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Identification of 5,6-*trans*-Epoxyeicosatrienoic Acid in the Phospholipids of Red Blood Cells*

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A novel eicosanoid, 5,6-*trans*-epoxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid (5,6-*trans*-EET), was identified in rat red blood cells. Characterization of 5,6-*trans*-EET in the *sn*-2 position of the phospholipids was accomplished by hydrolysis with phospholipase A_2 followed by gas chromatography/mass spectrometry as well as electrospray ionization-tandem mass spectrometry analyses. The electron ionization spectrum of 5,6-*erythro*-dihydroxyeicosatrienoic acid (5,6-*erythro*-DHET), converted from 5,6-*trans*-EET in the samples, matches that of the authentic standard. Hydrogenation of the extracted 5,6-*erythro*-DHET with platinum(IV) oxide/hydrogen resulted in an increase of the molecular mass by 6 daltons and the same retention time shift as an authentic standard in gas chromatography, suggesting the existence of three olefins as well as the 5,6-*erythro*-dihydroxyl structure in the metabolite. Match of retention times by chromatography indicated identity of the stereochemistry of the red blood cell 5,6-*erythro*-DHET *vis à vis* the synthetic standard. High pressure liquid chromatography-electrospray ionization-tandem mass spectrometry analysis of the phospholipase A_2-hydrolyzed lipid extracts from red blood cells revealed match of the mass spectrum and retention time of the compound with the authentic 5,6-*trans*-EET standard, providing direct evidence of the existence of 5,6-*trans*-EET in red blood cells. The presence of other *trans*-EETs was also demonstrated. The ability of both 5,6-*trans*-EET and its product 5,6-*erythro*-DHET to relax preconstricted renal interlobar arteries was significantly greater than that of 5,6-*cis*-EET. In contrast, 5,6-*cis*-EET and 5,6-*trans*-EET were equipotent in their capacity to inhibit collagen-induced rat platelet aggregation, whereas 5,6-*erythro*-DHET was without effect. We propose that the red blood cells serve as a reservoir for epoxides which on release may act in a vasoregulatory capacity.

Arachidonic acid can be metabolized to hydroxyeicosatetraenoic acids, epoxyeicosatrienoic acids (EETs),[1] prostaglandins, leukotrienes, and other biologically active mediators (1). The EETs are cytochrome P450 epoxygenase products that affect blood flow, mitogenesis, platelet aggregation, and Ca^{2+} signaling; EETs are also anti-inflammatory and regulate tyrosine kinase activity, cell migration, apoptosis, fibrinolysis, and steroidogenesis (2–8). Thus far, all EETs produced by cytochrome P450 epoxygenases are of the *cis* configuration (9–11); a *trans*-EET *in vivo* has not been described (Fig. 1).

The total EETs in plasma were reported to be 10.2 ± 0.4 ng/ml; greater than 90% of the plasma EETs was esterified to the phospholipids of circulating lipoproteins (12). As EETs are incorporated into cellular phospholipids (13) and bind to fatty acid-binding proteins (14), EETs may have long lasting effects subject to release from phospholipids in response to hormonal activation (15). EET phospholipids have been identified in human red blood cells by Nakamura *et al*. (16) who proposed that erythrocytes serve "as a reservoir from which EETs can be released." As EETs are vasoactive, they have been examined in terms of their effects on blood vessels and have been found to be essential components of key vasoregulatory mechanisms (17, 18).

The analysis of 5,6-EET poses problems because of its labile properties, *viz*. 5,6-EET is rapidly converted to a δ-lactone and/or dihydroxyeicosatrienoic acid (DHET) in buffers and during sample preparation (19). In this study, we have identified a 5,6-*trans*-EET in rat red blood cells with GC/MS and HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analyses. 5,6-*trans*-EET dilated renal interlobar arteries and inhibited platelet aggregation, suggesting a role for this eicosanoid in circulatory regulation.

EXPERIMENTAL PROCEDURES

Materials—Phospholipase A_2 (from *Naja mossambica mossambica*), diisopropylethylamine (DIPEA), pentafluorobenzyl (PFB) bromide, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), pyridine, and platinum(IV) oxide were obtained from Sigma. Acetonitrile, methanol, and chloroform (all HPLC grade) were purchased from Fisher. Eicosanoid standards were obtained from Cayman, and EET-d_8 standards were from Biomol. 14-Week-old male Sprague-Dawley rats were purchased from Charles River Laboratories, Wilmington, MA. Rats were maintained at 22 °C with alternating cycles of light and darkness and fed *ad libitum* with standard rat chow and water.

RBC Preparation—Sprague-Dawley rats were anesthetized with pentobarbital, 65 mg/kg intraperitoneal (20). Blood was collected from rats via cardiac puncture using EDTA-rinsed syringes and transferred

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[1] The abbreviations used are: EET, epoxyeicosatrienoic acid; RBC, red blood cell; HPLC, high pressure liquid chromatography; PFB, pentafluorobenzyl; TMS, trimethylsilyl; NICI, negative ion chemical ionization; EI, electron ionization; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GC/MS, gas chromatography/mass spectrometry; DHET, dihydroxyeicosatrienoic acid; DIPEA, diisopropylethylamine; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TPP, triphenylphosphine; BHT, butylated hydroxytoluene.

to Vacuette EDTA tubes (Fisher). Butylated hydroxytoluene (BHT) powder, 1.2 mg (final concentration 0.2 mM), was added to the 3-ml EDTA tube before blood collection, and the EDTA tubes with collected blood were gently inverted 4–6 times to mix the blood with EDTA and BHT. RBC (3 ml of blood) samples were stored in ice and separated within 20 min by centrifugation at 800 × *g* at 4 °C for 10 min. The supernatant and the buffy layer were removed by aspiration. Packed RBCs were washed three times and resuspended in a physiological salt solution (in mM: 4.0 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 15.7 Hepes, 11.1 dextrose, and 5 mg/ml bovine serum albumin, pH adjusted to 7.4) (21, 22) to the original volume. The purity of erythrocytes prepared this way was analyzed under the microscope and found to be 99% or greater.

