Published in final edited form as:

Pain. 2013 November ; 154(11): . doi:10.1016/j.pain.2013.07.040.

Role of Endogenous TRPV1 Agonists in a Post-Burn Pain Model of Partial-Thickness Injury

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Abstract

Oxidized linoleic acid metabolites (OLAMs) are a class of endogenous transient receptor potential vanilloid 1 (TRPV1) channel agonists released upon exposure of tissue to transient noxious temperatures. These lipid compounds also contribute to inflammatory and heat allodynia. As persistent pain after a burn injury represents a significant clinical challenge for treatment, we developed an *in vivo* rat model of partial thickness cutaneous thermal injury and examined whether TRPV1 and specific OLAM metabolites play a role in mediating post-burn pain injury. This peripheral model of burn injury had marked thermal allodynia peaking at 24 hours post thermal injury, with allodynia being maintained for up to 7 days. Immunohistochemical characterization of tissue taken from injury site revealed an increase of leukocyte/macrophage infiltration that was co-localized with TRPV1-positive fibers. Utilizing this peripheral thermal injury model we found that pharmacological blockade of peripheral TRPV1 receptors reduced thermal allodynia by about 67%. Moreover, there was a significant increase in OLAM levels compared to naïve controls in hindpaw skin biopsies. Additional studies on metabolism of [¹⁴C]-linoleic acid in skin biopsies revealed the role of the cytochrome P450 (CYP) system in mediating the metabolism of linoleic acid post thermal injury. Finally, we demonstrated direct inhibition of OLAMs using OLAM antibodies and indirect inhibition using the CYP inhibitor ketoconazole significantly reduced post-burn thermal allodynia. Collectively, these findings point to a novel role of the OLAMs and CYP-related enzymes in generating post-burn allodynia via activation of peripheral TRPV1.

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Endogenous TRPV1 agonists, oxidized linoleic acid metabolites, are released via the cytochrome P450 enzyme system and contribute to thermal allodynia in an *in vivo* model of burn injury.

INTRODUCTION

Although the mechanisms of post-burn pain are incompletely understood, recent research has shed light on one potentially important receptor system. TRPV1, a prominent member of the transient receptor potential (TRP) family of ion channels, plays a critical role in detecting various noxious physical and chemical stimuli, including noxious heat, and contributing to inflammatory heat hyperalgesia [2,30]. Although previously studies have implicated TRPV1 in transducing thermal allodynia in the first 60 min after thermal injury, the mechanism of activation is unclear [1].

Recent studies have characterized a novel class of endogenous TRPV1 agonists, consisting of 9- and 13-hydroxy-10E, 12Z-octadecadienoic acid (9-HODE and 13-HODE) as well their metabolites, 9-oxoODE and 13-oxoODE. These oxidized linoleic acid metabolites (OLAMs) are released upon transient thermal stimulation and prolonged inflammatory tissue injury, where they activate TRPV1 and contribute to thermal allodynia [22,23,25]. The mechanism for the formation of OLAMs includes enzymatic oxidative pathways such as the cytochrome P450 class of enzymes [5,26]. Interestingly, previous studies have demonstrated that oxidized forms of linoleic acid and arachidonic acid are elevated after burn injury. These oxidized lipids have been found both in burned tissue extracts [5,6,27] as well as circulating in the vascular compartment [4,11,20]. However, to our knowledge, no study has evaluated whether OLAMs contribute to post-burn pain.

Burn injuries trigger a distinct constellation of pain mechanisms. Preclinical studies indicate that burn injuries invoke both inflammatory and neuropathic pain mechanisms [15] as well as central changes including a rapid down-regulation in expression of mu opioid receptors [29]. Because of this unusual phenotype and the continued poor clinical outcomes in treating burn pain in patients [7,21], it is important to determine mechanisms of post-burn pain using a highly reproducible preclinical model. Here we present a peripheral model of partial-thickness cutaneous thermal injury that evokes a highly reproducible thermal allodynia. We then used this model to determine whether OLAMs contribute to the development of post-burn nociception.

