

Hydrolysis of *cis*- and *trans*-Epoxyeicosatrienoic Acids by Rat Red Blood Cells

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ABSTRACT

Erythrocytes serve as reservoirs for *cis*- and *trans*-epoxyeicosatrienoic acids (EETs). Incubation of rat red blood cells (RBCs) with *cis*- and *trans*-EETs produces *threo*- and *erythro*-dihydroxyeicosatrienoic acids, respectively. The V_{\max} of EET hydrolysis by rat intact RBCs (2.35 ± 0.24 pmol/min/ 10^9 RBCs for 14,15-*trans*-EET) decreased by approximately 2 to 3-fold sequentially from 14,15-, 11,12- to 8,9-EETs for both *cis*- and *trans*-isomers. The V_{\max} of *trans*-EET hydrolysis by RBCs is approximately 2 to 3 times that of the corresponding *cis*-EETs. Incubation of EETs with recombinant murine soluble epoxide hydrolase (sEH) yielded the same geometric and regio preferences of EET hydrolysis as with rat intact RBCs. The principal epoxide hydrolase activity for EET hydrolysis (approximately 90%) is present in the erythrocyte cytosol. Western blots of sEH suggested a concentration of sEH protein to be approximately

2 μ g/mg protein or 0.4 μ g/ 10^9 RBCs. The apparent K_m values of EETs were between 1 and 2 μ M, close to the K_m for purified sEH as reported. Erythrocyte hydration of *cis*- and *trans*-EETs was blocked by sEH inhibitors, 1,3-dicyclohexylurea and 4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid. Erythrocyte sEH activity was inhibited more than 80% by 0.2% bovine serum albumin in the buffer. Preferred hydrolysis of 14,15-EETs and *trans*-epoxides characterizes sEH activity in RBCs that regulates the hydrolysis and release of *cis*- and *trans*-EETs in the circulation. Inhibition of sEH has produced antihypertensive and antiinflammatory effects. Because plasma *trans*-EETs would increase more than *cis*-EETs with sEH inhibition, the potential roles of *trans*-EETs and erythrocyte sEH in terms of circulatory regulation deserve attention.

Epoxyeicosatrienoic acids (EETs) are arachidonic acid (AA)-derived vasoactive, antithrombotic, antiproliferative, and anti-inflammatory lipid mediators (Larsen et al., 2006; Fleming, 2007) that are inactivated by soluble epoxide hydrolase (sEH), producing dihydroxyeicosatrienoic acids (DHETs) (Inceoglu et al., 2007). Formation of EETs is catalyzed by cytochrome P450 epoxygenases (Capdevila et al., 1991), hemoglobin (Jiang et al., 2007), and lipid peroxidation (Nakamura et al., 1997). The substrate preferences for *cis*- and *trans*-epoxides by sEH vary according to cell type (Seidegård et al., 1984) and species

(Morisseau and Hammock, 2005). sEH is a therapeutic target for control of blood pressure (Sinal et al., 2000; Loch et al., 2007; Zhang et al., 2007), vascular inflammation (Davis et al., 2002; Schmelzer et al., 2005; Smith et al., 2005), and cancer progression (Morisseau and Hammock, 2005), as well as for cardiac (Seubert et al., 2006) and renal protection (Imig, 2005) by augmenting the levels of EETs in vivo. Inhibition of sEH also increases EET incorporation into phospholipids, "thereby modulating endothelial function in the coronary vasculature" (Weintraub et al., 1999). Increases in sEH activity have been associated with diabetes (Rodriguez and Clare-Salzler, 2006) and hypertension (Imig et al., 2002; Ai et al., 2007).

Epoxide hydrolases convert *cis*-EETs to *threo*-DHETs and *trans*-EETs to *erythro*-DHETs (Fig. 1). Cytochrome P450 epoxygenases generate *cis*-EETs, whereas EETs in vivo include both *cis*- and *trans*-EETs (Jiang et al., 2005). The 5,6-*erythro*-DHET was more potent in dilating precontracted renal interlobar arteries than the 5,6-*cis*-EET,

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ABBREVIATIONS: EET, epoxyeicosatrienoic acid; AA, arachidonic acid; sEH, soluble epoxide hydrolase; DHET, dihydroxyeicosatrienoic acid; RBC, red blood cell; LTA₄, leukotriene A₄; PSS, physiological salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; AUCB, 4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid; DCU, 1,3-dicyclohexylurea; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; HETE, hydroxyeicosatetraenoic acid; SC27716, 1-[2-(4-phenylphenoxy)ethyl]pyrrolidine.

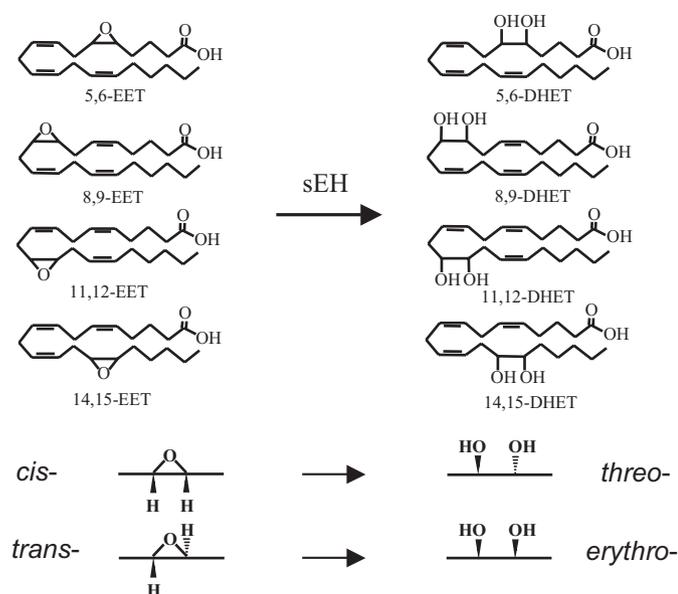


Fig. 1. Structures of EETs and DHETs, and illustrations of *cis*-, *trans*-, *threo*-, and *erythro*-configurations.

whereas, unlike *cis*- and *trans*-EETs, it did not inhibit platelet aggregation (Jiang et al., 2004). Red blood cells (RBCs) are reservoirs for both *cis*- and *trans*-EETs that can be released by ATP stimulation of erythrocyte P2X₇ receptors (Jiang et al., 2007). The hydrolysis of EETs by erythrocytes may represent an important mechanism involved in circulatory regulation.

Human erythrocytes were originally discovered to possess a sEH that hydrates leukotriene A₄ (LTA₄) and, to a much lesser degree, hydrates *cis*-EETs (McGee and Fitzpatrick, 1985). Because epoxide hydrolases occur in multiple forms and possess individual substrate specificities (Ota and Hammock, 1980; Thomas et al., 1990), an additional form of sEH for the hydrolysis of EETs in RBCs could not be excluded (McGee and Fitzpatrick, 1985). It was a surprise that human erythrocyte cytosol was not found to hydrolyze stilbene oxide in a previous study, whereas the erythrocyte membrane has epoxide hydrolase activities at less than 1% of that of the granulocyte in human blood (Seidegård et al., 1984). In our studies of erythrocyte-derived EETs (Jiang et al., 2007), we found significant hydrolysis of *cis*/*trans*-EETs by rat RBCs, which gives rise to questions regarding the nature of the epoxide hydrolase present in erythrocytes responsible for *cis*/*trans*-EET hydrolysis.

