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Molecules and Cells

Lipoxygenase Inhibitors Suppressed Carrageenan-Induced Fos-Expression and Inflammatory Pain Responses in the Rat

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Lipoxygenase (LO) metabolites are generated in inflamed tissues. However, it is unclear whether the inhibition of the LO activity regulates the expression of c-Fos protein, a pain marker in the spinal cord. Here we used a carrageenan-induced inflammation model to examine the role of LO in the development of c-Fos expression. Intradermally injected carrageenan caused elevated number of cells exhibiting Fos-like immunoreactivity (Fos-LI) in the spinal dorsal horn, and decreased the thermal and mechanical threshold in Hargreaves and von Frey tests. Pretreatment with an inhibitor of phospholipase A2, that generates the LO substrate, prior to the carrageenan injection significantly reduced the number of Fos-(+) cells. A general LO inhibitor NDGA, a 5-LO inhibitor AA-861 and a 12-LO inhibitor baicalein also exhibited the similar effects. Moreover, the LO inhibitors suppressed carrageenan-induced thermal and mechanical hyperalgesic behaviors, which inidcates that the changes in Fos expression correlates with those in the nociceptive behaviors in the inflamed rats. LO products are endogenous TRPV1 activators and pretreatment with BCTC, a TRPV1 antagonist inhibited the thermal but not the mechanical hypersensitivity. Overall, our results from the Fos-LI and behavior tests suggest that LO products released from inflamed tissues contribute to nociception during carrageenan-induced inflammation, indicating that the LO pathway is a possible target for modulating inflammatory pain.

INTRODUCTION

During inflammation, mediators like growth factors, kinins, lipid metabolites and other endogenous chemicals are released from tissues and cause variety of inflammatory reaction and pain (Vane and Botting, 1987). Of the various inflammatory mediators, arachidonic acid and its metabolites have been intensively studied for decades in this respect. Prostaglandins (PGs), the products of cyclooxygenase (COX) have attracted more attention than other arachidonic acid derivatives because they are known to play a potent proinflammatory role and to have hyperalgesic effects (Vane et al., 1988). It is now known that another arachidonic acid metabolizing pathway through lipoxygenase (LO) around inflamed tissues produces hydroxyeicosanoids and leukotrienes, which positively contribute to exacerbation of inflammation (Funk, 2001). Pain sensitivity was also heightened, as with PGs, when products of LO like leukotrienes were challenged in animal tissues (Levine et al., 1984; 1986). In some animal models of inflammatory hyperalgesia, LO inhibitors effectively reversed elevated pain threshold (Amann et al., 1996; Shin et al., 2002). Furthermore, LO metabolites are able to activate TRPV1, a noxious heat-activated ion channel mainly expressed in sensory C-fibers (Hwang et al., 2000). TRPV1 is not only a thermosensitive ion channel, but a promiscuous detector for extremely diverse sources of pain such as acidic pH, ethanol, cannabinoids, N-arachidonoyl dopamine and natural pungent chemicals like capsaicin (Caterina et al., 1997; Huang et al., 2002; Tominaga et al., 1998; Zygmunt et al., 1999). Moreover, the potent inflammatory mediators, bradykinin, nerve growth factor and histamine use TRPV1 as a downstream effector on their intracellular signaling to evoke pain (Chuang et al., 2001; Shin et al., 2002; 2007).

Previous studies have shown that TRPV1 mediates carrageenan-induced inflammatory pain (Jhaveri et al., 2005; Kwak et al., 1998). The former study suggested that an endogenous TRPV1 activating substance is released and sensitizes thermal pain reflexes during carrageenan inflammation. C-fos protein is an important pain marker (Honore et al., 1995; Hunt et al., 1987; Menétrey et al., 1989) and Kwak et al. (1998) have also shown that the induction of the spinal c-fos expression was evoked by the TRPV1 activating substance. Since LO products are not only inflammatory mediators but also pain inducers through TRPV1 activation, and because the roles of LO products have not been investigated with respect to Fos expression in carrageenan-inflamed animal models, we asked whether LO inhibition can negatively modulate carrageenan-evoked increases in Fos-expression. We also tested if correlated changes occurred by LO inhibition in heat and mechanical hyperalgesic behavior under carrageenan inflammation.

