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The Oxidized Linoleic Acid Metabolite-Cytochrome P450 System is Active in Biopsies from Patients with Inflammatory Dental Pain

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Abstract

Endogenous TRPV1 agonists such as oxidized linoleic acid metabolites (OLAMs) and the enzymes releasing them [e.g., cytochrome P450 (CYP)], are up-regulated following inflammation in the rat. However, it is not known if such agonists are elevated in human inflammatory pain conditions. Since TRPV1 is expressed in human dental pulp nociceptors, we hypothesized that OLAM-CYP machinery is active in this tissue type and is increased under painful inflammatory conditions such as irreversible pulpitis (IP). The aim of this study was to compare CYP expression and linoleic acid (LA) metabolism in normal versus inflamed human dental pulp. Our data showed that exogenous LA metabolism was significantly increased in IP tissues compared to normal tissues and that pretreatment with a CYP inhibitor, ketoconazole, significantly inhibited LA metabolism. Additionally, extracts obtained from LA-treated inflamed tissues, evoked significant inward currents in TG neurons, and were blocked by pretreatment with the TRPV1 antagonist, IRTX. Moreover, extracts obtained from ketoconazole-pretreated inflamed tissues significantly reduced inward currents in TG neurons. These data suggest that LA metabolites produced in human inflamed tissues act as TRPV1 agonists and that the metabolite production can be targeted by CYP inhibition. In addition, immunohistochemical analysis of two CYP isoforms, CYP2J and CYP3A1, were shown to be predominately expressed in immune cells infiltrating the inflamed dental pulp, emphasizing the paracrine role of CYP enzymes in OLAM regulation. Collectively, our data indicates that the machinery responsible for OLAM production is up-regulated during inflammation and can be targeted to develop potential analgesics for inflammatory-induced dental pain.

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Authors deny any conflicts of interest.

Keywords

Cytochrome P450; OLAM; Dental Pulp; TRPV1; Peripheral Pain

1. Introduction

Transient receptor potential vanilloid 1 (TRPV1) is the capsaicin-sensitive cation channel expressed on a major subclass of nociceptors. It is activated by heat as well as endogenous ligands including protons and certain lipids, and is sensitized by inflammatory mediators such as bradykinin, prostaglandins and nerve growth factor [6,9,16,36,41]. Recently, our group has reported a novel class of lipids as specific TRPV1 agonists released upon peripheral thermal and inflammatory insults in rodent models [33,34,39]. These lipids are oxidized metabolites of linoleic acid (OLAMs), namely, 9-HODE, 13-HODE and their corresponding oxo-metabolites. In addition, we have also shown that OLAM synthesis could be regulated by certain cytochrome P450 (CYP) enzymes in a rat model of inflammation [39,40]. Collectively, these preclinical studies indicate that inflammation triggers the release of endogenous oxidized lipids that act as TRPV1 agonists and contribute to allodynia and hyperalgesia.

However, it is not known whether the OLAM-CYP system is active in humans suffering from inflammatory pain. Accordingly, we evaluated activity of the OLAM system and CYP expression in dental pulp tissues collected from patients with either painful irreversible pulpitis or normal healthy teeth. Irreversible pulpitis originates as a localized inflammatory response to bacterial invasion of the pulp-dentin complex [3,13,18,31], leading to an influx of neutrophils, macrophages and other immune cells and local release of inflammatory mediators such as bradykinin, prostaglandins, nerve growth factor and cytokines [28]. Irreversible pulpitis is characterized by acute and often intense thermal and mechanical allodynia and hyperalgesia that may be difficult to control with analgesics [8,24,31]. Odontogenic pain mechanisms include the sensitization and activation of peripheral nociceptors, including those that express TRPV1 [4,10,11,15,20,22,23,26,28,31,32,42].

Based upon these factors, we hypothesized that the OLAMs are locally produced by oxidative CYP enzymes in human suffering from irreversible pulpitis and the specific aim of this study was to compare CYP expression and linoleic acid metabolism in normal versus inflamed human dental pulp. Our findings demonstrate that human inflamed dental pulp displays significantly increased metabolism of linoleic acid via CYP enzymes, release of endogenous TRPV1 agonists and accumulation of CYP enzymes in inflammatory cells as compared to normal, non-painful dental pulp. Our data indicates the potential role of OLAM-CYP system as targets for novel analgesic for human inflammatory pain.

