Effects of Bupivacaine and Tetrodotoxin on Carrageenan-induced Hind Paw Inflammation in Rats (Part 2)

Cytokines and p38 Mitogen-activated Protein Kinases in Dorsal Root Ganglia and Spinal Cord

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Background: The authors previously showed that bupivacaine and tetrodotoxin via a contralateral or ipsilateral sciatic block could attenuate the inflammatory hind paw edema and hyperalgesia induced by hind paw injection of carrageenan in rats. Systemic administration of either bupivacaine or tetrodotoxin was ineffective in preventing hind paw edema and hyperalgesia. Bupivacaine by all three routes inhibited lipopolysaccharide-stimulated production of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in cultures of circulating leukocytes and partially inhibited Staphylococcus aureus Cowan (SAC)-stimulated production of TNF-α, but it did not inhibit production of interleukin 10 (IL-10). Tetrodotoxin by all three routes had no effect on stimulated production of cytokines in cultures of circulating leukocytes. The mechanisms underlying contralateral effects of sciatic blockade remain unexplained.

Several lines of evidence suggest that peripheral inflammation elicits central changes in proinflammatory cytokines that mediate the cascade underlying hyperalgesia and allodynia. For example, up-regulation of spinal IL-1β expression seems to parallel the development and maintenance of formalin-induced mechanical allodynia. The mitogen-activated protein kinases (MAPKs) are a family of signaling molecules that transduce extracellular stimuli into intracellular responses in a wide variety of circumstances. The MAPK family includes extracellular signal–regulated kinases, p38, and c-Jun N-terminal kinase. p38 has been implicated in exaggerated pain states. p38 is activated in dorsal root ganglion (DRG) nociceptor neurons by peripheral inflammation and participates in the maintenance of inflammatory heat hyperalgesia.

Carrageenan-induced inflammation also triggers phosphorylation of spinal p38 MAPK. p38 MAPK plays a dual role in the generation of, and cellular response to, certain cytokines. It has a recognized role in TNF-α and IL-1β production. A sequential role for TNF-α and p38 in the induction of neuropathic pain was shown in a spinal nerve ligation model.

A variety of analgesics not commonly regarded as antiinflammatory agents may act in part by influencing cytokine production. For example, perineural administration of clonidine reduced both hyperalgesia and local cytokine production in a model of nerve injury. LA modulation of inflammatory responses could be a result of an action on MAPK pathways at a spinal cord and DRG level. To explore further action of LAs, we examined the effect of bupivacaine or tetrodotoxin sciatic block (ipsi-
lateral or contralateral to the side of hind paw inflammation) on the content of major proinflammatory cytokines (TNF-α and IL-1β) and p38 and phosphorylated p38 (p-p38) MAPK in the ipsilateral and contralateral DRGs and the ipsilateral and contralateral sides of the spinal dorsal horn after carrageenan-induced paw inflammation. Because our previous results showed similar effects of tetrodotoxin and bupivacaine sciatic blocks on local hyperalgesia and hind paw inflammation but different effects of the two drugs on systemic cytokine production, we included tetrodotoxin groups in the current study. Unlike conventional amino-amide and amino-ester LAs, the site 1 sodium channel–blocking toxin tetrodotoxin seems to act specifically on sodium channels.11

Materials and Methods

All procedures were conducted in accordance with the Children’s Hospital Animal Care and Use Committee (Boston, Massachusetts). Young adult male Sprague-Dawley rats weighing 250–300 g were used. The animals were kept on a 12-h light-dark cycle with free access to food and water. The rats were handled repeatedly over at least 3 days before experiments to habituate them to the investigators and the testing paradigm.

Solutions

Carrageenan was prepared fresh before each experiment (0.2 ml of 2% solution of lambda carrageenan in saline). Tetrodotoxin (50 μM)12 stock solutions were made by dissolving 1 mg tetrodotoxin (Sigma Chemical Co., St. Louis, MO) in 10 ml of 20 mM citrate buffer. Bupivacaine, 0.5% (Sigma Chemical), with epinephrine was used as the LA. Epinephrine from a commercial 1:100,000 (1 mg/ml) solution (American Regent Laboratories, Shirley, NY) was diluted to 55 μM (1:100,000) final concentration and prepared fresh for each set of injections.

