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INHIBITION OF SOLUBLE EPOXIDE HYDROLASE DOES NOT PROTECT AGAINST ENDOTOXIN-MEDIATED HEPATIC INFLAMMATION

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Abstract

Epoxyeicosatrienoic acids (EETs) are derived from cytochrome P450 (CYP)-catalyzed epoxidation of arachidonic acid and have emerged as important mediators of numerous biological effects. The major elimination pathway for EETs is through soluble epoxide hydrolase (sEH) catalyzed metabolism to dihydroxyeicosatrienoic acids (DHETs). Based on previous studies showing that EETs have anti-inflammatory effects, we hypothesized that chronic inhibition of sEH would attenuate a lipopolysaccharide (LPS)-induced inflammatory response *in vivo*. Continuous dosing of the sEH inhibitors 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), a polyethylene glycol ester of AUDA (AUDA-PEG), and 1-adamantan-1-yl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea (AEPU) resulted in robust exposure to the inhibitor and target engagement, as evidenced by significant increases in plasma EET/DHET ratios following six days of inhibitor treatment. However, sEH inhibitor treatment was not associated with an attenuation of LPS-induced inflammatory gene expression in the liver and AUDA did not protect from LPS-induced neutrophil infiltration. Furthermore, *Ephx2*^{−/−} mice that lack sEH expression and have significantly increased plasma EET/DHET ratios were not protected from LPS-induced inflammatory gene expression or neutrophil accumulation in the liver. LPS did have an effect on sEH expression and function, as evident from a significant downregulation of *Ephx2* mRNA and a significant shift in plasma EET/DHET ratios four hours after LPS treatment. In conclusion, there was no evidence that increasing EET levels *in vivo* could modulate an LPS-induced inflammatory response in the liver. However, LPS did have significant effects on plasma eicosanoid levels and hepatic *Ephx2* expression, suggesting that *in vivo* EET levels are modulated in response to an inflammatory signal.

Introduction

Eicosanoids have long been studied for their important roles in inflammation and vasoactivity, and more recently epoxyeicosatrienoic acids (EETs) have become a particularly exciting focus in eicosanoid research due to their numerous protective actions in the vasculature. EETs are products of cytochrome P450 (CYP) epoxidase-catalyzed metabolism of arachidonic acid and have been implicated as mediators of vascular tone and inflammatory processes (Spector and Norris, 2007). There is much evidence supporting EETs as antihypertensive compounds,

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including their ability to dilate arteries and their actions as endothelial-derived hyperpolarizing factors (Campbell and Falck, 2007). EETs also exert anti-inflammatory effects (Node et al., 1999), which in combination with the antihypertensive properties, have made EETs an attractive target for the treatment of chronic cardiovascular and inflammatory diseases.

There are three potential strategies for increasing endogenous EET levels: 1) increase EET production, 2) administer EETs or EET mimetics, or 3) inhibit EET degradation. Targeting specific isoforms of CYPs is difficult and generally avoided due to their extensive roles in xenobiotic metabolism. Administration of EETs is also problematic since they are poorly bioavailable and rapidly metabolized *in vivo*. Therefore, recent efforts have focused on increasing EET levels by inhibiting their degradation. The main pathway for metabolism of EETs is through soluble epoxide hydrolase (*Ephx2*; sEH), an enzyme that hydrolyzes the epoxide bond to convert EETs to dihydroxyecosatrienoic acids (DHETs) (Chacos et al., 1983; Zeldin et al., 1993). Several urea-based inhibitors of sEH with low nanomolar potency have been developed and used *in vivo* with outcomes that were associated with antihypertensive (Yu et al., 2000; Imig et al., 2002; Zhao et al., 2004; Imig et al., 2005; Jung et al., 2005; Loch et al., 2007) or anti-inflammatory activity (Schmelzer et al., 2005; Smith et al., 2005). Chronic treatment for 12–14 days with sEH inhibitors by injection or in drinking water attenuated angiotensin-II induced hypertension in mice and rats, and in some cases the decrease in blood pressure was associated with a decrease in measures of hypertension-induced inflammation (Zhao et al., 2004; Imig et al., 2005; Jung et al., 2005; Loch et al., 2007). To date, studies involving models of inflammation have used only two or three days of once-daily treatment (Schmelzer et al., 2005; Smith et al., 2005). Acute dosing of sEH inhibitors reduced tobacco smoke-induced inflammation in the lung and prevented lipopolysaccharide (LPS)-induced mortality in mice (Schmelzer et al., 2005; Smith et al., 2005). It is of interest to test whether chronic dosing of sEH inhibitors is also protective against inflammatory processes, since this would support a potential use for these inhibitors in chronic inflammatory diseases.