Phospholipid Extraction and Hydrolysis—Prepared RBC samples (0.1 ml) were spun down at 2,000 × *g*, and 2 ml of $CHCl_3/CH_3OH$ (2:1) containing 0.1 mM triphenylphosphine (TPP) were added to the RBC pellets, which were then homogenized with frequent vortex mixing for 1 h. The sample was centrifuged at 2,000 × *g* to remove the cell residues, and the supernatant was transferred to another tube with a Pasteur pipette. The extraction was repeated once. The supernatants were combined, dried under nitrogen, and subjected to hydrolysis using 50 units of phospholipase A_2 in 0.5 ml of Tris buffer, pH 8.9, at room temperature for 2 h. The mixture was adjusted to pH 4 with 10% acetic acid, and eicosanoids were extracted twice with 1 ml of ethyl acetate. The use of BHT or TPP is to quench free radicals and to prevent peroxyl radical-propagated transformations of polyunsaturated structures (23, 24).

The method of Rose and Oklander (25) for phospholipid extraction from RBC pellets was also used according to Murphy and co-workers (16). The phospholipid extracted in a final supernatant of chloroform/isopropyl alcohol/water (7:11:1) was dried under nitrogen and followed by phospholipase A_2 hydrolysis and ethyl acetate extraction as described above. No difference was observed in terms of identification whether using this method or direct extraction with chloroform/methanol (2:1) containing 0.1 mM TPP from the RBC pellets. The ethyl acetate extract was dried under nitrogen and dissolved in acetonitrile (20 μl) for LC/MS/MS analysis.

DIPEA Treatment—Mild alkali catalyzes the unique 5,6-EET conversion to DHET through an intermediary δ-lactone (19). For HPLC-GC/MS analysis, the dried ethyl acetate extract was added to a mixture of methanol/water/DIPEA (200:100:15 μl) and heated at 60 °C for 1 h to convert 5,6-EETs to 5,6-DHETs without affecting the other EETs. DIPEA treatment was also used to prepare the 5,6-*threo*-DHET-d_8 from 5,6-*cis*-EET-d_8 methyl ester, which was hydrolyzed according to demethylation protocol from Biomol. This is a moderate treatment compared with the sodium hydroxide hydrolysis of phospholipids that takes 1 h at 60 °C.

Hydrogenation—Extracted or standard samples in 0.5 ml of methanol were hydrogenated by bubbling hydrogen for 6 min in the presence of platinum(IV) oxide as a catalyst.

HPLC Separations—HPLC was carried out by using a Shimadzu LC-10AT liquid chromatograph with automatic sample injection and programmed fraction collection. A Beckman-Coulter Ultrasphere ODS column (25 cm × 4.6 mm × 5 μm) was used. UV absorbance from 200 to 400 nm was monitored. The four DHETs were completely separated with an isocratic gradient of acetonitrile/water/methanol/acetic acid (60:30:10:0.05) over 20 min, after which an 8-min elution with acetonitrile/methanol/acetic acid (90:10:0.05) was used to wash out all fatty acids in the samples from the column. The HPLC eluent from 8.8 to 10.0 min corresponding 5,6-DHET fraction was collected for further analysis. For separation of 5,6-DHET fraction to *threo*- and *erythro*-isomers, the solution of acetonitrile/water/methanol/acetic acid (40:50:10:0.05) was changed over 20 min to acetonitrile/water/methanol/acetic acid (60:30:10:0.05), which was continued for another 10 min.

Synthesis of 5,6-trans-EET—Diethyl azodicarboxylate (184 μl, 1.16 mmol) was added to a solution of TPP (303 mg, 1.15 mmol), 4-nitrobenzoic acid (172 mg, 1.03 mmol), methyl *threo*-5-bromo-6-hydroxyeicosa-8*Z*,11*Z*,14*Z*-trienoate and its 5-hydroxy-6-bromo regioisomer (200 mg, 0.48 mmol) in dry toluene (4.0 ml) under an argon atmosphere at −20 °C (26). After stirring for 4 h at 23 °C, the sample was dried *in vacuo*, and the residue was purified by SiO_2 column chromatography using ethyl acetate/hexane as eluent to give a mixture of inverted benzoate and its 5-(4-nitrobenzoyloxy)-6-bromo regioisomer (220 mg, 81% combined yield). For TLC: ethyl acetate/*n*-hexane (1:4), R_f = 0.31; ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.88 (t, *J* = 7.1 Hz, 3H), 1.22–1.38 (m, 7H), 1.65–2.10 (m, 5H), 2.36–2.39 (m, 2H), 2.56–2.89 (m, 7H), 3.65 (s, 1.2H), 3.66 (s, 1.8H), 4.22–4.28 (m, 1H), 5.25–5.43 (m, 5H), 5.45–5.56 (m, 1H), 8.20–8.24 (m, 2H), and 8.28–8.38 (m, 2H).

Sodium methoxide (191 mg, 3.54 mmol) was added to the above mixture and its regioisomer (200 mg, 0.35 mmol) in dry methanol (5 ml) at 23 °C under an argon atmosphere. After 30 min, the reaction mixture was diluted with ether (50 ml), and the organic layer was washed with water (three times, 8 ml) and saline (two times, 8 ml) and dried over Na_2SO_4. Removal of all volatiles *in vacuo* and purification of the residue by SiO_2 preparative thin layer chromatography using $CHCl_3/C_6H_6$ (1:9) furnished 5,6-*trans-epoxy*-8*Z*,11*Z*,14*Z*-eicosatrienoic acid (5,6-*trans*-EET) methyl ester (100 mg, 85%). For TLC: $CHCl_3/C_6H_6$ (1:9), R_f = 0.20; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, *J* = 7.0 Hz, 3H), 1.25–1.39 (m, 6H), 1.49–1.65 (m, 3H), 1.70–1.86 (m, 2H), 2.24–2.31 (m, 1H), 2.35–2.44 (m, 3H), 2.70–2.74 (m, 2H), 2.78 (q, *J* = 7.0 Hz, 4H), 3.67 (s, 3H), 5.29–5.45 (m, 5H), and 5.48–5.55 (m, 1H).

