Increased Expression of Cyclooxygenase and Nitric Oxide Isoforms, and Exaggerated Sensitivity to Prostaglandin E₂, in the Rat Lumbar Spinal Cord 3 Days after L5–L6 Spinal Nerve Ligation

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Background: Spinal prostaglandins seem to be important in the early pathogenesis of experimental neuropathic pain. Here, the authors investigated changes in the expression of cyclooxygenase and nitric oxide synthase (NOS) isoforms in the lumbar, thoracic, and cervical spinal cord and the pharmacologic sensitivity to spinal prostaglandin E₂ (PGE₂) after L5–L6 spinal nerve ligation (SNL).

Methods: Male Sprague-Dawley rats, fitted with intrathecal catheters, underwent SNL or sham surgery 3 days before experimentation. Paw withdrawal threshold was monitored for up to 20 days. Immunoblotting, spinal glutamate release, and behavioral testing were examined 3 days after SNL.

Results: Allodynia (paw withdrawal threshold ≤ 4 g) was evident 1 day after SNL and remained stable for 20 days. Paw withdrawal threshold was unchanged (P > 0.05) from baseline (> 15 g) after sham surgery except for a small but significant decrease on day 20. Cyclooxygenase 2, neuronal NOS, and inducible NOS were significantly increased in the ipsilateral lumbar dorsal horn after SNL. Expression in the contralateral dorsal horn and ventral horns (lumbar segments) or bilaterally (thoracic and cervical segments) was unchanged from sham controls. This was accompanied by a significant decrease in both the EC₅₀ of PGE₂-evoked glutamate release and the ED₅₀ of PGE₂ on brush-evoked allodynia. Enhanced sensitivity to PGE₂ was localized to lumbar segments of SNL animals and attenuated by SC-51322 or S(+)-ibuprofen, but not R(−)-ibuprofen (100 μg).

Conclusion: The increased expression of cyclooxygenase-2, neuronal NOS, and inducible NOS and the enhanced sensitivity to PGE₂ in spinal segments affected by SNL support the hypothesis that spinal prostanooids play an early pathogenic role in experimental neuropathic pain.

There is growing evidence that prostaglandins, generated in the spinal cord after nerve injury, play a contributory role in the pathogenesis of neuropathic pain. This was first suggested by the robust allodynia induced by intrathecal prostaglandins in otherwise normal (uninjured) conscious mice, an effect blocked by intrathecal prostaglandin receptor antagonists and later shown to occur in prostaglandin E₂ subtype (EP) 1 receptor, EP₃ receptor, and EP₃ receptor but not EP₁ receptor mice. Other studies have reported increased EP-receptor immunoreactivity in injured nerves after partial sciatic nerve ligation and brush-evoked increases in the concentration of prostaglandin E₂ (PGE₂) in spinal dialysate 2–8 days after L5–L6 spinal nerve ligation (SNL). The latter were temporally and spatially linked to brush-evoked allodynia and absent in sham-operated controls. Experimental allodynia was also attenuated by certain cyclooxygenase (COX) inhibitors or the EP-receptor antagonist, SC-51322, when given in the 2- to 8-day window after SNL.

Spinal nerve ligation triggers early and sustained activation of spinal N-methyl-D-aspartate receptors, a critical trigger for central prostaglandin and nitric oxide synthesis. This is accompanied by the enhanced expression of COX-1 and COX-2 and nitric oxide synthase (NOS) in the spinal cord. Nerve injury–induced changes in spinal COX-1 and COX-2 expression or immunoreactivity have been described, but little is known about the concurrent expression of neuronal NOS (nNOS) and inducible NOS (iNOS) or whether any of these changes are confined to the spinal segments affected by nerve injury. The expression of both iNOS and nNOS increases early after spinal cord injury. Furthermore, the genes coding for inducible NOS and COX have identical promoters and response elements to nuclear factor κB, which also has increased expression after spinal cord injury. In the current study, we compared the expression of COX-1, COX-2, nNOS, and iNOS in the lumbar, thoracic, and cervical spinal cord 3 days after SNL.

