Peripheral inflammatory hyperalgesia depends on the COX increase in the dorsal root ganglion

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It is well established that dorsal root ganglion (DRG) cells synthesize prostaglandin. However, the role that prostaglandin plays in the inflammatory hyperalgesia of peripheral tissue has not been established. Recently, we have successfully established a technique to inject drugs (3 μL) directly into the L5-DRG of rats, allowing in vivo identification of the role that DRG cell-derived COX-1 and COX-2 play in the development of inflammatory hyperalgesia of peripheral tissue. IL-1β (0.5 pg) or carrageenan (100 ng) was administered in the L5-peripheral field of rat hindpaw and mechanical hyperalgesia was evaluated after 3 h. Administration of a nonselective COX inhibitor (indomethacin), selective COX-1 (valeryl salicylate), or selective COX-2 (SC-236) inhibitors into the L5-DRG prevented the hyperalgesia induced by IL-1β. Similarly, oligodeoxynucleotide-antisense against COX-1 or COX-2, but not oligodeoxynucleotide-mismatch, decreased their respective expressions in the L5-DRG and prevented the hyperalgesia induced by IL-1β in the hindpaw. Immunofluorescence analysis demonstrated that the amount of COX-1 and COX-2, constitutively expressed in TRPV-1$^+$ cells of the DRG, significantly increased after carrageenan or IL-1β administration. In addition, indomethacin administered into the L5-DRG prevented the increase of PKCε expression in DRG membrane cells induced by carrageenan. Finally, the administration of EP1/EP2 (7.5 ng) or EP4 (10 μg) receptor antagonists into L5-DRG prevented the hyperalgesia induced by IL-1β in the hindpaw. In conclusion, the results of this study suggest that the inflammatory hyperalgesia in peripheral tissue depends on activation of COX-1 and COX-2 in C-fibers, which contribute to the induction and maintenance of sensitization of primary sensory neurons.

pain | cyclooxygenase | inflammation | prostaglandin-E2 | peripheral nervous system

During tissue injury, prostaglandin-E_2 (PGE_2) is produced by the activation of the enzyme cyclooxygenase (COX) to play an important role in inflammatory hyperalgesia. PGE_2 sensitizes peripheral nociceptors through the activation of PGE_2 receptors (EP) (1). This sensitization, characterized by a reduction of nociceptive threshold and by an increase in peripheral afferent neuron responsiveness, is the main feature of inflammatory hyperalgesia in the peripheral tissue. The widespread use of nonsteroidal anti-inflammatory drugs to control inflammatory hyperalgesia exemplifies the relevance of PGE_2 for the development of inflammatory hyperalgesia. These drugs decrease peripheral inflammatory hyperalgesia by inhibiting COX and, therefore, by preventing the synthesis of PGE_2 (2, 3).

The COX enzyme is expressed in two major isoforms, COX-1 and COX-2, with different biological functions (4), although both play a role in the inflammatory hyperalgesia (5, 6). The isoform of COX constitutively expressed in dorsal root ganglion (DRG) is still unclear. Although COX-1 mRNA and, to a lesser extent, COX-2 mRNA have been detected in DRG cultures (7) under basal conditions, immunohistochemical studies have shown that DRG cells express only COX-1, but not COX-2 protein (8), in contrast to the spinal cord cells that express COX-2 but not COX-1, protein (8). These data suggest a specific role for each COX isoform under normal conditions in the nervous system. On the other hand, during peri-DRG inflammation there is a prominent increase in COX-2 expression in DRG cells, suggesting that this isoform is more relevant for the inflammatory hyperalgesia (9).

Although it has been demonstrated that primary culture of trigeminal ganglia stimulated with IL-1β produce PGE_2 (10), the role that it plays in the development of inflammatory hyperalgesia in the peripheral tissue is unknown. PGE_2 released in the spinal cord can sensitize secondary afferent neurons (11–13), but data suggest that it can also retrogradely sensitize primary afferent neurons to contribute to the development of inflammatory hyperalgesia (6, 14, 15). Importantly, the development of inflammatory hyperalgesia also involves the activation of the protein kinase A (PKA) and the ϵ isoform of PKC (PKCε) signaling pathway in primary afferent neurons (16, 17).

The aim of this study was to test the hypothesis that inflammatory hyperalgesia in the peripheral tissue depends on PGE_2, synthesized by COX-1 and COX-2 activation in the DRG transient receptor potential cation channel, subfamily V, member 1 (TRPV-1$^+$) cells, and on its release in the DRG.

Results

COX-1 and COX-2 Activation in the DRG Is Required for the Development of Inflammatory Hyperalgesia in Peripheral Tissue. Indomethacin (100 μg) administered into the DRG-L5 prevented the mechanical hyperalgesia induced by IL-1β (0.5 pg) administered in the hindpaw (Fig. 1*A*). The same dose of indomethacin administered into the contralateral L5-DRG did not change the hyperalgesia induced by IL-1β administered in the hindpaw, ruling out a possible systemic effect (Fig. 1*A*). Neither indomethacin nor its vehicle (Tris) when administered alone had an effect on the mechanical threshold.