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MATERIALS AND METHODS

Inflammation

Inflammation was induced in male Sprague-Dawley rats weighing 250-350 g by intradermal injection of 6 mg carrageenan (λ-carrageenan) dissolved in 150 μl of saline into a hind paw foot pad. In some animals phospholipase A2 (PLA2), LO, COX or TRPV1 inhibitors were administered into the left foot and vehicle (150 μl saline containing 10% ethanol and 10% Tween 80) for the inhibitors was injected in the right foot just prior to carrageenan administration. In other experiments, quinacrine, a PLA2 inhibitor was subcutaneously administered (100 mg/kg) to animals with an inflamed left hind paw induced by carrageenan. λ-Carrageenan, quinacrine, indomethacin, and nordihydroguaiaretic acid were purchased from Sigma-Aldrich and other lipoxygenase inhibitors and BCTC were from Biomol.

Immunohistochemistry

C-Fos immunohistochemistry in spinal cord sections from inflamed animals treated with carrageenan has been previously described (Kwak et al., 1998). Briefly, 3 h post carrageenan injection, animals were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and cardiac perfusion was performed using ~300 ml of 0.05 M phosphate-buffered saline (PBS, pH 7.4) followed by 300 ml of 0.01 M PBS containing 4% paraformaldehyde. Lumbosacral regions of the animals' spinal cords (stored in 4% paraformaldehyde solution overnight at 4°C) were sectioned at 40 μm thickness using a vibratome (TPI, USA). Every fourth sections from the lumbar enlargement were collected in PBS and immunostained for Fos using previously described avidin-biotin-peroxidase method (Kwak et al., 1998). Sections were incubated for 1 h in PBS containing 3% normal rabbit serum and 0.3% Triton X-100 and then for 48 h at 4°C in PBS containing a sheep polyclonal antibody raised against c-Fos protein (pan-Fos antibody, Cambridge Research Biomedicals, UK; 1:4000 dilution). Sections were then washed and incubated in biotinylated rabbit anti-sheep IgG for 1 h at room temperature, rewashed and reincubated for 1 h in avidin-biotin-peroxidase complex (Vectastain, Vector Labs, USA), and washed again with 0.05 M PBS. Sections were then stained with 0.045% diaminobenzidine (Sigma-Aldrich) and 0.07% H_2O_2. Next, the sections were mounted on gelatin-coated slides, dried in air, dehydrated in xylene, and placed under a coverslip. Numbers of Fos-positive cells were counted regardless of the intensity of the staining using a bright-field microscope fitted with a camera lucida drawing tube. An average number from each animal was generateds in atusing at least 10 sections.

Tests of nociceptive behavior

The Hargreaves and von Frey test methods were used to examine the effect of the drugs that inhibit PLA2, LO, COX, or TRPV1. Sprague-Dawley rats weighing between 250-350 g were used for all behavioral assays. Hargreaves (Plantar Analgesia meter, for thermal hyperalgesia) and von Frey apparatus (Dynamic Plantar Aesthesiometer, for mechanical allodynia) were from UGO Basile (Italy). Assays for changes in mechanical or thermal behaviors induced by carrageenan were performed as described previously (Moqrich et al., 2005; Na et al., 2008). Briefly, animals were acclimated for ~60 min to their testing environment prior to conducting experiments. Baseline responses were then measured 5 min prior to inhibitor administration and then inhibitors (in 50 μl saline) were injected into the skin of left hind paws just prior to intradermal carrageenan injection (6 mg/100 μl) into both hind paws. Paw withdrawal latencies or von Frey thresholds were recorded at 3 h post carrageenan injection. Latencies or thresholds of the contralateral hind paws were also measured both before and after drug-carrageenan injection. The mean withdrawal latencies (or mean von Frey thresholds) are expressed as the percentage difference between values recorded before and after drug treatments using the following formula:

Withdrawal latency (%) = (Latency after drug injection - latency before drug injection) × 100/Latency before drug injection

Statistical analysis

Comparisons between pairs of means were performed using the paired Student's *t*-test and *P*-values less than 0.05 were considered significant. All values are expressed as means ± SEMs.

