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Authors

Goswami, Sumanta Kumar
Inceoglu, Bora
Yang, Jun
et al.

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Omeprazole increases the efficacy of a soluble epoxide hydrolase inhibitor in a PGE_2 induced pain model

Sumanta Kumar Goswami[1], **Bora Inceoglu**[1], **Jun Yang**[1], **Debin Wan**[1], **Sean D. Kodani**[1], **Carlos Antonio Trindade da Silva**[1,2], **Christophe Morisseau**[1], and **Bruce D. Hammock**[1]

[1]Department of Entomology and Nematology, and UC Davis Comprehensive Cancer Center, University of California, Davis

[2]Department of Genetics and Biochemistry, Federal University of Uberlandia, MG, Brazil

Abstract

Epoxyeicosatrienoic acids (EETs) are potent endogenous analgesic metabolites produced from arachidonic acid by cytochrome P450s (P450s). Metabolism of EETs by soluble epoxide hydrolase (sEH) reduces their activity, while their stabilization by sEH inhibition decreases both inflammatory and neuropathic pain. Here, we tested the complementary hypothesis that increasing the level of EETs through induction of P450s by omeprazole (OME), can influence pain related signaling by itself, and potentiate the anti-hyperalgesic effect of sEH inhibitor. Rats were treated with OME (100 mg/kg/day, p.o., 7 days), sEH inhibitor TPPU (3 mg/kg/day, p.o.) and OME (100 mg/kg/day, p.o., 7 days) + TPPU (3 mg/kg/day, p.o., last 3 days of OME dose) dissolved in vehicle PEG400, and their effect on hyperalgesia (increased sensitivity to pain) induced by PGE_2 was monitored. While OME treatment by itself exhibited variable effects on PGE_2 induced hyperalgesia, it strongly potentiated the effect of TPPU in the same assay. The significant decrease in pain with OME + TPPU treatment correlated with the increased levels of EETs in plasma and increased activities of P450 1A1 and P450 1A2 in liver microsomes. The results show that reducing catabolism of EETs with a sEH inhibitor yielded a stronger analgesic effect than increasing generation of EETs by OME, and combination of both yielded the strongest pain reducing effect under the condition of this study.

Keywords

Omeprazole; cytochome P450 1A; Time dependent P450 induction; epoxyeicosatrienoic acid; prostaglandin E_2 induced hyperalgesia; sEH inhibitor TPPU

Corresponding author: Bruce D. Hammock, Distinguished Professor, Department of Entomology & UCD Comprehensive Cancer Center, Director, NIEHS-UCD Superfund Research Program, PI, NIH Biotechnology Training Program, University of California - Davis, One Shields Ave, Davis, CA 95616, USA, Phone: 530) 752-7519, Fax: 530) 752-1537, bdhammock@ucdavis.edu.

Conflict of interest

The University of California holds patents on the sEH inhibitors used in this study as well as their use to treat inflammation, inflammatory pain, and neuropathic pain. B.D. Hammock and B. Inceoglu are co-founders of EicOsis L.L.C., a startup company advancing sEH inhibitors into the clinic.

Introduction

The arachidonic acid (ARA) cascade is essential in the homeostatic regulation of physiological processes including inflammation, vasotension, and nociception (Kuehl and Egan, 1980; Davies et al., 1984; Solomon et al., 1968; Williams and Peck, 1977). Branches of this pathway include metabolites such as prostaglandins, leukotrienes and the epoxyeicosatrienoic acids (EETs). Although the existence and biological relevance of prostaglandins have been known for over half a century, the contribution of EETs to the regulation of these processes has only been realized within the past two decades (Morisseau and Hammock, 2013). Compared to the pro-inflammatory prostaglandins, EETs are potently anti-inflammatory (Thomson et al., 2012), antihypertensive (Jiang et al., 2011), anticonvulsive (Inceoglu et al., 2013) and analgesic (Inceoglu et al., 2008; Inceoglu et al., 2006). Concentrations of EETs are reported to be regulated both by their synthesis by P450s, particularly −2J2 and −2C8 in addition to others, and by their hydrolysis by sEH (Chacos et al., 1983; Imig, 2012; Morisseau and Hammock, 2013). P450's are widely known to be involved in both the biosynthesis of chemical mediators such as steroids and epoxy-fatty acids, but also in the degradation of xenobiotics including pharmaceuticals. Here we test the hypothesis that some of the predominantly xenobiotic metabolizing P450s can contribute to the biosynthesis of biologically relevant levels of EETs.

Increasing local and systemic levels of EETs through inhibition of sEH is a well-established approach for studying the biology of these lipid mediators. Potent sEH inhibitors with sub-nanomolar potency demonstrate efficacy towards reducing pain (Inceoglu et al., 2011), hypertension (Ulu et al., 2014), fibrosis (Harris et al., 2015; Kim et al., 2015), among other biological effects (Kodani and Hammock, 2015). Interestingly, sEH inhibitors (sEHIs) are analgesic in both inflammatory (Inceoglu et al., 2006) and neuropathic pain models (Inceoglu et al., 2012), indicating they act on a pivotal mechanism of pain perception. This mechanism occurs downstream of prostaglandin formation as evidenced by the ability of sEH inhibitors to block PGE_2-induced pain (Inceoglu et al., 2011).

Given that analgesic effects of sEHIs are through increasing the levels of epoxy-fatty acids including EETs, an alternative approach to increase the titer of EETs is to increase their production by inducing P450s. P450s normally associated with xenobiotic metabolism can metabolize numerous lipophilic compounds. Thus, inducers and inhibitors of these P450 enzymes can alter physiological processes by modulating the production of EETs. P450 induction generally occurs through activation of various nuclear receptors or the arylhydrocarbon receptor (AhR) (Shivanna et al., 2011). Although P450 2C8 and P450 2J2 are generally considered to be the major isoforms responsible for EET production, growing evidence indicates a role in the synthesis of the oxylipins by the inducible P450 1A1 and P450 1A2 (Nebert and Karp, 2008). The potent AhR agonist 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for example, leads to increase epoxy and dihydroxy fatty acid concentrations in mammalian liver and lung (Yang et al., 2013). Furthermore, a number of AhR-activators including TCDD and OME increased levels of various epoxy and dihydroxy metabolites in chick embryos (Diani-Moore et al., 2006). This effect was enhanced by combining TCDD with 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), a well characterized sEHI (Diani-Moore et al., 2014) suggesting the feasibility of

modulating EET levels by augmenting their synthesis through inducing P450s and minimizing their metabolism by sEH inhibition.

