Spinal 12-lipoxygenase-derived hepoxilin A_3 contributes to inflammatory hyperalgesia via activation of TRPV1 and TRPA1 receptors

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Peripheral inflammation initiates changes in spinal nociceptive processing leading to hyperalgesia. Previously, we demonstrated that among 102 lipid species detected by LC-MS/MS analysis in rat spinal cord, the most notable increases that occur after intraplantar carrageenan are metabolites of 12-lipoxygenases (12-LOX), particularly hepoxilins (HXA_3 and HXB_3). Thus, we examined involvement of spinal LOX enzymes in inflammatory hyperalgesia. In the current work, we found that intrathecal (IT) delivery of the LOX inhibitor nordihydroguaiaretic acid prevented the carrageenan-evoked increase in spinal HXB_3 at doses that attenuated the associated hyperalgesia. Furthermore, IT delivery of inhibitors targeting 12-LOX (CDC, Baicalein), but not 5-LOX (Zileuton) dose-dependently attenuated tactile allodynia. Similarly, IT delivery of 12-LOX metabolites of arachidonic acid 12(S)-HpETE, 12(S)-HETE, HXA_3, or HXB_3 evoked profound, persistent tactile allodynia, but 12(S)-HpETE and HXA_3 produced relatively modest, transient heat hyperalgesia. The pronociceptive effect of HXA_3 correlated with enhanced release of Substance P from primary sensory afferents. Importantly, HXA_3 triggered sustained mobilization of calcium in cells stably overexpressing TRPV1 or TRPA1 receptors and in acutely dissociated rodent sensory neurons. Constitutive deletion or antagonists of TRPV1 (AMG9810) or TRPA1 (HC030031) attenuated this action. Furthermore, pretreatment with antihyperalgesic doses of AMG9810 or HC030031 reduced spinal HXA_3-evoked allodynia. These data indicate that spinal HXA_3 is increased by peripheral inflammation and promotes initiation of facilitated nociceptive processing through direct activation of TRPV1 and TRPA1 at central terminals.

eicosanoid | pain | central sensitization

Tissue injury and inflammation are associated with hyperalgesia mediated by facilitated spinal nociceptive processing that can be modulated by lipids derived from arachidonic acid (AA) and other polyunsaturated fatty acids (PUFA), including eicosanoids synthesized via three enzymatic pathways: (*i*) cyclooxygenase (COX)–prostaglandins (PG); (*ii*) 5-, 12-, and 12/15-lipoxygenases (LOX)–leukotrienes, hydroxyeicosatetraenoic acids (HETEs), hepoxilins (HXA_3 and HXB_3), lipoxins, resolvins, and protectins; and (*iii*) cytochrome P450–epoxyeicosatrienoic acids and HETEs (1). Substantial evidence indicates that peripheral injury or direct activation of spinal dorsal horn receptors [Neurokinin 1 (NK1), AMPA, and NMDA] increases eicosanoid formation and that spinal delivery of COX inhibitors reduces the associated hyperalgesia (2, 3). Recently, we reported that paw carrageenan increases spinal production of both COX and 12-LOX metabolites of AA, including 12(S)-HETE in cerebrospinal fluid (CSF) and hepoxilins in the lumbar spinal cord (4).

Several groups point to a peripheral role for 5- and 12-LOX in nociception, as shown by antihyperalgesic actions of LOX inhibitors administered via systemic routes (5–9). It has been suggested that spinal 12-LOX may play a role in morphine physical dependence (10), yet there is little work defining its involvement in spinal nociception. The chemosensors Transient Receptor Potential Vanilloid 1 (TRPV1) and Ankyrin 1 (TRPA1) have been implicated in peripheral inflammation-evoked hyperpathic states (11). Irritants, such as formalin and oxidized lipids, can cause hyperalgesia via activation of spinal TRPA1 (12–14). For example, 4-hydroxy-2-nonenal (HNE) elicits spinal release of Substance P (SP) and triggers nocifensive behaviors via activation of TRPA1 receptors (15). Both 12(S)-HpETE and its reduction product 12(S)-HETE directly stimulate TRPV1 in cultured dorsal root ganglion (DRG) neurons (16). Noxious heat or depolarization releases 12-LOX metabolites of linoleic acid that activate spinal TRPV1 and contribute to complete Freund's adjuvant (CFA)-induced mechanical allodynia (17, 18). Given that linoleic acid is used in vivo for biosynthesis of AA and that HNE is generated by peroxidation of 12/15-LOX products of *n*-6 PUFA, we hypothesized that spinal 12-LOX metabolites of AA, particularly hepoxilins, also contribute to inflammatory hyperalgesia through stimulation of nociceptive afferents. Although hepoxilins increase intracellular Ca^{2+} in neutrophils (19, 20) and neurons (21) and enhance vascular permeability in rat skin (22, 23), its receptors remain undefined. Our findings demonstrate that peripheral inflammation increases spinal HXA_3, which produces hyperesthesia via activation of TRPV1 and TRPA1 and spinal SP release.