To investigate the existence of sEH and to compare hydrolysis of *cis*- and *trans*-EETs in RBCs, we analyzed kinetics of rat RBCs in hydrolyzing *cis*- and *trans*-EETs and compared the regioselectivity and geometric selectivity with recombinant murine sEH. The comparable K_m and geometric selectivity between rat erythrocyte cytosol and purified mouse liver sEH in hydrolyzing EETs suggested the presence of a typical sEH in RBCs as in hepatocytes. Rat sEH demonstrated preferred hydrolysis for *trans*- over *cis*-EETs with similar regioselectivity for both *cis*- and *trans*-EETs in a decreasing order from 14,15-, 11,12-, 8,9- to 5,6-EETs.

Materials and Methods

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of New York Medical College

and conformed to the Institute of Laboratory Animal Resources (1996). Eight-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were maintained at 22°C with alternate light/dark cycles and fed ad libitum with standard rat chow and water.

Blood Cell Preparation. Sprague-Dawley rats (9–12 weeks old) were anesthetized with pentobarbital (65 mg/kg i.p.), and 10 ml of blood was drawn from the inferior vena cava after midline laparotomy using heparin-rinsed syringes and transferred to Vacuette heparin tubes (Thermo Fisher Scientific, Waltham, MA). After inverting four to six times, the blood was centrifuged at 800g at 4°C for 10 min. The supernatant was removed by aspiration, and the buffy layer was collected in some experiments. Packed RBCs were washed four times in an ice-cold physiological salt solution (PSS) with centrifugation at 400g for 10 min, and the washing buffer and any residual buffy layer were discarded after each wash. The PSS contained 5.0 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 128 mM NaCl, 20 mM HEPES, 5 mM dextrose, and 2 mg/ml bovine serum albumin (BSA), and pH was adjusted to 7.4. Washed RBCs were suspended in PSS, examined, and counted using hemacytometers under the microscope.

Incubation and Eicosanoid Extraction. Given a total of approximately 40 ng/ml EETs in rat plasma, less than 5% is free, whereas over 95% is esterified in phospholipids (Karara et al., 1992; Jiang et al., 2005). To test hydrolysis of EETs by RBCs, 6 ng of *cis*- and/or *trans*-EETs was added to 2 ml of prewarmed RBCs (2 × 10⁹ RBCs/ml) in PSS at 37°C for up to 30 min with shaking around a 3-mm orbit at 600 rpm in a VWR Incubating Mini Shaker (VWR, West Chester, PA). The nonenzymatic conversion of 5,6-EETs to DHETs was tested with control incubations using 2 ml of PSS buffer under the same conditions.

To determine the proportion of EET hydrolysis by RBCs in blood, 100 μl of plasma, the buffy layer diluted in phosphate-buffered saline (PBS), and diluted RBCs in PBS were incubated with *trans*-EETs (1 μM) for 5 min at 37°C, respectively. EET and DHET extraction for electrospray ionization (ESI)-liquid chromatography (LC)/mass spectrometry (MS) analyses were performed as described previously (Jiang et al., 2004).

Separation of Membrane and Soluble Fractions. Washed rat erythrocytes (4 ml) were lysed hypotonically in ice-cold sterile water and vortexed for 2 min. After restoration to isotonic buffer conditions, the crude lysate was centrifuged at 100,000g for 1 h with a Beckman Ultracentrifuge (Beckman Coulter, Fullerton, CA) at 4°C. The supernatant was filtered through a 0.45-μm nylon syringe filter to obtain the cytosol fraction of RBCs. Both the pellet and the cytosol were diluted in PSS buffer without BSA to correspond to a cellular concentration of 2 × 10⁹ RBCs/ml.

Western Blots of Erythrocyte sEH. Total protein concentration was quantified with the Pierce BCA assay (Pierce, Rockford, IL), using Fraction V BSA as the calibrating standard. After thawing the frozen erythrocyte cytosol, sEH activity was measured using racemic [³H]*trans*-1,3-diphenylpropene oxide as described previously (Morisseau and Hammock, 2007). For each cytosolic sample, 50 μg of protein was loaded on a 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a polyvinylidene difluoride membrane, and sEH was detected using a rabbit serum raised against recombinant mouse sEH (Davis et al., 2002). Detection was done using a goat antibody raised against rabbit IgG labeled with horseradish peroxidase. Bands were revealed using the ECL kit (Amersham, Piscataway, NJ). Recombinant purified mouse sEH (500 ng) was used as a positive control.

Kinetic Studies of EET Hydrolysis. Because BSA was found to have inhibitory effects on EET hydrolysis, PSS buffer without BSA was used in the last wash of rat RBCs, and RBCs were diluted to 2 × 10⁹ RBCs/ml in PSS without BSA. To achieve substrate saturation, 14,15-, 11,12-, and 8,9-*cis*- or *trans*-EETs (0.1–4 μM) in 50 μl of RBCs or cellular fractions were hydrolyzed at 37°C for 10 min. Endogenous EETs and DHETs released from 1 × 10⁸ RBCs in a buffer without BSA were negligible (Jiang et al., 2007), which was

confirmed by the insignificant alternative EET/DHET peaks in LC/MS analyses. Freshly prepared rat intact RBCs were used for the incubations because freeze-thawing of RBCs reduced EET hydrolysis by 50% as tested. After incubation, 2 ml of ice-cold ethyl acetate/hexanes (1:1) was added to each tube to stop the reaction, the mixtures of which were extracted after adding internal standards of EET-d8 and DHET-d8 and adjusting pH to 4 with 10% acetic acid. The kinetics of 5,6-EET were not measured because of nonenzymatic conversion to 5,6-DHET (Jiang et al., 2004).

IC₅₀ Assay. IC₅₀ of sEH inhibitors were determined based on National Institutes of Health Chemical Genomics Center enzymatic assay guidance manual. Rat erythrocyte cytosol (corresponding to 5×10^8 RBCs) was incubated with *cis*- and *trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid (AUCB) (Hwang et al., 2007) as well as 1,3-dicyclohexylurea (DCU), respectively, for 5 min in PBS, pH 7.4, at 37°C before substrates of 1 μM 14,15-, 11,12-, and 8,9-*trans*-EETs were added. Activity of sEH was assessed by analyzing *erythro*-DHETs formed with LC/MS after ethyl acetate extraction. Assays were performed in triplicate. IC₅₀ and S.E. were determined by regression of at least eight data with a minimum of two points in the linear region of the curve on either side of the IC₅₀.

