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Interaction of Spinal Nitric Oxide and Prostaglandins after L5–L6 Spinal Nerve Ligation in the Rat

An Isobolographic Analysis

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SPINAL prostaglandins seem to be important in the pathogenesis and early maintenance (hours to days after nerve injury) of experimental allodynia.[1–4] This relation is consistent with (1) cyclooxygenase and prostanoid receptor localization in the outer laminae of the spinal dorsal horn,[5] (2) exogenous prostaglandins (*i.e.*, intrathecal) eliciting allodynia-like behavior in otherwise normal animals,[6] and (3) brush-evoked allodynia and spinal prostaglandin E_2 (PGE_2) release in nerve-injured but not sham-operated rats.[3,4] It is also supported by recent studies showing that intrathecal cyclooxygenase inhibitors given 2–8 h after L5–L6 spinal nerve ligation prevented the development of allodynia for at least 20 days in the rat.[2,4] A similar connection between spinal nitric oxide (NO) and neuropathic pain has emerged,[7–10] including the increased expression of neuronal nitric oxide synthase (NOS) in the spinal cord after L5–L6 spinal nerve ligation.[7] These reports, combined with the apparent regulation of cyclooxygenase expression and prostaglandin synthesis by NO,[11,12] suggest that spinal NO and prostaglandins interact to effect allodynia early after nerve injury. To test this hypothesis and to determine the nature of the pharmacologic interaction, we used isobolographic analysis with selective NOS inhibitors and cyclooxygenase inhibitors in the L5–L6 spinal nerve ligation 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, NL, Canada).

Animals

Male Sprague-Dawley rats (weight, 100–130 g) were obtained from the Vivarium, Memorial University of Newfoundland, and were housed in standard cages with wood chip bedding. Animals had free access to food and water and were housed singly after surgery. A 12/12 h light/dark cycle (lights on at 07:00 h) was used throughout.

Intrathecal Catheterization

Intrathecal catheters (6.5 cm long, 6-μl dead volume) were implanted during halothane anesthesia for termination near the lumbar enlargement.[3,4] Only rats exhibiting normal gait, feeding, and grooming behavior were used in subsequent experiments.

Spinal Nerve Ligation

After a 3- to 4-day recovery period, rats were reanesthetized with halothane, and a dorsal midline incision was made from L3 to S2. The left posterior interarticular process (L5) was resected, the L6 transverse process was partially removed to expose the L4 and L5 spinal nerves, and the L5 spinal root was tightly ligated with 6-0 silk thread.[13,14] The L6 spinal root was then located medial and caudal to the sacroiliac junction and ligated in the same manner. The wound was closed with 4-0 silk sutures in two layers and cleaned with 70% alcohol, and a 5-ml bolus of lactated Ringer's solution was injected intraperitoneally. After recovery, the animal was returned to the animal care facility for 2 days before experimentation.

Testing

Rats were placed in a plastic cage with a wire-mesh bottom to allow access to the plantar surface of the hind paws. After a 20-min acclimatization period, the 50% paw withdrawal threshold (control PWT; the applied force evoking a brisk hind paw withdrawal) was determined using von Frey filaments.[14] Drug or vehicle was then injected intrathecally, and PWTs were determined every 20 min for up to 2 h. All experiments were performed during the daylight portion of the circadian cycle (08:00–18:00 h); drug testing occurred 2–12 days after nerve ligation. Drugs were administered on alternate days such that no animal received more than six drug injections in total. In all cases, the investigator was blinded to the drug treatment. Allodynia was defined as a PWT of 4 g or less; nerve-ligated animals with values greater than 4 g were excluded.[14]