OME is often used as an experimental probe to induce P450s (Shivanna et al., 2011; Masubuchi et al., 1997). Equally interestingly, it is a first-in-class proton pump inhibitor and one of the most commonly prescribed drugs internationally. In a number of countries OME is available as an over the counter drug and is used by millions of people with more than 720 million prescriptions issued within 15 years from the year of approval (Kaunitz, J.D., 2014; Raghunath et al., 2005; Shaheen et al., 2006). Given the roles of P450s in the synthesis of epoxy-fatty acids, their induction by OME could have functional consequences.

Therefore, in this study we asked if OME treatment would alter the physiology of pain signaling through positively modulating EET levels in a rat model. The potent sEH inhibitor TPPU was used in key experiments to slow the degradation of de novo produced EETs. To test the hypothesis that P450 induction will increase the levels of EETs and this increase in turn will functionally alter pain perception, a stringent model of pain was used. Intraplantar injection of PGE_2 results in intense pain. Although this pain is resistant to treatment with steroids and nonsteroidal anti-inflammatory agents (NSAIDs), earlier we demonstrated that inhibition of sEH effectively blocks this pain (Inceoglu et al., 2011). Rats were treated with OME for 7 days and tested for pain thresholds with and without TPPU. To negate the inhibitory effect of OME on certain isoforms of P450s, the effect of TPPU on pain was also studied during the OME washout period. Subsequently, P450 activity was monitored *in vitro* with general selective substrates; epoxygenase and hydroxylase activities were monitored *in vivo* using levels of EETs+dihydroxyeicosatrienoic acid (DHETs) and hydroxyeicosatrienoic acids (HETEs) as respective biomarkers.

Materials and methods

Materials

OME, PGE_2, PEG400, methoxyresorufin, ethoxyresorufin, pentoxyresorufin, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were purchased from Fisher Scientific (Houston, TX). 7-Methoxy-4-(trifluoromethyl)coumarin (MFC), 7-hydroxy-4-(trifluoromethyl)coumarin (HFC), 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin (AHMC), dibenzylfluorescein (DBF) from Corning Inc. (New York, NY); 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) from ChemBridge Corporation (San Diego, CA); 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) from Molecular probes; fluorescein from Arcos Organic (New Jersey, NJ); 7-(4-methoxybenzyloxy)-4-trifluoromethylcoumarin (MOBFC) from Life Technologies (Carlsbad, CA) were used in the assay. N-[1-(1-Oxopropyl)-4-piperidinyl]-N'-[4-(trifluoromethoxy)phenyl)-urea or TPPU, 2-^{3}H]-*trans*-1,3-diphenylpropene oxide (or [^{3}H]*t*-DPPO), [^{3}H]-*cis*-stilbene oxide (or *c*-SO), cyano(6-methoxy-2-naphthyl)methyl acetate (or CMNA) and N-(6-methoxypyridin-3-yl) octanamide (Octanoyl-MP) were synthesized in-house (Borhan et al., 1995; Shan and Hammock, 2001; Huang et al., 2007).

Animals and treatment

Male Sprague Dawley (SD) rats, 5–6 months old and weighing 400–500 grams were used for this study. Animals were housed in a temperature and humidity controlled room and had free access to rat chow and drinking water. The study protocol was approved by the institutional animal care and use committee of University of California, Davis, and all animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Animals were divided into 7 groups each containing 5–6 rats (Fig. 1). Stock solution of OME (40 mg/mL) and TPPU (10 mg/mL) were prepared in 100% PEG400 and were administered orally, the same day. The final doses of OME and TPPU were 100 mg/kg and 3 mg/kg respectively administered in a volume of 2 mL/kg of PEG400. An intermediate dose of OME was selected based on available literature on P450 1A and P450 2B induction in rats (Masubuchi et al., 1997), and the dose of TPPU was selected based on literature on the anti-hyperalgesic effect of TPPU and similar sEH inhibitors in rats (Sasso et al., 2015; Inceoglu et al., 2011; Inceoglu et al., 2012).

Effect of OME and TPPU on PGE_2 induced pain

Nociceptive withdrawal threshold was quantified with the von Frey assay using an electronic analgesiometer (IITC Inc; Woodland hills, CA) as described earlier (Inceoglu et al., 2006; Inceoglu et al., 2012). Before the start of treatment, the response threshold of each rat was determined. Rats were then treated with vehicle (7 days), OME (7 days) and TPPU (3 days) as outlined in Fig 1. On the days when PGE_2 induced pain behavior was measured, TPPU was administered 2 hours before intraplantar administration of PGE_2. OME and vehicle were administered 1 hour before injection of PGE_2. Although OME is an inducer of P450 1A isoforms, it is also an inhibitor of certain isoforms of P450 2C and P450 2D (Caraco et al., 1996). To reduce the effects of possible short term inhibition of P450 enzymes, in a second experiment we introduced a washout period after the last dose of OME and tested the efficacy of TPPU up to 7 days following the last OME dose. We expected that during the washout period, levels of OME would decrease below the inhibitory levels and induced P450s would still contribute to the EET pool. This experiment also provided a time course of cessation of P450 induction mediated by OME. For this second experiment, pain behavior was measured 2, 4 or 7 days after the last dose of OME. For pain behavior measurements, the withdrawal threshold was measured before PGE_2 and 15, 30, 45, and 60 minutes following PGE_2 administration.

Measurement of oxylipins in plasma

Samples were collected 2h after dose with PGE_2. For sample collection, animals were anesthetized with isofluorane, blood was collected by cardiac puncture, and livers were excised for subsequent analysis of activities of P450, esterase and hydrolase enzymes. Plasma was separated and stored at - 0 C until analysis by LC-MS/MS as described earlier (Yang et al., 2009). Epoxygenase activity was monitored using levels of EETs and their DHET metabolites in plasma. Similarly, hydroxylase activity was monitored using levels of HETEs in plasma.

Preparation of liver S9 and microsomal fractions

Rat liver samples were homogenized with a Polytron® bench top homogenizer (Kinematica GmbH, Luzern, Switzerland) in 4 volumes of 0.1 M Tris/HCl buffer (pH 7.4) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 150 mM potassium chloride and 0.1 mM dithiothreitol (DTT) for 30 seconds. The homogenates were centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatants (S9 fractions) were collected, aliquoted and stored at −80 °C for later use.

Rat liver microsomes were prepared separately in the same buffer, but containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The S9 fractions were collected using the procedure discussed above and centrifuged for 1 h at 4 °C and 106,000 g. Supernatants were discarded and pellets were suspended in 1 mL buffer as mentioned above. he microsomes ere stored at – 0 C until analyzed (Lee et al., 2007A). The protein concentrations of the S9 and microsomal fractions were measured using bicincinic acid (BCA) assay protocol utilizing bovine serum albumin as standard (Brown et al., 1989; Walker J.M., 1994).