Results

Inhibition of Spinal 12-LOX Attenuates Inflammatory Hyperalgesia. First, we examined involvement of spinal LOX in the development of hyperalgesia following intraplantar (IPLT) carrageenan. Intrathecal (IT) pretreatment with the LOX inhibitor nordihydroguaiaretic acid (NDGA) dose-dependently attenuated tactile allodynia as depicted by timecourse (Fig. 1*A*) and area under the curve (AUC), or percent hyperalgesic index (Fig. 1*C*), without overt toxicity. An antihyperalgesic dose of IT NDGA at the upper limit of solubility (60 μg) also prevented the development of thermal hyperalgesia (Fig. S1 *A* and *B*) and partially reversed established tactile allodynia (Fig. S1*C*). IT NDGA did not alter paw volume at any of the doses tested (Fig. S1*D*), indicating that spinal lipoxygenases contribute centrally to inflammatory hyperesthesia.

Recently, we demonstrated that IPLT carrageenan increases spinal levels of AA metabolites of 12-LOX but not of 5-LOX (4). Therefore, we reasoned that spinal 12-LOX likely contributes to inflammatory hyperesthesia. To address this question, we examined the effect of IT pretreatment with 5- or 12-LOX inhibitors on

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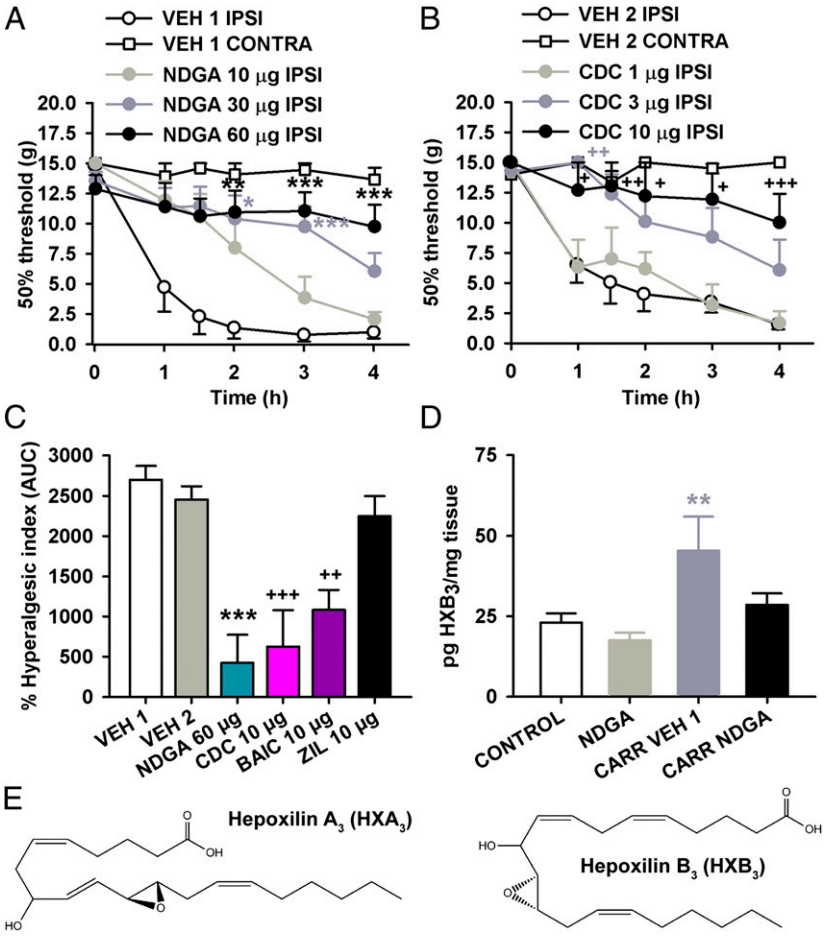

Fig. 1. Inhibition of spinal 12-LOX attenuates carrageenan-induced tactile allodynia and increased spinal formation of hepoxilins (HXB_3). (*A* and *B*) IT pretreatment with (*A*) NDGA or (*B*) CDC dose-dependently attenuates the carrageenan-induced reduction in tactile thresholds. (*C*) Percent hyperalgesic index, or AUC, of tactile allodynia after IT pretreatment with vehicle (VEH) or maximum doses of NDGA, CDC, Baicalein (BAIC) or Zileuton (ZIL) reveals reduced nociceptive behavior after spinal inhibition of 12-LOX but not 5-LOX. (*D*) IT pretreatment with an antihyperalgesic dose of NDGA (60 μg) prevents the increase in spinal HXB_3 4 h postcarrageenan. (*E*) Structures of HXA_3 (*Left*) and HXB_3 (*Right*). CONTRA, contralateral; CONTROL, naïve + IT VEH-treated; IPSI, ipsilateral; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. VEH 1; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. VEH 2; n = 7–10.