Anecdotal reports suggest that nonsteroidal antiinflammatory drugs provide little, if any, clinical benefit in relieving neuropathic pain. However, work in our laboratory has shown that the timing of drug administration after nerve injury and the COX isoforms affected are important variables governing the effectiveness of these drugs in experimental neuropathy. For example, COX-1-selective inhibitors, given intrathecally 2–4 h after SNL, prevented the emergence of allodynia in rats for up to 25 days but were ineffective in reversing established allodynia. These results, and those of previous reports, strongly suggest that COX inhibitors must be given early after nerve injury and not for the relief of neuropathic pain per se, but to prevent the emergence of allodynia. They, in turn, support a pathogenic role for...
spinal prostaglandins early after SNL, although the relationship of spinal prostanoids to nerve injury and allodynia remains unclear.

One possibility is that nerve-injured animals develop abnormal sensitivity to the pathophysiologic effects of endogenous prostaglandins in the spinal cord. This could trigger, or at least facilitate (i.e., through a pathogenic cascade), further adaptations that effect long-term disturbances in sensory processing. If correct, such a change in sensitivity should be greatest in, and possibly restricted to, those spinal segments most affected by nerve injury. It should also be attenuated by pharmacologic agents that disrupt spinal prostaglandin activity. To test this hypothesis, we compared the concentration- and dose-response effects of PGE2 in the lumbar and thoracic spinal cord of SNL, sham-operated, and naive rats. We also investigated the effects of the EP-receptor antagonist, SC-51322, and the δ(+) and R(−)-isomers of ibuprofen in this model.

Materials and Methods

All studies were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland, St. John’s, Newfoundland and Labrador, Canada.

Animals

Male Sprague-Dawley rats (approximately 130 g on the day of experimentation) were obtained from the Vivarium of Memorial University of Newfoundland and were housed in standard cages with woodchip bedding. Animals had free access to food and water and were housed singly after surgery. A 12-h light–dark cycle (lights on at 07:00 h) was used throughout.

Intrathecal Catheterization

Intrathecal catheters (6.5-cm length terminating near the lumbar enlargement or 4.0-cm length terminating in the midthoracic segments) were implanted as previously described.7 Rats with normal motor, grooming, and feeding behavior were housed separately and allowed to recover for 3 days before SNL or sham surgery.

Neuropathy

Neuropathy was induced using the method of Kim and Chung,20 as previously described.7 Rats were anesthetized with halothane, and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham controls, the L5 and L6 spinal nerves were isolated but not ligated. All animals were allowed to recover for 3 days before experimentation. Allodynia, defined as a paw withdrawal threshold (PWT) of 4 g or less, was confirmed using von Frey filaments.

Western Blotting

The spinal cord was extracted hydraulically,21 immediately frozen in 2-methylbutane (Sigma Chemical, St. Louis, MO), and stored at −80°C. The spinal cord was separated into cervical, thoracic, and lumbar regions (L2–L6) and further subdivided into the left and right, ventral and dorsal quadrants. Spinal cord was homogenized in ice-cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet [Roche Diagnostics, Laval, Canada], 1 mm sodium vanadate, 1 mm sodium fluoride, and 0.025% SDS) and centrifuged at 10,000 rpm for 5 min (4°C). Samples, diluted to achieve equal protein concentrations (30 μg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (25 mM Tris, 192 mM glycine, 200 mL methanol, pH 8). Prestained protein markers were used for molecular weight determination. The blots were then stained with Ponceau red to assess the equivalency of protein loading and were subsequently probed with the following antibodies: polyclonal rabbit anti–COX-1 (1:250), polyclonal rabbit anti–COX-2 (1:1,000), polyclonal rabbit anti–nNOS (1:1,000), and polyclonal rabbit anti–iNOS (1:1,000) primary antibodies (Cayman Chemical, Ann Arbor, MI). Membranes were incubated overnight at 4°C with primary antibodies and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000; Chemicon International Inc., Temecula, CA) for 1 h at room temperature, washed for 30 min in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA), and exposed to x-ray film (Cronex MRF Clear base; Agfa Corp., Greenville, SC).

