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Anti-Ulcer Efficacy of Soluble Epoxide Hydrolase Inhibitor TPPU on Diclofenac-Induced Intestinal Ulcers[S]

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ABSTRACT

Proton pump inhibitors such as omeprazole (OME) reduce the severity of gastrointestinal (GI) ulcers induced by nonsteroidal anti-inflammatory drugs (NSAIDs) but can also increase the chance of dysbiosis. The aim of this study was to test the hypothesis that preventive use of a soluble epoxide hydrolase inhibitor (sEHI) such as TPPU can decrease NSAID-induced ulcers by increasing anti-inflammatory epoxyeicosatrienoic acids (EETs). Dose- [10, 30, and 100 mg/kg, by mouth (PO)] and time-dependent (6 and 18 hours) ulcerative effects of diclofenac sodium (DCF, an NSAID) were studied in the small intestine of Swiss Webster mice. Dose-dependent effects of TPPU (0.001–0.1 mg/kg per day for 7 days, in drinking water) were evaluated in DCF-induced intestinal toxicity and compared with OME (20 mg/kg, PO). In addition, the effect of treatment was studied on levels of Hb in blood, EETs in plasma, inflammatory markers such as myeloperoxidase (MPO) in intestinal tissue homogenates, and tissue necrosis factor-α (TNF-α) in serum. DCF dose dependently induced ulcers that were associated with both a significant ($P < 0.05$) loss of Hb and an increase in the level of MPO and TNF-α, with severity of ulceration highest at 18 hours. Pretreatment with TPPU dose dependently prevented ulcer formation by DCF, increased the levels of epoxy fatty acids, including EETs, and TPPU's efficacy was comparable to OME. TPPU significantly ($P < 0.05$) reversed the effect of DCF on the level of Hb, MPO, and TNF-α. Thus sEHI might be useful in the management of NSAID-induced ulcers.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively used for the management of inflammation and pain at the expense of gastrointestinal adverse events, among other side effects. NSAIDs are known to suppress pain and inflammation by inhibiting cyclooxygenase (COX) enzymes and decreasing levels of prostaglandins (PGs), such as prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) (Gottlieb et al., 2008; Mallen et al., 2011; Ricciotti and FitzGerald, 2011; Kellner et al., 2012; Radner et al., 2012). However, PGE_2 protects the gastrointestinal (GI) mucosa by increasing mucus production and the release of bicarbonates. The adverse effects of NSAIDs in the form of ulceration in lumen, inflammation, and bleeding of intestinal mucosa are collectively known as NSAID-induced enteropathy. Though inhibition of COX is considered a major factor contributing to GI toxicity, other factors such as inflammation and endoplasmic reticulum (ER) stress are also involved (Wallace, 2008; Ramirez-Alcantara et al., 2009; Castellsague et al., 2012). These effects may be caused either directly by the NSAIDs or as downstream effects of COX inhibition.

COX-1-selective inhibitors (such as SC-560) and COX-2-selective inhibitors (such as celecoxib) were developed with the promise of less gastrointestinal toxicity than nonselective

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ABBREVIATIONS: BCA, bicinchoninic acid; BSA, bovine serum albumin; CES, carboxylesterase; CMNA, cyano(6-methoxy-2-naphthyl)methyl acetate; COX, cyclooxygenase; DCF, diclofenac; DHA, docosahexaenoic acid; EDPs, epoxydocosapentaenoic acids; EETs, epoxyeicosatrienoic acids; EpODE, epoxyoctadecadienoic acid; EpOME, epoxyoctadecenoic acid; ER, endoplasmic reticulum; GI, gastrointestinal; HTAB, hexadecyltrimethylammonium bromide; IL, interleukin; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NSAIDs, nonsteroidal anti-inflammatory drugs; OME, omeprazole; PG, prostaglandin; PGE_2, prostaglandin E_2; PO, by mouth; PPI, proton pump inhibitor; sEH, soluble epoxide hydrolase; sEHI, sEH inhibitor; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tissue necrosis factor-α; TPPU, 1-(1-propanoylpiperidin-4-yl)-3-(4-trifluoromethoxy)phenyl)urea; *trans*-AUCB, 4-[[trans-4-[[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)carbonyl]amino]cyclohexyl]oxy]-benzoic acid.

NSAIDs (Silverstein et al., 2000; Wallace et al., 2000). Even though COX-2 inhibitors have better gastrointestinal tolerability than traditional nonselective NSAIDs, these agents still exert GI toxicity that includes bleeding, ulceration, and dyspepsia (Silverstein et al., 2000; Crawford and White, 2002; Chan et al., 2010; Wallace et al., 2011), and patients taking high doses of these compounds are closely monitored for cardiovascular toxicity (myocardial infarction, angina, venous thrombosis, stroke, etc.), hepatotoxicity (elevated serum alanine aminotransferase, aspartate aminotransferase, etc.), and renal toxicity (peripheral edema, hypertension, increased creatinine level, etc.) (Silverstein et al., 2000; Mukherjee et al., 2001; Antman et al., 2005; Grosser et al., 2006; Liu et al., 2010a). In spite of these side effects, NSAIDs remain key components for the management of pain and inflammation.

Inhibitors of soluble epoxide hydrolase (sEH), which reduce the metabolism of epoxyeicosatrienoic acids (EETs) generated from arachidonic acid, alleviate inflammatory bowel disease and other inflammatory and fibrotic diseases in animal models (Schmelzer et al., 2005; Zhang et al., 2012; Harris et al., 2015). Similarly, sEH gene deletion also reduces inflammatory bowel disease and ulcerative colitis (Zhang et al., 2012, 2013). These data suggest that a potent sEH inhibitor such as TPPU might be effective in the management of NSAID-induced intestinal enteropathy. Therefore, we hypothesized that prophylactic administration of TPPU might decrease intestinal ulcers induced by diclofenac (DCF) through increasing the level of the anti-inflammatory epoxy –fatty acids, including EETs.

