Central activation of TRPV1 and TRPA1 by novel endogenous agonists contributes to mechanical and thermal allodynia after burn injury

Dustin P Green, PhD1, Shivani Ruparel, PhD2, Xiaoli Gao, PhD3, Nikita Ruparel, DDS, PhD2, Mayur Patil, PhD4, Armen Akopian, PhD2,4 and Kenneth M Hargreaves, DDS, PhD1,2,4

Abstract
The primary complaint of burn victims is an intense, often devastating spontaneous pain, with persistence of mechanical and thermal allodynia. The transient receptor potential channels, TRPV1 and TRPA1, are expressed by a subset of nociceptive sensory neurons and contribute to inflammatory hypersensitivity. Although their function in the periphery is well known, a role for these TRP channels in central pain mechanisms is less well defined. Lipid agonists of TRPV1 are released from peripheral tissues via enzymatic oxidation after burn injury; however, it is not known if burn injury triggers the release of oxidized lipids in the spinal cord. Accordingly, we evaluated whether burn injury evoked the central release of oxidized lipids. Analysis of lipid extracts of spinal cord tissue with HPLC-MS revealed a significant increase in levels of the epoxide and diol metabolites of linoleic acid: 9,10-DiHOME, 12,13-DiHOME, 9(10)-EpOME, and 12(13)-EpOME, that was reduced after intrathecal (i.t.) injection of the oxidative enzyme inhibitor ketoconazole. Moreover, we found that these four lipid metabolites were capable of specifically activating both TRPV1 and TRPA1. Intrathecal injection of specific antagonists to TRPV1 (AMG-517) or TRPA1 (HC-030031) significantly reduced post-burn mechanical and thermal allodynia. Finally, i.t. injection of ketoconazole significantly reversed post-burn mechanical and thermal allodynia. Our data indicate that spinal cord TRPV1 and TRPA1 contributes to pain after burn and identifies a novel class of oxidized lipids elevated in the spinal cord after burn injury. Since the management of burn pain is problematic, these findings point to a novel approach for treating post-burn pain.

Keywords
burn, pain, OLAM, TRPV1, TRPA1, AMG-517, HC-030031

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Introduction
Burn pain is an especially devastating form of pain that can have acute and chronic components, with presentations of both mechanical and thermal allodynia.1,2 Two well-studied ion channels involved in detecting tissue damage and noxious environmental stimuli and irritants are transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1). TRPV1 plays a major role in detecting noxious heat, as well as endogenous and exogenous soluble factors, and its activation contributes to heat hyperalgesia.3 TRPA1 is a chemo sensor capable of detecting chemicals such as formalin, hydrogen peroxide, and acrolein, as well as natural products such as allyl isothiocyanate (mustard oil)
and cinnamaldehyde.4–7 Both TRPV1 and TRPA1 are implicated in the peripheral transduction of noxious signals during inflammation.8 Lipid agonists released after inflammation or noxious heat have been shown to activate both channels, contributing to the development of thermal and mechanical allodynia.9–12

Oxidized linoleic acid metabolites (OLAMs) were recently identified as endogenous agonists of TRPV1.10 Both 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE) as well their metabolites, 9-oxoODE and 13-oxoODE, were first found to be released from skin upon acute thermal stimulation.10,12 Importantly, intrathecal (i.t.) injection of antibodies to both 9- and 13-HODE attenuated mechanical allodynia in CFA-induced inflammation.13 More recently, the OLAMs 9- and 13-HODE were shown to be elevated in rat skin biopsies collected after a partial-thickness burn injury.13 Yet, there is a complete gap in knowledge about the role of spinal OLAMs and TRP channels in mediating central pain mechanisms after peripheral burn injury.

In this study, we utilized a previously established model of peripheral partial-thickness burn injury13 to test the hypothesis that burn injury triggers increases in TRP-active oxidized lipids in the spinal cord, leading to TRPV1- and TRPA1-dependent mechanical and thermal allodynia.

Materials and methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used for all studies. Animals were housed for at least seven days prior to the experiments.

