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-8,11Z,14Z-ecosatrienoic 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 A₂ 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. 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 epoxide products that affect blood flow, mitogenesis, platelet aggregation, and Ca²⁺ 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 epoxigenases are of the cis configuration (9–11); a trans-EET in vivo has not been described (Fig. 1).

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 (10). 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 A₂ (from Naja mossambica mossambica), diisopropylfluorophosphate (DFP), 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-d₅ standards were from Biomol. 14-Week-old male Sprague-Dawley rats were purchased from Charles River Laboratories, Wilmington, MA. Rats were main-

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5,6-trans-Epoxycosatetraenoic Acid

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to Vacutte EDTA tubes (Fischer). 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. Packaged RBCs were washed three times and resuspended in a physiological salt solution (in mM: 4.0 KCl, 2.0 CaCl2, 1.2 MgSO4, 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. Prepared RBC samples (4 mL) were centrifuged at 2,000 g 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 A2 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).

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/isopropanol alcohol/water (7:11:1) was dried under nitrogen and followed by phospholipase A2 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/methylene chloride. RBC (3 mL of blood) samples were stored in ice and separated using a myograph (J. P. Trading) bathed in Krebs buffer (37 °C for 10 min) gassed with 95% O2 and 5% CO2. The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28). The myograph was operated in isometric mode at calculated resting intramural pressure of 100 mm Hg. The interlobar arteries were cut into segment rings 2 mm in length and mounted on a myograph (J. P. Trading) bathed in Krebs buffer (37 °C) gassed with 95% O2 and 5% CO2 for measurement of isometric tension (mm Hg) (28).
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 MgCl₂, 2 mM KCl, 5.5 mM glucose, and 12 mM NaHCO₃ at a concentration of 2 × 10⁹/ml. Washed platelets were used for aggregation studies within 3 h of isolation. Aggregation reactions (300 platelets were used for aggregation studies within 3 h of isolation.

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-DHET—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-trans-DHET (Fig. 2A).

Without DIPEA treatment, the GC/MS analysis from RBC 5,6-DHET fraction showed only trace amounts of 5,6-trans-DHET and a new peak of similar intensity (Fig. 2B). However, after treatment with DIPEA, the GC/MS ions of 5,6-trans-DHET and the new peak were significantly increased (Fig. 2C), 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 (5,6-erythro-DHET) from RBC phospholipids. TMS = Si(CH₃)₃; PFB = (C₆F₅)CH₂.

HPLC 5,6-DHET fraction was further separated with this gradient. Fractions A and B, corresponding to the 5,6-erythro- and 5,6- thermo-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- thermo-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- thermo-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- thermo-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 C₁₀-C₁₁ position; m/z 215 indicates the breakdown at C₆-C₇.

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

Fig. 2. A, DIPEA conversion of standard 5,6-cis-EET. B, trace amounts of 5,6- thermo-DHET and a new peak from RBCs without DIPEA conversion. C, 5,6- thermo-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- thermo-DHET-d₄ 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(CH₃)₃; PFB = (C₆F₅)CH₂.
position of a loss of a TMS group, and m/z 305 is from the breakdown at the C₆—C₇ 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. 7A). 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. 7B). 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 CO₂ (m/z 275), and the loss of both water and CO₂ (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-EETs (Fig. 8A). 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. 8B). 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. 9A) and the sample (Fig. 9B) 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. 9C). 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...
11,12-, 8,9-, and 5,6-trans-EETs from RBC samples (see "Experimental Procedure"). Two peaks for each of these three regioisomers indicated the presence of 14,15-, 11,12-, 8,9-, and 5,6-trans-EETs from RBC samples (see "Experimental Procedure").

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 t1 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/10⁹ 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. 7B; 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. ED₅₀ 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.

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
cis-structure produced by phospholipid oxidation contains a 5,6-arachidonic acid. A prostaglandin E-like epoxyisoprostane trans-epoxide structure (31). Formation of a triene A4, the formation of which also requires the presence of (32). The 5,6-epoxide structure is also present in leuko-epoxide of styrenes by sperm whale myoglobin-EET. However, the abundance of trans-trans-the other three original trans-epoxide metabolite from arachidonic acid, which retains trans-EET in relaxing DHET have greater potencies than 5,6-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-

![Diagram of 5,6-trans-EET formation mechanism](image-url)
tive stress (54, 55), have crucial roles in disease. EET regioiso-
mers are readily incorporated into cellular phospholipids; the rate of release of 14,15-EET from phosphatidylcholine and phosphatidylserine 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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