To test the involvement of COX-1 and COX-2 located in the DRG in the development of inflammatory hyperalgesia of the peripheral tissue, the selective COX-1 inhibitor valeryl salicylate or the selective COX-2 inhibitor SC-236 was administered in the L5-DRG. Valeryl salicylate (3, 10, or 30 μg) (Fig. 1*B*) or SC-236 (30, 70, 100, or 300 μg) (Fig. 1*C*) prevented in a dose–response manner the mechanical hyperalgesia induced by IL-1β (0.5 pg) administered in the hindpaw 30 min later ($P < 0.05$; one-way ANOVA followed by Bonferroni test). Neither inhibitor changed the mechanical threshold when administered alone (Fig. 1 *B* and *C*).

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The authors declare no conflict of interest.

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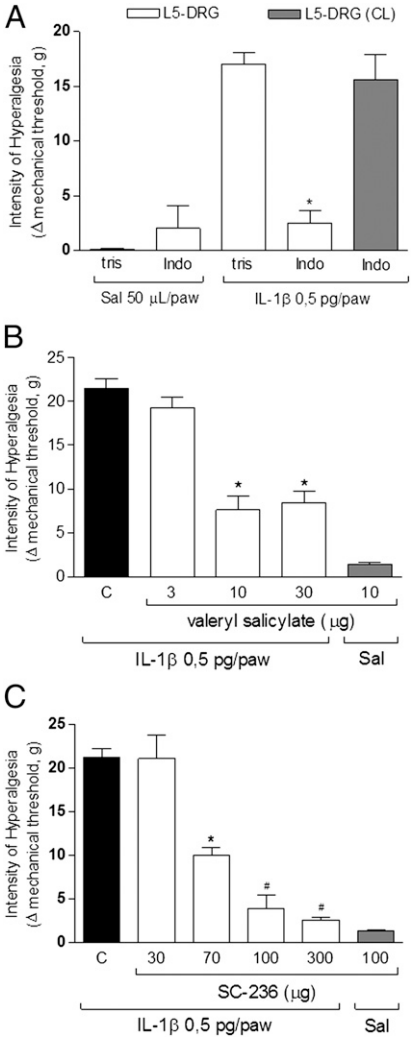

Fig. 1. COX-1 or COX-2 inhibitors administered into the L5-DRG prevented the hyperalgesia induced by IL-1β in the L5 peripheral field. Indomethacin (*A*) (Indo, 100 μg), valeryl salicylate (*B*) (3, 10, or 30 μg), or SC-236 (*C*) (30, 70, 100, or 300 μg) administered into the L5-DRG prevented the hyperalgesia induced by IL-1β (0.5 pg per paw) in the L5 peripheral field. Indomethacin, valeryl salicylate, SC-236, or their vehicles were administered 30 min before IL-1β and the hyperalgesia was evaluated 3 h after its administration. Indomethacin, valeryl salicylate (10 μg), SC-236 (100 μg), saline (3 μL), or Tris (3 μL) into the L5-DRG had no effect on the nociceptive mechanical threshold in rats treated with saline (Sal, 50 μL) in the hind-paw. Indomethacin administered in the contralateral (CL) L5-DRG did not change the hyperalgesia induced by the administration of IL-1β in the L5 peripheral field. The asterisk (*) indicates a response significantly lower ($P < 0.05$, one-way ANOVA followed by the Bonferroni test) than that induced by vehicle administration (C or Tris) in rats treated with IL-1β in the L5 peripheral field, and the hash-tag (#) indicates a response significantly lower ($P < 0.05$, one-way ANOVA followed by the Bonferroni test) than that induced by SC-236 (70 μg). Results are expressed as the mean ± SEM of five rats per group.

A submaximal dose of the COX-1 (10 μg) or COX-2 (100 μg) inhibitor was used in subsequent experiments.

To confirm that activation of COX-1 and COX-2 in the DRG is important for the development of inflammatory hyperalgesia induced by an inflammatory agent administered in the peripheral tissue, valeryl salicylate (10 μg/3 μL) or SC-236 (100 μg/3 μL) was administered into the L5-DRG 30 min before the administration of carrageenan (100 μg) in the rat's hindpaw. Both valeryl salicylate and SC-236 significantly reduced the hyperalgesia induced by carrageenan (Fig. 2).