Spinal Glutamate Release

The spinal cord was extracted hydraulically,21 and the dorsal and arachnoid membranes were carefully removed. The lumbar region (L2–L6) was excised, mounted on cutting blocks, and immersed in sucrose-modified artificial cerebrospinal fluid (aerated with 95% O2 and 5% CO2). Slices (600–800 μm) were cut with a vibratome and placed in aerated artificial cerebrospinal fluid at room temperature. They were immersed in HEPES buffer oxygenated with 100% O2 throughout the experiment. Glutamate released from the tissue was immediately oxidized to α-ketoglutarate by glutamate dehydrogenase, thereby preventing neuronal reuptake of glutamate.22 The reduced form of nicotinamide adenine dinucleotide phosphate generated from this reaction was quantitated using spectrophotometry (excitation: 335 nm; emission: 430 nm).22 Basal and PGE2-evoked glutamate release was quantified using standard curves constructed with L-glutamate (0–560 pmol; Sigma-Aldrich, Oakville, Canada) on each day of analysis. Basal glutamate release (i.e.,
release in the absence of any drug stimulus) was determined for 15 min before the introduction of drug into the cuvette. Drug-evoked release was defined as total release (i.e., after a drug stimulus) minus basal release. Protein content in each slice was determined using a modified Lowry protein assay kit (Pierce, Rockford, IL).

Behavioral Testing

Spontaneous (no brushing) and brush-evoked behavior were continuously graded using a modified scoring system.\(^2^3\) Spontaneous behavior (0 = normal behavior, bright, alert, and exploring; 1 = huddling, burrowing, or hiding; 2 = one of the following: piloerection, occasional vocalization, or favoring the affected side; 3 = two or more of the following: piloerection, occasional vocalization, favoring the affected side; and 4 = any of the following: frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes) was graded over a 4-min interval. The hair on the back, flanks, limbs, and hind paws was then brushed with a cotton-tipped applicator sufficient to deflect the pelage. This was continued for 1 min, and the behavior was graded as follows: 0 = normal behavior, curious, alert, and exploring; 1 = avoidance of stimulus source or protection of the affected dermatomes; 2 = one of the following: piloerection, paw withdrawal, or occasional vocalization; 3 = two or more of the following: piloerection, paw withdrawal, or occasional vocalization; and 4 = any of the following: attacking the applicator, frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes. This sequence was repeated every 5 min for the first hour and every 30 min thereafter up to 6 h or until no responses were detected. The investigator was blinded to the nature of the treatment (i.e., vehicle, drug, concentration) in all behavioral experiments.

Drugs

Prostaglandin E\(_2\), SC-51322, and \(S(\pm)\)- and \(R(-)\)-ibu-profen were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI). PGE\(_2\) solutions were prepared according to Nishihara et al.\(^2^4\) Briefly, PGE\(_2\) was initially dissolved in ethanol and evaporated under nitrogen gas. It was then redissolved in normal saline and diluted with the same to yield the desired concentrations. All other drugs were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%. For the in vitro experiments, drugs were added directly into the cuvette containing the slice using a microsyringe. Each concentration of PGE\(_2\) was tested using a separate slice so that a full PGE\(_2\) concentration–response curve was determined in each animal. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into conscious, unrestrained rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 \(\mu\)l followed by 5 \(\mu\)l sterile saline. The intrathecal catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after death in randomly selected animals. In three animals, methylene blue (10 \(\mu\)l) was injected intrathecally at the midthoracic level (i.e., 2 cm rostral to the midthoracic enlargement) to assess the extent of staining below the thoracic cord.

Data Analysis

Data are reported as the mean \(\pm\) SEM. Western blots were analyzed by optical density using ImageQuant \(^R\) software (Amersham Biosciences Corp., Piscataway, NJ). Expression data are presented as nanograms of protein, based on a single COX and NOS standard (50 ng), run in separate lanes on each gel, and corrected for background optical density. The area under the curve was calculated using trapezoidal integration. Concentration–response analysis was performed using methods from Tallarida and Murray.\(^2^5\) Comparisons within each treatment group were performed using one-way, repeated-measures analysis of variance, followed by the Newman–Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized Analysis of variance, followed by the Newman–Keuls test (SigmaStat\(^R\) 2.0; Systat Software, Inc., Point Richmond, CA).

Results

Rats undergoing SNL displayed a significant decrease in PWT from 15 g or more (baseline) to 4 g or less (fig. 1). This change in sensitivity remained stable for at least 20 days and was confined to the plantar surface of the left hind paw (ipsilateral to nerve ligation). By day 20, a modest but significant \((P < 0.01)\) decrease in PWT developed on the contralateral side of nerve-ligated animals (fig. 1). Generally, the affected hind paw was kept in an elevated and cupped position, thereby minimizing contact with the cage floor. All nerve-ligated rats were otherwise healthy, showed normal feeding and grooming behavior and regular weight gain. Neither intrathecal catheterization nor sham surgery had any effect on PWT compared with presurgical values (fig. 1).

