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Original Reports

Neuronal Nitric Oxide Synthase Suppression Confers the Prolonged Analgesic Effect of Sciatic Nerve Block With Perineural Dexamethasone in Postoperative Pain Model Mice

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Abstract: **Dexamethasone supplementation to local anesthetics prolongs its action, yet the underlying mechanism is unclear. Previous studies have reported that increased p-p38 mitogen-activated protein kinase (MAPK) in the dorsal root ganglia (DRG) is associated with pain-associated behavior and that nitric oxide (NO), which is known to be a pronociceptive substance, directly inhibits sciatic nerve conduction. Here, we investigated the temporal changes in the hyperalgesic effect and p-p38 MAPK and NO synthase (NOS) expression levels in the DRG when dexamethasone was added to ropivacaine used for a sciatic nerve block (SNB) in postoperative pain model mice. Dexamethasone supplementation to ropivacaine significantly prolonged the analgesic effect of SNB via glucocorticoid receptor activation. Histological examination revealed that ropivacaine suppressed p-p38 MAPK expression in the DRG regardless of dexamethasone supplementation, suggesting that p-p38 MAPK was not involved in the prolonging effect of dexamethasone on nerve block. Contrastingly, plantar incision markedly increased the expression of neuronal NOS (nNOS) in DRG, and dexamethasone supplementation to ropivacaine significantly suppressed nNOS expression. Supplementation of L-NAME, an inhibitor of NOS, to ropivacaine markedly prolonged the effect of SNB, similar to dexamethasone. These results suggest that dexamethasone supplementation to local anesthetics prolongs the analgesic effect by inhibiting nNOS activity.**

Perspective: ***The current study revealed that dexamethasone supplementation to local anesthetics prolongs the analgesic effect by inhibiting the activity of neuronal NOS and that p-p38 MAPK may not be involved in this phenomenon. Our findings offer a new target for the discovery of long-acting local anesthetics.***

Key words: *Dexamethasone, local anesthetic, nitric oxide synthase, prolonged analgesic effect, nerve block.*

Introduction

Recently, increasing attention has been paid to opioid-sparing anesthesia. Peripheral nerve block is considered one of the most useful methods of providing perioperative analgesia and has been shown to reduce perioperative opioid use and promote postoperative recovery.[1] However, patients who have received a peripheral nerve block consisting of only local anesthetics experience more pain after the analgesic effect disappears than those without a nerve block; this is known as rebound pain.[2] Therefore, attempts have been made to extend the duration of action of local anesthetics by supplementing them with other drugs or

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using liposomal (sustained-release) local anesthetics, such as Exparel (Pacira BioSciences, Inc, Parsippany-Troy Hills, NJ).

Several drugs, including dexamethasone, can supplement local anesthetics and prolong their effects.[3] The effects of dexamethasone-supplemented peripheral nerve blocks can reportedly last longer than those when dexamethasone is administered systemically.[4,5]

Several behavioral experiments in rodents have demonstrated that dexamethasone supplementation to local anesthetics prolongs anesthesia.[6-8] Histological examinations of the sciatic nerve in these studies have also shown that dexamethasone reduces the neurotoxicity of local anesthetics. However, no studies have been conducted to evaluate the histological changes in peripheral nerves involved in the prolonged analgesic effect induced by dexamethasone supplementation to local anesthetics. Previous studies have shown that an enhancement of the nerve conduction inhibition of local anesthetics by glucocorticoids was observed in vivo but not in vitro,[9,10] suggesting that the prolonging effect of dexamethasone on local anesthetics may not involve a direct effect on axonal conduction. Moreover, cortexolone, a glucocorticoid receptor antagonist, counteracts the prolonging effect of dexamethasone on local anesthetics, indicating that the mechanism is mediated by glucocorticoid receptors.[7]

Mitogen-activated protein kinases (MAPKs) in the spinal dorsal horn and the dorsal root ganglion (DRG) are widely recognized to have crucial roles in signal transduction in pain perception and development. In particular, the phosphorylation of p38 MAPK has been reported to increase in the DRGs of postoperative pain model rodents by plantar incision. Previous studies have shown that the inhibition of p38 MAPK activity suppressed pain behaviors.[11,12] Moreover, sciatic nerve blockade by bupivacaine microsphere before nerve injury inhibits the increase of phosphorylated-p38 MAPK (p-p38 MAPK) activity in spinal microglia, whereas nerve blocks after established nerve injury did not reverse the activation of p38 MAPK.[13] These results suggest that the conduction block only before nociceptive input prevents p38 MAPK activation.

Nitric oxide (NO) is a neurotransmitter in both acute and chronic (neuropathic) pain in the peripheral and central nervous systems via direct and indirect pathways.[14,15] A previous study suggested that peripheral tissue damage induced the expression of NO synthase (NOS) in DRG.[16] The chronic administration of dexamethasone suppresses neuronal and endothelial NOS in various tissue including neural tissue, resulting in the pharmacological phenotype of dexamethasone.[17-19] However, since NO directly inhibits nerve conduction,[20,21] an increase in NO activity due to tissue damage may additively enhance the local anesthetic effect. Previous studies suggest that p38MAPK and NOS activation are associated.[22-25]

This study investigated the molecular mechanism underlying the prolonging effect of adding dexamethasone to local anesthetics in a postoperative pain mouse model, mimicking clinical peripheral nerve block for perioperative pain management. Histological assessments focused on p-p38 MAPK, which is involved in postoperative pain development,[11,12] and NOS in the DRG upon peripheral tissue damage. We hypothesized that dexamethasone supplementation to local anesthetics suppressed the phosphorylation of p38 MAPK (p-p38 MAPK) and NOS expression in the DRG when a sciatic nerve block (SNB) was used for analgesia for a hind paw incision.

Methods

Animals

The experiments presented here were approved by the Ethics Committee for Animal Experiments of the Niigata University Graduate School of Medicine and Dentistry (approval number: SA00234) and were performed according to the guidelines of the Science Council of Japan. Male C57BL/6N mice, aged 6 to 8 weeks, were used in this study (Charles River Japan, Yokohama, Japan). All mice were housed at room temperature (20−25°C) under a 12 to12 hours light-dark cycle with unrestricted access to food and water. All efforts were made to minimize the use of animals and reduce pain and discomfort.

Drug Preparation, SNB, and Plantar Incision

The solutions used for SNB were prepared by diluting ropivacaine (Aspen Japan, Tokyo, Japan) and dexamethasone (Fuji Pharma, Toyama, Japan) in saline to create a standardized volume of 40 μL. Dexamethasone was added to the ropivacaine solution immediately before SNB.

All invasive procedures were performed under general anesthesia with 2% isoflurane. SNB was performed based on previously described methods.[6] Briefly, the mice were placed in the supine position, and a 5-mm incision was made on the posterior surface of the left thigh. Under aseptic conditions, a 2-mm incision was made in the fascia over the sciatic nerve, and the drug was administered in the perineural space using a Hamilton syringe with a 30-gauge needle. After drug administration, the surgical wound was sutured with a single 5 to 0 nylon suture.

The plantar incision was made 10 minutes after SNB according to a previous report with some modifications.[26] A 5-mm incision was made on the left hind paw starting 2 mm from the proximal edge of the heel. The underlying flexor muscle was elevated and incised transversely. The surgical wound was closed with a single 5 to 0 nylon suture.

