# Inhibition of soluble epoxide hydrolase reduces LPS-induced thermal hyperalgesia and mechanical allodynia in a rat model of inflammatory pain

Bora Inceoglu [a], Steven L. Jinks [b], Kara R. Schmelzer [a],
Troy Waite [a], In Hae Kim [a], Bruce D. Hammock [a,*]

[a] *Department of Entomology, University of California Davis Cancer Center, One Shields Avenue, Davis, CA 95616, United States*
[b] *Department of Anesthesiology and Pain Medicine, School of Medicine, University of California Davis, Davis, CA 95616, United States*

## Abstract

Soluble epoxide hydrolases catalyze the hydrolysis of epoxides in acyclic systems. In man this enzyme is the product of a single copy gene (EPXH-2) present on chromosome 8. The human sEH is of interest due to emerging roles of its endogenous substrates, epoxygenated fatty acids, in inflammation and hypertension. One of the consequences of inhibiting sEH in rodent inflammation models is a profound decrease in the production of pro-inflammatory and proalgesic lipid metabolites including prostaglandins. This prompted us to hypothesize that sEH inhibitors may have antinociceptive properties. Here we tested if sEH inhibitors can reduce inflammatory pain. Hyperalgesia was induced by intraplantar LPS injection and sEH inhibitors were delivered topically. We found that two structurally dissimilar but equally potent sEH inhibitors can be delivered through the transdermal route and that sEH inhibitors effectively attenuate thermal hyperalgesia and mechanical allodynia in rats treated with LPS. In addition we show that epoxydized arachidonic acid metabolites, EETs, are also effective in attenuating thermal hyperalgesia in this model. In parallel with the observed biological activity metabolic analysis of oxylipids showed that inhibition of sEH resulted with a decrease in $PGD_2$ levels and sEH generated degradation products of linoleic and arachidonic acid metabolites with a concomitant increase in epoxides of linoleic acid. These data show that inhibition of sEH may become a viable therapeutic strategy to attain analgesia.
© 2006 Published by Elsevier Inc.

*Keywords:* Soluble epoxide hydrolase; Arachidonic acid; EETs; Inflammation; Pain

## Introduction

Tissue injury results in the release of a diverse group of inflammatory mediators that sensitize nociceptors and spinal nociceptive neurons to mechanical and thermal stimuli, leading to heightened pain transmission. Local, systemic, or neurogenic release of inflammatory mediators include $K^+$, neuropeptides such as substance P, peptides such as bradykinin, cytokines, monoamines, and ATP, which activate or sensitize peripheral nociceptors (Coutaux et al., 2005). Furthermore, peripheral sensitization of nociceptors can, in turn, lead to central sensitization in the spinal cord, producing secondary hyperalgesia and allodynia through processes that include activation of NMDA glutamate receptors, nitric oxide production, as well as spinal upregulation of COX and resulting prostaglandin synthesis (Vadivelu and Sinatra, 2005).

Long chain fatty acids, prominently arachidonic acid (AA), are molecules that lie at a pivotal point of important inflammatory cascades that result in peripheral sensitization of nociceptors. It has been well documented that AA release activates two classes of enzymes: the cyclooxygenases (COX) and the lipoxygenases, which lead to the production of the pro-inflammatory mediators including prostaglandins (PG) and leukotrienes (Oliw et al., 1982; Cohen, 2002; Coutaux et al., 2005). These enzymes have been the focus of intense research during the last decades, and inhibitors of these enzymes are major therapeutic agents for inflammatory pain (Vane, 1971; Fabien et al., 2004).

Another branch of the arachidonate cascade is the cytochrome P450-catalyzed conversion of AA and linoleic acid (LA)

---

* Corresponding author. Tel.: +1 530 751 7519; fax: +1 530 752 1537.
*E-mail address:* bdhammock@ucdavis.edu (B.D. Hammock).

to a conspicuous group of metabolites including epoxyeicosatrienoic acid (EET), hydroxyeicosatrienoic acids (HETEs) and epoxyoctadecenoic acids (EpOMEs). Among these metabolites, EET is the putative endothelium-derived hyperpolarization factor, which exerts anti-inflammatory and antihypertensive effects in the cardiovascular system (Makita et al., 1994; Node et al., 1999; Kozak et al., 2000; Zeldin, 2001; Spector et al., 2004; Spiecker and Liao, 2005; Schmelzer et al., 2005; Fleming and Busse, 2006). EETs and EpOMEs are short-lived AA and LA metabolites that are converted by the enzyme soluble epoxide hydrolase (sEH) to pro-inflammatory dihydroeicosatrienoic acids (DHETs) and dihydroxyoctadecenoic acids (DiHOMEs), respectively (Zeldin et al., 1993; Fang et al., 2004). Inhibition of sEH increases detectable concentrations of EETs, decreasing blood pressure only under hypertensive conditions and reducing vascular inflammatory responses (Yu et al., 2000; Fang et al., 2004; Oliver et al., 2005, Schmelzer et al., 2005). The sEH enzyme has thus been identified as a therapeutic target for inflammation and hypertension, and therefore might serve to treat inflammatory pain (Schmelzer et al., 2005). We have previously shown that increases in PG synthesis by the inflammatory agent lipopolysaccharide (LPS), also known as endotoxin, are inhibited by sEHIs via a COX-independent as well as an indirect COX dependant mechanism (Schmelzer et al., 2005).

Because sEHIs have been shown to possess vascular anti-inflammatory properties, the present study addresses the hypothesis that sEHIs have anti-nociceptive properties in a rat model of LPS-induced local inflammatory hyperalgesia (Kanaan et al., 1996), suggesting that sEHIs might serve as novel anti-inflammatory agents for pain relief. Because sEHIs raise endogenous EET levels by preventing EET degradation, we further hypothesized that exogenous EETs would reduce hyperalgesia when applied directly to the hind paw.