Sciatic Blockade Technique

Before nerve block injections, rats were anesthetized briefly with isoflurane (2–4% inspired concentration in 100% oxygen) by facemask. The block was initiated by introducing a 23-gauge needle posteromedially to the greater trochanter, pointed in an anteromedial direction. When bone was touched, the needle was withdrawn 1 mm and the drug was injected. To provide a prolonged effect, bupivacaine and tetrodotoxin were injected with 1:100,000 epinephrine, and reinjections were performed 6 h later. This paradigm was chosen in preference to a previous approach using sustained release of LAs from biodegradable microspheres, because previous work showed that the microspheres induced inflammation, and the coincorporated dexamethasone could produce confounding local and systemic antiinflammatory effects.13 The final volume of injectate was 0.2 ml test solution. The left leg was always used for blocks.

Experimental Groups

Animals were assigned to 1 of 10 experimental groups (table 1). Each animal received two types of injections: a left sciatic block (with bupivacaine plus epinephrine, tetrodotoxin plus epinephrine, or saline plus epinephrine) and a subcutaneous paw injection (with either carrageenan or saline). The animals in the ipsilateral sciatic block groups received their hind paw injections (with either carrageenan or saline) on the left, whereas animals in the contralateral block groups received hind paw injections (with either carrageenan or saline) on the left, whereas animals in the contralateral block groups received hind paw injections (with either carrageenan or saline). The animals in the forepaw injection groups received a right subcutaneous forepaw injection (with either carrageenan or saline) and a left sciatic block (with saline plus epinephrine). The animals received a second left perisciatic injection 6 h later to provide a prolonged effect of bupivacaine, tetrodotoxin, or saline.

Tissue Preparation and Assessment of TNF-α, IL-1β, p38, and Phosphorylated p38 in the DRGs and Spinal Cord

Animals were deeply anesthetized with pentobarbital and then killed by decapitation 15 h after the hind paw or forepaw injection. Ipsilateral and contralateral L4, L5 DRGs and the corresponding right and left ventral and dorsal quadrants of the spinal cord were removed. The

Table 1. Summary of the Treatment Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Sciatic Area</th>
<th>Left Hind Paw (SC)</th>
<th>Right Hind Paw or Forepaw (SC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hind paw control</td>
<td>Saline + epi</td>
<td>Saline</td>
</tr>
<tr>
<td>2</td>
<td>Hind paw carr</td>
<td>Saline + epi</td>
<td>Carr</td>
</tr>
<tr>
<td>3</td>
<td>Contra bupi block</td>
<td>Bupi + epi</td>
<td>Bupi</td>
</tr>
<tr>
<td>4</td>
<td>Contra bupi block + carr</td>
<td>Bupi + epi</td>
<td>Carr</td>
</tr>
<tr>
<td>5</td>
<td>Ipsi bupi block + carr</td>
<td>Bupi + epi</td>
<td>Carr</td>
</tr>
<tr>
<td>6</td>
<td>Contra TTX block</td>
<td>TTX + epi</td>
<td>Saline</td>
</tr>
<tr>
<td>7</td>
<td>Contra TTX block + carr</td>
<td>TTX + epi</td>
<td>Carr</td>
</tr>
<tr>
<td>8</td>
<td>Ipsi TTX block + carr</td>
<td>TTX + epi</td>
<td>Carr</td>
</tr>
<tr>
<td>9</td>
<td>Forepaw control</td>
<td>Saline + epi</td>
<td>Saline</td>
</tr>
<tr>
<td>10</td>
<td>Forepaw carr</td>
<td>Saline + epi</td>
<td>Carr</td>
</tr>
</tbody>
</table>

Bupi = bupivacaine; carr = carrageenan; contra = contralateral; epi = epinephrine; ipsi = ipsilateral; SC = subcutaneous; TTX = tetrodotoxin.
wet weight of each sample was determined, and the tissues were frozen at 