Thermal injury

We employed a rat model of peripheral burn injury as previously described.13 Animals were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL, USA), and a surgical plane of anesthesia was confirmed by a negative response to tail pinch. Thermal injury was induced by standardized exposure of a 1 × 2 cm area of plantar hind paw skin to a 100°C thermal stimulus for 30 s. A stable stimulus temperature was maintained by using a heating block (Fischer Scientific, Pittsburgh, PA, USA) and consistent hind paw contact with the heated surface was achieved by placing a 30 g weight onto the dorsal hind paw. Silver sulfadiazine cream (1%) was applied daily on the injured area to prevent infection. The injury was well tolerated and normal feeding and drinking behavior was maintained. No piloerection or chromodacryorrhea was observed.

Drugs

Ketoconazole was purchased from Toecris (Ellsville, MO, USA) and was diluted in 32% methylpyrrolidinone (MPL)/phosphate buffered saline (PBS) to make a stock of 18 mM and further diluted in saline on the day of each experiment. The TRPV1 antagonist AMG 517 and TRPA1 antagonist was diluted with 5% DMSO/2% Tween in PBS. The goat anti-9-HODE and anti-13-HODE antibodies were purchased from Oxford Biomedical Research (Rochester Hills, MI, USA). As a control, a nonspecific goat IgG antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Behavioral testing

All observers were blinded to treatment allocation, with \(n = 4\) to 9 per group. Paw-withdrawal latencies to radiant heat were tested using methods previously described.14 A dynamic plantar anesthesiometer was used to measure mechanical allodynia.15 Post-thermal injury baseline measurements were followed with intrathecal (i.t.) injections of drug or vehicle administered under brief isoflurane anesthesia. Drugs were administered in a 30 μL volume by lumbar puncture between the L4 and L5 vertebrae. To assess the effects of spinal TRPV1 inhibition on peripheral burn injury, AMG 517 (15, 50, or 165 μg in 30 μL) was used, for TRPA1, HC-030031 (15, 50, or 165 μg in 30 μL) was used. A combination of anti-9-HODE and anti-13-HODE antibodies (25 or 60 μg each) or control nonspecific IgG antibodies (60 and 120 μg) was used to assess OLAM inhibition on post burn thermal and mechanical allodynia. To examine the effects of CYP inhibition on peripheral burn injury, ketoconazole (1.2, 4, 8, and 25 μg), a broad CYP inhibitor15 was used.

High performance liquid chromatography – Electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS)

PUFAs were extracted from weighed samples of spinal cord using 100% ice cold methanol. Stable isotope-labeled standards (Arachidonic Acid-d8, and 9S-HODE-d4) were added at the time of extraction for absolute quantification. Tissue samples with the solvents were homogenized with Omni Bead Ruptor Homogenizer (OMNI International). MS analyses were conducted on a Thermo Fisher Q Exactive fitted with a PicoChip nanospray source (New Objective) and a PicoChip column (Waters Atlantis dC18 column; 150 μm × 105 mm; 3 μm particle). A 55-min water/ acetonitrile/isopropanol/ammonium acetate gradient was run at the flow rate of 1 μL/min. Mobile phase A is
acetonitrile/water (40:60) containing 10 mM ammonium acetate and mobile phase B is acetonitrile/isopropanol (10:90) containing 10 mM ammonium acetate. Data-dependent analyses were conducted using one full MS scan (70,000 resolution) followed by six tandem-MS scans with electrospray negative ion detection. Targeted-MS$_2$ analyses were also performed to distinguish the isomers. Standard curves were generated for all targeted PUFAs using appropriate stable isotope labeled internal standards and authentic fatty acids. Quantitative results were obtained by reference of the experimental peak area ratios to the standard curves.