Western analysis revealed a significant increase in the expression of COX-2 (45%), nNOS (25%), and iNOS (45%) compared with sham controls in the lumbar cord 3 days after SNL (fig. 2). These changes were localized to the left dorsal horn (i.e., ipsilateral to SNL). In contrast, COX-2, nNOS, and iNOS expression remained unchanged across the dorsoventral axis and between the ipsilateral and contralateral sides in the thoracic and cervical segments (figs. 2A, C, and D). Although COX-1
expression increased by 11% in the left lumbar dorsal horn of ligated rats, this was not statistically different (P > 0.05) from sham controls (fig. 2B). There was also no difference in COX-1 across the dorsoventral axis or between the ipsilateral and contralateral sides (fig. 2B).

The relative expression of COX-1, COX-2, nNOS, and iNOS varied within each spinal region. However, there were no significant differences in the rostrocaudal expression of each protein (P > 0.05) in ligated and sham-operated animals, except in the left lumbar dorsal horn after SNL. Representative immunoblots of COX-2 and iNOS from the lumbar cord of sham-operated and nerve-ligated animals and their corresponding protein standards are shown in figure 2E.

Prostaglandin E₂ resulted in a concentration-dependent release of glutamate (EC₅₀ = 2.37 × 10⁻¹¹ M; table 1) from lumbar slices harvested 3 days after sham surgery (fig. 3). PGE₂ was equipotential using slices from naive (no surgery) and sham-operated animals (fig. 3A and table 1). In contrast, the PGE₂ concentration–response curve using lumbar slices from nerve-ligated rats was significantly shifted to the left compared with the sham and naive groups (fig. 3A). The EC₅₀ in the nerve-ligated group was 8.30 ± 0.43 pmol·min⁻¹·mg protein⁻¹ (table 1), representing a 24–fold increase in the EC₅₀ of PGE₂ in sham-operated preparations (fig. 3A and table 1).

To determine whether the enhanced sensitivity to PGE₂ had relevance to allodynia, the dose–response effect of intrathecal PGE₂ was compared in nerve-ligated and sham-operated rats. Brushing the hind limbs of sham-operated rats treated with intrathecal PGE₂ evoked robust, nociceptive-like behavior (i.e., brush-evoked vocalizations, defensive posturing, licking the affected dermatomes, and biting the cotton-tipped applicator). Peak allodynia (90% of maximum possible score) was observed 10 min after injection and declined thereafter (fig. 6A). The duration of allodynia (0.1 μg) was 210 min. In nerve-ligated rats, the same dose of PGE₂ elicited peak allodynia (100% of maximum possible score) at 10 min and remained unchanged for 90 min (fig. 6A). The duration of allodynia was greater than 6 h. There were no significant differences between sham-operated and nerve-ligated animals for spontaneous behavior (figs. 6A and B). Dose–response analysis revealed a 362-fold decrease in the ED₅₀ of PGE₂ in nerve-ligated compared with sham-operated rats (fig. 6B and table 2). In the absence of PGE₂, identical brushing of SNL plus vehicle-treated (302 ± 93% maximum possible score · min), SNL plus untreated (262 ± 82), sham plus vehicle-treated (154 ± 31), or sham plus untreated (116 ± 50) rats had no significant effect (P > 0.05; fig. 6B). In addition, there was no dose-response effect of PGE₂ on spontaneous behavior (P > 0.05) in either nerve-ligated or sham-operated animals (fig. 6B).

Area-under-the-curve analysis revealed a significant re-
duction in brush-evoked allodynia (63% decrease) and spontaneous behavior (53% decrease) in nerve-ligated animals when PGE$_2$ (0.1 µg) was injected into the thoracic as compared with the lumbar subarachnoid space (fig. 7). A similar trend was observed in sham-operated controls. Both brush-evoked and spontaneous behaviors were significantly lower after thoracic as compared with lumbar delivery in all groups. In three animals, methylene blue (10 µl) was injected intrathecally at the level of the midthoracic cord. No staining was observed below the thoracic cord.