## Materials and methods

### Animals

The UC Davis Animal Care and Use Committee approved this study. Male Sprague–Dawley rats weighing 275–350 g were obtained from Charles River Inc., and maintained in UC Davis animal housing facilities with *ad libitum* water and food on a 12h:12h light–dark cycle with lights on at 07:00. Data were collected during the same time of day for all groups.
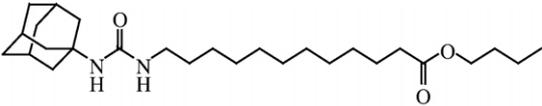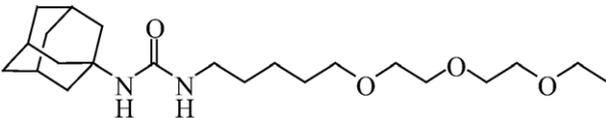
### Chemicals

The sEH inhibitors 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE) and 1-adamantan-3-(5-(2-(2-ethylethoxy) ethoxy)pentyl)urea (IK 950), were synthesized in our laboratory as described previously (Morisseau et al., 2002; Kim et al., 2004). Methyl ester EETs were synthesized using arachidonic acid methyl ester (AAme) as described previously (Campbell et al., 1991). Briefly, 7 g AAme was epoxidized with $m$-chloro-perbenzoic acid (mCPBA) at room temperature in a $CH_2Cl_2$/phosphate buffer (pH 7.4) biphasic system for 2 h, the

organic phase was isolated, treated with hexane, anhydrous potassium fluoride and celite, and filtered through celite to remove precipitated residual mCPBA and the corresponding acid. The solution was partitioned against sodium bicarbonate and saline then dried over anhydrous sodium sulfate. Upon evaporation of the organic phase the residue was purified by flash chromatography. The mixture was characterized by LC–MS/MS as described (Smith et al., 2005). LPS was from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline for administration. Inhibitors were formulated into Vanicream™ (Pharmaceutical Specialties, Inc. Rochester, MN) by first heating 1 ml of Vanicream™ until it liquefied and mixing the cream with the inhibitors dissolved in 1 ml ethanol. This dissolved mixture was then added into 20 g of Vanicream™ and mixed well by forcing the Vanicream through a narrow bore between syringes. The methyl ester forms of EETs were formulated by first dissolving the oil in 1 ml ethanol and mixing with 9 ml Vanicream™.

### Treatments and behavioral nociceptive tests

Behavioral nociceptive testing was conducted by assessing thermal hindpaw withdrawal latencies (TWL) using a commercial Hargreaves (Hargreaves et al., 1988) apparatus (IITC, Woodland Hills, CA), or by determining mechanical hindpaw withdrawal thresholds (MWT) using a series of calibrated von Frey filaments with graded bending forces (Stoelting, Wood Dale, IL). On the day of the experiment, rats were transferred to a quite room, acclimated for 1 h and their baseline responses were measured. In pilot experiments, the intensity of the thermal stimulus was set to produce a baseline TWL of 7–8 s. Following baseline measurements, rats were first treated with 200 µl of vehicle or compound-formulated cream (IK 950, AUDA-be, or EETs) by topical application to one hindpaw. The cream was thoroughly massaged across the entire hindpaw surface over a 2 min period. Within 10 min of cream application, LPS (10 µg in 50 µl 0.9% NaCl) or saline was injected subcutaneously into the plantar surface of the treated paw. Immediately following LPS injection, animals were placed in acrylic chambers on a glass platform maintained at a temperature of $30 \pm 1$ °C for TWL measurement. During TWL measurement, a beam of radiant heat was focused onto the mid-portion of the plantar surface of the treated hind paw until the rat moved its stimulated hindpaw abruptly away from the heat stimulus. The duration of heat application necessary to elicit a withdrawal was designated as TWL. A maximum stimulus duration of 22 s was imposed to prevent tissue damage. Five TWL measurements were taken at 3–4 min interstimulus intervals for each of the 30, 60, 120 and 240-min time points following treatment. The three median TWLs were averaged for each animal at each time point. For MWT measurement, the series of filaments (1.2–180 g bending force) were applied to the plantar hind paw surface through the wire-mesh platform in order of increasing bending force. The filament with the lowest bending force necessary to elicit a hindpaw withdrawal was designated as threshold. MWT measurement was repeated two more times, starting with a bending force two steps weaker than the filament that elicited a

Table 1
Structure and potency of sEHIs used in this study

| Inhibitor | Structure | Human IC$_{50}$ |
|---|---|---|
| AUDA-be | | 0.8 nM |
| IK 950 | | 4.3 nM |

Both inhibitors contain a urea primary pharmacophore. Although IK-950 is more water soluble compared to AUDA-be both inhibitors were effective topically when formulated into a cream. The IC$_{50}$ refers to the potency of inhibition of the indicated compound on the recombinant human enzyme *in vitro*.

withdrawal on the previous trial. Bending force was increased or decreased according to the observed response in order to obtain MWT. The three MWT measurements were averaged for each animal at 60 min and 120 min following treatment.

*Sample collection, extraction, analysis*

Blood samples for lipid analysis were collected by cardiac puncture under deep halothane anesthesia. Plasma was immediately separated by centrifuging at 4000 rpm for 10 min in a cold microfuge. Rats were terminated by deep halothane anesthesia followed by KCl injection.

Lipid extractions and oxylipin concentrations were measured according to Schmelzer et al. (2005). Briefly, oxylipins were extracted from plasma via Oasis SPE, dried under nitrogen and reconstituted in 50 μl MeOH:water (75:25) containing internal standards. Oxylipids extracts were separated by reverse-phase HPLC on an XTerra MS C$_{18}$ column [30×2.1 mm i.d., 3.5 μm (Waters, Milford, MA)] and quantified with a Quattro Ultima tandem quadrupole mass spectrometer (Micromass, Manchester, UK) in negative mode electrospray ionization and multiple reaction monitoring.