RESULTS

Effect of quinacrine on carrageenan-evoked Fos-LI increases

In order to test the effect of inhibitors of the LO pathway on nociceptive neuronal input to the spinal cord, Fos-like immunoreactivity (Fos-LI) was determined in the spinal cord. Intraplantar injection of carrageenan into the left hind paws resulted in a marked increase in the number of neurons expressing Fos-like immunoreactivity in the ipsilateral (relevant to the carrageenan-injected hind paw) dorsal horn in the lumbar spinal cord (Figs. 1A-1C). Ipsilateral portions of the spinal cord sections possessed 71 ± 4 cells/section expressing Fos-LI on average whereas the contralateral sections, corresponding to saline administration, showed 11 ± 3 cells/section (n = 5). Consistent with the previous study (Kwak et al., 1998), neurons densely expressing Fos-LI were clustered in the superficial layer as shown in Fig. 1B. Of the total Fos-(+)-neurons in ipsilateral regions, 56% were found in the superficial lamiae I and II, and 44% in the deep laminae (n = 5).

PLA2, an enzyme upstream of LO, supplies arachidonic acid as an LO substrate on the inflammatory signaling. Thus, we first examined the effect of PLA2 inhibition on the Fos-LI before testing for the effect of LO inhibition. The systemic administration of quinacrine, a PLA2 inhibitor (100 mg/kg s.c.) suppressed the development of ipsilateral Fos-LI induced by intraplantar injection of carrageenan in the left hind paw (44 ± 9, $p < 0.05$ and n = 4, Figs. 1A, 1B, and 1D). Contralateral Fos-LI was not affected by quinacrine injection (10 ± 5, $p = 0.83$). Pretreatment with intradermal quinacrine on the same spot where carrageenan was injected, was also effective at significantly reducing an increase of number of Fos-(+)-cells under the carrageenan inflammation (58 ± 15, n = 5, 37 ± 1% reduction, Figs. 1C and 1D) compared to vehicle pretreatment followed by carrageenan (89 ± 16, $p < 0.005$).

Effect of lipoxygenase inhibitors on carrageenan-evoked Fos-LI increases

We then asked whether the action of LO is also important for Fos-LI development by carrageenan-induced hind paw inflammation. Nordihydroguaiaretic acid (NDGA; a nonspecific inhibitor of 5-, 12- and 15-LO) significantly reduced the increase in number of the Fos-(+)-cells when intradermally administered into a hind paw immediately prior to carrageenan injection into both hind paws (50.1 ± 8.7% reduction, n = 5, Figs. 2A and 2B). However, its vehicle injection did not affect the carrageenan-induced increase of Fos-LI (Figs. 2A and 2B). NDGA was tested at several doses and was found to attenuate Fos-LI increase dose-dependently (Figs. 2B and 2C). Thus, these results indicate that the LO pathway is involved in the Fos-LI