Endotoxin, or more specifically LPS, is an inflammatory stimulus that exerts effects on major organs, including the liver and to a lesser extent the lung, spleen and kidney (Mathison and Ulevitch, 1979). LPS binds to CD14 and Toll-like receptor 4 on the cell surface to trigger activation of NF- κ B, a transcription factor normally sequestered in the cytoplasm that upon stimulation translocates to the nucleus to drive transcription of target genes, including cytokines, chemokines and cellular adhesion molecules (Van Amersfoort et al., 2003). EETs have been shown to disrupt signaling of NF- κ B in bovine aortic endothelial cells (Node et al., 1999; Liu et al., 2005), human umbilical vein endothelial cells (Fleming et al., 2001), and cardiomyocytes (Xu et al., 2006). While the exact mechanism for this disruption is unknown, 11,12-EET has been shown to inhibit TNF- α -induced nuclear translocation of NF- κ B by interfering with I κ B kinase activity and thus preventing degradation of the NF- κ B inhibitor (Node et al., 1999). Based upon the reported anti-inflammatory properties of EETs and the previous findings that acute dosing with sEH inhibitors is anti-inflammatory, the current studies used a model of LPS-induced systemic inflammation to test whether chronic inhibition of sEH with chemical inhibitors or genetic disruption of sEH could attenuate an inflammatory response *in vivo*.

Methods

Reagents

iNOS antibody was purchased from Cayman Chemical (Ann Arbor, MI), and VCAM-1 and GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit anti-mouse sEH antibody was raised against mouse recombinant sEH (Davis et al., 2002). The rabbit anti-rat mEH antibody was purchased from Oxford Biomedical Research (Oxford, MI). Donkey anti-goat and rabbit anti-goat HRP-conjugated secondary antibodies

were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA) and goat anti-rabbit HRP-conjugated secondary antibody was purchased from Bio-Rad (Hercules, CA). Sterile saline for injection and IsoFlo (isoflurane, USP) were purchased from Abbott Laboratories (Chicago, IL). Sterile saline for priming of pumps was prepared by the University of California, San Francisco Cell Culture Facility. ALZET osmotic pumps, model 2001, were purchased from DURECT Corporation (Cupertino, CA). Pharmaceutical grade β -hydroxypropyl cyclodextrin was purchased from Cyclodextrin Technologies Development, Inc. (High Springs, FL). EDTA was purchased from Teknova (Hollister, CA). Oasis HLB 3 cc (60 mg) solid phase extraction columns were purchased from Waters Corporation (Milford, MA). Nembutal (pentobarbital sodium) was obtained from the Moffitt Hospital Pharmacy (San Francisco, CA). Dithiothreitol (DTT), phenylmethanesulphonylfluoride (PMSF), Tris-HCl, LPS serotype 055:B5 (1×10^6 endotoxin units/mg) and all other reagents were purchased from Sigma Aldrich (St. Louis, MO), unless specifically stated.

sEH inhibitors

The synthesis of 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) and 1-adamantan-1-yl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea (AEPU) has been described previously (Morisseau et al., 2002; Kim et al., 2007a; Kim et al., 2007b) and the synthesis of a polyethylene glycol ester of AUDA (AUDA-PEG) is described in the Supplementary Materials. The structures, IC_{50} values and physical properties of the compounds used in these studies are provided in Supplemental Table 1.

Although AUDA is a very potent sEH inhibitor, the compound is high melting and lipophilic. AUDA-PEG is quickly cleaved by esterases *in vivo* to release AUDA (Kim et al., 2007a). The formation of a PEG ester of AUDA resulted in the material going from a high melting crystal to an oil that would not crystallize out of solution. The PEG ester also dramatically increased water solubility and reduced lipophilicity. Compared to AUDA, AEPU is more potent on the recombinant murine sEH enzyme, and has a lower melting point and increased water solubility.

Mouse studies

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Male C57BL/6J mice were purchased from the Jackson Laboratory at 5–7 weeks of age and allowed to acclimate for one week prior to undergoing any procedures. Maximal daily dosing of AUDA was limited by its poor solubility in a vehicle compatible with ALZET mini-pumps. Sterile saline containing 30% β -hydroxypropyl cyclodextrin (W/V) was used as the vehicle to deliver a continuous infusion of AUDA at a dosing rate of 3 mg AUDA/kg/day. AUDA-PEG was dosed at 10 mg AUDA/kg/day in 30% β -hydroxypropyl cyclodextrin (W/V) via the osmotic pumps and AEPU was dosed at ~ 10 mg/kg/day. Pumps were filled with either vehicle or sEH inhibitor sterile-filtered solutions and allowed to prime overnight in sterile saline at 37°C.

