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Plasticity of Cytochrome P450 Isozyme Expression in Rat Trigeminal Ganglia Neurons During Inflammation

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Abstract

Recently, specific oxidized linoleic acid metabolites (OLAMs) have been identified as transient receptor potential vanilloid 1 (TRPV1) channel agonists that contribute to inflammatory and heat hyperalgesia mechanisms, yet the specific mechanism responsible for OLAM synthesis in sensory neurons is unknown. Here, we use molecular, anatomical, calcium imaging and perforated patch electrophysiology methods to demonstrate the specific involvement of cytochrome P450 enzymes (CYPs) in the oxidation of linoleic acid leading to neuronal activation and show that this is enhanced under inflammatory conditions. Additional studies evaluated CYP expressions in the native rat trigeminal ganglia (TG) tissue and cultures as well as changes in their expression pattern following the induction of peripheral inflammation. Fourteen of twenty candidate transcripts were detected in native TG and seven of these displayed altered expression under cultured conditions. Moreover, complete Freund's adjuvant-induced inflammation of vibrissal pad selectively increased expression of CYP3A23/3A1 and CYP2J4 transcripts in TG. In situ hybridization studies demonstrated broad expression pattern of CYP3A23/3A1 and CYP2J4 within TG neurons. Anatomical studies characterized the expression of CYP3A1 and the CYP2J families within TG sensory neurons including those with TRPV1, with about half of all TRPV1 positive neurons showing more prominent CYP3A1 and CYP2J expression. Together, these findings show that CYP enzymes play a primary role in mediating linoleic acid-evoked activation of sensory neurons and furthermore implicate the involvement of specific CYPs as contributing to the formation OLAMs that act as TRPV1 agonists within this subpopulation of nociceptors.

Keywords

Cytochrome P450; TRPV1; sensory neurons

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INTRODUCTION

Recently, our group has identified a family of endogenous oxidized linoleic acid metabolites (OLAMs) that specifically activate the transient receptor potential vanilloid 1 (TRPV1) channel in sensory neurons in response to acute thermal stimuli [32] and in the spinal cord following CFA inflammation [34]. These compounds include 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE, as well as their metabolites, 9-oxoODE and 13-oxoODE. We have shown that these compounds act as endovanilloids by activating TRPV1 on nociceptive neurons, leading to generation of inward currents, accumulation of intracellular calcium, exocytosis of neuropeptides and thermal hyperalgesia. Further, these data indicate that the OLAMs can be produced by sensory neurons themselves, thereby implying that these neurons have intrinsic regulatory machinery to synthesize these compounds in response to noxious stimuli. However, the specific metabolic pathway responsible for the conversion of linoleic acid to OLAMs in nociceptive neurons is unknown.

Linoleic acid is an essential fatty acid and a component of lipids located in membranes. Most studies on linoleic acid metabolism have been conducted in the liver where it is known to be oxidized by either enzymatic pathways, such as lipoxygenases or certain cytochrome P450 enzymes (CYPs), or spontaneously by non-enzymatic mechanisms [23; 35]. Here, we evaluated the involvement of different pathways responsible for the oxidation of linoleic acid by sensory neurons in an attempt to further understand mechanisms responsible for the activation of TRPV1 class of nociceptors by the OLAMs.

EXPERIMENTAL PROCEDURES

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, Inc., Wilmington, MA, USA) were used for all the studies except for the immunohistochemical studies that also included the use of female rats.

TG Cultures

Experiments were performed on 1-day-old cultures of trigeminal ganglia (TG) plated on poly-D-lysine coated 6-well plates (BD Biosciences, San Jose, CA, USA) and grown in the presence of 10% FBS and 100 ng/mL nerve growth factor (NGF; Harlan, Indianapolis, IN, USA) as described previously [5; 33]. TGs from three rats were used per plate. For calcium imaging and electrophysiology experiments, cells were plated on poly-D-lysine/laminin coated glass coverslips and grown as described above except the cells used for electrophysiology experiments were acutely cultured in media containing 10% FBS without NGF and were used 4 hours after culturing.