Study Groups

The following experimental groups were used to confirm the prolonging effect of dexamethasone on local anesthetics: 1) control group, which received perineural injection with saline; 2) R group, which received

perineural injection with ropivacaine (15 mg/kg); 3) RSD group, which received perineural injection with ropivacaine (15 mg/kg) and the intramuscular administration of dexamethasone (1.5 mg/kg) into the right thigh; 4) RPD group, which received perineural injection with ropivacaine (15 mg/kg) supplemented with dexamethasone (1.5 mg/kg); and 5) PD group, which received perineural injection with saline supplemented with dexamethasone (1.5 mg/kg). The dose of dexamethasone (1.5 mg/kg) was calculated according to a previous report.[27]

To confirm that the prolonging effect of dexamethasone on local anesthetics is mediated by glucocorticoid receptor activation, mifepristone (MIF; 50 mg/kg, Sigma, St. Louis, MO) was administered intraperitoneally to block glucocorticoid receptors[28] 30 minutes before SNB. The following groups containing mice pretreated with MIF were subsequently prepared: 6) MIF-control group, which received perineural injection with saline; 7) MIF-R group, which received perineural injection with ropivacaine (15 mg/kg); and (8) MIF-RPD group, which received perineural injection with ropivacaine (15 mg/kg) supplemented with dexamethasone (1.5 mg/kg).

In this study, the immunohistochemical experiment results suggested that the prolonging effect of SNB supplemented with dexamethasone was associated with the suppression of NO synthesis in the DRG after plantar incision. To confirm that NOS inhibition mediated the prolonging effect of dexamethasone on local anesthetics, we first examined whether pan-NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Sigma, St Louis, MO) had an analgesic effect. To determine the maximal dose of L-NAME without an analgesic effect, several doses of L-NAME (range: 3.125−50 mg/kg, dissolved in saline, total volume 40 μL) were perineurally administered to naïve mice in a similar fashion as the administration of SNB. To confirm that NOS inhibition was associated with the prolonging effect of local anesthetics, the non-analgesic dose of L-NAME was determined, and pain threshold was evaluated in the following groups: P-L-NAME 6.25, which received perineural injection with saline supplemented with L-NAME (6.25 mg/kg), and RP-L-NAME 6.25, which received perineural injection with ropivacaine (15 mg/kg) supplemented with L-NAME (6.25 mg/kg). Each group of naïve mice contained 4 mice, and each group with mice undergoing plantar incision comprised 6 mice.

Behavioral Assessment

All experiments were performed at the same time of day to ensure that circadian rhythms were unaffected. The nociceptive hind paw threshold for thermal stimulus was tested, as described previously.[29] The mice were habituated to the experimental room for 30 minutes. The experimental animals were subsequently anesthetized with isoflurane, which was followed by perineural drug administration. The postoperative pain model mice underwent a left hind plantar incision 10 minutes after perineural drug administration. Behavioral experiments were performed 30 minutes after the end of isoflurane anesthesia. The pain threshold was assessed by paw withdrawal latency (PWL) against radiant heat stimuli (model 7,370, Ugo Basile, Comerio, Italy).[30] The mice were placed on a glass plate in a red acrylic chamber and PWLs were measured at 30, 60, 120, 240, and 360 minutes after SNB. The time until licking, tapping the left hind paw, or avoiding weight bearing on the left hind paw was recorded as the PWL time. At each time point, the heat hypersensitivity score was averaged for 3 measurements at 2-minutes intervals. The cut-off time was set to 20 seconds to avoid damage to the hind paw.

Immunohistochemistry

The mice were euthanized by isoflurane overdose and thereafter were immediately perfused transcardially with 25 mL of saline followed by 25 mL of 4% paraformaldehyde (Mildform 10N, Fujifilm Wako Pure Chemical Company, Osaka, Japan). Neuronal NOS (nNOS) expression in the DRG of each group was compared. Harvested DRGs were post-fixed at 4°C overnight in the same fixative solution and subsequently equilibrated in 20% sucrose in 0.1 M phosphate-buffered saline (PBS) overnight at 4°C for cryoprotection. Tissues were embedded in FSC22-frozen section medium (Leica Biosystems, Wetzlar, Germany) and cryopreserved at -70°C until sectioning. DRG sections with a thickness of 5 μm, prepared via thin-slicing using a frozen microtome (CM1520, Leica Biosystems, Wetzlar, Germany), were mounted on aminopropyltriethoxysilane-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan). These DRG sections were then cryopreserved at -70°C until immunohistochemistry was performed.

Immunofluorescence Against p-p38 MAPK

Immunohistological procedures were performed as described in a previous study.[29] The primary and secondary antibodies used in this study were phospho-p38 MAPK (Thr 180/Tyr 182) antibody (1:1,000; Cat. No. 4,511; Cell Signaling Technology, Danvers, MA) and Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:2,000; Cat. No. 111-167-003; Jackson ImmunoResearch Laboratories Inc, West Grove, PA), respectively. To verify the co-localization with the neuronal nuclear marker NeuN, double immunostaining with p-p38MAPK and NeuN was performed. The L5 DRG specimen model at 240 minutes after plantar incision was incubated with phospho-p38 MAPK antibody and NeuN antibody (1:800; Cat. No. MAB377; Millipore, Burlington, MA) and the secondary antibody Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated donkey anti-mouse antibody (1:400; Cat. No. AP192F; Millipore), as described above. The sections were embedded using the VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and were visualized using a fluorescence microscope

(BX 53, Olympus, Tokyo, Japan) equipped with a digital camera system (DP73, Olympus, Tokyo, Japan).

Diaminobenzidine (DAB) Staining Against NOS Subtypes

Sections were washed twice with PBS (10% 1 M phosphate buffer, 5% 3 M NaCl) for 10 minutes, and tissue blocking was performed as described above. After rinsing with PBS, the sections were incubated with an anti-nNOS antibody (1:400, Cat. No. ab15203; Abcam, Cambridge, UK), anti-endothelial NOS (eNOS) antibody (1:400; Cat. No. ab5589; Abcam), or anti-inducible NOS (iNOS) antibody (1:200; Cat. No. ab15,323; Abcam) at 4° C for 2 days. The sections were subsequently incubated with biotinylated anti-rabbit IgG (1:1,000, Cat. No. BA-1,000; Vector Labs Inc, Burlingame, CA) for 4 h at room temperature, followed by avidin-biotin complex (1:1,000, Vector Laboratories) for 1 h at room temperature. The sections were rinsed with PBS and reacted with liquid DAB+ substrate (Dako North America, Inc, Carpinteria, CA). Finally, the sections were visualized as described above. Slices that were not incubated in primary antibodies were used as negative controls and did not show any significant staining. The tissues after blocking with normal saline (mock block) were evaluated using the same methods to confirm that the SNB procedure itself did not affect the results. There was no difference between the mock block group and naïve mice (n = 3, data not shown).

Cell Count in Immunohistochemistry in DRGs

Individuals unaware of the experimental protocol counted the cells in a double-blinded manner. The images were analyzed using the "Fiji" version of ImageJ.[31] Four sections sized ≥60 μm separated from each mouse were selected, and only neurons with clearly visible nuclei were used to calculate the results of p-p38 MAPK. To validate our counting method, double immunostaining with p-p38 MAPK and NeuN was performed on selected samples to confirm that p-p38MAPK-positive nuclei co-localized with NeuN. In one DRG image, 80 to 120 neurons were counted. The proportion of neurons expressing p-p38 MAPK was determined by counting the number of neurons that were clearly labeled in the DRG section compared to those in the background. The ratio of immunopositive neurons to the total number of neurons with nuclei was calculated to identify the expression of p-p38 MAPK in the DRG.

Regarding immunostaining for eNOS, nNOS, and iNOS, sections were selected in the same manner as described above, and only neurons with clearly visible nuclei were counted. The number of neurons stained with each antibody in the DRG section was counted to obtain the total number of neurons. Among these, neurons that were labeled with a clear difference were regarded as immunopositive neurons, and the ratio to the total number was calculated.