2. Methods

2.1 Human Subjects

This study was approved by the Human Subjects Institutional Review Board at the University of Texas Health Science Center at San Antonio and patient consent was obtained following IRB protocol guidelines. The inclusion criteria consisted of patients with a pre-existing indication for tooth extraction. Study participants received standard dental treatment for tooth extraction including the use of local anesthetics. Extracted teeth utilized in this study included normal healthy third molar teeth with fully formed apices and lacking a past history of pain and pathology, and approximately 90% of painful irreversible pulpitis teeth were molars or pre-molars. To appropriately diagnose irreversible pulpitis (IP), Endo Ice™ Refrigerant spray was applied onto a cotton tip applicator and placed on the tooth in

question to test response to a cold stimulus. Patients were diagnosed with irreversible pulpitis following a positive and lingering pain response to this cold stimulation. All teeth with irreversible pulpitis had the presence of a well-defined carious lesion, indicative of a bacterial etiology for pulpal inflammation. Ten normal teeth and ten teeth with irreversible pulpitis were utilized in the anatomical studies, whereas 3-10 normal and IP pulp samples were used per group for the HPLC and the electrophysiology experiments. The teeth were extracted using standard dental methods and were immediately frozen on dry ice.

2.2 Drugs

Ketoconazole and iodo-resiniferatoxin (I-RTX) were purchased from Tocris (Ellsville, MO). Ketoconazole is an FDA-Approved anti-fungal drug that inhibits cytochrome P450 enzymes. I-RTX is an iodinated form of resiniferatoxin and is a competitive inhibitor of TRPV1. Linoleic acid (LA) was purchased from Cayman Chemicals (Ann Arbor, MI) and was diluted in Hank's balanced salt solution (HBSS) immediately prior to use. The pH of this solution was confirmed to be 7.4 to ensure no activation of TRPV1 by protons. [C^{14}]-linoleic acid was purchased from Perkin Elmer (Waltham, MA). Ketoconazole was diluted in 32% methylpyrrolidinone (MPL)/ HBSS to make a stock of 18 mM and further diluted in HBSS on the day of each experiment. I-RTX stock (2 mM) was made in 100% ethanol that was further diluted in HBSS on the day of use. Vehicles for ketoconazole and I-RTX were 0.25% MPL and 0.005% ethanol.

2.3 Evaluation of Linoleic Acid Metabolism

Frozen human dental pulp tissue biopsies were thawed and transferred to HBSS at 37°C for 20 min to equilibrate and transferred to modified HBSS buffer (CaCl₂ 2 mM, NaHCO₃ 4 mM, HEPES 10 mM, dextrose 10 mM, pH 7.4) containing either vehicle or ketoconazole 150 μ M for 40 min at 37°C. The tissues were then transferred to fresh HBSS buffer containing [C^{14}]-linoleic acid 100 μ M and either vehicle or ketoconazole (150 μ M); the biopsies were incubated at 37°C for 1 h. The identical procedure was followed in patch-clamp electrophysiology experiments, except that samples were incubated with non-radioactive linoleic acid. The superfusates were collected and subjected to lipid extraction using the C₁₈-SepPak™ column (Waters, Inc, MA) and dried with N₂ gas [33]. The dried samples were then resuspended in HBSS and subjected to either HPLC/radiometric analysis or patch clamp electrophysiology studies.

2.4 High Performance Liquid Chromatography (HPLC)/Radiometric Analysis

[C^{14}]-linoleic acid metabolites were measured as described [30] with the following modifications. Dried linoleic acid/metabolite mixtures were resuspended in 300 μ l of 50% methanol in water. Metabolites and substrate (120 μ l) were separated by reverse phase HPLC using a Gemini 5 μ M C₁₈ column (Phenomenex, CA) on a Shimadzu Liquid Chromatograph equipped with a β -RAM flow-through radiometric HPLC detector (IN/US Systems, FL). The elution is isocratic with a 48:52 mixture of 100% acetonitrile:water. Application of [C^{14}]-linoleic acid standards eluted at 25-28 min.