\(-80^\circ C\). Each sample was homogenized in an ice bath with a lysis buffer containing proteinase inhibitors and phosphatase inhibitors, with the following composition: 1 mM EDTA, 0.5% (wt/vol) Triton X-100, 5 mM sodium fluoride, 6 mM urea, 10 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin, 100 \(\mu\)M phenylmethanesulfonyl fluoride, 3 \(\mu\)g/ml aprotinin, 2.5 mM sodium pyrophosphate, and 1 mM sodium orthovanadate in phosphate-buffered saline 14 (Roche Boehringer, Mannheim, Germany) using an ultrasound sonicator. The homogenates were centrifuged (15,000 g) for 30 min at 4°C, and the supernatants were aliquoted and stored at 

\(-80^\circ C\). Concentrations of TNF-\(\alpha\), IL-1\(\beta\), p38 MAPK, and p-p38 MAPK were measured with commercially available sandwich enzyme-linked immunosorbent assays (DuoSet; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Statistical Analysis**

The distribution of cytokines and MAPK concentrations in each unit were checked for normality using the Shapiro-Wilk test. The difference between groups was assessed using a one-way analysis of variance with post hoc analysis via Fisher protected least significant difference test. Data are presented in figures as mean \(\pm\) SD. A \(P\) value below 0.05 was considered as the minimum level of statistical significance.

**Results**

**TNF-\(\alpha\) in Spinal Cord and DRGs (n = 4 or 5/Group)**

A significant increase in TNF-\(\alpha\) content in ipsilateral and contralateral dorsal spinal cord (fig. 1A) and bilateral DRGs (fig. 1B) was observed at 15 h in the hind paw carrageenan group (right hind paw carrageenan plus left periscatic saline) compared with the control group (right hind paw saline plus left periscatic saline). In the groups receiving bupivacaine or tetrodotoxin sciatic blocks with hind paw saline (right hind paw saline plus left periscatic bupivacaine or tetrodotoxin block), TNF-\(\alpha\) content in ipsilateral and contralateral dorsal spinal cord (fig. 1A) and bilateral DRGs (fig. 1B) was not significantly different from that observed in the corresponding sites in the control group. In the groups receiving hind paw carrageenan and either an ipsilateral or a contralateral sciatic block with either bupivacaine or tetrodotoxin, TNF-\(\alpha\) content was significantly higher in bilateral spinal cord and bilateral DRGs than that observed in the control group but not significantly different from corresponding sites in the hind paw carrageenan group.

**IL-1\(\beta\) in Spinal Cord and DRGs (n = 4 or 5/Group)**

A significant increase in IL-1\(\beta\) content was observed in ipsilateral and contralateral dorsal spinal cord (fig. 2A) and in bilateral DRGs (fig. 2B) at 15 h in the hind paw carrageenan group compared with the control group. In the groups receiving hind paw saline along with sciatic blocks with either bupivacaine or tetrodotoxin, IL-1\(\beta\) content in either ipsilateral or contralateral dorsal spinal cord (fig. 2A) or bilateral DRGs (fig. 2B) was not significantly different from that observed in the corresponding sites in the control group. In the groups receiving hind paw carrageenan and either ipsilateral or contralateral sciatic blocks with either bupivacaine or tetrodotoxin, IL-1\(\beta\) levels were significantly higher in bilateral spinal cord and DRGs than those observed in the corresponding sites in the control group but not significantly different from the corresponding sites in the carrageenan group.

**p38 and p-p38 MAPK in Spinal Cord and DRGs (n = 3-5/Group)**

Total p38 (nonphosphorylated and phosphorylated) content was not increased at 15 h in the hind paw...
carrageenan groups compared with the control groups in either ipsilateral or contralateral dorsal spinal cord (fig. 3A) or in bilateral DRGs (fig. 3B). Phosphorylated p38 content did not increase in the spinal cord in the hind paw carrageenan group (fig. 4A) compared with control, but it did increase significantly in bilateral DRGs (fig. 4B). In the groups receiving hind paw carrageenan and an ipsilateral or contralateral block with either bupivacaine or tetrodotoxin, p-p38 content in bilateral DRGs was significantly greater than at corresponding sites for the control group but not significantly different from that at corresponding sites in the hind paw carrageenan group (fig. 4B).