Mice were randomly assigned to receive either vehicle-filled or inhibitor-filled pumps, such that in each cage two mice received sEH inhibitor and three mice received vehicle. Under isoflurane anesthesia, a pump was implanted subcutaneously on the dorsal side of each mouse to administer sEH inhibitor or vehicle continuously for six days. On day six, mice received 1 mg/kg LPS or saline (i.p.) and were sacrificed four hr later by pentobarbital overdose (>200 mg/kg) for tissue harvest. Blood was collected via cardiac puncture in an EDTA-rinsed syringe, a small aliquot was taken for quantitation of sEH inhibitors, and the remaining blood was spun for ten minutes at 400g to separate plasma. Blood and plasma were flash frozen in liquid nitrogen and stored at -80°C until analysis. After ice-cold saline perfusion through the heart, the liver was removed, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Ephx2^{+/+} and *Ephx2*^{-/-} littermates of a C57BL/6 background were bred from heterozygote mice obtained from Darryl Zeldin (NIEHS, Research Triangle Park, NC), and genotypes were determined by PCR analysis of genomic DNA isolated from tail snips. Tail snips (0.5 – 1 cm) were lysed overnight in 100–200 μ L DirectPCR Lysis Reagent (Viagen, Los Angeles, CA) containing 2.5% V/V Proteinase K Solution (Roche Applied Science, Indianapolis, IN) in a shaking water bath at 55°C. Samples were then heat inactivated for 45–60 minutes at 85–90°C, centrifuged briefly to pellet debris, and the supernatant was used directly in a PCR reaction. PCR primer sequences for *Ephx2* were previously described (Sinal et al., 2000) and were purchased from Invitrogen (Carlsbad, CA). PCR reactions (50 μ L) contained 50 nM of each primer, 0.25 mM dNTPs (Promega, Madison, WI), and 5 units GoTaq DNA Polymerase (Promega, Madison, WI) in 1.5 mM MgCl₂, and 0.5 μ L template DNA from tail lysis supernatant. PCR conditions were as follows: two minutes at 94°C, 35 cycles of 30 seconds at 94°C, 60 seconds at 60°C, and 30 seconds at 72°C, followed by one minute at 72°C. PCR products were run on a 2% agarose gel in Tris-Acetate-EDTA Buffer and stained with ethidium bromide for visualization under ultraviolet light. *Ephx2*^{+/+} and *Ephx2*^{-/-} littermates were used in the LPS studies at 6–9 weeks of age and were treated with LPS and harvested exactly as described above.

AUDA, AEPU and metabolite quantitation

Quantitation of sEH inhibitors in the blood was performed as described previously (Xu et al., 2006). Briefly, 10 μ L of whole blood was liquid-liquid extracted twice with ethyl acetate and analytes were detected and quantified by LC/MS/MS. AUDA and its ester were both monitored as the active metabolite AUDA due to the very short half-life of the AUDA esters (Kim et al., 2007a)

Oxylipin quantitation

Oxylipins in plasma were quantified as described previously (Newman et al., 2002). Briefly, plasma samples (250 μ L) were extracted using Waters SPE columns and eluted with ethyl acetate. Samples were then quantified by LC/MS/MS as described previously (Newman et al., 2002).

Real-time quantitative PCR

Tissues (50–100 mg) were homogenized in TRIZOL (Invitrogen, Carlsbad, CA) or prepared with a PARIS kit (Ambion, Austin, TX) for extraction of RNA according to each manufacturer's protocol. RNA (1 μ g) was reverse transcribed to cDNA using random hexamers (Roche Applied Science, Indianapolis, IN), M-MLV reverse transcriptase (Promega, Madison, WI) and dNTPs (Promega, Madison, WI) in the presence of RNase Out (Invitrogen, Carlsbad, CA). All Taqman gene assays were purchased as Assays-on-Demand from ABI, with the exception of Gapdh. The gene assays used from ABI included inducible nitric oxide synthase (iNos), cyclooxygenase 2 (Cox-2), tumor necrosis factor α (Tnf- α), interleukin 6 (Il-6), monocyte chemoattractant protein 1 (Mcp-1), vascular cellular adhesion molecule 1 (Vcam-1), E-selectin (E-Sel), α_1 -acid glycoprotein (Agp) and fibrinogen (Fbg). The following primers used to detect Gapdh were purchased from Invitrogen (Carlsbad, CA): TGCACACCAACTGCTTAG (forward) and GGATGCAGGGATGATGTTC (reverse). The Gapdh probe was purchased from Integrated DNA Technologies, Inc. (San Diego, CA) as 5'-(6-carboxyfluorescein)-AGAGTGGATGGCCCCCTCA-(black hole quencher 1)-3'. Gene expression in each sample was normalized to expression of Gapdh by calculating a ΔC_t for each gene in each animal, where $\Delta C_t = C_{t, \text{gene}} - C_{t, \text{Gapdh}}$. In order to compare gene expression across all mice, $\Delta\Delta C_t$ values were calculated, where $\Delta\Delta C_t = \Delta C_{t, \text{mouse}} - \Delta C_t$, average of saline-treated mice and relative fold expression was calculated as $2^{\Delta\Delta C_t}$.

Western blotting

Tissues (50–100 mg) were homogenized in 50 mM Tris HCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.15 M KCl and 0.25 M sucrose and S9 fractions were prepared by differential centrifugation. Protein concentrations were determined with BCA assays (Pierce, Rockford, IL) and 20–50 µg protein was loaded onto Criterion 10% Tris-HCl gels (Bio-Rad, Hercules, CA). After separation by electrophoresis, proteins were transferred to nitrocellulose membranes according to the manufacturer's protocol using a wet transfer method in a buffer containing 192 mM glycine, 25 mM Tris base, and 10% methanol at 70 V for two hrs at 4°C. Membranes were blocked in 5% milk overnight at 4°C, and then probed for iNOS, VCAM-1, sEH, mEH and GAPDH. The working dilution for each primary antibody was selected according to the manufacturer's recommendation for that particular lot. Appropriate HRP-conjugated secondary antibodies were used at 1:10,000 to 1:20,000 dilutions. Membranes were developed using an ECL detection system (Millipore, Billerica, MA). Quantitation of Western blots was performed using Image Quant 5.2 and protein expression is normalized to GAPDH expression.