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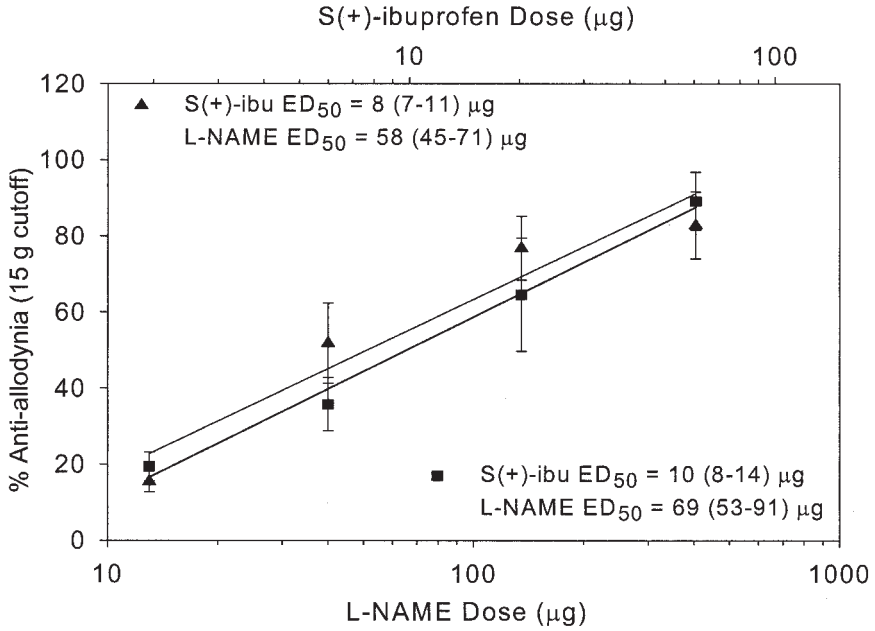

Fig. 1. Addition of intrathecal *S*(+)-ibuprofen enhanced the antiallodynic effect of *N*-nitro-L-arginine methylester (L-NAME) in the L5–L6 spinal nerve ligation model. Each *point* represents the mean ± SEM of six to eight animals. Dose–response experiments were conducted 2–12 days after nerve ligation. *S*(+)-ibuprofen was administered either 20 min before (■) or 20 min after (▲) intrathecal L-NAME. Drug-induced changes in left-paw withdrawal thresholds are expressed as percent antiallodynia (see Materials and Methods). The ED_{50}s and corresponding 95% confidence intervals for each dose–response curve are shown.

Experimental Protocols

Dose–response curves for *S*(+)-ibuprofen and *N*-nitro-L-arginine methylester (L-NAME) were determined in separate groups of rats. For combination studies, a molar ratio of 1:5 was used based on the ED_{40} values of each agent (*S*(+)-ibuprofen = 87 nmol [18 μg]; L-NAME = 445 nmol [120 μg]).[3] Dose ratios of *S*(+)-ibuprofen:L-NAME were 10:50, 30:150, 100:500, or 300:1,500 nmol (2:13, 6:40, 21:135, or 62:404 μg, respectively). Each drug was delivered intrathecally, with a 20-min delay between *S*(+)-ibuprofen and L-NAME (to correct for the difference in the time of peak effect). In a separate experiment, the order was reversed to control for the sequence of administration. *S*(+)-ibuprofen (active stereoisomer) was dissolved in 100% dimethyl sulfoxide and diluted with saline on injection for a final dimethyl sulfoxide concentration of 50%; L-NAME and aminoguanidine were dissolved in saline and diluted with the same. All drugs were purchased from Research Biochemicals International (Natick, MA).

Data Analysis

Paw withdrawal thresholds are presented as percent antiallodynia (% Antiallodynia = (Test Value − Control Value)/(15 − Control Value) × 100), with 100% representing complete reversal (PWT ≥ 15 g). Dose–response curves were calculated using the peak effect of each dose from the time-course data. Statistical testing was performed using Sigmastat 2.03 for Windows (SPSS, Inc., Chicago, IL). Multiple comparisons were performed using one-way analysis of variance followed by Newman-Keuls test. Results are shown as mean ± SEM; $P < 0.05$ was considered to be statistically significant.