In nerve-ligated rats, intrathecal S(+) ibuprofen (100

Table 1. EC$_{50}$ Values and 95% CIs of PGE$_2$ or Arachidonic Acid on Glutamate Release from Lumbar or Thoracic Spinal Cord Slices

<table>
<thead>
<tr>
<th>Experimental Treatment</th>
<th>EC$_{50}$, M</th>
<th>95% CI, M</th>
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<tr>
<td>Naive + PGE$_2$ (L)</td>
<td>2.33 x 10$^{-11}$</td>
<td>1.23 x 10$^{-11}$ to 4.41 x 10$^{-11}$</td>
</tr>
<tr>
<td>Sham operated + PGE$_2$ (L)</td>
<td>2.37 x 10$^{-11}$</td>
<td>9.25 x 10$^{-12}$ to 6.08 x 10$^{-11}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + PGE$_2$ (L)</td>
<td>8.30 x 10$^{-10}$</td>
<td>1.65 x 10$^{-10}$ to 4.18 x 10$^{-10}$</td>
</tr>
<tr>
<td>Naive + arachidonic acid (L)</td>
<td>5.80 x 10$^{-9}$</td>
<td>2.52 x 10$^{-9}$ to 1.34 x 10$^{-8}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + PGE$_2$ (T)</td>
<td>2.48 x 10$^{-10}$</td>
<td>1.61 x 10$^{-10}$ to 3.82 x 10$^{-10}$</td>
</tr>
<tr>
<td>Sham operated + S(+) ibuprofen + PGE$_2$</td>
<td>1.05 x 10$^{-10}$</td>
<td>4.89 x 10$^{-12}$ to 2.26 x 10$^{-10}$</td>
</tr>
<tr>
<td>Sham operated + R(−) ibuprofen + PGE$_2$</td>
<td>4.86 x 10$^{-12}$</td>
<td>1.28 x 10$^{-12}$ to 1.84 x 10$^{-11}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + S(+) ibuprofen + PGE$_2$</td>
<td>6.20 x 10$^{-13}$</td>
<td>2.00 x 10$^{-13}$ to 1.92 x 10$^{-12}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + R(−) ibuprofen + PGE$_2$</td>
<td>7.15 x 10$^{-15}$</td>
<td>3.42 x 10$^{-15}$ to 1.49 x 10$^{-14}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + DMSO vehicle + PGE$_2$</td>
<td>2.59 x 10$^{-14}$</td>
<td>2.00 x 10$^{-14}$ to 3.35 x 10$^{-14}$</td>
</tr>
<tr>
<td>Spinal nerve ligated + SC-51322 + PGE$_2$ (L)</td>
<td>6.31 x 10$^{-12}$</td>
<td>2.95 x 10$^{-12}$ to 1.35 x 10$^{-11}$</td>
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Cl = confidence interval; DMSO = dimethyl sulfoxide; EC$_{50}$ = concentration producing 50% of maximum response; L = lumbar region; PGE$_2$ = prostaglandin E$_2$; T = thoracic region.

Fig. 2. The expression of cyclooxygenase 2 (COX-2; A), cyclooxygenase 1 (COX-1; B), neuronal nitric oxide synthase (nNOS; C), and inducible nitric oxide synthase (iNOS; D) in the left (L) and right (R) ventral (V) and dorsal (D) horns of the cervical, thoracic, and lumbar spinal cord 3 days after spinal nerve ligation or sham surgery. Representative immunoblots (E) of COX-2 and iNOS in the lumbar spinal cord (lanes 1 and 4; sham-operated control; lanes 2 and 5; corresponding protein standard; lanes 3 and 6; nerve ligation). Asterisks indicate a significant difference between sham-operated and nerve-ligated animals (* P < 0.05, ** P < 0.01). Each bar represents the mean ± SEM of 5–8 animals.
injected 20 min before PGE$_2$ significantly decreased brush-evoked behavior by 62% and spontaneous behavior by 46% compared with DMSO plus PGE$_2$ (fig. 8). In sham-operated rats, S(+)ibuprofen had no significant effect on either brush-evoked or spontaneous behaviors ($P > 0.05$). R(−)ibuprofen was without effect in all treatment groups (fig. 8). In nerve-ligated animals, pretreatment with intrathecal SC-51322 (100 μg), 20 min before intrathecal PGE$_2$ (0.01 μg), significantly attenuated brush-evoked (39%) and spontaneous behavior (28%) compared with DMSO plus PGE$_2$ (fig. 9). SC-51322 had no effect on brush-evoked or spontaneous behaviors in sham-operated animals ($P > 0.05$).