NaOH (1 M aqueous solution, 120 μl, 0.12 mmol) was added to a solution of 5,6-*trans*-EET methyl ester (10 mg, 0.03 mmol) in THF/H_2O (4:1, 2.5 ml). After 12 h, all of the THF was removed *in vacuo*, and a slurry of SM-2 Bio-Beads (500 mg, 20–50 mesh, Bio-Rad) in water (2 ml) was added to the remaining aqueous solution. After stirring for 30 min, the beads were collected by filtration and rinsed gently with water (100 ml), and then the eicosanoid adhering to the beads was stripped off by using ethanol (25 ml). Concentration of the ethanol washes *in vacuo* yielded the sodium salt of 5,6-*trans*-EET (10 mg, 98%).

GC/MS Analyses—For quantitation and scan using negative ion chemical ionization (NICI) GC/MS, DHETs and the hydrogenated products were derivatized to form trimethylsilyl (TMS) ether PFB ester (27). The GC column (DB-1ms, 10 m length, 0.25 mm inner diameter, 0.25-μm film thickness, Agilent Technologies Inc.) was temperature programmed from 180 to 300 °C at a rate of 25 °C/min for DHET detection. Methane was used as the reagent gas. The ions *m/z* of 481 and 489 were monitored for endogenous and deuterium-labeled DHETs. 5,6-EETs were converted to DHETs with DIPEA treatment and a standard curve (r^2 >0.99) was constructed by using 5,6-*erythro*-DHET-d_8 as internal standard for the quantitation.

For electron ionization (EI) GC/MS analysis, the 5,6-DHETs were derivatized as TMS ether methyl esters with diazomethane (400 μl) at room temperature for 10 min and then dried and treated with 60 μl of BSTFA and 20 μl of pyridine at 60 °C for 5 min. The derivative was dried, dissolved in isooctane, and subjected to GC/MS analysis by using the same column as mentioned above. The column temperature was programmed from 180 to 250 °C at a rate of 15 °C/min. The scan range was from *m/z* 100 to *m/z* 550.

HPLC-ESI-MS/MS Experiment—Direct identification of EETs was done using a Finnigan LCQ Advantage quadrupole ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an electrospray ionization (ESI) source run by Xcalibur software. Instrument lens parameters were tuned with syringe infusion at 5 μl/min of 14,15-EET (0.1 ng/μl) dissolved in HPLC eluent in the negative ionization mode. HPLC was run with a Luna C18 (2) 250 × 2.0-mm column (Phenomenex, Torrance, CA) with an isocratic gradient of acetonitrile/water/methanol/acetic acid (60:30:10:0.05) at a flow of 0.30 ml/min. ESI was carried out at an ion transfer tube temperature of 280 °C, a spray voltage of 4.5 kV, a sheath gas flow of 34 units, and an auxiliary gas flow of 5 units (units refer to arbitrary values set by the LCQ software). In the MS/MS experiments, the deprotonated precursor molecular ions $[M - H]^-$ of EETs (*m/z* = 319) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 34%. The mass spectra resulting from these fragmentations were acquired with selected reaction monitoring at *m/z* = 319 for EETs. For quantitative assay of EETs, 2 ng of d_8-11,12-EET was added to 100 μl (8×10^8 RBCs) of RBC preparation as internal standard (*m/z* = 327). A calibration curve for EETs in the range of 0.5 to 8 ng was obtained that showed good correlation (*r* = 0.99).

LC/MS/MS analyses of hydrogenated EETs were carried out under the same conditions except that an isocratic gradient of acetonitrile/water/methanol/acetic acid (75:15:10:0.05) was used, and the precursor ion of hydrogenated EETs (*m/z* 325) was selected for tandem mass monitoring.

Renal Vascular Relaxation—Renal interlobar arteries from Sprague-Dawley rats were cut into ring segments 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O_2 and 5% CO_2 for measurement of isometric tension (mN/mm) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar artery rings were preconstricted with phenylephrine (1 μM) to test the arterial relaxation caused by 5,6-*trans*-EET, 5,6-*erythro*-DHET, and 5,6-*cis*-EET at concentrations ranging from 0.001 to 5 μM.

Platelet Aggregation—Blood (10 parts), obtained from Sprague-Dawley rats, was drawn into citrate/dextrose (1 part). Platelet-rich plasma was obtained after centrifugation at 1,100 × *g* for 2 min at 21 °C. A

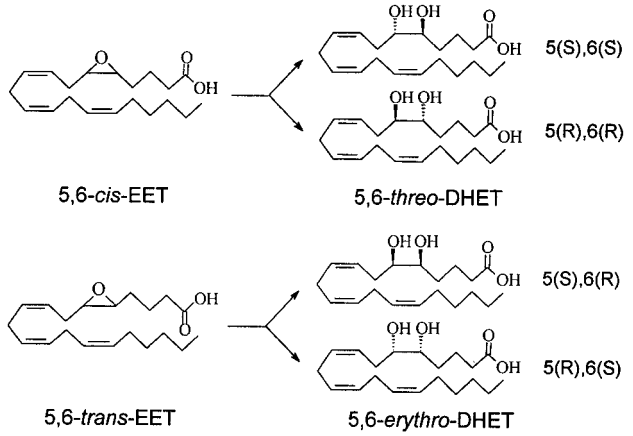

FIG. 1. **Structures of the 5,6-*cis*-EET, 5,6-*trans*-EET, and their corresponding DHET enantiomers.**

suspension of apyrases (12.5 milliunits/ml) was added, and platelets were removed by centrifugation at 1,100 × *g* for 15 min at 21 °C. Platelets were resuspended in a modified Tyrode's buffer, pH 6.4, containing 5 mM Hepes, 140 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5.5 mM glucose, 12 mM $NaHCO_3$, 0.2% bovine serum albumin, and 0.2 mM EDTA, recentrifuged at 1,100 × *g*, and suspended in a buffer, pH 7.4, containing 10 mM Hepes, 140 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5.5 mM glucose, and 12 mM $NaHCO_3$ at a concentration of 2×10^8/ml. Washed platelets were used for aggregation studies within 3 h of isolation. Aggregation reactions (300 μl) using washed platelets (3×10^8/ml) were conducted at 37 °C using a Chronolog Lumiaggregometer (model 600) in the presence of stirring (800 rpm). Platelets were preincubated with vehicle, 5,6-*erythro*-DHET, or 5,6-*trans*-EET 2 min prior to the addition of collagen. Both 5,6-*trans*-EET and 5,6-*erythro*-DHET were dried from acetonitrile and suspended into platelet buffer prior to the experiments.

