nerve injury. This finding clearly indicates that NO plays a critical role in the development of the thermal hyperalgesia following nerve injury. Exogenous NO may function as a trigger in the early stages of the development of hyperalgesia. It is possible that a large amount of exogenous NO derived from NOC-18 diffuses to enter the spinal postsynaptic dorsal horn neuron and thus facilitate the nociceptive processing. A portion of NO may diffuse into the presynaptic neuron, increasing the release of glutamate, substance P and the calcitonin gene-related peptide and also produce facilitation of the nociceptive responses to stimuli [34]. The lack of effect of 0.1  $\mu\text{g}$  of NOC-18 may indicate an existence of a threshold level of NO needed to induce hypersensitivity of the dorsal horn neuron.

Hemoglobin acts as a NO scavenger [45], but cannot permeate through cell membranes into the interior of the cell [37,45]. It is thus unable to scavenge intracellular NO produced by NO synthase in the postsynaptic dorsal horn neuron, but it is able to scavenge extracellular NO released from NOC-18 as well as that which has diffused from neighboring cells. We found that pretreatment with Hb alone delayed the development of thermal hyperalgesia from 2 days to about 4 days after the sciatic nerve ligation (Fig. 4). These results indicate that scavenging of extracellular NO which has diffused into the synaptic cleft from neuron cells may attenuate the initiation of hyperalgesia. While the effect of NOC-18 on the rate of development of hyperalgesia was prevented by Hb, neither MK-801, L-NAME, nor MB showed any effects.

The NMDA receptor is involved in multisynaptic nociceptive transmission and plasticity in the spinal cord [1,22,28,32,33,40]. Many effects of NMDA receptor activation seem to be ultimately mediated through production of NO. Activation of the postsynaptic NMDA receptor results in an influx of  $\text{Ca}^{2+}$  which activates a calmodulin-sensitive site on NO synthase to produce NO from L-arginine [7].

It has been reported that pretreatment with either MK-801, an NMDA antagonist which inhibits  $\text{Ca}^{2+}$  influx through the ion channel and subsequently decreases the production of NO or L-NAME, a NO synthase inhibitor, also blocked the development of thermal hyperalgesia [47,56]. Intrathecal injection of NOC-18 produces NO in the synaptic cleft, which may travel into presynaptic and postsynaptic neural cells, as well as glial cells. Hb can scavenge extracellular NO released from NOC-18 and thus can completely prevent the NO-induced development of thermal hyperalgesia following nerve injury. In these experiments, neither MK-801 nor L-NAME could influence the rate of development of thermal hyperalgesia induced by exogenous NO released from NOC-18. These findings provide further support for the role of the NMDA recep-

tor–NO pathway in the natural development of thermal hyperalgesia following nerve injury and suggest that exogenous NO derived from NOC-18 can decrease the time before development of hyperalgesia in the absence of endogenous NO.

NO plays an important physiological role in local transcellular communication by facilitating cGMP formation in adjacent cells through the activation of soluble guanylate cyclase [9,29], which is present in the neurons of spinal cord and brain regions. Cyclic GMP modifies several intracellular processes, including direct activation of K<sup>+</sup> channels [16] and Ca<sup>2+</sup> current enhancement via a cGMP-dependent protein kinase [39]. This NO–cGMP pathway modifies several intracellular processes, including activation of protein kinases, ion channels and phosphodiesterases [9,36]. MB is an inhibitor of a soluble guanylate cyclase, and as such it decreases the production of cGMP induced by NO in the cell and may also play an important role in the development of hyperalgesia following the nerve injury.

The current study shows that pretreatment with MB alone delays the development of thermal hyperalgesia from 2 days to about 5 days after nerve ligation (Fig. 4). However, we found that intrathecal administration of MB did not antagonize the rapid development of hyperalgesia induced by exogenous NO released from NOC-18. This finding indicates that alternative pathway(s) apart from the NO–cGMP pathway may also be involved in the rapid development of hyperalgesia in the spinal level.

Recently, NO has been shown to be involved in the activation of synthesizing enzymes for eicosanoids and for gene modification [31]. Another in vitro study reported that the NO-mediated cGMP-independent activation of Ca<sup>2+</sup>-dependent K<sup>+</sup> channel (K<sub>Ca</sub><sup>+</sup>) may circumvent the cGMP-dependent pathway in vascular smooth muscle cells [6]. These reports demonstrate a new pathway for NO regulation of physiological function in the absence of cGMP. Our results also suggest that NO might directly activate K<sup>+</sup> channels or trigger the expression of early genes such as *c-fos* and *c-jun* which are involved in central plasticity associated with hyperalgesia.

## 5. Conclusion

Intrathecal administration of NOC-18 accelerated the development of thermal hyperalgesia following application of the unilateral sciatic nerve ligation in the rat. The NOC-18-induced rapid development of thermal hyperalgesia was abolished by Hb, but was not affected by either L-NAME, MK-801 or MB. These findings suggest that NO plays an important role in the rapid development of thermal hyperalgesia following the nerve injury, and the alternative pathway(s) except the NO–cGMP may be involved in the facilitation of the nociceptive processing in the spinal cord [11].

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