nociceptive behaviors. Consistent with our hypothesis, IT pretreatment with 12-LOX inhibitors cinnamyl 3,4-dihydroxy-(α)-cyanocinnamate (CDC) (Fig. 1 *B* and *C*) or Baicalein (Fig. 1*C* and Fig. S2*A*) dose-dependently attenuated carrageenan-induced tactile allodynia. In contrast, pretreatment with the 5-LOX inhibitor Zileuton (Fig. 1*C* and Fig. S2*B*) did not prevent allodynia, even at the maximum soluble dose (10 μg). These results suggest involvement of 12-LOX in the development of inflammatory hyperalgesia.

Inflammation-Evoked Spinal 12-LOX Activation Produces Hyperesthesia. Paw carrageenan produces a time-dependent, bilateral elevation in levels of 12-LOX metabolites 12(S)-HETE and hepoxilins in rat CSF and L4/L5 spinal cord, respectively, which is prevented by systemic pretreatment with NDGA (4). The 12(S)-HpETE can undergo reduction to form 12(S)-HETE or isomerization by a hepoxilin synthase to form hepoxilins A_3 and B_3. HXA_3 and HXB_3 are synthesized concurrently, yet most of the bioactive effects are attributed to HXA_3 (1). Because HXA_3 is labile, it is eventually degraded in our assay, so we used HXB_3 as a marker of hepoxilin synthase activity. Thus, we examined if an antihyperalgesic dose of IT NDGA (60 μg) would prevent the increase in spinal HXB_3 during inflammation. As predicted, pretreatment with IT NDGA prevented the carrageenan-induced increase in spinal HXB_3 at 4 h (Fig. 1 *D* and *E*). These data indicate that spinal 12-LOX metabolites are elevated in direct correlation with hyperesthesia following peripheral inflammation.

Because commercially available 12-LOX inhibitors are limited in terms of selectivity and systemic administration exerts multiple actions, we investigated the effect of spinal delivery of 12-LOX metabolites of AA on nociceptive tactile and thermal thresholds. We observed that IT HXA_3 (Fig. 2 *A* and *B*), 12(S)-HpETE (Fig. 2 *C* and *D*), HXB_3 (Fig. S3 *A* and *B*), or 12(S)-HETE (Fig. S3 *C* and *D*) rapidly elicited a profound, dose-dependent tactile allodynia that persisted for up to several hours. Interestingly, IT HXA_3 and 12(S)-HpETE each induced a relatively modest, transient thermal hyperalgesia only at a dose exceeding that required to elicit tactile allodynia (Fig. S4 *A* and *B*). In contrast, thermal (heat) thresholds were unchanged following IT 12(S)-HETE or HXB_3 at the same dose (Fig. S4 *C* and *D*). These results demonstrate that bioactive 12-LOX metabolites of AA contribute directly to hyperesthesia, with a markedly greater effect on tactile versus thermal nociceptive thresholds.

HXA_3 Activates TRPV1 and TRPA1 on Sensory Neurons and Triggers Spinal SP Release Concurrent with Hyperesthesia. As discussed above, other products of lipid peroxidation contribute to spinal facilitated states via activation of TRPV1 or TRPA1 and release of neuropeptides in dorsal horn (15, 17). Therefore, we asked if HXA_3 activates TRPV1- or TRPA1-mediated calcium mobilization in DRG neurons at concentrations previously shown to evoke calcium flux and AA release in human neutrophils (19, 24). Superfusion of HXA_3 (1 μM) of acutely dissociated adult rat DRG cells significantly increased free-Ca^{2+} levels (Fig. 3 *A* and *B*). This effect was restricted to a subpopulation of Fura-2–loaded cells (41 of 146, 28.1%); of these, 47.6% were capsaicin-responsive. Remarkably, HXA_3-induced Ca^{2+}-mobilization was diminished by the TRPA1-selective antagonist HC030031 at 10 μM (Fig. 3*C*), a concentration approaching its IC_{50} of ∼5–7.5 μM (25, 26) that also blocks mechanically evoked currents in mouse DRG neurons (27). Exposure to the parent compound 12(S)-HpETE (1 μM) also increased free-Ca^{2+} levels in a subpopulation of Fura-2–loaded cells (31 of 177, 17.5%); of these, 55.2% were capsaicin-sensitive

