Inhibition of Soluble Epoxide Hydrolase Does Not Protect against Endotoxin-Mediated Hepatic Inflammation

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ABSTRACT

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 (P450) epoxygenase-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 anti-inflammatory agents and vasodilators, including their ability to inhibit leukotriene synthesis, reduce platelet aggregation, and attenuate vascular tone and inflammatory processes (Spector and Norris, 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 P450s is difficult and generally avoided due to their extensive roles in xenobiotic metabolism.

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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 (P450) epoxygenase-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 anti-inflammatory agents and vasodilators, including their ability to inhibit leukotriene synthesis, reduce platelet aggregation, and attenuate vascular tone and inflammatory processes (Spector and Norris, 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 P450s is difficult and generally avoided due to their extensive roles in xenobiotic metabolism.

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Administration of EETs is also problematic because 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 dihydroxyicosatetraenoic 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 anti-hypertensive (Yu et al., 2000; Imig et al., 2002, 2005; Zhao et al., 2004; Jung et al., 2005; Loch et al., 2007) or anti-inflammatory activity (Schmelzer et al., 2005; Smith et al., 2005). Chronic treatment for 12 to 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 2 or 3 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, because 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-κB, 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-κB 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). Although the exact mechanism for this disruption is unknown, 11,12-EET has been shown to inhibit tumor necrosis factor-α (TNF-α)–induced nuclear translocation of NF-κB by interfering with inhibitor of nuclear factor-κB kinase activity and thus preventing degradation of the NF-κB 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.

**Materials and Methods**

**Reagents.** Inducible nitric-oxide synthase (iNOS) antibody was purchased from Cayman Chemical (Ann Arbor, MI), and vascular cellular adhesion molecule (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., 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 (Abbott Park, IL). Sterile saline for priming of pumps was prepared by the San Francisco Cell Culture Facility (University of California, San Francisco, CA). Alzet osmotic pumps (model 2001) were purchased from Durect Corporation ( Cupertino, CA). Pharmaceutical grade hydroxypropyl β-cyclodextrin was purchased from Cyclodextrin Technologies Development, Inc. (High Springs, FL). EDTA was purchased from Teknova (Holister, CA). OASIS HLB 3 cc (60 mg) solid phase extraction columns were purchased from Waters (Milford, MA). Nembutal (pentobarbital sodium) was obtained from the Mof- fitt Hospital pharmacy (San Francisco, CA). Dithiothreitol, phenylmethylsulfonyl fluoride, 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)-dodecanic acid (AUDA) and 1-adamantan-1-yl-3-(5-(2-(2-ethoxy ethoxy)ethoxy)pentyl)urea (AEPU) has been described previously (Morisseau et al., 2002; Kim et al., 2007a,b). The synthesis of a polyethylene glycol ester of AUDA (AUDA-PEG) is described in Supplemental Data. 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 with 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 (Bar Harbor, ME) at 5 to 7 weeks of age and allowed to acclimate for 1 week before 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% hydroxypropyl β-cyclodextrin (w/v) was used as the vehicle to deliver a continuous infusion of AUDA at a dosing rate of 3 mg of AUDA/kg/day. AUDA-PEG was dosed at 10 mg of AUDA/kg/day in 30% hydroxypropyl β-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 6 days. On day 6, mice received 1 mg/kg LPS or saline (intraperitoneal) and were sacrificed 4 h 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 10 min 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 (National Institute of Environmental Health Sciences, Research Tri-
angle 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 to 200 μl of 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 to 60 min 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 described previously (Sinal et al., 2000) and were purchased from Invitrogen (Carlsbad, CA). PCR reactions (50 μl) contained 50 nM of each primer, 0.25 mM dNTPs (Promega, Madison, WI), and 5 units of GoTaq DNA polymerase (Promega, Madison, WI) in 1.5 mM MgCl₂, and 0.5 μM of template DNA from tail lysis supernatant. PCR conditions were as follows: 2 min at 94°C, 35 cycles of 30 s at 94°C, 60 s at 60°C, and 30 s at 72°C, followed by 1 min 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−/− littersmates were used in the LPS studies at 6 to 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 μl 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 for the amount of NF-κB activity. Significant 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 study used urea-based inhibitors with low nanomolar potency, including AUDA, AUDA-PEG, and AEPU. Continuous administration of AUDA for 6 days resulted in blood concentrations that were greater than the IC₅₀ value 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 2-fold greater than the previously reported in vitro IC₅₀ value (Hwang et al., 2007). Although a 3-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, 29–105 nM; n = 8), with most mice reaching inhibitor levels at least 10-fold greater than the IC₅₀ value (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 IC₅₀ value (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 epoxycotitadecanoic acid (EpOME)/dihydroyxyoctadecanoic 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 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 enhanced chemiluminescence detection system (Milipore Corporation, Billerica, MA). Quantitation of Western blots was performed using ImageQuant 5.2 (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and protein expression is normalized to GAPDH expression.