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) were used for all studies. Animals were housed for at least 7 days prior to the experiments.

Thermal Injury

Animals were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL) and a surgical plane of anesthesia was confirmed with a negative response to tail pinch. Thermal injury was induced by exposing an area of plantar hindpaw skin to a 100°C thermal stimulus for 30 sec. To promote reproducible thermal injuries, the same 1 cm × 2 cm region of the hindpaw was exposed in each animal (Fig 1A), a stable stimulus temperature was maintained by a heating block (Fischer Scientific Pittsburgh, PA) and consistent hindpaw contact with the heated surface was achieved by placing a 30 g weight onto the dorsal hindpaw. 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.

Immunohistochemistry

One day following the hindpaw burn injury, animals were sacrificed and the hindpaw removed. The hindpaw was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 45 min, rinsed in 0.1 M PB, and placed in 0.1 M PB with 30% sucrose overnight at 4°C. The next day the hindpaw tissues were removed, embedded in Neg-50 (Richard-Allan Scientific; Kalamazoo, MI) and the skin serially sectioned with a cryostat at 30 µm sections in the transverse plane. Sections were placed onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air dried and stored at -20°C until stained with the indirect immunofluorescence method as previously described [12]. This staining included the use of primary antibodies against TRPV1 (rat-specific guinea pig polyclonal from Neuromics, GP14100; 1:2000 dilution) in combination with primary antibodies against specific immune cell-type markers including CD45 (to identify leukocytes; rat-specific mouse monoclonal from AbD Serotec, MCA43R; 1:100 dilution) or CD68 (to identify macrophages/monocytes; rat-specific mouse monoclonal from AbD Serotec, MCA341R; 1:100 dilution). Sections were incubated in Alexa Fluor secondary antibodies (1:200; Molecular Probes, Eugene, OR) including 488 goat anti-mouse to visualize immune cell markers and 568 goat anti-rabbit to visualize TRPV1. TO-PRO-3 iodide (Topro, Invitrogen, Carlsbad, CA; 1:5,000 dilution) was used as a nuclear stain and was added with secondary antibodies. Hindpaw skin specimens were evaluated and optical images collected with a Nikon Eclipse 90i microscope equipped with a C1si laser scanning confocal imaging system (Nikon Corporation, Melville, NY). Images were processed for illustration purposes with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). Control preparations included samples stained like experimental preparations but with the following exceptions: 1) no primary and no secondary antibody, and 2) no primary but with secondary antibody. Control preparations revealed typical autofluorescence in the stratum corneum but otherwise lacked fluorescence signal within other regions when obtained with the same laser and microscope settings used to evaluate experimental specimens.

Drugs

Ketoconazole was purchased from Tocris (Ellsville, Missouri) and was diluted in 32% methylpyrrolidinone (MPL)/phosphate buffered saline (PBS) to make a stock of 18mM and further diluted in saline on the day of each experiment. The TRPV1 antagonist AMG 517 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). As a control, a nonspecific goat IgG antibody was purchased from Sigma Aldrich (St. Louis, MO).

Behavioral Testing

All observers were blinded to treatment allocation with an n=5–7 per group. Paw withdrawal latencies to radiant heat were tested using methods previously described [10]. At 24 hours post thermal injury, a combination of anti-9- and anti-13-HODE antibodies (25 µg each) or control non-specific IgG antibodies (50 µg) were injected into the intraplantar (ipl) skin of the injured hindpaw in a 50 µl volume. Thermal thresholds were measured 60 minutes after injection. To examine the effects of CYP inhibition on peripheral burn injury, ketoconazole (4 µg in 50 µl), a broad CYP inhibitor [13], was injected ipl 24h after thermal injury, with testing at 60 min after injection. To assess the effects of TRPV1 inhibition on peripheral burn injury, AMG 517 (50 µg in 30 µl) was injected into the ipl skin of the injured hindpaw. Thermal thresholds were measured in an area adjacent to the site of burn injury at 15, 30, 60, and 120 minutes after injection (Fig 1A).