Neutrophil infiltration

Frozen liver slices were prepared at 8 µm thickness on a cryostat. Slides were fixed in acetone and stored at –20°C until staining with Hematoxylin & Eosin using a standard protocol. Image capture was performed on a Nikon Microphot-FXA equipped with 10× and 20× objective lenses using Spot Advanced 2.2.1 software. Neutrophils were quantified in five 20× fields per mouse by an observer blinded to the treatment groups.

sEH activity assay

sEH activity assays were carried out as described previously (Borhan et al., 1995). Briefly, hydrolysis of [³H]-dPPO was measured in hepatic S9 fractions using liquid scintillation counting for detection of the diol.

NF-κB activity assay

NF-κB activity was measured using a Quantitation NF-κB EIA kit (Oxford Biomedical Research, Oxford, MI). Assays were run in triplicate exactly as described by the manufacturer. The amount of NF-κB was normalized to protein concentration.

Statistics

Data were analyzed by one-way ANOVA followed by Bonferroni post-hoc multiple comparison testing using GraphPad Prism 4.03. Significance was set at $p < 0.05$.

Results

sEH inhibitor concentrations in the blood after chronic dosing

Several structurally similar inhibitors of sEH have been used *in vivo* to increase EET concentrations. The present studies utilized urea-based inhibitors with low nanomolar potency, including AUDA, AUDA-PEG, and AEPU. Continuous administration of AUDA for six days resulted in blood concentrations that were greater than the IC₅₀ in all treated mice. The mean concentration of AUDA was 44 nM (range 19–70 nM, $n = 8$) and most had inhibitor levels at least two-fold greater than the previously reported *in vitro* IC₅₀ (Hwang et al., 2007). While a three-fold greater dosing rate was achieved in the AUDA-PEG study, the mean of inhibitor concentrations in the blood increased only ~60% from those in the AUDA-treated mice. This difference was not significant. The mean concentration of AUDA in AUDA-PEG-treated mice was 70 nM (range of 29–105 nM, $n = 8$), with most mice reaching inhibitor levels at least ten-fold greater than the IC₅₀ (Hwang et al., 2007). Uncleaved AUDA-PEG was not detected in

the blood. AEPU is structurally similar to AUDA, but more water soluble and not subject to metabolism by β -oxidation (Xu et al., 2006). Analysis of AEPU in blood samples ($n = 7$) confirmed that inhibitor levels were 16- to 66-fold greater than the IC_{50} (Hwang et al., 2007) in AEPU-treated mice.

Effect of continuous dosing of sEH inhibitors on plasma EET/DHET ratios

A change in the ratio of epoxides to their corresponding diol products, including EET/DHET and epoxyoctadecanoic acid (EpOME)/dihydroxyoctadecanoic acid (DHOMe), is commonly used as an indication of sEH activity. Plasma oxylipins were quantified by LC/MS/MS to confirm that the sEH inhibitors effectively decreased sEH activity *in vivo*. In general, mice treated with sEH inhibitors had greater EET and EpOME plasma levels and corresponding increases in EET/DHET and EpOME/DHOMe ratios when compared to the LPS-treated group (Figure 1, Supplemental Figure 1 and Supplemental Tables 2–4).

AUDA-treated mice were protected from a LPS-induced decrease of EETs and increase of DHETs in the plasma. After LPS treatment, average 14,15-, 11,12-, and 8,9-EET concentrations were about two- to four-fold greater in AUDA-treated and about three- to six-fold greater in AUDA-PEG-treated mice relative to the respective LPS vehicle-treated mice (Supplemental Tables 2 and 3). These changes are consistent with robust inhibition of sEH activity. Average 14,15-, 11,12-, and 8,9-DHET levels were decreased about 20–60% in AUDA-treated mice, and about 40–60% in AUDA-PEG-treated mice, relative to the respective LPS vehicle-treated mice (Supplemental Tables 2 and 3), which is also consistent with sEH inhibition. 5,6-EET and 5,6-DHET are excluded from analysis because of the significant formation of the δ -lactone of 5,6-EET through a non-sEH-mediated mechanism (Chacos et al., 1983; Zeldin et al., 1993). Mice treated with AEPU + LPS had slightly greater EET concentrations and significantly lower DHET concentrations compared to mice treated only with LPS (Supplemental Table 4).

