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REGIOSPECIFIC AND ENANTIOSELECTIVE METABOLISM OF 8,9-EPOXYEICOSATRIENOIC ACID BY CYCLOOXYGENASE

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Summary: 8(S),9(R)-epoxyeicosatrienoic acid, a major product of the renal cortex, was found to be a substrate for cyclooxygenase from human platelets and ram seminal vesicles. 11(R)-hydroxy-8(S),9(R)-epoxyeicosatrienoic acid was the sole metabolic product. The 8(R),9(S)-enantiomer formed both C-11 and C-15 hydroxylated metabolites. These novel findings suggest that the cyclooxygenase-dependent renal vasoconstrictor activity of 8(S),9(R)-epoxyeicosatrienoic acid may be due to the 11(R)-hydroxy metabolite.

Arachidonic acid oxygenation by the microsomal cytochrome P-450 system leads to the formation of EETs and HETEs (1-4). EETs have a diversity biological activities (5) including their ability to inhibit arachidonate-induced human platelet aggregation (6). It has been proposed that EETs are involved in the pathophysiology of human disease (7). In support of this proposal it was found that 8(S),9(R)-EET, a major product of rat kidney renal cortex, was a potent renal vasoconstrictor substance (8). During recent studies on the inhibition of human platelet aggregation by 8,9-EET enantiomers, we observed that four metabolites were formed. Two of the metabolites were absent when platelet cyclooxygenase was inhibited with indomethacin or aspirin. 5,6-EET is known to be a substrate for cyclooxygenase where it serves as a precursor for a number of PGs (9-12). However, 8,9-EET is unable to undergo cyclization to an endoperoxide and it has been assumed in the past that it is not metabolized by cyclooxygenase (5,6,13). We report that

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Abbreviations. DHET, dihydroxyeicosatrienoic acid; EC, electron capture negative ion chemical ionization; EI, electron ionization; EET, epoxyeicosatrienoic acid; GC, Gas chromatography; HETE, hydroxyeicosatetraenoic acid; ID, internal diameter; MS, mass spectrometry; ME, methyl ester; PFB, pentafluorobenzyl; 4-PCO, 4-phenylchalcone oxide; PG, prostaglandin; TMS, trimethylsilyl.

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8,9-EET is in fact a good substrate for human platelet cyclooxygenase and for the cyclooxygenase purified from ram seminal vesicles.

Materials and Methods. [1-^{14}C]EETs were prepared from [1-^{14}C]arachidonic acid (5 mCi/mmol) using standard procedures. Enantiomers of [1-^{14}C]8,9-EET (5 mCi/mmol) were separated by a modification of our previously described HPLC method (14). Chemicals and solvents were of the best grades that were commercially available.

Platelet incubations: Human platelets supplied by the Vanderbilt Blood Bank, were isolated as described previously (15) and suspended in buffer (145 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 1 mM $MgCl_2$, 0.2 mg/ml BSA, 100 mM HEPES, pH 7.4). Incubations were carried out with 5 ml platelet suspensions (10^9 platelets/ml buffer) in the presence of individual [1-^{14}C]EET enantiomers (15 nmol, 3 μM). Platelets were activated with thrombin (1 unit/ml) or ionophore A-23187 (2 μM), in the presence or absence of 4-PCO (60 μM) and the appropriate [1-^{14}C]8,9-EET-enantiomer was added immediately. Inhibition studies were carried out by pre-incubation of the platelets for 2 min with aspirin (0.5 mM) or indomethacin (0.2 mM). All incubations were carried out at 37 °C for 1 hr in a shaking water bath under an atmosphere of normal laboratory air. They were terminated by the addition of ice-cold methanol (5 ml). Chloroform (10 ml) was added and the samples centrifuged at 2000 x g for 10 min. The lower phase was collected and the aqueous upper phase extracted three times with 5 ml of chloroform/methanol/acetic acid (200:100:1, v/v/v). The organic phases were combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 40 μl chloroform/methanol (2:1, v/v) and lipids separated by TLC on a LK6D TLC plate (Whatman) developed with diethyl ether/hexane/acetic acid (160:40:1, v/v/v). Non-esterified lipids (Rf 0.25-0.75) were extracted with ethyl acetate and the solvent evaporated to dryness. The residue was dissolved in methanol (30 μl) and carboxylic acids converted to ME derivatives by treatment with ethereal diazomethane (1 ml). The ether was evaporated and the residue dissolved in methanol. Recovery of radioactivity was 60-80 %, depending on the amount of incorporation into phospholipids. PFB-ester derivatives were prepared as described previously (16).