The effect of TPPU in the DCF-induced intestinal ulcer model was evaluated. First, dose- and time-dependent effects of DCF in Swiss Webster mice were studied, and next, the effects of TPPU on DCF-generated intestinal toxicities were studied. Release of myeloperoxidase (MPO) from infiltrated neutrophils at the site of ulceration further damages the tissues, and an increase in the level of tumor necrosis factor-α (TNF-α) is associated with NSAID-induced ulcers (Sánchez et al., 2002; Kinsey et al., 2011; Chatterjee et al., 2012). Therefore, levels of MPO and TNF-α were quantified and compared with those in vehicle-treated control animals. The effects of sEH inhibition and knockout ($^{-/-}$) on DCF-induced intestinal ulcer and hematologic and biochemical parameters are reported here. Omeprazole (OME) was used as a positive control and standard of care.

Materials and Methods

Materials. Diclofenac (Sigma-Aldrich, St. Louis, MO), OxySelect myeloperoxidase activity assay kit (Cell Biolabs, Inc., San Diego, CA), hemoglobin assay kit (Sigma-Aldrich), Mouse TNF-α ELISA Kit (Thermo Scientific, Rockford, IL), COX Activity Assay Kit (Cayman Chemicals, Ann Arbor, MI), and Pierce bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) were purchased. 1-(1-propanoylpiperidin-4-yl)-3-(4-trifluoromethoxy)phenyl)urea or TPPU, 2-[^{3}H]*trans*-1,3-diphenylpropene oxide (or [^{3}H]*trans*-DPPO or *trans*-DPPO), and cyano(6-methoxy-2-naphthyl)methyl acetate (CMNA) were synthesized previously in this laboratory (Borhan et al., 1995; Shan and Hammock, 2001; Rose et al., 2010). All other reagents were of analytical grade.

Animals. Male Swiss Webster mice from Charles River Laboratory and sEH knockout mice in a *C57BL6* background raised at University of California, Davis weighing 35–40 g and aged 5–6 months were used for this study. A colony of the sEH knockout mouse strain has been maintained at the "Mouse Biology Program" facility of the University of California, Davis, under standard rearing conditions as described previously (Luria et al., 2009). The sEH knockout mouse strain has a targeted disruption in exon 1 of the *Ephx2* gene and was obtained from Dr. Christopher J. Sinal (National Cancer Institute, Bethesda, MD) (Miyata et al., 1999; Sinal et al., 2000). The homologous sEH null mice were obtained by more than 10 back-crosses with C57BL6 mice and maintained with repeated back-crosses (Jackson Laboratories, Bar Harbor, ME).

The animals were maintained in standard laboratory conditions, and approval for the animal experiment was obtained from the institutional animal care and use committee, University of California, Davis, CA. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Animals were divided into 13 groups each containing 4–5 animals. Animals were fasted with free access to water for 12 hours before euthanasia for determining effect of treatment on intestine. Wire meshes were kept above the bedding in the cages of animals during fasting to prevent animals from eating bedding and feces.

Drug Administration. An aqueous solution of DCF was prepared and administered to mice at doses of 10, 30, and 100 mg/kg by the oral route (Ramirez-Alcantara et al., 2009). A common therapeutic dose of DCF in mice is 30 mg/kg. The effects on ulcer formation in the intestine were evaluated at 6 and 18 hours after DCF administration in anesthetized animals. After selection of a dose of DCF that would cause significant intestinal ulcer formation in mice, pretreatments with TPPU and OME were tested on DCF-induced ulcers. TPPU was administered at a dose range of 0.001–0.1 mg/kg in drinking water containing 1% of PEG400 for 7 days, and a single dose of OME (20 mg/kg) was used (Kinsey et al., 2011). The last doses of TPPU and OME were administered 1 hour before DCF administration.

Effect of sEH gene deletion on DCF-induced ulceration was studied by administering 100-mg/kg doses of DCF to sEH-knockout mice. Vulnerability toward DCF-induced ulcer of Swiss Webster and *C57BL6* mice is similar (Ramirez-Alcantara et al., 2009).

Quantification of Ulcers. Animals were anesthetized with isoflurane after 18 hours of DCF administration, and blood was collected for drug (DCF, TPPU, and OME), cytokine, and eicosanoid quantitation. The intestines were removed, lumens were washed with saline, cut longitudinally, and the ulcers were counted (Ramirez-Alcantara et al., 2009; Yang et al., 2009; Zhang et al., 2012). A portion of intestines were then stored at –80°C until further biochemical analysis. The other portion of intestines (2–3 cm) were transferred to 10% neutral buffered saline, stored for 48 hours, and then transferred to 70% ethanol until further histopathology. Intestines were embedded in paraffin and 5-μm tissue slices were cut and stained in hematoxylin and eosin for histopathological evaluation (Zhang et al., 2012).

Quantification of Drugs in Blood. From each animal, 10 μl of blood was collected and added to 50 μl of an aqueous solution containing 0.1% EDTA and acetic acid each. These solutions were immediately vortexed and stored at –80°C until drug concentrations were determined by liquid chromatography–tandem mass spectrometry.

Estimating Effect of Treatment on Eicosanoid Profile. Blood samples were collected in tubes containing K_2 EDTA and centrifuged at 4000 rpm for 10 minutes at 4°C for separating plasma. Plasma samples (500 μl) were treated with antioxidant solutions (10 μl) containing 1 mg/ml EDTA, triphenyl phosphite (TPP; 0.2 mg/ml), and butylhydroxytoluene (BHT; 0.2 mg/ml). Samples were stored at –80°C until analysis and were analyzed as previously reported (Yang et al., 2009).