**Discussion**

Tight ligation of the left L5–L6 spinal nerves, but not sham surgery, induced localized hypersensitivity to both brushing and von Frey filaments. Allodynia was accompanied by the increased expression of COX-2, nNOS, and iNOS and an exaggerated sensitivity to spinal PGE$_2$ in the lumbar cord 3 days after SNL. Although the extent of change along the spinal cord was more difficult to define in vivo compared with the slice experiments, the be-

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Fig. 3. The effect of prostaglandin E$_2$ (PGE$_2$) on glutamate release from lumbar slices of naive, sham-operated, or nerve-ligated rats (A), and thoracic slices from nerve-ligated or lumbar slices from sham-operated rats (B), 3 days after surgery. Each point represents the mean ± SEM of at least 5 animals (4–12 slices/dose). Asterisks indicate a significant difference between spinal nerve–ligated, sham-operated, and/or naive animals ($* P < 0.05$, **$P < 0.001$) at the corresponding PGE$_2$ concentration. Daggers indicate a significant difference between naive and sham-operated animals after treatment with arachidonic acid ($† P < 0.05$, $†† P < 0.001$). Double daggers indicate a significant difference between the PGE$_2$-treated and vehicle-treated slices at the corresponding PGE$_2$ concentration ($‡ P < 0.05$, $‡‡ P < 0.01$, $‡‡‡ P < 0.001$).

Fig. 4. The effect of pretreatment with S(+) or R(−)ibuprofen (1.0 μM; IBU) on prostaglandin E$_2$ (PGE$_2$)-evoked glutamate release. Spinal (lumbar) slices were harvested 3 days after spinal nerve ligation or sham surgery. Each point represents the mean ± SEM of at least 6 animals (4–10 slices/dose). DMSO = dimethyl sulfoxide.

Fig. 5. The effect of SC-51322 (1.0 μM) on prostaglandin E$_2$ (PGE$_2$)-evoked glutamate release from lumbar slices harvested 3 days after spinal nerve ligation. Each point represents the mean ± SEM of at least 5 animals (4–12 slices/dose). Asterisks indicate a significant difference from sham-operated controls at the corresponding PGE$_2$ concentration ($* P < 0.01$, **$P < 0.001$). Daggers indicate a significant difference from sham-operated animals at the corresponding PGE$_2$ concentrations ($‡ P < 0.05$, $†† P < 0.01$, $††† P < 0.001$). DMSO = dimethyl sulfoxide.
Table 2. ED₅₀ Values and 95% CIs of Intrathecal PGE₂ on Brush-evoked Allodynia in Spinal Nerve–ligated and Sham-operated Rats

<table>
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<tr>
<th>Experimental Group</th>
<th>ED₅₀  µg</th>
<th>95% CI µg</th>
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<tbody>
<tr>
<td>Sham operated + brushing</td>
<td>3.84 × 10⁻¹</td>
<td>1.49 × 10⁻¹ to 9.93 × 10⁻¹</td>
</tr>
<tr>
<td>Spinal nerve ligated + brushing</td>
<td>1.06 × 10⁻³</td>
<td>5.71 × 10⁻⁴ to 1.96 × 10⁻³</td>
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CI = confidence interval; ED₅₀ = dose producing 50% of maximum response; PGE₂ = prostaglandin E₂.
12 h to 3 days after injury. A progressive increase in iNOS expression, beginning 24 h after spinal trauma and peaking on day 7, has also been reported. Neurons exhibiting increased iNOS immunoreactivity are located in the superficial laminae of L4–L6 segments ipsilateral to nerve or spinal cord injury. Collectively, the expression data indicate that COX and NOS isozymes are upregulated in the spinal cord after SNL, but with different time courses. nNOS and possibly COX-1 are affected within hours of injury (COX-1 expression is increased in the lumbar dorsal horn 12 h after SNL—unpublished results, DD O’Rielly, B.Sc., and CW Loomis, Ph.D., 2005). COX-2 and iNOS exhibit a more delayed effect (i.e., days after injury). The genes coding for inducible NOS and possibly COX-1 are affected within hours of injury (COX-1 expression is increased in the lumbar dorsal horn 12 h after SNL—unpublished results, DD O’Rielly, B.Sc., and CW Loomis, Ph.D., 2005). COX-2 and iNOS exhibit a more delayed effect (i.e., days after injury). The genes coding for inducible

**Fig. 7.** Brush (BR)–evoked and spontaneous behaviors after intrathecal prostaglandin E₂ (0.1 µg) into the lumbar or thoracic subarachnoid space 3 days after spinal nerve ligation or sham surgery. Each bar represents the mean ± SEM of 6–15 animals. Asterisks indicate a significant difference from thoracic delivery within each treatment group (**P < 0.01, ***P < 0.001).