Statistical Analysis

The sample sizes were determined based on previous publications on behavioral and immunohistological analyses. No *a priori* statistical power calculation was conducted. A post-hoc power analysis was conducted using G* power version 3.1.7.,[32] which indicated that a sample of 4 or 6 mice per group was sufficient for behavioral experiments and immunohistochemical analysis to reach statistical significance with power (1 - β) set at 0.8 and α-value of 0.05.

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using 2-way analysis of variance (ANOVA) or repeated measure (RM) 2-way ANOVA, followed by Tukey's test for each test with GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA). *P*-values of <.05 were considered statistically significant.

Results

Effect of SNB With Dexamethasone

The postoperative pain model mice demonstrated reduction in PWL to thermal stimuli for 30 to 360 minutes after surgery. When ropivacaine SNB alone was administered to the model mice, the analgesia duration was ≤120 minutes. In contrast, when dexamethasone (1.5 mg/kg) was added to ropivacaine, the analgesia duration was prolonged to >360 minutes. Systemic administration of the same dose of dexamethasone had no obvious prolonging effect on the SNB. Perineural administration of only dexamethasone did not demonstrate an analgesic effect in the model mice (n = 6 for each group; Time: $F(5, 30) = 48.73$, $P < .0001$; Group: $F(2.879, 86.36) = 62.09$, $P < .0001$; Time * Group: $F(20, 120) = 11.03$, $P < .0001$ by RM 2-way ANOVA, Fig 1A).

The prolonged effect of dexamethasone on local anesthetics was completely abolished by the intramuscular administration of MIF (50 mg/kg) 30 minutes before dexamethasone-supplemented SNB administration. In contrast, MIF itself did not influence local anesthetic potency in this experimental setting (n = 6 for each group; Time: $F(5, 30) = 86.07$, $P < .0001$; Group: $F(1.854, 55.63) = 29.44$, $P < .0001$; Time * Group: $F(15, 90) = 5.203$, $P < .0001$ by RM 2-way ANOVA, Fig 1B).

Effects of SNB on p-p38 MAPK and NOS Subtypes in L5 DRGs After Plantar Incision

The results of the behavioral experiment revealed that the SNB analgesic effect was highest after 30 minutes and the analgesic effect was prolonged beyond 240 minutes only in the RPD group. Hence, DRGs were collected from each group 30 and 240 minutes after the SNB for immunostaining to further evaluate the roles of p-p38 MAPK and the 3 NOS subtypes in prolonging the local anesthetic effect with dexamethasone.

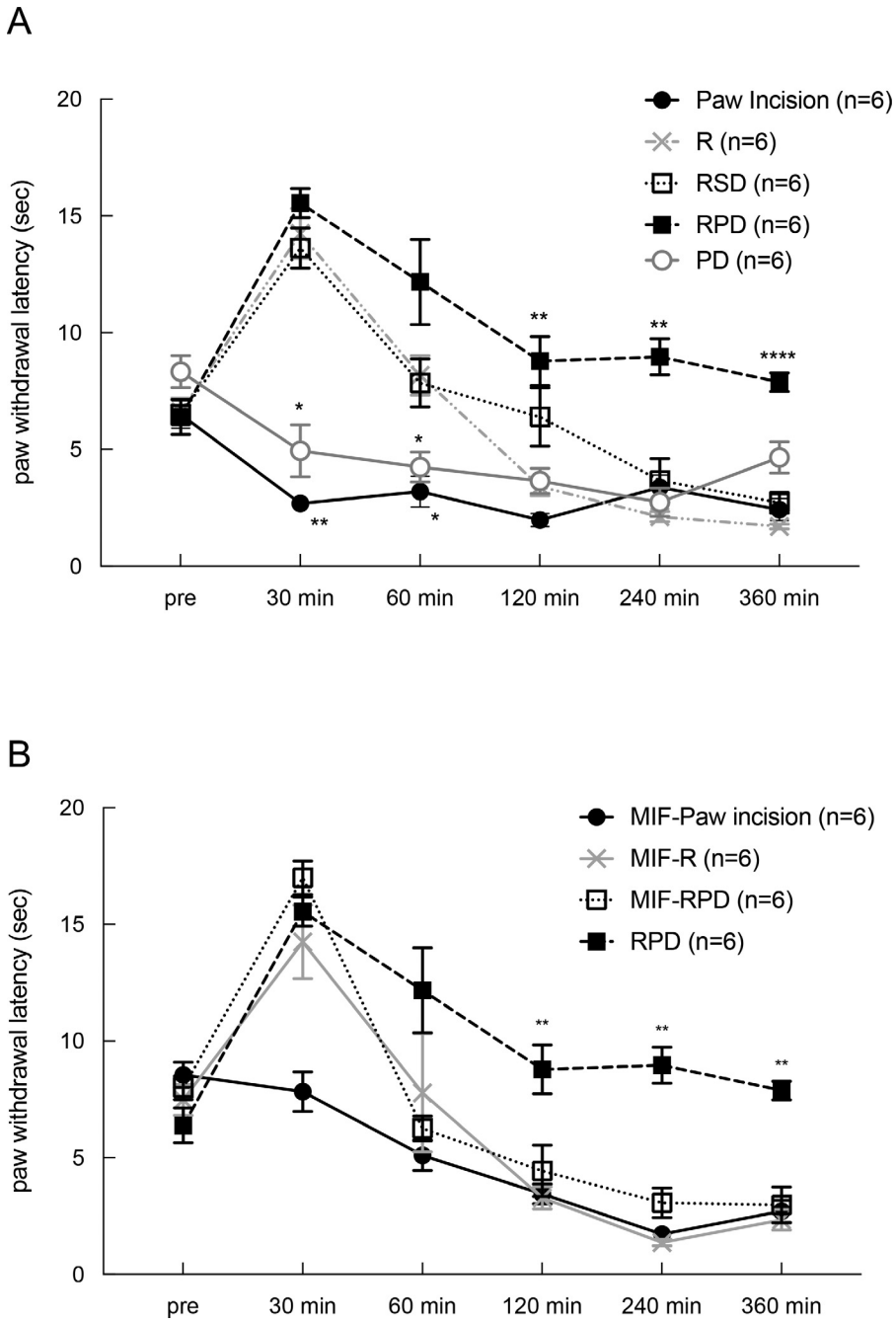

Figure 1. (A) Time course plots showing changes in paw withdrawal latencies (PWLs) after sciatic nerve block (SNB) with or without dexamethasone supplementation using radiant heat stimuli in postoperative pain model mice. Plantar Incision, model mice that received mock SNB with saline; R, model mice that received SNB with ropivacaine (15 mg/kg, 40 μL); RPD: model mice that received SNB with ropivacaine supplemented with dexamethasone (1.5 mg/kg); RSD: model mice that received SNB with ropivacaine and an intramuscular injection of dexamethasone (1.5 mg/kg); and PD, model mice that received perineural dexamethasone (1.5 mg/kg). * $P < .05$, ** $P < .01$, and **** $P < .0001$ compared with the R group by an RM 2-way ANOVA followed by the Tukey's test. (B) Time course plots showing changes in PWLs after SNB with or without dexamethasone supplementation using radiant heat stimuli in postoperative pain model mice pretreated with a glucocorticoid receptor antagonist, mifepristone (MIF). MIF-Plantar Incision, model mice that were pretreated with mifepristone (50 mg/kg) and received mock SNB with saline; MIF-R, model mice that were pretreated with mifepristone (50 mg/kg) and received SNB with ropivacaine; and MIF-RPD, model mice that were pretreated with mifepristone (50 mg/kg) and received SNB with ropivacaine supplemented with dexamethasone. ** $P < .01$ compared with the MIF-R group by the RM 2-way ANOVA followed by the Tukey's test.