2.5 Whole cell Patch Clamp Electrophysiology

The use of animals in this study was approved by the UTHSCSA Institutional Animal Care and Use Committee and adhered to guidelines of the National Institutes of Health and the International Association for the Study of Pain. Male rat trigeminal ganglia (TG) were dissected, plated on poly-D-lysine/laminin-coated coverslips (BD Biosciences, San Jose, CA) and grown in the presence of 10% FBS and 100 ng/mL NGF (Harlan, Indianapolis, IN) for one day [1]. Recordings were made in whole-cell voltage clamp (holding potential (V_h) of -60 mV) configurations at 22-24°C from the somata of neurons (15-40 pF) as previously

described [1]. Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP 9.0 software (Axon Instruments, Union City, CA). Borosilicate pipettes (Sutter, Novato, CA) were polished to 3–4 M Ω in whole-cell pipette solution. Access resistance (R_s) was compensated (40–80%) when appropriate up to the value of 7–10 M Ω . Data were rejected when R_s changed >20% during recording, leak currents were >50 pA, or input resistance was <300 M Ω . Standard external solution (SES) contained (mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 D-glucose and 10 Hepes, pH 7.4. The standard pipette solution (SIS) for the whole-cell configurations contained (mM): 140 KCl, 1 MgCl₂, 1 CaCl₂, 10 EGTA, 10 D-glucose, 10 Hepes, pH 7.3. Lipid extracts from dental pulp were resuspended in 3 mL of HBSS buffer or HBSS with iRTX and applied on to the neurons for 2 min. The neurons were pretreated with 30 μ M ketoconazole for 15 min prior to extract application, to exclude potential responses obtained from unmetabolized linoleic acid present in the extracts. To determine whether the lipid extract-evoked inward current was TRPV1 dependent, the neurons were also pretreated with 100 nM iRTX for 5 min prior to extract application. Also, 100 nM capsaicin was applied to neurons after extract application as an additional verification that extract currents were recorded in TRPV1-positive neurons.

2.6 Immunohistochemistry

Extracted teeth were collected in 0.1 M phosphate buffer (PB), split longitudinally and the pulpal tissues were removed and fixed in 4% paraformaldehyde in 0.1 M PB for 20 min, rinsed in 0.1 M PB, placed in 0.1 M PB with 30% sucrose overnight at 4°C, and stored at –20°C the next day. Pulpal samples were thawed and embedded in Neg-50 (Richard-Allan Scientific; Kalamazoo, MI), with a normal specimen placed adjacent to an irreversible pulpitis specimen, and serially sectioned with a cryostat at 30 μ m in the longitudinal 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 [2]. This staining included the use of primary antibodies against CYP2J2 peptide 3 (rabbit polyclonal kindly provided by Dr. Darryl Zeldin at the National Institutes of Health; 1:500 dilution) or CYP3A4 (Millipore, Billerica, MA, rabbit polyclonal, AB1254; 1:200 dilution), in combination with primary antibodies against specific immune cell-type markers including CD45 (to identify leukocytes; Dako, Denmark, mouse monoclonal, M0701; 1:200 dilution) and CD68 (to identify macrophages/monocytes; Dako mouse monoclonal [17], M0718; 1:200 dilution). The CYP2J2 antibody identifies multiple isoforms in the CYP2J superfamily [44,45] and is used as a pan CYP2J marker. Sections were incubated in Alexa Fluor secondary antibodies (1:200; Molecular Probes, Eugene, OR) including Alexa Fluor 488 goat anti-mouse to visualize immune cell markers and Alexa Fluor 568 goat anti-rabbit to visualize CYP isoforms. TO-PRO-3 iodide (Topro, Invitrogen, Carlsbad, CA; 1:5,000 dilution) was used as a nuclear stain and was added with the secondary antibodies. Pulp samples were evaluated and images were collected with a Nikon Eclipse 90i microscope equipped with a C1si laser scanning confocal imaging system (Nikon Corporation, Melville, NY). Control preparations included samples with no primary and no secondary antibodies, and samples with only the secondary antibodies. Specimen blinding was not possible due to the substantial difference in cellular density and CYP expression between the normal and inflamed samples. This was not considered a limitation to the present study since the histological results are descriptive and was uniformly observed across all specimens.