To quantify compound plasma levels, serial tail bleed samples of 20–50 μl blood were collected into heparin-treated tubes. For urine quantification, 500 μl of urine was collected at various time points (2 to 24 h) and immediately frozen. The samples were then transferred to a 1.5 ml microcentrifuge tube, weighed and mixed with 100 μl of purified water and 25 μl of surrogate (100 ng/ml CUDA; *N*-cyclohexyl-*N*-dodecanoic acid urea) and vortexed. The samples were extracted with 500 μl of ethyl acetate. The organic layer was then transferred to a 1.5 ml microcentrifuge tube, and dried under nitrogen. The residues were reconstituted in 25 μl of methanol and 10 μl aliquots were injected to the LC/MS/MS system. An XTerra™MS C$_{18}$ column (30×2.1 mm i.d., 3.5 μm; Waters Corporation) was used with a flow rate of 0.3 ml/min at ambient temperature. The ESI mass spectrometer was operated in the positive ion mode with a capillary voltage at 1.0 kV. Cone gas (N$_2$) and desolvation gas (N$_2$) were maintained at flow rates of 130 and 630 l/h, respectively. The source and the desolvation temperature were set at 100 and 300 °C, respectively. Optimum cone voltages were

set at 80 V for AUBA, AUDA, IK 950 and 950-OH and 85 V for CUDA.

*Statistical analyses*

For TWL and MWT measurements, within group comparisons were performed using a two-factor ANOVA (animal × timepoint) followed by two-sided Dunnett multicomparisons using commercial statistical software (SPSS, Chicago, IL). Between group comparisons of TWLs and MWTs were performed using a two-tailed Student's *t*-test. Because von Frey gradations consist of non-linear increases in bending force, a log-transform was
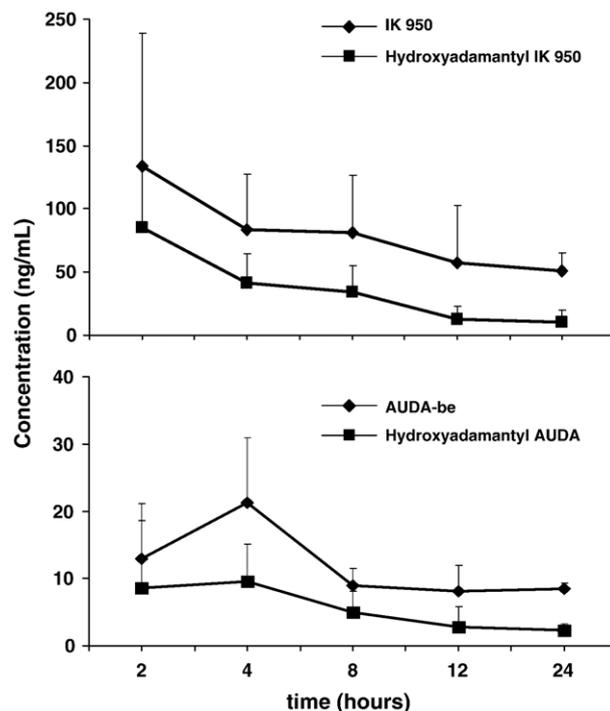


Fig. 1. Urine concentrations of IK 950 (■) and its metabolite hydroxyadamantyl-IK 950 (♦, top), AUDA-be (♦) and its metabolite hydroxyadamantyl-AUDA (■, bottom). Urine samples are collected at times indicated on the graph and extracted and quantified as described in the Methods section. Both compounds are present in significant quantities in the urine (*n*=4).

performed on the MWT data before running statistical analyses. Results are depicted as mean±SEM.

## Results

### Pharmacokinetics of topical sEHIs

AUDA-be and IK 950 are stable in this cream formulation at room temperature for at least 12 months (data not shown). The characteristics of sEHIs can be seen in Table 1. Topical application of 100 mg/kg of sEHIs results in significant blood and urine levels of both sEHIs AUDA-be and IK 950 and their corresponding hydroxy-adamantyl primary metabolites (Fig. 1).

### Effects of sEHIs on LPS-induced thermal hyperalgesia and mechanical hyperalgesia

Injection of 10 μg LPS into the plantar hind paw skin caused TWLs to decrease significantly at all timepoints across a 4-h period (Fig. 2A, solid squares; $p < 0.0001$ in all cases), which had a peak effect at two hours where TWL decreased to 48% of baseline. Vehicle-treated controls showed no significant changes in TWL over the same 4-h testing period (Fig. 2A, open squares). Pro-

phylactic topical administration of the AUDA-be to the LPS-treated hindpaw alleviated thermal hyperalgesia across the 4-h testing period (Fig. 2A, open circles). At the earliest, 30-min timepoint, AUDA-be-treated animals showed a small but significant decrease in TWL, however, TWL was significantly elevated above that for LPS control animals ($p < 0.001$). Over the ensuing 60–240-min time points, AUDA-be-treated animals (100 mg/kg) showed no significant decreases in TWL compared to their pre-treatment baseline TWL. IK 950-treated animals (100 mg/kg) showed significantly longer TWL compared to LPS control animals only at the 120-min time point (Fig. 2A, open triangles; $p < 0.004$), which at this time was also not significantly different from the pre-treatment baseline TWL. Thus, AUDA-be more effectively blocked LPS-induced thermal hyperalgesia compared to IK 950 across the 4-h testing period.

For both sEHIs, a significant dose–response relationship was found when tested at the 4-h time point (Fig. 2B). The effect of the 300 mg/kg dose was significantly greater than the effect of 50 mg/kg dose in reversing LPS-induced thermal hyperalgesia, for both AUDA-be ($p < 0.043$) and IK 950 ($p < 0.018$).