The expression of p-p38 MAPK significantly increased in NeuN-positive DRG neuronal nuclei in the model mice 240 minutes after plantar incision. Perineuronal dexamethasone administration without ropivacaine did not alter the expression profile of p-p38MAPK in L5 DRG after plantar incision. The increased expression of p-p38 MAPK in L5 DRG was suppressed by the SNB despite dexamethasone supplementation, and there was no temporal increase in the R or RPD groups (n = 6 for each group; Time: $F(1, 50) = 17.15$, $P = .0001$; Group:

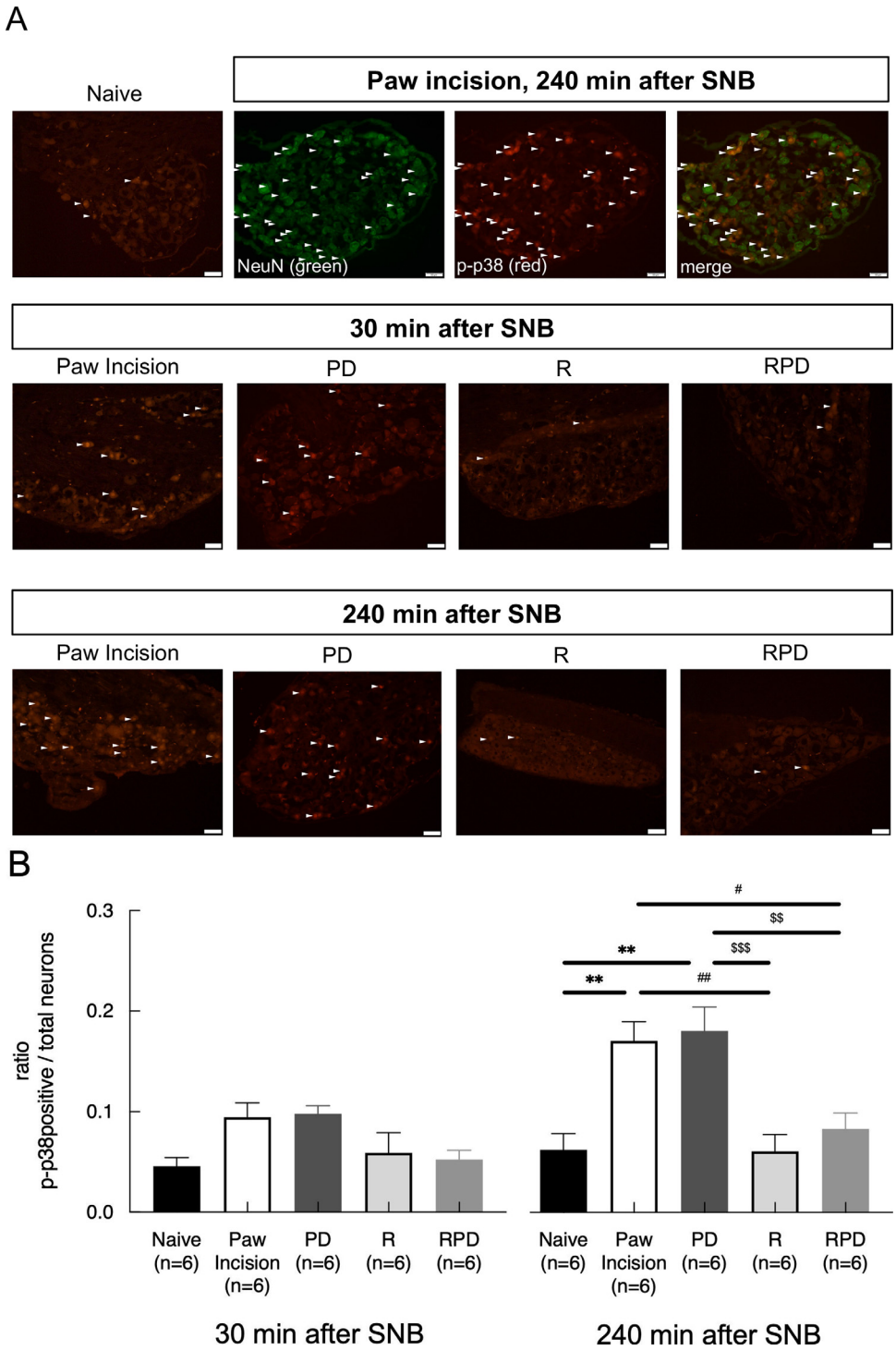

Figure 2. Enhancement of the phosphorylation of p38 mitogen-activated protein kinase (p-p38 MAPK) in the L5 dorsal root ganglion (DRG) of postoperative pain model mice. (A) p-p38 MAPK is expressed in the L5 DRG to some extent. Double immunostaining shows that p-p38MAPK clearly co-localizes with the NeuN-positive nuclei of DRG neurons in model mice. The expression of p-p38 MAPK is significantly increased in L5 DRG 240 minutes after plantar incision in model mice that received a mock block. An increase in p-p38 MAPK in the L5 DRG in model mice that received a sciatic nerve block (SNB) is suppressed similarly to the manner in naïve mice 240 minutes after plantar incision under local anesthesia with or without dexamethasone supplementation. Arrowheads indicate immunopositive neurons. Scale bar: 50 μm. (B) Summarized data of changes in p-p38 MAPK expression after plantar incision and SNB. * $P < .01$ compared with the naive group, # $P < .05$, ## $P < .01$ compared with the Plantar Incision group and $$ $P < .01$ and $$$ $P < .001$ compared with the PD group by RM 2-way ANOVA followed by the Tukey's test. Plantar Incision: model mice that received mock SNB with saline; R, model mice that received SNB with ropivacaine (15 mg/kg, 40 μL); and RPD, model mice that received SNB with ropivacaine supplemented with dexamethasone (1.5 mg/kg).