ESI LC/Tandem Mass Spectrometry Analyses. ESI LC/tandem mass spectrometry (MS/MS) analyses of EETs and DHETs were carried out as described previously (Jiang et al., 2005). In brief, a Finnigan LCQ Advantage quadrupole ion-trap mass spectrometer (Thermo Fisher Scientific) equipped with ESI source run by Xcalibur software was used. Reversed-phase high-performance liquid chromatography (HPLC) was run with a Luna C18(2) 250 × 2.0-mm column (Phenomenex, Torrance, CA) maintained at 30°C with an isocratic eluent of acetonitrile/water/methanol/acetic acid (60:30:10:0.05) at a flow rate of 0.30 ml/min. For EET hydrolysis kinetic studies, the isocratic eluent was kept for 5 min and followed by a gradient to a final composition of acetonitrile/water/methanol/acetic acid (75:15:10:0.05) in 15 min. ESI was carried out at an ion transfer tube temperature of 260°C, a spray voltage of 4.5 kV, a sheath gas flow of 34 units, and an auxiliary gas flow of 20 units (units refer to

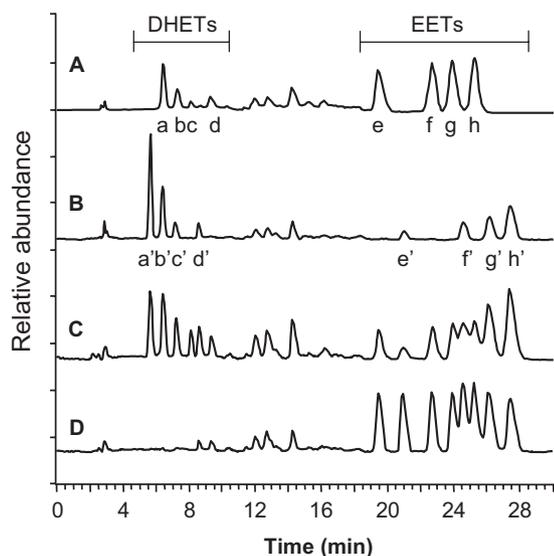


Fig. 2. Representative LC/MS monitoring ($n = 6$, m/z 337 + m/z 319) of *cis*- and *trans*-EET (10 nM each) hydrolysis after 2-min incubation at 37°C with 4×10^9 rat RBCs in 2 ml of PSS. A, 14,15-, 11,12-, 8,9-, and 5,6-*threo*-DHETs were produced by incubation of 14,15-, 11,12-, 8,9-, and 5,6-*cis*-EETs, corresponding to peaks a to h from left to right, respectively. B, 14,15-, 11,12-, 8,9-, and 5,6-*erythro*-DHETs were produced by incubation of 14,15-, 11,12-, 8,9-, and 5,6-*trans*-EETs, corresponding to peaks a' to h' from left to right, respectively. C, incubation of EET isomers together resulted in formation of both *threo*- and *erythro*-DHETs. D, conversion of EETs to DHETs by rat RBCs was blocked by 10 μM DCU, a sEH inhibitor.

arbitrary values set by the LCQ software). MS/MS breakdown for m/z 337 was at an energy level of 30% set by the instrument, and a 7-point Gaussian smoothing was applied in the mass data processing.

Statistical Analysis. Results are presented as mean \pm S.E.M. Parameters of V_{max} , K_m , and IC₅₀ were analyzed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) using Michaelis-Menten kinetics. One-way analysis of variance followed by a Bonferroni test for selected groups were used to analyze for differences. A P value less than 0.05 was regarded as statistically significant.

Reagents. Standard *cis*-EETs and LTA₄ hydrolase were purchased from Cayman Chemical (Ann Arbor, MI). Standard racemic *trans*-EETs were synthesized by Dr. John R. Falck (Falck et al., 2003; Jiang et al., 2004). Potent sEH inhibitors, *cis*- and *trans*-AUCB, were prepared as described previously (Hwang et al., 2007). SC22716, DCU, and AA were ordered from Sigma-Aldrich (St. Louis, MO). AA was used immediately after HPLC purification. Fatty acid-free BSA and HPLC grade organic solvents were obtained from Thermo Fisher Scientific, and EET-d8 standards were from Biomol Research Laboratories (Plymouth Meeting, PA). Recombinant murine sEH was produced in a baculovirus expression system (Grant et al., 1993) and purified by affinity chromatography. The preparations were at least 97% pure as judged by SDS-polyacrylamide gel electrophoresis and scanning densitometry. No detectable esterase or glutathione transferase activity, which can interfere with this sEH assay, was observed.

Results

Hydrolysis of EETs by Rat RBCs in PSS. Incubation of 6 ng of each *cis*- and *trans*-EETs with 4×10^9 rat RBCs in 2 ml of PSS at 37°C for 2 min revealed conversion of EETs to corresponding DHETs (Fig. 2). LC/MS chromatography resulted in complete separation of individual EETs and DHETs when *cis*- or *trans*-EETs were incubated separately (Fig. 2, A and B). *cis*-EETs were converted to *threo*-DHETs (Fig. 2A), whereas *trans*-EETs were converted to *erythro*-DHETs (Fig. 2B). When eight EET isomers were incubated together with RBCs, the chromatogram was similar to a combination of individual incubations (Fig. 2C), indicating no inhibition of hydrolysis by EET isomers at 10 nM. The hydrolysis of *cis*- and *trans*-EETs by rat RBCs can be blocked by 10 μM DCU (Fig. 2D), a traditional sEH inhibitor, as well as by 100 nM *cis*- and *trans*-AUCB (similar to Fig. 2D), novel potent

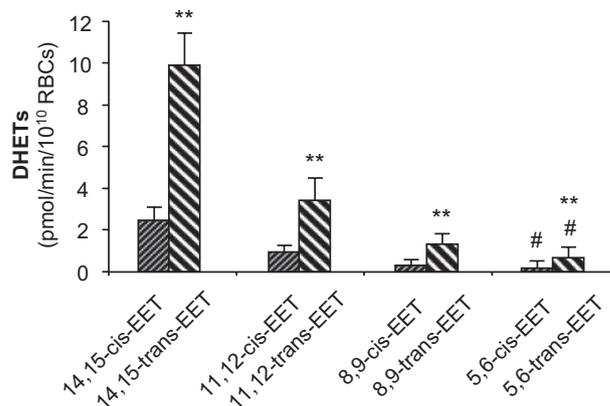


Fig. 3. Specific epoxide hydrolase activities of rat intact RBCs for *cis*- and *trans*-EETs calculated by the release of free DHETs from RBCs. EET hydrolysis was carried out with incubation of 10 nM *cis*- or *trans*-EETs, respectively, with 4×10^9 rat RBCs in 2 ml of PSS at 37°C for 2 min ($n = 5$). #, after correction for nonenzymatic conversion. **, $P < 0.01$ versus corresponding *cis*-isomers.