Measurement of total P450 level

Two aliquots of microsomal suspensions (100 uL) were added to 2,900 μL of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 20% glycerol (vol/vol), 0.5% sodium cholate (wt/vol), and 0.4% non-ionic detergent Triton N-101 (wt/vol) in two quartz cuvettes. One cuvette was bubbled with carbon monoxide (CO) for 1 minute with the speed of 1 bubble per second through a fine pipette. Sodium dithionite (1 mg) was added into both control and CO treated cuvettes. Absorbances of the microsomes were recorded at wavelengths from 500-400 nm and the concentrations of P450s per mg of protein were determined as described (Guengerich et al., 2009).

Measurement of P450 activity

Microsomal proteins (100 μg) were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 35 μM $MgCl_2$ and 10 μM EDTA. The mixtures were incubated with substrates and NADPH regenerating system (Petrulis et al., 2001; Baer-Dubowska et al., 1998; Donato et al., 2004; Stresser et al., 2002). The reactions were run for 30 minutes in kinetic mode at 30 °C and the fluorescences were recorded (supplementary data Table 1). The linear region of the plot of the standards shown in supplementary data Table 1 was used for calculating the products formed.

Hydrolase activity assay

Enzyme assays such as hydrolysis of [^{3}H]*t*-DPPO), *c*-SO, CMNA and octanoyl-MP were performed to determine the effect of treatment on hydrolases such as sEH (Borhan et al., 1995), microsomal epoxide hydrolase (mEH) (Gill et al., 1983; Morisseau et al., 2008), carboxylesterase 1 (CES1) (Morisseau et al., 2009) and fatty acid amide hydrolase (FAAH) (Morisseau et al., 2009), respectively. Briefly, the S9 fractions were diluted in 0.1M of sodium phosphate or Tris/HCl buffer containing 0.1 mg/mL of BSA. The reactions were started by adding 50 μM of substrates such as t-DPPO, c-SO, CMNA, or octanoyl-MP respectively and run at 37°C.

Results

Effect of OME and TPPU on PGE_2 induced pain

To test the hypothesis that induction of P450s enhances the anti-hyperalgesic efficacy of sEHI, we used PGE_2 administration in the paw as a transient model of severe pain (Inceoglu et al. 2011). Consistent with previous studies, treatment with the TPPU (3 mg/kg/day, 3 days) resulted in a significant increase in withdrawal threshold relative to the vehicle, indicating a reduction in overall pain (Fig. 2A). The dose of PGE_2 used here (600 ng/paw) was selected to be higher than the earlier 100 ng/paw dose to ensure a robust and sustained period of increased pain related behavior (Inceoglu et al., 2011). The group treated with OME alone at 100 mg/kg for 7 days surprisingly displayed a minor but significant reduction in pain related behavior 30 minutes post intraplantar PGE_2 administration. On the other hand, OME treatment prolonged the pain response when compared with the vehicle 60 minutes post PGE_2 treatment (Fig. 2A). Consistent with our hypothesis that OME induces P450 1A which biosynthesize EETs, we found that the combined treatment of OME and TPPU to be the most effective in relieving pain at all time points after PGE_2 administration.

OME is known to have short term effects on P450 activity through inhibition of select isoforms (Shirasaka et al., 2013; Funck-Brentano et al., 1997), and have long term effects on P450s through AhR activation (Shivanna et al., 2011; Kanebratt et al., 2008). Therefore, we designed a follow-up experiment to test if inhibition of P450s by OME influenced the results of the first experiment (Fig. 2B). Interestingly, compared to TPPU alone, co-treatment with OME significantly reduced pain at zero, two and four days after discontinuing OME therapy. By the 7th day of washout, the enhancing effect of OME completely disappeared. The efficacy of OME to increase anti-hyperalgesic effects of TPPU was the highest at 2 days post OME cessation. Given the short half-life of OME in rats (< 1 hr) (Lee et al, 2007A; Lee et al., 2007B; Watanabe et al., 2002) this observation suggests that the effect of OME lasted longer than the inhibition of P450s such as P450 2D2, and likely includes its ability to induce activities of P450s such as P450 1A and P450 2B (Masubuchi et al., 1997). The vehicle, PEG400 did not have any significant effect on pain threshold compared to PGE_2 treated controls.

Effect of treatment on the level of oxylipins in plasma

Epoxy-fatty acids were previously shown to mediate an increase in withdrawal thresholds implying their role in anti-hyperalgesic activity of sEH inhibitors (Inceoglu et al., 2006; Inceoglu et al. 2007). Thus, the plasma concentrations of these epoxides and their sEH metabolites, the diols or DHETs, were measured in plasma immediately after bioassay. Compared to the PEG400 vehicle control, OME significantly increased ($p < 0.05$) levels of 14, 15-EET and 12,13-EpOME in plasma. It has been reported that P450 1A and 2B generate more hydroxy metabolites than epoxy metabolites *in vitro* (El-Sherbeni and El-Kadi, 2014). Similarly, TPPU alone resulted in a significant increase ($p < 0.05$) in the level of 12,13-EpOME, a linoleic acid epoxy metabolite (Table 1). Additionally, as expected, many of the diol metabolites were decreased in TPPU treated animals. Specifically, 8,9-DHET, 14,15-DHET and 12,13-DiHOME metabolites were significantly ($p < 0.05$) reduced compared to the vehicle controls. In the rats treated with OME+TPPU, this effect was

enhanced with levels of EETs and EpOMEs 50–400% higher than vehicle treated controls. From these data, it is clear that increasing the generation of EETs and minimizing metabolism of these epoxides increases the levels of EET significantly *in vivo*. Generally, one would expect that treatment with a sEHI would increase the levels of all the epoxide metabolites of all the polyunsaturated fatty acids because they are all substrates for sEH (Morisseau et al, 2010). However, epoxy-fatty acids and their diols are only qualitative biomarkers of the action of sEH inhibitors. Surprisingly, the levels of 19,20-EpDPE from DHA were unchanged with treatment of TPPU alone, OME alone or a combination of both TPPU and OME without washout. These data illustrate the complexity of a system where the EETs and their diol metabolites have dramatically different polarities and both can be metabolized by multiple pathways. In addition, the activity of the sEH on a substrate in tissues results from a combination of affinity, access to the epoxide and its' concentration. While 19,20-EpDPE compartmentalization is not known, its concentration is low in animals fed normal chow (Ulu et al, 2013) and sEH has a low affinity for it (Morisseau et al, 2010), thus its hydrolysis will be limited even in absence of TPPU.