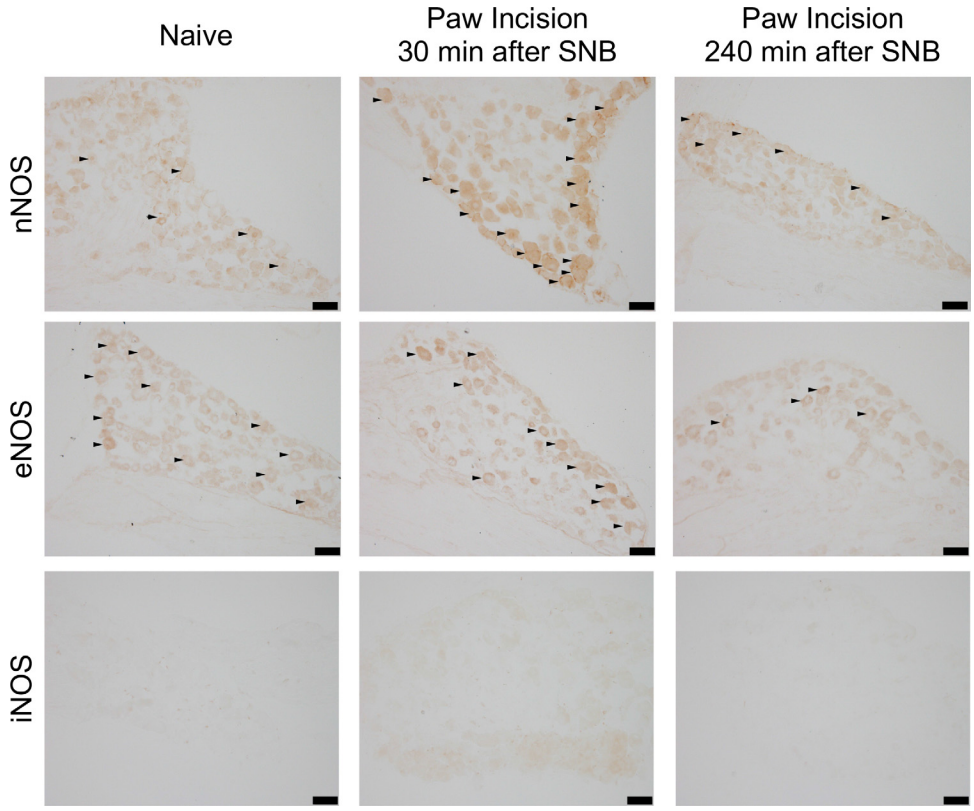

Figure 3. Expression changes in 3 subtypes of nitric oxide synthase (NOS) (neuronal NOS [nNOS], endothelial NOS, [eNOS], inducible NOS, [iNOS]) in the L5 DRG) after sciatic nerve block (SNB) and plantar incision. Arrowheads indicate immunopositive DRG neurons. Scale bar: 50 μm.

F(4, 50) = 13.67, $P < .0001$; Group * Time: F(4, 50) = 2.566, $P = .0494$ analyzed by RM 2-way ANOVA, Fig 2).

Among the 3 NOS subtypes, nNOS and eNOS were constitutively expressed in L5 DRG of naïve mice, and both were increased 30 minutes after plantar incision. The expression of both nNOS and eNOS quickly decreased at 240 minutes. Contrary to nNOS and eNOS expression, the expression of iNOS in the L5 DRG was very low, and plantar incision did not influence iNOS expression in L5 DRG (Fig 3). Based on these results, the following experiment evaluated the expression of nNOS and eNOS in each group.

Constitutive nNOS expression was observed in naïve mice. In contrast to the p-p38 MAPK expression changes, nNOS expression was significantly increased 30 minutes after plantar incision. SNB with ropivacaine alone significantly suppressed nNOS expression in the DRG. Interestingly, dexamethasone supplementation with ropivacaine suppressed nNOS expression to the same level as that in naïve mice. In contrast, perineural dexamethasone administration alone did not inhibit the induction of nNOS expression by plantar incision. The expression of nNOS quickly decreased 240 minutes after plantar incision in the control group to the same level as that in naïve mice. The enhancement of nNOS expression in the DRG by plantar incision was suppressed by SNB with ropivacaine, and dexamethasone supplementation with ropivacaine enhanced the suppressive effect of ropivacaine on nNOS expression (n = 6 for each group; Time: F(1, 40) = 109.1, $P < .0001$; Groups: F(3, 40) = 47.97, $P < .0001$; Groups * Time: F(3, 40) = 40.34, $P < .0001$, analyzed by 2-way ANOVA, Fig 4).

Constitutive eNOS expression was observed in naïve mice, similar to nNOS expression. However, unlike nNOS expression, eNOS expression did not significantly increase 30 minutes after plantar incision. Perineural administration of dexamethasone alone did not inhibit the induction of eNOS expression by plantar incision. SNB with ropivacaine alone did not suppress eNOS expression, whereas SNB supplemented with dexamethasone strongly suppressed eNOS expression 30 minutes after foot incision (n = 6 for each group; Time: F(1, 40) = 24.12, $P < .0001$; Groups: F(3, 40) = 14.18, $P < .0001$; Groups * Time: F(3, 40) = 6.718, $P = .0009$ analyzed by 2-way ANOVA, Fig 5), as noted with nNOS.

Effect of Perineural L-NAME Administration With Local Anesthetics on PWL

Perineurally administered L-NAME prolonged PWLs in naïve mice in a dose-dependent manner, and <6.25 mg/kg of L-NAME did not affect PWLs (n = 4 for each group; Time: F(3.832, 80.48) = 11.62, $P < .0001$; Dose: F(6, 21) = 18.25, $P < .0001$; Time * Dose: F(30,