Rats were then pretreated with ganglionar injections of oligodeoxynucleotide (ODN) antisense (AS) against COX-1 or COX-2, and control animals were treated with a ODN-mismatch or saline. Ganglionar treatment with ODN-AS against either COX-1 (Fig. 3*A*) or COX-2 (Fig. 3*B*), but not with ODN-mismatch, significantly reduced the mechanical hyperalgesia induced by IL-1β. ODN-AS against COX-1 (Fig. 3 *C* and *E*) or COX-2 (Fig. 3 *D* and *F*), but not mismatch, inhibited protein expression, quantified by Western blot analysis of L5-DRG tissue, confirming that this approach is appropriate for selective decrease of COX-1 and COX-2 expression in L5-DRG cells. Ganglionar administration of ODN-AS against COX-1 did not reduce the COX-1 expression in the L1-T13 spinal cord segment (Fig. S1). This finding demonstrates that ganglionar administration does not reach the spinal cord, and was limited to the primary afferent neurons because secondary afferent neurons from L5-DRG are located at the L1-T13 spinal cord segment (18).

Inflammation of Peripheral Tissue Increases the Expression of COX-1 and COX-2 in DRG Cells. Local administration of IL-1β (0.5 pg) or carrageenan (100 μg) in the rat's hindpaw significantly increased the expression of COX-1 and COX-2 in L5-DRG (Fig. 4). The double-labeling immunostaining of rat L5-DRG sections detected by laser-scanning confocal microscopy demonstrated that COX-1 and COX-2 are constitutive (Fig. 4 *A* and *B*) and colocalized in L5-DRG cells (Fig. 4 *C*, *F*, and *I*). Furthermore, COX-2 is expressed on TRPV-1$^+$ cells of L5-DRG (Fig. 4*M*).

EP4 or EP1/EP2 Receptor Antagonists Administered into the L5-DRG Prevents the Hyperalgesia Induced by IL-1β Administered in the Peripheral Tissue. To verify whether PGE_2 synthesized in DRG acts on the DRG cells, AH23848 (EP4 receptor antagonist; 10 μg) or AH6809 (EP1/EP2 receptor antagonist; 7.5 ng) was administered into the L5-DRG 30 min before IL-1β (0.5 pg) in the hindpaw. AH23848 or AH6809 significantly reduced the mechanical hyperalgesia induced by IL-1β (Fig. 5). Administration of AH23848 or AH6809 alone had no effect on the mechanical nociceptive threshold (Fig. 5).

Inflammatory Hyperalgesia in Peripheral Tissue Induces PKCε Translocation That Depends on COX Activation in DRG. Local administration of carrageenan (100 μg) in the rat hindpaw significantly increased PKCε expression in L5-DRG membrane cells. This increase was blocked by administration of indomethacin (100 μg), but not its vehicle Tris (2 μL) into the L5-DRG (Fig. 6). Because inflammatory hyperalgesia involves PKCε translocation to the membrane of primary afferent neurons (16, 17), this

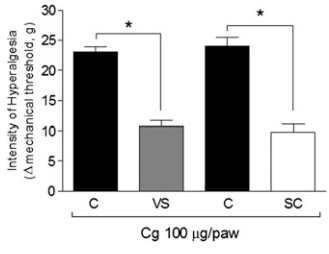

Fig. 2. COX-1 or COX-2 inhibitors administered into the L5-DRG significantly decreased hyperalgesia induced by carrageenan in the L5 peripheral field. Valeryl salicylate (VS, 10 μg) or SC-236 (SC, 100 μg) administered into the L5-DRG significantly decreased the hyperalgesia induced by carrageenan (100 μg per paw) in the L5 peripheral field. Valeryl salicylate, SC-236, or their vehicles were administered 30 min before carrageenan and the hyperalgesia was evaluated at 3 h after its administration. The asterisk (*) indicates a significant difference ($P < 0.05$, unpaired *t* test) between the groups indicated (C; 3 μL). The results are expressed as the mean ± SEM of five rats per group.