Some specimens were stained with the use of the immunoperoxidase method and diaminobenzidine (DAB) as the substrate to further evaluate specificity of staining seen with the indirect immunofluorescence method. This staining involved 0.1 M PB rinses, 30 min treatment with 3% H₂O₂ (Sigma, St. Louis, MO) in 0.1 M PB followed by 0.1 M PB rinses, a 2 h incubation in blocking solution consisting of 4% normal goat serum (Vector Laboratories, Burlingame, CA), 2% bovine serum albumin (Sigma), and 0.3% Triton X-100

(Sigma) in 0.1 M PB, followed by a 2 h incubation in primary antibody (1:2500 dilution of CYP2J2 or 1:1000 dilution of CYP3A4) diluted in blocking solution. Sections were rinsed in 0.1 M PB with 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA), incubated for 2 h in biotinylated secondary antibody (1:200 dilution; Vectastain Elite ABC Kit Rabbit IgG, Vector) diluted in blocking solution, rinsed in 0.1 M PB with 0.1% Tween-20, and incubated for 30 min in avidin-peroxidase conjugate (1:500 dilution; Vectastain Kit) diluted in blocking solution. Sections were rinsed in 0.1 M PB with 0.1% Tween-20, incubated in 10 mg of DAB (Sigma) and 45 μ l of 3% H₂O₂ in 40 ml of 0.1 M PB with 0.1% Tween-20 for 10 min, rinsed in 0.1 M PB, dried, dehydrated and coverslipped. Stained specimens were evaluated with brightfield optics and images acquired with a Nikon Eclipse 90i microscope. The immunoperoxidase control preparations were processed exactly like experimental sections but lacked primary antibody.

2.7 Statistics

Data were analyzed by ANOVA (>2 groups) with Bonferroni post-hoc test or Student's t-test (2 groups). A $p < 0.05$ was considered significant and error bars are S.E.M.

3. Results

3.1 Linoleic acid metabolism is increased in inflamed dental pulp and is mediated by CYP enzymes

To investigate whether the OLAM-CYP system is biochemically active in human peripheral tissues, we incubated normal and inflamed human dental pulp tissue with [C¹⁴]-linoleic acid and used HPLC/radiometric analysis to monitor metabolite production (Fig. 1). Inflamed human tissue significantly increased formation of oxidized, hydrophilic metabolites of [C¹⁴]-linoleic acid by ~60% compared to normal tissue samples (Fig. 1A and B). Moreover, the application of ketoconazole, a broad CYP inhibitor, abolished the increased [C¹⁴]-linoleic acid metabolism from inflamed tissue. Taken together, these data indicate that a ketoconazole-dependent oxidative enzyme system, capable of forming OLAMs, is significantly increased in inflamed human tissues.

3.2 Linoleic acid metabolites formed by inflamed dental pulp generate TRPV1-mediated inward currents in sensory neurons

To study the functional relevance of the OLAMs formed from inflamed dental pulp, we incubated linoleic acid with un-inflamed and inflamed dental pulp tissues and determined whether extracts from these human tissues activated TRPV1 in cultured sensory neurons. To avoid possible neuronal formation of OLAMs [40], all neurons were pre-treated with ketoconazole prior to application of the dental pulp extracts. As seen in Fig. 2A and B, the application of extracts obtained from un-inflamed and inflamed tissue exposed to linoleic acid evoked inward currents in TG neurons. However, the inward current generated by application of the extracts from inflamed tissue was significantly greater than that observed with extracts from normal tissue. Interestingly, this increase in current was significantly reduced by pretreatment with the TRPV1 antagonist, iRTX (Fourth Bar on Fig 2A). Moreover, incubation of inflamed dental pulp with ketoconazole significantly reduced the ability of inflamed human tissue to form substances capable of generating inward currents in cultured sensory neurons (Third Bar on Fig 2A). Taken together, these data provide functional evidence for the formation of linoleic acid-derived TRPV1 agonists from inflamed human tissues that is mediated, at least in part, by a ketoconazole-dependent mechanism.