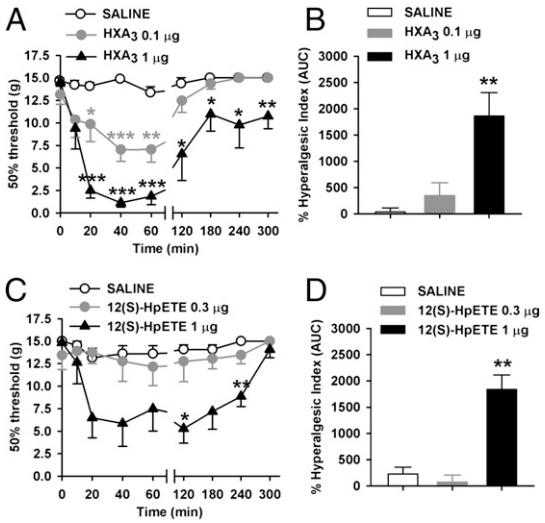

Fig. 2. Bioactive 12-LOX metabolites produce robust tactile allodynia after spinal delivery. (*A–D*) Dose–response depicting timecourses (*Left*) and AUC values (*Right*) reveal sustained reduction of tactile thresholds after a single IT administration of nanogram to microgram amounts of (*A* and *B*) HXA_3 or (*C* and *D*) 12(S)-HpETE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline vehicle; $n = 5–6$.

(Fig. S5 *A* and *B*). This response was attenuated by treatment with 10 μM HC030031 (83%) (Fig. S5*C*). Application of the TRPV1-selective antagonist AMG9810 at 1 μM, a concentration that exceeds its IC_{50} of ~0.1 μM (28), reduced HXA_3-evoked Ca^{2+}-responses by 61% and 12(S)-HpETE–evoked Ca^{2+}-responses by 55% (Fig. 3*C* and Fig. S5*D*). HXA_3 (1 μM) also elicited intracellular Ca^{2+} accumulation in cells heterologously overexpressing TRPA1 (HEK-TRPA1-tet) or TRPV1 (CHO-TRPV1) compared with their respective controls (Fig. 3*D*). Similarly, superfusion of HXA_3 (1 μM) on acutely dissociated adult mouse DRG cells increased free-Ca^{2+} levels, a response that was dampened in both TRPA1 and TRPV1 knockout mice (Fig. 3 *E* and *F*). It is important to note that although the majority of cells recorded from either TRPA1 or TRPV1 KO mice were not considered HXA_3-sensitive according to our pre-established criteria (see *SI Materials and Methods*), some cells were very weakly responsive. Of this population, the percent sensitivity to capsaicin was as follows: WT, 36%; TRPA1 KO, 20%; TRPV1 KO, 0%. Thus, HXA_3-mediated calcium mobilization was drastically reduced but not completely abolished in the absence of either TRPA1 or TRPV1. These data indicate that HXA_3, as well as its parent compound 12(S)-HpETE, produce potent activation of both TRPA1 and TRPV1 receptors on sensory neurons.

We then examined whether the hyperalgesic activity of HXA_3 is related to its activation of primary sensory afferents and subsequent release of pronociceptive neurotransmitters. We investigated in rat spinal cord in vivo if IT HXA_3 increases SP release from peptidergic primary afferents by measuring NK1 receptor internalization in L4, L5, and L6 levels of lumbar spinal dorsal horn. We found no difference in the percentage of internalized NK1 receptors at 10 min after IT HXA_3 (1 μg), before the onset of allodynia (Fig. S6*A*). In contrast, NK1 receptor internalization is significantly increased in lamina I of L4, L5, and L6 at 30 min after spinal administration of HXA_3 (Fig. S6*B*), concurrent with the onset of tactile allodynia. Thus, IT HXA_3 evokes spinal release of SP from nociceptive primary sensory afferents in direct temporal correlation with hyperesthesia.

HXA_3-Induced Activation of TRPV1 and TRPA1 Contributes to Tactile Allodynia. Next, we sought to determine if HXA_3-evoked tactile allodynia is mediated via stimulation of TRPV1 or TRPA1 receptors on nociceptive primary sensory afferents. IT pretreatment with the maximum soluble dose (10 μg) for antagonists of TRPA1 (HC030031) or of TRPV1 (AMG9810) significantly attenuated IPLT carrageenan-evoked tactile allodynia and thermal hyperalgesia (Fig. S7 *A* and *B*). These observations corroborate other studies that use the same doses of these antagonists in different rodent models of inflammatory pain (14, 29, 30). Similarly, IT HXA_3-induced tactile allodynia is significantly attenuated by spinal pretreatment with antihyperalgesic doses of HC030031 or AMG9810 (Fig. 4). Taken together, the present study provides strong evidence that HXA_3 produces hyperesthesia at the spinal level by activating TRPA1 and TRPV1 receptors in a subpopulation of primary sensory afferents and by facilitating release of nociceptive mediators in dorsal horn (Fig. 5).