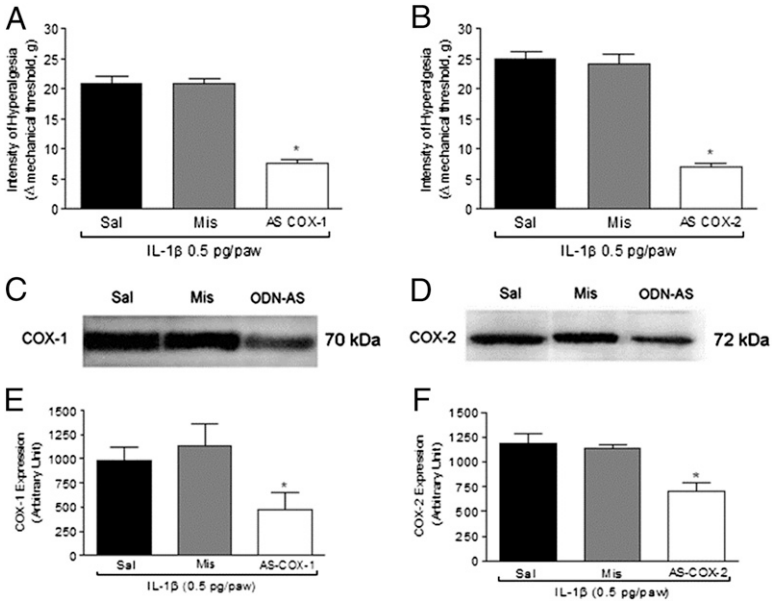

Fig. 3. COX-1 or COX-2 knock-down in the L5-DRG significantly decreased the hyperalgesia induced by IL-1β in the L5 peripheral field. Treatment (20 μg/5 μL per day, for 4 d) with ODN-AS against COX-1 (*A*) or COX-2 (*B*), but not with their respective ODN-mismatch (Mis, 20 μg/5 μL per day, for 4 d) or saline (Sal; 5 μL/d, for 4 d) administered into the L5-DRG, significantly decreased the mechanical hyperalgesia induced by IL-1β (0.5 pg per paw) administered in the L5 peripheral field. Specific ODN-AS, but not ODN-mismatch or saline significantly decreased the expression of COX-1 (*E*) or COX-2 (*F*). *C* and *D* show, respectively, a representative image of COX-1 or COX-2 knock-down induced by ODN-AS. The asterisk (*) indicates a response significantly lower than that of other groups (*A* and *B*, $P < 0.05$, one-way ANOVA followed by the Bonferroni test; and *E* and *F*, $P < 0.05$, unpaired *t* test). The results are expressed as the mean ± SEM of five rats per group.

provides further evidence that COX-1 and COX-2 activation in DRG cells is involved in the inflammatory hyperalgesia in peripheral tissue.

Discussion

In this study we provide pharmacological and molecular evidence that COX-1 and COX-2 activation in primary afferent neurons and the subsequent release of PGE_2 into the DRG is essential to the development of inflammatory hyperalgesia in peripheral tissue.

We found that administration of the nonselective COX inhibitor indomethacin, the selective COX-1 inhibitor valeryl salicylate, or the selective COX-2 inhibitor SC-236 into the L5-DRG inhibited the inflammatory hyperalgesia induced by the administration of IL-1β in L5-peripheral field. These findings indicate that both COX isoforms in DRG cells are important to inflammatory hyperalgesia. In fact, it has been demonstrated that in the primary culture of DRG cells, IL-1β increases expression of the COX-2 gene (19).

Our results showed that the magnitude of the antihyperalgesic effect induced by a COX inhibitor administered to the DRG is similar to that induced by a COX inhibitor administered systemically or intracerebroventricularly (20). In fact, unlike IL-1β, carrageenan-induced inflammatory hyperalgesia was only partially inhibited by selective COX-1 and COX-2 inhibitors. This result can be explained by the fact that only the prostaglandin component of carrageenan-induced hyperalgesia depends on COX activation in DRG cells.

We recognize that our assertion that both COX isoforms in the DRG are important to the development of inflammatory hyperalgesia in peripheral tissue hinges in part on a limited pharmacological assessment using submaximal doses of single drugs (valeryl salicylate or SC-236). We therefore supported our findings by demonstrating that the administration of ODN-AS against COX-1 and COX-2 in the L5-DRG region significantly reduced IL-1β–induced inflammatory hyperalgesia. The effectiveness of ODN antisense was demonstrated by the decrease of protein expression in L5-DRG. In agreement with our results, it has been demonstrated that daily intrathecal administration of ODN-AS accumulates in the DRG in sufficient quantity to knock down the target protein (21). Furthermore, recent data from our laboratory has demonstrated that administration of ODN-AS into the L5-DRG region significantly decreases the target-protein in L5-DRG without changing its expression in the spinal cord. In contrast, intrathecal administration of the same ODN-AS decreases the target-protein in DRG and spinal cord (22).

We have also provided molecular evidence that both COX-1 and COX-2 are constitutively expressed in DRG because both COXs isoforms were expressed in rats receiving saline in the L5-peripheral field, in agreement with the findings of Yaksh et al. (23). However, we found that the expression of COX2 was significantly higher than that of COX-1. Importantly, although the constitutive expression of COX-1 in DRG cells has been previously detected by different techniques, such as in situ hybridization (24), Northern blotting (25–27), immunohistochemistry (8, 26), and Western blotting (8, 26, 28), there is little data demonstrating the constitutive expression of COX-2 in DRG cells (23).

