Role of Oxidized Lipids and TRP Channels in Orofacial Pain and Inflammation

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Abstract

Acute or chronic inflammation comprises a highly prevalent type of orofacial pain and is mediated by the generation of endogenous agonists that activate numerous receptors expressed on terminals of trigeminal (TG) nociceptive afferent neurons. One such studied receptor is transient receptor potential vanilloid subtype 1 (TRPV1). TRPV1 is a ligand-gated cation channel that is expressed on a major subclass of nociceptors and is found in many orofacial tissues, including dental pulp. Antagonists to TRPV1 reveal an important role for this channel in mediating hypersensitivity in preclinical models of inflammatory or neuropathic pain. Recent studies have demonstrated that endogenous TRPV1 agonists are generated by oxidation of omega-6 polyunsaturated fatty acids, including both linoleic acid and arachidonic acid. A major mechanism triggering the release of oxidative linoleic acid metabolites (OLAMs) and oxidative arachidonic acid metabolites (OAAMs) is the action of oxidative enzymes. Oxidative enzymes such as cytochrome P450 isozymes are rapidly upregulated in TG neurons after orofacial inflammation and increase the capacity of TG neurons to generate OLAMs. Cytochrome P450 isozymes are also increased in immune cells in irreversibly inflamed human dental pulp, and extracts of this tissue have significantly increased capacity to generate OLAMs. Together, these studies point to a novel pain mechanism involving the enzymatic generation of endogenous OLAM and OAAM agonists of TRPV1. This finding provides a rationale for an entirely new class of analgesics by inhibition of oxidative enzyme activity.

Keywords: linoleic acid, arachidonic acid, cytochrome P450, pulpitis, TRPV1, capsaicin

Introduction

Chronic orofacial pain is a major medical and social problem. This issue was emphasized in a recent US Surgeon General's report on orofacial health by concluding that "oral health means much more than healthy teeth. It means being free of chronic oral-facial pain conditions" (US Department of Health and Human Services 2000). Community-based surveys indicate that many participants commonly report pain in the orofacial region, with estimates of >39 million, or 22% of Americans older than 18 years of age, in the United States alone (Lipton et al. 1994). These reported rates are similar to other population-based surveys conducted in the United Kingdom (Macfarlane et al. 2002; Macfarlane et al. 2004), Germany (John, LeResche, et al. 2003), or other locations (Dworkin et al. 1990). Importantly, chronic widespread body pain, patient sex and age, and psychosocial factors appear to serve as risk factors for chronic orofacial pain (LeResche 1997; Aggarwal et al. 2003; John, Miglioretti, et al. 2003; Portenoy et al. 2004; Aggarwal et al. 2010). Therefore, it is imperative that basic research on orofacial pain mechanisms continues to develop novel therapeutics (Gibbs and Hargreaves 2013).

Orofacial pain is derived from many unique target tissues such as the meninges, cornea, tooth pulp, oral/nasal mucosa, and temporomandibular joint (Hargreaves 2011; Gibbs and Hargreaves 2013). Indeed, the trigeminal sensory system has several physiologic characteristics that are distinct from the spinal nociceptive system under either basal (Bereiter et al. 2008) or postinjury (Hargreaves 2011) conditions. Given these

considerations, it is not surprising that accurate diagnosis and effective management of orofacial pain conditions represent a significant health care problem. Orofacial structures such as dental pulp are heavily innervated (Fig. 1A) and may have a distinct class of nociceptive afferent neurons not found in other tissues (Fried et al. 2011).

Inflammatory Pain Transduction

Tissue damage, due to noxious physical, chemical, or mechanical stimuli, constitutes a major challenge to homeostasis that disrupts tissue integrity and triggers a coordinated and diverse response mediated by effector cells such as immune cells, peripheral neurons, fibroblasts, odontoblasts, and endothelium. A hallmark feature of such damage is the release of inflammatory mediators that activate receptors leading to altered cellular activities. As illustrated in Figure 1B, many of these mediators bind to receptors expressed on afferent terminals, leading to dramatic changes in neuronal excitability (Henry and Hargreaves 2007). For example, human dental pulp and trigeminal ganglion (TG) neurons express the TLR-4 receptor, and application of bacterial