LPS caused epoxide-to-diol ratios to decrease relative to saline-treated mice in all the studies (Figure 1 and Supplemental Figure 1). In general, mice treated with sEH inhibitors were significantly protected from the LPS-induced decrease in epoxide-to-diol ratios (Figure 1 and Supplemental Figure 1). The relative pattern of epoxide-to-diol ratios in response to LPS and sEH inhibition was consistent across all the experiments. The 11,12-EET regioisomer has the highest anti-inflammatory activity *in vitro* (Node et al., 1999). In these studies the 11,12-EET/11,12-DHET ratio was 6.3-fold greater in AUDA + LPS-treated compared to vehicle + LPS-treated mice, 9.8-fold greater in AUDA-PEG + LPS-treated relative to vehicle + LPS-treated mice, and 2.5-fold greater in AEPU + LPS-treated relative to vehicle + LPS-treated mice. Thus, sEH inhibitor treatment shifted EET levels such that their anti-inflammatory effects were expected to be enhanced.

Effects of chronic dosing of sEH inhibitors on LPS-induced hepatic expression of inflammatory genes in the liver

A moderate dose of LPS was chosen for these studies and all of the animals administered LPS survived the treatment period. In preliminary studies, transcript levels of iNos, Cox-2, Tnf- α and Il-6 in the liver all peaked four to six hrs after a 1 mg/kg dose of LPS (data not shown) and a four hr time point was selected for subsequent studies. In the chronic AUDA and AUDA-PEG studies, a robust induction of inflammatory gene mRNA was observed in the liver four hrs after LPS treatment (Figure 2). While there was a significant attenuation of LPS-induced iNos mRNA by AUDA-PEG, there was no effect of AUDA on hepatic mRNA levels of Cox-2, Tnf- α , Il-6, Mcp-1, Vcam-1, or E-selectin after AUDA or AUDA-PEG treatment (Figure 2). Similarly, there was no effect of AEPU on the LPS-induced hepatic expression of Tnf- α , Il-6, Cox-2 or iNos (Supplemental Figure 2). No attenuation of LPS-induced expression of the

hepatic acute phase response genes Agp or Fbg was observed (Figure 2 and Supplemental Figure 2). The sum of this mRNA data shows that in this model, chronic inhibition of sEH does not attenuate the LPS-induced increase in hepatic inflammatory gene transcription. Similarly, LPS induced hepatic expression of iNOS and VCAM-1 protein, which was unaffected by treatment with AUDA or AUDA-PEG (Figure 3). The mRNA and protein results are concordant and suggest that the measured increase in EETs provided no significant protection from induction of inflammatory gene expression in the liver. Surprisingly, activation of NF- κ B could not be detected in nuclear fractions collected four hrs after LPS treatment (Supplemental Figure 3). Attenuation of inflammatory gene expression by sEH inhibition was also not observed in kidney (Supplemental Figure 4).

LPS-induced leukocyte accumulation in the liver sinusoids

Liver slices were stained by hemotoxylin & eosin and examined for accumulation of neutrophils in sinusoids (Figure 4). The mean neutrophil count in saline-treated mice was 90 ± 18 ($n = 3$), and was significantly greater in LPS-treated mice (143 ± 18 , $n = 6$; $p < 0.01$ versus the saline-treated group) and in LPS + AUDA-treated mice (145 ± 10 , $n = 6$; $p < 0.001$ versus the saline-treated group). Following LPS treatment in *Ephx2*^{+/+} and *Ephx2*^{-/-} littermates, there was a small but insignificant increase in neutrophil accumulation over saline-treated littermate controls. LPS-treated *Ephx2*^{+/+} mice had a mean neutrophil count of 118 ± 10 ($n = 5$), and this was similar in the *Ephx2*^{-/-} mice (128 ± 18 ; $n = 5$). Based on quantitation of neutrophil accumulation in the liver, disruption of sEH inhibition does not appear to protect against LPS-induced inflammation.

Effects of genetic disruption of *Ephx2* on LPS-induced expression of inflammatory genes in the liver

Ephx2^{-/-} mice were used to determine the effects of the loss of sEH expression and function on inflammatory gene induction in the liver. Following LPS treatment, plasma EET/DHET ratios were increased in the *Ephx2*^{-/-} mice compared to *Ephx2*^{+/+} mice (Figure 5 and Supplemental Table 5), consistent with loss of sEH activity. A survey of the expression of Il-6, iNos, Cox-2, Tnf- α , Mcp-1, Vcam, and E-sel in the liver showed that *Ephx2*^{-/-} mice were not significantly protected from LPS-induced inflammatory gene expression (Figure 6). There was also no protection from LPS-induced expression of Agp or Fbg (Figure 6). Western blotting confirmed lack of sEH expression in the livers of *Ephx2*^{-/-} mice (Figure 6). iNOS protein levels were similar in *Ephx2*^{+/+} and *Ephx2*^{-/-} mice (Figure 6).