Preparation of Skin and Plasma Lipid Extracts

For measurement of skin lipids, a total of six 6 mm hindpaw biopsy punches were collected from three animals (two biopsies/hindpaw), pooled and diced into small (~3–4mm³) pieces in Hank's Balanced Salt Solution (HBSS) containing CaCl₂ 2 mM, NaHCO₃ 4 mM, and HEPES 10 mM buffer. As described previously [8], a chloroform: methanol solution (1ml of 4:1) was added to a tube containing the skin sample and a 7mm stainless steel bead (Qiagen Inc, Valencia CA). The tissue was lysed in a Qiagen Tissue Lyser™ for 5 min at 50 oscillations/sec, followed by a second incubation with an additional 6 ml of chloroform:methanol for 1.5 hr on a shaker. The extract solution was washed with 0.9% saline (1.6 ml), and centrifuged (1200 g for 5 min), with collection of the lower organic layer followed by drying under N₂. The dried skin extracts were analyzed by Cayman Chemicals (Ann Arbor, Michigan) for high performance liquid chromatography/mass spectrometry (HPLC-Mass Spec analysis) using a Thermo Electron Quantum Ultra™ MS unit. Using synthetic 9-HODE, 13-HODE, 9-oxoODE, 13-oxoODE, the resulting standard curves were linear ($r^2 = 0.988 - 0.999$) over a range of 0.25–2500 ng/ml.

Lipophilic Extract Preparation with C¹⁴ radio labeled linoleic acid ([C¹⁴]-LA) and HPLC Analysis

To determine the rate and mechanism of OLAM formation, biopsy punches (6 mm) of either control or burned (24h post-injury) rat hindpaws were diced into smaller pieces (~3–4mm³), transferred to 24 well tissue culture plates containing HBSS (2ml), washed (20 min at 37°C) and then transferred to wells containing ketoconazole (150 uM) or vehicle (32% MPL) in HBSS (2 ml) for 40 min at 37°C. Each group was then transferred to separate wells containing [C¹⁴]-LA (100 µM in 2ml) made in HBSS with vehicle or ketoconazole (150 uM) for 1 hr at 37°C. The superfusates were collected using C₁₈-SepPak™ column (Waters, Inc) as described previously [22] and dried under a stream of N₂. The dried samples were then subjected to HPLC/radiometric analysis for evaluating metabolite formation.

The [C¹⁴]-labeled linoleic acid metabolites were measured using HPLC as described [19] with the following modifications. Dried linoleic acid/metabolite mixtures were re-suspended in 300 µl of 50% methanol in water. Metabolites (120 µl) were separated from the parent [C¹⁴]-LA by reverse phase HPLC using a Gemini 5µm C18 column (Phenomenex) on a Shimadzu Liquid Chromatograph equipped with a -RAM radiometric HPLC detector (IN/US Systems). The elution was isocratic with a 48:52 mixture of 100% acetonitrile: water. Control experiments indicated that application of [C¹⁴]-LA standards eluted at 25 min. Data were analyzed by subtracting area under the curve of hydrophilic C¹⁴ metabolites from the area under the curve of the [C¹⁴]-LA parent compound.

Statistics

Data are presented as mean ± SEM. Depending upon experimental design, statistical analyses were performed using either a 2-tailed Student's *t* test (2 groups) or one- or two-way ANOVA with Bonferroni's post-hoc test (>2 groups). Data were analyzed by GraphPad area under the curve analyses. A statistically significant difference was defined as $P < 0.05$. Error bars are S.E.M.