During the OME washout period, with concurrent administration of TPPU, the levels of 11,12-EET and 14,15-EET were ($p < 0.05$) elevated at all the time points (Table 1). This pattern was consistent with an increase in levels of EpOMEs from linoleic acid observed during the same time period (Table 1). Finally, levels of the docosahexaenoic acid (omega-3 fatty acid) metabolite 19,20-EpDPE were increased ($p < 0.05$) during the washout period, which had not been seen in any other groups. Overall epoxide metabolites of ARA, LA and DHA were elevated by 170–5800% of vehicle; strongly suggesting that OME induced P450s produced more epoxy-fatty acids.

Effect of treatment on P450 levels and activities

As expected (Masubuchi et al., 1997) the total level of P450, measured by carbon monoxide difference spectra, in all of the groups treated with OME were increased ($p < 0.05$) by approximately 2-fold (Table 2) compared to PEG400. Interestingly, total levels of P450 were also significantly increased ($p < 0.05$) with TPPU treatment. Although total P450 levels were increased up to 7 days after the last dose of OME, the activities of P450s we investigated did not seem to follow the same time course. As determined using isoform-selective substrates, several of the P450s exhibited time dependent induction and inhibition relative to the last day of OME administration. EROD, MROD, PROD and BFCOD activities, consistent with P450 1A1, P450 1A2, P450 2B1 and P450 3A1 + P450 1A2 (Yang et al., 2003; Masubuchi et al., 1997; Petrulis et al., 2001; Baer-Dubowska et al., 1998; Donato et al., 2004) were induced with OME; however, this induction was short lived (2 days). These isoforms of P450s also generate EETs (Diani-Moore et al., 2006; El-Sherbeni and El-Kadi, 2014; Mitra et al., 2011) in addition to P450 2D (Thompson et al., 2000). In contrast, AMMCOD activity, indicative of P450 2D2 activity, was significantly suppressed by OME because the proton pump inhibitor at different concentrations (10 nM-1 μM) did not inhibit formation of the product AHMC when AMMC (substrate) was incubated with liver microsomes from untreated rats *in vitro*. At concentrations of 10 and 50 μM, OME marginally inhibited 18 and 29% production of AHMC respectively. In addition, OME also did not quench the AHMC signal at concentrations up to 50 μM *in vitro*. However, this

suppression of P450 2D2 activity was not observed at 2 days washout of OME as expected from the pharmacokinetic half life of OME. As shown in previous studies OME inhibits P450 2D6 in humans, which is orthologous to P450 2D2 in rats (Watanabe et al., 2002; Caraco et al., 1996; Ko et al., 1997; Makaji et al., 2010). OME inhibits some P450s such as P450 2C (Caraco et al., 1996) and P450 2D (Caraco et al., 1996; Ko et al., 1997; Makaji et al., 2010), therefore we cannot rule out that the anti-hyperalgesic effect seen in Fig. 2A is due in part to enzyme inhibition by an alternate substrate. Interestingly, the activities of P450 2C23 and 2C6, orthologous to human P450 2C8 and P450 2C9, respectively known to produce epoxy-fatty acids (Imig, 2012; Stresser et al., 2002; Donato et al., 2004), was not altered significantly by OME or TPPU in our assay (Table 2) though inhibitory effects of these chemicals on other P450 2C isoforms cannot be ruled out.

In addition to using fluorescent substrates selective for specific P450 isoforms, we also used microsome preparations from rat liver to measure *in vitro* formation of epoxy-fatty acids and hydroxyl-fatty acids from ARA (Table 2). Surprisingly the formation of these metabolites remained unchanged when expressed as the sum of regioisomers (Table 2) or expressed as separate regioisomers (supplementary data Table 2). As observed previously, the 11, 12-EET and 20-HETE are the predominant products from the epoxygenase and hydroxylase activities, respectively.

Rate of epoxidation/hydroxylation of ARA by rat recombinant P450 1A1, P450 1A2, P450 2B1, P450 2C6 and P450 2C11 is approximately 1/11, 1.5/6.5, 0.5/1.5, 3/7, and 12/8 pmole/pmole P450/min respectively (El-Sherbeni and El-Kadi et al., 2014). The rate of epoxidation/hydroxylation by P450 3A4 is reported as ~ 2/0.1 pmol/pmol CYP3A4/min (Mitra et al., 2011). These observations support the role of P450 1A, P450 2B and P450 3A in generating EETs in addition to P450 2C and thereby influencing physiology and disease. The significant contribution of P450s other than P450 2C in generating EETs would be greater when these are induced. It also cautions against the use of the term epoxygenase to describe these oxidase enzymes which can produce many hydroxylated products as well.

Effect of treatment on hydrolase activities

The activity of several hydrolases involved in the metabolism of oxylipins were measured in liver S9 fractions of treated rats (Table 3). Compared to PEG400, the sEH activity tended to be lower in the animals treated with TPPU, but was not affected by OME treatment. The mEH activity, determined using the selective substrate [^{3}H]*c*-SO, was not affected by TPPU and OME administrations. The treatment of OME and TPPU alone did not significantly alter the esterase or amidase activities. Interestingly, the octanoyl-MP-hydrolase activity, representative of fatty acid amide hydrolase activity, was significantly lowered ($p < 0.05$) at 2 and 4 days after OME discontinuation and in the presence of TPPU. The anti-hyperalgsic effect of TPPU during the 2 and 4 day washout period of OME might be at least in part due to altering FAAH activity (Sasso et al., 2012, Sasso et al., 2015).

Discussion

EETs and sEH inhibition have been shown to decrease pain associated with different pathological conditions, including inflammation and neuropathy (Inceoglu et al, 2006;