Discussion

Rat lipoxygenases are classified as 5-, 12-, and 12/15-LOX, named according to the stereospecific insertion of O_2 into PUFA (1). To date, most reports that examine the role of LOX in hyperalgesia focus on 5-LOX, which catalyzes the rate-limiting step in the synthesis of leukotrienes (LT), potent regulators of innate immune cell functions and chemoattractants for neutrophils (31). Systemic treatment with 5-LOX inhibitors or LT receptor antagonists attenuated inflammatory hyperalgesia (5, 8, 32). However, spinal 5-LOX inhibition did not prevent carrageenan-induced allodynia, which supports our previous observation that spinal 5-LOX metabolites are either not present or are at levels below the assay limit of detection (4). Nonetheless, our data do not preclude the possibility of inflammation-induced activation of 5-LOX in brain, DRG, or peripherally at the site of injury. For example, intradermal administration of LTB_4 produces hyperalgesia that is attenuated by depletion of neutrophils (33). In addition, systemic administration of inhibitors targeting 5-LOX or LT receptors reduces mechanical allodynia and, respectively, prevents increased levels of LTB_4 (8) and neutrophil infiltration (32) in paw tissue during inflammation. Thus, it appears that induction of 5-LOX activity occurs largely outside of the lumbar spinal cord.

In contrast, we found that spinal inhibition of 12-LOX significantly attenuated hyperesthesia. Conversely, spinal delivery of either HXA_3 or HXB_3, the parent molecule 12(S)-HpETE, or its reduction product 12(S)-HETE, produced persistent tactile allodynia. Our findings regarding thermal responses to 12(S)-HpETE are consistent with Trang et al., who found that IT delivery at doses up to 1 μg has no effect on thermal (heat) nociceptive thresholds in rats (34). Similar responses were observed following IT administration of COX metabolites PGE_2 or $PGF_{2\alpha}$, which elicit robust mechanical but weak thermal hyperalgesia (35). Others have illustrated that activation of 12-LOX in the paw produces mechanical allodynia (36) as well as thermal hyperalgesia (6), which is attenuated by local administration of LOX inhibitors (7). Furthermore, several groups have shown that during inflammation, spinal TRPV1 receptors contribute significantly to both tactile allodynia and thermal hyperalgesia, but TRPV1 on peripheral terminals preferentially mediates the latter (7, 37–39). Peripheral activation of TRPA1 produces some thermal hyperalgesia, but the resulting tactile allodynia is the more robust response (40). Thus, the current work demonstrates that spinal HXA_3 contributes to the development of inflammatory hyperalgesia, preferentially initiating tactile allodynia via activation of TRPV1 and TRPA1 on central terminals. Although mechanisms underlying thermal and mechanical hypersensitivity remain to be elucidated, differential expression of these phenomena have been described in other animal models of pain syndromes (41, 42).

An important question relates to the mechanisms by which hepoxilins cause inflammatory hyperalgesia. Both HXA_3 and its precursor 12(S)-HpETE stimulated TRPV1 and TRPA1 on nociceptive sensory neurons. However, in an aqueous environment, lipid hydrogen peroxides (such as 12-HpETE) have a short $T_{1/2}$ on the order of minutes (43); in contrast, HXA_3 and structurally similar compounds created by 15-LOX have a half-life on the order of hours (20, 44). Moreover, the in vivo degradation of each of these metabolites is handled by two distinct enzyme classes, glutathione peroxidase for 12-HpETE and soluble