Quantification of Hemoglobin Levels in the Blood. Hb level in blood was quantified using a hemoglobin assay kit (Sigma-Aldrich). The assay is an improved version of the Triton/NaOH method in which Hb is converted to a colorimetric product proportional to the level of Hb. Briefly, 25 μl of blood samples diluted 20 times was mixed with

225 μl of reagent in a microplate and incubated for 5 minutes followed by measurement of absorbance at 400 nm. Concentration of Hb was determined by fitting data with a standard graph plotted with calibrator solutions.

Measurement of Myeloperoxidase Activity. MPO activity was determined in intestinal tissue homogenates as published with slight modifications using the OxySelect myeloperoxidase activity assay kit (Sánchez et al., 2002, Chatterjee et al., 2012). Approximately 150 mg of intestinal tissue from each mouse was homogenized in 20 volumes of phosphate-buffered saline containing 0.5% HTAB. The homogenates were centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatants were aliquoted and protein concentrations were measured using a NanoDrop Lite Spectrophotometer (Thermo Scientific). Supernatants were then stored at –80°C until MPO estimation after one freeze-and-thaw cycle. Briefly, 25 μl of supernatant was mixed with 25 μl of 1 mM H_2O_2 solution and incubated for 30 minutes. Stop solution (50 μl) was added, and after 15 minutes 50 μl of 1 mM TMB solution was added. Absorbance was read at 405 nm. The concentration of TMB used gives an estimation of H_2O_2 consumed.

Quantification of TNF-α in Serum. Blood samples were centrifuged at 4000 rpm for 10 minutes at 4°C, the supernatants (serum samples) were stored at –80°C until cytokine estimation. The levels of TNF-α were quantified according to the Mouse TNF-α ELISA Kit protocol at room temperature (Li et al., 2014). For TNF-α estimation, 50 μl of serum or standard sample was added to the wells of precoated plates in duplicate. An equal volume of TNF-α biotinylated antibodies was added and plate was incubated for 2 hours. After washing plates with wash buffer, 100 μl of streptavidin–horseradish peroxidase solution was added, and plate was incubated for 30 minutes. After careful washing of the plate, 100 μl TMB substrate solution was added, and plate was incubated in dark for 30 minutes for development of color. The reactions were stopped by addition of stop solutions. Absorbance was measured at 450 nm and concentrations of TNF-α were determined from a standard graph.

COX Inhibition Assay. The possible inhibition of COX by TPPU was studied using the COX Activity Assay Kit according to the procedure provided by the manufacturer. Indomethacin, a better COX-1 and COX-2 inhibitor than DCF, was used as the standard inhibitor (Blanco et al., 1999).

Effect of Treatment on CMNA Hydrolysis and *trans*-DPPO Ex Vivo. For the CMNA hydrolysis assay to determine carboxylesterase (CES) activity, approximately 30 mg of intestinal tissues were homogenized in 2 ml of 0.1 M ice-cold sodium phosphate buffer, pH 7.4, for 30 seconds using an homogenizer (Polytron benchtop homogenizer; Kinematica GmbH, Lucerne, Switzerland). The homogenates were centrifuged at 10,000*g* (4°C) for 10 minutes, and the supernatant solutions (S9 fractions) were used for estimating CES activities (Morisseau et al., 2009). Protein concentrations of intestinal S9 fractions were determined using BCA assay with bovine serum albumin (BSA) used as standard (Walker JM, 1994). The S9 fractions were diluted 3000 times in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 mg/ml BSA. The reactions were started with addition of 50 μM CMNA solution at 37°C and CMNA hydrolysis was determined for 10 minutes in fluorescence mode with 330-nm excitation and 465-nm emission wavelength (Morisseau et al., 2009).

For the *trans*-DPPO assay, approximately 30 mg of intestinal tissues was homogenized in 2 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and protease inhibitor phenylmethylsulfonyl fluoride for 30 seconds using an homogenizer (Polytron). The homogenates were centrifuged at 10,000*g* (4°C) for 10 minutes, and the supernatant solutions were used for estimating sEH activity using the *trans*-DPPO hydrolysis assay (Borhan et al., 1995). S9 fractions were diluted 50 times in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mg/ml of BSA. The reactions were started with addition of 50 μM *trans*-DPPO at 37°C, and *trans*-DPPO hydrolysis was determined after 20 minutes (Borhan et al., 1995).

Results

Diclofenac Creates Ulcers in Swiss Webster Mice. DCF created ulcers in the small intestines of Swiss Webster mice in a dose- and time-dependent manner. Ulcerative effects of DCF after a single oral administration were seen at 30 mg/kg (a reported therapeutic dose for DCF in mice) with the highest number of ulcers noticed at 100 mg/kg (Fig. 1A). Therefore, a 100-mg/kg dose of DCF was selected for subsequent studies. The number of ulcers seen after 18 hours of DCF dosing was slightly higher (Fig. 1B) and with greater severity in comparison with ulcers at 6 hours. Therefore, further studies were performed at 18 hours post-dosing. No mortality was observed in diclofenac-treated mice.