Calcium Imaging

Fluorescence imaging to measure calcium accumulations in sensory neurons was performed as described previously [5]. The TG cultures were incubated with the calcium-sensitive dye, Fura-2 AM (2 μ m; Molecular Probes, Carlsbad, CA, USA) in Hanks modified buffer. Fluorescence was detected with a Nikon TE 2000U microscope fitted with a $\times 40/1.35$ NA Fluor objective. Data were collected and analyzed with MetaFluor Software (Universal Imaging Corporation, Downingtown, PA, USA). The net changes in calcium influx were calculated by subtracting the intracellular calcium $[Ca^{2+}]_i$ level (mean value collected for 60 s prior to agonist addition) from the peak $[Ca^{2+}]_i$ value achieved after exposure to the agonist. Cells were pretreated with vehicle or one inhibitor (Table 1) for 15 minutes followed by the addition of 1mM linoleic acid (Cayman Chemicals Inc., Ann Arbor,

Michigan, USA) while in the presence of the same inhibitor for 2 minutes.. Saturating concentrations of the each of the inhibitors was used. A solution containing 250mM KCl was applied after linoleic acid application to ensure sampling of viable neurons

Induction of Inflammation

Male rats were anesthetized with isoflurane and injected with 50 μ l of a 1:1 mixture of complete Freund's adjuvant (CFA; Sigma) in saline into the left and right vibrissal pads. At 3, 6, 24, 48 and 72 hour post-injection time points, the TG were removed and subjected to RNA isolation for RT-PCR experiments or at 24 hours the TG were used to culture sensory neurons for electrophysiology experiments.

Electrophysiology

Recordings were made in perforated patch (holding potential (V_h) of -60 mV) configurations at $22-24^{\circ}\text{C}$ from the somata of sensory neurons ($15-40$ pF) as described previously (Akopian et al., 2007). Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP9.0 software (Axon Instruments, Union City, CA, USA). 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, USA) were polished to resistances of $4-7$ M Ω in the perforated patch pipette solution. Access resistance (R_s) was compensated ($40-80\%$) when appropriate up to the value of $13-18$ M Ω for perforated patch configurations. Data were rejected when R_s changed $> 20\%$ during recording, leak currents were > 50 pA, or input resistance was < 300 M Ω . Currents were considered positive when their amplitudes were 5-fold bigger than displayed noise (in root mean square).

RNA isolation

The TG were collected in RNase Later (Qiagen, Valencia, CA, USA) and immediately frozen at -80°C . RNA was extracted from the tissue using the RNeasy Mini Kit (Qiagen) per manufacturer's instructions. For RNA isolation from cultured TG, the cells were lysed with lysis buffer provided in the kit. The isolated RNA was then treated with a DNA-free reagent (DNase I; Ambion, Austin, TX, USA) and used for cDNA synthesis that was analyzed by real-time (RT)-PCR. cDNA was prepared using Superscript III First Strand Synthesis kit (Invitrogen, CA, California, USA).

Real-Time PCR

Fixed amounts of cDNA were used as the template in the RT-PCR protocol. Amplification of target sequences was detected by a sequence detector ABI 7700 (Applied Biosystems, Foster City, CA, USA) utilizing TaqMan Gene Expression Assays on Demand and specific primers as listed in Table 2. The reactions were run in triplicates of $25\mu\text{l}$, including the endogenous control, 18S ribosomal subunit, for each individual gene expression assay. For quantitative analysis, comparative delta-delta Ct (ddCt) was utilized to normalize the data based on the endogenous reference, and to express it as the relative fold change, after the exclusion criteria were verified by comparing primer efficiencies.

For qualitative analysis, the PCR products were run on a 1% agarose gel containing $0.5\mu\text{g/ml}$ ethidium bromide in 1X TAE buffer (40mM Tris, 20mM glacial acetic acid, 1mM EDTA) and the bands were visualized using a UV transilluminator. Data were acquired from the TG collected from three rats and from three independent neuronal cultures.