Results

Tight ligation of the L5–L6 spinal nerves produced touch-evoked allodynia manifested as a significant reduction in the PWT (from ≥ 15 g to 1–3 g) of the ipsilateral hind paw and posturing to protect the sensitized area. PWTs in all sham-operated rats remained unchanged from presurgical values (≥ 15 g). Intrathecal L-NAME produced dose-dependent (ED_{50} [95% confidence interval] = 212 [112–402] μg) but incomplete reversal of allodynia (57% of maximum) over the 30- to 300-μg range; a dose of 1,000 μg yielded no further reversal of allodynia (data not shown). The time of peak effect ranged from 40–60 min with a duration of action of 60–80 min. Neither intrathecal L-NAME (100 μg; midcervical injection) nor D-NAME (100 μg; lumbar injection) had any effect on PWTs in ligated rats. Intrathecal lumbar injection of dimethyl sulfoxide and saline[3] or aminoguanidine (30–300 μg) were also without effect; the slope of the aminoguanidine dose–response curve was not significantly different from zero (data not shown).

The combination of intrathecal *S*(+)-ibuprofen and L-NAME attenuated allodynia in a dose-dependent manner, irrespective of treatment order (fig. 1); the dose–response curves were statistically indistinguishable. *S*(+)-ibuprofen significantly reduced the ED_{50} of L-NAME from 212 μg to 69 μg (L-NAME followed by ibuprofen) and increased the maximum antiallodynic effect to 89% (fig. 1). There was also a modest increase in the duration of action (from 80 to 120 min; data not shown). Isobolographic analysis revealed an additive interaction with the ED_{50}s of the combination falling within the 95% confidence intervals of the theoretical additive line (fig. 2). Unlike their sequential injection, the coinjection of

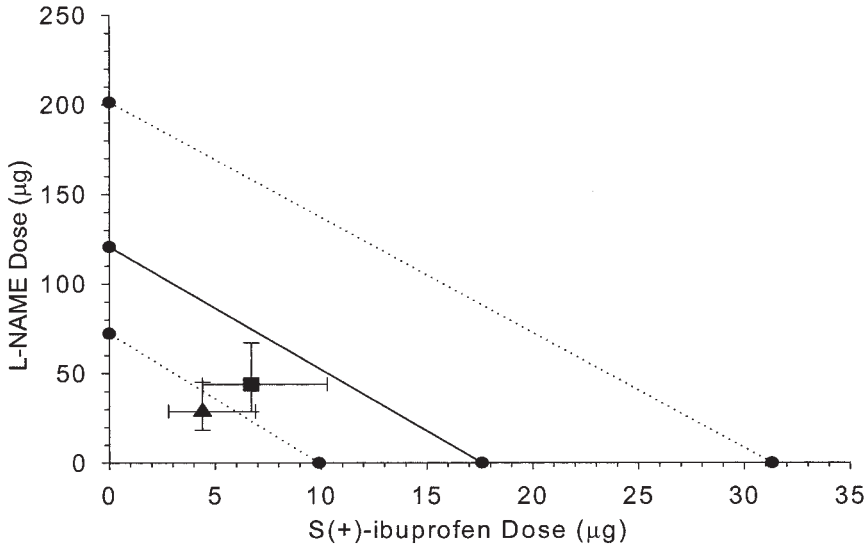

Fig. 2. Isobologram illustrating the additive interaction between intrathecal *N*-nitro-L-arginine methylester (L-NAME) and *S*(+)-ibuprofen in the L5–L6 spinal nerve ligation model. The *points* on the x- and y-axes are the ED_{40}s and 95% confidence intervals of *S*(+)-ibuprofen and L-NAME, respectively. ▲ = ED_{40} and corresponding 95% confidence interval for the combination of L-NAME followed by *S*(+)-ibuprofen; ■ = ED_{40} and corresponding 95% confidence interval for the combination of *S*(+)-ibuprofen followed by L-NAME.