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Figure 1. Examples of innervation patterns of human dental pulp and an illustration of major receptor classes expressed on trigeminal nociceptive neurons. **(A)** Confocal micrographs of nerve fibers in human dental pulp as identified with the neuronal marker PGP9.5 (red) located within fiber bundles (large arrow) and small axons that traverse the odontoblastic layer (small arrow). Scale bar = 100 μ m. **(B)** Nerve fibers within the radicular pulp express sodium channels (red) that are prominent at nodes of Ranvier (arrow) as identified by paranodal staining of caspr (green). Scale bar = 20 μ m. The lower panel is a cartoon depicting major classes of receptor or ion channels proposed to be present on peripheral terminals of sensory neurons that serve to transduce external stimuli into altered neuronal function. Not all receptors/ion channels are present on all neurons, and several have been shown to be altered during inflammation or nerve injury. CGRP, calcitonin gene-related peptide; GABA (B), Gamma Aminobutyric Acid receptor subtype B; IL1-R, interleukin 1-R; PG, prostaglandin; NPY 1, Neuropeptide Y Receptor subtype 1; PAR-2, protease-activated receptor subtype 2; TNF α , tumor necrosis factor α ; TRPA1, transient receptor potential A1; TRPM8, transient receptor potential M8; TRPV1, transient receptor potential vanilloid subtype 1 (aka the capsaicin receptor); VGCC, voltage-gated calcium channel; VGKC, voltage-gated potassium channel; VGSC, voltage-gated sodium channel. Figures modified from Henry and Hargreaves (2007).

endotoxins directly activates and sensitizes TG neurons, providing a mechanism for pain due to orofacial infections (Wadachi and Hargreaves 2006; Diogenes et al. 2011; Ferraz et al. 2011). In this review on inflammatory orofacial pain mechanisms, we focus on one example of this response: the release of endogenous soluble factors that lead to activation of the TRP vanilloid subtype 1 (TRPV1) receptor.

Transient Receptor Potential Channels

The family of transient receptor potential (TRP) receptors is an evolutionarily conserved group of ligand-gated ion channels that respond to environmental stimuli, including soluble factors, temperature (heating or cooling), oxidative stressors, and mechanical stimuli (Mickle et al. 2015; Smani et al. 2015; Parenti et al. 2016). Members of the TRP family are expressed in orofacial structures, including TG neurons, odontoblasts, vasculature, immune cells, fibroblasts, and mesenchymal stem cells (Park et al. 2006; Fehrenbacher et al. 2009; Son et al. 2009; El Karim et al. 2011; Kim et al. 2012; Yajima et al. 2015). TRPV1 has received major attention as it is expressed on a large subclass of nociceptive afferent neurons (Julius 2013), and antagonists to this receptor are effective in preclinical models of inflammatory or neuropathic pain (Gavva et al. 2005; Biggs et al. 2008; Green et al. 2013; Lima et al. 2014; Uchytlova et al. 2014). As illustrated in Figure 2, human

dental pulp is heavily innervated with neurons that coexpress both calcitonin gene-related peptide (CGRP) and the TRPV1 receptor (Fehrenbacher et al. 2009). Moreover, application of capsaicin to human dental pulp leads to a concentration-dependent release of CGRP that is blocked by pretreatment with a TRPV1 antagonist (Fig. 3). Collectively, these data are consistent with the hypothesis that orofacial tissues may respond to inflammation via transduction mediated by TRP channels.

Endogenous TRP Agonists

TRPV1 was originally reported to be a thermoreceptor with an activation threshold of about 43°C (Caterina et al. 1997). However, more recent studies indicate that this ligand-gated ion channel responds not only to exogenous substances, such as capsaicin, but also to endogenous factors released during tissue injury. For example, oxidation of omega-6 polyunsaturated fatty acids (PUFAs) releases lipid metabolites that potentially activate TRPV1 (Patwardhan et al. 2010; Sisignano et al. 2014). These substances include oxidative linoleic acid metabolites (OLAMs) as well as oxidative arachidonic acid metabolites (OAAMs) (Patwardhan et al. 2010; Sisignano et al. 2014). The OLAMs comprise 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE, and their corresponding oxo metabolites, 9-oxoODE and 13-oxoODE. Heating isolated rat skin biopsy specimens leads to a temperature-dependent release of

9-HODE (Fig. 4A), and application of this OLAM generates inward currents in capsaicin-sensitive TG neurons (Fig. 4B), triggers intracellular calcium accumulation in TG neurons (Fig. 4C), produces thermal hyperalgesia in wild-type but not TRPV1^{-/-} mice (Fig. 4D), and evokes CGRP release (Fig. 4E) (Patwardhan et al. 2010). Research from our group demonstrates that tissue injuries as diverse as acute heating of isolated skin, partial-thickness burn injuries, irreversible pulpitis due to bacterial infection, and injection of complete Freund's adjuvant (CFA) or nerve growth factor (NGF) all lead to activation of the OLAM system both in the injured tissues and in the central nervous system (Patwardhan et al. 2009; Patwardhan et al. 2010; Ruparel, Green, et al. 2012; Ruparel, Henry, et al. 2012; Ruparel et al. 2013; Green et al. 2013; Eskander et al. 2015). In addition, certain oral squamous cell carcinoma tumors release lipid TRPV1 agonists, suggesting a novel mechanism for oral cancer pain (Ruparel et al. 2015). Collectively, these studies have led to a new perspective on inflammatory pain due to the release of endogenous agonists that activate TRP channels such as TRPV1.