RESULTS

Rat model of thermal injury results in persistent thermal allodynia

The thermal injury model was established by standardized exposure of the plantar hindpaw to a 100°C stimulus for 30 sec. Compared to baseline data taken pre-injury, the injury produced a significant thermal allodynia that peaked at 24h after injury and lasted for at least 7 days, returning to baseline values by day 14 (Fig 1A). At the primary site of injury,

encompassing the digits and metatarsal pads, the paw displayed hyperemia and blistering with discoloration of the digits. The tarsal pad region was at the proximal transition area to un-injured skin and exhibited hyperemia and slight edema without blisters; this was the area where behavioral tests were performed (Fig 1A insert). Histological evaluation of the hindpaw skin 24 hours after burn injury revealed the common presence of a blister (Fig 1B). The blister was typically located on the anterior part of the plantar hindpaw in the region covered by the metatarsal pads and located just posterior to the toes. The blister resulted from the splitting of the epidermis from the dermis (Fig 1B). Staining of the tissue with antibodies to immune cell-type markers demonstrated that the dermis was heavily infiltrated with CD68⁺ macrophages/monocytes (Fig 1B). This infiltration was extensive throughout the injured hindpaw tissues and also was seen within the center of the foot (just posterior to the metatarsal pads and blister) where the behavioral sensory testing was performed.

Immune cell infiltration and TRPV1 positive nerve fiber expression at site of thermal injury

Additional immunohistochemical studies evaluated for the presence of TRPV1⁺ nerve fibers and immune cells within the area where sensory testing was performed. At 24h after burn injury, many TRPV1⁺ nerve fibers were located among a dense infiltration of both CD45⁺ cells (a panleukocyte marker; Fig 2A) and CD68⁺ macrophages/monocytes (Fig 2B). The TRPV1⁺ fibers were common at the epidermis-dermis interface and within the epidermis, while the inflammatory cells were mostly located throughout the dermis. Together, these results demonstrate that this model produces a consistent thermal allodynia due to a superficial partial-thickness burn injury [14] with blister formation, a robust inflammatory cell response and an intact epithelium with TRPV1⁺ nerve fibers within the center of the hindpaw where sensory testing is performed. the contralateral, , the**0 (Fig 3D) Similarly when evaluating the area of the dermis above the blister, PGP9.5 nerve fibers 24 hours after burn injury are significantly reduced (1.44% 0.97; *p<0.05), compared to the contralateral hindpaw dermis (2.05% 0.79).Theses are present and that the observed are F

Peripheral TRPV1 contributes to thermal allodynia after burn injury

We next evaluated the role of TRPV1 in mediating post-burn thermal allodynia at 24h after injury using AMG517, a specific TRPV1 antagonist [9]. Peripheral injection of the TRPV1 antagonist post-burn blocked ~67% of thermal allodynia largest (Fig 3). largest, In addition, the TRPV1-dependent post-burn thermal allodynia is due to a peripheral mechanism in the burned tissue since injection of AMG517 into the contralateral hindpaw was without effect Fig 1. Together, these data indicate, that while non-TRPV1 post-burn pain mechanisms may exist, the activation of peripheral TRPV1 has a major, dominant role in mediating post-burn thermal allodynia.

Oxidized Linoleic Acid Metabolites (OLAMs) are elevated in tissue and plasma post thermal injury

Levels of OLAMs have been found clinically to be elevated following severe burn injury, with increasing levels of OLAMs correlating to increasing burn surface area in patients [16]. Consequently, we examined levels of OLAMs in skin at the site of injury 24 h post thermal injury using HPLC-MS. The results indicate a significant increase in skin levels of 9- and 13- HODE compared to amounts found in skin from naïve control animals (Fig 4A,B, p<0.05). For oxoODE levels, there was a significant increase in both 9,10- and 12,13-oxoODE in skin following thermal injury (Fig 4C,D, p<0.05).