TPPU Protects Intestine from Ulcerative Effect of DCF. Pretreatment of TPPU for 7 days in drinking water significantly ($P < 0.001$) decreased the number of ulcers in murine intestine caused by DCF in a dose-dependent manner (Fig. 1C, Supplemental Fig. 1). OME at the 20-mg/kg dose was equally effective in reducing the DCF-induced enteropathy, though the potency of sEH inhibitor (sEHI) in reducing ulcers was much higher. Deletion of the sEH gene also reduced the ulcerative effect of DCF, suggesting the role of sEH in reducing

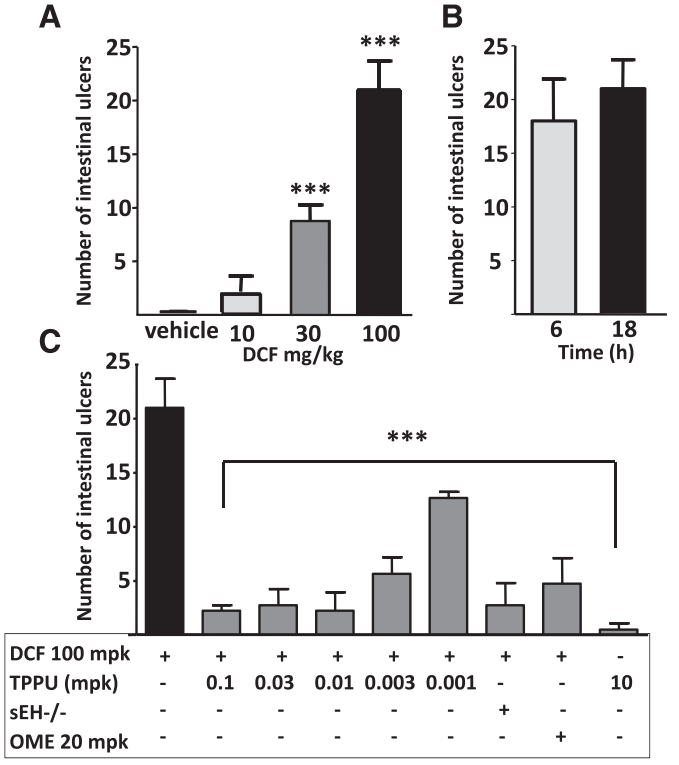

Fig. 1. Effect of drug treatment on intestinal ulcers. (A) Number of intestinal ulcers in mice treated with different doses of DCF after 18 hours. DCF at the higher doses of 30 and 100 mg/kg increased intestinal ulcers significantly (***$P < 0.001$) in comparison with the vehicle. (B) The number of intestinal ulcers increased at 18 hours compared with 6 hours post-treatment with 100 mg/kg DCF. (C) Pretreatment with TPPU (0.001–0.1 mg/kg per day, PO, 7 days) and OME (20 mg/kg, PO, single dose) or sEH knockout (sEH$^{-/-}$, in a C57BL6 background) decreased ulcerative potential of DCF significantly (***$P < 0.001$). Note DCF causes similar levels of ulceration when used alone in control C57B and Swiss Webster mice. When used alone TPPU at 10 mg/kg produced significantly fewer ulcers than DCF at 100 mg/kg (***$P < 0.001$). Values are presented as mean ± standard deviation, $n = 4$.

ulcer formation (Fig. 1C). Ramirez-Alcantara et al. (2009) showed that C57BL6 mice have similar number of ulcers to the Swiss Webster mice used here; thus, the sEH knockout mice in a C57 background were compared with the Swiss Webster mice.

Intestinal ulcers were associated with significant inflammation of the mucosal layer, which consists of epithelium, lamina propria, and muscularis mucosae (Rao and Wang, 2010). The number of ulcers in the lumen was proportional to the histologic damage. Hematoxylin and eosin staining of tissues revealed extensive damage to the epithelium and extension of ulcers to muscular mucosae in the intestine of DCF-treated animals (Fig. 2). Pretreatment with sEHI and OME, and sEH gene deletion protected epithelial cells from the damaging effect of DCF (Fig. 2), though a few ulcers were still observed under microscope in the intestinal tissues. TPPU was 200 times more potent than OME in reducing ulcers.