Using mechanical stimulation with graded von Frey filaments, IK 950 and AUDA-be had an approximately equal effect in reversing LPS-induced mechanical allodynia (Fig. 2C). For both types of sEHIs, MWTs at 60- and 120-min time points were not
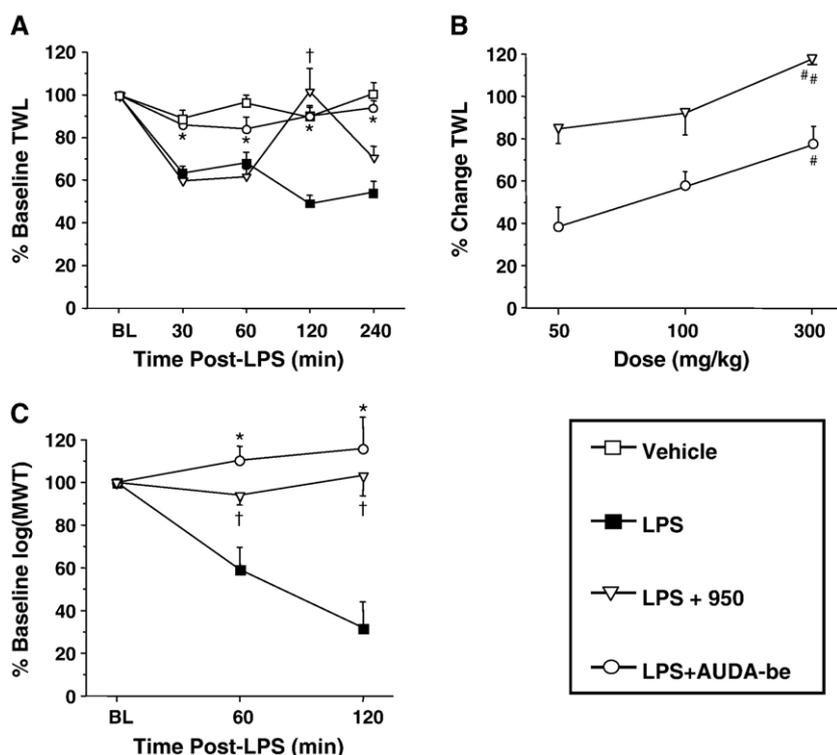


Fig. 2. Line graphs showing mean effects of soluble epoxide hydrolase inhibitors (sEHI) on LPS-induced thermal hyperalgesia and mechanical allodynia. The legend for Figs. 2A–C is shown in the lower right panel. (A) Intraplantar injection of LPS (solid squares; $n=8$) significantly decreased thermal hind paw withdrawal latencies (TWL) during the entire 4-h testing period. The sEHIs AUDA-be (open circles; $n=6$) and IK 950 (open triangles; $n=8$) blocked thermal hyperalgesia, although AUDA-be was more effective. Vehicle-treated controls (open squares; $n=8$) showed no significant change in TWLs. (B) The effects of the sEHIs AUDA-be ($n=4$) and IK 950 ($n=4$) on LPS-induced thermal hyperalgesia were doses dependent when three doses were tested at the 4-h timepoint. (C) Intraplantar LPS injection caused a significant decrease in mechanical withdrawal threshold ($n=8$), which was also blocked by the sEHIs AUDA-be ($n=8$) and IK 950 ($n=8$). * AUDA-be group, significantly different from LPS-only group. † IK 950 group, significantly different from LPS-only group. # dose–response, significantly different from low (50 mg/kg) dose. Values are shown as mean±SEM.
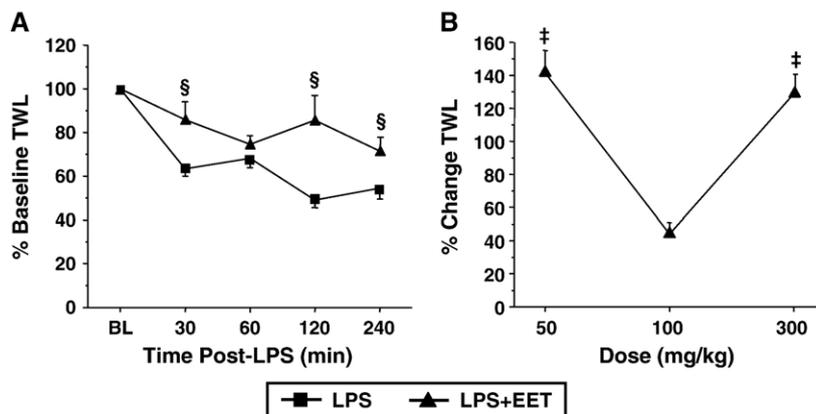
Fig. 3. Line graphs showing mean effects of EETs on LPS-induced thermal hyperalgesia. (A) EETs (solid triangles; $n=8$) partially blocked thermal hyperalgesia at the 30, 60, and 240-min time points as indicated by significant increases in TWL over the LPS-only group (solid squares). (B) The dose response curve for EETs was biphasic within the dose range tested, where the middle dose ($n=4$) was less effective than the lower ($n=4$) or higher dose ($n=4$). § EET group, significantly different from LPS-only group. ‡ Significantly different from middle (100 mg/kg) dose. Values are shown as mean±SEM.

significantly different from their pre-treatment baselines, and were significantly elevated above the LPS control group at each respective time point ($p<0.014$ in all cases).

### Effects of EETs on thermal hyperalgesia

Based on the previous findings that EETs are endogenous substrates for sEH, we tested if administration of EETs would also alleviate LPS-induced thermal hyperalgesia. Animals receiving topical application of EETs to the LPS-treated hindpaw showed significantly longer TWL compared to LPS control animals at 30 min ($p<0.018$), 120 min ($p<0.006$), and 240 min ($p<0.011$) post-LPS (Fig. 3A). EET-treated animals showed no significant change in TWL compared to pre-treatment baseline at 30 and 120 min following LPS injection. The effect of EETs on LPS-induced thermal hyperalgesia at the 2-h time point exhibited a biphasic dose–response relationship (Fig. 3B), with greater efficacies at low-dose (50 mg/kg) and high-dose (300 mg/kg) compared to the intermediate 100 mg/kg dose.

### Effects of sEHIs and EETs in the absence of hyperalgesia

We tested for potential analgesic effects of sEHIs and EETs in the absence of inflammatory pain (Fig. 4). The sEHIs AUDA-be and IK 950 did not significantly change TWL. In contrast to the effect of topically-applied EETs during inflammatory hyperalgesia, EETs (100 mg/kg) were algesic, as indicated by a moderate reduction (∼25%) in TWL at the 60- and 120-min time points ($p<0.01$ in both cases; Fig. 3).