**Chinese hamster ovary cell transfection**

We used the following expression constructs: enhanced green fluorescent protein (pEGFP-N1 from Clontech, Mountain View, CA), rat TRPV1, rat TRPA1, rat TRPV2, rat TRPV3, rat TRPV4, and rat TRPM8 in pcDNA3 (Invitrogen, Carlsbad, CA). The expression constructs were delivered into Chinese hamster ovary (CHO) cells using PolyFect (Qiagen, Valencia, CA) according to the manufacturers' protocol. CHO cells were subjected to experimental procedures within 24–48 h after transfection.

**Electrophysiology**

The whole-cell voltage-clamp (Vh = -60 mV) from trigeminal ganglia neurons or CHO cells recordings were performed at 22–24°C from the somata of TG neurons (15–45 pF) or CHO cells. Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP9.0 software (Axon Instruments, Union City, CA). Recording data were filtered at 0.5–2.5 kHz and sampled at 2–10 kHz depending on current kinetics. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances 3–6 MΩ in whole-cell pipette solution. Access resistance (Rs) was compensated (40%–80%) when appropriate up to the value of 7–10 MΩ for whole-cell configurations. Data were rejected when Rs changed >20% during recording, leak currents were >50 pA, or input resistance was <300 MΩ. Standard external solution for whole-cell recording contained (mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 D-glucose, and 10 Hepes, pH 7.4. The standard pipette solution for whole-cell configuration contained (mM): 140 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 EGTA, 0.2 GTP, 2 ATP, 20 Hepes, pH 7.3. Drugs were applied using a fast, pressure-driven, and computer-controlled 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA). All compounds were applied for a duration of at least 1 min. Responses from transfected cells were excluded if they did not respond to capsaicin (TRPV1 transfected) or mustard oil (TRPA1 transfected) after lipid application.

**Statistics**

Data are presented as mean ± SEM. Depending upon experimental design, statistical analyses were performed using either a two-tailed Student $t$ test (two groups) or one-way or two-way repeated measures analysis of variance with the Newman-Keuls post hoc test or Dunnett’s Multiple Comparison Test when comparing to vehicle group. Data were analyzed by GraphPad Prism, including determination of area under the curve analyses. A statistically significant difference was defined as $p < 0.05$. Error bars are SEM.

**Results**

**Central TRPV1, TRPA1, and oxidative enzymes contribute to mechanical and thermal alldynia after burn injury**

As described previously, the thermal-injury model was established by standardized exposure of the plantar hind paw of an anesthetized rat to a 100°C stimulus for 30 s. Compared to baseline values, the injury produced a significant mechanical (Figure 1(a)) and thermal (Figure 1(b)) alldynia that peaked at 24 h after injury and lasted for at least seven days, returning to baseline values by day 14.

Using this preclinical burn model, we evaluated the role of spinal TRPV1 and TRPA1 in mediating post-burn mechanical and thermal alldynia at 24 h after injury using the TRPV1 antagonist AMG 517 and the TRPA1 antagonist HC-030031. Intrathecal injection of AMG 517 produced a dose-related inhibition of post-burn mechanical and thermal alldynia, with the largest i.t. dose of AMG 517 reversing post-burn
Figure 2. The role of spinal TRPV1, TRPA1, and oxidative enzymes in burn injury-induced mechanical and thermal allodynia. For antagonist studies, the paw withdrawal latencies were measured under basal conditions and at 24-h post-burn injury. Rats were then injected i.t. between lumbar vertebrae #4–5 with either the TRPV1 antagonist AMG 517 or HC-030031 (15, 50, or 165 μg in 30 μL) or saline vehicle (30 μL). Time of peak effect of antagonists is shown, 60 min for mechanical allodynia, 75 min for thermal allodynia, AMG-517 (a, b); HC-030031 (c, d) (***p < 0.001, **p < 0.01, *p < 0.05 for comparison to vehicle group, n = 4 to 8/group, error bar: SEM). (e–f) At 24-h post-thermal injury (100°C×30s), animals were injected with either control nonspecific antibody (vehicle, IgG anti-goat antibody) or
mechanical allodynia by 81% and inhibiting thermal allodynia by 98% at time of peak effect (Figure 2(a) and (b)). In addition, the largest i.t. dose of the TRPA1 antagonist HC-030031 reversed post-burn mechanical allodynia by 68% and thermal allodynia by 79% at time of peak effect (Figure 2(c) and (d)). Together, these data indicate that the activation of spinal TRPV1 and TRPA1 has a major role in mediating post-burn thermal and mechanical hypersensitivity.