S(+)-ibuprofen and L-NAME yielded inconsistent results (data not shown), reflecting their incompatibility in solution. The inactive *R*(−)-isomer of ibuprofen was without effect (data not shown).

Discussion

S(+)-ibuprofen enhanced the effect of L-NAME in the L5–L6 spinal nerve ligation model as indicated by the significant increase in both the potency of L-NAME (greater than threefold) and the maximum reversal of allodynia (57–89%) as compared with L-NAME alone. The stereospecificity of L-NAME and *S*(+)-ibuprofen suggests that their antiallodynic effects can be attributed to NOS inhibition and cyclooxygenase inhibition, respectively. Fixed-molar isobolographic analysis revealed an additive interaction at a molar ratio of 1:5 (ibuprofen:L-NAME), indicating the convergence of spinal NO- and prostaglandin-mediated alterations in sensory processing. That this interaction was not significantly affected by the order of drug injection indicates that cyclooxygenase inhibition and NOS inhibition was of sufficient duration to correct for the difference in time of peak effect and that spinal NOS and cyclooxygenase pathways are both activated early after L5–L6 spinal nerve ligation.

A spinal NO–prostanoid interaction is consistent with reports of their individual effects in neuropathic pain models. For example, allodynia induced by intrathecal *N*-methyl-D-aspartate was blocked by NOS and cyclooxygnease-2 inhibitors.[8] Intrathecal L-NAME attenuated the hyperalgesia elicited by spinal PGE_2 and allodynia induced by spinal strychnine.[9,10] Allodynia was also partially reversed by spinal cyclooxygenase inhibitors or prostaglandin receptor antagonists in the chronic constriction, intrathecal strychnine, intrathecal bicuculline, and the spinal nerve ligation models.[1,3,15,16] These results are in agreement with recent findings that nanomolar concentrations of PGE_2 reduced inhibitory (glycinergic) transmission in rat spinal cord,[17] and normally innocuous brushing elicited spinal prostaglandin synthesis and nocifensive behaviors in nerve-ligated (allodynic) but not sham-operated animals.[3,4] The latter effects were both blocked by intrathecal *S*(+)-ibuprofen. These studies underscore the relevance of spinal prostanoids and NO to experimental allodynia and thus their ability to effect, in combination, this abnormal state.

While the mechanisms underlying this interaction are currently being investigated, previous studies have identified numerous sites of intersection between NO and prostanoids. The genes coding for NOS and cyclooxygenase have identical promoters and response elements to nuclear factor κB and nuclear factor–interleukin 6,[18,19] and their expression is triggered by similar mediators.[20] Prostaglandin synthesis was triggered in human fetal fibroblasts, bovine endothelial cells, and mouse macrophages exposed to NO donors.[21] NO also potentiated the interleukin 1β–induced up-regulation of cyclooxygenase-2 messenger RNA (mRNA) and protein in cultured dorsal root ganglion cells.[22] Inducible NOS has also been implicated in the regulation of hepatic cyclooxygenase 2.[23] Conversely, NOS activity and nitrite concentrations were increased in murine macrophages treated with PGE_2,[24] and ibuprofen inhibited the synthesis of inducible NOS (mRNA and protein) in glial cell cultures at concentrations blocking PGE_2 production.[25] These reports indicate a bidirectional relation between distinct pathways that contribute to allodynia early after nerve injury and are consistent with the additive interaction reported in the current study. The latter may also include the exacerbation of nerve injury–induced cell damage through the local formation of peroxynitrite,[26] the reaction product of NO plus superoxide anion (derived from cyclooxygenase).

In summary, the interaction between spinal NO and

prostanoids has been characterized in a well-established model of neuropathic pain using isobolographic analysis. To the extent that similar mechanisms are at work in clinical allodynia, these results have important bearing on future strategies in the early treatment of neuropathic pain.

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