Incubations with cyclooxygenase from ram seminal vesicles: Purified cyclooxygenase (100 μg) from ram seminal vesicles (17) was suspended in 0.1 M Tris-HCl (1 ml; pH 8.0) containing 2 mM *p*-hydroxymercuribenzoate, 5 μM hematin and 2 mM tryptophan. The suspension was bubbled with oxygen for 2 min and [1-^{14}C]8,9-EET (25 μg; 78 μM) added in ethanol (10 μl). The incubation was allowed to proceed for 5 min and stopped by the addition of 10 μl of citric acid. The incubation mixture was loaded on a C_{18} Sep-Pak, washed with water (5 ml) and products eluted with ethyl acetate (5 ml). The ethyl acetate was evaporated and ME derivatives prepared as described above. Recovery of radioactivity was > 95 %.

HPLC purification and analysis of metabolites: ME and PFB derivatives were analyzed on an Econosphere silica column (4.6 x 250 mm, 5 μm; Alltech) using a linear gradient from hexane/isopropanol/acetic acid (98.5:1.5:0.05, v/v/v) to hexane/isopropanol/acetic acid (95:5:0.05, v/v/v) over 40 min with a flow rate of 1 ml/min. Detection was carried out using a Radiomatic (FLO-ONE Beta) radioactive flow detector. Fractions corresponding to the peaks eluting from the HPLC column were collected ready for GC/MS analysis.

GC/MS analysis of metabolites: ME and PFB derivatives were converted to their TMS-ethers (16). GC/EIMS was carried out on a Finnigan INCOS 50 instrument using a 15 m DB-1 fused silica column (0.25 mm ID, 0.25 μM coating thickness; J & W). Helium was used as the carrier gas and injections were made in the splitless mode at a flow rate of 1 ml/min. The column was temperature programmed from 150 °C to 320 °C at 15 °C/min.

Retention times (min:sec) of metabolite-ME, TMS-derivatives were as follows: 1 (8:06), 2 (8:23), 3 (8:06), 4 (8:24), 5 (8:27). GC/ECMS was carried out on a Nermag R1010C instrument (16) using a 15 m SPB-1 fused silica column (0.32 mm ID, 0.25 μM coating thickness; Supelco). The column was held at 190 °C for 1 min then programmed to 320 °C at 20 °C/min. Retention times of the metabolite-PFB, TMS derivatives were as follows: 1 (5:19), 2 (5:31), 3 (5:19), 4 (5:31), 5 (5:35). Hydrogenation of EET-MEs was carried out as described previously (18).

Results and Discussion. HPLC analysis of the products from incubations of [1-^{14}C]8(S),9(R)-EET with human platelets revealed the presence of two metabolites (metabolites 1 and 2; Figure 1a). Stimulation of the platelets with thrombin or ionophore A23187 resulted in a decrease in the amount of metabolite 1 and a concomitant increase in the amount of metabolite 2. When platelets were stimulated in the presence of 4-PCO (an epoxide hydrolase inhibitor) metabolite 2 was the sole metabolite formed (Figure 1b). The ME, TMS derivative of metabolite 1 had a mass spectrum and a GC retention time that were identical with *bis*-TMS-ME-8,9-DHET. Metabolite 2 was a conjugated diene based on its UV spectrum (λ_{max} 235 nm). The EC mass spectrum of its PFB,TMS derivative (16) showed an intense [M-PFB] negative ion at *m/z* 407, which was consistent with the attachment of a single hydroxyl group to the EET molecule. GC/EIMS of metabolite 2 as its ME, TMS derivative revealed that the site of hydroxylation was C-11 (Figure 2a). There was an intense fragment ion at *m/z* 225, resulting from an α-cleavage at C-11 (19). After hydrogenation, this fragment ion appeared at *m/z* 229 (Figure 2b)