Effect of LPS on *Ephx2* expression and sEH function

Hepatic *Ephx2* mRNA expression was significantly decreased in LPS-treated mice relative to saline controls in all inhibitor studies (Figure 7). sEH inhibitor treatment had no additional effect on hepatic *Ephx2* levels. In preliminary studies the downregulation of *Ephx2* mRNA was time-dependent, with expression decreasing progressively for at least eight hrs following LPS treatment (data not shown). Despite the decrease in mRNA levels four hrs after LPS treatment, a change in hepatic sEH protein was not detected in the AUDA study (Supplemental Figure 5). A small but significant decrease in hepatic sEH activity was observed in LPS + AUDA-treated animals (Supplemental Figure 6). In the *Ephx2*^{-/-} study, a significant decrease in sEH protein levels was observed in *Ephx2*^{+/+} mice following LPS treatment (Supplemental Figure 5). No change in mEH expression in response to LPS or disruption of sEH activity was detected in either of the studies (Supplemental Figure 5).

Discussion

EETs are CYP epoxygenase-derived eicosanoids with numerous beneficial properties, including vasodilatory and anti-inflammatory roles (Spector and Norris, 2007). EET levels can

be increased by limiting their degradation by sEH, and as a result sEH has emerged as a promising target for the modulation of blood pressure and inflammation (Yu et al., 2000; Imig et al., 2002; Zhao et al., 2004; Imig et al., 2005; Jung et al., 2005; Schmelzer et al., 2005; Smith et al., 2005; Loch et al., 2007). In the current studies, continuous dosing of AUDA, AUDA-PEG, and AEPU via osmotic pumps results in excellent exposure (blood levels several fold higher than the IC₅₀) and robust target engagement (increased EET/DHET ratios consistent with *in vivo* inhibition of sEH). Based on the reported anti-inflammatory effects of EETs and earlier studies with sEH inhibitors, it was predicted that the current treatments would attenuate LPS-induced inflammation. However, there was no significant attenuation of LPS-induced inflammatory gene expression or leukocyte accumulation in the liver or kidney under these experimental conditions. In addition, EET/DHET ratios were increased in *Ephx2*^{-/-} mice compared to *Ephx2*^{+/+}, but this did not protect against hepatic inflammatory gene induction and neutrophil infiltration in response to LPS. Previous studies have suggested that EETs disrupt NF- κ B signaling to exert anti-inflammatory effects (Node et al., 1999; Fleming et al., 2001). The results from the current studies suggest the mechanism by which EETs may attenuate inflammation is more complex than is currently proposed.

A number of questions are raised by the results of this study, particularly in the context of a previous report of acute treatment with AUDA-BE completely preventing LPS-induced mortality with an associated reduction in hepatic iNOS and COX-2 levels (Schmelzer et al., 2005). There were several key differences between the studies that might contribute to the discordant outcomes. First, lethal (10 mg/kg) and non-lethal (1 mg/kg) doses of LPS activate inflammatory pathways with differential severity, which may variably trigger responses from a mediator(s) potentially sensitive to EETs (Xie et al., 2002). The strains of LPS in the two studies also differed. Additionally, the endpoint in the current studies corresponded to the initial peak of inflammatory gene transcription, while the previous studies measured later endpoints (Schmelzer et al., 2005). It is possible that the beneficial effects of sEH inhibition are only detected at later time points, for example during the resolution phase of the inflammatory response. The oxylipin profiles associated with a protective effect in the earlier studies were most apparent in mice given the sEH inhibitor both on the day prior to and immediately preceding LPS treatment, suggesting that the anti-inflammatory effect could be enhanced by initiating sEH inhibition sooner (Schmelzer et al., 2005). The current studies provided six days of continuous dosing prior to LPS treatment, which resulted in presumably steady-state inhibitor concentrations that were confirmed to be well above the IC₅₀. In addition, the inhibitor blood concentrations in this study were relatively high, in contrast to a previous study where beneficial effects were achieved at AEPU concentrations far below the IC₅₀ (Xu et al., 2006). Thus, it is puzzling why the increased exposure to sEH inhibitors in these studies did not afford a similar or better protection from inflammatory insult. Confounding results due to estrogen-related differences in sEH expression between males and females is not a concern, since both the previous and the current study involved only male mice.

The most effective means for disrupting sEH activity is to disrupt the gene itself, and thus the *Ephx2*^{-/-} mouse is a powerful tool for testing the effects of increased EET levels in models of inflammation. However, with a hypertension end point it is hypothesized that the mice adapted to compensate for increased EETs by increasing 20-hydroxycosatetraenoic acid (Luria et al., 2007). The current studies examined the inflammatory response in *Ephx2*^{-/-} mice and found they were not protected from LPS-induced inflammatory gene expression in the liver. Thus, the results from the genetic model are consistent with the chronic chemical inhibition studies. EET/DHET ratios were significantly increased by either chemical inhibition or genetic disruption of sEH, and the failure to observe an anti-inflammatory effect in these studies strongly suggests that EETs do not affect the LPS-induced inflammatory response in the liver. The discordance in results between acute and chronic dosing of sEH inhibitors raises the possibility of tolerance developing in the anti-inflammatory pathway following sustained

elevations in EET levels. That is, similar to the compensation seen by ω -hydroxylase upregulation in the *Ephx2*^{-/-} mice (Luria et al., 2007), the inflammatory pathways may become less sensitive to EET-mediated inhibition following chronically increased levels of EETs. This hypothesis of compensation would be consistent with both the previous reports of acute dosing of sEH inhibitors being anti-inflammatory and the current results in which there was a lack of effect after continuous dosing of sEH inhibitors or genetic disruption of sEH. It is worth considering whether the peak-to-trough swings of sEH inhibitors dosed by subcutaneous injection or in drinking water might not trigger such compensatory changes. Interestingly, in a previous study, loss of sEH afforded some protection from LPS-induced death in *Ephx2*^{-/-} mice (Luria et al., 2007). This protection was not as dramatic as that following acute administration of sEH inhibitors (Schmelzer et al., 2005), and may further support the idea of a desensitization in the anti-inflammatory properties of EETs.