Statistical Analyses—Data are presented as mean ± S.E. An unpaired Student's *t* test and a one-way analysis of variance were performed to test for differences between groups. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

Conversion of 5,6-trans-EETs to 5,6-DHETs—Conversion of standard 5,6-*cis*-EET under mild organic alkaline conditions: methanol/water/DIPEA (200:100:15 μl) at 60 °C for 1 h produced a single peak in GC/MS corresponding to 5,6-*threo*-DHET (Fig. 2*A*).

Without DIPEA treatment, the GC/MS analysis from RBC 5,6-DHET fraction showed only trace amounts of 5,6-*threo*-DHET and a new peak of similar intensity (Fig. 2*B*). However, after treatment with DIPEA, the GC/MS ions of 5,6-*threo*-DHET and the new peak were significantly increased (Fig. 2*C*), demonstrating the origin of the DHET peaks from corresponding 5,6-EETs in the RBC samples. There were no other detectable DHETs from RBCs either before or after DIPEA treatment of these samples.

The NICI GC/MS spectrum of the TMS ether PFB ester of the new peak (Fig. 3) was identical to the GC/MS spectrum of 5,6-*threo*-DHET. All the evidence suggested that the new peak was 5,6-*erythro*-DHET that originated from the 5,6-*trans*-EET in the *sn*-2 position of phospholipids of RBCs.

GC/MS Confirmation of 5,6-trans-EET—To obtain additional confirmation of the structure, 5,6-*trans*-EET was synthesized and analyzed by NMR to be authentic. DIPEA treatment of 5,6-*trans*-EET produced a single peak of 5,6-*erythro*-DHET both in HPLC and GC/MS.

By using a gradient of acetonitrile/water/methanol/acetic acid from 40:50:10:0.05 to 60:30:10:0.05 over 20 min, which was continued for another 10 min, the 5,6-*erythro*-DHET eluted ahead of 5,6-*threo*-DHET. The retention time was 21.7 min for 5,6-*erythro*-DHET and 22.6 min for 5,6-*threo*-DHET (*peaks A* and *B*, respectively, in the HPLC *inset* in Fig. 4). The RBC

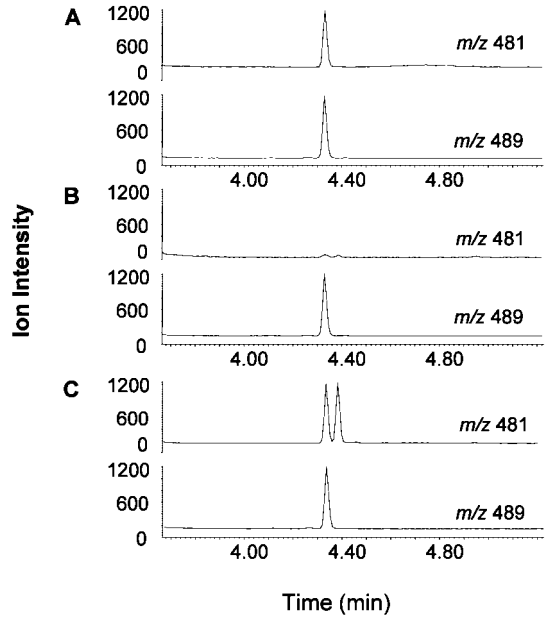

FIG. 2. *A*, DIPEA conversion of standard 5,6-*cis*-EET. *B*, trace amounts of 5,6-*threo*-DHET and a new peak from RBCs without DIPEA conversion. *C*, 5,6-*threo*-DHET and the new peak from RBCs with DIPEA conversion. DHET was converted to TMS ether PFB ester for NICI GC/MS analysis (see "Experimental Procedures"). *m/z* 481 represents endogenous DHET; *m/z* 489 represents the 5,6-*threo*-DHET-d_8 internal standard.

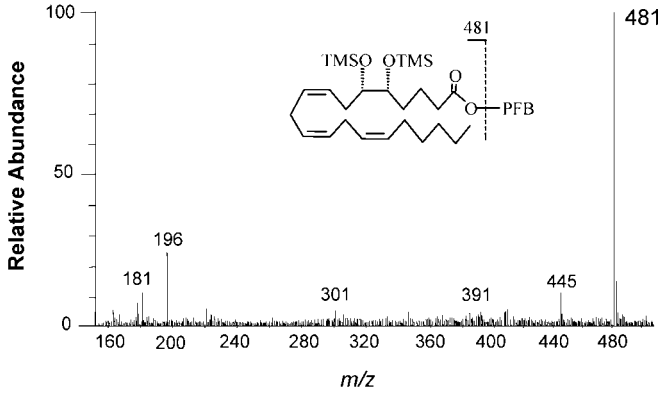

FIG. 3. **NICI GC/MS spectrum of the TMS ether PFB ester of the new peak (5,6-*erythro*-DHET) from RBC phospholipids.** *TMS* = $Si(CH_3)_3$; *PFB* = $(C_6F_5)CH_2$.

HPLC 5,6-DHET fraction was further separated with this gradient. Fractions A and B, corresponding to the 5,6-*erythro*- and 5,6-*threo*-DHET from RBC samples, were collected and analyzed by GC/MS. The retention times of ion *m/z* 481 of the TMS ether PFB esters of the two HPLC fractions matched with those of the authentic 5,6-*erythro*-DHET and 5,6-*threo*-DHET, respectively (Fig. 4), demonstrating that a pure 5,6-*erythro*-DHET can be obtained from RBC samples. The minor peak in Fig. 4, *Fraction B*, indicated that its HPLC fraction may have included a tail of 5,6-*erythro*-DHET.