Activation of TRPV1 in the spinal cord by OLAMs, specifically 9-HODE and 13-HODE, contributes to CFA-evoked mechanical allodynia as revealed by intrathecal injection of a combination of anti-HODE antibodies. More recently, we found that intraplantar injection of a combination of anti-9-HODE and anti-13-HODE antibodies at the site of burn injury also significantly reduced burn injury-evoked thermal allodynia. Based on these studies, we next evaluated whether intrathecal injection of a combination of anti-9-HODE and anti-13-HODE antibodies could reduce post-burn thermal and mechanical allodynia. Interestingly using the highest reported efficacious dose (60 μg) of anti-9-HODE and anti-13-HODE antibodies, we found no effect on either mechanical or thermal allodynia (Figure 2(e) and (f)). Nor did doubling the dose (120 μg) have an effect on post-burn thermal and mechanical allodynia. Previous reports have identified other OLAMs that are capable of activating TRPV1 as well as inducing mechanical allodynia. Unfortunately, antibodies against other OLAMs are not presently available. Therefore, we next examined the effect of intrathecal administration of ketoconazole on mechanical and thermal allodynia post-burn injury. We found that ketoconazole significantly reduced mechanical (Figure 2(g)) and thermal (Figure 2(h)) allodynia in a dose-dependent manner. The largest i.t. dose of ketoconazole reversed post-burn mechanical allodynia by 72% and inhibited thermal allodynia by 85%, revealing a pivotal role for spinal oxidative enzymatic machinery in the production of oxidized TRP-active lipids in mediating post-burn pain.

Additionally, the TRPV1- or TRPA1-dependent post-burn mechanical allodynia appears to be due to a spinal mechanism, as peripheral (intraplantar) injection of AMG 517 or HC-030031 had no effect on mechanical allodynia (Figure 2(i)). Furthermore, intraplantar injection of the largest dose of OLAM antibodies or ketoconazole at the site of burn injury in the periphery had no effect on altering mechanical allodynia.

**Burn injury increases certain subsets of oxidized metabolites of linoleic acid in the spinal cord**

No prior study has determined a lipidomic profile in the spinal cord after burn injury. Therefore, we next examined spinal cord lipids post-burn using HPLC/mass spectrometry. Unlike previous studies showing an increase of HODEs and oxoODEs in skin after burn injury, we saw no significant elevation in 9-HODE and 13-HODE or 9-oxoODE and 13-oxoODE (Figure 3(a)). However these results are consistent with the behavior data in Figure 2(e) and (f), as intrathecal injection of the antibodies to the HODEs had no effect on burn injury. The hydroxy metabolites of linoleic acid, namely, 9,10-dihydroxy-12octadecenoic acid (9,10-DiHOME) and 12,13-dihydroxy-12octadecenoic acid (12,13-DiHOME) were significantly increased in spinal cord tissue after burn injury (Figure 3(b)). Moreover, epoxide metabolites of linoleic acid, specifically 9(10)-epoxy-12-octadecenoic acid (9(10)-EpOME) and 12(13)-epoxy-12-octadecenoic acid (12(13)-EpOME), were also significantly elevated in spinal cord tissues after burn injury. Interestingly, both 9(10)-EpOME and 12(13)-EpOME were previously identified in peripheral tissues from human burn patients and circulating levels are directly related to the percentage of total body surface area damaged by the burn injury. We previously found that peripheral injection of the broad oxidative enzyme inhibitor ketoconazole was capable of significantly reducing skin levels of 9- and 13-HODE at 24 h after burn injury. When injected i.t., ketoconazole significantly reduced both 9- and 13-HODE as well as 9- and 13-oxoODE levels (Figure 3(a) and (b), gray bar), moreover, ketoconazole was also able to significantly reduce both diol and epoxide classes of linoleic acid metabolites (Figure 3(b), gray bar). Collectively, these data indicate that peripheral