3.3 CYP isoforms are elevated in inflamed human dental pulp tissue

Control confocal experiments revealed minimal nonspecific staining/autofluorescence in images from normal human pulp specimens under identical laser and microscope conditions used to obtain images in the experimental sections that included primary antibody (compare Fig. 3A-B to Fig. 5 E-F). In contrast, evaluation of the same control sections in inflamed human pulp specimens revealed the presence of a broadly evident signal for nonspecific staining/autofluorescence (Fig. 3 C-D). The intensity of this autofluorescence signal varied among the different inflamed specimens and appeared highest in the region of specimens where many inflammatory cells were present. Even so, the examination of experimental sections using both primary and secondary antibodies revealed specific and discrete CYP staining within CD-identified immune cells (compare Fig. 3C-D to E-F). Special care was taken to evaluate all specimens for autofluorescence due to these findings. Since the autofluorescence was most common in inflamed specimens and in areas with high cellular density, this autofluorescence was interpreted as originating from both nonviable and viable immune cells as previously described [14,43].

The inflamed human tissue had several notable distinctions from the normal tissue. First, there was a striking increase in the density of CD45-identified leukocytes and CD-68 labeled macrophages/monocytes seen in inflamed as compared to normal tissues (Figs. 4-5A,C,E,G). The shape of the cells varied with the CD45⁺ cells appearing round, while processes were common on CD68⁺ cells. Some of the CD68⁺ cells had highly polarized and prominent processes. Although CD68⁺ cells were seen at low density throughout the pulp, the ones with highly polarized processes were most common just below the odontoblast layer (Figs. 4E and 5E). These same cells may correspond to antigen-presenting dendritic immune cells that are common in this location and that are stained with the CD68 antibody [29]. Second, the CYP2J and CYP3A4 expressions were especially enhanced in inflamed specimens. Both CYP isoforms were heavily expressed in CD45⁺ and CD68⁺ immune cells. Whereas the expression of both CYPs was variable in CD45⁺ cells (see boxed insert in Figs. 4C-D and 5 C-D), the expression of CYP2J and CYP3A4 in CD68⁺ cells was highly consistent and prominent (see boxed inserts in Figs. 4G-H and 5 G-H).

To verify the specificity of the CYP signals, we repeated these studies using the immunoperoxidase method (Fig. 6). Similar patterns were observed, with a broadly enhanced pattern of expression of both CYPs in inflamed specimens (Fig. 6C-D) as compared to the minimal CYP3A4 and CYP2J expression patterns observed within normal specimens (Fig. 6 A-B). In contrast, the no primary antibody control preparation lacked staining (Fig. 6E). In normal tissues, the CYP staining was most common in cells located just below the odontoblast layer, corresponding with the location of the CD68⁺ cells exhibiting prominent CYP expression seen with the immunofluorescence technique. The enhanced CYP staining seen in inflamed specimens was seen in both small round cells like the CD45⁺ identified ones, and in larger cells with processes like those identified with the CD68 antibody. These results obtained with the immunoperoxidase method confirm the specificity of CYP staining seen in immune cells with the immunofluorescence technique. Together, these results demonstrate an enhanced immune cell expression of both CYP2J and CYP3A4 within inflamed human pulp specimens.

4. Discussion

9-HODE, 13-HODE as well 9-oxoODE and 13-oxoODE comprise a class of OLAMs that were recently reported by our group to be specific TRPV1 agonists, released in response to noxious thermal stimuli as well as under inflammatory conditions [33,34]. Upon release in the periphery or in the spinal dorsal horn, these lipids are shown to be activators of TRPV1 leading to thermal or mechanical hyperalgesia in the rodent model of inflammation. Thus,

OLAMs are endogenous nociception-producing compounds that, if targeted, may provide a useful approach for reversing injury-related nociception. We have also reported that cytochrome p450 enzymes regulate LA metabolism to OLAMs in rat TG neurons as well as play a role in mediating OLAM-induced inflammatory hyperalgesia [39,40]. Moreover, inhibiting these enzymes partially blocked OLAM-mediated thermal hyperalgesia in the periphery. Thus, the CYP enzymes involved in OLAM synthesis could be potential targets to inhibit the peripheral nociceptive pathway following inflammatory insults. The current study builds on this foundation by demonstrating the translational significance of the OLAM-CYP system in human inflamed tissue. Thus, certain oxidative enzyme inhibitors could potentially be used to inhibit OLAM production during injury and inflammation for treating pain. The inflamed human tissues showed an increase in LA metabolism compared to normal tissues that was repressed by CYP inhibition, indicating that increase in LA metabolism was primarily due to increased CYP activity during inflammation.