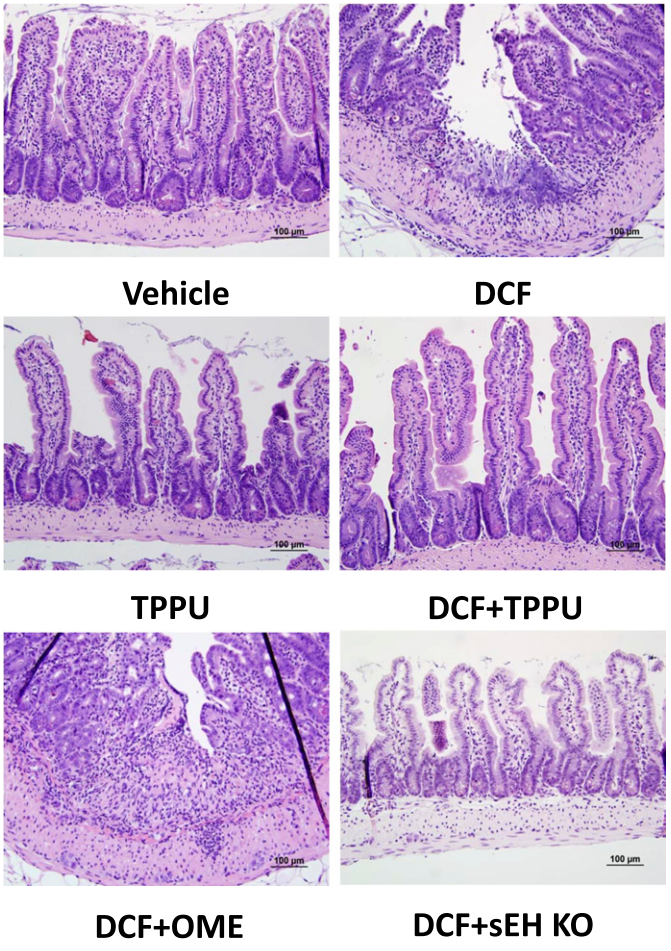

Fig. 2. Inhibition and genetic deletion of sEH protects intestine from the ulcerative effect of diclofenac. The mucosal layer consists of epithelium, lamina propria, and muscularis mucosa. Hematoxylin and eosin staining reveals that the vehicle had no effect on intestine and a healthy mucosal layer is clear. A single dose of DCF at 100 mg/kg created severe disruption of the mucosal layer, and ulcers extended from the epithelial layer to the muscularis mucosa. Pretreatment with TPPU (0.1 mg/kg per day, 7 days) decreased ulceration in DCF-treated animals. sEH gene deletion and OME pretreatment significantly prevented ulcer formation in DCF-treated mice (versus DCF-only treatment) though occasional ulcers are seen in epithelial layers. TPPU alone at 10 mg/kg per day (7 days at ~1000× therapeutic dose) did not create ulcers in intestine of mice. Bars represent 100 μm. Photomicrograph is shown at 100×.

Eighteen hours after DCF administration, the animals were sacrificed and blood samples were collected for determining the levels of drugs used. Concentrations of DCF, TPPU, and OME were between 100–700 ng/ml, 100–235 ng/ml, and 3–23 ng/ml in plasma, respectively (Table 1). Neither the 0.1-mg/kg dose of TPPU nor a much higher dose (10 mg/kg per day, 7 days) created ulcers in intestines of mice compared with those of vehicle-treated mice (Fig. 2). This is 100–1000 times the dose needed to significantly reduce ulcers, whereas even a therapeutic dose of 30 mg/kg DCF causes significant ulceration in mice (Fig. 1A).

Anti-Ulcer Effects of TPPU Correlate with Levels of EETs and EDPs in Plasma. Cytochrome P450s are known to metabolize arachidonic acid, linoleic acid, α-linolenic acid, and docosahexaenoic acid (DHA) to EETs, epoxyoctadecenoic acids (EpOMEs), epoxyoctadecadienoic acids (EpODEs), and epoxydocosapentaenoic acids (EDPs). respectively. DCF had no effect on the plasma levels of these metabolites, whereas TPPU treatment significantly increased the levels of EETs and EDPs in plasma of animals (Fig. 3). An increase in epoxy fatty acids or an increase in the ratio of epoxy fatty acids to their corresponding diols are commonly used markers of target engagement by sEH inhibitors (Zhang et al., 2012). In this study the anti-ulcer effect of TPPU is marked with a significant increase in the levels of EETs ($^*P < 0.05$, TPPU versus vehicle) and EDPs ($^{**}P < 0.01$, TPPU versus vehicle). Ulcer resolution seen with sEH gene deletion is associated with a significant increase in levels of EDPs ($^{**}P < 0.01$, sEH knockout versus vehicle) and EpOMEs ($^*P < 0.05$, sEH knockout versus vehicle, Fig. 3). Though OME treatment increased levels of EETs and EDPs by 50–100% in comparison with vehicle, the increase was not statistically significant. OME treatment significantly increased ($^*P < 0.05$, OME versus vehicle) epoxide metabolites of α-linolenic acid (EpODEs) (Fig. 3). This increase in epoxide-containing lipids may result from increased biosynthesis by induced P450 enzymes (Goswami et al., 2015), whereas the increase caused by TPPU is from reduced metabolism. Levels of other quantified metabolites were not altered significantly and are presented in Supplemental Table 1.

TPPU Prevents Decrease in Blood Hb Levels in DCF-Treated Animals. Acute treatment of DCF led to a small but significant decrease in the level of Hb in the blood of Swiss Webster mice and sEH gene knockout mice compared with vehicle-treated animals. The level of Hb in animals treated with TPPU and OME before administration of DCF was

TABLE 1
Concentration of drugs in the plasma of treated mice at the time of termination
Values are expressed as mean ± S.E.M. of observations for four animals.

Group	Drug Concentration (ng/ml)		
	DCF (100 mg/kg)	TPPU (0.1 mg/kg)	OME (20 mg/kg)
DCF[a]	331 ± 110	—	—
DCF[a] + TPPU[b]	464 ± 110	152 ± 28	—
DCF[a] + OME[c]	178 ± 79	—	8 ± 4
sEH KO	496 ± 223	—	—

[a]Plasma was collected 18 hours after DCF (PO, single dose) administration.
[b]TPPU was administered to mice for 7 days in drinking water and administration was terminated 1 hour before DCF administration.
[c]OME (PO, single dose) was administered to mice 1 hour before DCF administration.