The EI GC/MS spectrum was used to confirm the molecular breakdown patterns of the 5,6-*erythro*-DHET. Fraction A was derivatized to TMS ether methyl ester for the analysis. The EI GC/MS spectrum (Fig. 5) of the purified fraction A from RBCs is almost identical to that of the authentic 5,6-*erythro*-DHET; the EI spectrum does not differ with that of authentic 5,6-*threo*-DHET. The only difference is the GC retention time of the peaks (Fig. 5, *inset*). Ions at *m/z* 203 indicate the molecular breakdown of the TMS ether methyl ester of 5,6-DHET at the C_5–C_6 position; *m/z* 215 indicates the breakdown at C_6–C_7

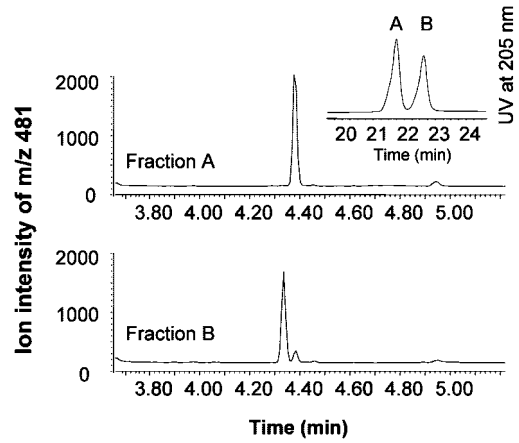

FIG. 4. **Purified 5,6-*erythro*-DHET from RBC phospholipids.** *Fraction A*, the GC/MS peak (*m/z* 481) of the HPLC fraction A from RBCs; *Fraction B*, GC/MS peak (*m/z* 481) of the HPLC fraction B from RBCs. *Inset*, HPLC peaks of standard 5,6-*erythro*-DHET derived from 5,6-*trans*-EET (*peak A*) and standard 5,6-*threo*-DHET (*peak B*).

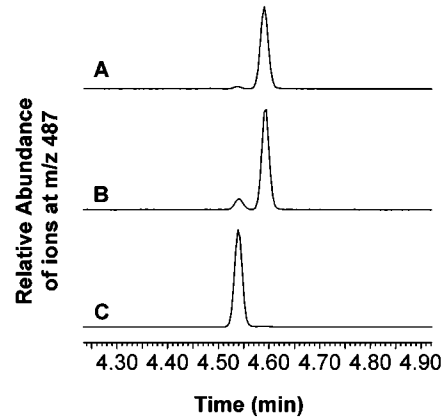

FIG. 6. **Hydrogenation produced peaks at *m/z* 487 instead of *m/z* 481 for the DHET TMS ether PFB esters and match of the GC/MS retention times.** *A*, fraction A from RBC phospholipids; *B*, authentic 5,6-*erythro*-DHET; *C*, authentic 5,6-*threo*-DHET. The hydrogenated products were derivatized to TMS ether PFB esters for NICI GC/MS analysis (see "Experimental Procedures").

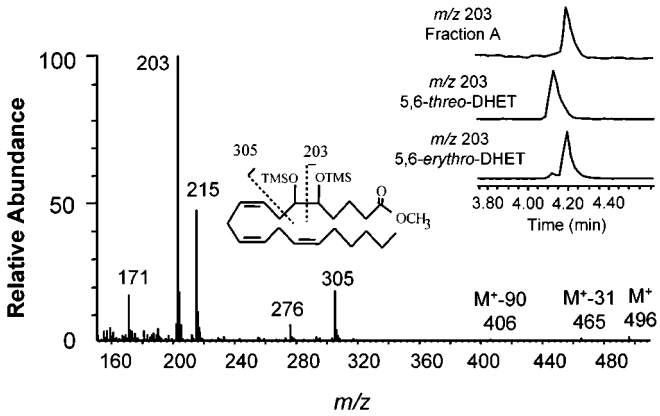

FIG. 5. **Representative EI GC/MS spectrum of the 5,6-*erythro*-DHET methyl derivative from RBC samples.** The spectrum matches with that of authentic 5,6-*erythro*-DHET, and there is no difference between the spectra corresponding to 5,6-*erythro*- and 5,6-*threo*-DHET. The only difference is at the GC retention time as shown in the *inset*. *TMS* = $Si(CH_3)_3$.

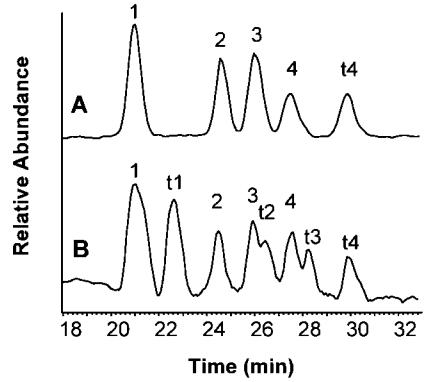

FIG. 7. **LC/MS chromatograms of EETs from authentic standards (*A*) and from Sprague-Dawley rat RBCs (*B*).** Selected ion chromatograms at *m/z* 319 are shown. *Peaks 1–4* and *t4* correspond to authentic 14,15-*cis*-, 11,12-*cis*-, 8,9-*cis*-, 5,6-*cis*-, and 5,6-*trans*-EETs, respectively (*A*). Isomeric EET peaks were observed in RBC samples pretreated with 0.2 mM BHT (*B*) (see "Experimental Procedures").

position with a loss of a TMS group, and *m/z* 305 is from the breakdown at the C_6–C_7 position.

Hydrogenation of the purified 5,6-*erythro*-DHET fraction from RBCs resulted in the disappearance of ion at *m/z* 481 and appearance of ion at *m/z* 487 for the PFB ester TMS ether derivatives, which is identical to the hydrogenation for authentic 5,6-*erythro*-DHET. The GC/MS retention times of the hydrogenated products also matched correspondingly (Fig. 6), suggesting the existence of three olefins as well as the 5,6-*erythro*-dihydroxy structure in the metabolite from RBCs.

HPLC-ESI-MS/MS Direct Identification of 5,6-trans-EET—Authentic *cis*-EETs and the 5,6-*trans*-EET were well separated with reversed phase HPLC in the experiment (Fig. 7*A*). LC/MS analysis of the extracts from RBC samples pretreated with 0.2 mM BHT revealed a chromatogram showing eight EET peaks, labeled as *1–4* and *t1* to *t4* (Fig. 7*B*). The HPLC retention times of peaks 4 and t4 in RBC samples matched that of authentic 5,6-*cis*-EET and 5,6-*trans*-EET, respectively.