### Effects of sEHIs, EETs and sEHIs+EETs on plasma oxylipins

Treatment with sEHI IK 950 and AUDA-be resulted in a significant increase ($p<0.01$) in plasma levels of oxidized linoleic acid metabolites EpOMEs (Fig. 5A). No significant changes in oxidized arachidonic acid metabolites (EETs) were observed (Fig. 5B). However, in line with the increase in EpOMEs in animals treated with either sEHI or a combination of

sEHIs and EETs, there was a significant reduction ($p<0.01$) in the plasma levels of EpOME degradation products DiHOMEs (dihydroxyoctadecenoic acids), suggesting that EpOME degradation is attenuated by sEHIs (Fig. 5A). Similarly, the levels of arachidonic diols, DHETs are significantly lower ($p<0.01$) in animals treated with sEHIs than control group, indicative of their slower production when sEH is inhibited (Fig. 5B).

### Effects of sEHIs, EETs and sEHIs+EETs on $PGE_2$, $PGD_2$

To test whether the observed antinociceptive effects were mediated at least in part by inhibiting production of inflammatory mediators, we measured LPS-induced changes in prostanoid and oxylipid production in the presence and absence of sEHIs and/or EETs. Consistent with the behavioral analyses, sEHIs attenuated LPS-induced increases of two key prostanoids: $PGE_2$ and $PGD_2$ (Fig. 5C), which were significantly higher in LPS-treated rats that received vehicle only ($p<0.01$). However, EETs alone had no significant effect in decreasing $PGE_2$ or
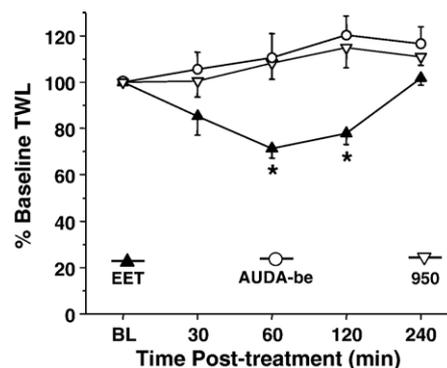


Fig. 4. Line graph showing effects of sEHIs and EETs on TWL in the absence of LPS-induced inflammatory pain. Neither AUDA-be (open circles; $n=4$) nor IK 950 (open triangles; $n=6$) significantly changed TWL over the 4-h testing period. However, EETs (solid triangles; $n=4$) were slightly pro-nociceptive, causing significant reductions in TWL at the 60- and 120 min time points. ∗ EET group, significantly different from untreated vehicle controls. Values are shown as mean±SEM.
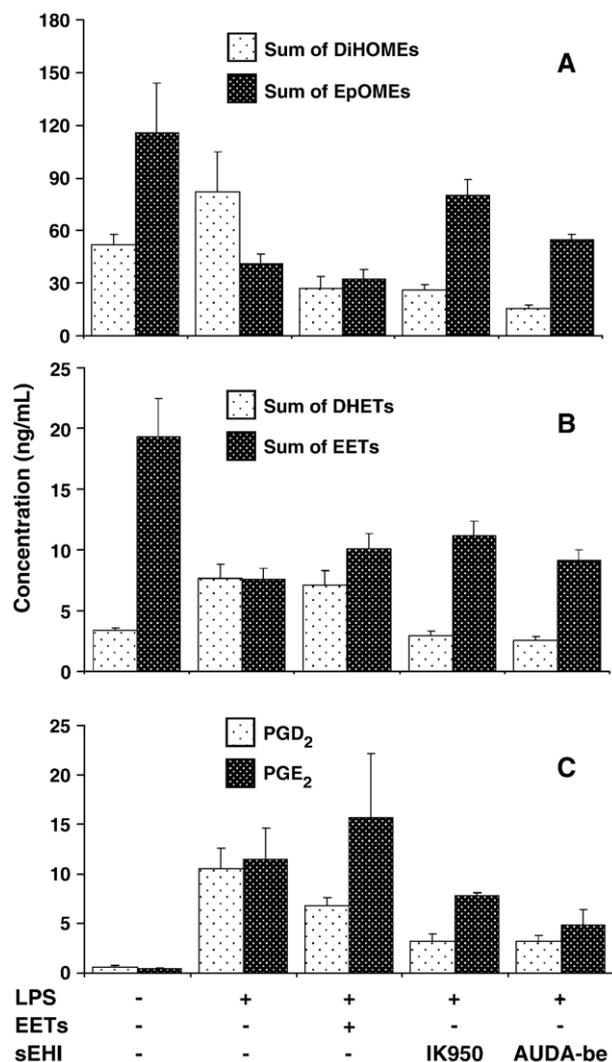
Fig. 5. (A) Administration of LPS causes an increase in linoleic acid metabolites DiHOMEs with a concomitant decrease in EPOMEs. sEHIs stabilize the levels of EPOMEs while causing a decrease in the levels of DiHOMEs. (B) Arachidonic acid metabolites in LPS injected animals follow the same trend; LPS causes an increase in DHETs with a concomitant decrease in EETs. sEHIs but not EETs decrease the levels of pro-inflammatory DHETs. No significant changes are observed in EET levels in any of the groups compared to the LPS treated animals. (C) LPS treatment also causes dramatic increases in levels of two key prostanoids PGD2 and PGE2. These increases are attenuated by both sEHIs whereas EETs cause a significant decrease only in PGD2 levels.

$PGD_2$ blood concentrations, possibly indicating that they are acting through an independent mechanism.

## Discussion

The main finding of the present study was that increasing EET levels through sEH inhibition or exogenous EET application was effective in an LPS model of inflammatory hyperalgesia in rats. The data suggest that sEHIs might serve as novel agents for the treatment of inflammatory pain. However, the present data also suggest that the bioavailability of certain sEHIs (i.e., IK 950) and the pronociceptive action of EETs at certain doses/time points are factors that can limit the efficacy of these agents.