Fig. 8. The effect of intrathecal S(+) or R(−)-ibuprofen (100 µg; IBL) on brush (BR)–evoked and spontaneous behaviors elicited by intrathecal prostaglandin E₂ (PGE₂; 0.01 µg). All drugs were injected 3 days after spinal nerve ligation or sham surgery. Each bar represents the mean ± SEM of 6–12 animals. Asterisks indicate a significant difference (***P < 0.001) from the both dimethyl sulfoxide (DMSO) plus PGE₂ or IBL-ibuprofen plus prostaglandin E₂ groups.

Fig. 9. The effect of intrathecal SC-51322 (100 µg) on brush (BR)–evoked and spontaneous behaviors elicited by intrathecal prostaglandin E₂ (PGE₂; 0.01 µg). All drugs were injected 3 days after spinal nerve ligation or sham surgery. Each bar represents the mean ± SEM of 6–12 animals. Asterisks indicate a significant difference from the corresponding SC-51322 plus PGE₂–treated group (**P < 0.01, ***P < 0.001). DMSO = dimethyl sulfoxide.

NOS and COX have identical promoters and response elements to nuclear factor κB, a factor known to be increased in the spinal cord after injury. Experiments are currently in progress to investigate the connection between increased nuclear factor κB, NOS, COX, and EP receptors in the SNL model.

In vitro, PGE₂ yielded a consistent bell-shaped concentration–response curve in all slice preparations. This was previously described using synaptosomes and astrocytes from normal animals and may reflect the preferential activation of EP receptors inhibiting glutamate release (e.g., EP₃) and/or the desensitization of excitatory EP receptors (i.e., EP₁, EP₂, and EP₄) at high PGE₂ concentrations. The former possibility seems most likely in view of the effect of SC-51322 to increase glutamate release at high PGE₂ concentrations. The blockade of EP₃ receptors, some of which are coupled to inhibitory G proteins on the central terminals of primary afferent fibers, would disinhibit this release. The parallel leftward shift in the PGE₂ concentration–response curve (including the descending phase) 3 days after SNL suggests that all EP-receptor subtypes eliciting or inhibiting glutamate release in the affected lumbar segments must have undergone comparable increases in sensitivity after SNL. Alterations in the spinal expression of EP receptors after SNL are currently being investigated in our laboratory. Although arachidonic acid also evoked glutamate release, it was significantly less potent than PGE₂. Arachidonic acid does not seem to activate inhibitory mechanisms at high concentrations because its peak effect was identical to that of PGE₂ in the presence of SC-51322.

Prostanoids are known to be active in a variety of nerve injury models. PGE₂ (10 µm), administered as part of an inflammatory cocktail, increased spontaneous activity in dorsal root fibers and evoked activity in a sub-
population of previously “silent” fibers 2–4 weeks after chronic constriction injury. In axotomized rats, a mixture containing PGE\(_2\) (10 \(\mu\)M) enhanced ectopic mechanical excitability, reduced PWT, and increased the response magnitude in most (77%) severed mechanosensitive C fibers and some (46%) mechanosensitive A fibers. Unlike the current study, however, these reports provided no direct information on the degree to which nerve injury alters the pharmacodynamic response to prostanooids.

The effects of COX inhibitors in neuropathic pain models vary depending on the time of administration (i.e., before or after treatment). Isoform-selective and -nonselective COX inhibitors, given systemically or intrathecally near the time of injury (i.e., before to 1–2 days after), attenuated thermal hyperalgesia and/or mechanical allodynia in various neuropathic pain models. In contrast, their effectiveness declined as the time of administration increased after nerve injury (i.e., > 7 days). The results of the current study (i.e., 3 days after SNL) are consistent with this overall time course profile.