The hypothesis that tissue injury evokes the release of oxidized TRPV1-active lipids has novel therapeutic implications. For example, application of antibodies to OLAMs significantly reduces thermal and mechanical hypersensitivity after either peripheral or intrathecal injections (Patwardhan et al. 2009; Patwardhan et al. 2010; Ruparel, Green, et al. 2012; Green et al. 2013). In addition, the formation of oxidized TRPV1 lipids appears to be enzymatically mediated as administration of antioxidants such as N-acetyl-cysteine has little to no effect in preclinical pain models (Ruparel, Henry, et al. 2012; Eskander et al. 2015). Instead, enzymatic oxidative mechanisms for the generation of endogenous TRPV1 agonists have been implicated. For example, the application of linoleic acid (LA) to TG cultures induces a TRPV1-mediated increase in intracellular calcium as demonstrated by blockade with I-RTX, a TRPV1 antagonist (Fig. 4F) (Ruparel, Henry, et al. 2012). This provides a useful assay to determine mechanisms for generation of TRPV1-active OLAMs. Importantly,

Figure 2. A representative confocal image evaluating the expression patterns of calcitonin gene-related peptide (CGRP) and transient receptor potential vanilloid subtype I (TRPV1) in nerve fibers located in the human coronal dental pulp. The inset indicates the area of the pulp represented by the image in panel A. (B–D) Magnified images from the area enclosed by the white square in panel A. TRPV1 (red) and CGRP (green) immunoreactivities were often coexpressed in the same nerve fibers (C). Nerve fibers containing N52 are represented in blue. Figure taken from Fehrenbacher et al. (2009).

Figure 3. Effect of capsaicin applied to acutely isolated human dental pulp on release of calcitonin gene-related peptide (CGRP). (A) Effect of calcium-free buffer and the capsaicin receptor antagonist capsazepine on basal (BL) and capsaicin (CAP)-evoked immunoreactive calcitonin gene-related peptide (iCGRP) release. The first pair of columns represents basal and stimulated release elicited by capsaicin (60 µM). The second pair of columns corresponds to basal and capsaicin-stimulated release in calcium-free buffer containing ethylene glycol tetraacetic acid (EGTA) (10 mM). The last pair of columns represents basal and stimulated release in the presence of the transient receptor potential vanilloid subtype I (TRPV1) antagonist capsazepine (300 µM). (B) Concentration-response curve of capsaicin-induced iCGRP release from human dental pulp. Each biopsy was exposed to only 1 concentration of capsaicin (to avoid TRPV1 desensitization). Figure taken from Fehrenbacher et al. (2009). n.s., nonsignificant; **P* < 0.05 vs BL (Panel A) or 0 (Panel B); ***P* < 0.01 vs 0; † < 0.05 vs Veh/Cap group.

LA-induced generation of inward $[Ca^{2+}]$ is dependent on heme enzymes as indicated by application of carbon monoxide, requires the CYPOR enzyme as indicated by pretreatment with DPI, and is blocked by ketoconazole, a broad cytochrome

Figure 4. Characterization of release and actions of 9-hydroxyoctadecadienoic acid (9-HODE). **(A)** Temperature-dependent release of 9-HODE from heated skin as measured by high-performance liquid chromatography/mass spectrometry. **(B)** Generation of inward currents induced by application of 9-HODE and capsaicin (cap) to cultured rat trigeminal (TG) neurons as measured by patch-clamp electrophysiology. **(C)** Comparison of 9-HODE-evoked accumulation of intracellular Ca^{2+} applied to TG neurons from wild-type (WT) or TRPV1 $^{-/-}$ mice. **(D)** Evaluation of 9-HODE on thermal hyperalgesia after peripheral injection into either wild-type (WT) or TRPV1 $^{-/-}$ mice. **(E)** Evaluation of various enzymes inhibitors on linoleic acid-induced accumulation of intracellular Ca^{2+} applied to rat TG neurons. BL, basal; iCGRP, immunoreactive calcitonin gene-related peptide; KO, knockout; TRPV1, transient receptor potential vanilloid subtype 1. Panels A to D taken from Patwardhan et al. (2010). Panel E modified from Ruparel, Henry, et al. (2012). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.005$.