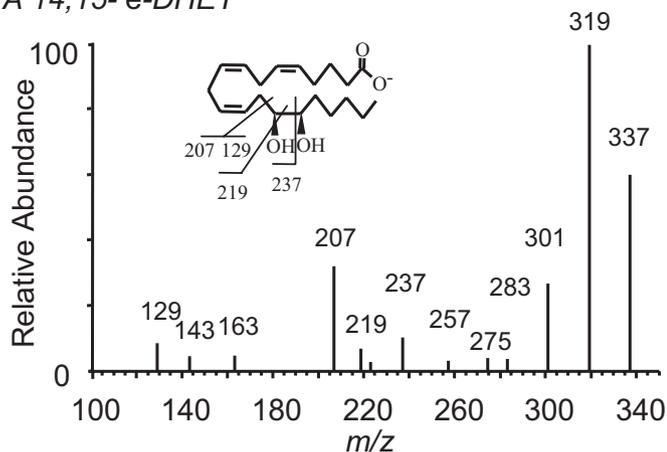
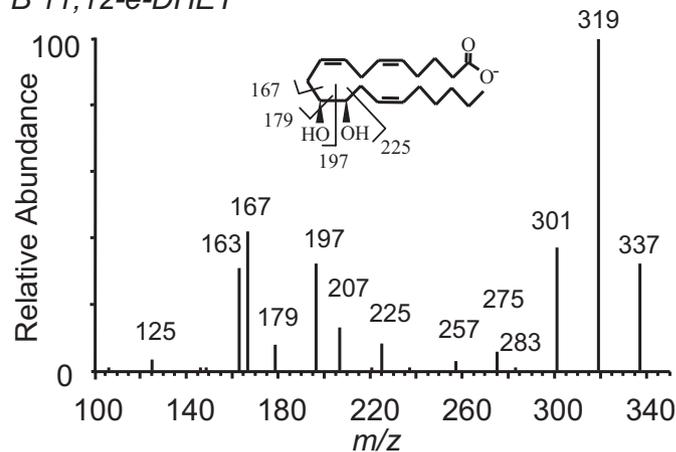
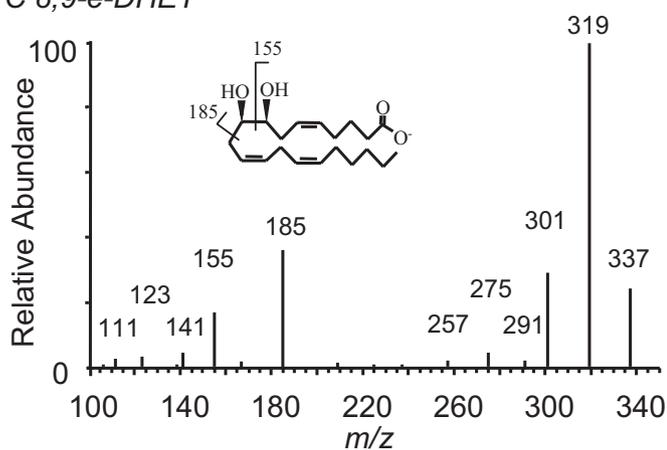
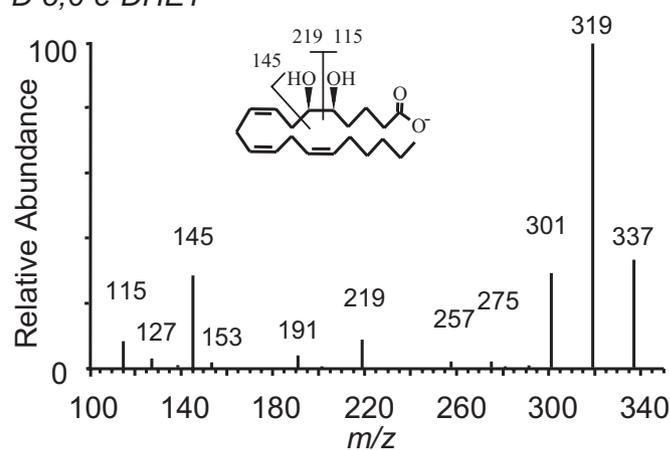
A 14,15-*e*-DHETB 11,12-*e*-DHETC 8,9-*e*-DHETD 5,6-*e*-DHET

Fig. 4. LC/MS/MS spectra of *erythro* (*e*)-DHETs. A, 14,15-*erythro*-DHET. B, 11,12-*erythro*-DHET. C, 8,9-*erythro*-DHET. D, 5,6-*erythro*-DHET. Peaks mostly resulted from $[M - H]^-$, breakdown around the hydroxyl group and neutral losses of H_2O (-18) or CO_2 (-44) molecules. The spectra of *threo*-DHETs are similar, with the exception of slight variations in relative intensities of breakdown ions.

sEH inhibitors. A cell-permeable LTA_4 hydrolase inhibitor, SC22716, did not inhibit EET hydrolysis by rat RBCs, and commercial LTA_4 hydrolase (25 $\mu\text{g}/\text{ml}$) did not hydrolyze *cis*- or *trans*-EETs when tested under similar conditions of incubation.

Selectivity of *cis*- and *trans*-EET Hydrolysis by Recombinant Murine sEH. When incubating recombinant murine sEH (0.5 $\mu\text{g}/\text{ml}$) with *cis*- or *trans*-EETs, preferred hydrolysis for *trans*- over *cis*-EETs and for 14,15-EETs over other regioisomers similar to the hydrolysis of RBCs (Fig. 3) was also observed. Comparative hydration rates are 121.4 ± 18.2 , 55.6 ± 9.6 , and 29.6 ± 4.7 nmol/min/mg protein for 14,15-, 11,12-, and 8,9-*cis*-EET, respectively, and 230.6 ± 35.1 , 174.3 ± 31.4 , and 85.8 ± 13.7 nmol/min/mg protein for 14,15-, 11,12-, and 8,9-*trans*-EET, respectively, for 1 μM EETs in PBS at 37°C as averaged from at least three incubations.

Active Formation and Hydrolysis of EETs by Rat RBCs. The presence of *erythro*-DHETs *in vivo* was expected as *trans*-EETs were identified in plasma and phospholipids (Jiang et al., 2005). LC/MS/MS spectra of *erythro*-DHETs produced with the hydrolysis of *trans*-EETs by RBCs (Fig. 4) provided the starting point for individual identification of *erythro*-DHETs. Incubation of AA (20 μM) with 4×10^9 RBCs in 2 ml of PSS produced both *threo*- and *erythro*-DHETs as

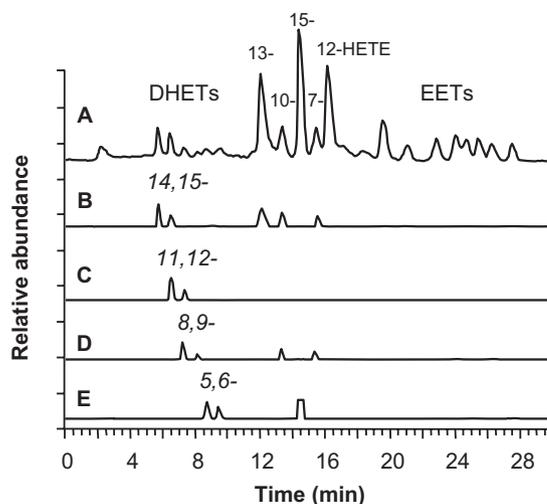


Fig. 5. Incubation of AA (20 μM) with 4×10^9 RBCs in 2 ml of PSS for 20 min resulted in the formation and release of *cis*- and *trans*-EETs as well as *threo*- and *erythro*-DHETs from rat RBCs. Identification of *threo*- and *erythro*-DHETs was shown by the LC/MS/MS breakdown of ion m/z 337, $[M - 1]^-$ for DHETs. A, m/z 337 + m/z 319; B, m/z 337 \rightarrow m/z 207; C, m/z 337 \rightarrow m/z 197; D, m/z 337 \rightarrow m/z 185; and E, m/z 337 \rightarrow m/z 145. DHET peaks from left to right in each duplex from B to E represent *erythro*- and *threo*-DHET, respectively, consistent with peaks of standards in Fig. 2. LC/MS/MS analyses were carried out as described under *Materials and Methods*, and identification of HETEs and EETs was as reported (Jiang et al., 2007).