Inceoglu et al., 2007, Inceoglu et al, 2008, Inceoglu et al., 2011, Wagner et al., 2011). These effects seem to occur through action on both the central and peripheral nervous system (Inceoglu et al., 2007, Terashvili et al., 2008, Sisignano et al., 2012). The main findings from this study demonstrate that increasing production of EETs by P450 induction increases the efficacy of a sEH inhibitor in the PGE_2 induced pain model (Fig. 2). P450 induction was achieved by using OME, a well-known AhR activator and a major pharmaceutical (Hayashi et al., 2012; Jin et al., 2012, Jin et al., 2014). While, as expected, we observed a moderate induction of P450s by OME in our rat model, OME is known to show a stronger P450 induction effect on human (Lu et al, 2001). Thus, small effects in rats may translate to much larger effects in men. Data from other research groups, suggests that P450s other than the commonly discussed P450 2C can also generate EETs, though to a lesser extent. Specifically, involvement of P450 1A and P450 2B in generation of EETs was reported by El-Sherbeni and El-Kadi (2014). Therefore, their induction should elevate levels of EETs. This observation was supported by Diani-Moore et al., (2014) who demonstrated that the P450 1A inducer TCDD can increase the production of EETs and its metabolite DHETs in livers of chick embryos. Further, they reported that cotreatment with the sEH inhibitor AUDA increases the ratio of EET: DHET suggesting that stabilizing EETs can alter biology influenced by the epoxides. Our observation supports this hypothesis. Although OME pretreatment alone had only a small and variable effect on pain, it significantly enhanced the efficacy of TPPU. Potentiation of anti-hyperalgesic effect of TPPU by OME correlated with increase in activity of P450 1A2 (Table 2), and production of EETs such as 8, 9-EET, 11, 12-EET and 14, 15-EET in plasma of rats (Table 1). Intra-plantar administrations of the epoxides of arachidonic, eicosapentaenoic and docosahexaenoic acids (EETs, EpETEs and EpDPEs respectively) significantly reduced inflammatory pain in the paws of rat (Morisseau et al, 2010). This observation implies the significance of induction of P450 1A2 in modulating levels of EETs and thereby affecting biologies such as hyperalgesia (Masubuchi et al., 1997; Inceoglu et al., 2011) though there may be other mechanisms involved.

This study suggests that the beneficial effects of increasing the production of EETs by induction of P450s will be observed if these lipid mediators are protected from metabolism by sEH inhibitors. This probably reflects the observation that the activity of the epoxy-fatty acid catabolic enzyme (sEH) (Table 3) is much higher than the activity of the enzymes producing them (Table 2). Hydrolysis by sEH was previously shown to be the major route of metabolism of the EETs (Chacos et al., 1983; Spector et al., 2004). Thus, one would anticipate an additive or synergistic effect resulting from the enhancement of biosynthesis and reduction of degradation. Interestingly, the combined effect of OME and sEH inhibitors was enhanced if analgesia was measured even 2 days after the last OME dose. This long term effect is probably due to a sustained presence of high levels of induced P450s in the tissues, and to the absence of direct inhibition/suppression of P450s by the rapidly metabolized OME (Lee et al. 2007b). It is interesting to note that although the anti-hyperalgesic effect peaked two days after the last OME dose (Fig. 2B), the plasma EET concentrations remained increased up to seven days after the last dose (Table 1). This underlines that there is a complex relationship between the levels of EETs in the plasma and their pain reducing activities. We previously observed in a model of neuropathic pain, that pain reduction was not tightly correlated with plasma levels of EETs but with their levels in

the targeted organ (Inceoglu et al. 2012). This is especially true when the targeted biology represent a small area/volume of the body, such as here the peripheral nerve fibers innervating the skin of paw, while plasma concentrations are more representative of the whole-animal status. Another consideration is that when more epoxy-fatty acids are produced, more incorporation into membranes may have occurred. Subsequently, the basal rate of EET release from membranes might have shifted upwards leading to a longer duration of increase in the levels of epoxy-fatty acids. EpOME's were not seen to have a direct effect on pain perception (unpublished data from our lab) although their diols are proinflammatory in increasing vascular permeability (Slim et al., 2001).

This study highlighted that drug-drug interaction is possible when both of the agents administered affect arachidonic acid/ P450/ sEH pathways. Omeprazole has been shown to affect the pharmacokinetics of many drugs through induction and inhibition of many isoforms of P450s and through change in drug bioavailability from decrease in intragastric acidity (Labenz et al., 2003; Andersson, 1991). Apart from pharmacokinetic drug interaction, this study suggests that pharmaco-dynamic drug interaction is possible when any compound affecting the metabolism of EETs is administered with OME. Other pharmaceuticals and a wide variety of chemicals can affect P450 expression. For example, PPAR agonists, such as phthalates or fibrates, are well known to change the expression levels of several P450s such as P450 4A (Hardwick et al., 2009). Interestingly, this class of xenobiotics dramatically alters levels of sEH (Hammock and Ota, 1983) and can alter EET levels *in vivo* in rodents (Wheelock et al., 2007). Similarly drug-drug and drug-xenobiotic pharmacodynamic interaction is possible when both of the agents affecting arachidonic acid/ P450/ sEH pathway are administered together. For example, resveratrol, a natural stilbene compound found in a variety of foods and phenobarbital, an anti-convulsant and sedative hypnotic induces P450 1A and increase P450 expression respectively (Chow et al., 2010, Masubuchi et al., 1997). These studies suggest that interaction between sEHIs and resveratrol and phenobarbital may affect clinical outcome by altering levels of EETs. sEH inhibitors are considered for treatment of neuropathic pain in humans and animals (Inceoglu B., 2014), reducing hypertension (Ulu et al., 2014) and reducing chronic obstructive pulmonary disorder (Podolin et al., 2013). Evidence reported here suggests efficacy of sEH inhibitors may be significantly modified by P450 induction through their effects on production of epoxy-fatty acids as well as by drug metabolism. The combined effect resulting in increased EET concentrations may therefore represent an important drug-drug and drug-xenobiotic interaction to consider when comparing the efficacy of these inhibitors in humans. Several metabolites of linoleic acid formed by oxidation including of note 9-HODE and 13-HODE have been reported to invoke allodynia (a painful rseponse to innocuous stimuli). Also the broadly active P450 inhibitor ketoconazole which decreases HODE-induced hyperalgesia by inhibiting P450s also likely influences the efficacy of sEH inhibitors (Patwardhan et al., 2009; Ruparel et al., 2012. Thus, we can anticipate that a variety of xenobiotics which alter biosynthesis and degradation of oxylipins will influence the efficacy of sEH inhibitors.

Overall, our data demonstrate that OME treatment itself can influence pain signaling, and that OME mediated increases in activity of P450s enhance the anti-hyperalgesic efficacy of

sEH inhibition. The observed increase in P450 1A1 and P450 1A2 activity, and in systemic levels of EETs support the argument that modulation of P450s may be an alternative approach for regulating the levels of EETs and biological effects associated with EETs. This study suggests that both EET synthesis and hydrolysis are important for the physiologic regulation of nociception. The data presented here indicate that, in addition to P450s usually thought to be selective for epoxidation of unsaturated fatty acids, the less specialized P450s, normally thought to be involved in xenobiotic metabolism, can contribute significantly to EETs titer. This is particularly true if these P450s are induced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The soluble epoxide hydrolase (sEH) inhibitor TPPU is anti-hyperalgesic.
- Omeprazole potentiates the anti-hyperalgesic actions of TPPU.
- This potentiation is associated with increased P450 activity.
- The potentiation is associated with an increase in fatty acid epoxide/diol ratio.
- Joint use of sEH inhibitors and P450 inducers could result in drug-drug interactions.