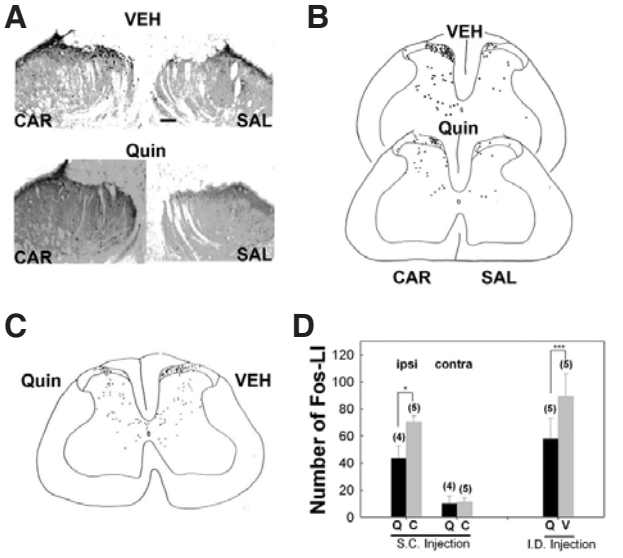

Fig. 1. A phospholipase A2 inhibitor quinacrine suppressed carrageenan-induced increase of the number of cells with Fos-LI. (A) A typical example of photomicrographs that illustrate Fos-LI in the dorsal horn of the lumbar spinal cord of rats in which carrageenan was injected (6 mg/150 μl) intradermally into the left hind paw. Quinacrine (upper, 100 mg/kg s.c.) or vehicle (lower) was administered. Scale bar = 0.1 mm. (B) Camera Lucida drawings illustrating the distribution of cells with Fos-LI 3 h after the carrageenan injection. Quinacrine (upper, 100 mg/kg s.c.) or vehicle (lower) was administered. (C) Camera Lucida drawings illustrating the distribution of cells with Fos-LI 3 h after intradermal injection of carrageenan (6 mg/150 μl) in both hind paws. In the left hind paw, quinacrine (4.5 mg/150 μl) was intradermally injected prior to carrageenan injection while vehicle injected in the right hind paw. (D) Summary of the effect of quinacrine treatment on the carrageenan-induced increase of number of Fos-(+) cells (*P < 0.05, **P < 0.01, **P < 0.005). Numbers of each case are parenthesized on the relevant bars. Each data point represents the mean ± SEM. Abbreviations: CAR, carrageenan; SAL, saline; Quin, quinacrine; VEH, vehicle SC, subcutaneous injection; ID, intradermal injection.

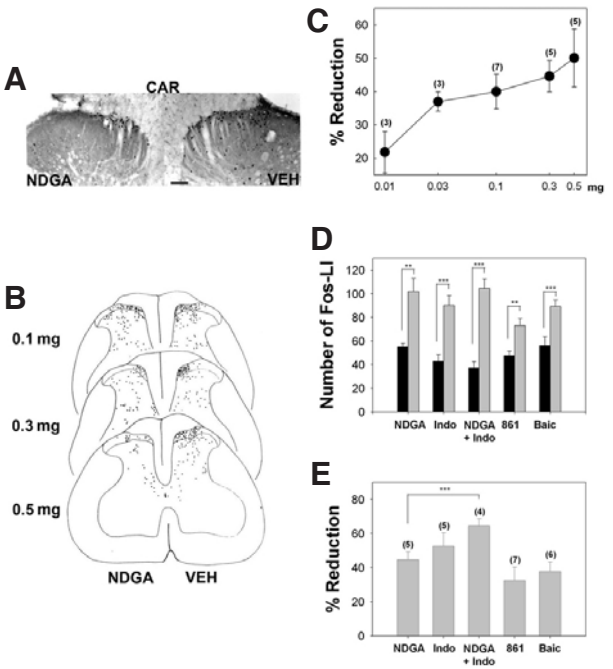

Fig. 2. Lipoxygenase (LO) inhibitors suppressed carrageenan-induced increase of the number of cells with Fos-LI. (A) A representative example of photomicrograph that illustrates a attenuating effect of NDGA, a nonspecific LO inhibitor on a carrageenan-induced increase of the number of Fos-(+) cells. The Fos-LI was observed 3 h after intradermal injection of carrageenan (6 mg/150 μl) in both hind paws. In the left hind paw, NDGA (0.5 mg/150 μl) was intradermally injected prior to carrageenan injection and in the right hind paw, the vehicle for NDGA was injected. Scale bar = 0.1 mm. (B-C) NDGA suppressed the carrageenan-induced Fos-LI increase in a dose-dependent manner. (B) Examples of Camera Lucida drawings illustrating the distribution of cells with Fos-LI 3 h after the carrageenan injection into both hind paws. NDGA (0.1, 0.3 or 0.5 mg/150 μl upper to lower drawings) into the left hind paw and vehicle into the right hind paw were intradermally administered respectively. (C) Summary of the effect of NDGA pretreatment at each dose on the carrageenan-induced increase of number of cells exhibiting Fos-LI. Values were obtained by averaging percent reductions of cell numbers with Fos-LI upon NDGA treatment compared to the vehicle effect at each case. (D) Summary of the effect of treatment with NDGA (0.3 mg) alone, indomethacin (0.3 mg) alone, NDGA (0.3 mg) plus indomethacin (0.3 mg), AA-861 (0.5 mg) or baicalein (0.03 mg) on the carrageenan-induced increase of number of Fos-(+) cells (*P < 0.05, **P < 0.01, ***P < 0.005). Black bars and gray bars represent results of drug-treatment and vehicle-treatment, respectively. Each sample size is parenthesized on the relevant bars. (E) Percent reductions of Fos-(+) cell numbers upon each individual drug treatment compared to its vehicle effect at each case from (D) were averaged. The co-administration of NDGA plus indomethacin elicited a significantly greater reduction in increases of number of cells with Fos-LI than that of NDGA alone under carrageenan inflammation (**P < 0.05). *Abbreviations*: Indo, indomethacin; 861, AA-861; Baic, baicalein.