Oxidative Enzyme Activity is Elevated in Thermally Injured Tissue Mediating OLAM Production

Recent work by our group has shown that the family of cytochrome P450 oxidative enzymes is involved in mediating linoleic acid metabolism in cultured sensory neurons [26] and that pharmacological inhibition of P450 enzymes significantly reduces thermal allodynia in the CFA model of peripheral inflammation [25]. We next determined if OLAM-CYP machinery was increased in a model of thermal injury. Biopsies of either post-burn (24h) or control hindpaw skin were pretreated with either the CYP inhibitor ketoconazole or vehicle, and then exposed to [C^{14}]-linoleic acid with subsequent HPLC/radiometric analysis of [C^{14}]-tagged metabolites (Fig 5A–D). At 24hr after burn injury, there was a significant, near ten-fold increase in metabolism of C^{14} -linoleic acid (Fig 5D), that was characterized by increased formation of hydrophilic [C^{14}]-metabolites (Fig 5A–B). Pretreatment with ketoconazole reduced the metabolism of linoleic acid (Fig 5C), to levels comparable to baseline values (Fig 5D). These data suggests that the enzymatic machinery for the oxidation of LA into its hydrophilic metabolites is upregulated after burn injury.

Immunoneutralization of OLAMs as well as CYP inhibition reduces thermal allodynia in a model of thermal injury

To determine the role of the HODEs in mediating post-burn thermal allodynia, we injected ipl a combination of anti-9- and anti-13-HODE antibodies (25 μ g each) or control IgG antibodies (50 μ g) at the site of thermal injury. Peripheral administration of the anti-HODE antibodies significantly reduced thermal allodynia compared to animals injected with nonspecific IgG control antibodies (Fig 6A, $**p<0.01$). time-response analyzed In contrast, injection of the largest dose of antibodies into the uninjured contralateral paw had no effect on altering thermal allodynia in the ipsilateral injured paw (Supplemental Fig 1). These data confirm the *in vivo* role that OLAMs play in mediating post-burn allodynia at the site of tissue injury. We next examined if inhibition of oxidative enzyme activities could reduce thermal allodynia caused by a peripheral burn injury. Similar to the antibody data, ketoconazole significantly reduced thermal allodynia (Fig 6B largest, $*p<0.05$). In contrast, injection of the largest dose of ketoconazole into the uninjured contralateral paw had no effect on altering thermal allodynia in the ipsilateral burned hindpaw (Supplemental Fig 1). Taken together, these data implicate a pivotal role for a peripheral mechanism of oxidative enzymatic production of OLAMs in mediating post-burn pain.

DISCUSSION

OLAMs have been found to be elevated in tissue of burn patients, including the burn eschar [3,4]. The OLAMs serve as endogenous agonists of TRPV1, and are acutely released due to transient application of noxious heat [22]. Together, these data are consistent with the hypothesis that these oxidized lipids may contribute to post-burn thermal allodynia. Accordingly, we tested this hypothesis by establishing a model of persistent thermal injury. The data presented herein has expanded the role of OLAMs to not only mediate acute responses to transient, noninjurious thermal stimulation [22,23] and inflammation [25], but also contribute to peripheral mechanisms of thermal allodynia after cutaneous burn injury.

The exposure to a 100°C stimulus for 30 sec evoked a highly reproducible superficial partial-thickness second degree burn injury that was spatially restricted and well tolerated by the animals. The partial-thickness injury was histologically confirmed by separation of dermis from epidermis. By 24 h, the site of thermal injury was densely populated by CD45⁺ and CD68⁺ positive cells with an intact epidermis. This staining revealed a striking co-localization with TRPV1⁺ neurons. Since prior studies have shown that immune cells contain oxidative enzymes and release OLAMs [17,28], it is possible that localized

accumulation of immune cells in burned tissue contributes to the development of post-injury allodynia/hyperalgesia. Indeed, at the local burn site, afferent terminals may be exposed to a variety of leukocyte-derived substances [24]. Although our previously published studies primarily focused on the autocrine action of OLAMs in neurons [22,25], here it appears that peripheral nociceptor activation may occur via a paracrine OLAM mechanism. This is further strengthened by the finding that ipl injection of anti-OLAM antibodies are capable of reducing post-burn thermal allodynia since these proteins are unlikely to cross plasma membranes.