### Effects of topical sEHIs and EETs in an LPS model of inflammatory pain

We topically applied sEHIs and EETs to rat hind paws. Topical administration is an alternative and in many cases a favored method for delivering therapeutics. The transdermal delivery of sEHIs and EETs here was investigated for several reasons. One reason is that the physico-chemical properties of these compounds enable easy formulation into already available creams. A second reason is that the topical route is non-invasive and targets the compound to the inflamed/injured site and furthermore bypasses first-pass metabolism. In addition, the skin provides a natural slow release medium. These benefits are proposed to increase patient compliance, and reduce treatment costs (Hadgraft and Lane, 2005). We observed in the present study that topical delivery of sEHIs result in quantifiable urine levels of these compounds (Fig. 1). However, because of this finding we cannot determine how much of the effect was attributed to a local versus systemic action. Nonetheless, the ability to administer a therapeutic agent systemically by topical application has advantages, especially in the case of poor oral availability.

We tested the effects of sEHIs and EET in an LPS model of inflammatory pain. LPS (endotoxin) is present in the cell wall of Gram-negative bacteria and capable of producing hyperalgesic and pyrogenic effects associated with the febrile responses during illness. A previous study has shown in rats that intra-plantar injections of 0.6–40 µg LPS significantly decreases thermal hot-plate latencies and ipsilateral (but not contralateral) mechanical paw-pressure thresholds, which return to baseline after an approximate 24-h period. In the present study we used a mid-range dose of 10 µg LPS that reduced ipsilateral TWL and MWT to $\leq 50\%$ of baseline 2 h post-injection (Fig. 2A and 2B). Therefore, the decreases in TWL and MWT we observed were likely to be largely attributed to local inflammatory pain, although systemic fever responses were not tested.

Topical application of the sEHIs AUDA-be and IK 950 alleviated LPS-induced inflammatory hyperalgesia and mechanical allodynia. While AUDA-be significantly reduced thermal hyperalgesia across the entire 4-h testing period, IK 950 was effective only at the 2-h time point (Fig. 2A). However, the effect of both sEHIs at 4 h post-LPS was dose-related (Fig. 2B). We selected AUDA-be and IK 950 for several reasons. They have similar potency as inhibitors of the murine recombinant enzyme but they have quite different structures. The polyether of IK 950 makes it both less lipophilic and much more water soluble. The similar reduction in pain when using two quite different compounds supports the hypothesis that sEH inhibition is involved in the response. The increase in EET analgesia with both compounds supports the hypothesis that the sEHI are acting at least in part through stabilizing EETs. Because the $IC_{50}$ of both inhibitors are equivalent the difference between AUDA-be and IK 950 in reducing thermal hyperalgesia might be explained by possible differences in the pharmacokinetic properties of the two compounds such as penetration rate and renal clearance rate (Fig. 1). In contrast to IK 950's moderate effect using thermal stimulation, IK 950 blocked the LPS-induced reduction in MWT at the 1-h time point (Fig. 2C). From the present data we cannot

conclude why IK 950 appeared more effective using mechanical stimulation, however, this difference could be explained by differences in the recruitment of primary afferent fiber types with thermal versus mechanical stimulation. While the slowly ramping heat stimulus selectively activates unmyelinated C fiber nociceptors (Yeomans and Proudfit, 1996), the von Frey filaments would have necessarily recruited both mechanosensitive Aδ and C nociceptors as well as low-threshold tactile Aβ fibers. Thus, the greater effect of IK 950 using mechanical compared to noxious thermal stimulation could have resulted from combined effects on multiple fiber types that culminated in a relatively greater reduction in primary afferent input converging on spinal nociceptive dorsal horn neurons.

While sEHIs attenuate LPS-induced hyperalgesia, they did not alter TWLs in normal rats not treated with LPS (Fig. 4), similar to what has been found in the case of COX inhibition by NSAIDs (Ferreira et al., 1971; Moncada et al., 1973; Dirig et al., 1998). Thus, metabolites generated by sEH do not appear to play an important role in responses to acute noxious stimuli, but are rather involved in hyperalgesic inflammatory responses following tissue injury. Indeed the free intracellular AA content is low under basal conditions, but it is released from membrane phospholipids pools by phospholipases in response to tissue injury (Needleman et al., 1986).

Topical hindpaw application of EETs caused a significant reversal of LPS-induced thermal hyperalgesia at the 30-min, 2-h and 4-h timepoints, but not at the 1-h timepoint (Fig. 3A). In contrast to sEHIs, EETs had a biphasic dose–response effect on LPS-induced decreases in TWL (Fig. 3B). This effect could be at least partly attributed to our finding that EETs had a slightly pro-nociceptive action on TWL when administered to LPS-naïve animals (Fig. 4). This difference could be explained by the fact that we used a mixture of four regioisomers of EETs, one or more of which could have exerted a relatively greater pro-nociceptive versus anti-nociceptive action. Potential pro-nociceptive properties of EETs include activation of the heat-sensitive, mechanosensitive and osmosensitive TRPV4 receptor (Nilius et al., 2004; Vriens et al., 2005; Vriens et al., 2004; Watanabe et al., 2003), or in the case of 5,6-EET, conversion to prostaglandins by COX (Carroll et al., 1993). We are not certain why EETs were anti-hyperalgesic in LPS-treated rats and pro-nociceptive in LPS-naïve animals. However, if inflammatory pain and associated motor responses to noxious stimulation are already close to saturation, then the pro-nociceptive properties of EETs might be limited and their anti-hyperalgesic properties unmasked.