Generation of CYP riboprobes

Riboprobes for CYP3A23/3A1 and CYP2J4 were generated as described previously [36]. Briefly, CYP3A23/3A1 and CYP2J4 fragments were amplified from commercially available

rat liver cDNA (BioChain, Hayward, CA, USA) and subsequently subcloned into pGEM T-easy vector (Promega, Madison, WI, USA) and sequenced for verification. Dig-labeled riboprobes were synthesized from the linearized fragment-containing constructs using the Riboprobe Combination System SP6/T7 (Promega) incorporating 1mM ATP, CTP and GTP with 650 μ M unlabeled UTP and 350 μ M Dig-labeled UTP (Roche, Indianapolis, IN, USA). Riboprobes were purified by G-50 column chromatography (Roche), aliquoted and stored at -80°C .

In-situ Hybridization

The TG and liver were removed from young adult male rats, 30 μ m thick frozen sections were cut with a cryostat and collected onto Superfrost Plus glass slides (Fisher Scientific, Pittsburg, PA, USA). Sections were fixed in ice-cold 3.7% formaldehyde (Fisher, Fairlawn, New Jersey) for one hr, followed by three rinses in phosphate buffered saline (PBS) and permeabilization with 0.5% Triton X-100 in 0.1 M Tris/0.05M EDTA for 15 min. Sections were incubated in 1% active DEPC (Sigma, St Louis, MO) for 30 min, rinsed, and incubated for 15 min in 5X SSC. Sections were placed in a humidified chamber with hybridization buffer for 2 hrs and then riboprobes in hybridization buffer were applied to the sections for 16–18h at 58°C . Sections were washed four times with 4X SSC and treated with RNase I (1 μ g/ml, Roche) for 30 min at 37°C . Washes were performed in decreasing concentrations of SSC, culminating in a final high stringency wash in 0.1X SSC at 55°C for 30 min. Sections were blocked in B1 Buffer (NaCl, KCL, 1M Tris pH 7.4) containing 4% normal goat serum (NGS, Gibco, Rockville, MD) for 30 min, incubated with anti-Dig-Fab Ab fragments (Roche) for 1 hr and rinsed quickly with detection B2 Buffer (5M NaCl, 1M Tris pH 9.5, 4.9M MgCl_2). The alkaline phosphatase reaction was developed with BCIP/NBT solution in B2 Buffer (Roche) for 15–18 hr at RT. Slides were rinsed 3 times in PBS, then H_2O , dried for 5 min and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). Sections were evaluated and images were obtained with a Nikon Eclipse 90i microscope. Control sections were processed identically as described above but lacked riboprobes.

Immunohistochemistry

Two male and two females rats were deeply anesthetized with an intraperitoneal injection of pentobarbital (25mg) and transcardially perfused with 100ml of 0.9% saline followed by 200ml of fixative consisting of 4% paraformaldehyde in 0.1M phosphate buffer (PB). The TG were removed, post-fixed for 20 min, rinsed in PB and placed in cold 0.1M PB with 30% sucrose overnight. Tissues were embedded in Neg-50 (Richard Allan, Kalamazoo, MI, USA) and sectioned in the horizontal plane at 30 μ m with the use of a cryostat. Sections were placed onto Superfrost Plus slides (Fisher Scientific), dried and stored at -20°C until stained. Immunostaining was performed as described previously [18]. Briefly, tissue sections were permeabilized and blocked for non-specific protein binding with blocking solution consisting of 4% normal goat serum (Sigma, St. Louis, MO), 2% bovine gamma-globulin (Sigma) and 0.3% Triton X-100 (Fisher Scientific) in PBS for 90 min prior to incubation with primary antibodies in blocking solution for 16 hr. Primary antibodies included guinea pig anti-rat TRPV1 (1:2000; Neuromics, Bloomington, MN, USA) and either rabbit anti-rat CYP3A1 (1:300, Millipore, MA, USA), or rabbit anti-CYP2Jrec (95718) which is an anti-pan-CYP2J (1:300, kindly provided by Dr. Darryl Zeldin/NIH); this latter antibody identifies multiple isoforms in the CYP2J superfamily including the rat CYP2J3 and rat CYP2J4 isoforms [37; 41; 42; 44]. Sections were rinsed with PBS, incubated in secondary antibody with TO-PRO-3 iodide as a nuclear stain (1:5000, Invitrogen, Carlsbad, CA, USA) in blocking solution for 90 min, rinsed in PBS and H_2O , dried and coverslipped with Vectashield. Secondary antibodies included Alexa Fluor 488 to visualize TRPV1 and Alexa Fluor 568 to visualize either CYP2J or CYP3A1 (1:200; Molecular Probes, Eugene, OR, USA). All immunostaining procedures were performed at