The ESI-MS/MS spectrum of EETs from RBCs has been reported by Murphy and co-workers (16). The ESI mass spectrum of regioisomeric EETs consisted of a single carboxylate anion $[M - H]^-$ at *m/z* 319. Collisional activation of the carboxylate anion resulted in product ions characteristic of each of the regioisomers of the EETs and also common product ions formed by loss of water (*m/z* 301), loss of CO_2 (*m/z* 275), and the loss of both water and CO_2 (*m/z* 257) from the $[M - H]^-$ ion. The relative abundance of these peaks may vary with different instrumentation and electrospray conditions. There is no consistent difference of the ESI-MS/MS spectra among peaks 4, t4, authentic 5,6-*cis*-EET, and 5,6-*trans*-EET (Fig. 8*A*). Selected reaction monitoring of the unique fragmentation for 5,6-EETs, from *m/z* 319 → *m/z* 191, revealed two clear 5,6-EET peaks (Fig. 8*B*). The match of both the HPLC retention time and ESI-MS/MS spectrum of the peak in RBC samples with authentic 5,6-*trans*-EET is strong evidence of the existence of 5,6-*trans*-EET in RBCs.

To eliminate the possibility that peak t4 in the sample may be 5,6-*cis*-epoxide with a combination of *trans*-double bonds that resulted in the same retention time shift as that exhibited by 5,6-*trans*-EET, the EET fraction of RBC sample was purified, hydrogenated, and subjected to LC/MS/MS analysis. A match of both the retention times and MS/MS spectra between the authentic standards (Fig. 9*A*) and the sample (Fig. 9*B*) for the two reduced 5,6-EET peaks was obtained. The four hydrogenated EET regioisomers can be differentiated with mass spectra as has been done with the nonhydrogenated EETs (16). Selected reaction monitoring of the fragmentation, from *m/z* 325 → *m/z* 225, revealed twin peaks for hydrogenated 5,6-EETs (Fig. 9*C*). The clear separation of these two reduced molecules without double bonds leaves only the geometry of the epoxide being either 5,6-*cis* or 5,6-*trans*. The ESI-MS/MS spectra of

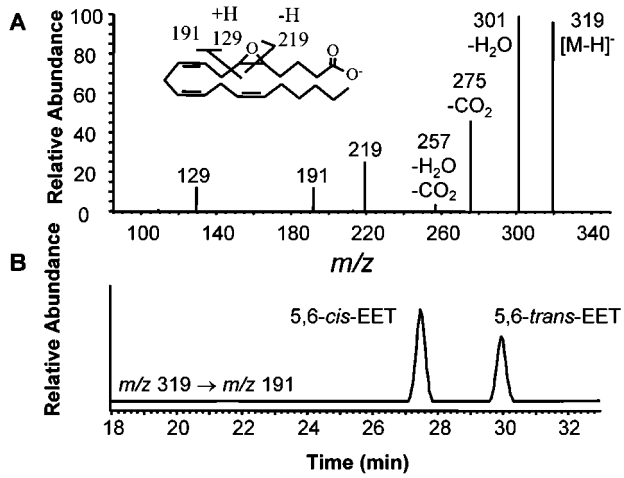

FIG. 8. **ESI-MS/MS analysis of 5,6-EETs.** No consistent differences of the ESI-MS/MS spectrum between 5,6-*cis*- and 5,6-*trans*-EETs were observed. *A*, representative spectrum of 5,6-EETs; *B*, the characteristic selected reaction monitoring (*m/z* 319 → *m/z* 191) of 5,6-EETs from rat RBCs.

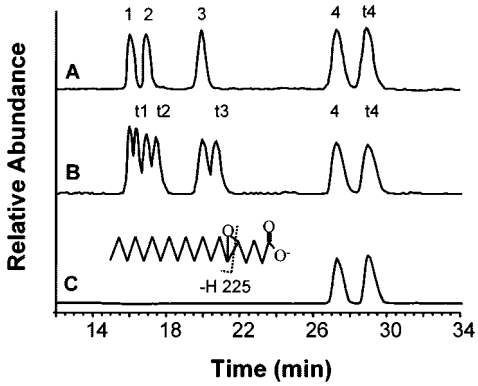

FIG. 9. **LC/MS/MS analysis of hydrogenated EETs.** *A*, total ion chromatogram of hydrogenated EET standards. *B*, total ion chromatogram of purified EET fraction from Sprague-Dawley rat RBCs. *C*, selected reaction monitoring (*m/z* 325 → *m/z* 225) of reduced 5,6-EETs from rat RBCs. *Peaks 1–4*, and *t4* in *A* correspond to hydrogenated authentic 14,15-*cis*-, 11,12-*cis*-, 8,9-*cis*-, 5,6-*cis*-, and 5,6-*trans*-EETs, respectively. *Peaks t1*, *t2*, *t3* and *t4* in *B* represent hydrogenated 14,15-, 11,12-, 8,9-, and 5,6-*trans*-EETs from RBC samples (see "Experimental Procedures").

peaks t1, t2, and t3 matched with corresponding 14,15-, 11,12-, and 8,9-EETs, respectively, both before and after hydrogenation (data not shown). Two peaks for each of these three regioisomers indicated the presence of 14,15-, 11,12-, and 8,9-*trans*-EETs, respectively.

The levels of 5,6-*cis*- and 5,6-*trans*-EET in rat red blood cell total phospholipids were 2.23 ± 0.69 and 2.12 ± 0.83 ng/10^9 RBCs, respectively, as quantitated with LC/MS analysis for three separate samples. The results indicate that there are about the same amount of 5,6-*cis*- and 5,6-*trans*-EETs in normal Sprague-Dawley rat red blood cells. The presence of other *trans*-EETs are evident in Fig. 7*B*; the levels of 14,15-*trans*-EET are a little higher than those of 11,12-*trans*- and 8,9-*trans*-EETs, which resemble those of the 5,6-*trans*-EET. The ratios of *cis*/*trans* EETs for each regioisomers in the erythrocytes are approximately 1:1.