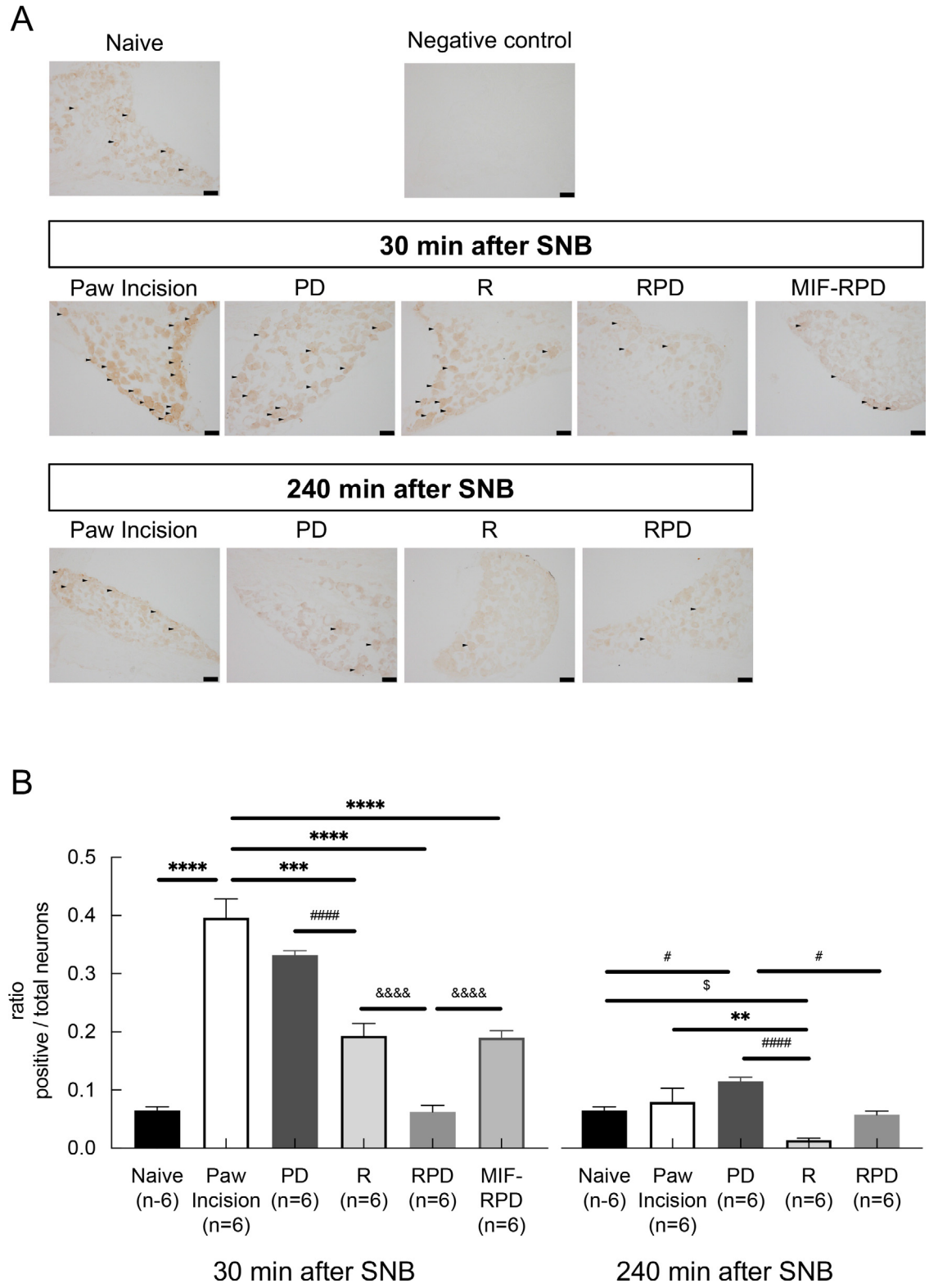

Figure 4. Early enhancement of neuronal nitric oxide synthase (nNOS) expression in the L5 DRG of postoperative pain model mice. (A) Constitutive expression of nNOS is seen in the L5 DRGs of naïve mice. There are no immunopositive signals seen for secondary antibodies; only negative control staining is observed. Thirty minutes after plantar incision, nNOS expression is upregulated in the L5 DRG. nNOS expression following plantar incision is inhibited by sciatic nerve block (SNB), and profound inhibition is seen in mice that received an SNB supplemented with dexamethasone. In contrast, perineural administration of dexamethasone only does not suppress nNOS expression. Pretreatment with mifepristone (MIF), a glucocorticoid receptor antagonist, abolished the effect of dexamethasone in DRG. nNOS expression after plantar incision quickly decreased within 240 minutes. Arrowheads indicate immunopositive neurons. Scale bar: 50 μm. (B) Statistical analysis reveals that plantar incision induced nNOS expression in the L5 DRG only 30 minutes later and that SNB with ropivacaine only significantly suppressed the increase in nNOS 30 and 240 minutes after plantar incision. In addition, the increase in nNOS expression in the L5 DRG 30 minutes after SNB is significantly more suppressed when SNB is supplemented with dexamethasone than when SNB contains ropivacaine alone. Pretreatment with MIF completely

105) = 3.421, $P < .0001$, analyzed by RM 2-way ANOVA followed by the Tukey's test, Fig 6A). Subsequently, L-NAME (6.25 mg/kg) alone and ropivacaine supplemented with L-NAME (6.25 mg/kg) were perineurally administered in the postoperative pain model mice, as described above, and PWLs were compared across the Control, R, and RPD groups. The analgesic effect lasted up to 120 minutes after administration of the SNB supplemented with 6.25 mg/kg of L-NAME. In contrast, perineural administration of L-NAME (6.25 mg/kg) alone did not demonstrate analgesic effects, similar to dexamethasone (n = 6 for each group; Time: F(3.384, 84.59) = 57.97, $P < .0001$; Group: F(4, 25) = 181.0, $P < .0001$; Time * Group: F(20, 125) = 15.25, $P < .0001$ by RM 2-way ANOVA. Fig 6B).

Discussion

Herein, we demonstrated that SNB supplemented with 1.5 mg/kg of dexamethasone significantly prolonged the duration of the local anesthetic effect in postoperative pain model mice, similar to the effects seen in naïve mice in previous studies.[6,8] Moreover, we observed that the prolonged effect of local anesthetics by dexamethasone is mediated by glucocorticoid receptors.[7] Histological analyses indicated that the expression of p-p38 MAPK in DRGs was upregulated after plantar incision, as previously shown.[12] However, SNB suppressed the expression of p-p38 MAPK regardless of dexamethasone supplementation. Contrary to our hypothesis, this suggests that the activation of p-p38 MAPK in DRG is not related to the prolonging effect of local anesthetic action by dexamethasone supplementation. In contrast, plantar incision induced the expression of nNOS but not eNOS or iNOS in DRG, and dexamethasone supplementation to local anesthetic significantly suppressed the expression of nNOS and eNOS compared with local anesthetic alone. Finally, perineural administration of L-NAME, a pan-NOS inhibitor, prolonged the duration of the local anesthetic effect of SNB, which is the same as dexamethasone supplementation to local anesthetics. These results suggest that NO production in DRGs after plantar incision affected the duration of local anesthetic effect in SNB and that the suppression of nNOS expression after plantar incision mediated the prolonging effect of local anesthetic supplemented with dexamethasone.

Several drugs have been reported to extend the pharmacological duration of local anesthetics in experimental and clinical settings, whereas the mechanisms by which glucocorticoids prolong local anesthetic effects have not yet been clarified. Our behavioral analysis showed that NOS inhibition in DRGs after peripheral tissue injury was associated with the prolonging effect of the local anesthetic by dexamethasone supplementation, even though NO itself has been reported to inhibit nerve conduction.[20,21] To the best of our knowledge, this is the first report to clarify the relationship between the effect of local anesthetic duration and NO production in peripheral nerve block and show that the prolonging effect of local anesthetics by dexamethasone is related to the inhibition of the upregulation of constitutively expressed NOS.

Corticosteroids, including dexamethasone, inhibit transcription elicited by inflammation. A previous in vitro study demonstrated that dexamethasone inhibits the phosphorylation of p38MAPK.[33] Other previous studies reported that plantar incision induced the phosphorylation of p38MAPK in L5 DRG neurons and that the inhibition of its phosphorylation attenuated mechanical hypersensitivity, indicating that p-p38 MAPK plays a crucial role in the development of postoperative pain.[11,12] Here, we showed that SNB before plantar incision suppresses p-p38 MAPK expression in DRG neurons, regardless of dexamethasone supplementation. This suggests that the conduction block before plantar incision is sufficient to suppress the phosphorylation of p-38 MAPK in the DRG and that the prolonging effect of local anesthetic with supplemented dexamethasone is independent of p38MAPK phosphorylation.

NO is biosynthesized when L-arginine is oxidized by NOS and plays an important role in vascular signaling, immune response, neurotransmission, pain perception, and sensitization. It is also known to exacerbate pain behavior in vivo.[34,35,16] Among the 3 NOS subtypes, nNOS has been shown to be closely related to pain. Its expression in the DRG is increased by peripheral nerve injury, and NO generated by nNOS is involved in the development of neuropathic pain.[36] Furthermore, while eNOS is thought to be less involved in pain, iNOS may be involved in inflammatory pain.[37,24] On the other hand, NO itself has been reported to inhibit nerve conduction when administered directly to the nerve bundle and may enhance the local anesthetic effect.[20,21] In this study, we demonstrated that a plantar incision increased nNOS, but not eNOS or iNOS expression in L5 DRG. The expression of nNOS was significantly and transiently increased in the DRG only 30 minutes after plantar incision and was significantly suppressed by SNB with ropivacaine alone, suggesting that the conduction blockade of peripheral nerves by local anesthetics partially suppresses signaling in DRG. However, dexamethasone supplementation to ropivacaine significantly suppressed the expression of nNOS and eNOS in DRGs compared to ropivacaine alone within a short time after plantar incision. Pretreatment with

abolishes the effects of dexamethasone. ** $P < .01$, *** $P < .001$, and **** $P < .0001$ compared with the control group; $ $P < .05$ compared with the naïve group; # $P < .05$ and #### $P < .0001$ compared with the PD group; and &&&& $P < .0001$ compared with the RPD group analyzed by a repeated measure 2-way ANOVA followed by the Tukey's test. Plantar Incision, model mice that received mock SNB with saline; R, model mice that received SNB with ropivacaine (15 mg/kg, 40 μL); RPD, model mice that received SNB with ropivacaine supplemented with dexamethasone (1.5 mg/kg); PD: model mice that received perineural dexamethasone (1.5 mg/kg); and MIF-RPD, model mice that were pretreated with mifepristone (50 mg/kg) and received SNB with ropivacaine supplemented with dexamethasone.