development during carrageenan-inflammation. Indomethacin, a COX inhibitor, also suppresses Fos-LI increase induced by carrageenin-inflammation (Honore et al., 1995). Similar results were obtained with our intradermal indomethacin pretreatment (50.8 ± 8.9%, n = 5, Figs. 2D and 2E). We compared the effects of NDGA alone, indomethacin alone and indomethacin plus NDGA on the Fos-LI development. The mean percent (%) reduction of Fos-LI by concomitant intradermal administration of NDGA and indomethacin (64.4 ± 4.1%, n = 4, Figs. 2D and 2E) was larger than that of NDGA alone (44.7 ± 4.7%, n = 5, by 19.7% point) and also than that of the indomethacin alone (by 13.6% point), but only between NDGA plus indomethacin and NDGA alone, the significant difference was detected ($p < 0.005$). As well, Fos-LI suppression by indomethacin alone was larger than that of NDGA alone (by 6.1%) but those are not significantly different ($p = 0.51$). These data suggests that the effects of COX inhibition and LO inhibition were comparable, but that it is unclear whether those effects can be additive on the Fos-LI induction during the carrageenan-induced inflammation.

In addition, we tested whether or not LO subtypes are involved in this Fos-LI upregulation by carrageenan inflammation using specific 5-LO or 12-LO inhibitors. Intradermal pretreatment with AA-861 (a specific 5-LO inhibitor) significantly reduced carrageenan-induced increase in the number of cells exhibiting Fos-LI (32.4 ± 7.9%, n = 7, Figs. 2D and 2E). Baicalein is a specific 12-LO inhibitor and it also suppressed Fos-LI development (37.7 ± 5.6%, n = 6, Figs. 2D and 2E) when in-

jected at 0.03 mg in the same way to that of AA-861. These results indicate that products from LO subtypes contribute to the inflammatory increase of the Fos-LI induced by the local carrageenan injection.

Effect of lipoxygenase inhibitors on carrageenan-evoked behavioral changes

Fos protein is an important nociceptive marker in the spinal cord dorsal horn (Coggeshall, 2005; Harris, 1998). Thus, the suppression of carrageenan-induced Fos-LI development by LO inhibition, would reflect relief of nociceptive responses *in vivo* induced by carrageenan inflammation. We examined this possibility of a correlation using the Hargreaves and von Frey methods to assess thermal and mechanical hypersensitivity behaviors in adult rats upon LO inhibition. Carrageenan-inflamed hind paws showed significantly reduced withdrawal latency upon plantar heat stimulation (4.4 ± 0.4 s) compared to the latency measured before the carrageenan treatment or that of saline-treated hind paws (9.8 ± 0.3 s and 8.5 ± 0.6 s respectively, Fig. 3A left), This suggested that carrageenan inflammation produces thermal hyperalgesia. Mechanical allodynia was also developed in the same way of carrageenan injection (control 28.7 ± 1.5 g, carrageenan 12.3 ± 0.7 g and saline 24.2 ± 1.5 g in von Frey thresholds, Fig. 3A right). Pretreatment with a nonspecific LO inhibitor NDGA significantly attenuated carrageenan-induced thermal hypersensitivity (2.3 ± 11.1% versus 31.4 ± 5.9% {control} in reduction of latency, Fig. 3B). In addition, pretreatment of AA-861 (a 5-LO inhibitor) or baicalein (a 12-LO inhibitor) also inhibited the shortening of paw-withdrawal latency upon the heat stimulation with carrageenan-induced hind paw inflammation (9.0 ± 11.5% *versus* 65.8 ± 6.4% and 43.0 ± 9.8% *versus* 64.3 ± 4.6% respectively, Fig. 3B). The analgesic effects of these LO inhibitors were comparable to that of indomethacin alone (11.4 ± 12.7% *versus* 38.1 ± 9.4%) and to that of NDGA plus indomethacin (6.7 ± 5.6% *versus* 38.3 ± 8.7%, Fig. 3B).