room temperature. Sections were evaluated and images obtained with a Nikon Eclipse 90i microscope equipped with a C1si laser scanning confocal imaging system. One image was acquired from two different TG sections per animal for each antibody combination with a 10x objective from the middle of the section with identical laser gain settings. The staining patterns were analyzed with the use of quantitative imaging techniques [27; 28]. ImageJ software was used to determine the percentage of sensory neurons with expressions of CYP3A1 and CYP2J and colocalization patterns of each with the TRPV1+ subpopulation of neurons. Analysis was limited to those neurons with a clearly identifiable nucleus. Only those neurons that showed 100 or more contiguous pixels above threshold levels were counted as positive for either TRPV1 or the CYPs. To estimate CYP expression based on neuronal size, TG's from one male and one female were used. The size was determined by measuring the longest (x) and the shortest (y) axis of each cell and then recording the average of these two values.

Statistics

Data are presented as mean $\pm$ SEM. Statistical analyses were performed using either a 2-tailed Student's *t* test or ANOVA with Neuman-Keul's post-hoc test. A statistically significant difference was defined as $P < 0.05$.

RESULTS

Cytochrome P450 Enzymes are involved in Mediating Linoleic Acid response in Sensory Neurons

Since linoleic acid (LA) can be converted into its oxidized metabolites by either enzymatic pathways (i.e., cytochrome P450s or lipoxygenases) or by non-enzymatic mechanisms, we tested inhibitors for each of these pathways against LA-evoked calcium influx as a method to determine the pathway/s that play a role in LA metabolism in sensory neurons. As shown in Figure 1, the application of LA to sensory neurons in culture caused a significant accumulation of $[Ca^{2+}]_i$ that was blocked by pretreatment with the TRPV1 antagonist iRTX, indicating that Ca^{2+} accumulation results from TRPV1 activation. However, pretreatment with cytochrome P450 (CYP) inhibitors (nordihydroguaiaretic acid [4], diphenyliodonium [38], and carbon-monoxide [25]) almost completely blocked the LA-induced Ca^{2+} influx response. Since at least some of these inhibitors also inhibit the nitric oxide pathway, additional cultures were pretreated with nitric oxide synthase (NOS) pathway inhibitors L-NNA [31] and L-NAME [29]. However, neither of these compounds blocked the LA response. Additionally, neither pretreatment of lipoxygenases inhibitors (BW-70C [22] and PD146176 [7]) nor antioxidants (TROLOX [16], TEMPOL [26]) that deplete free radicals from the cells, were able to reduce Ca^{2+} accumulation after LA application. Collectively, these results suggest that cytochrome p450 (CYP) enzymes represent a primary class of enzymes responsible for mediating the LA-induced Ca^{2+} responses in nociceptive neurons.

Linoleic Acid Response is Increased Under Inflammatory Conditions and is Blocked by CYP Inhibitors

To study the role of OLAM synthesis during inflammation, rat vibrissal pads were injected with CFA to induce inflammation. One day later, TG's were dissected from inflamed and control uninflamed rats and the TG neurons were acutely cultured for 4 hrs without NGF. Perforated patch clamp recordings show that LA response increased in TG neurons from inflamed rats compared to normal rats suggesting that the machinery responsible for OLAM production from LA may be elevated in TG neurons from inflamed rats (Fig. 2). Treatment with carbon monoxide and diphenyliodonium inhibited this LA response implicating cytochrome P450s as a dominant mechanism in OLAM production from LA in TG neurons after peripheral tissue inflammation.