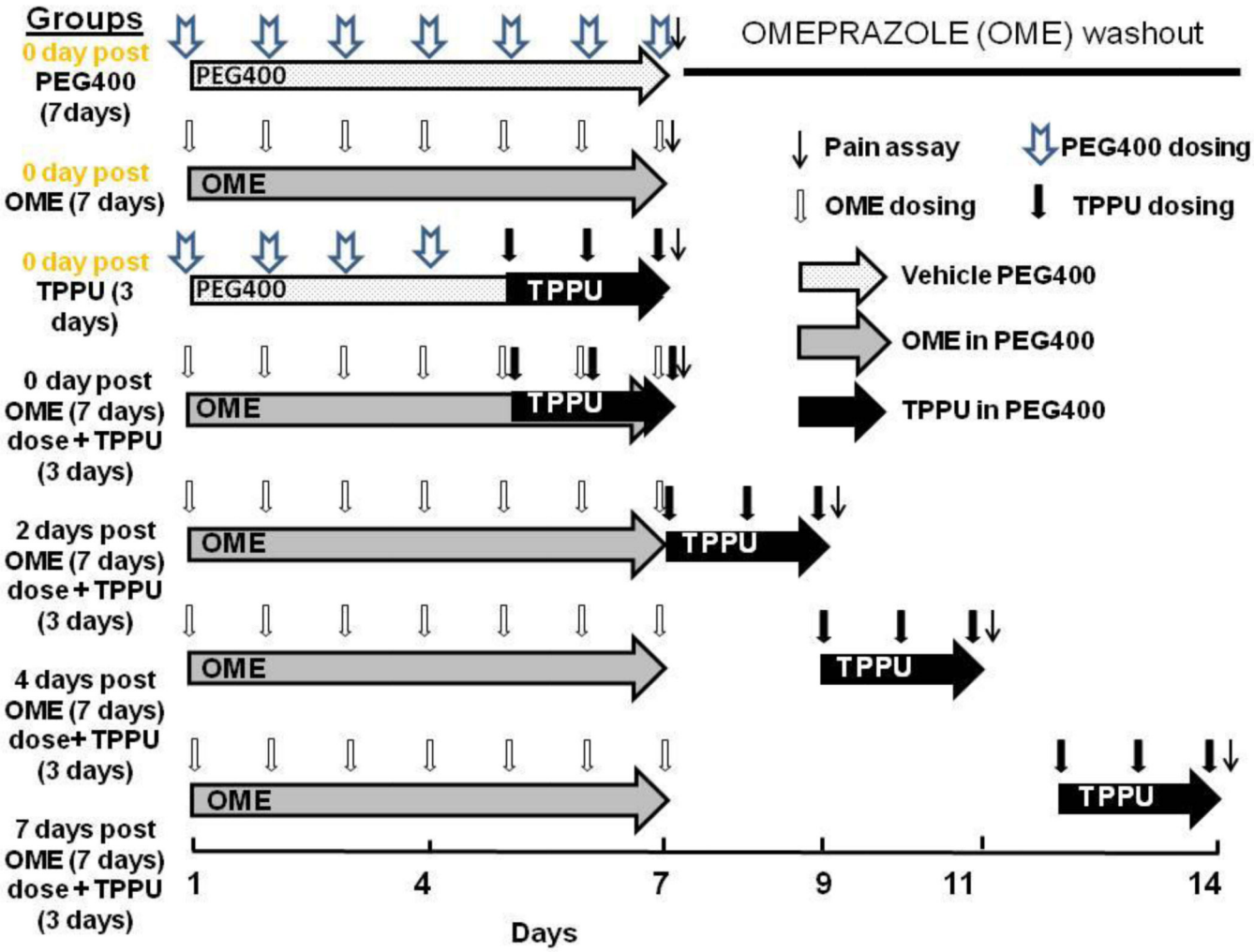

Fig. 1.

Dosing schedule of vehicle, omeprazole (OME) and soluble epoxide hydrolase inhibitor TPPU. Male rats were treated with vehicle PEG400 (2 mL/kg/day, p.o.), OME (100 mg/kg/day, p.o., 7 days), TPPU (3 mg/kg/day, p.o., 3 days) or OME (100 mg/kg/day, p.o., 7 days) + TPPU (3 mg/kg/day, p.o., last 3 days of OME dose). Effect of treatment was evaluated in PGE_2 (600 ng/20 μL, intraplantar) induced model of hyperalgesia. TPPU was administered before 2 hour of PGE_2 whereas OME and PEG400 were administered 1 h before PGE_2 injection. Effect of TPPU (3 mg/kg/day, p.o., 3 days) on hyperalgesia was also examined after 2, 4 and 7 days of washout period of OME.