sEH inhibitors have proven beneficial in several tissue injury models, including renal injury and monocyte infiltration associated with hypertension (Zhao et al., 2004; Imig et al., 2005), cardiac hypertrophy (Xu et al., 2006), and stroke (Dorrance et al., 2005; Zhang et al., 2007). Similarly, hearts from *Ephx2*^{-/-} mice have improved recovery and less infarction following ischemia (Seubert et al., 2006). However, decreased sEH activity is not always beneficial. In a model of hypertension, sEH inhibition provided some protection from increased blood pressure and cardiac and endothelial dysfunction, but did not attenuate inflammatory cell infiltration (Loch et al., 2007). Furthermore, sEH inhibition potentiated hypoxia-induced pulmonary vasoconstriction (Pokreisz et al., 2006) and *Ephx2*^{-/-} mice had reduced survival after cardiac arrest and cardiopulmonary resuscitation (Hutchens et al., 2007). Thus, the anti-inflammatory benefits of sEH inhibition are insult- and tissue-specific.

LPS triggers activation of cytosolic phospholipase A₂ (cPLA₂) (Rodewald et al., 1994), which releases arachidonic acid from phospholipids (Clark et al., 1991) and thus facilitates its metabolism into numerous inflammatory eicosanoids. EETs are also largely incorporated in phospholipids and quickly released by cPLA₂ (Fang et al., 2003). In the current studies, EET/DHET ratios in the plasma decreased dramatically in response to LPS, which was likely a result of cPLA₂ activation since EETs released from the cellular membrane are subject to sEH-mediated metabolism. Over 90% of the EETs in the plasma are esterified into the phospholipids of lipoproteins (Karara et al., 1992). Since lipoproteins are assembled in the liver and facilitate an exchange of lipids with extrahepatic tissues, it thus appears that plasma levels of EETs are a good biomarker for their relative abundance in tissues.

Ephx2 mRNA was significantly downregulated by LPS in all inhibitor studies and sEH protein was significantly decreased by LPS in *Ephx2*^{+/+} mice. It is interesting to speculate on the implications of this innate regulation in response to an inflammatory stimulus since downregulation of sEH would increase levels of EETs, which are hypothesized to be important in inflammation. There is precedence in the linking of sEH gene regulation with anti-inflammatory signaling, as activation of PPAR γ is associated with downregulation of sEH under conditions of laminar flow (Liu et al., 2005). sEH is also regulated by the vasoactive and pro-inflammatory molecule angiotensin II, however here it is upregulated (Imig et al., 2002; Ai et al., 2007). More work will be needed to characterize and understand the implications of sEH downregulation in response to LPS.

The current studies utilized complementary genetic and chemical approaches to study the effects of disrupting sEH activity on the inflammatory response. The findings support the conclusion that there is no reduction in the LPS-induced hepatic inflammatory response following continuous chemical inhibition or genetic disruption of sEH, even though EET/DHET ratios indicated robust sEH inhibition. Importantly, these studies also show that osmotic pumps are an effective route for administering continuous infusions of precise doses of sEH

inhibitors to mice that result in plasma levels well above the IC₅₀. Several previous reports have identified inhibition of sEH as a target for improving outcome following tissue injury or inflammatory insult *in vivo* (Zhao et al., 2004; Dorrance et al., 2005; Schmelzer et al., 2005; Smith et al., 2005; Luria et al., 2007; Seubert et al., 2007; Zhang et al., 2007). There have been fewer reports of unfavorable outcomes associated with sEH inhibition or genetic deletion (Pokreisz et al., 2006; Hutchens et al., 2007). Only more recently have limits to the benefits of decreased sEH activity been identified, and indicate that favorable outcomes are insult- and tissue-specific. Further research is needed to characterize the effects of EETs on inflammatory signaling pathways in order to improve understanding of the spectrum of outcomes following modulation of sEH activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

EETs, epoxyeicosatrienoic acids
 CYP, cytochrome P450
 DHETs, dihydroxyeicosatrienoic acids
 sEH, soluble epoxide hydrolase
 LPS, lipopolysaccharide
 AUDA, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid
 AUDA-PEG, polyethylene glycolester of AUDA
 AEPU, 1-adamantan-1-yl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea
 EpOME, epoxyoctadecanoic acid
 DHOME, dihydroxyoctadecanoic acid
 cPLA₂, cytosolic phospholipase A₂