Our data also demonstrated that the amount of current evoked by the extracts from each tissue type was in accordance to the relative amounts of linoleic acid metabolized (as shown in Fig. 1). Extracts from inflamed tissue evoked significantly greater inward currents in cultured neurons compared to currents generated by application of extracts of control tissue. Importantly, I-RTX blocks this effect, demonstrating the inflamed human tissue can synthesize TRPV1 agonists. Moreover, application of ketoconazole also blocked the ability of human inflamed tissue to form these compounds, implicating a role for CYPs in LA metabolism and subsequent activation of TRPV1. Since it has been previously shown that TRPV1 is expressed in the nerve terminals innervating human as well as rat dental pulp tissues [10,20,32], we speculate that OLAM release from the immune cells during local inflammation of pulp tissues cause TRPV1 activation, leading to increased transduction of pain signals. It would be useful to study whether OLAM production is restricted to only local peripheral injuries or whether it can also be a systemic event during prolonged, persistent inflammation.

To characterize CYP expression in dental pulp tissues, we studied the expression pattern of two CYP isoforms; CYP2J and CYP3A4 in normal and IP tissues. These two isoforms were shown previously to be upregulated in rat trigeminal ganglia under inflammatory conditions [40]. Our data pointed out two primary features: 1) Expression of both the CYP isoforms was prominent in inflammatory cells suggesting that immune cells may play a pivotal role in OLAM formation. Among other studies, this finding indicates an additional mechanism of interaction between immune cells and nociceptors upon injury [38]; 2) Expression of both isoforms was elevated under inflamed conditions compared to normal conditions in accordance with our functional experiments (Fig. 1), implicating the role of these particular isoforms in OLAM formation. There are several reports of CYP2J and CYP3A expression in immune cells where these enzymes are believed to play a role in drug metabolism, steroid synthesis, angiogenesis, mitogenesis, cell signaling, etc [5,7,21,27,35]. Our study is the first to report that these CYPs in immune cells may also regulate peripheral pain pathways, thus, being potential candidates for peripherally acting analgesics for inflammatory conditions. Moreover, targeting these enzymes peripherally will not affect the global function of these enzymes thereby minimizing adverse effects.

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Summary

Oxidized Linoleic Acid Metabolites (OLAMs) are novel TRPV1 agonists that are released upon heat injury and during tissue inflammation leading to injury-related allodynia and/or hyperalgesia. In rat model of inflammation, cytochrome P450 enzymes regulate the synthesis of these compounds in the periphery. Current study shows that the OLAM-CYP machinery may play a role in activating peripheral pain pathways even in human tissues such as the dental pulp under inflammatory conditions, thereby suggesting a novel strategy of targeting the OLAMS to inhibit inflammatory pain conditions.

Fig. 1.

Biochemical analysis of linoleic acid metabolites generated by inflamed human dental pulp tissues. Normal human dental pulp as well as human dental pulp with irreversible pulpitis (IP) were pretreated with either vehicle or ketoconazole 150 μ M for 40 min at 37°C prior to application of buffer containing [C^{14}]-linoleic acid 100 μ M with either ketoconazole 150 μ M or vehicle for 1 h at 37°C. The superfusates were collected, lipids extracted and subjected to HPLC/radiometric analysis to quantify the amount of linoleic acid metabolized by each group of tissues. (A) Data are plotted as the mean of the sum of % area under the curve. n=3/group. Error Bar: S.E.M. (B) Representative traces for each treatment group is shown with the peak of unmetabolized [C^{14}]-linoleic acid and oxidized [C^{14}]-labeled linoleic acid metabolites.

Fig. 2.

Functional analysis of linoleic acid metabolites generated by inflamed human dental pulp tissues. Normal human dental pulp as well as human dental pulp with irreversible pulpitis (IP) were pretreated with either vehicle or ketoconazole 150 μ M for 40 min at 37°C prior to application of buffer containing linoleic acid 100 μ M with either ketoconazole 150 μ M or vehicle for 1 h at 37°C. The superfusates were collected, lipids extracted and applied onto one-day old rat trigeminal ganglion neurons for 2 min with inward currents measured using whole-cell patch clamp electrophysiology. Neurons were pretreated with either ketoconazole 30 μ M or the combination of ketoconazole 30 μ M and the TRPV1 antagonist I-RTX 100nM for 15 min prior to application of tissue extracts. n= 8-10 tissue samples were used and extracts were applied over five different neuronal cultures. (A) Data are plotted as the mean of all the peak currents generated by each group after extract application. Error Bar: S.E.M. (B) Representative traces for each group is shown.