well as hydroxyeicosatetraenoic acids (HETEs) and *cis/trans*-EETs (Fig. 5), suggesting that erythrocytes function as a potential source of AA-derived eicosanoids in plasma. Identification of individual *erythro*-DHETs by LC/MS/MS is shown in Fig. 5, whereas identification of HETEs and *cis/trans*-EETs was as reported previously (Jiang et al., 2007). Breakdown of *m/z* 337 to *m/z* 237 is ideal for the MS/MS identification of 14,15-DHETs (Fig. 4). The selection of *m/z* 207 for the identification of 14,15-DHETs (Fig. 5) is based on the observation that MS/MS product ions with weaker abundances, such as *m/z* 237 for 14,15-DHETs and *m/z* 207 for 11,12-DHETs, do not show up in the analysis of barely detectable amounts of DHETs. Using the same method of analysis, both free *erythro*- and *threo*-DHETs were identified in rat plasma with the total amount at 1.0 ± 0.2 ng/ml; *erythro*-DHETs accounted for approximately one fourth of the total free DHETs ($n = 3$).

EET Hydrolysis by Rat RBCs Compared with Other Blood Fractions. Comparison of EET hydrolysis by rat plasma, buffy layer, and RBCs revealed predominant EET hydrolysis by RBCs in blood. EETs were not hydrolyzed by plasma, particularly when there was no hemolysis. The buffy layer containing most of the leukocytes and platelets in blood



Fig. 6. Western blots of sEH in the cytosol of rat RBCs. Recombinant murine sEH (C, 500 ng) and 50 μ g of protein of the rat erythrocyte cytosolic samples were loaded for analysis. The last rat erythrocyte cytosol (sample 3) was freshly prepared and frozen, whereas samples 1 and 2 had been stored and frozen at -20°C for 5 months. Western blots were carried out as described under *Materials and Methods*.

hydrolyzed EETs, approximating $9.1 \pm 2.2\%$ ($n = 4$) of the

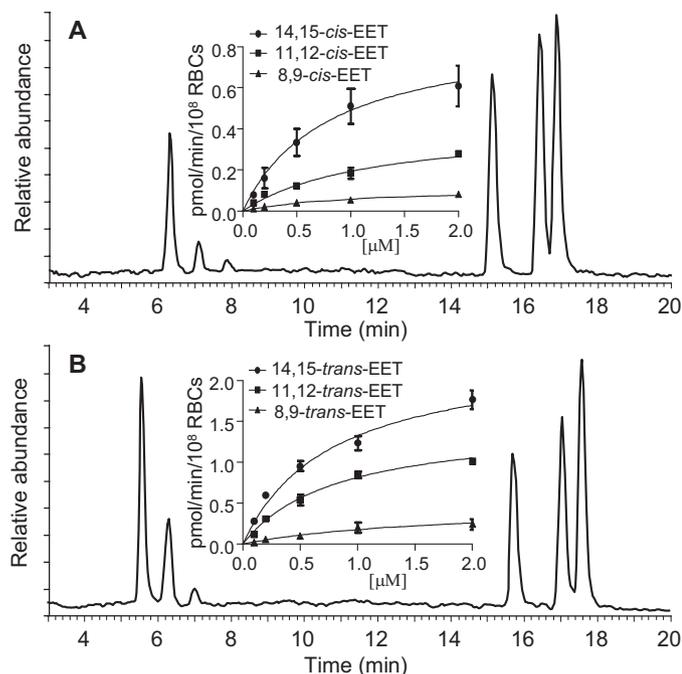


Fig. 7. Representative LC/MS chromatograms (*m/z* 337 + *m/z* 319) of the kinetic analysis of rat RBCs on the hydrolysis of 14,15-, 11,12-, and 8,9-*cis*-EETs (A) and 14,15-, 11,12-, and 8,9-*trans*-EETs (B). Insets in the middle are the Michaelis-Menten data. Studies were carried out as described under *Materials and Methods*.

hydrolysis by rat RBCs as calculated from the hydrolysis of 14,15-, 11,12-, and 8,9-*cis*- or *trans*-EETs ($1 \mu\text{M}$) for 5 min at 37°C .

Localization of Epoxide Hydrolases in Rat RBCs. Localizing the specific epoxide hydrolase for EET hydrolysis in rat RBCs disclosed its major existence in the cytosol of RBCs. The rat RBC membrane residue accounted for 11.9 ± 5.1 and $9.4 \pm 4.5\%$ ($n = 4$) of the total hydrolysis of *trans*- and *cis*-EETs by RBCs, respectively, as identified by the conversion of $1 \mu\text{M}$ 14,15-EETs to their respective DHETs. When 0.2% BSA was included in the buffer for the study, the specific activity of rat RBCs or cytosol to hydrolyze EETs at $1 \mu\text{M}$ fell to an approximately 10 to 20% level ($n = 4$), indicating considerable inhibition of EET hydrolysis by BSA binding of EETs.

Protein Analysis of Erythrocyte sEH. Total protein concentration of the prepared cytosol of rat RBCs corresponding to 2 or 4×10^9 RBCs/ml was 4.8 ± 0.6 mg/ 10^9 RBCs ($n = 6$), indicating less than 50% recovery of hemoglobin in the prepared cytosol after centrifugation and filtration. Whereas a dense band was detected for the positive control in Western blots of sEH (Fig. 6), weaker bands were observed for the samples, suggesting a concentration of sEH protein to be approximately 2 $\mu\text{g}/\text{mg}$ protein or 0.4 $\mu\text{g}/10^9$ RBCs based on densitometry comparisons. The last rat erythrocyte cytosol (sample 3) was freshly prepared and frozen, whereas samples 1 and 2 had been stored and frozen at -20°C for 5 months, which may explain the lighter sEH band than the one observed for sample 3.

Kinetics of Erythrocyte sEH on EET Hydrolysis. Kinetic studies of the sEH activities of rat RBCs were tested using 50 μl of RBCs (2×10^9 RBCs/ml) in PSS without BSA for 0.1 to 4 μM 14,15-, 11,12-, and 8,9-*cis*- or *trans*-EETs (Fig. 7). The specific EET hydrolysis at 4 μM were excluded in the kinetic analysis (Table 1) because of distortion of the kinetic parameters caused by apparent inhibition of the hydrolysis of

TABLE 1
Kinetics of EET (0.1–2 μM each in groups of *trans*- or *cis*-EETs) hydrolysis by rat intact RBCs ($n = 8$ –15)

EETs	V_{max}	K_m
14,15- <i>trans</i> -EET	2.35 ± 0.24	0.77 ± 0.18
11,12- <i>trans</i> -EET	1.48 ± 0.21	0.82 ± 0.24
8,9- <i>trans</i> -EET	0.68 ± 0.39	3.27 ± 2.58
14,15- <i>cis</i> -EET	0.86 ± 0.23	0.84 ± 0.50
11,12- <i>cis</i> -EET	0.26 ± 0.08	1.17 ± 0.54
8,9- <i>cis</i> -EET	0.17 ± 0.10	2.06 ± 1.82

TABLE 2
Kinetics of EET (0.2–4 μM individually) hydrolysis by rat erythrocyte cytosol ($n = 7$ –11)

EETs	V_{max}	K_m
14,15- <i>trans</i> -EET	3.59 ± 0.37	1.29 ± 0.35
11,12- <i>trans</i> -EET	2.72 ± 0.22	1.15 ± 0.25
8,9- <i>trans</i> -EET	1.24 ± 0.24	2.24 ± 0.89
14,15- <i>cis</i> -EET ^a	3.06 ± 0.90	0.91 ± 0.65
11,12- <i>cis</i> -EET	0.77 ± 0.10	0.69 ± 0.30
8,9- <i>cis</i> -EET	0.36 ± 0.06	1.55 ± 0.61

^a Values were obtained by analyzing substrate concentrations from 0.2 to 2 μM . Hydrolysis of 14,15-*cis*-EET at 4 μM by erythrocyte cytosol was greatly enhanced, not conforming to Michaelis-Menten kinetics possibly due to further hydrolysis by LTA₄ hydrolase (McGee and Fitzpatrick, 1985).