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Figure 1. Epoxide/Diol ratios confirm inhibition of sEH after chronic dosing of AUDA and AUDA-PEG

Plasma samples collected at harvest were analyzed for oxylipin concentrations. sEH activity is calculated as the ratios of epoxide regioisomers to their diol products and was compared after LPS with or without AUDA (A) or AUDA-PEG (B) treatment. Clear bars indicate values for saline-treated mice. Filled bars represent LPS + vehicle-treated mice. Hatched bars represent mice treated with LPS + AUDA (A) or LPS + AUDA-PEG (B). Values shown are the mean $\pm$ SD of three to six plasma samples in each treatment group, where each plasma sample was pooled from one to two mice. Statistical significance is marked: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 2. Effects of chronic AUDA or AUDA-PEG treatment on the induction of inflammatory gene mRNA in liver four hrs after 1 mg/kg LPS
 Liver mRNA from vehicle-, LPS-, and AUDA (A and B) and vehicle-, LPS-, and AUDA-PEG (C and D) treated mice was reverse transcribed for quantitative real-time PCR analysis of expression of Il-6, iNos, Cox-2, and Tnf- α (A and C) and Vcam, E-sel, Mcp-1, Agp and Fbg (B and D). Expression is normalized to *Gapdh* and reported as the fold-expression relative to the saline control group. Values shown are the mean $\pm$ SD of three or four mice from the saline control group (open bars), and three or eight mice each from the LPS + vehicle (filled bars) and LPS + inhibitor-treated (hatched bars) groups. Statistical significance is marked: * $p < 0.05$ compared to LPS + vehicle.

Figure 3. Effects of chronic AUDA or AUDA-PEG treatment on the induction of inflammatory proteins in liver four hrs after 1 mg/kg LPS

S9 fractions were prepared from frozen liver from vehicle-, LPS-, and AUDA (A) and vehicle-, LPS-, and AUDA-PEG (D) treated mice. Samples were analyzed by Western blotting for expression of iNOS, VCAM and GAPDH. iNOS and VCAM expression is normalized to respective GAPDH expression, and the values are presented for comparison (B, C, E, and F). Analysis was performed on four mice from the saline control group, and eight mice each from the LPS + vehicle and LPS + inhibitor-treated groups.

Figure 4. Chronic AUDA treatment does not attenuate LPS-induced leukocyte accumulation in liver sinusoids

Eight micron sections were cut from frozen liver and stained with hematoxylin and eosin. Sections were analyzed for the presence of leukocytes in the sinusoids. Analysis was performed on three mice from the saline control group and six mice each from the LPS + vehicle and LPS + AUDA-treated groups. Representative pictures from saline (A and D), LPS + vehicle (B and E), and LPS + AUDA-treated mice (C and F) are shown. Images A–C are at 10× magnification, and D–F are at 40× magnification. Arrows indicate neutrophils in the sinusoids.

Figure 5. Epoxide/Diol ratios in *Ephx2*^{+/+} and *Ephx2*^{-/-} mice after LPS treatment
 Plasma samples collected four hrs after LPS treatment were analyzed for oxylipin concentrations. To compare sEH activity between genotypes, the ratios of epoxide regioisomers to their diol products were calculated. Open bars represent LPS-treated *Ephx2*^{+/+} mice. Shaded bars represent LPS-treated *Ephx2*^{-/-} mice. Values shown are the mean $\pm$ SD of three or four plasma samples in each treatment group, where each plasma sample was pooled from one to two mice. Statistical significance is marked: *** $p < 0.001$, ** $p < 0.01$.

Figure 6. LPS-induced inflammatory gene expression in the liver of *Ephx2*^{-/-} mice

Liver mRNA was reverse transcribed for quantitative real-time PCR analysis of E-sel, Mcp-1, Il-6, Tnf-α and iNos (A) and Vcam, Cox-2, Agp and Fbg (B). Expression is normalized to Gapdh and reported as the fold expression over the saline-treated mice. Values shown are the mean ± SD of three mice from saline-treated *Ephx2*^{+/+} (open bars), LPS-treated *Ephx2*^{+/+} (filled bars), saline-treated *Ephx2*^{-/-} (checkered bars) and LPS-treated *Ephx2*^{-/-} (striped bars) groups, respectively. S9 fractions were prepared from frozen liver and samples were analyzed by Western blotting for expression of iNos, sEH, and Gapdh (C). The expression of iNOS relative to GAPDH is shown in panel D as the mean ± SD of five or six mice per group.

Figure 7. LPS downregulates *Ephx2* mRNA four hrs after LPS-treatment

Liver mRNA was reverse transcribed for quantitative real-time PCR analysis of *Ephx2*, which is normalized to *Gapdh*. Expression was measured after chronic AUDA (filled squares), chronic AUDA-PEG (filled circles), and chronic AEPU (filled triangles). Each point represents a single mouse. Analysis was performed on four to eight samples per treatment group in each study. Statistical significance is marked: *** $p < 0.001$; ** $p < 0.01$.