We have also provided molecular evidence that inflammation of peripheral tissue significantly increases the expression of both COX-1 and COX-2 in DRG cells. It has been previously demonstrated that IL-1β induces COX-2 expression with a subsequent increase in prostaglandins synthesis in different cells, including DRG cells (9). In this study, we not only confirmed these findings but have extended them by showing that the administration of IL-1β, as well as of the inflammatory agent carrageenan, in the peripheral tissue significantly increased the expression of COX-1 and COX-2. Because COX-1 and COX-2 are colocalized in the same DRG cells, our findings that COX-2 is expressed on $TRPV\text{-}1^+$ cells of L5-DRG cells suggest that the inflammation of peripheral tissue increases the expression of

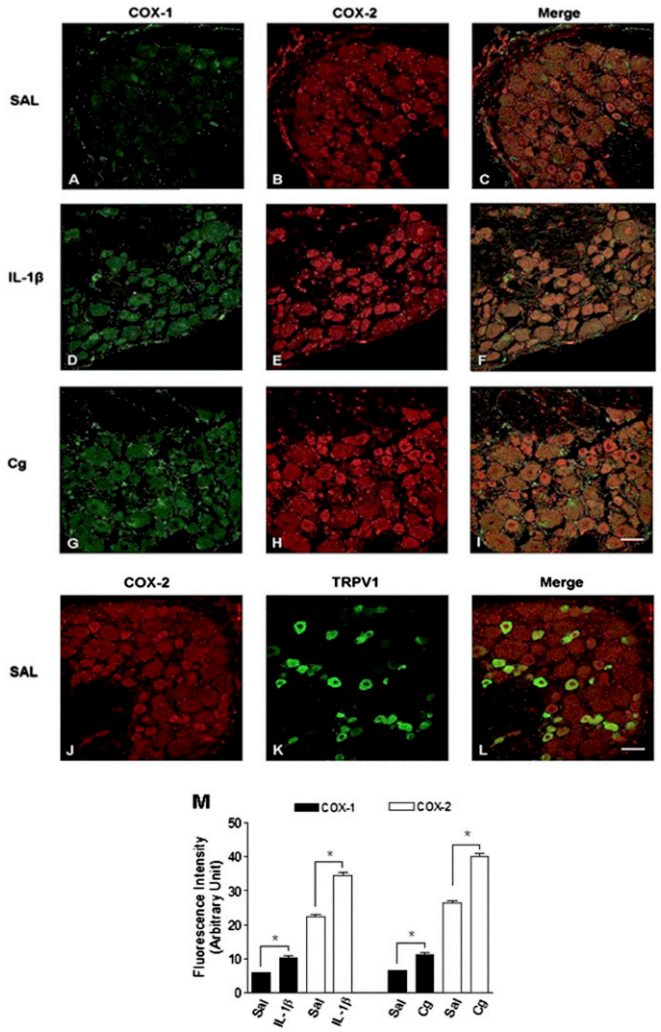

Fig. 4. IL-1β or carrageenan in peripheral tissue significantly increased the expression of COX-1 and COX-2 in TRPV-1$^+$ cells of L5-DRG. IL-1β (0.5 pg per paw) or carrageenan (Cg, 100 μg per paw), but not saline (50 μL) in L5 peripheral field significantly increased the expression of COX-1 and COX-2 in the L5-DRG (M). COX-1 and COX-2 in normal conditions (*A–C*) or induced by IL-1β (*D–F*) or carrageenan (*G–I*) are colocalized in L5-DRG. COX-2 is colocalized with the TRPV-1 receptor in the L5-DRG (*J–L*). The asterisk (*) indicates a response significantly different between the groups indicated ($P < 0.001$, unpaired *t* test). The results are expressed as the mean ± SEM of 50 cells per group. (Scale bars, 25 μm.)

COX-1 and COX-2 in peripheral afferent neurons, especially in C-fibers.

The pharmacological evidence that activation of COX-1 and COX-2 in the DRG is important to the development of inflammatory hyperalgesia in peripheral tissue was also supported by molecular evidence. Because it is now generally accepted that inflammatory hyperalgesia in the peripheral tissue depends on PKCε translocation to the membrane of primary afferent neurons (16, 17), in this study we also demonstrated that the administration of indomethacin in the DRG prevents the increase of PKCε expression in DRG cells membrane induced by carrageenan administration in the peripheral tissue. Therefore, this result indicates that the activation of PKCε pathway depends on COX activation and on the subsequent release of prostaglandin by peripheral afferent neurons. It is therefore plausible to hypothesize that, besides the release of prostaglandin in the peripheral tissue, its release into the DRG is also important to sensitize peripheral afferent neurons. This hypothesis was confirmed by the finding that administration of EP1/EP2 or EP4 receptor antagonists into the L5-DRG before the administration of IL-1β in the peripheral tissue prevented the inflammatory hyperalgesia of the peripheral tissue. Taken together, our findings suggest that prostaglandin released in the DRG activates an autocrine signaling mechanism in primary afferent neurons to sensitize them. Indeed, prostanoid receptors are present either in the spinal cord or in the DRG (29) and intrathecal administration of the EP receptor agonists induced hyperalgesia (30). However, it is important to point out that the total volume of injection of COX-inhibitors or EP antagonists into the L5-DRG was 2 μL. This volume is smaller than that originally described to inject drugs into the L5-DRG (31). Therefore, it is unlikely that the administration of COX-inhibitors or EP antagonists into the L5-DRG reached secondary afferent neurons in the spinal cord.