Rat TG and TG Cultures Express Cytochrome P450 Enzymes

To further study the role of CYP isoforms during inflammation, we first determined basal expression levels of twenty different CYP isoforms by RT-PCR in native rat TG and in TG cultures. The candidate enzymes were selected for detailed examination based on meeting any of three criteria: 1) enzyme transcripts that were detected in rat TG based on data obtained from a prior Affymetrix Chip analysis by our group [10]; 2) enzyme isoforms that are known to be present and/or upregulated in afferent neurons after peripheral nerve injury [6; 20; 24]; or 3) isoforms that are believed to be involved in linoleic acid metabolism [3; 13; 23; 40]. The enzymes tested were CYP2A1, CYP2A2, CYP2C7, CYP2C13, CYP2C23, CYP2J3, CYP2J4, CYP3A23/3A1, CYP4A1, CYP4A2, CYP4A3, CYP4B1, CYP4F6, CYP7B1, CYP17A1, CYP27A1, CYP51, Epxh2, LOX-5 and LOX-15. The results (Fig. 3) demonstrated that native TG tissue express 11 of the 20 enzymes evaluated, with no detectable amounts of CYP2A2, CYP2C7, CYP2C13, CYP4A1, CYP4A2, or CYP4A3 present in native tissue. Interestingly, we observed that 1 day culturing of rat TG resulted in substantial increases in expression of LOX-5 and LOX-15, with de novo induction of CYP2C7, CYP2C13, CYP4A1, CYP4A2 and CYP4A3 transcripts compared to expression levels observed in native TG. The upregulation of these enzymes may result from injury since others have shown the expression of nerve injury markers in sensory neurons grown under culturing conditions [11].

CYP3A23/3A1 and CYP2J4 Expression Increases in the TG Following CFA-Induced Inflammation of the Vibrissal Pad

To evaluate whether or not neuronal CYP enzymes may have a role in tissue injury, we studied the effect of inflammation on CYP expression in TG neurons. Inflammation was induced with bilateral injections of CFA into the vibrissal pads and the expression of CYPs in the TG was determined 3, 6, 24, 48 and 72 hours later. CYP P450 subtypes that showed detectable basal levels in the native TG tissues (Fig. 3) were selected for this experiment. The results (Fig. 4) indicate that CYP2J4 and CYP3A1 expression significantly increased at 3 and 6 hours after CFA injection respectively. These data indicate that certain neuronal CYPs may be regulated after peripheral tissue inflammation and therefore demonstrates plasticity of CYP expression in sensory neurons.

CYP3A23/3A1 and CYP2J Expression in Trigeminal Sensory Neurons and Colocalization Patterns with TRPV1

We next employed parallel in situ hybridization and immunohistochemistry analyses to evaluate the sensory neuron expression of selected CYP isoforms. In situ hybridization used riboprobes specific to the CYP2J4 and CYP3A1 isoforms, while immunohistochemistry used a pan-CYP2J antibody [37; 41; 42; 44] and an antibody against the CYP3A1 isoform. The specificity of the riboprobes was first confirmed in rat liver sections and these results demonstrated basal expression that was greater for the CYP3A1 isoform than for CYP2J4 (Fig. 5A and B). We also employed liver sections to verify the immunohistochemical analyses. The anti-CYP antibodies displayed selective expression patterns within the liver, with an overall pattern quite similar to that seen with the use of the riboprobes (Fig. 5A and B). The use of the CYP antibodies showed a broad expression of each CYP within the cytoplasm of most trigeminal sensory neurons that included a subpopulation of more brightly stained sensory neurons in each (Fig. 5C and D) which included small to medium sized neurons indicating C-fiber origin. Fig. 5F shows that about 70% of all CYP positive neurons for CYP 3A1 as well as CYP2J were in the size range of 10–30 μ m. The results of the quantitative analysis performed on the subpopulation of sensory neurons with bright staining showed that 35% of all neurons showed bright staining for CYP3A1, while 28% were positive for CYP2J. The results of the colocalization analysis of neurons with bright CYP staining with TRPV1⁺ neurons revealed that 43% of TRPV1⁺ neurons co-expressed

CYP3A1, whereas 61% of TRPV1⁺ neurons co-expressed pan-CYP2J. Control images for immunohistochemistry showing minimal non-specific binding of the secondary antibodies for TRPV1 as well as for CYP staining are shown in Fig 5E. The results of these studies demonstrate the broad and specific differential expression of the CYP3A1 isoform and of the CYP2J superfamily within trigeminal sensory neurons, including prominent expression patterns within the TRPV1 subclass of nociceptive neurons.