Fig. 3.

(A and B) Images from a normal pulp section adjacent to section shown in Figs. 5 E and F. Images from both sections were obtained with identical laser and microscope settings. These images are from a section stained with 488 and 568 secondary antibodies while lacking primary antibodies and shows a lack of specific staining as obtained with the 488 and 568 lasers (A) and 568 laser alone (B). This lack of specific staining contrasts sharply with the specific CYP3A1 staining seen in CD68-positive immune cells located in the experimental section shown in Fig. 5. (C-F) Images from adjacent pulp sections from a tooth with irreversible pulpitis (IP) obtained with identical laser and microscope settings shows nonspecific staining/autofluorescence in the section stained with secondary antibodies that lacked primary antibody (C-D) and the specific staining seen in the section stained with both primary and secondary antibodies (E-F). The nonspecific staining/autofluorescence seen in the control section with 488 and 568 lasers (C) is most prominent as seen with the 568 laser (D) and is located in small cellular profiles that appear similar to immune cells (arrowhead). In contrast, the specific CYP2J staining (red) seen in the experimental section (E and F) is especially prominent within CD68-positive immune cells (green; arrow). Topro (blue) identifies nuclei in A,C,E, while scale bar in A applies to A-F.

Fig. 4.

Confocal images of CYP2J expression (red) in CD45 and CD68-identified immune cells (green) in normal and irreversible pulpitis (IP) pulp specimens. (A-D) CYP2J expression in CD45-identified leukocytes in a normal pulp specimen (A-B) and an IP specimen (C-D). CD45-positive cells are rare in the normal specimen (A) and common in the IP specimen (C). The CD45-positive cells in both include some without (arrowheads) and others with prominent CYP2J expression (arrows; and see boxed insert at higher magnification in C and D). (E-H) CYP2J expression in CD68-identified macrophages/monocytes in a normal pulp specimen (E-F) and an IP specimen (G-H). CD68-positive cells are common and seen at low density throughout the normal specimen (E), and are especially prominent in the IP specimen (G). The CD68-positive cells in both include many with prominent CYP2J expression (arrows; and see boxed insert at higher magnification in G and H). Topro (blue) identifies nuclei, while scale bar in A applies to A-H.

Fig. 5.

Confocal images of CYP3A4 expression (red) in CD45 and CD68-identified immune cells (green) in normal and irreversible pulpitis (IP) pulp specimens. (A-D) CYP3A4 expression in CD45-identified leukocytes in a normal pulp specimen (A-B) and an IP specimen (C-D). CD45-positive cells are rare in the normal specimen (A), and common in the IP specimen (C). The CD45-positive cells in the normal specimen show minimal CYP3A4 expression (arrowhead), whereas these same cells in the IP specimen show varying levels of expression ranging from minimal (arrowheads) to prominent (arrows; and see boxed insert at higher magnification in C and D). (E-H) CYP3A4 expression in CD68-identified macrophages/monocytes in a normal pulp specimen (E-F) and an IP specimen (G-H). CD68-positive cells are common and seen at low density in the normal specimen (E) and more prominent in the IP specimen (G). The CD68-positive cells in both include many with prominent CYP3A4 expression (arrows; and see boxed insert at higher magnification in G and H). Topro (blue) identifies nuclei, while scale bar in A applies to A-H.

Fig. 6. Light photomicrographs of CYP3A4 and CYP2J expression in normal (A and B) and irreversible pulpitis (IP; C and D) pulp specimens as performed with the DAB immunoperoxidase method confirms the specificity of staining within immune cells seen with immunofluorescence technique. (A-B) CYP expression is minimal in normal pulp specimens (A and B) but does include expressions in cells (arrows) that appear like CD68-identified immune cells as seen with the immunofluorescence method. (C-D) CYP expression is more prominent within the IP specimens (C and D) and common in immune-like cells (arrows). (E) The control specimen that lacked primary antibody was processed in parallel with the experimental specimens and shows a lack of specific staining. Scale bar in A applies to A-E.