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**Figure 2.** Continued

a mixture of anti-9 and 13-HODE antibodies (25, 60 μg each), and mechanical (e) or thermal allodynia was measured (f) (60 min for mechanical allodynia, 75 min for thermal allodynia, NS = not significant, n = 4/group, error bar: SEM). (g, h) To test the effect of spinal oxidative enzyme inhibition on post-burn mechanical and thermal allodynia, at 24-h post-thermal injury, animals were injected intrathecal between L4-L5 with either vehicle or ketoconazole (1.2, 4, or 13 μg in 30 μL). Time of peak effect of antagonist (60 min for mechanical allodynia, 75 min for thermal allodynia) is shown in panels g and h, respectively (**p < 0.05, ***p < 0.01, ****p < 0.001) for comparison to vehicle group, n = 4 to 8/group, error bar: SEM). (i) To test peripheral inhibition of TRPV1, TRPA1 or oxidative enzymes on post-burn mechanical allodynia, rats were injected ipl with either the TRPV1 antagonist AMG 517. TRPA1 antagonist HC-030031 (165 μg in 50 μL), oxidative enzyme inhibitor ketoconazole (13 μg in 50 μL), or saline vehicle (50 μL) into the injured (ipsilateral) hind paw. Graph represents data of time of peak effect of drugs (75 min) (**p < 0.001, NS = not significant, 4 to 7/group, error bar: SEM). Observers were blinded to treatment group.
burn injury triggers the selective elevation of epoxide and hydroxy metabolites of linoleic acid in the spinal cord via an oxidative enzymatic mechanism.

**Activation of sensory neurons by EpOMEs and DiHOMEs is TRPV1 and TRPA1 dependent**

Although the present results demonstrate significant increases in spinal levels of 9,10-DiHOME, 12, 13-DiHOME, 9(10)-EpOME, and 12(13)-EpOME after burn injury, their role in activating TRP channels was completely unknown. Using whole cell electrophysiology, the application of these metabolites triggered inward currents in sensory neurons (Figure 4(a)). We next evaluated whether these metabolites could activate various TRP channels. Interestingly, all four metabolites selectively gated TRPV1 and TRPA1 activities in expression systems but were inactive against TRPV2, -V3, -V4, or M8, (Figure 4(c)–(f)). Moreover, the intrathecal injection of a combination of these four linoleic acid metabolites (25 μg each) triggered significant mechanical and thermal allodynia, an effect blocked partially with either TRPV1 antagonist AMG (50 μg) or TRPA1 antagonist HC (50 μg), and blocked completely by combination of both antagonists (Figure 4(g) and (h)). These data reveal that the DiHOMEs and EpOME comprise a family of OLAMs released after thermal injury and capable of activating TRPV1 and TRPA1, and triggering mechanical and thermal allodynia via a spinal mechanism.

**Discussion**

Comparatively, little research has been conducted on burn pain mechanisms with comparatively little advancement in effective pain management. Pain after burn injury can persist for more than a decade and is worsened by the daily procedures patients undergo.1,22–25 Although opioids are considered a critical analgesic for treating burn-pain26 spinal levels of the mu opioid receptor are down-regulated after burn injuries,27 and rapid tolerance and opioid-induced hyperalgesia have been reported in post-burn patients.28 Thus, there is a critical need for novel approaches to treating chronic pain after burn injuries.

We previously established a role for OLAM-mediated post-burn thermal allodynia via activation of peripheral TRPV1 at the site of the burn injury.13 Ample evidence is
emerging for the role of oxidative enzyme-derived lipids as contributors to peripheral mechanisms of inflammatory and post-burn thermal and mechanical allodynia, with considerably less known about spinal mechanisms. Surprisingly, there was no change in spinal cord levels of 9-HODE or 13-HODE, or their metabolites, 9-oxoODE or 13-oxoODE after burn injury. Instead, peripheral burn injuries selectively triggered a two-fold increase in spinal cord levels of hydroxy metabolites of linoleic acid, 9,10-DiHOME and 12,13-DiHOME. Moreover, comparable increases were also found for the epoxide metabolites of linoleic acid, 9(10)-EpOME and 12(13)-EpOME.