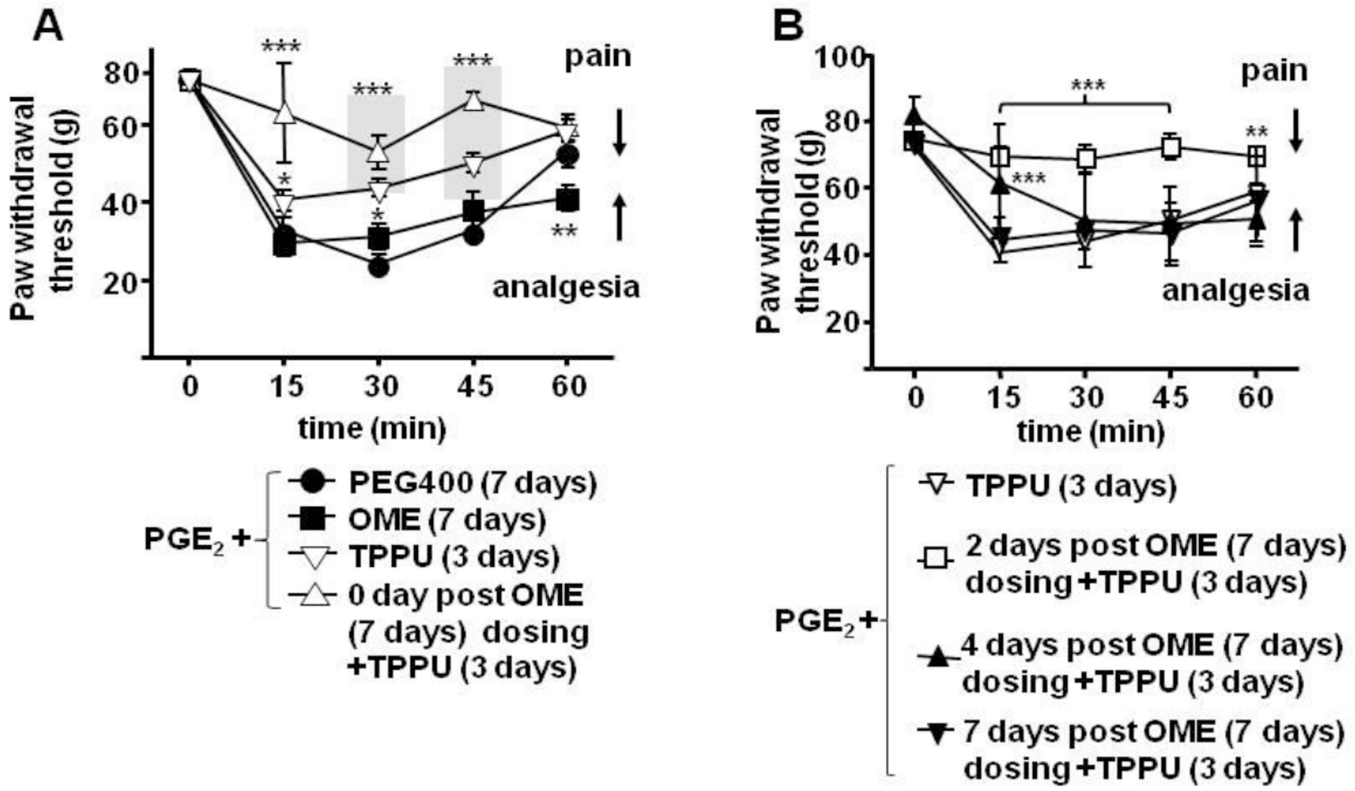

Fig. 2.

Omeprazole (OME) induction of P450s increases the anti-hyperalgesic efficacy of the sEH inhibitor TPPU in a PGE_2 induced model of hyperalgesia in rats. PGE_2 was administered after treatment of vehicle (1 h), OME (1 h or 2, 4 and 7 day washout period) and TPPU (2 h) (Fig. 1). Effect of treatment on hyperalgesia was evaluated using von Frey method within 60 minutes of PGE_2 intraplantar injection. Decrease in paw withdrawal threshold (PWT) means increase in pain and reversal of this phenomenon represents anti-hyperalgesic effect. Values are mean ± standard deviation (n =5–6). One- way ANOVA followed by Bonferroni's post hoc comparison test was used for statistical significance. (**A**) Intraplantar PGE_2 (600 ng/paw) resulted in a rapid decrease in PWT of rats. Pretreatment of OME (100 mg/kg, p.o., 7 days) significantly (*p<0.05, OME *vs.* vehicle) reversed decrease in PWT at 30 minutes post PGE_2 administration, but at the last time point, displayed n decrease in PWT (**p<0.01, vehicle *vs.* OME). Rats treated daily with TPPU (3 mg/kg, p.o.) for 3 days displayed the expected and significant (*p<0.01 and *** p <0.001, OME *vs.* vehicle) decrease in PWT (Inceoglu et al., 2011). Treatment of both OME (100 mg/kg, 7 days) and TPPU (3 mg/kg, last 3 days) was better effective in reversing PWT (*** P < 0.001, OME and TPPU *vs.* vehicle). Similarly the combined treatment was better efficacious than TPPU alone group (**p < 0.01, significance not shown in graph), with the exception of the last time point and compared to OME (p < 0.001, significance not shown in graph), at all time points tested after PGE_2 administration. (**B**) OME-treatment is also expected to inhibit certain P450s, therefore pain assays were performed at various times following the cessation of OME treatment to minimize this possibility. TPPU results from panel **A** are re-plotted to provide a comparison. Last dose of TPPU (3 mg/kg, 3 days) was administered 2 h before the pain

assay. Two days after cessation of OME treatment, TPPU exhibited best effect in reversing PWT. Enhancement of the anti-hyperalgesic efficacy of OME+TPPU treatment gradually diminished to the level of TPPU alone over the course of 7 days following the cessation of OME with 2 days post OME being the most efficacious. Significance is represented as *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ compared to TPPU.

Table 1

Effect of treatment on levels of epoxy fatty acids in plasma.

Groups	PEG400 (7 days)	OME (7 days)	TPPU (3 days)	0 day post OME (7 days) dosing	2 days post OME (7 days) dosing	4 days post OME (7 days) dosing	7 days post OME (7 days) dosing
				+ TPPU (3 days)			
	Plasma level (nM)						
8, 9-EET	8.5 ± 4.0	7.3 ± 1.5	6.5 ± 2.8	13 ± 6$^{\$\#}$	15 ± 3$^{*\$\#}$	7.4 ± 2.2	8.6 ± 1.9
11, 12-EET	5.1 ± 1.5	9.0 ± 3.7	8.8 ± 3.4	18 ± 11	64 ± 14	81 ± 41$^{*\$\#}$	130 ± 70$^{*\$\#}$
14, 15-EET	3.2 ± 0.6	8.2 ± 2.3	7.6 ± 2.1	14 ± 6	44 ± 6$^{*\#}$	53 ± 25$^{*\$\#}$	55 ± 29$^{*\$\#}$
8, 9-DHET	0.88 ± 0.33	0.82 ± 0.19	0.40 ± 0.18	0.55 ± 0.26	0.66 ± 0.21	0.49 ± 0.16	0.19 ± 0.20$^{*\$}$
11, 12-DHET	2.6 ± 0.2	2.8 ± 0.9	2.2 ± 0.6	2.0 ± 0.8	3.3 ± 1.5	2.2 ± 0.8	2.5 ± 1.4
14, 15-DHET	3.7 ± 0.3	3.2 ± 1.3	1.8 ± 0.7*	1.6 ± 0.6*	2.9 ± 1.1	1.3 ± 0.4*	1.6 ± 0.7*
9, 10-EpOME	15 ± 6	31 ± 16	38 ± 9	62 ± 29	160 ± 50	110 ± 60	840 ± 620$^{*\$\#}$
12, 13-EpOME	19 ± 6	58 ± 40	83 ± 18	100 ± 10	240 ± 80	160 ± 50	1100 ± 700$^{*\$\#}$
9, 10-DiHOME	30 ± 10	15 ± 11	11 ± 5	11 ± 4	7.1 ± 1.1	7.2 ± 1.0	37 ± 29$^{\#}$
12, 13-DiHOME	120 ± 80	71 ± 55	16 ± 9*	15 ± 5*	22 ± 5*	15 ± 4*	79 ± 58$^{\#}$
19, 20-EpDPE	1.1 ± 0.3	1.7 ± 0.6	1.5 ± 0.5	2.1 ± 1.3	32 ± 8$^{*\$\#}$	19 ± 7	26 ± 19$^{*\$\#}$
19, 20-DiHDPE	1.1 ± 0.1	1.3 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.5	1.2 ± 0.9	0.42 ± 0.53

Co-treatment of omeprazole (OME, 100 mg/kg/day, p.o., 7 days) and TPPU (3 mg/kg/day, p.o., 3 days) significantly increases levels of epoxy metabolites of arachidonic acid (epoxyeocosatrienoic acid or EET), linoleic acid (*epoxy*-octadecenoic acid or EpOME) and docosahexaenoic acid (*epoxy docosapentaenoic acid or* EpDPE or EDP) in plasma of treated animals. Blood was collected 1 h after PGE_2 administration i.e. after evaluating effect of treatment on pain. Values are mean ± standard deviation (n = 5–6). One way ANOVA followed by Tukey's multiple comparison test as used for statistical significance.