The mechanisms underlying the marked sensitivity to PGE\(_2\) remain to be determined, but increased EP receptor–effector coupling in the affected spinal cord (i.e., receptor supersensitivity) and/or increased spinal EP receptor expression are obvious possibilities. SNL resulted in a parallel leftward shift in the PGE\(_2\) concentration–response curve, whereas pretreatment with SC-51322 yielded an opposite rightward shift. SC-51322 also inhibited brush-evoked and spontaneous behavior in vivo, confirming that these effects are mediated by spinal EP receptors. The sheer magnitude of the increase in potency of PGE\(_2\) after SNL also argues strongly for amplification of the pharmacodynamic response at the EP receptor–effector level. SC-51322 had no effect on spontaneous or brush-evoked behavioral responses in sham-operated animals. This is because an inactive dose of intrathecal PGE\(_2\) (0.01 \(\mu\)g) in sham controls was selected for these experiments. Moreover, endogenous spinal prostaglandins have no effect on behavior in sham-operated as compared with nerve-ligated rats early (i.e., post 3 days) after nerve injury.

However, additional mechanisms that could have important pathophysiologic implications in the nerve-injured state seem to be involved. Pretreatment with \(S(+)\)-ibuprofen, but not the \(R(-)\) isomer, attenuated exogenous PGE\(_2\)-evoked glutamate release from slices of nerve-ligated animals, as well as brush-evoked and, to a lesser degree, spontaneous behavior in vivo. \(S(+)\)-ibuprofen had a similar effect on electrically evoked flexor afferent responses recorded from the rat biceps femoris 3 days after SNL. In every experiment, the effect of ibuprofen was stereospecific, indicating that the attenuation of exogenous PGE\(_2\) was related to COX inhibition. These results strongly suggest that spinal prostaglandin biosynthesis is activated by exogenous PGE\(_2\) in the nerve-injured state and that the resulting products contributed to the observed COX-dependent effects of spinal PGE\(_2\) in this model. The up-regulation of COX and NOS, as suggested by the expression data 3 days after SNL, would provide the enhanced biosynthetic capacity in the lumbar spinal cord by which to generate such products. The preferential coupling and synergistic interaction between the inducible forms of COX-2 and membrane-bound PGE\(_2\) synthase also represent a well-regulated system by which the biosynthesis of PGE\(_2\) could be specifically up-regulated after SNL.

In fact, the enzymes responsible for PGE\(_2\) biosynthesis and at least one of the EP receptor subtypes used by PGE\(_2\) are strategically colocated within the cell. Functional EP\(_1\) receptors, like those in the plasma membrane, are colocalized with the inducible forms of COX-2 and membrane-bound PGE\(_2\) synthase in the nuclear membrane and endoplasmic reticulum. Moreover, COX-1 and COX-2 are also monotoically inserted in the endoplasmic reticulum and nuclear membrane with the substrate-binding pocket precisely orientated to take up arachidonic acid as it is liberated from the membrane by phospholipase A\(_2\) or phospholipase C. Phospholipase A\(_2\) isoforms are up-regulated after sciatic nerve injury, and prostaglandins rapidly increase the free cytosolic calcium concentration from internal stores in a concentration-dependent manner. The latter initiates Ca\(^{2+}\)-dependent phospholipase activity as evidenced by the effect of prostaglandin F\(_{2\alpha}\) on phospholipase C activity. These biochemical studies confirm the presence of the prostanoan catalytic cascade and a requisite receptor subtype within the cell by which PGE\(_2\) could elicit its own synthesis in an up-regulated state. Whether a similar relation extends to nitric oxide remains to be determined, but it is noteworthy that (1) iNOS and nNOS are up-regulated in the ipsilateral lumbar dorsal horn 3 days after SNL, (2) prostaglandins and nitric oxide are positively and reciprocally coupled in many cell types, and (3) PGE\(_2\) and nitric oxide exhibit additive proalldynic effects in the SNL model.

In summary, the results of the current study support the hypothesis of early and abnormal sensitivity to spinal PGE\(_2\) 3 days after SNL in the rat, effects that are mediated by spinal EP receptors, COX-dependent, and localized to spinal segments most likely affected by SNL. They provide further evidence for an early pathogenic role of spinal PGE\(_2\) in experimental allodynia and indicate that...
spinal prostaglandins, in the nerve-injured state, are capable of triggering their own biosynthesis. These results have important pathophysiologic implications and are in accordance with previous reports on the functional relation between spinal prostanoids and experimental allodynia in the SNL model.7,8,15

References


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