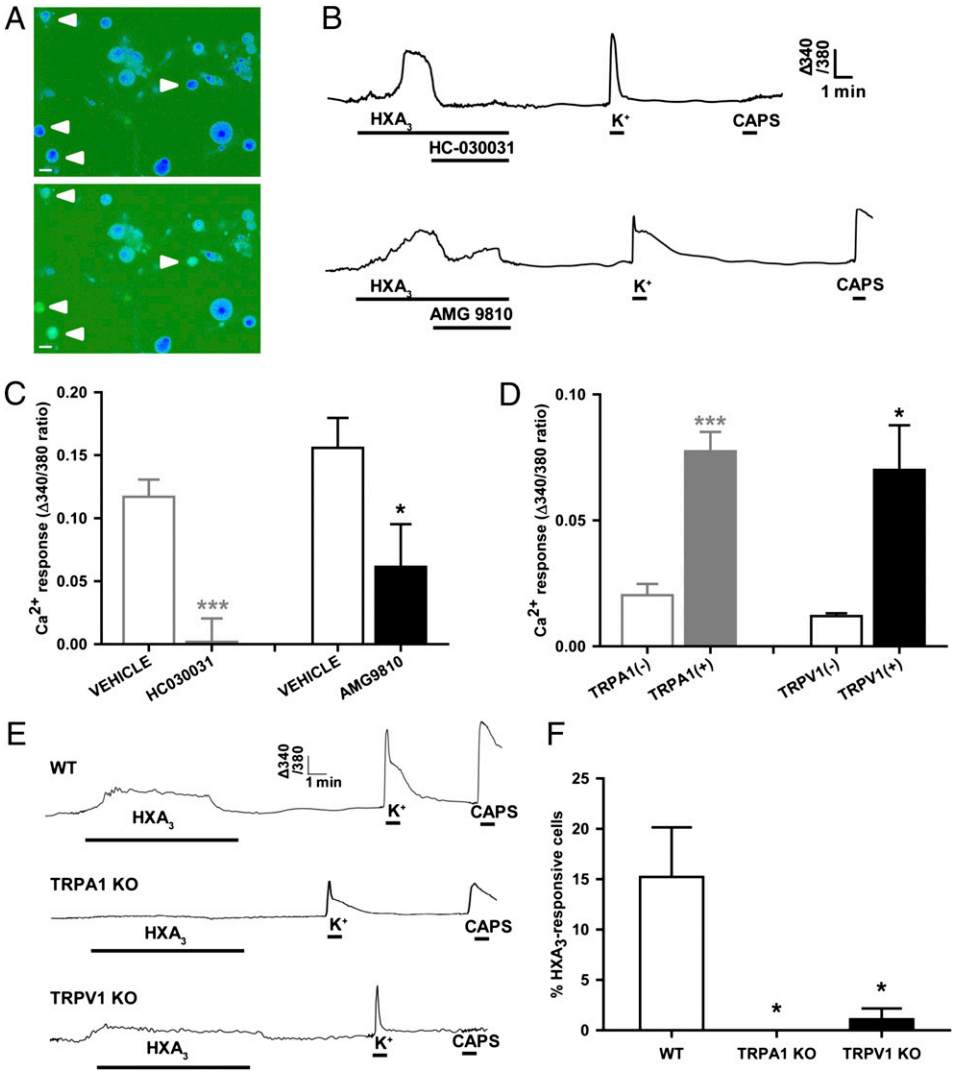

Fig. 3. HXA_3 produces direct activation of TRPV1 and TRPA1 in sensory neurons. (*A*) Representative micrographs of cultured rat sensory neurons before (*Upper*) and after (*Lower*) application of 1 μM HXA_3. Arrowheads, Fura-2–loaded cells exhibiting Ca^{2+} mobilization in response to HXA_3. (Scale bars, 50 μm.) (*B*) Representative traces of HXA_3-evoked calcium responses in rat sensory neurons and attenuation by the TRPA1 antagonist HC030031 (*Upper*) or the TRPV1 antagonist AMG9810 (*Lower*). Peak Ca^{2+} responses represent mean change in fluorescence ratio (Δ340/380) ± SEM. (*C*) HXA_3-evoked Ca^{2+}-responses were reduced by HC030031 (10 μM) (VEH, 0.117 ± 0.01 vs. HC030031, 0.001 ± 0.02; $***P <= 0.001$, $n = 16$ cells, 4 rats) and AMG 9810 (1 μM) (VEH, 0.159 ± 0.03 vs. AMG9810 0.061 ± 0.03; $*P < 0.05$, $n = 5$ cells, 3 rats). (*D*) HXA_3 (1 μM) also increased calcium mobilization in HEK-TRPA1-tet [TRPA1(+) Induced, 0.077 ± 0.008 vs. TRPA1(–) Uninduced control, 0.020 ± 0.004; $***P < 0.001$, $n = 4$ cells] and in CHO-TRPV1 cells [TRPV1(+) 0.070 ± 0.001 vs. TRPV1(–) CHO control, 0.012 ± 0.001; $*P < 0.05$, $n = 3$–5 cells]. (*E*) Representative traces of HXA_3-evoked calcium responses in mouse sensory neurons from WT (*Top*) and attenuation in TRPA1 (*Middle*), or TRPV1 knockout (KO) (*Bottom*). (*F*) Percent of WT, TRPA1, or TRPV1 KO neurons in which 1 μM HXA_3 increased free intracellular Ca^{2+} levels. (WT, 15.2 ± 4.9% $n = 42$ cells; TRPA1 KO, 0.0 ± 0.0%, $n = 34$ cells; TRPV1 KO, 1.1 ± 1.1%; $n = 51$ cells; $*P <= 0.05$, 3 mice per group). Cell viability was confirmed using 50 mM K^+; functional TRPV1 or TRPA1 receptors were verified with 500 nM capsaicin or 5 μM icilin, respectively. VEH, artificial CSF.

epoxide hydrolase for HXA_3 (1). Thus, although 12-HpETE and HXA_3 may interact with these channels via a similar mechanism, their bioavailability in vivo may differ.

Collectively, our findings are intriguing given the differential effect of spinally delivered 12-LOX products on thermal and tactile sensitivity. Several groups have demonstrated that TRPA1 modulates nociceptive tactile thresholds, triggers release of the neuropeptides from primary sensory afferents, and mediates the mechanical allodynia produced by inflammatory agents and chemical irritants (13, 15, 25). Similarly, we observed a time-dependent increase in spinal SP release following IT administration of HXA_3 at a dose that produces hyperalgesia. Although others have demonstrated that systemic or IT delivery of the TRPA1 antagonist HC030031 attenuates mechanical allodynia induced by formalin, mustard oil, or CFA (14, 25, 26), the spinal actions of this agent have not yet been examined following carrageenan or after IT delivery of 12-LOX metabolites. In this study, we found that TRPA1 receptors are required for the observed effects of HXA_3 on calcium mobilization in sensory neurons and tactile allodynia.