Knock-out studies have implicated TRPV1 mediating development of acute thermal allodynia that occurs within an hour after thermal injury [1]. Our findings using the specific TRPV1 antagonist AMG517 confirm a role for TRPV1 in mediating thermal allodynia at 24h after injury. Indeed, peripheral TRPV1 activation appears to be a dominant post-burn mechanism since ipl injection of AMG517 reversed ~67% of the thermal allodynia. Based on the role of peripheral TRPV1 in mediating post-burn pain, we next evaluated whether the OLAM system was activated and functional after thermal injuries.

Tissue levels of 9- and 13-HODE and their oxoODE metabolites were significantly elevated in hindpaw skin at 24h after burn injury. All four of these OLAMs are efficacious in activating TRPV1 [22]. The differences in tissue levels of the various OLAMs could be due to upregulation of oxidative enzymes with differing oxidative mechanisms [18,26] or to differences in lipid stability. Based on these findings, we next evaluated whether oxidative enzymes may be involved in mediating the release of OLAMs after thermal injury. As mounting evidence has implicated the oxidative CYP system in mediating the release of OLAMs from neurons or inflamed tissue [25,26], we tested this hypothesis with the CYP inhibitor ketoconazole. Analysis of [C^{14}]-tagged linoleic acid revealed a significant increase in linoleic acid metabolism after burn injury that was blocked by pretreatment with ketoconazole. Although these findings provide strong support for an oxidative enzyme-mediated formation of linoleic acid metabolites after burn injury, future studies will be required to identify which enzyme(s) mediate this effect.

Finally, we found that direct inhibition of OLAMs via ipl injection of 9- and 13-HODE antibodies reduced post-burn thermal allodynia. Moreover ipl injection of ketoconazole significantly reduced thermal allodynia post injection. These findings point to two possible classes of novel analgesics, with direct blockade of OLAMs by immunoneutralization and indirect inhibition via the enzymes responsible for their formation for treating post-burn allodynia/hyperalgesia. Interestingly, the AMG517 experiment indicated about a 67% reversal of post-burn thermal allodynia, and this was similar to the magnitude of the anti-OLAM and ketoconazole interventions.

Taken together the studies have established a role for OLAMs in mediating post-burn thermal allodynia via a paracrine mechanism. Future studies will investigate the cellular origins of OLAMs as well as the specific oxidative enzymes involved in their formation. Identification of the oxidative enzymes-OLAM pathway may provide novel targets for developing analgesics useful for treating post-burn pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported by NCATS 8UL1TR000149, R01NS72890, R01DA019585, the US Army W45MW30167N901, the Owens Foundation and the USAA Foundation President's Distinguished University Chair in Neurosciences (KMH), and F31NS082019 (DG).

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Figure 1. Characterization of peripheral thermal injury on thermal allodynia

Thermal injury was induced by exposing a 1×2 cm area of the plantar surface of the hindpaw of isoflurane-anesthetized rats to a metal heating block maintained at 100°C for 30 sec, (A) paw withdrawal thresholds to a beam of radiant heat were measured daily after injury Behavioral testing was performed on the injured (ipsilateral) and uninjured (contralateral) hindpaws. (** $p < 0.01$, **** $p < 0.0001$, $n = 6-9$ /group, error bar: S.E.M.) Image inset illustrates the area of injury and site of behavioral testing. (B) Confocal image of the rat hindpaw 24 hours following burn injury shows prominent CD68⁺ macrophage/monocyte expression (green) throughout the dermis. Nuclei are identified with Topro (blue). The burn injury produces a blister that is associated with a splitting of the epidermis from the dermis (arrow). The arrowhead indicates the region where behavioral sensory testing is performed.