### Effects of sEHIs and EETs on prostaglandins and oxylipins

In parallel with the anti-nociceptive activity of sEHIs we see that plasma levels of $PGD_2$, DHETs and DiHOMEs are reduced in the treated animals. Unlike other studies, where a higher dose of LPS were used or compounds were administered systemically (Schmelzer et al., 2005), we did not see a significant decrease in $PGE_2$ formation or a significant increase in EETs in plasma when sEHIs and/or EETs are administered. This might be due to the local nature of the induced inflammation. Another possibility is that due to the differences in administration routes (injection vs.

topical) and the rapid metabolism of EETs by beta-oxidation and epoxide hydration, we were unable to see significant differences in plasma levels of EETs. On the other hand, the degradation products of EpOMEs and EETs, DiHOMEs and DHETs, respectively are clearly lower when sEHIs are administered (Fig. 5). These data support the use of lipid epoxide to diol ratios as a biomarker of sEH activity.

### Potential anti-hyperalgesic mechanisms of sEHIs and EETs

So far, studies investigating mechanisms of EETs and sEHIs have been mainly restricted to cardiovascular effects. The mechanisms by which sEHIs and EETs exert their anti-inflammatory, anti-hypertensive, and anti-proliferative effects in vasculature are still in the midst of elucidation. Although no specific EET receptor has been characterized or cloned, several studies suggest there are both intracellular and membrane bound EET high affinity binding sites (Spector et al., 2004). EETs have been implicated as the endothelium-derived hyperpolarization factor. It has been proposed that release of EETs from endothelial cells acts to hyperpolarize arterial smooth muscle by increasing the open probability of large-conductance calcium-activated potassium channels ($BK_{Ca}$), thereby causing vasodilation (Campbell et al., 1996; Fisslthaler et al., 1999; Hu and Kim, 1993; Harder et al., 1995). Effects of EETs on other membrane ion channels have also been reported, including inhibition of voltage-activated $Na^+$ channels (Lee et al., 1999), and either potentiation or inhibition of L-type voltage-activated $Ca^{++}$ channels (Chen et al., 1999; Fang et al., 1999). The anti-inflammatory mechanisms of EETs that may play a role in alleviating inflammatory pain are their ability to inhibit $PGE_2$ production (Fang et al., 1998; Kozak et al., 2003) as well as NF-κB-mediated gene transcription and cell adhesion molecule 1 expression induced by TNFα, IL-1α, or LPS (Node et al., 1999). Thus, the ability of sEHIs and EETs to modulate ion channel function and block many aspects of the inflammatory process suggest potential mechanisms by which these compounds might suppress peripheral and perhaps central sensitization of pain pathways. However, the mechanisms by which sEHIs and EETs affect pain processing per se have not been investigated, yet the present study warrants further investigation into the mechanisms and potential clinical application of sEHIs and EETs in pain treatment.

The roles of sEH and its proposed substrates and products are still emerging. It is highly likely that many yet unidentified epoxides of polyunsaturated fatty acids and related mediators exist. In the presence of cytochrome P450 epoxygenases, polyunsaturated fatty acids and related molecules including endocannabinoids are likely to yield epoxides similar to EETs with ethanolamide, glycerol, glycine, dopamine etc. groups attached to the carboxylic acid moiety of the fatty acids. Therefore inhibition of sEH should impact the production of multiple epoxides, in general, with diverse biological implications.

## References

Campbell, W.B., Brady, M.T., Rosolowsky, L.J., Falck, J.R., 1991. Metabolism of arachidonic acid by rat adrenal glomerulosa cells: synthesis of hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids. Endocrinology 128, 2183–2194.

Campbell, W.B., Gebremedhin, D., Pratt, P.F., Harder, D.R., 1996. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circulation Research 78, 415–423.

Carroll, M.A., Balazy, M., Margiotta, P., Falck, J.R., McGiff, J.C., 1993. Renal vasodilator activity of 5,6-epoxyeicosatrienoic acid depends upon conversion by cyclooxygenase and release of prostaglandins. Journal of Biological Chemistry 268, 12260–12266.

Chen, J., Capdevila, J.H., Zeldin, D.C., Rosenberg, R.L., 1999. Inhibition of cardiac L-type calcium channels by epoxyeicosatrienoic acids. Molecular Pharmacology 55, 288–295.

Cohen, J., 2002. The immunopathogenesis of sepsis. Nature 420, 885–891.

Coutaux, A., Adam, F., Willer, J.C., Le, B.D., 2005. Hyperalgesia and allodynia: peripheral mechanisms. Joint Bone Spine 72, 359–371.

Dirig, D.M., Isakson, P.C., Yaksh, T.L., 1998. Effect of COX-1 and COX-2 inhibition on induction and maintenance of carrageenan-evoked thermal hyperalgesia in rats. Journal of Pharmacological and Experimental Therapeutics 285, 1031–1038.

Fabien, J., Dogne, J.M., Pirotte, B., de Leval, X., 2004. Recent development in the field of dual COX/5-LOX inhibitors. Mini Reviews in Medicinal Chemistry 4, 633–638.

Fang, X., Moore, S.A., Stoll, L.L., Rich, G., Kaduce, T.L., Weintraub, N.L., Spector, A.A., 1998. 14,15-Epoxyeicosatrienoic acid inhibits prostaglandin E2 production in vascular smooth muscle cells. American Journal of Physiology 275, H2113–H2121.

Fang, X., Weintraub, N.L., Stoll, L.L., Spector, A.A., 1999. Epoxyeicosatrienoic acids increase intracellular calcium concentration in vascular smooth muscle cells. Hypertension 34, 1242–1246.

Fang, X., Weintraub, N.L., McCaw, R.B., Hu, S., Harmon, S.D., Rice, J.B., Hammock, B.D., Spector, A.A., 2004. Effect of soluble epoxide hydrolase inhibition on epoxyeicosatrienoic acid metabolism in human blood vessels. American Journal of Physiology 287, H2412–H2420.

Ferreira, S.H., Moncada, S., Vane, J.R., 1971. Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature New Biology 231, 237–239.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D.R., Fleming, I., Busse, R., 1999. Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 401, 493–497.

Fleming, I., Busse, R., 2006. Endothelium-derived epoxyeicosatrienoic acids and vascular function. Hypertension 47, 629–633.