Renal Vascular Effects—The capacity of 5,6-*trans*-EET to relax rat interlobar arteries preconstricted with phenylephrine exceeded that of 5,6-*cis*-EET by a significant degree, *viz.* ED_{50} was lower by 1 log unit (~10^{-8} *versus* 10^{-7} M) and maximum relaxation produced by 5,6-*trans*-EET was ~2-fold greater (Fig. 10). The renovascular activity of 5,6-*erythro*-DHET fell between those of the 5,6-*cis*- and *trans*-EETs.

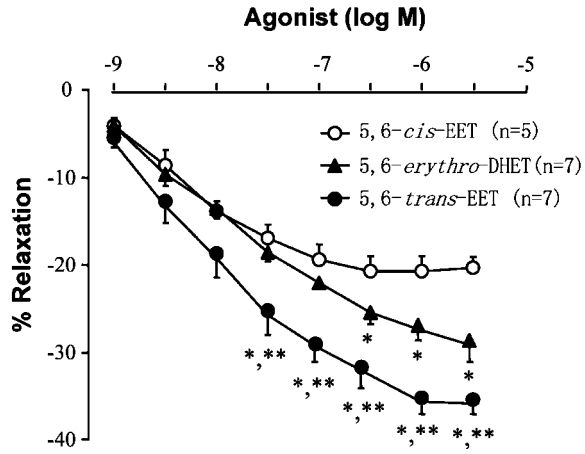

FIG. 10. **Vasorelaxation effects of 5,6-*trans*-EET and 5,6-*erythro*-DHET compared with 5,6-*cis*-EET.** Renal interlobar artery rings of Sprague-Dawley rats were preconstricted by phenylephrine (1 μM), and isometric tension was measured. *, $p < 0.05$, compared with 5,6-*cis*-EET; **, $p < 0.05$, compared with 5,6-*erythro*-DHET.

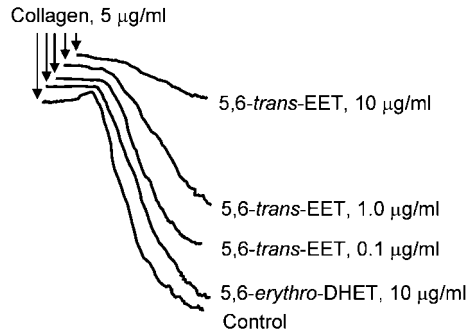

FIG. 11. **5,6-*trans*-EET inhibits Sprague-Dawley rat platelet aggregation.** Platelets were preincubated with buffer (*Control*), 5,6-*erythro*-DHET, or 5,6-*trans*-EET at the indicated concentrations for 2 min at 37 °C. Collagen was added, and platelet aggregation was allowed to proceed for 4 min. Presented in the figure are representative results of at least four different experiments.

Inhibition of Platelet Aggregation—5,6-*trans*-EET and 5,6-*erythro*-DHET were tested for their ability to inhibit collagen-induced platelet aggregation at concentrations from 0.1 to 10 μg/ml. At the highest concentration, 5,6-*trans*-EET inhibited collagen-induced platelet aggregation, whereas 5,6-*erythro*-DHET was ineffective (Fig. 11). 5,6-*cis*-EET produced similar effects to those of 5,6-*trans*-EET; namely each inhibited collagen-induced rat platelet aggregation (data not shown). Greater than 90% inhibition was consistently observed with 10 μg/ml 5,6-*trans*-EET. 50% inhibition was usually achieved at a concentration of 1 μg/ml or less.

DISCUSSION

The criteria used to identify 5,6-*trans*-EET in the *sn*-2 position of the phospholipids in RBCs can be summarized as follows. 1) Without mild alkaline DIPEA treatment to convert 5,6-*cis*- and *trans*-EETs to the respective DHETs, there were only trace amounts of 5,6-*threo*- and 5,6-*erythro*-DHET; with DIPEA treatment, the increased levels of 5,6-*threo*- and 5,6-*erythro*-DHETs indicated their origin from corresponding 5,6-EETs. 2) DIPEA treatment of standard 5,6-*cis*-EET generated only 5,6-*threo*-DHET, and standard 5,6-*trans*-EET generated only 5,6-*erythro*-DHET. 3) The NICI GC/MS spectrum of the 5,6-*erythro*-DHET TMS ether PFB ester proved the molecular weight. 4) The EI GC/MS spectrum of the 5,6-*erythro*-DHET

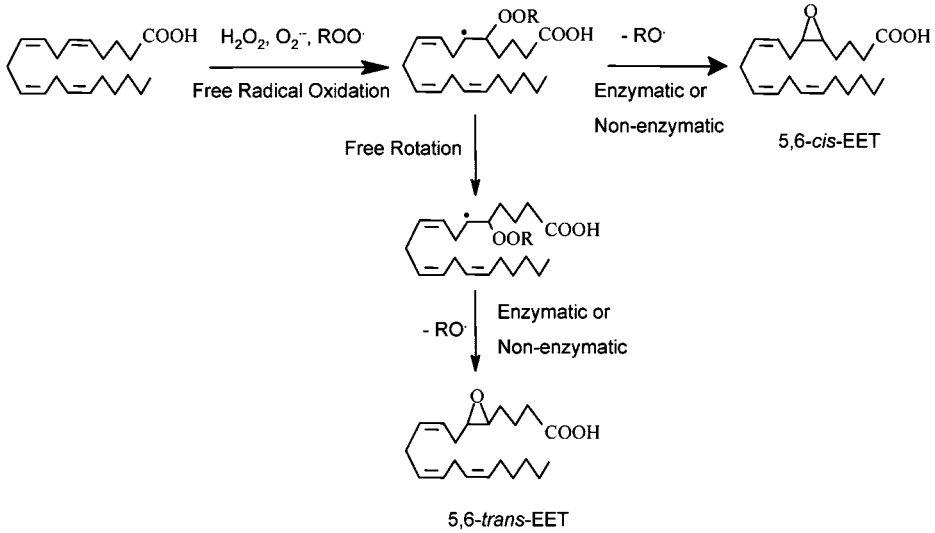

FIG. 12. **A proposed 5,6-*trans*-EET formation mechanism *in vivo*.** Other *cis*- and *trans*-EETs can be generated under the same mechanism. Red blood cells are constantly exposed to free radicals in tissues and organs. An energy-favorable rotation of the peroxidation intermediate leads to the isomerization. *R* = H, lipid, nucleic acid, peptide, protein, and other molecules.

methyl ester confirmed its correct 5,6-DHET fragmentation. 5) Hydrogenation experiments confirmed the existence of three olefins and the *erythro*-dihydroxy structure. 6) The match of retention times in HPLC and GC/MS experiments indicated the identity of the stereochemistry of RBC 5,6-*erythro*-DHET *vis à vis* the synthetic standard. 7) Direct identification of 5,6-*trans*-EET was performed by HPLC-ESI-MS/MS analysis. This study is the first report of the presence in nature of 5,6-*trans*-EET and 5,6-*erythro*-DHET.