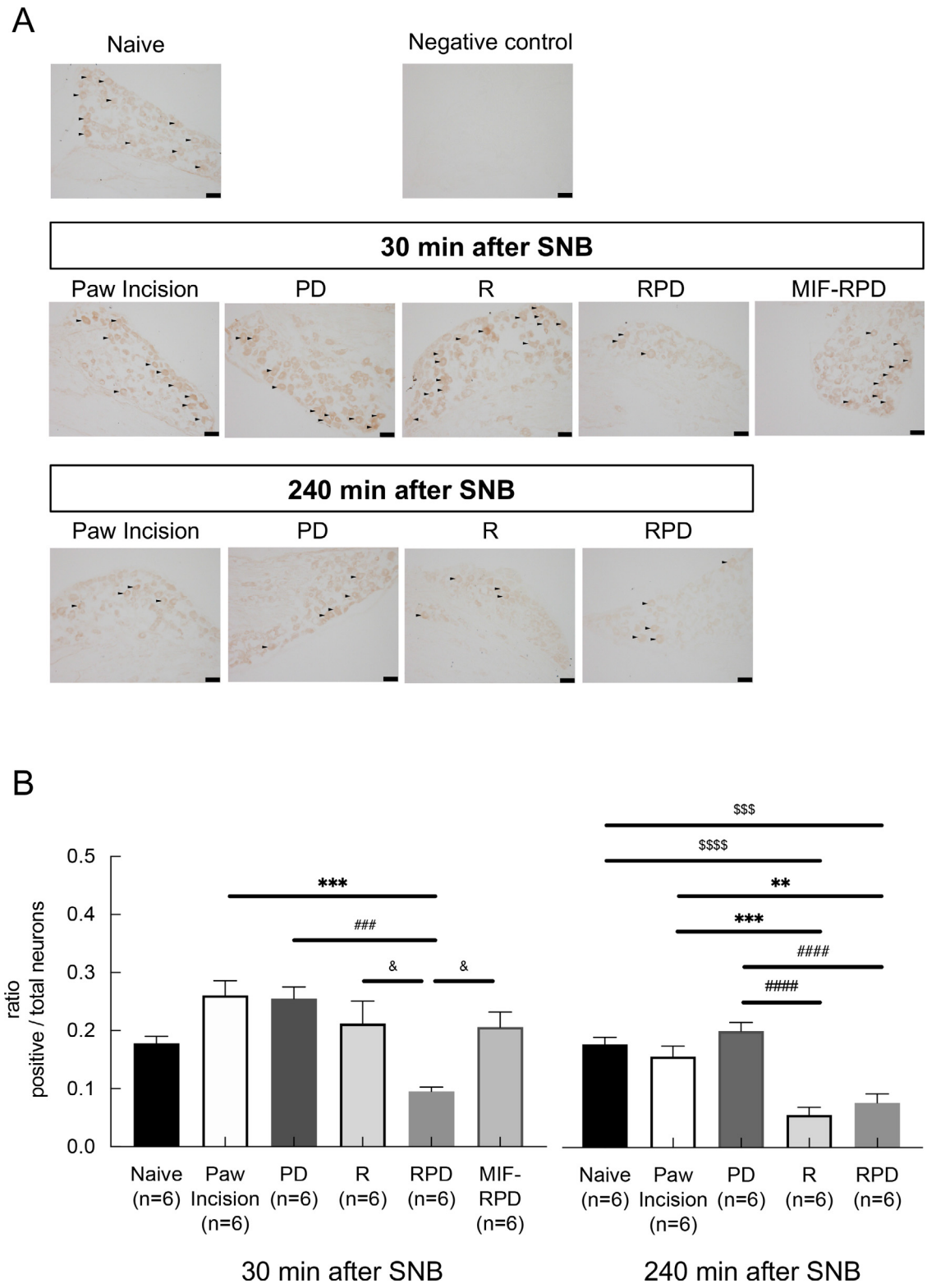

Figure 5. (A) Constitutive expression of endothelial nitric oxide synthase (eNOS) is observed in the L5 dorsal root ganglion (DRG) of naive mice. There are no immunopositive signals observed with secondary antibodies; only negative control staining is observed. Thirty minutes after plantar incision, eNOS expression is slightly but significantly upregulated in the L5 DRG. eNOS expression by plantar incision is only inhibited by the administration of a sciatic nerve block (SNB) supplemented with dexamethasone. In contrast, perineural administration of dexamethasone only does not suppress neuronal NOS (nNOS) expression. Pretreatment with mifepristone (MIF), a glucocorticoid receptor antagonist, abolishes the effect of dexamethasone on DRG. eNOS expression after plantar incision quickly decreases 240 minutes after plantar incision. Scale bar: 50 μm. Arrowheads indicate immunopositive neurons in the DRG. (B) Statistical analysis reveals that the plantar incision induced eNOS expression changes in the L5 DRG only 30 minutes later. The enhancement of eNOS expression in the L5 DRG 30 minutes after plantar incision is insignificant. However, it is significantly suppressed by dexamethasone-supplemented SNB, such as enhancing nNOS expression by plantar incision. Moreover, eNOS expression 240 minutes after plantar incision is significantly suppressed by SNB with or without dexamethasone supplementation. Pretreatment with MIF completely abolishes the effects of dexamethasone. ** $P < .01$ and *** $P < .001$ compared with the control group; $$