Significance is represented as * $P < 0.01$ compared to PEG400 group;

$^{\$}P < 0.01$ compared to OME group;

$^{\#}P < 0.01$ compared to TPPU group.

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Table 2

Effect of treatment on the activity of cytochrome P450s, epoxygenase, hydroxylase and level of cytochrome P450.

Group		PEG400 (7 days)	OME (7 days)	TPPU (3 days)	0 day post OME (7 days) dosing	2 days post OME (7 days) dosing	4 days post OME (7 days) dosing	7 days post OME (7 days) dosing
					+ TPPU (3 days)			
P450 level (nmoles/ mg microsomal protein)		0.34 ± 0.20	0.58 ± 0.07*	0.62 ± 0.07**	0.41 ± 0.04$^{\#}$	0.59 ± 0.15*	0.59 ± 0.13*	0.52 ± 0.08
Specific activity (pmol.min^{-1}.mg^{-1})	P450 1A1	2.6 ± 0.6	5.2 ± 0.6	4.5 ± 1.9	8.1 ± 2.2$^{*\$\#}$	4.9 ± 1.2	3.3 ± 1.5	2.9 ± 1.1$^{\#}$
	P450 1A2	1.6 ± 0.6	5.6 ± 2*	3.4 ± 1.7	12 ± 4$^{*\$\#}$	2.0 ± 0.4	1.0 ± 0.4$^{\$}$	1.0 ± 0.6$^{\$}$
	P450 2B1	1.0 ± 0.4	2.0 ± 0.2	1.6 ± 0.2	2.2 ± 0.6*	2.5 ± 1.1**	1.6 ± 0.6	1.4 ± 0.6
	P450 2C23	7.0 ± 2.7	4.7 ± 2.7	3.8 ± 3.1	4.9 ± 3.1	4.5 ± 2.9	6.9 ± 1.3	6.5 ± 1.6
	P450 2C6	96 ± 30	100 ± 10	110 ± 30	80 ± 17	100 ± 20	120 ± 40	82 ± 19
	P450 2D2	19 ± 7	0.8 ± 1.4*	23 ± 5$^{\$}$	1.5 ± 1.3$^{*\$}$	18 ± 5$^{\$}$	28 ± 2$^{\$}$	21 ± 5$^{\$}$
	P450 2J3	26 ± 9	39 ± 8	32 ± 8	28 ± 4	23 ± 7	29 ± 13	21 ± 8$^{\$}$
	P450 3A1, P450 1A2	8 ± 5	26 ± 3*	18 ± 6	25 ± 11*	12 ± 7$^{\$}$	9 ± 6$^{\$}$	9 ± 5$^{\$}$
	Epoxygenase activity	1800 ± 600	1800 ± 600	1800 ± 300	1400 ± 300	1700 ± 800	1900 ± 600	1600 ± 400
	Hydroxylase activity	1100 ± 200	1400 ± 200	1100 ± 200	1300 ± 300	1200 ± 600	1100 ± 300	1000 ± 400

In vitro enzyme activities were performed using microsome of rat liver collected immediately after evaluating paw withdrawal threshold. Epoxygenase and hydroxylase activities were measured using ARA as substrate while P450 activities were studied using separate specific fluorescent substrates. Assays were performed in quadruplicate. DBF-O-debenzylation assay and MOBFC-O-demethoxybenzyloxylase assay representative of P450 2C23 activity and P450 2J3 activity respectively were performed using substrates meant for respective orthologs in humans. The specificity of these substrates may vary for rat P450s. Values are mean ± standard deviation (n = 5–6). One ay ANOVA followed by Tukey's multiple comparison test as used for statistical significance.

Significance is represented as * $P < 0.05$ compared to PEG400 group;

$^{\$}P < 0.05$ compared to OME group;

$^{\#}P < 0.05$ compared to TPPU group. See supplementary data table 1 for details of methodology.

Table 3

Effect of treatment on hydrolase activity of S9 liver fractions *in vitro*.

Groups	PEG400 (7 days)	OME (7 days)	TPPU (3 days)	0 day post OME (7 days) dosing	2 days post OME (7 days) dosing	4 days post OME (7 days) dosing	7 days post OME (7 days) dosing
				+ TPPU (3 days)			
	Specific activity (product formed expressed as nmol.min^{-1}.mg^{-1})						
t-DPPO hydrolysis (sEH)	3.0 ± 1.3	3.5 ± 2.8	2.5 ± 1.1	2.1 ± 0.5	2.6 ± 1.1	2.8 ± 0.4	1.9 ± 0.8
c-SO hydrolysis (mEH)	2.5 ± 0.7	3.5 ± 0.4	2.0 ± 0.4$^{\$}$	2.4 ± 0.3	3.6 ± 0.8$^{\#}$	2.7 ± 0.7	2.8 ± 0.9
CMNA hydrolysis (CES)	180 ± 60	170 ± 50	180 ± 40	180 ± 30	230 ± 40	170 ± 30	150 ± 40
Octanoyl-MP hydrolysis (FAAH)	6.8 ± 1.6	5.9 ± 0.8	6.3 ± 1.8	6.2 ± 0.6	3.8 ± 1.1$^{*\#}$	3.4 ± 1.5$^{*\$\#}$	4.7 ± 0.7

TPPU treatment significantly decreased mEH activity as evident by decrease in c-SO hydrolysis using S9 liver fraction *in vitro*. Enzyme activities were performed using rat liver S-9 fraction. Assays were performed in triplicate. Values are mean ± standard deviation (n = 5–6). One ay ANOVA followed by Tukey's multiple comparison test as used for statistical significance.

Significance is represented as * $P < 0.05$,

$^{\$}P < 0.05$,

$^{\#}P < 0.05$ when compared to PEG400, OME and TPPU group respectively.