8,9-EETs by 14,15-EETs that have a greater affinity for the sEH in RBCs. The V_{\max} of rat RBCs for *trans*-EET hydrolysis was greater by 3-fold or more than the V_{\max} for *cis*-EETs.

Erythrocyte EETs were mostly esterified in RBC membranes; the presence of cytosolic EETs and DHETs was minimal. Kinetic analyses of individual EETs were carried out using 50 μ l of RBC cytosol corresponding to 1×10^8 RBCs to exclude factors affecting EET uptake and DHET release, as well as endogenous EETs. A more accurate V_{\max} and K_m for the *cis*-/*trans*-EET hydrolysis was obtained using erythrocyte cytosol (Table 2) instead of intact RBCs. The exception is 14,15-*cis*-EET that does not conform to the Michaelis-Menten kinetics when the substrate concentration is at 4 μ M, possibly revealing the participation of LTA₄ hydrolase in hydrolyzing 14,15-*cis*-EET (McGee and Fitzpatrick, 1985).

IC₅₀ of sEH Inhibitors. IC₅₀ of *cis*-AUCB, *trans*-AUCB, and DCU on *trans*-EET hydrolysis by rat erythrocyte cytosol was determined as 12.5 ± 1.5 , 5.1 ± 1.3 , and 156.5 ± 1.3 nM, respectively (Fig. 8). Substrate selection and experimental conditions may have contributed to the different relative potencies of *cis*- and *trans*-AUCB compared with the IC₅₀ reported for *cis*-AUCB (0.89 nM) and *trans*-AUCB (1.3 nM) (Hwang et al., 2007). The IC₅₀ of DCU is comparable with the reported IC₅₀ of 160 nM on recombinant human sEH and 90 nM on recombinant murine sEH (McElroy et al., 2003). However, nanosuspension of DCU greatly enhanced the potency of DCU (Ghosh et al., 2008).

Discussion

The preferred hydrolysis of 14,15-EETs and *trans*-epoxides characterizes sEH activities in rat RBCs. Incubation of rat RBCs with 10 nM EETs in PSS demonstrated that hydration of *trans*-EETs by RBCs was at rates that differ by more than three folds from hydration rates of the corresponding *cis*-EETs (Fig. 3). The rates of EET hydrolysis by rat RBCs decreased at approximately 2 to 3-fold sequentially from 14,15-, 11,12-, and 8,9- to 5,6-EETs for both the *cis*- and *trans*-isomers. The results are in accord with the report that 14,15-*cis*-EET was found to be hydrolyzed faster than other *cis*-regioisomers by sEH (Zeldin et al., 1995). 5,6-EETs are hydrolyzed the least by rat RBCs, consistent with the inability of the 5,6-EET to serve as a suitable substrate for liver sEH (Chacos et al., 1983).

The 3 to 5-fold greater specific hydrolysis of *trans*- relative

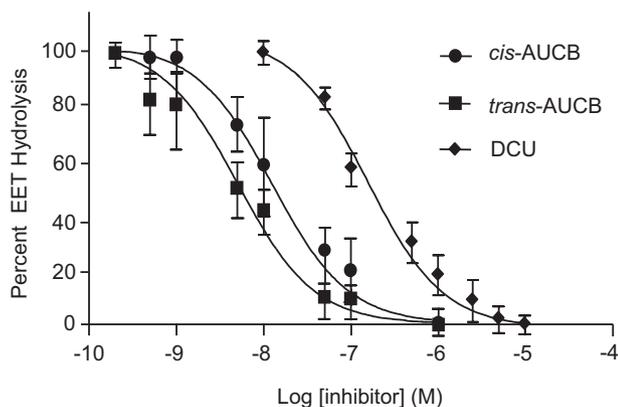


Fig. 8. Concentration-response curves of sEH inhibitors, *cis*-AUCB and *trans*-AUCB as well as DCU on the hydrolysis of *trans*-EETs (1 μ M). Each point is the mean \pm S.E.M. of $n = 3$ to 4.

to *cis*-EET by rat RBCs at 10 nM (Fig. 2 and 3) presumably is a combined result of sEH, membrane epoxide hydrolase, membrane transport, inhibition by albumin, as well as contributions of endogenous EETs. Nevertheless, the result may reflect de facto EET hydrolysis by RBCs in vivo. The RBC LTA₄ hydrolase that has a K_m of 20 μ M for 14,15-*cis*-EET (McGee and Fitzpatrick, 1985) will probably not play a significant role in hydrolyzing EETs at low nanomolar concentrations. Hydrolysis of EETs by RBCs was immediate (Fig. 2), indicating rapid uptake of EETs and rapid release of DHETs by RBCs. The uptake of *trans*-EETs by RBCs may be faster than that of *cis*-EETs, considering that *trans*-EETs are more hydrophobic than *cis*-EETs as was also evidenced by the later elution of *trans*-EETs than *cis*-EETs, respectively, in reversed-phase HPLC separations (Fig. 2). We have reported that the cystic fibrosis transmembrane conductance regulator and pannexin-1 are involved in the secretion of EETs from rat RBCs (Jiang et al., 2007). Fatty acid-binding proteins are known to inhibit hydrolysis of EETs by sEH (Widstrom et al., 2003), as was manifested by inclusion of 0.2% fatty acid-free BSA in the incubations that resulted in over 80% inhibition of the rate of EET hydrolysis.

The V_{\max} for *trans*-EET hydrolysis is approximately 3-fold or greater than that of *cis*-EETs, respectively, when testing incubations of 0.1 to 2 μ M of the three *cis*- or *trans*-EETs together with rat intact RBCs (Table 1). The diminution of the hydrolysis of 8,9-EETs when incubating the three *cis*- or *trans*-EETs together at 4 μ M, respectively, suggested substrate saturation and greater affinity of 14,15- and 11,12-EETs than 8,9-EETs for the sEH, as was also confirmed by individual EET kinetics obtained with the erythrocyte cytosol (Table 2). Nonconformance to the Michaelis-Menten kinetics for the hydrolysis of 14,15-*cis*-EET at concentrations of 4 μ M is probably caused by involvement of LTA₄ hydrolase in the erythrocyte cytosol (McGee and Fitzpatrick, 1985).