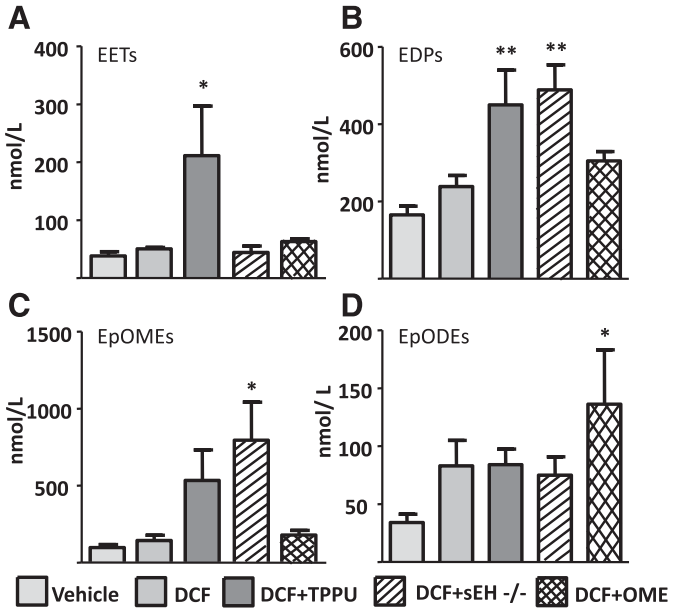

Fig. 3. Effect of drug treatment on levels of epoxy fatty acids in plasma samples of mice. A) Combined EETs (8, 9-EET; 11, 12-EET; 14, 14-EET), metabolites of arachidonic acid; B) combined EDPs (7, 8-EDP; 10, 11-EDP; 13, 14-EDP; 16, 17-EDP; 19, 20-EDP), metabolites of docosahexaenoic acid; C) combined EpOMEs (9, 10-EpOME and 12, 13-EpOME), metabolites of linoleic acid; and D) combined EpODEs (9, 10-EpODE and 15, 16-EpODE), metabolites of α-linolenic acid. DCF and OME have no effect on epoxy metabolites of arachidonic acid (EETs) and docosahexaenoic acid/DHA (EDPs) and linoleic acid (EpOMEs) and α-LA (EpODEs) in comparison with vehicle. OME significantly increased metabolite of αLA (EpODE) in plasma of animals versus vehicle-treated animal. The anti-ulcer effect of TPPU strongly correlated with the increase in the level of EETs and EDPs. The sEH knockout ($sEH^{-/-}$) significantly increased levels of EDPs and EpOMEs in comparison with vehicle-treated animals. Values are presented as mean ± S.E.M. of observation of four animals. One-way analysis of variance followed by Dunnett's test was used for statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle. See Supplemental Table 1 for full analytical data.

significantly higher than in animals treated with only DCF (Fig. 4A). It is known that chronic administration of DCF decreases the level of Hb in the blood of animals and humans (Sanchez et al., 2002; Goldstein et al., 2011), and the small decrease in hemoglobin observed here was probably attributable to the short term nature of the model. The decrease in the significant loss of Hb in TPPU+DCF-treated animals compared with DCF-only–treated animals is directly proportional to the decrease in number of ulcers. This could result from a decrease in blood loss at the site of ulcerations.

TPPU Decreases the Level of MPO and TNF-α. DCF administration significantly increased the level of inflammatory markers in this experimental model, including intestinal MPO activity and TNF-α in serum, compared with vehicle-treated mice (Fig. 4, B and C). This is in accord with the effect of NSAIDs on levels of MPO and TNF-α in the gastrointestinal system and other models (Bertrand et al., 1998; Sanchez et al., 2002; Page at al., 2010; Zhang et al., 2012). TPPU pretreatment significantly decreased the level of MPO and TNF-α in DCF-treated mice compared with mice treated with only DCF. This anti-inflammatory effect of sEHI was expected on the basis of previous observations (Schmelzer et al., 2005; Zhang et al., 2012; Kodani and Hammock, 2015). OME was also reported to decrease the levels of MPO and TNF-α in indomethacin-induced ulcers (Chatterjee et al., 2012; Yadav et al., 2013). In our study, pretreatment with OME decreased the level of MPO and TNF-α significantly, consistent with OME having significant anti-inflammatory activity in addition to inhibiting proton pumps (Fig. 4, B and C). Fornai et al. (2014) reported an increase in MPO level in the intestine of Wistar rats orally fed with DCF (8 mg/kg per day, 14 days) + OME (0.7 mg/kg per day, 14 days) in comparison with control rats. This 2-fold increase in the level of MPO by OME could be attributable to the difference in dose of OME and duration of treatment.

TPPU Does Not Significantly Inhibit COX. TPPU at 100 μM inhibited approximately 10% of both COX-1 and COX-2 activity in vitro, whereas indomethacin at the same concentration inhibited approximately 95% of both COX isoforms.

Treatment Does Not Affect CES and sEH Activity Ex Vivo. DCF and DCF along with TPPU and OME did not significantly alter carboxylesterase activity (data not shown) or sEH activity in murine intestinal S9 fraction when evaluated in terms of CMNA hydrolysis and *trans*-DPPO hydrolysis, respectively. As expected, no sEH activity was observed in the intestines of sEH knockout animals (Fig. 4D).

Discussion

In this murine model of DCF-induced ulceration, TPPU pretreatment decreased the number of ulcers associated with an increase in the level of inflammatory markers at such local sites of ulceration as intestine and systemically in the serum. The decrease in ulceration is directly correlated with an increase in levels of anti-inflammatory bioactive lipids, such as epoxides of arachidonic acid, DHA, and linoleic acid, in plasma of mice. Further, TPPU treatment decreased Hb loss owing to ulceration. TPPU was 200 times more potent than OME in reducing intestinal inflammation and ulceration in this model. This study illustrates the potential value of sEHI as an alternative to OME and related proton pump inhibitors in management of intestinal ulcers induced by NSAIDs. Since sEH inhibitors dramatically synergize the analgesic and anti-inflammatory effects of NSAIDs (Schmelzer et al., 2005; Liu et al., 2010b), they could be combined with NSAIDs both to synergize efficacy and reduce side effects of NSAIDs (Schmelzer et al., 2005; Liu et al., 2010a, b; Kodani and Hammock, 2015).