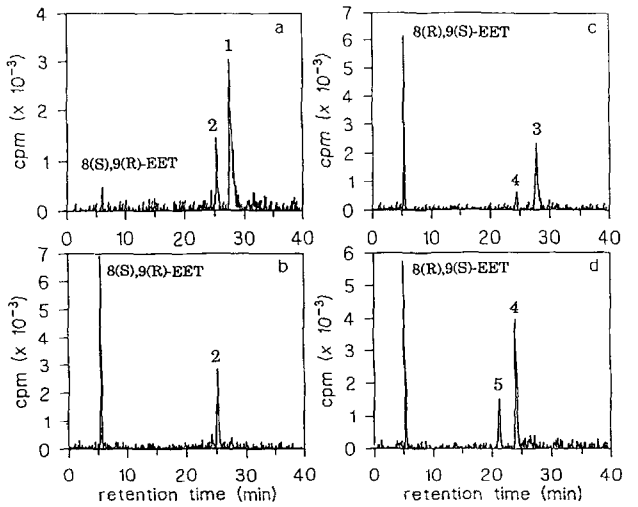

Fig. 1. HPLC radiochromatograms of ME derivatives from incubations of human platelets with: (a) 8(S),9(R)-EET. (b) 8(S),9(R)-EET + ionophore A-23187 (2 μM) and 4-PCO (60 μM). (c) 8(R),9(S)-EET. (d) 8(R),9(S)-EET + ionophore A-23187 (2 μM) and 4-PCO (60 μM).

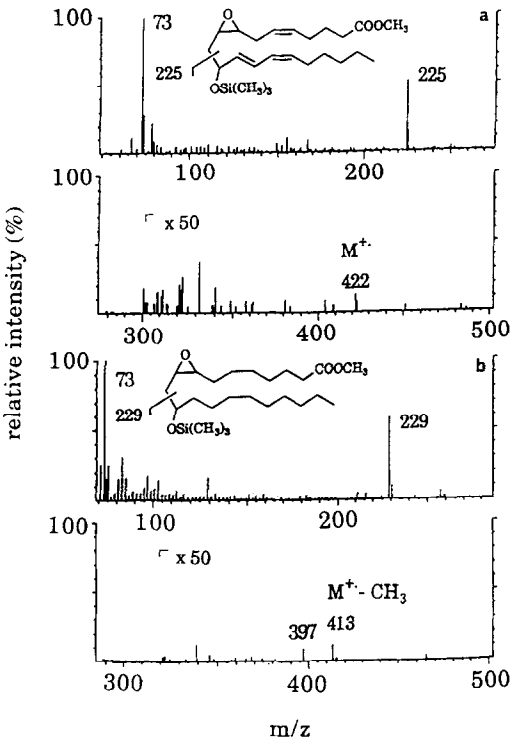

Fig. 2. EI mass spectra of (a) ME, TMS-derivative of metabolite 2 [11(R)-hydroxy-8(S),9(R)-EET]. (b) ME, TMS-derivative of metabolite 2 after hydrogenation.

providing evidence that the structure of metabolite 2 was 11-hydroxy-8(S),9(R)-EET. The formation of metabolite 2 was completely inhibited by indomethacin or aspirin and this suggested that it was a product of the cyclooxygenase enzyme rather than a lipoxygenase. Cyclooxygenase is known to hydroxylate eicosanoids at C-11 as a consequence of incomplete PG-endoperoxide formation (20-25). The epoxide moiety in 8,9-EET would serve to prevent cyclization to an endoperoxide and so the formation of an 11-hydroxy metabolite is in keeping with the known regioselectivity of the cyclooxygenase enzyme.