The highly similar regio and geometric selectivity of EET hydrolysis by recombinant murine sEH and rat erythrocyte cytosol suggests either identity or close similarity of erythrocyte and hepatic sEH. Western blots of sEH suggested that a concentration of sEH protein is approximately 2 μ g/mg protein or 0.4 μ g/ 10^9 RBCs (Fig. 6). The apparent K_m of 1 to 2 μ M EETs for rat RBCs is comparable with the K_m of 3 to 5 μ M EETs for the purified mouse liver sEH (Zeldin et al., 1995). The apparent K_m and V_{\max} of rat erythrocyte for the hydrolysis of *cis*-/*trans*-EETs approximate those of the sEH in human leukocytes for the hydrolysis of *cis*-stilbene oxide (Seidegård et al., 1984). Purification of enzymes involves procedures that can increase or decrease specific activities of an enzyme. Thus, it may not be appropriate to compare the V_{\max} of erythrocyte cytosol with V_{\max} of purified sEH.

The divergent abilities of LTA₄ hydrolase to hydrolyze LTA₄ and *cis*-EETs seem to preclude erythrocyte LTA₄ hydrolase as an effective sEH that hydrolyzes *cis*-EETs (McGee and Fitzpatrick, 1985). This is consistent with the inability of the LTA₄ hydrolase inhibitor, SC22716, to affect EET hydrolysis as well as the inability of commercial LTA₄ hydrolase to hydrolyze EETs as tested in the present study. The LTA₄ sEH purified from human erythrocytes hydrolyzes 14,15-*cis*-EET with a K_m of 20 μ M (McGee and Fitzpatrick, 1985), which is 20-fold greater than the K_m of sEH for 14,15-*cis*-EET in our study. Despite LTA₄ and *trans*-EETs sharing the *trans*-epoxide configuration, sEH demonstrated extraordi-

nary specificity for substrate selection. The presence of sEH in RBCs is further supported by inhibition of EET hydrolysis by sEH inhibitors, *cis*- and *trans*-AUCB (Fig. 8).

This study mainly addressed the comparison of added *cis*- and *trans*-EET hydration by rat erythrocytes because comparable amounts of *cis*- and *trans*-EETs are present in plasma and erythrocyte phospholipids (Jiang et al., 2005). The conversion of AA to EETs and HETEs by rat RBCs (Fig. 5) is a function of hemoglobin-activating oxygen in a monooxygenase-like fashion (Starke et al., 1984). Peroxy radicals of AA may mediate the formation of more *trans*- than *cis*-EETs in RBCs (Jiang et al., 2004). EETs in vivo demonstrate chiral prevalences (Wei et al., 2006), and regio- and enantioselectivity have been identified for the enzymatic hydration of *cis*-EET enantiomers (Zeldin et al., 1993, 1995). Analysis of the chirality of *trans*-EET isomers and their enantioselectivity for hydration has yet to be carried out.

It is a challenge to estimate the relative contributions to the hydrolysis of EETs by RBCs and by specific organs. However, the role of erythrocyte sEH in the regulation of circulating EETs may be particularly significant when considering potential effects of EETs on the rheological and hemodynamic determinants of the circulation, such as in cardiovascular and hematological diseases, as well as physiological implications for regulating blood flow in the microcirculation. Release of EETs from RBCs into the circulation in response to ATP stimulation of the erythrocyte P2X₇ receptor (Jiang et al., 2007) will contribute to activation of endothelial peroxisome proliferator-activated receptor γ transcription by EETs (Liu et al., 2005), which is magnified through elevating EETs at the blood-endothelial interface in response to inhibition of sEH in RBCs. Activation of peroxisome proliferator-activated receptor γ inhibits nuclear factor κ B-mediated expression of adhesion molecules and endothelin that promotes vascular wall damage and atherogenesis (Liu et al., 2005). Furthermore, localization of sEH in the RBC and the increase of EETs resulting from its inhibition presumably contributes to elevating the positive effects of EETs on regional blood flows to a greater degree than sEH localized "in the smooth muscle layers of the arterial wall" (Yu et al., 2004).

This study revealed preferential hydrolysis of *trans*- over *cis*-EETs and the presence of sEH in rat RBCs. Angiotensin II up-regulates sEH expression in the vascular endothelium (Ai et al., 2007); sEH inhibition greatly lowered systolic blood pressure in angiotensin II-induced hypertensive rats (Imig et al., 2002; Ghosh et al., 2008). Consistent with the positive effects of EETs on the circulation, sEH has been proposed as a novel therapeutic target for control of blood pressure (Sinal et al., 2000), prevention of renal damage (Imig, 2005) and stroke (Zhang et al., 2007), as well as amelioration of inflammation (Schmelzer et al., 2005). Because plasma *trans*-EET levels would increase more than *cis*-EETs with sEH inhibition, the potential functional roles of *trans*-EETs and erythrocyte sEH in terms of contributing to the regulation of the circulation deserve attention.