Neutrophil Infiltration. Frozen liver slices were prepared at 8 μm in thickness on a cryostat. Slides were fixed in acetone and stored at −20°C until staining with hematoxylin and eosin using a standard protocol. Image capture was performed on a Microphot-FXA (Nikon, Tokyo, Japan) equipped with 10× and 20× objective lenses using SPOT Advanced 2.2.1 software (Diagnostics Instruments, Sterling Heights, MI). 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]trans,1,3-diphenylpropene oxide 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). 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 analysis of variance followed by Bonferroni post hoc multiple comparison testing using GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA). Significance was set at p < 0.05.

Western Blotting. Tissues (50–100 mg) were homogenized in 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 nM phenylmethylsulfonyl fluoride, 0.15 M KCl, and 0.25 M sucrose, and S9 fractions were prepared by differential centrifugation. Protein concentrations were determined with bicinchoninic acid assays (Pierce Chemical, Rockford, IL) and 20 to 50 μg of protein was loaded onto Criterion 10% Tris-HCl gels (Bio-Rad). 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 2 h 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

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GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA).
LPS caused epoxide-to-diol ratios to decrease relative to saline-treated mice in all the studies (Fig. 1; Supplemental Fig. 1). In general, mice treated with sEH inhibitors were significantly protected from the LPS-induced decrease in epoxide-to-diol ratios (Fig. 1; Supplemental Fig. 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 regiosomer 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 with 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 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 4 to 6 h after a 1-mg/kg dose of LPS (data not shown) and a 4-h 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 4 h after LPS treatment (Fig. 2). Although 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 (Fig. 2). Similarly, there was no effect of AEPU on the LPS-induced hepatic expression of Tnf-α, Il-6, Cox-2, or iNos (Supplemental Fig. 2). No attenuation of LPS-induced expression of the hepatic acute phase response genes App or Fbg was observed (Fig. 2; Supplemental Fig. 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 that was unaffected by treatment with AUDA or AUDA-PEG (Fig. 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-κB could not be detected in nuclear fractions collected 4 h after LPS treatment (Supplemental Fig. 3). Attenuation of inflammatory gene expression by sEH inhibition was also not observed in kidney (Supplemental Fig. 4).

LPS-Induced Leukocyte Accumulation in the Liver Sinusoids. Liver slices were stained by hematoxylin and eosin and examined for accumulation of neutrophils in sinusoids (Fig. 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). After 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
Based on quantitation of neutrophil accumulation in the liver, disruption of sEH inhibition does not seem 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. After LPS treatment, plasma EET/DHET and EpOME/DHOME ratios were increased in the Ephx2–/– mice compared with Ephx2+/+ mice (Fig. 5; Supplemental Table 5), consistent with loss of sEH activity. A survey of the expression of Il-6, iNos, Cox-2, Tnf-α, 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 ± S.D. 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.

**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 (Fig. 7). sEH inhibitor treatment had no additional effect on hepatic Ephx2 levels. In preliminary

(128 ± 18; n = 5). Based on quantitation of neutrophil accumulation in the liver, disruption of sEH inhibition does not seem to protect against LPS-induced inflammation.

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studies, the down-regulation of Ephx2 mRNA was time-dependent, with expression decreasing progressively for at least 8 h following LPS treatment (data not shown). Despite the decrease in mRNA levels 4 h after LPS treatment, a change in hepatic sEH protein was not detected in the AUDA study (Supplemental Fig. 5). A small but significant decrease in hepatic sEH activity was observed in LPS/AUDA-treated animals (Supplemental Fig. 6). In the Ephx2 study, a significant decrease in sEH protein levels was observed in Ephx2 mice after LPS treatment (Supplemental Fig. 5). No change in mEH expression in response to LPS or disruption of sEH activity was detected in either of the studies (Supplemental Fig. 5).