TRPA1 receptors are expressed by a subpopulation of peptidergic somatosensory neurons that often coexpress TRPV1 (45). It has been suggested that 12-LOX–mediated activation of TRPV1 in hippocampal neurons mediates long-term depression (46) and in primary afferent nociceptors contributes to inflammatory pain hypersensitivity (6). Until recently, TRPV1 receptors were viewed primarily as sensors of noxious heat, the activation of which underlies thermal hyperalgesia (47). However, data are now emerging in support of TRPV1-mediated involvement in tactile allodynia during persistent inflammation by CFA. For example, it has been reported that 9-HODE and 13-HODE contribute to mechanical hypersensitivity via stimulation of TRPV1 (17, 18). Similarly, we found that TRPV1 receptors also are required for the observed effects of HXA_3 on calcium mobilization in sensory neurons and tactile allodynia.

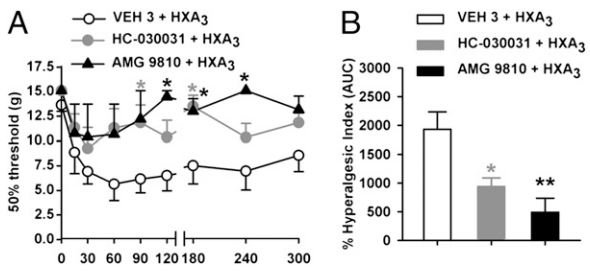

Fig. 4. HXA_3-evoked tactile allodynia is mediated by spinal TRPV1 and TRPA1. (*A*) Timecourse and (*B*) corresponding AUC reveals significant attenuation of tactile allodynia following IT HXA_3 (1 μg) by IT pretreatment with antihyperalgesic doses (10 μg) of antagonists of TRPA1 (HC030031) or TRPV1 (AMG9810). $*P < 0.05$, $**P < 0.01$ vs. VEH 3; $n = 5$–7.

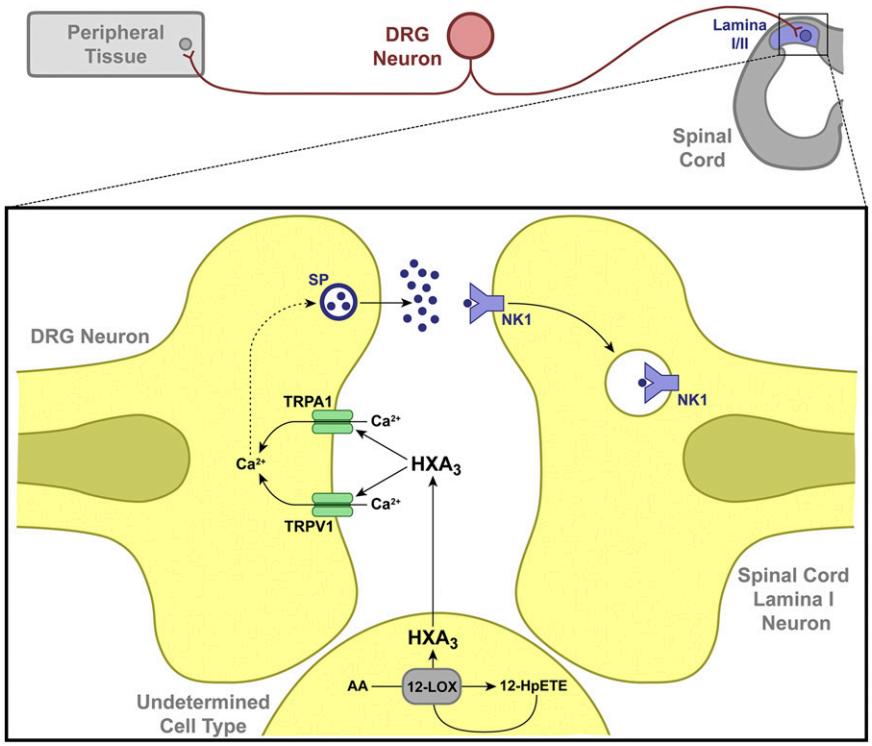

Fig. 5. Model of HXA_3-mediated hyperalgesic effects at the spinal level. HXA_3 is produced through 12-LOX either from arachidonic acid or via 12-HpETE. Cellular sources of HXA_3 may represent DRG neurons or satellite cells, spinal neurons, or glia, and circulating leukocytes or platelets. Spinally generated HXA_3 activates TRPV1 and TRPA1, resulting in calcium mobilization and release of SP from nociceptive afferents, internalization of NK1 receptors in dorsal horn, and ultimately tactile allodynia.