**Effects of Carrageenan Injections in the Forepaw**

Because of the remarkable finding of increased content of cytokines and p-p38 MAPK in contralateral lumbar DRGs after hind paw carrageenan injection, an additional control experiment was added with groups receiving forepaw carrageenan injections, to evaluate the possibility that activation of cytokines and p-p38 MAPK in lumbar DRGs on the side contralateral to hind paw injection might reflect a systemic, rather than segmental, inflammatory response. Animals receiving carrageenan injections in a forepaw did not evoke any increases in production of either TNF-α (fig. 1), IL-1β (fig. 2), p38 MAPK (fig. 3), or p-p38 MAPK (fig. 4) in either lumbar spinal cord (part A of each figure) or bilateral L4–L5 DRGs (part B of each figure).

**Discussion**

In the current study, we examined two primary hypotheses: (1) that carrageenan-induced hind paw inflam-
information could activate inflammatory pathways ipsilaterally and/or contralaterally in both spinal cord and DRGs and (2) that sciatic blockade with either bupivacaine or tetrodotoxin could inhibit these inflammatory responses. TNF-α and IL-1β content showed bilateral significant increases in the dorsal spinal cord and lumbar DRGs after a unilateral hind paw challenge with carrageenan. In parallel, p-p38 MAPK content was bilaterally increased in the lumbar DRGs after unilateral hind paw carrageenan injection, although not in spinal cord. Total p38 (nonphosphorylated and phosphorylated) content was not increased in the carrageenan groups, compared with the control groups, either in spinal cord or in DRGs. This suggested that the increase in p-p38 in bilateral lumbar DRGs may reflect increased phosphorylation rather than increased substrate. Neither ipsilateral nor contralateral sciatic blockade with bupivacaine or tetrodotoxin had any effect on the evoked production of the two cytokines or p-p38 MAPK.

Activation of TNF-α, IL-1β, and p-p38 MAPK in contralateral DRGs was a remarkable observation, because somatic afferent peripheral fibers project to ipsilateral DRGs and are not thought to have contralateral connections at the level of DRGs. Ji et al. reported similar activation of p-p38 MAPK in bilateral DRGs, but not in spinal cord, after Freund complete adjuvant hind paw injections. A trivial cause for this contralateral DRG activation could have been due to a systemic, rather than a regional or segmental, mechanism. To test this possibility, additional groups of animals received carrageenan injections into a forepaw. Forepaw injections did not increase TNF-α, IL-1β, or p-p38 MAPK content in lumbar spinal dorsal horn or lumbar DRGs. This latter observation supports the view that hind paw carrageenan-induced activation of cytokines and p-p38 MAPK in bilateral lumbar DRGs involves a regional or segmental, rather than systemic, mechanism.

Bilateral, segmental activation of inflammatory mechanisms in DRGs after a unilateral injury or inflammatory stimulus may be relevant to mirror-image syndromes noted in some patients with chronic pain. Patients with complex regional pain syndromes not infrequently develop bilateral pain and neurovascular signs and symptoms after a unilateral injury. Oaklander et al. have provided evidence that unilateral nerve injury produces contralateral distal sensory loss and bilateral down-regulation of messenger RNA encoding the SCN10A sodium channel in DRGs in rats, and contralateral neurite loss in the skin was observed in patients with unilateral postherpetic neuralgia. As noted by Oaklander, these findings are remarkable because “There are no known anatomical connections between neurons that innervate homologous right and left body parts.” The mechanisms underlying contralateral activation of cytokines and kinases in DRGs, contralateral down-regulation of sodium channels, and contralateral cutaneous neurite loss merit further study and may be relevant to clinical mirror-image syndromes. Contralateral mirror-image pain syndromes and contralateral-directed treatments for phantom limb pain and complex regional pain syndromes may involve a complex interplay of supraspinal, spinal, and peripheral mechanisms.