The formation of 5,6-*trans*-EET is possibly through radical-driven reactions (16). Whether an enzyme is involved has yet to be investigated. Cytochrome P450 epoxygenases produce only *cis*-EETs from arachidonic acid (2, 29, 30). It is very likely that cytochrome P450 incubation with 5,6-*trans*-arachidonic acid could form 5,6-*trans*-EET. However, the abundance of *trans*-arachidonic acid *in vivo* is only nominal compared with that of arachidonic acid. A prostaglandin E-like epoxyisoprostane structure produced by phospholipid oxidation contains a 5,6-*trans*-epoxide structure (31). Formation of a *trans*-epoxide from a *cis*-olefin has also been observed in hydrogen peroxide-dependent epoxidation of styrenes by sperm whale myoglobin (32). The 5,6-*trans*-epoxide structure is also present in leukotriene A_4, the formation of which also requires the presence of peroxide (33, 34). It is possible that a hydroperoxide free radical serves as an intermediate in the arachidonic acid oxidation to form EETs; an energy-favorable free rotation of the intermediate may lead to the formation of 5,6-*trans*-EET (Fig. 12). Chiral analysis of the product will be needed to determine whether an enzyme is involved in the formation mechanism. Identification of 5,6-*trans*-EET in erythrocytes represents the first report of a *trans*-epoxide metabolite from arachidonic acid, which retains the other three original *cis*-olefin structures. Red blood cell 5,6-*trans*-EET cannot be formed during sample preparations because of the use of BHT or TPP. The importance of free radical oxidation of erythrocyte phospholipids in elevating EET and hydroxyeicosatetraenoic acid levels in human red blood cells was striking and has been lucidly characterized by Nakamura *et al.* (16). Lipid peroxidation produced by *tert*-butyl hydroperoxide in red blood cells increased esterified EETs in excess of 30-fold and approached 60-fold for phosphatidylcholine. Three of the regioisomer EETs were formed in almost equal amounts; the 5,6-EET was formed in greatest abundance.

It is worth noting that both 5,6-*trans*-EET and 5,6-*erythro*-DHET have greater potencies than 5,6-*cis*-EET in relaxing renal interlobar arteries. 14,15-*trans*-EET has been reported to be less potent than 14,15-*cis*-EET in relaxing bovine coronary arteries (35). Among the *cis*-EETs derived from cytochrome P450 epoxygenases, 5,6-*cis*-EET has unique biological activities in regulating regional vascular tone (36–39). It has been shown to dilate (37–39) as well as to constrict (40) blood vessels, each of which may be cyclooxygenase-dependent depending on the vascular bed (40, 41). Other effects include modulation of angiotensin II-induced sodium transport activity (42, 43) and epithelial growth factor signaling in renal proximal tubules (44), releasing somatostatin from hypothalamic nerve terminals (45), and stimulating prolactin secretion (46). Comparison of the agonist and antagonist functions of 5,6-*trans*-EET corresponding to those actions of 5,6-*cis*-EET are the subjects of future studies. Because leukotriene A_4 hydrolase has a narrow substrate specificity (47), if there is a specific epoxide hydrolase responsible for 5,6-*trans*-EET hydrolysis, it should be investigated. Whether cyclooxygenase plays a role in 5,6-*trans*-EET metabolism and function as it does for 5,6-*cis*-EET (48, 49) is a distinct possibility that may confer a larger functional role on this epoxide.

EETs have been proposed as therapeutic targets in the control of blood pressure (50, 51). Treatment of spontaneously hypertensive rats with a selective soluble epoxide hydrolase inhibitor decreased blood pressure significantly but had no effect on blood pressure in normotensive Wistar-Kyoto rats (52). Soluble epoxide hydrolase inhibition can also lower arterial blood pressure in angiotensin II-induced hypertension (53). The vasodilator activity and ability to inhibit platelet aggregation of 5,6-*trans*-EET demonstrated the potential range of biological responses to these novel eicosanoids. The potency of both 5,6-*cis*- and 5,6-*trans*-EETs in inhibiting platelet aggregation is in line with the reported inhibition of human platelet aggregation by 14,15-*cis*-, 14,15-*trans*-, 11,12-*cis*-, and 8,9-*cis*-EET isomers, namely the action is not stereospecific (11). Human platelet aggregation induced by arachidonic acid was shown to be inhibited by 14,15-*cis*-, 14,15-*trans*-, 11,12-*cis*-, and 8,9-*cis*-EET isomers at concentrations from 1 to 10 μM with no evident stereospecificity. The identification of a 5,6-*trans*-EET in erythrocyte phospholipids suggests a vasoregulatory role of RBCs through the release of vasoactive eicosanoids.

In view of the biological activity of 5,6-*trans*-EET and its corresponding 5,6-*erythro*-DHET, the definition of the full range of their biological effects will be of interest whether or not 5,6-*trans*-EET formation derives from free radical oxidation alone and/or enzymatic generation. Isoprostanes, which are derived from free radical oxidation under conditions of oxida-

tive stress (54, 55), have crucial roles in disease. EET regioisomers are readily incorporated into cellular phospholipids; the rate of release of 14,15-EET from phosphatidylcholine and phosphatidylinositol exceeded that for arachidonic acid (13). The rate of incorporation into and the release from phospholipids of *trans*-EETs remains to be established. Definition of the formation, storage, and release of *trans*-EETs together with characterizing their biological profile and range of activities in vascular mechanisms should uncover potential contributions of *trans*-EETs to circulatory regulation in health and in disease.

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Identification of 5,6-*trans*-Epoxyeicosatrienoic Acid in the Phospholipids of Red Blood Cells

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