Pretreatment with NDGA also significantly reduced carrageenan-induced mechanical allodynia (4.0 ± 15.6%, *versus* 47.1 ± 11.6% {control} in threshold reduction, Fig. 3C). The above mentioned LO subtype inhibitors were also effective at preventing mechanical allodynia. AA-861 and baicalein exhibited reversing effects on the decreased von Frey threshold induced by carrageenan inflammation (26.6 ± 11.5%, *versus* 54.6 ± 8.2% and 25.5 ± 17.0%, *versus* 56.5 ± 5.0% respectively, Fig. 3C). Indomethacin alone (29.8 ± 9.1%, *versus* 56.6 ± 9.2%) and indomethacin plus NDGA (109.7 ± 50.1% increase of threshold *versus* 36.0 ± 12.3% decrease of threshold) also significantly reversed the paw withdrawal latency upon von Frey mechanical stimulation (Fig. 3C). These results indicate that both thermal and mechanical hyperalgesic behaviors are closely related to the action of lipoxygnases during carrageenan inflammation as well as to the cyclooxygenase pathway. Since TRPV1 is an important component in carrageenan-induced thermal hyperalgesia and LO products are endogenous TRPV1 activators (Kwak et al., 1998), we tested BCTC, a specific TRPV1 antagonist for the elevated thermal and mechanical pain sensitivity induced by carrageenan inflammation. As shown in Fig. 3B, intradermally injected BCTC (0.05 mg/50 μl) reversed paw-withdrawal latency upon thermal stimulation but it did not affect the mechanical hypersensitivity even in doses up to 0.15 mg.