Although, it is generally assumed that intrathecally administered drugs act in the spinal cord (11–13), they can also act in the DRG cells (13). In fact, it has been demonstrated that molecules leak from the subarachnoidal space to the DRG (32), which is consistent with the demonstration that intrathecal administration of PGE_2 induces hyperalgesia, at least in part, by acting on DRG cells, instead of by a retrograde action in central terminals of the primary afferent neuron, as previously stated (6, 14). Although this study indicates the importance of COX activation in C-fibers to the development of inflammatory hyperalgesia, it does not exclude the involvement of other cells of the DRG in this process. It is timely to comment that, although the primary afferent neuron extends from the peripheral tissues to the central nervous system, both COX-1 and COX-2 are more abundantly found around the cell nucleus, attributing to the DRG a special role in the development of inflammatory hyperalgesia of the peripheral tissue.

In summary, the results of this study demonstrate that activation of COX-1 and COX-2 in the DRG is essential to the development of inflammatory hyperalgesia in the peripheral tissue. These finding emerges as a new line of studies involving the role of DRG cells in the sensitization of primary afferent neurons and opens new perspectives for the development of safer drugs to control inflammatory pain.

Materials and Methods

Animals. Experiments were performed with male Wistar rats (180–200 g), obtained from the Multidisciplinary Center for Biological Investigation at the State University of Campinas (UNICAMP) and kept in a 12-h light/dark cycle, with controlled humidity (60–80%) and temperature (22–25 °C). Food and water were available ad libitum. Rats were taken to the testing area at least 1 h before testing. Each experiment used five rats per group. All behavioral testing was performed between 9:00 AM and 4:00 PM. Animal

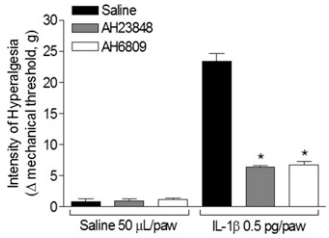

Fig. 5. EP4 or EP1/EP2 receptor antagonists administered into the L5-DRG prevented the hyperalgesia induced by IL-1β in the L5-peripheral field. The administration of AH23848 (EP4 receptor antagonist; 10 μg) or AH6809 (EP1/EP2 antagonist; 7.5 ng) into the L5-DRG prevented the mechanical hyperalgesia induced by IL-1β (0.5 pg/paw) administered in the L5-peripheral field. The asterisk (*) indicates a response significantly lower than that of rats treated with IL-1β (0.5 pg per paw) and with saline in the L5-DRG ($P < 0.001$, one-way ANOVA followed by the Bonferroni test). The results are expressed as mean ± SEM of five animals per group.

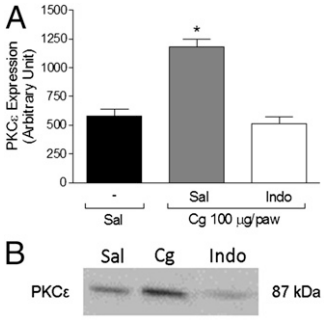

Fig. 6. The administration of indomethacin into the L5-DRG blocked the PKCε translocation induced by carrageenan in the L5-peripheral field. The administration of carrageenan (Cg, 100 μg per paw) in the L5 peripheral field significantly increased PKCε expression in the membrane of L5-DRG cells. This increase was blocked by the administration of indomethacin (100 μg/3 μL) into the L5-DRG (*A*). (*B*) A representative figure of carrageenan-induced PKCε expression in the membrane of L5-DRG cells and its decrease by indomethacin. The asterisk (*) indicates a response significantly greater ($P < 0.05$, unpaired t test) than that of other groups. The results are expressed as the mean ± SEM of five rats per group.

care and handling procedures were in accordance with International Association for the Study of Pain guidelines for the use of animals in pain research and with the approved by the institutional Committee for Ethics in Animal Experimentation at UNICAMP (CEUA/IB-UNICAMP), São Paulo, Brazil. All efforts were made to minimize discomfort for the animals.