The role of acidic pH in aggravating stomach ulcers associated with decreases in the level of PGE_2 is well documented, and a proton pump inhibitor that decreases the release of acid in the stomach is useful in decreasing stomach ulcers, including NSAID-induced ulcers (Wallace, 2008; Wallace et al., 2011). However, the role of acid in the generation and aggravation of intestinal ulcers is negligible, because the pH of intestine is alkaline. However, the decrease in the level of PGE_2 is still a risk factor (Fornai et al., 2014). Unlike NSAIDs, which exhibit their anti-inflammatory effect because of COX inhibition, anti-inflammatory properties of sEH inhibitors in this experiment result from mechanisms other than COX inhibition, because TPPU does not significantly inhibit COX at up to 100 μM in vitro. Other mechanisms for the causes of intestinal ulcers by NSAIDs are discussed elsewhere in detail (Boelsterli et al., 2013). The OME- and TPPU-mediated anti-ulcer effects appear anti-inflammatory and involve decreases in levels of TNF-α and MPO. The anti-inflammatory effect of at least TPPU seems to be modulated by epoxides of fatty

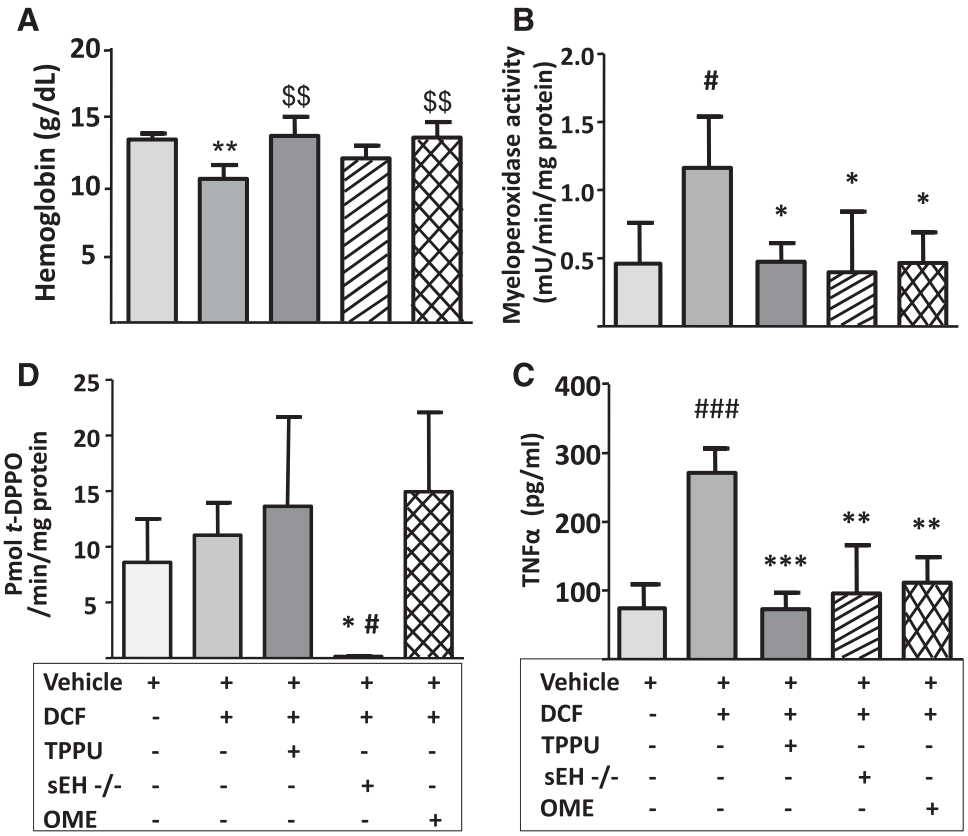

Fig. 4. Effect of drug treatment on levels of hemoglobin and TNF-α, and activities of MPO and sEH. (A) DCF treatment significantly decreased ($^{**}P < 0.01$) Hb level in the blood of mice in comparison with vehicle-treated mice. Pretreatment of TPPU ($^{\$\$}P < 0.01$) and OME ($^{\$\$}P < 0.01$) prevented loss of Hb in DCF-treated animals significantly in comparison with DCF-only–treated animals. (B) Level of myeloperoxidase (MPO) in intestinal homogenate was significantly higher ($^{\#}P < 0.05$) in the DCF-treated group in comparison with vehicle-treated group. Pretreatment with TPPU and OME or sEH knockout significantly decreased ($^{*}P < 0.05$ versus DCF) the effect of DCF on MPO. (C) DCF treatment significantly ($^{\#\#\#}P < 0.001$) increased TNF-α level in serum of treated mice in comparison with vehicle-treated mice. Pretreatments of TPPU, OME or sEH knockout significantly ($^{**}P < 0.01$; $^{***}P < 0.001$ versus DCF) decreased TNF-α level. (D) The treatment did not affect sEH activity in vitro as evidenced by *trans*-DPPO hydrolysis. But as expected, sEH activity was negligible in intestine of sEH knockout animals and it was significantly less in comparison with animals treated with DCF+TPPU ($^{*}P < 0.05$) and DCF+OME ($^{\#}P < 0.05$). One-way analysis of variance followed by Tukey's multiple comparison tests were used for statistical significance. Values are presented as mean ± standard deviation of value from analyzing four to five animals in each group.

acids. Earlier studies reported beneficial effect of polyunsaturated fatty acids such as DHA, linoleic acid, and γ-linolenic acid in ulcer relief, including NSAID-induced ulcers (al-Shabanah, 1997; Pineda-Peña et al., 2012). The beneficial effects of these fatty acids in ulcer models could be attributable in part to their epoxide metabolites, as suggested by this study.