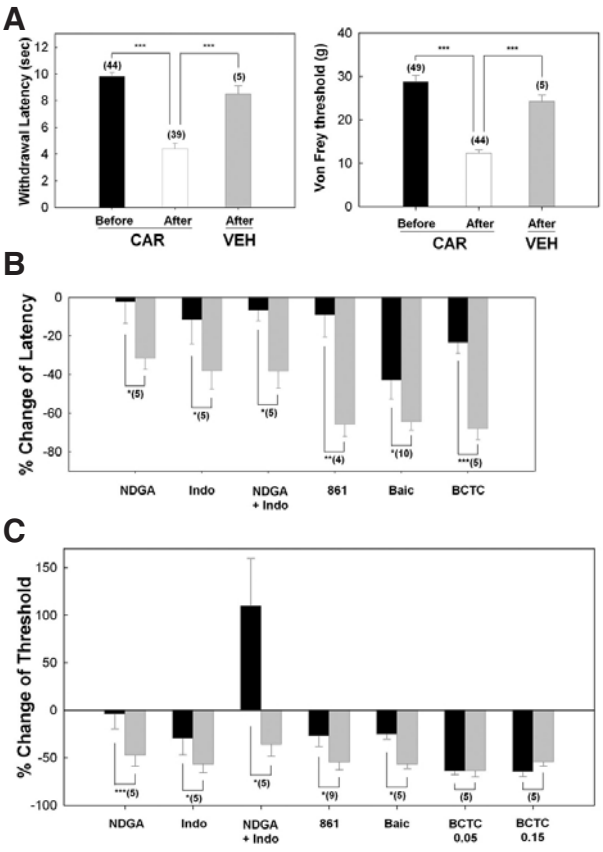

Fig. 3. Lipoxygenase inhibitors or a TRPV1 antagonist blocked carrageenan-induced thermal and mechanical hypersensitivity. (A) Carrageenan-injected hind paw demonstrated a significant reduction in heat withdrawal latency (left) and decreased mechanical threshold (right). Filled bars: latencies or thresholds measured immediately before the intradermal carrageenan injection. Open bars: latencies or thresholds measured 3 h after the intradermal carrageenan injection. Gray bars: latencies or thresholds measured 3 h after the intradermal saline (100 μl) injection. (B) Results of percentage changes in paw-withdrawal latency from Hargreaves tests with rats upon intradermal injection of LO inhibitors prior to carrageenan injection (6 mg/150 μl). The TRPV1 antagonist BCTC was also tested using the same procedure to that for LO inhibitors. Data are expressed as percentage changes (see "Materials and Methods" for the calculating formula) from control values recorded before drug administration in each case (*P < 0.05, **P < 0.01, ***P < 0.005). Black bars represent averaged percentage changes from control latency values in the group treated with inhibitors and gray bars without inhibitors. (C) Results of percentage changes in paw-withdrawal threshold from von Frey tests with inflamed rats in the same way to those described in (B).

DISCUSSION

The contributions of different LOs to the spinal FOS-LI development have not been tested previously in the carrageenan-induced inflammatory pain model. The present study demonstrated that local LO inhibition using three different LO inhibitors suppressed carrageenan-induced Fos expression in the spinal cord. Induction of Fos-LI in the spinal dorsal horn well reflects the peripheral nociception (Bullitt et al., 1990; Hunt et al., 1987). The utility of the spinal Fos-LI as a marker for pain development under carrageenan inflammation were reported earlier (Honore et al., 1995; Kwak et al., 1998). As in these previous studies, here we demonstrated that the intraplantar injection of carrageenan induced increases in the Fos-(+) cells in the rat dorsal horn. Local pretreatment with NDGA, AA-861 or Baicalein reduced the increases of Fos-LI during carrageenan inflammation

(Fig. 2), implying that the reduction of the Fos-LI would reflect the antinociceptive effects of the LO inhibitors. Indeed, treatment with the LO inhibitors reduced thermal and mechanical hyperalgesic responses and the effects of these inhibitors were comparable to that of indomethacin, a COX inhibitor. Behavioral data in this study also corresponds to the following previous behavioral, electrophysiological and biochemical observations on the role of LOs in inflammatory pain. Increases in LO products were observed in carrageenin-induced inflamed areas as well as increases in other inflammatory mediators such as PGs (Peskar et al., 1991). LO products exerted hyperalgesic effects by sensitizing the sensory terminal of C and Aδ fibers (Martin et al., 1987; 1988). Inhibition of 5-LO prevented some types of hyperalgesic behaviors under carrageenan-induced knee joint inflammation and hind paw inflammation (Singh et al., 2005; Tonussi et al., 1999). LO products are also involved in other models of inflammatory pain such as a complete adjuvant-injection model and a nerve growth factor treatment model (Amann et al., 1996; Szabo et al., 2005). Recently two groups reported that the LO pathway is also involved in bradykinin-induced hyperalgesia (Ferreira et al., 2004; Shin et al., 2002).

Early studies have suggested that LO subtypes contribute to inflammatory hyperalgesic responses. Local treatment with a 5-LO inhibitor AA-861 was able to significantly suppress the carrageenan-induced thermal and mechanical hypersensitivities (Figs. 3B and 3C). Similar effects were observed in a previous study concerning the systemic effect of orally administered zileuton, another 5-LO inhibitor using the Randall-Selitto test and the acetic acid-induced writhing test in the carrageenan-induced inflammation (Singh et al., 2005). Direct intradermal injection of 12-LO was able to evoke mechanical hyperalgesia (Aley et al., 2003) and a 12-LO inhibitor baicalein was reported to have an antinociceptive effect in acute hyperalgesia models based on epinephrine or bradykinin administration (Aley et al., 2003; Ferreira et al., 2004; Shin et al., 2002). The present study demonstrates that treatment with baicalein also effectively prevents carrageenan-induced thermal and mechanical hypersensitivity. These findings suggest that action of 12-LO is widely involved in acutely heightened pain sensitivity during inflammation. Ethyl 3,4-dihydroxybenzylidenecyanoacetate has an inhibitory activity on 15-LO and during the course of the present study it was found to upregulate Fos-LI (data not shown). Further study using a more specific inhibitor should answer how much 15-LO contributes to the carrageenan-induced hyperalgesia. Collectively, together with earlier reports, the present study suggests that the signalings of LO subspecies participate in pain development over numbers of types of acute inflammation.