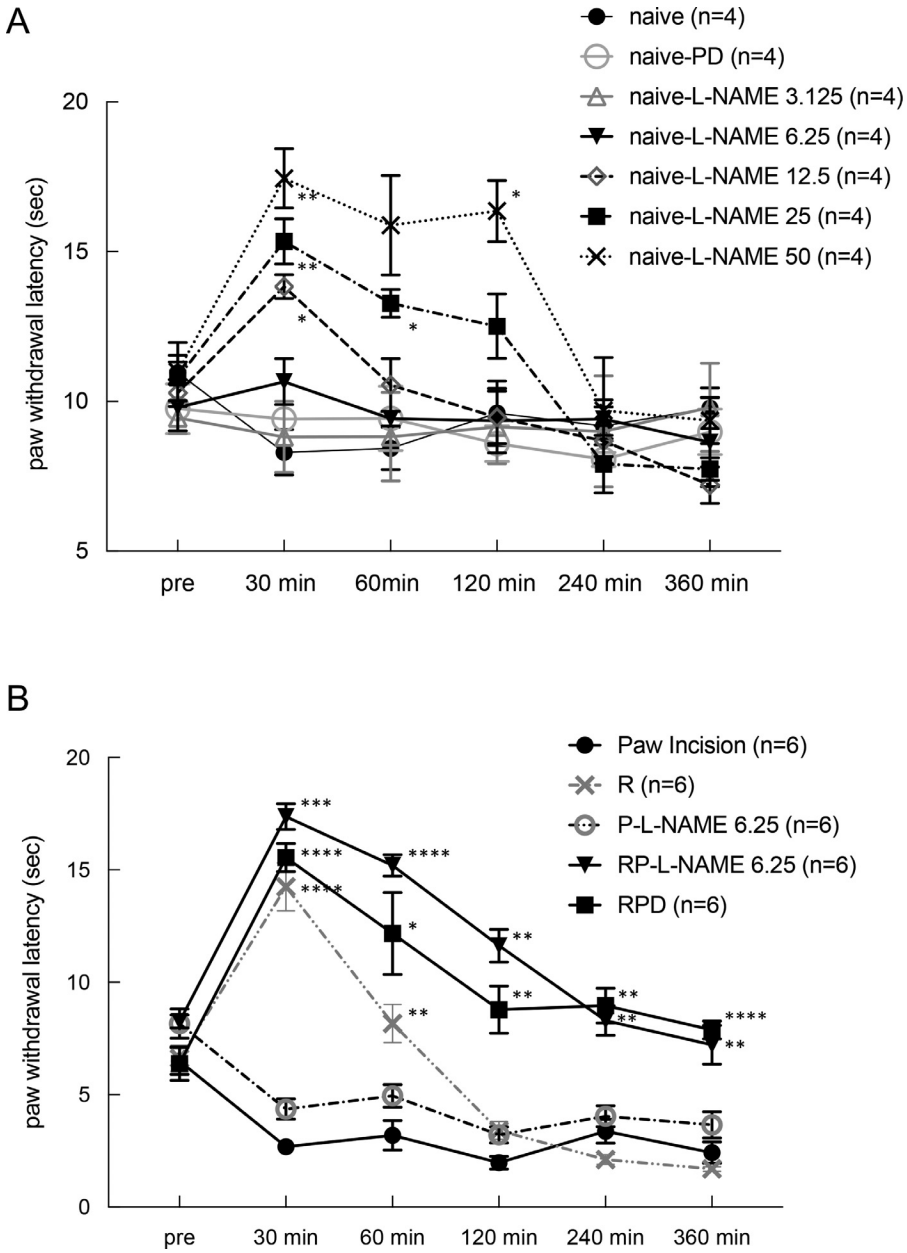

Figure 6. Effect of perineural administration of N^G-nitro-L-arginine methyl ester (L-NAME), a pan-nitric oxide synthase (NOS) inhibitor, on antinociceptive response to radiant heat stimuli. (A) Perineural administration of L-NAME prolongs paw withdrawal latencies to heat stimuli in naïve mice in a dose-dependent manner. Perineurally administered dexamethasone (PD; 1.5 mg/kg) does not show an analgesic effect in naïve mice. * $P < .05$, ** $P < .01$ compared with naïve mice in corresponding time by RM 2-way ANOVA followed by the Tukey's test. (B) Supplementation of local anesthetics with a non-analgesic dose of L-NAME prolongs the analgesic effect of SNB. Plantar Incision, postoperative pain model mice that received mock SNB with saline; R, postoperative pain model mice that received SNB with ropivacaine (15 mg/kg, 40 μL); P-L-NAME 6.25, postoperative pain model mice that received L-NAME (6.25 mg/kg) only; PR-L-NAME 6.25, postoperative pain model mice that received SNB with ropivacaine supplemented with L-NAME (6.25 mg/kg); and RPD, postoperative pain model of mice that received SNB with ropivacaine supplemented with dexamethasone (1.5 mg/kg). * $P < .05$, ** $P < .01$ and **** $P < .0001$ compared with control mice in corresponding time using an RM 2-way ANOVA followed by the Tukey's test.

MIF, a potent glucocorticoid receptor inhibitor, abolished the prolonged SNB effect of dexamethasone supplementation to ropivacaine and the suppression of nNOS and eNOS expression 30 minutes after plantar incision. These results suggest an association between the inhibition of nNOS and eNOS expression in DRGs by SNB with dexamethasone-supplemented ropivacaine 30 minutes after plantar incision and the

$ $P < .001$ and $$$$ $P < .0001$ compared with the naïve group; ### $P < .001$ and #### $P < .0001$ compared with the PD group; and $ $P < .05$ compared with the RPD group. Plantar Incision, model mice that received mock SNB with saline; R, model mice that received SNB with ropivacaine (15 mg/kg, 40 μL); RPD, model mice that received SNB with ropivacaine supplemented with dexamethasone (1.5 mg/kg); PD, model mice that received perineural dexamethasone (1.5 mg/kg); and MIF-RPD, model mice that were pretreated with mifepristone (50 mg/kg) and received SNB with ropivacaine supplemented with dexamethasone.

prolonging effect of ropivacaine by dexamethasone supplementation. These effects are suggested to be mediated by glucocorticoid receptors.

Previous studies have shown that pain behavior is suppressed when L-NAME is administered peripherally[38] and centrally[39,40] in inflammatory pain model rodents. Another study also demonstrated that L-NAME and methylene blue, a soluble guanylate cyclase inhibitor, ameliorate prostaglandin-evoked allodynia.[41] These previous results indicated that NO has properties as a pain-producing substance and that NO signaling is involved in the pain pathway. We found that the perineuronal L-NAME dose-dependently increased the withdrawal latencies to heat stimuli in naïve mice. Moreover, we demonstrated that perineural administered L-NAME, even with a non-analgesic dose, significantly prolonged the duration of SNB, similar to dexamethasone. These results suggest that the peripheral activity of NO is directly involved in antinociception and prolongs the efficacy of local anesthetics.

Our results of histological examination also indicated that the transient decrease in the expression of NOS, which produces NO with a very short half-life, has a crucial role in the prolonging effect of local anesthetics. Moreover, even in the group that received SNB with ropivacaine alone, the expression levels of nNOS and eNOS at 240 minutes after plantar incision were similar to or even lower than those in naïve mice. This result suggested that NOS activity 240 minutes after plantar incision does not affect the prolonging effect of local anesthetics by dexamethasone and that NOS inhibition before the increase in NOS expression by plantar incision is essential for the prolonging effect of local anesthetics. Previous in vitro studies have shown that long-term potentiation in the spinal cord does not occur when guanylate cyclase, a signaling pathway downstream of NO, is inhibited *a priori* and that once long-term potentiation occurs, it is not reversed by inhibiting NOS with L-NAME.[25] This phenomenon may explain our results that the transient inhibition of nNOS affects the long-lasting prolonging effect of local anesthetics.

In our study, the co-administration of dexamethasone and ropivacaine suppressed nNOS expression in the DRG after plantar incision very rapidly within 30 minutes, whereas a previous in vitro study showed that the suppression of nNOS expression by dexamethasone is needed for several hours and that there is a lack of an acute inhibitory effect of glucocorticoids on NO production.[42] This discrepancy suggests that unidentified mechanisms exist in the suppression of NOS expression by glucocorticoids when used with local anesthetics. Moreover, the upregulation of nNOS has been shown to precede phosphorylation of p-38 MAPK after plantar incision, which suggests that NO signaling occurs upstream of p-38 MAPK. Previous studies demonstrate that NO donor sodium nitroprusside induces the phosphorylation of p38 MAPK in cultured cells.[22,23] This finding indicates that peripheral tissue damage induces expression of constitutive expressing nNOS; NO is produced by nNOS and NO phosphorylate p38 MAPK.

This study has some limitations. Since this study did not measure the NO concentration in the perineural tissue, it is arguable whether NO concentration increased around the nerve due to tissue damage. However, our results indicate an inverse correlation between nNOS expression in DRG and PWL in postoperative pain model mice. The supplementation of L-NAME to a local anesthetic had the same effect as dexamethasone. Therefore, the prolonging effect of dexamethasone on local anesthetics could be due to the inhibition of NOS. Additionally, dexamethasone has a vasoconstrictive effect when administered perineurally,[43] and this effect may delay the absorption and washout of local anesthetics. Further studies are needed to clarify the detailed mechanisms.

In conclusion, dexamethasone supplementation with local anesthetics may prolong the efficacy of SNB via glucocorticoid receptor activation. Moreover, dexamethasone suppressed nNOS in the DRG soon after tissue injury; like dexamethasone, NOS inhibition also prolongs the effects of local anesthetics. Previous studies indicate that perineurally administered dexamethasone suppresses axonal damage due to local anesthetics. Considering our findings in conjunction with those of previous studies, we believe that nerve blocks supplemented with dexamethasone have increased efficacy and safety and may be a powerful tool to support opioid-sparing anesthesia.

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Data Statement

The data produced and analyzed in this study are available from the corresponding author upon reasonable request.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jpain.2022.06.001.

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