Drugs. The agents used in this study were obtained as follows: PGE_2, AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), AH23848 ((4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-Biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid hemicalcium salt hydrate) and Complete Freund's Adjuvant from Sigma; Valeryl Salicylate, from Cayman; indomethacin from Prodrome; SC-236 ((4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-Biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3 oxocyclopentyl]-4-heptenoic acid hemicalcium salt hydrate) from Axxora; IL-1β from the National Institute for Biological Standards and Control (United Kingdom); carrageenan (Cg) from FMC corporation; and 2% Lidocaine chloride, from Cristalia. The specific activity of IL-1β was 100,000 IU μg^{-1} ampoule^{-1}. AH6809 and AH23848 were diluted in 0.9% NaCl (saline) and 2% dimethyl sulfoxide (Sigma). PGE_2 was dissolved in saline and 1% ethanol (Merck). Carrageenan and IL-1β were diluted in saline. Indomethacin was diluted in Tris•HCl buffer (pH 8.0). SC-236 and valeryl salicylate were dissolved in 2% Tween 80 and resuspended in saline.

Drug Administration. ***Administration in L5-peripheral field of rat hindpaw.*** A hypodermic 26-gauge needle was inserted in the subcutaneous tissue of the plantar surface of rat's hindpaw, which is responsively to L5-DRG area, L5 peripheral field. At the same place where mechanical stimulus was applied, the drugs were administered in a volume of 50 μL (33). The L5 peripheral field was mapped by testing the mechanical nociceptive threshold in different points of plantar hindpaw after injection of 2% lidocaine (3 μL) in L5-DRG (Fig. S2).

Ganglionar administration. In this study the L5-DRG injection technique originally described by Ferrari et al. (31) was minimally modified. Briefly, after shaving the fur over the lower back, rats were put under light halothane anesthesia (1– 2%) and placed over a small cylinder to elevate the lumbar region. The peri-ganglionar injection (L5-DRG) was performed using a 30-cm PE-10 catheter (Intramedic; Clay Adams; internal diameter 0.28 mm and external diameter 0.61 mm), calibrated in such way that 25 mm of the catheter corresponded to an injected volume of 1 μL. The injection site was 1.5 cm laterally to the vertebral column, and ~0.5 cm caudal from a virtual line passing over the rostral borders of the iliac crests. Delicate movements of the needle were made until the bone resistance was diminished and a paw-flinch reflex was observed. The paw-flinch reflex was used as a sign that the needle tip penetrated the L5-DRG distal nerve insertion of the fifth lumbar spinal nerve located underneath the transversal process of the fifth lumbar vertebra (Fig. S3). After the needle reached the ganglion, 3 μL of solution was injected. ODN-AS or their mismatched controls were injected in the ganglionar region of L5-DRG in a volume of 5 μL. Ganglionar L5 administration is restricted to L5-DRG and it did not reach the spinal cord L1-T13 segments (Fig. S4).

Evaluation of Mechanical Hyperalgesia. The mechanical nociceptive threshold was measured by the electronic von Frey method as previously described (34). In a quiet room, rats were placed in acrylic cages (12 × 20 × 17 cm) with wire grid floors, 15–30 min before the start of testing. During this adaptation period, the paws were tested (probed) three times. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer adapted with a 0.7-mm^2 polypropylene tip (electronic von Frey hair; IITC Life Science). A tilted mirror placed under the grid provided a clear view of the rat hindpaw. The investigator was trained to apply the tip in between the five distal footpads with a gradual increase in pressure. The stimulus was automatically discontinued, and its intensity was recorded when the paw was withdrawn. The maximal force applied was 80 *g*. The endpoint was characterized by the removal of the paw in a clear flinch response after the paw withdrawal. The animals were tested before and after treatments. A different investigator performed each test, prepared the solution, and administered the intraplantar and ganglionar injections. The results are expressed by the Δ mechanical threshold (grams) that was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before treatments.

ODN-AS. The functional blockade of COX-1 and COX-2 enzymes expression on peripheral sensory neurons was realized by the ganglionar injection of ODN-AS. The following COX-1 and COX-2 ODN-AS sequence of 19-mer was used: 5′-TCC ATG GCG ATG CGG TTG C-3′ and 5′-GAA CAG TCG CTC GTC ATC C-3′, respectively. The mismatch-ODN sequence to COX-1, 5′-TC**T** ATG **C**CG **T**TG **AGC** T**T**C C-3′ and COX-2, 5′- GA**T** CAG **C**CG **T**TC **ATG** AT**G** C-3′, corresponded to the antisense sequence except that six bases were changed (denoted by boldface type). The corresponding GenBank accession number and ODN position within the cDNA sequence to COX-1 is U04300 (927-945) and to COX-2 is S67721 (1179–1197). A search of the National Center for Biotechnology Information database to *Rattus norvegicus* identified no other sequences homologous to that used in this experiment. The ODN was purchased from Erviegas, lyophilized, and reconstituted in 0.9% NaCl. The ODN was aliquoted and stored at –20 °C. Rats received a ganglionar (L5) injection of either antisense one time a day (20 μg/5 μL per day), saline (5 μL/d) or mismatch (20 μg/5 μL per day) for 4 d. On the day after the ODN treatments, the mechanical hyperalgesia induced by IL-1β (0.5 pg/50 μL per paw) was evaluated (von Frey Electronic) followed by the removal of DRG (L5) of the ipsilateral side of IL-1β injection. COX-1 and COX-2 expressions in DRG-L5 from antisense, saline, and mismatch were evaluated using Western blot analyses.