DISCUSSION

Cytochrome P450 enzymes are well recognized for their role in the metabolism of numerous drugs and xenobiotic compounds. These enzymes also oxidize endogenous substances such as cholesterol, steroid hormones and certain fatty acids including linoleic acid [14; 15; 39]. We recently have shown that the linoleic acid metabolites, 9-HODE, 13-HODE, 9-oxoODE and 13-oxoODE are TRPV1 agonists that are produced in response to thermal stimuli in the periphery as well as in the dorsal spinal cord in the CFA model of inflammation [32; 34]. However, the regulation of OLAM production from linoleic acid still remains to be elucidated. It has been speculated that linoleic acid can be metabolized into the HODEs by both enzymatic (i.e., cytochrome P450 enzymes or lipoxygenases) and non-enzymatic mechanisms (e.g., oxidation by free radicals). Our data shows that linoleic acid-evoked calcium influx in sensory neurons is completely abolished by broad CYP inhibitors, NDGA, CO and DPI, whereas the LA response remains unaffected when cells were pretreated with LOX inhibitors, NOS inhibitors or antioxidants. Thus, it is possible that the CYPs regulate the synthesis of OLAMs in sensory neurons, thereby playing a major role in regulating TRPV1 activities and pain. To further study the role of OLAM production and CYPs during inflammation, we measured linoleic acid-evoked inward current from neurons acutely cultured from inflamed rats versus control rats. Neurons were acutely cultured since longer culturing conditions triggers changes in CYP expression (note that Fig. 3 shows that some CYPs may be induced after 24 hour cultures). Interestingly after CFA inflammation, the application of linoleic-acid generated a highly significant, 8-fold increase in evoked-current compared to TGs taken from control rats. These data suggest that either the TRPV1 channel that is activated by LA is sensitized by inflammation or that the machinery involved in metabolizing LA is upregulated under inflammatory conditions. Interestingly, pretreatment with CYP inhibitors completely abolished LA response suggesting that the CYPs play a crucial role in regulating LA response and possibly OLAM synthesis during inflammation.

Although CYPs have been shown to be expressed in several tissue types, there are relatively few reports on their expression in sensory neurons. Iliff *et al.* reported the expression of CYP families; 2C and 2J in rat trigeminal ganglia and indicated that the CYP2J family may play a role in cap-evoked CGRP release from TG neuronal cultures [19; 20]. However, there are no reports indicating whether other CYP subtypes play a role in nociceptor activation. Using RT-PCR, we detected 12 of 20 targeted transcripts in native rat trigeminal ganglia and 19 of 20 targeted transcripts in one day old TG cultures. This differential expression pattern was consistent with the hypothesis of dynamic mechanisms regulating enzyme expression. Since neuronal cultures resemble a model of nerve injury [11], it is possible that these isoforms may be regulated following injury.

To evaluate the possible regulation of cytochrome P450 expression in response to inflammation, we employed the CFA model of vibrissal pad inflammation. The vibrissal pad is a useful area for investigation since the infraorbital nerve, which innervates the vibrissal pad, is the largest sensory nerve in the rat with ~33,000 axons [21], while the entire rat TG has ~55,000 neurons [17]. Thus, the vibrissal pad model of inflammation is a reasonable sampling approach for detecting moderate changes in the entire TG [17; 21].

To test the hypothesis of inflammation-induced alterations in enzyme expression, we determined the expression of these enzymes in TG at various times after CFA-induced inflammation of the vibrissal pad. Our data showed that CYP2J4 and CYP3A23/3A1 were up-regulated in the rat TG's under inflammatory conditions, suggesting a possible role of these enzymes in mediating inflammatory pain. Interestingly, prior studies have implicated the CYP2J family in modulating tissue inflammation [8; 9; 30], although to our knowledge, this is the first report of inflammation-induced changes in CYP mRNA expression in trigeminal afferent neurons. Conversely, LOX-5, LOX15, and CYP2C7, CYP2A2, CYP4A1, CYP4A2, CYP4A3 and CYP2C13 were up-regulated in cultured vs. native TG, and yet there was no change in their expression levels in the CFA model of inflammation. Thus, the present findings suggest multiple and independent mechanisms regulating CYP expression in sensory neurons.