**Discussion**

EETs are P450 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, 2005; Zhao et al., 2004; Jung et al., 2005; Schmelzer et al., 2005; Smith et al., 2005; Loch et al., 2007). In the current study, continuous dosing of AUDA, AUDA-PEG, and AEPU via osmotic pumps results in excel-
lent exposure (blood levels severalfold higher than the IC50 value) 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 with Ephx2+/+ controls, 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-κB 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.

Several 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 nonlethal (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, whereas 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 inflam-
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Dorrance et al., 2005; Zhang et al., 2007). Similarly, hearts
injury models, including renal injury and monocyte infiltra-
tion from LPS-induced death in male mice (Luria et al., 2007),
which resulted in presumably steady-state inhibitor concen-
trations that were confirmed to be well above the IC50 value. 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 value (Xu et al., 2006). Thus, it is puzzling
why the increased exposure to sEH inhibitors in these stud-
ies did not afford a similar or better protection from inflam-
matory insult. Confounding results due to estrogen-related
differences in sEH expression between males and females is
not a concern, because 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 compen-
sate for increased EETs by increasing 20-hydroxecisatet-
aenoic acid (Luria et al., 2007). The current studies examined the inflammatory response in Ephx2−/− mice and found they were not protected from LPS-induced inflam-
matory gene expression in the liver. Thus, the results from
the genetic model are consistent with the chronic chemical inhibition studies. EET/DHET ratios were signifi-
cantly 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 possi-
bility of tolerance developing in the anti-inflammatory
pathway following sustained elevations in EET levels.
That is, similar to the compensation seen by ω-hydroxylase
up-regulation 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 drink-
ing water might not trigger such compensatory changes. Inter-
estingly, in a previous study, loss of sEH afforded some protec-
tion 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 infiltra-
tion 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 in-
farction 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 dys-
fuction 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 car-
diac arrest and cardiopulmonary resuscitation (Hutchens et
al., 2008). Thus, the anti-inflammatory benefits of sEH inhibi-
tion 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 facil-
itates its metabolism into numerous inflammatory eico-
sanoids. 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 probably a result of
cPLA2 activation because EETs released from the cellular
membrane are subject to sEH-mediated metabolism. More
than 90% of the EETs in the plasma are esterified into the
phospholipids of lipoproteins (Karara et al., 1992). Because
lipoproteins are assembled in the liver and facilitate an ex-
change of lipids with extrahepatic tissues, it thus seems that
plasma levels of EETs are a good biomarker for their relative
abundance in tissues.

Ephx2 mRNA was significantly down-regulated by LPS in
all inhibitor studies, and sEH protein was significantly de-
creased by LPS in Ephx2−/+ mice. It is interesting to spec-
ulate on the implications of this innate regulation in response
to an inflammatory stimulus because down-regulation 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 sig-
naling, because activation of peroxisome proliferator-acti-
vated receptor-γ is associated with down-regulation of sEH
under conditions of laminar flow (Liu et al., 2005). sEH is
also regulated by the vasoactive and proinflammatory mole-
cule angiotensin II; however, here it is up-regulated (Imig et
al., 2002; Ai et al., 2007). More work will be needed to char-
acterize and understand the implications of sEH down-regu-
lation in response to LPS.

The current study used complementary genetic and chem-
ical approaches to study the effects of disrupting sEH activity
on the inflammatory response. The findings support the con-
clusion that there is no reduction in the LPS-induced hepatic
inflammatory response following continuous chemical inhibi-
tion or genetic disruption of sEH, even though EET/DHET
ratios indicated robust sEH inhibition. Importantly, this
study also shows 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 value. Several previous reports have identified in-
hibition 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 out-
comes associated with sEH inhibition or genetic deletion
(Pokreisz et al., 2006; Hutchens et al., 2008). Only more
recently have limits to the benefits of decreased sEH activity been identified and indicate that favorable outcomes are insulin- and tissue-specific. Further research is needed to characterize the effects of EETs on inflammatory signaling pathways to improve understanding of the spectrum of outcomes following modulation of sEH activity.

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References


Kim IH, Tsai HJ, Nishi K, Kasagami T, Morisseau C, and Hammock BD (2007b) 1,3-Di substituted ureas functionalized with ether groups are potent inhibitors of the soluble epoxide hydrolase with improved pharmacokinetic properties. J Med Chem 50:5217–5226.


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