We demonstrate here that low micromolar concentrations of HXA_3 produce potent activation of TRPV1 and TRPA1 receptors on sensory neurons and increased spinal SP release concurrent with the development of tactile allodynia. Our observations of HXA_3-mediated activation of TRPV1 or TRPA1 in heterologous overexpression systems indicate a direct interaction with these channels. However, HXA_3 may also exert effects via direct binding and gating of TRPV1/TRPA1 multimeric complexes, binding to TRPV1 and transactivation of TRPA1, or binding to a G protein-coupled receptor and signaling via second messenger systems to increase membrane trafficking of TRPV1/TRPA1. Recent studies demonstrate functional interaction of these receptors in sensory neurons (48, 49). Similar to HXA_3, bradykinin, nitric oxide, and BH4 all require both TRPV1 and TRPA1 receptor function for hyperalgesia (12, 50). Phospholipase C-dependent sensitization and increased plasma membrane trafficking of TRPA1 has been described for bradykinin and mustard oil, respectively (51, 52). Accordingly, it has been shown in neutrophils that HXA_3 produces a biphasic increase in levels of intracellular calcium through G protein-coupled receptor-mediated signaling via phospholipase C (20, 53).

Ultimately, although the local concentration of individual 12-LOX products may be subthreshold for activation of TRP channels, it is possible that several metabolites could function in concert to produce hyperalgesia, as suggested previously (17). The identities, temporal profiles, concentrations, and targets of these mediators also may vary with different pain states. In summary, this work is unique in providing evidence of hepoxilin receptors in the CNS and that HXA_3-mediated activation of TRPA1 and TRPV1 contributes to hyperesthesia at the spinal level. These findings highlight the importance of developing novel anti-inflammatory therapeutics targeting this pathway. Furthermore, these findings advance our understanding of distinct mechanisms contributing to tactile allodynia versus heat hyperalgesia.

Materials and Methods

Animals. Male Sprague-Dawley rats (300–350 g; Harlan) or mice (25–30 g; C57BL/6 and TRPV1 knockout, Jackson Laboratories, or TRPA1 knockout, University of Kentucky) were used in accordance with protocols approved by the Institutional Animal Care and Use Committies of the University of California at San Diego and the University of Kentucky. All testing was performed by a blinded observer. IT catheter implantation of rats and drug delivery was performed as described previously (54).

Drugs. NDGA, Baicalein, 12(S)-HpETE, and 12(S)-HETE (Cayman); CDC, hepoxilins HXA_3 and HXB_3 (Enzo); Zileuton, HC-030031, and AMG9810 (Tocris) were prepared in vehicles (VEH): NDGA in 20% (wt/vol) β-cyclodextrin/saline; Baicalein, CDC, and Zileuton in 3% (vol/vol) DMSO/3% Cremaphor-EL/saline; HC030031 and AMG9810 in 3% (vol/vol) DMSO/3% Tween-80/saline.

Behavioral Testing. Peripheral inflammation and hyperalgesia were assessed as previously described (4) after IT delivery of drugs 30 min before or 90 min after 2% (wt/vol) carrageenan injected IPLT into the left hind paw. The 12-LOX metabolites were prepared fresh before use (16) and IT injections were performed immediately after dilution followed by measurement of nociceptive behaviors. Vehicle or antagonists of TRPA1 or TRPV1 were given IT 30 min before HXA_3. Data were expressed as response latency vs. time and as AUC, or hyperalgesic index (percent change from baseline × minute).

Immunohistochemistry. At 10 or 30 min after IT delivery of saline or HXA_3 (1 μg), rats were perfused and the lumbar spinal cord was processed for NK1 receptor internalization (55, 56).

Heterologous Overexpression Systems, DRG Cell Preparation, and Ca^{2+} Imaging. Inducible HEK293 cells stably overexpressing TRPA1 (HEK-TRPA1-tet) and CHO cells stably overexpressing TRPV1 (CHO-TRPV1) were maintained as previously described (15, 17). L4 and L5 DRGs were dissected from male Sprague-Dawley rats at 4–5 wk old or mice (WT, TRPV1, or TRPA1 knockout) at 8 wk old, dissociated and used within 24 h of culture, as previously described (57), with modifications by S.D. and B.K.T. (*SI Materials and Methods*).

LC-MS/MS. LC-MS/MS of L4/L5 rat spinal cord was conducted using a tandem quadrupole mass spectrometer (ABI 4000 Q-Trap) as previously described (4, 58, 59).

Statistics. Normalized raw data of spinal eicosanoids were filtered using the Grubbs' test (4). Data were expressed as the mean ± SEM, with $P < 0.05$ as significant. *P* values were determined using SPSS software (PASW version 18, SPSS) as follows: *t* test for two-group analysis, standard or repeated-measures ANOVA for multiple group analysis with Bonferroni or Dunnett's post hoc for behavioral or biochemical data, respectively.

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