Figure 4. Activation of sensory neurons by EpOMEs and DiHOMEs is TRPV1 and TRPA1 dependent. (a) Whole-cell currents in rat TG neurons were generated by 9,10-EpOME; 12,13-EpOME; 9,10-DiHOME and 12,13-DiHOME (100μM each), n = 5–10. (b) Representative traces of whole-cell currents evoked by 9,10-EpOME; 12,13-EpOME; 9,10-DiHOME; and 12,13-DiHOME (100 μM each). Application times are marked by horizontal lines. (c-f) Activation of TRP channels by EpOMEs and DiHOMEs. Whole-cell current densities at Vh = −60 mV activated by 9,10-EpOME (b); 9,10-DiHOME (c); 12,13-EpOME (d); and 12,13-DiHOME (e) (100 μM each) were measured from CHO cells expressing indicated TRP channels. At right, representative current-voltage (I-V) curves are shown for each drug and represented TRP channel expressed in CHO cells. (g) Mechanical allodynia or (h) thermal allodynia was measured after spinal injection of a combination of 9,10-EpOME; 9,10-DiHOME; 12,13-EpOME; and 12,13-DiHOME (25 μg each) versus vehicle (Veh) treatment. Allodynia, using methods described in Figure 1, was measured in the hind paw after injection of HC-030031 (HC, 50 μg), AMG-517 (CPZ, 50 μg), or combination of both antagonists, followed by injection of 9,10-EpOME; 9,10-DiHOME; 12,13-EpOME; and 12,13-DiHOME (25 μg each). Observers were blinded to treatment group. *p < 0.05, **p < 0.01, ***p < 0.001 for comparison to vehicle group, n = 4–6/group.
Prior studies have demonstrated that the activity of oxidative enzymes is increased after inflammation, and in the CFA model of inflammatory thermal allodynia, the broad oxidative enzyme inhibitor ketoconazole blocked linoleic acid-induced elevation of intracellular calcium in cultured neurons and significantly reduced linoleic acid-induced nociceptive behavior in rats. From this perspective, ketoconazole is a useful pharmacologic tool for probing the actions of key oxidative enzymes in mediating biological responses to tissue injury. Here, we found that i.t. injection of ketoconazole both reduced levels of epoxide and diol metabolites of linoleic acid and triggered significant reductions in post-burn thermal and mechanical allodynia. Since ketoconazole is FDA approved drug for treating fungal infections, these data suggest that it may have efficacy in treating post-burn pain.

Converging evidence indicates that oxidized lipids, derived from linoleic or arachidonic acid, are released during tissue injury and activate TRPV1 and/or TRPA1. To our knowledge, the studies reported here constitute the first report that the diol and epoxide metabolites of linoleic acid potently and selectively activate TRPV1 and TRPA1 in expression systems and trigger mechanical and thermal allodynia after intrathecal injection by activation of these TRP channels. Collectively, these data reveal that the DiHOMES and EpOMEs comprise another family of TRP-active OLAMs, the EpOMEs, and DiHOMEs. The results here and those of previous studies further establish the importance of oxidative enzymes in contributing to post-injury hypersensitivity with peripheral mechanisms contributing to post-burn thermal allodynia and spinal mechanisms contributing to both thermal and mechanical allodynia. Since clinical studies indicate that ketoconazole does not impose opioid-like adverse effects such as dependence, or TRPV1 antagonist-like adverse effects such as hyperthermia, it is possible that oxidative enzymes inhibitors may comprise a novel approach for treating post-burn pain.

Declaration of Conflicting Interests
The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The University of Texas has claimed intellectual property on this discovery. The authors have declared that no other conflict of interest exists.

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