P450 (CYP) enzyme inhibitor (Fig. 4F) (Ruparel, Henry, et al. 2012). In contrast, inhibitors to nitric oxide, lipoxygenase, or free radicals had no effect on LA. Collectively, these data indicate that TG cells generate TRPV1 agonists via CYP oxidation of LA.

Demonstration that CYP mediates the generation of endogenous TRPV1 agonists has several important physiologic implications. First, since ketoconazole blocks the *in vitro* generation of TRPV1 agonists from LA (Ruparel, Henry, et al. 2012), it is possible that this drug has unexpected *in vivo* analgesic activities. Indeed, several studies demonstrate that ketoconazole blocks thermal hyperalgesia in CFA and postburn models of pain (Ruparel, Henry, et al. 2012; Green et al. 2013). In addition, nordihydroguaiaretic acid (NDGA) also blocks the formation of OLAMs and reverses thermal hyperalgesia due to NGF administration (Eskander et al. 2015). Finally, injury models lead to a PLA2-dependent release of OLAMs and OAAMs, as indicated by inhibition of lipid metabolite formation with PLA2 inhibitors such as bromoenol lactone (Eskander et al. 2015). These findings have led to the hypothesis that tissue injury leads to a PLA2-dependent release of LA or arachidonic acid from the cellular membranes followed by enzymatic formation of OLAMs or OAAMs. Taken together, these studies suggest that enzymatic inhibitors of OLAM and OAAM formation constitute a novel class of analgesic drugs for treatment of inflammatory pain.

While CYPs are an extremely well-characterized class of oxidative enzymes, nearly all studies to date have focused on nonneuronal cells such as the liver. To identify potential CYPs in orofacial pain, we injected CFA into the vibrissal pad of rats and determined changes in CYP expression in the TG (Ruparel, Henry, et al. 2012). Interestingly, among a large array of measured enzymes, only CYP 2J4 and CYP 23/3A1 were increased in TG cells after orofacial inflammation (Fig. 5A). Interestingly, about 60% of TRPV1 $^{+}$ TG neurons coexpressed CYP2J and about 40% coexpressed CYP 3A1 (Fig. 5B, C). Thus, the capsaicin-sensitive subpopulation of trigeminal neurons expresses CYP oxidative enzymes that are dramatically and significantly upregulated after orofacial inflammation (Ruparel, Henry, et al. 2012). But what is the functional significance of this observation? Does orofacial inflammation trigger any changes in the ability of TG neurons to generate TRPV1-active lipids? To address these important questions, TG neurons were cultured 24 h after injection of CFA or saline vehicle into the vibrissal pads. The TG cultures were then exposed to LA, and inward currents were determined by whole-cell patch-clamp electrophysiology. As indicated in Figure 5D, E, the results indicate that orofacial inflammation significantly increases LA-induced inward currents. Moreover, this effect was due to increased oxidative enzyme activities as it was blocked by pretreatment with either carbon monoxide or diphenyliodonium (Fig. 5E). Taken together, these studies indicate that the TRPV1 $^{+}$ subpopulation

Figure 5. Effect of orofacial inflammation in rats on expression of cytochrome P450 in TG neurons and ability to oxidize linoleic acid into neuronally active metabolites. (A) Time-response effect of orofacial inflammation (complete Freund's adjuvant [CFA] into the vibrissal pads) in rats on expression of transcripts encoding CYP 2J4 and CYP 23A/3A1 in trigeminal (TG) cells. (B, C) Anatomical analysis of the coexpression pattern of CYP2J2 and transient receptor potential vanilloid subtype 1 (TRPV1) in TG neurons (B) or the coexpression pattern of CYP3A1 and TRPV1 in TG neurons (C). (D, E) Effect of inflammation on linoleic acid (LA)-evoked inward current. Perforated patch was performed on TG neurons 24 h after vibrissal pad injection of saline (Veh) or CFA. Linoleic acid (LA, 1 mM) was applied to the cells for 2 min after 15-min exposure to vehicle, carbon monoxide (CO), or 10 μ M diphenyliodonium (DPI). (D) Mean values for each group are plotted. (E) Traces for each group are shown. Figure modified from Ruparel, Henry, et al. (2012). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.005$.

of TG neurons expresses CYPs that are selectively upregulated after orofacial inflammation, leading to increased ability to generate PUFA-derived TRPV1 agonists (Ruparel, Henry, et al. 2012). But does clinical orofacial inflammation in humans lead to similar increases in the generation of oxidized TRPV1 active lipids?