Figure 2. TRPV1 fibers remain in areas occupied by immune cells (A–B)

Confocal images of the rat hindpaw 24 hours following burn injury shows many cells expressing the CD45⁺ pan-immune cell marker (A) and CD68⁺ macrophages/monocytes (B) immune cells (green) in the dermis and the presence of many TRPV1-positive nerve fibers (red; arrows) within the epidermis and at the epidermis-dermis interface. Topro (blue) identifies nuclei and scale bar applies to A–B. Blister appears as a space between dermis and epidermis (white arrow) 24 hours after burn injury (C). (D) Graph summarizing PGP9.5 positive nerve fiber reduction in normal versus burn tissue (** $p < 0.01$) and innervation in normal vs blister dermis (* $p < 0.05$)

Figure 3. The role of TRPV1 in thermal injury induced thermal allodynia

Paw withdrawal latencies were measured under basal conditions and at 24h post-burn injury. Rats were then injected ipl with either the TRPV1 antagonist AMG 517 (50 μg) or saline vehicle (50μL) into either the injured (ipsilateral) or un-injured (contralateral) hindpaws and were tested 45 min later. (**p<0.01, n = 5–7/group, error bar: S.E.M.).

Figure 4. The effect of thermal injury on OLAM levels in skin
 (A–B) 9- and 13-HODE as well as (C–D) 9- and 13-oxoODE concentrations were measured in skin biopsies collected 24 h after thermal injury (* $p < 0.05$, $n = 5-7$ /group, error bar: S.E.M.) using HPLC/mass spectrometry. Skin was harvested using a 6 mm biopsy punch taken at site of thermal injury with lipids extracted by chloroform:methanol. Data was analyzed using 2-tailed Student's t-test. (* $p < 0.05$, $n = 5-7$ /group, error bar: S.E.M.).

Figure 5. Effect of CYP inhibition on the formation of linoleic acid metabolites in a model of thermal injury

Biopsies (6mm) from thermally injured rat hindpaw skin were pretreated with either vehicle or the CYP inhibitor ketoconazole and exposed to [C^{14}]-linoleic acid for 1h at 37°C and the superfusates were evaluated for [C^{14}]-labeled metabolite formation by HPLC/radiometric analysis. (A) HPLC chromatogram with a peak corresponding to linoleic acid at a retention time of 25 minutes in normal biopsy tissue, and at 24 hours post thermal injury (B). HPLC chromatogram of thermally injured tissue exposed to C14-LA with 150 μ M ketoconazole (C). A graphical representation of the HPLC chromatograms displays data as percent area under the curve on the y-axis (D) (* p <0.05, n = 4/group, error bar: S.E.M.).

Figure 6. The effects of inhibition of OLAms by immunoneutralization and inhibition of oxidative enzymes by ketoconazole on post-burn thermal allodynia
(A) At 24h post-thermal injury (100°C × 30s), animals were injected with either control nonspecific antibody (“vehicle”, IgG anti-goat antibody) or a mixture of anti-9 and 13-HODE antibodies (25ug each) and thermal allodynia measured one hour later (**p<0.01, n = 5–6, error bars: S.E.M.). **(B)** At, 24 hours after thermal injury rats were injected with vehicle or ketoconazole (4 ug), and tested one hour later (*p < 0.05, n = 5–6, error bars: S.E.M.). Observers were blinded to treatment group. **Ipsilateral pw lies at 24h after burn injury and ipl injection of into the uninjured contralateral paw. All measures collected by observers blinded to treatment allocation.** **A** „when injected into the un-injured contralateral hindpaw, resulted in reduction in thermal allodynia in the thermally injured ipsilateral hindpaw. **B** Graphical representation plotted as a each compound (NS=not significant).

Figure 7.