In the present study, the PLA2 inhibitor quinacrine potently attenuated Fos-LI development during carrageenan inflammation. In addition to LO, COX also processes arachidoninc acid, the PLA2 product (Uchida, 2008). PGs, COX metabolites, were produced when tissues are inflamed and it has been well characterized *in vitro* and *in vivo* that nociceptors are greatly sensitized by PGs (Moriyama et al., 2005; Samad et al., 2002). Two recent reports have suggested that PGs and LO products cooperate to develop inflammatory hyperalgesia (Aley et al., 2003; Singh et al., 2004). In the present study, the addition of indomethacin improved the activity of NDGA in terms of Fos-LI reduction (Fig. 2E). Interestingly, combined inhibition of COX and LO was found to exhibit an increasing effect on the threshold beyond the normal range in the von Frey assay (Fig. 3C). These results suggest that COX and LO act additively, which is consistent with the previous findings. However, the mechanism underlying the potent mechanical analgesia over the normal threshold in the von Frey assay remains to be elucidated.

TRPV1 is a noxious heat sensitive ion channel which is expressed in small-diameter nociceptive nerve fibers (Caterina et al., 1997). Earlier, two groups generated TRPV1-knockout mice that exhibited impaired thermal hyperalgesic behavior under mustard oil, complete Freund's adjuvant or carrageenan-induced inflammation (Caterina et al., 2000; Davis et al., 2000). In addition, capsazepine, a competitive TRPV1 antagonist was found to have an inhibitory effect on inflammatory pain induced by complete Freund's adjuvant or carrageenan-induced inflammation (Kwak et al., 1998; Walker et al., 2003). BCTC, a novel TRPV1 antagonist was also effective at reducing pain in complete Freund's adjuvant model but has not been challenged to carrageenan-inflamed animals (Pomonis et al., 2003). Here we show that BCTC also suppresses carrageenan induced inflammatory heat hyperalgesia in the same way as capsazepine (Kwak et al, 1998). Leukotrienes, HPETE and HETE, the products of LO are endogenous activators of TRPV1 (Hwang et al., 2000; Shin et al., 2002). It is likely that LO metabolites produced during carrageenan-induced inflammation affect TRPV1 activity and lead to heightened pain sensitivity. PGs possess a sensitizing effect on TRPV1 activity via the protein kinase C pathway *in vitro* (Moriyama et al., 2005). The suppression of nociceptive behavior by treatment of indomethacin in this study suggests that PGs might contribute to TRPV1-mediated thermal nociception in this carrageenan inflammation. According to von Frey behavior assays in the present study, indomethacin, NDGA and baicalein attenuated behavioral threshold changes, but BCTC did not. This finding is consistent with previous findings in TRPV1-deficient mice, which showed no change in mechanical pain behaviors (Caterina et al., 2000; Davis et al., 2000). Noxious mechanosensor molecules expressed in sensory nerves are still unknown but a candidate is TRPA1, another TRP ion channel as its knockout animals exhibited blunted von Frey sensitivity (Kwan et al., 2006; Petrus et al., 2007). It is possible that lipoxygenase products also affect TRPA1 activity and thus reduce mechanical pain thresholds, but further study is needed to understand whether the TRPA1 and/or other unknown sensor molecules are involved in the carrageenan-induced mechanical hypersensitivity.

Another possible mechanism of contribution of LO products to inflammatory pain is the activation of cysteinyl leukotriene receptor in addition to the direct modification of TRPV1 activity. In a recent study, it was suggested that a cysteinyl leukotriene receptor antagonist zafirlukast interferes with inflammatory pain development (Jain et al., 2001). However, the identities of molecules downstream of cysteinyl leukotriene receptor signaling and how these molecules affect responses of sensory nerve fibers remain to answer.

The present study shows that LO inhibition relieves carrageenan-induced nociceptive behavior and that this is well associated with a reduction in the number cells with Fos-LI in the spinal cord. These findings suggest that lipoxygenase products may play an important role in the development of carrageenan-induced inflammatory pain.

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