HPLC analysis of the products from incubations of [1-^{14}C]8(R),9(S)-EET with human platelets revealed that two metabolites were formed (metabolites 3,4; Figure 1c). Stimulation of the platelets with thrombin or ionophore A23187 resulted in decreased formation of metabolite 3, with a concomitant increase in metabolite 4 and the appearance of metabolite 5. Formation of metabolite 3 was completely inhibited by 4-PCO and metabolite 5 became much more prominent (Figure 1d). Metabolites 4 and 5 were inhibited by indomethacin and aspirin. The EI mass spectrum of the ME, TMS-derivative of metabolite 3 was identical with that observed for metabolite 1, indicating that it was 8,9-DHET. Metabolite 4 was a conjugated diene (λ_{max} 235 nm). It had identical EC and EI

mass spectra to metabolite 2, but the HPLC retention time of its ME was slightly shorter. This suggested that metabolite 4 was 11-hydroxy-8(R),9(S)-EET, a diastereomer of metabolite 2. Metabolite 5 was also a conjugated diene (λ_{max} 235 nm). GC/EIMS analysis of its ME, TMS-derivative was consistent with the assignment of its structure as 15-hydroxy-8(R),9(S)-EET. In particular, there was a fragment ion at *m/z* 351, which is typical of an α-cleavage at C-15 (19). The molecular weight of this fragment was unchanged on hydrogenation, providing confirmation that the molecule had undergone hydroxylation at C-15. Cyclooxygenase-mediated hydroxylation of arachidonic acid at C-15 has been observed previously (23,25).

Hydroxylation at C-11 and C-15 can be mediated by lipoxygenase enzymes (24,26-28) and so it was important to definitively establish that the 11- and 15-hydroxy-EETs arose from cyclooxygenase. 8,9-EET turned out to be an excellent substrate for the cyclooxygenase purified from ram seminal vesicles. Three major products were observed and they corresponded to the cyclooxygenase-dependent metabolites obtained in the incubations of 8,9-EET with human platelets. This confirmed that the platelet products were indeed formed from cyclooxygenase and that 8(R),9(S)-EET selectively underwent oxygenation at C-15. There did not appear to be a significant difference in the rate of cyclooxygenase-mediated 11-hydroxylation between the two EET enantiomers, although there was a clear enantioselective preference of the platelet epoxide hydrolase for the 8(S),9(R)-enantiomer (Figure 1).

Previous studies have shown that cyclooxygenase specifically hydroxylates arachidonic acid in the (R)-configuration at C-11 (24,25) so that oxygen is delivered antarafacially from the site of hydrogen abstraction (13-L hydrogen). If hydroxylation of 8,9-EET follows the same anatarafacial rule (24) then the hydroxylated metabolites would also be in the 11(R)-configuration. To test this possibility, 11-hydroxy-8,9-EETs were prepared from 11(R)-HETE by reaction with *m*-chloroperbenzoic acid. The two diastereomeric 11-hydroxy-8,9-EETs isolated from the reaction mixture were identical (HPLC, GC/MS) with metabolites 2 and 4 from the platelet incubations. Based on these data, and the known stereoselectivity of cyclooxygenase (24,25), metabolites 2 and 4 were identified as 11(R)-hydroxy-8(S),9(R)-EET and 11(R)-hydroxy-8(R),9(S)-EET, respectively.

8(S),9(R)-EET is a renal vasoconstrictor (8). However, its activity is abolished in the presence of indomethacin, suggesting that 8(S),9(R)-EET could undergo further metabolism by cyclooxygenase to a vasoconstrictor substance. This raises the exciting prospect that 11(R)-hydroxy-8(S),9(R)-EET is the substance responsible for the vasoconstrictor activity of 8(S),9(R)-EET. Studies are currently under way to test this possibility.

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