Co-localization of CYP3A23/3A1 and CYP2J with TRPV1 in TG neurons supports the hypothesis that these enzymes may have an important role in regulating pain pathways. Immunohistochemical and in-situ hybridization demonstrated that these enzymes are predominantly expressed in the cytoplasm of sensory neurons. It is known that like most CYP enzymes, CYP3A23/3A1 and CYP2J are expressed in the membranes of the endoplasmic reticulum [12; 43] [1; 2]. However, it would be interesting to determine whether these enzymes are also expressed at the plasma membrane where the 9-HODE and 13-HODEs are presumably synthesized in nociceptive neurons.

The role of cytochrome P450 enzymes in the regulation of afferent neurons in general, and in the regulation of endogenous TRPV1 agonists in particular, is incompletely understood. These enzymes could provide targets for developing novel analgesics. The expression and role of these enzymes in the peripheral tissues such as skin and immune cells could provide further insight about the action of these enzymes in regulating OLAM production.

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Fig. 1. Linoleic Acid-evoked Calcium Influx in sensory neurons

Rat TG cultures were exposed to 2 minutes of 1mM Linoleic acid after 15 minutes pretreatment of either vehicle, TRPV1 antagonist, iRTX (5'-Iodoresiniferatoxin) or inhibitors to CYP enzymes, lipoxygenases enzymes, NOS pathway or antioxidants. CYP enzymes included NDGA (Nordihydroguaiaretic Acid), CO (Carbon Monoxide) and DPI (Diphenyliodonium). Argon was used as an inert control for gaseous substances like CO. Inhibitors to NOS pathway used were L-NAME [L-N^G-Nitroarginine methyl ester (hydrochloride)] and L-NNA (N^G-nitro-L-Arginine; L-N^G-Nitroarginine). Lipoxygenase enzymes were inhibited by BW-70C {N-[3-[3-(-Fluorophenoxy)phenyl]-1-methyl-2-propenyl]-N- hydroxyurea} and PD146176 {6,11-Dihydro[1]benzothiopyrano[4,3-b]indole}. Antioxidants used were TROLOX {6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid} and TEMPOL {1-Oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine}. (A). Mean responses to LA for each group are plotted. Each group was repeated three times with three independent TG cultures. Data were analyzed using 2-tailed Student 't' test. Error bars: S.E.M. (B), (C) and (D). Traces for LA responses after vehicle, CO and DPI pretreatment are shown. 250mM KCL solution was applied after LA application to confirm integrity of the neurons after drug treatment.

Fig. 2. Effect of Inflammation on Linoleic Acid-evoked Inward Current

Perforated Patch was performed on neurons cultured from TG's from inflamed and control rats. 24 hours after bilateral 1:1CFA/saline injections into the vibrissal pads, neurons were cultured for 4 hours in media containing 10% FBS without NGF. 1mM Linoleic acid was applied to the cells for 2 minutes after 15 minutes exposure to vehicle, CO or 10uM DPI. (A). Mean values for each group are plotted. Data were analyzed using one-way ANOVA and Neuman-Keuls post-hoc test. Error Bar: S.E.M. (B). Traces for each group are shown.

Fig. 3. Basal Expression of CYP isoforms in native TG and TG cultures

RT-PCR analysis of 20 P450 subtypes and related enzymes was performed on native rat TG and TG cultures. TG1, TG2 and TG3 represents TG tissues from 3 independent animals and culture 1, 2 and 3 represents three independent neuronal cultures. The neurons were cultured for 24 hours after which they were subjected to RNA isolation and subsequently RT-PCR. NTC= No template control. Expressions of each of the CYP subtypes are shown in rat liver and the brain (Biochain, Hayward, CA, USA) as positive controls. 18s RNA was used as an internal control.