Anti-inflammatory effects of EETs have been reported by numerous authors (Node et al., 1999; Chiamvimonvat et al., 2007; Thomson et al., 2012; Kodani and Hammock, 2015). Inhibition or gene deletion of sEH exert anti-inflammatory effects in different animal models and are associated with an increase in endogenous epoxy fatty acids or the ratio of epoxy/dihydroxy derivatives (Schmelzer et al., 2005; Liu et al., 2010b; Zhang et al., 2012, 2013; Askari et al., 2014). sEH enzyme inhibition caused by the sEHI *trans*-AUCB and the sEH knockout decrease piroxicam (NSAID)-induced ulcer formation and transmural inflammation in intestine of interleukin IL-$10^{-/-}$ mice in comparison with only piroxicam-treated IL-$10^{-/-}$ mice (Zhang et al., 2012). The sEH gene deletion significantly decreases dextran sodium sulfate–induced colitis (and adenocarcinoma). In addition, 14, 15-EET reduces the lipopolysaccharide (LPS)-induced increase in expression of IL-1β and TNF-α in macrophages in vitro (Zhang et al., 2013). The sEHIs are known to be anti-inflammatory in vivo and in vitro (Schmelzer et al., 2005, Liu et al., 2010b; Askari et al., 2014), presumably by stabilizing fatty acid epoxides.

Downstream components of TNF-α-mediated signaling, such as nuclear factor-κB (NF-κB), c-Jun N-terminal kinases (JNK), and caspase 3, are involved in the development of NSAID-induced intestinal toxicity, including ulceration (Ramirez-Alcantara et al., 2009; Fukumoto et al., 2011; Zhang et al., 2012). TNF-α-facilitated ulceration involves apoptosis of enterocytes in NSAID-treated animals. Administration of a TNF-α synthesis inhibitor (type-IV phosphodiesterase inhibitor RO 20-1724), TNF-α inhibitor (infliximab), JNK inhibitor (SP600125), or TNF-α gene deletion all decrease indomethacin-induced toxicity in intestine (Bertrand et al., 1998; Cury et al., 2008; Ramirez-Alcantara et al., 2009; Fukumoto et al., 2011; Boelsterli et al., 2013). Further, it is known that NSAIDs including DCF increase the production of TNF-α in human blood challenged by LPS. The decrease in production of TNF-α by PGE_2 is mediated through its EP_2 receptor (Page et al., 2010). A significant decrease in number of ulcers in murine intestine associated with a lower level of TNF-α in serum was also observed. Though the role of sEHI in minimizing NSAID-induced ulceration through TNF-α/NF-κB is established, its effect on other downstream regulators such as JNK and caspase 3 cannot be ruled out, because sEH inhibition in HepG2 cells and adipocytes decreases activation of JNK, caspase 3, p38, and cell death at least in part by decreasing ER stress (Bettaieb et al., 2013). Certain types of white blood cell also contribute to ulcer formation through production of cytokines such as TNF-α. Activated macrophages differentiated from monocytes mainly release these cell-signaling proteins, although the roles of neutrophils and eosinophils have also been discussed (Page et al., 2010).

Neutrophils are known to aggravate the ulceration caused by NSAIDs. Administration of rat antineutrophil serum or methotrexate, an anticancer drug that decreases neutrophil function, before indomethacin treatment decreases gastric ulceration (Wallace et al., 1990; Cronstein et al., 1991). Prostaglandins generated in endothelial cells of blood vessels

modulate neutrophil function. Prostaglandin I_2 inhibits adhesion of neutrophils to blood vessel and prostaglandins are reported to inhibit activation of polymorphonuclear leukocytes and the release of reactive superoxide ions from neutrophils. Therefore, decreases in the level of PGs by NSAIDs enhance neutrophil-driven damage (Wong and Freund, 1981; Gryglewski et al., 1987; Wallace et al., 1990). The level of neutrophil infiltration at inflammatory ulcer sites is measured by the level or activity of the inflammatory enzyme MPO released by the granular leukocytes (Sanchez et al., 2002; Chatterjee et al., 2012; Seo et al., 2012; Zhang et al., 2013). We observed an increase in MPO activity in ulcerated intestine of mice 18 hours after DCF administration. Pretreatment with TPPU and OME and sEH gene deletion significantly decreased the activity MPO after DCF administration compared with DCF-only–treated group.

In addition to the direct reduction of inflammation, TPPU might also work through other mechanisms, such as reducing ER stress and apoptosis to decrease the ulcerative effect of DCF. ER stress and apoptosis are involved in NSAID-induced ulcers in stomach and intestine (Tsutsumi et al., 2004; LoGuidice et al., 2010; Ohyama et al., 2012; Harada et al., 2015). TPPU might have decreased the number of ulcers by suppressing ER stress, because sEH inhibition and sEH gene deletion are reported to decrease ER stress in different disease models (Bettaieb et al., 2013; Harris et al., 2015). NSAID-induced intestinal ulcers are characterized by apoptosis of intestinal epithelial cells (Harada et al., 2015). EETs, sEH inhibition, and sEH gene deletion decrease apoptosis of cells (Luo et al., 2010; Liu et al., 2013). Therefore, an antiapoptotic role of TPPU in intestinal epithelial cells may be expected. An anti-inflammatory role of omeprazole in decreasing ulcer has been reported (Biswas et al., 2003). The reduction of ulcers by OME is attributable, at least in part, to induction of key P450 oxidases, thus increasing the biosynthesis of anti-inflammatory epoxy fatty acids (Goswami et al., 2015). Thus, the efficacy of TPPU, which stabilizes epoxy fatty acids, on prevention of NSAID-induced ulcers is not surprising. TPPU is a noncharged compound. Therefore, it is unlikely to induce dysbiosis.

In conclusion, sEHIs such as TPPU can decrease NSAID-induced enteropathy, at least in part, through anti-inflammatory effects driven by epoxy fatty acid.

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Authorship Contribution

Participated in research design: Goswami, Hammock.

Conducted experiments: Goswami, Kodani, Wan, J. Yang, Trindade da Silva, Morisseau.

Contributed new reagents or analytic tools: Wan, J. Yang.

Performed data analysis: Goswami and G-Y. Yang

Wrote or contributed to the writing of the manuscript: Goswami, Morisseau, Inceoglu, Hammock.

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