Hadgraft, J., Lane, M.E., 2005. Skin permeation: the years of enlightenment. International Journal of Pharmaceutics 305, 2–12.

Harder, D.R., Campbell, W.B., Roman, R.J., 1995. Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. Journal of Vascular Research 32, 79–92.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77–88.

Hu, S., Kim, H.S., 1993. Activation of K$^+$ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. European Journal of Pharmacology 230, 215–221.

Kanaan, S.A., Saade, N.E., Haddad, J.J., Abdelnoor, A.M., Atweh, S.F., Jabbur, S.J., Safieh-Garabedian, B., 1996. Endotoxin-induced local inflammation and hyperalgesia in rats and mice: a new model for inflammatory pain. Pain 66, 373–379.

Kim, I., Morisseau, C., Watanabe, T., Hammock, B.D., 2004. Design, synthesis, and biological activity of 1,3-Disubstituted ureas as potent inhibitors of the

soluble epoxide hydrolase of increased water solubility. Journal of Medicinal Chemistry 47, 2110–2122.

Kozak, W., Kluger, M.J., Kozak, A., Wachulec, M., Dokladny, K., 2000. Role of cytochrome P-450 in endogenous antipyresis. American Journal of Physiology 279, R455–R460.

Kozak, W., Aronoff, D.M., Boutaud, O., Kozak, A., 2003. 11,12-epoxyeicosatrienoic acid attenuates synthesis of prostaglandin E2 in rat monocytes stimulated with lipopolysaccharide. Experimental Biology and Medicine (Maywood) 228, 786–794.

Lee, H.C., Lu, T., Weintraub, N.L., VanRollins, M., Spector, A.A., Shibata, E.F., 1999. Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. Journal of Physiology 519, 153–168.

Makita, K., Takahashi, K., Karara, A., Jacobson, H.R., Falck, J.R., Capdevila, J.H., 1994. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. Journal of Clinical Investigation 94, 2414–2420.

Moncada, S., Ferreira, S.H., Vane, J.R., 1973. Prostaglandins, aspirin-like drugs and the oedema of inflammation. Nature 246, 217–219.

Morisseau, C., Goodrow, M.H., Newman, J.W., Wheelock, C.E., Dowdy, D.L., Hammock, B.D., 2002. Structural refinement of inhibitors of urea-based soluble epoxide hydrolases. Biochemical Pharmacology 63, 1599–1608.

Needleman, P., Truk, J., Jakschik, B.A., Morrison, A.R., Lefkowith, J.B., 1986. Arachidonic acid metabolism. Annual Review of Biochemistry 55, 69–102.

Nilius, B., Vriens, J., Prenen, J., Droogmans, G., Voets, T., 2004. TRPV4 calcium entry channel: a paradigm for gating diversity. American Journal of Physiology 286, C195–C205.

Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D.C., Liao, J.K., 1999. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285, 1276–1279.

Oliver, J., Brandes, R.P., Kim, I.H., Schweda, F., Schmidt, R., Hammock, B.D., Busse, R., Fleming, I., 2005. Soluble epoxide hydrolase is a main effector of angiotensin II-induced hypertension. Hypertension 45, 759–765.

Oliw, E.H., Guengerich, F.P., Oates, J.A., 1982. Oxygenation of arachidonic acid by hepatic monooxygenases. Isolation and metabolism of four epoxide intermediates. Journal of Biological Chemistry 257, 3771–3781.

Smith, K.R., Pinkerton, K.E., Watanabe, T., Pedersen, T.L., Ma, S.J., Hammock, B.D., 2005. Attenuation of tobacco smoke-induced lung inflammation by treatment with a soluble epoxide hydrolase inhibitor. Proceedings of the National Academy of Sciences 102, 2186–2191.

Schmelzer, K.R., Kubala, L., Newman, J.W., Kim, I., Eiserich, J.P., Hammock, B.D., 2005. Soluble epoxide hydrolase is a therapeutic target for acute inflammation. Proceedings of the National Academy of Sciences 102, 9772–9777.

Spector, A.A., Fang, X., Snyder, G.D., Weintraub, N.L., 2004. Epoxyeicosatrienoic acids (EpETrEs): metabolism and biochemical function. Progress in Lipid Research 43, 55–90.

Spiecker, M., Liao, J.K., 2005. Vascular protective effects of cytochrome p450 epoxygenase-derived eicosanoids. Archives of Biochemistry and Biophysics 433, 413–420.

Vadivelu, N., Sinatra, R., 2005. Recent advances in elucidating pain mechanisms. Current Opinion in Anaesthesiology 18, 540–547.

Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biology 231, 232–235.

Vriens, J., Watanabe, H., Janssens, A., Droogmans, G., Voets, T., Nilius, B., 2004. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. Proceedings of the National Academy of Sciences 101, 396–401.

Vriens, J., Owsianik, G., Fisslthaler, B., Suzuki, M., Janssens, A., Voets, T., Morisseau, C., Hammock, B.D., Fleming, I., Busse, R., Nilius, B., 2005. Modulation of the Ca$^2$ permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. Circulation Research 97, 908–915.

Watanabe, H., Vriens, J., Prenen, J., Droogmans, G., Voets, T., Nilius, B., 2003. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. Nature 424, 434–438.

Yeomans, D.C., Proudfit, H.K., 1996. Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: electrophysiological evidence. Pain 68, 141–150.

Yu, Z., Xu, F., Huse, L.M., Morisseau, C., Draper, A.J., Newman, J.W., Parker, C., Graham, L.R., Engler, M.M., Hammock, B.D., Zeldin, D.C., Kroetz, D.L., 2000. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. Circulation Research 87, 992–998.

Zeldin, D.C., 2001. Epoxygenase pathways of arachidonic acid metabolism. Journal of Biological Chemistry 276, 36059–36062.

Zeldin, D.C., Kobayashi, J., Falck, J.R., Winder, B.S., Hammock, B.D., Snapper, J.R., Capdevila, J.H., 1993. Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. Journal of Biological Chemistry 268, 6402–6407.