Fig. 4. Effect of CFA-induced inflammation on CYP isoform expression in the TG
 50ul of 1:1 CFA in saline was injected into the left and right vibrissal pads and the TG were harvested 3, 6, 24, 48 and 72 hours later. CYP expression within the TG was determined with quantitative RT-PCR. Seven animals were used at each time point. The data from the '0' hour time point were from control (un-treated) animals. Each data set was normalized to the expression seen in uninflamed/control animals. Data were analyzed using one-way ANOVA and Neuman-Keuls as the post-hoc test. Error bars = S.E.M

Figure 5. Anatomical Analysis of CYP3A23/3A1 and CYP2J in rat TG neurons

A and B. In-situ hybridization from normal rat liver and trigeminal ganglia (TG) with CYP3A23/3A1 (**A**) and CYP2J4 riboprobes (**B**) and 'No probe controls' show a lack of staining in the liver and TG. Immunohistochemistry of normal rat liver with rat specific CYP3A23/3A1 (**A**) and pan-CYP2J (**B**) antibody. TOPRO was used to identify nuclei. **C and D.** Immunohistochemistry from normal rat TG's for TRPV1 and CYP3A23/3A1 (**C**) and TRPV1 and CYP2J (**D**). Quantitative analysis for both P450 subtypes is shown in a graphical representation as well as in a tabular form.

E. Images of a control preparation stained with 488 and 568 Alexa Fluors but lacking primary antibody and obtained with identical parameters as used to obtain images in experimental sections. 488 laser used to visualize CYP staining and 561 laser used to visualize TRPV1 staining.

F. CYP3A1 and CYP2J expression pattern were determined in neurons of 4 different size categories. TG tissues from one male and one female rat were used for each CYP isoform. Total CYP3A1 neurons measured were 181 (84/181 from female 97/181 from male) and

total CYP2J2 neurons measured were 118 (61/118 from female 57/118 from male). Error bars= S.E.M.

Table 1**Inhibitors used for Figure 1**

Saturating concentrations of the each of the inhibitors was used as pretreatment for 15 minutes and as co-treatment with 1mM LA for 2 mins.

Inhibitor	Abbrev	Conc	Solvent for Stock Preparation	Vendor
5'-Iodoresiniferatoxin	iRTX	1uM	Ethanol	Tocris, Ellsville, Missouri, USA
Nordihydroguaiaretic Acid	NDGA	30uM	Ethanol	Cayman Chemical, Ann Arbor, Michigan, USA
Diphenyliodonium	DPI	10uM	DMSO	Tocris, Ellsville, Missouri
Carbon Monoxide	CO	Saturated Buffer	HBSS buffer was injected with CO gas for 5 minutes in a vacutainer	Praxair, Danbury, CT, USA
Argon	Ar	Saturated Buffer	HBSS buffer was injected with Argon for 5 minutes in a vacutainer	Praxair, Danbury, CT, USA
L- ^{NG} -Nitroarginine methyl ester (hydrochloride)	L-NAME	1mM	Water	Kindly provided by Beatty Sue Masters
^{NG} -nitro-L-Arginine; L- ^{NG} -Nitroarginine	L-NNA	1mM	Water	Kindly provided by Beatty Sue Masters
N-[3-[-(Fluorophenoxy)phenyl]-1-methyl- 2-propenyl]-N- hydroxyurea	BW-70C	20uM	Ethanol	Tocris, Ellsville, Missouri, USA
6,11-Dihydro[1]benzothiopyrano[4,3- b]indole	PD146176	54uM	Ethanol	Tocris, Ellsville, Missouri, USA
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	TROLOX	2mM	Ethanol	Sigma, St Louis, MO, USA
1-Oxyl-2,2,6,6-tetramethyl-4- hydroxypiperidine	TEMPOL	5mM	Ethanol	Tocris, Ellsville, Missouri, USA

Table 2

Primers used for RTPCR for Figure 4.

Gene Name	Assay #
CYP2A1	Rn00562200_m1
CYP2A2	Rn00562207_m1
CYP2C7	Rn01529602_mH
CYP2C13	Rn00593388_g1
CYP2C23	Rn00582954_m1
CYP2J3	Rn0059800_m1
CYP2J4	Rn00576482_m1
CYP3A23/3A1	Rn01412959_g1
CYP4A1	Rn00598510_m1
CYP4A2	Rn01417066_m1
CYP4A3	Rn00598416_gH
CYP4F6	Rn00685847_m1
CYP4B1	Rn00566515_m1
CYP7B1	Rn01461859_m1
CYP17A1	Rn00664858_m1
CYP27A1	Rn00710297_m1
CYP51	Rn01526553_m1
EPHX2	Rn00674566_g1
LOX-5	Rn00563172_m1
LOX-15	Rn00696151_m1
18S RNA	Hs99999901_s1