Previous studies, using a variety of inflammatory substances, including carrageenan, formalin, zymosan, or Freund complete adjuvant, have shown that peripheral inflammation is associated with activation in spinal astrocytes and microglia and increased concentrations of cytokines, MAPK, or both in either spinal cord or DRGs. Pharmacologic interventions to block actions of IL-1β or TNF-α, or blockade of p38 MAPK
phosphorylation can diminish either inflammatory edema or hyperalgesia in these models.\textsuperscript{27,29,30} Cytokines may act directly on neurons \textit{via} their receptors or indirectly by stimulating the local release of arachidonic acid, cyclooxygenase products, and nitric oxide.\textsuperscript{31,32} Peripheral hind paw inflammation can activate cytokines both spinally and supraspinally\textsuperscript{33} and can also activate descending modulatory systems from the locus coeruleus and the nucleus subcoeruleus bilaterally.\textsuperscript{34} Roles of the MAPK family, especially p-38, have been examined in several different pain models.\textsuperscript{7,8,28,35} Injection of formalin into the paw induces p38 activation in bilateral DRGs.\textsuperscript{28} Peripheral inflammation and nerve injury induce p38 MAPK activation in DRGs, and the corresponding nociceptive behaviors are prevented by p38 MAPK inhibition.\textsuperscript{7,8} Moreover, p38 MAPK is known to regulate the synthesis of cytokines, cyclooxygenase 2, and inducible nitric oxide synthase.\textsuperscript{36} Other members of the MAPK family, including extracellular signal-regulated protein kinases, are also implicated in pain signaling.\textsuperscript{37–41}

Previous studies of effects of LAs on inflammatory hyperalgesia and local, systemic, and spinal inflammatory markers have produced conflicting results\textsuperscript{42–50} and have involved a variety of different models, assays, and methods of LA administration. In our companion study,\textsuperscript{2} as in other studies,\textsuperscript{42,43} the local inflammatory edema and hyperalgesia induced by carrageenan was inhibited by a bupivacaine sciatic block. LA actions on spinal inflammation seem complex and may reflect differential effects on microglia \textit{versus} astroglia.\textsuperscript{46}

Contralateral intramuscular injection of bupivacaine microspheres in mice inhibited carrageenan-evoked increases in stimulated production of cytokines in circulating blood cell cultures.\textsuperscript{45} Previous studies by Bilevi-cute-Ljungar \textit{et al.}\textsuperscript{44,49,50} using both inflammatory and nerve injury models found antihyperalgesic effects of either bupivacaine or lidocaine administered in several different locations in the limb contralateral to the site of injury. In addition, these investigators reported that contralateral lidocaine administration inhibited activity in wide-dynamic-range dorsal horn neurons on the side ipsilateral to a chronic constriction injury.\textsuperscript{49} They suggested the possible involvement of crossed spinal reflexes.

We attempted to avoid some of the confounding factors in previous studies and to elucidate local, contralateral, systemic, and spinal mechanisms of LAs on inflammatory responses by using ipsilateral and contralateral sciatic blocks and remote systemic injections of either bupivacaine or tetrodotoxin in combination with epinephrine, with repeat injections 6 h later. This approach permits dense sensory and motor blockade over the 15-h period required to permit evolution of inflammatory edema and hyperalgesia in the carrageenan model.

Because this method of prolonged sciatic blockade, using either bupivacaine or tetrodotoxin, was able to suppress local hind paw edema and hyperalgesia in the first part of this two-part study,\textsuperscript{2} and because either perisciatic or systemic bupivacaine was able to suppress a systemic inflammatorymarker, namely evoked cytokine production in whole blood cultures, it was somewhat surprising that neither bupivacaine nor tetrodotoxin suppressed any aspect of spinal cytokine or MAPK activation in the current study. Future studies may examine higher doses of bupivacaine or tetrodotoxin, to achieve even more intense degrees of blockade, concomitant saphenous nerve blockade, or other interventions.

In conclusion, this two-part investigation showed that actions of LAs on inflammation are complex and reflect interplay of local, contralateral, systemic, and spinal mechanisms. Neither bupivacaine nor tetrodotoxin by any route was effective in blunting increases in cytokines or pp38 MAPK in spinal cord or DRGs. Future animal studies and clinical intervention studies will need to consider multiple sites and mechanisms of LA action that can influence success or failure in the treatment of acute or chronic pain.
LOCAL ANESTHETICS, SPINAL CYTOKINES, AND KINASES