To address this question, we conducted studies on normal dental pulp versus dental pulp from patients with a diagnosis of symptomatic irreversibly pulpitis (Ruparel et al. 2013). As indicated in Figure 6, there is a substantial increase in both CYP 2J and 3A4 in the dental pulp from participants with a diagnosis of symptomatic irreversibly pulpitis. Interestingly, many of the cells expressing these oxidative enzymes are of immune origin, suggesting that the immunologic response to orofacial inflammation includes generation of endogenous TRPV1 agonists. To directly test this hypothesis, homogenates of dental pulp from normal or inflamed conditions were mixed with C14-LA. The results indicated that irreversible pulpitis increases the capacity to oxidize LA and that this effect is

blocked by pretreatment with LA (Ruparel et al. 2013). The next experiment determined the functional significance of this effect. Homogenates of normal versus inflamed human dental pulp were incubated with LA and the applied onto TG neurons with results measured by whole-cell patch-clamp electrophysiology (Ruparel et al. 2013). The results demonstrate that a clinical model of orofacial inflammation leads to increased capacity to generate TRPV1 agonists. This mechanism is due to oxidative enzyme activity as indicated by blockade with enzymatic inhibitors such as ketoconazole and is mediated by TRPV1 as indicated by reversal with I-RTX, a TRPV1 receptor antagonist (Fig. 6C). These studies demonstrate that orofacial inflammation in humans leads to an increased capacity to generate TRPV1 agonists.

Conclusions

Collectively, studies conducted over the past decade indicate that orofacial pain is a major and ongoing medical and social

Figure 6. Confocal images of CYP expression (red) in CD45-identified immune cells (green) in normal human dental pulp and irreversible pulpitis (IP) pulp specimens. (A) CYP2J expression in CD45-identified leukocytes; CD45-positive cells are rare in the normal specimen (left) and common in the IP specimen (right). (B) CYP3A4 expression in normal and IP human dental pulp. The CD45-positive cells include some without (arrowheads) and others with prominent CYP2J expression (arrows; and see boxed insert at higher magnification). (C) Functional analysis of linoleic acid (LA) metabolites generated by inflamed human dental pulp tissues. Normal human dental pulp and human dental pulp with irreversible pulpitis (IP) were pretreated with either vehicle or ketoconazole before application of buffer containing LA 100 μM with either ketoconazole or vehicle. The superfusates were collected; lipids were extracted and applied onto 1-d-old rat trigeminal (TG) neurons for 2 min with inward currents measured using whole-cell patch-clamp electrophysiology. Neurons were pretreated with either ketoconazole or the combination of ketoconazole and the transient receptor potential vanilloid subtype 1 (TRPV1) antagonist I-RTX 15 min before application of tissue extracts; $n = 8$ –10 tissue samples were used, and extracts were applied over 5 different neuronal cultures. Figure modified from Ruparel et al. (2013). HBSS, Hank's Balanced Salt Solution. * $P < 0.05$. ** $P < 0.01$.

problem. Orofacial inflammation is a dominant subclass of pain and involves the generation of endogenous agonists that activate numerous receptors expressed on terminals of TG afferent neurons. One heavily studied receptor is TRPV1, and studies indicate that it is expressed on orofacial tissues such as dental pulp. More recent studies have demonstrated that endogenous TRPV1 agonists are generated by oxidation of omega-6 PUFAs. A major mechanism for this effect is due to oxidative enzymes. This finding provides a rationale for an entirely new class of analgesics for treating orofacial pain. The identification of a novel target for analgesic drug development may offer considerable clinical potential as currently available analgesics often display either limited efficacy or substantial adverse effects. For example, opioids in formulations that contain hydrocodone or oxycodone are recognized to have much greater potential for adverse effects than previously believed. Another clinical implication is that identification of oxidized lipids as pain mediators provides the potential for biomarker development of diagnostic tests for pain patients. Taken together, the development of novel, nonopioid analgesics for treating chronic orofacial and somatic pain conditions and the use of certain oxidized lipids as diagnostic biomarkers may offer substantial utility in diagnosing and treating chronic orofacial pain.

Author Contributions

K. Hargreaves, contributed to conception, design, data analysis, and interpretation, drafted the manuscript; S. Ruparel, contributed to design, data analysis, and interpretation, critically revised the

manuscript. Both authors gave final approval and agree to be accountable for all aspects of the work.

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