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References

- Ai D, Fu Y, Guo D, Tanaka H, Wang N, Tang C, Hammock BD, Shyy JY, and Zhu Y (2007) Angiotensin II up-regulates soluble epoxide hydrolase in vascular endothelium in vitro and in vivo. *Proc Natl Acad Sci U S A* **104**:9018–9023.
- Capdevila JH, Dishman E, Karara A, and Falck JR (1991) Cytochrome P450 arachidonic acid epoxygenase: stereochemical characterization of epoxyeicosatrienoic acids. *Methods Enzymol* **206**:441–453.
- Chacos N, Capdevila J, Falck JR, Manna S, Martin-Wixtrom C, Gill SS, Hammock BD, and Estabrook RW (1983) The reaction of arachidonic acid epoxides (epoxyeicosatrienoic acids) with a cytosolic epoxide hydrolase. *Arch Biochem Biophys* **223**:639–648.
- Davis BB, Thompson DA, Howard LL, Morisseau C, Hammock BD, and Weiss RH (2002) Inhibitors of soluble epoxide hydrolase attenuate vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A* **99**:2222–2227.
- Falck JR, Krishna UM, Reddy YK, Kumar PS, Reddy KM, Hittner SB, Deeter C, Sharma KK, Gauthier KM, and Campbell WB (2003) Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation. *Am J Physiol Heart Circ Physiol* **284**:H337–H349.
- Fleming I (2007) Epoxyeicosatrienoic acids, cell signaling and angiogenesis. *Prostaglandins Other Lipid Mediat* **82**:60–67.
- Ghosh S, Chiang PC, Wahlstrom JL, Fujiwara H, Selbo JG, and Roberds SL (2008) Oral delivery of 1,3-dicyclohexylurea nanosuspension enhances exposure and lowers blood pressure in hypertensive rats. *Basic Clin Pharmacol Toxicol* **102**:453–458.
- Grant DF, Storms DH, and Hammock BD (1993) Molecular cloning and expression of murine liver soluble epoxide hydrolase. *J Biol Chem* **268**:17628–17633.
- Hwang SH, Tsai HJ, Liu JY, Morisseau C, and Hammock BD (2007) Orally bioavailable potent soluble epoxide hydrolase inhibitors. *J Med Chem* **50**:3825–3840.
- Imig JD (2005) Epoxide hydrolase and epoxygenase metabolites as therapeutic targets for renal diseases. *Am J Physiol Renal Physiol* **289**:F496–F503.
- Imig JD, Zhao X, Capdevila JH, Morisseau C, and Hammock BD (2002) Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension* **39**:690–694.
- Inceoglu B, Schmelzer KR, Morisseau C, Jinks SL, and Hammock BD (2007) Soluble epoxide hydrolase inhibition reveals novel biological functions of epoxyeicosatrienoic acids (EETs). *Prostaglandins Other Lipid Mediat* **82**:42–49.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals* 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.
- Jiang H, McGiff JC, Quilley J, Sacerdoti D, Reddy LM, Falck JR, Zhang F, Lerea KM, and Wong PY (2004) Identification of 5,6-*trans*-epoxyeicosatrienoic acid in the phospholipids of red blood cells. *J Biol Chem* **279**:36412–36418.
- Jiang H, Quilley J, Reddy LM, Falck JR, Wong PY, and McGiff JC (2005) Red blood cells: reservoirs of *cis*- and *trans*-epoxyeicosatrienoic acids. *Prostaglandins Other Lipid Mediat* **75**:65–78.
- Jiang H, Zhu AG, Mameczur M, Falck JR, Lerea KM, and McGiff JC (2007) Stimulation of rat erythrocyte P2X₇ receptor induces the release of epoxyeicosatrienoic acids. *Br J Pharmacol* **151**:1033–1040.
- Karara A, Wei S, Spady D, Swift L, Capdevila JH, and Falck JR (1992) Arachidonic acid epoxygenase: structural characterization and quantification of epoxyeicosatrienoates in plasma. *Biochem Biophys Res Commun* **182**:1320–1325.
- Larsen BT, Guterman DD, and Hatoum OA (2006) Emerging role of epoxyeicosatrienoic acids in coronary vascular function. *Eur J Clin Invest* **36**:293–300.
- Liu Y, Zhang Y, Schmelzer K, Lee TS, Fang X, Zhu Y, Spector AA, Gill S, Morisseau C, Hammock BD, et al. (2005) The antiinflammatory effect of laminar flow: the role of PPARgamma, epoxyeicosatrienoic acids, and soluble epoxide hydrolase. *Proc Natl Acad Sci U S A* **102**:16747–16752.
- Loch D, Hoey A, Morisseau C, Hammock BO, and Brown L (2007) Prevention of hypertension in DOCA-salt rats by an inhibitor of soluble epoxide hydrolase. *Cell Biochem Biophys* **47**:87–98.
- McElroy NR, Jurs PC, Morisseau C, and Hammock BD (2003) QSAR and classification of murine and human soluble epoxide hydrolase inhibition by urea-like compounds. *J Med Chem* **46**:1066–1080.
- McGee J and Fitzpatrick F (1985) Enzymatic hydration of leukotriene A₄. Purification and characterization of a novel epoxide hydrolase from human erythrocytes. *J Biol Chem* **260**:12832–12837.
- Morisseau C and Hammock BD (2005) Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol* **45**:311–333.
- Morisseau C and Hammock BD (2007) Measurements of soluble epoxide hydrolase (sEH) activity, in *Techniques for Analysis of Chemical Biotransformation. Current Protocols in Toxicology* (Bus JS, Costa LG, Hodgson E, Lawrence DA, and Reed DJ eds) pp 4.23.1–4.23.18, John Wiley & Sons, New Jersey.
- Nakamura T, Bratton DL, and Murphy RC (1997) Analysis of epoxyeicosatrienoic and monohydroxyeicosatetraenoic acids esterified to phospholipids in human red blood cells by electrospray tandem mass spectrometry. *J Mass Spectrom* **32**:888–896.
- Ota K and Hammock BD (1980) Cytosolic and microsomal epoxide hydrolases: differential properties in mammalian liver. *Science* **207**:1479–1481.
- Rodriguez M and Clare-Salzler M (2006) Eicosanoid imbalance in the NOD mouse is related to a dysregulation in soluble epoxide hydrolase and 15-PGDH expression. *Ann N Y Acad Sci* **1079**:130–134.
- Schmelzer KR, Kubala L, Newman JW, Kim IH, Eiserich JP, and Hammock BD (2005) Soluble epoxide hydrolase is a therapeutic target for acute inflammation. *Proc Natl Acad Sci U S A* **102**:9772–9777.
- Seidgard J, DePierre JW, and Pero RW (1984) Measurement and characterization of membrane-bound and soluble epoxide hydrolase activities in resting mononuclear leukocytes from human blood. *Cancer Res* **44**:3654–3660.
- Seubert JM, Sinal CJ, Graves J, Degraff LM, Bradbury JA, Lee CR, Goralski K, Carey MA, Luria A, Newman JW, et al. (2006) Role of soluble epoxide hydrolase in posts ischemic recovery of heart contractile function. *Circ Res* **99**:442–450.

- Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, and Gonzalez FJ (2000) Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. *J Biol Chem* **275**:40504–40510.
- Smith KR, Pinkerton KE, Watanabe T, Pedersen TL, Ma SJ, and Hammock BD (2005) Attenuation of tobacco smoke-induced lung inflammation by treatment with a soluble epoxide hydrolase inhibitor. *Proc Natl Acad Sci U S A* **102**:2186–2191.
- Starke DW, Blisard KS, and Miesel JJ (1984) Substrate specificity of the monooxygenase activity of hemoglobin. *Mol Pharmacol* **25**:467–475.
- Thomas H, Schladt L, Doehmer J, Knehr M, and Oesch F (1990) Rat and human liver cytosolic epoxide hydrolases: evidence for multiple forms at level of protein and mRNA. *Environ Health Perspect* **88**:49–55.
- Wei S, Brittin JJ, Falck JR, Anjaiah S, Nithipatikom K, Cui L, Campbell WB, and Capdevila JH (2006) Chiral resolution of the epoxyeicosatrienoic acids, arachidonic acid epoxide metabolites. *Anal Biochem* **352**:129–134.
- Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, and Spector AA (1999) Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. *Am J Physiol Heart Circ Physiol* **277**:H2098–H2108.
- Widstrom RL, Norris AW, Van Der Veer J, and Spector AA (2003) Fatty acid-binding proteins inhibit hydration of epoxyeicosatrienoic acids by soluble epoxide hydrolase. *Biochemistry* **42**:11762–11767.
- Yu Z, Davis BB, Morisseau C, Hammock BD, Olson JL, Kroetz DL, and Weiss RH (2004) Vascular localization of soluble epoxide hydrolase in the human kidney. *Am J Physiol Renal Physiol* **286**:F720–F726.
- Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, and Capdevila JH (1993) Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J Biol Chem* **268**:6402–6407.
- Zeldin DC, Wei S, Falck JR, Hammock BD, Snapper JR, and Capdevila JH (1995) Metabolism of epoxyeicosatrienoic acids by cytosolic epoxide hydrolase: substrate structural determinants of asymmetric catalysis. *Arch Biochem Biophys* **316**:443–451.
- Zhang W, Koerner IP, Noppens R, Grafe M, Tsai HJ, Morisseau C, Luria A, Hammock BD, Falck JR, and Alkayed NJ (2007) Soluble epoxide hydrolase: a novel therapeutic target in stroke. *J Cereb Blood Flow Metab* **27**:1931–1940.

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