Western Blot Analyses. For the analysis PKCε translocation to a membrane-enriched fraction, the protocol described by Young et al. (35), was used with minor modifications. Immediately after the behavioral test, the DRG-L5 of anesthetized rats were removed and separated in samples, to allow detectable levels of protein. Samples were homogenized with an ultrasonic homogenizer (Sonic), in a buffer containing 50 mM phosphate buffer, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1% complete protease inhibitor mixture (Sigma; P8340), at 4 °C. The homogenate was centrifuged at 100 × *g* for 5 min, at 4 °C. The resulting supernatant was transferred to a new tube and was centrifuged at 20,000 × *g* for 30 min, at 4 °C. The supernatant was removed, and the pellet, that consisted in a membrane-enriched fraction, was resuspended in a buffer containing 1% Triton X-100, 50 mM phosphate buffer, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1% complete protease inhibitor mixture (Sigma; P8340), at 4 °C. After 20 min incubation at 4 °C, the membrane enriched fraction was centrifuged at 12,000 × *g* for 15 min at 4 °C, and the resulting supernatant was transferred to a new tube. Protein concentration was determined by the Bradford method, and 35 μg of total proteins from each sample were separated by SDS/PAGE and transferred to nitrocellulose membranes according to standard techniques.

For the analysis of COX-1 and COX-2 expression, total protein, samples were homogenized in 1% Triton X-100, 50 mM phosphate buffer pH 7.4, 5 mM EDTA, 1% protease inhibitor mixture (P8340; Sigma), 7 M urea, and 2 M thiourea, [10% (wt/vol)]. Sample homogenization was carried out at 4 °C using a Ultrasonic Homogenizer for 5 s. Insoluble materials were removed by centrifugation (12,000 × *g*, 4 °C, 15 min). Protein concentration was determined by the Bradford method, and 70 μg of total proteins from each sample were separated by SDS/PAGE and transferred to nitrocellulose membranes, according to standard techniques.

All membranes were stained with Ponceau S, and digital images were acquired for the control of protein loading by densitometric analysis (36, 37). Membrane was blocked in PBS-Tween containing 5% nonfat dry milk at room temperature, followed by incubation with COX-1 goat polyclonal (1:500; Santa Cruz), COX-2 mouse polyclonal (1:1,000; Cayman), and PKCε rabbit polyclonal antibody (1:1,000; Santa Cruz) overnight at 4 °C, rinsed six

times with TBST, and then incubated for 40 min in donkey anti-goat IgG peroxidase conjugate (1:20,000; Jackson ImmunoResearch), donkey anti-mouse IgG peroxidase conjugate (1:20,000; Jackson ImmunoResearch), and goat anti-rabbit IgG peroxidase conjugate (1:10,000; Zymed), respectively. Reactive bands were detected with the Super Signal West Pico chemiluminescent kit. Results are expressed as the ratio between PKCε and the corresponding Ponceau S optical densities. These data were analyzed by unpaired *t* test.

DRG Immunohistochemistry. Animals were terminally anesthetized with urethane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, DRG-L5 were removed and postfixed in the same fixative for 2 h, which was then replaced overnight with 30% sucrose. All of the DRG were embedded in optimum cutting temperature, and DRG sections (12 μm) were cut in a cryostat and processed for immunofluorescence. All of the sections were blocked with 2% BSA in 0.3% Triton X-100 for 1 h at room temperature and incubated for 2 h at 4 °C with a mixture of polyclonal goat anti-COX-1 (1:200; Santa Cruz) and polyclonal mouse anti-COX-2 (1:200; Cayman), or a mixture of polyclonal goat anti–TRPV-1 (1:500; Santa Cruz) and polyclonal mouse anti-COX-2 (1:200; Cayman) antibodies. Finally, sections were incubated with a mixture of Alexa Fluor-488 and Alexa Fluor-594 conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature. Sections were examined using a confocal laser-scanning microscope (Leica SP5). The specificity for antibodies was confirmed by loss of staining in the absence of primary antibodies and single bands in Western blotting.

Statistical Analysis. To determine if there were significant differences ($P < 0.05$) between treatment groups, one-way ANOVA or unpaired *t* test was performed. The statistical analysis of the results obtained was performed in the GraphPad Prism v4.00 for Windows (GraphPad Software). The results were presented as the mean ± SEM of five rats per group. The differences between the groups were compared using one-way ANOVA to obtain the degree of significance, followed by the Bonferroni multiple comparison test to compare the groups and doses (behavioral experiments). The results from Western blot and immunohistochemistry assays were expressed in arbitrary units.

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