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Arachidonic acid epoxygenase

Stereochemical analysis of the endogenous epoxyeicosatrienoic acids of human kidney cortex

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Mass spectral and chromatographic analysis demonstrates the presence of 14,15-, 11,12- and 8,9-epoxyeicosatrienoic acids (44%, 33% and 23% of the total, respectively) in human kidney cortex. Chiral analysis of the human renal expoxyeicosatrienoic acids shows the formation of 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids in a 1:1, 4:1 and 2:1 ratio of antipodes, respectively. These results demonstrate the biosynthetic origin of the human kidney 11,12- and 14,15-epoxyeicosatrienoic acids and suggest a role for renal cytochrome P-450 in the bioactivation of endogenous pools of arachidonic acid.

Cytochrome P-450; Epoxygenase; Arachidonic acid; Epoxyeicosatrienoic acid

1. INTRODUCTION

The metabolism of arachidonic acid by cyclooxygenase or lipoxygenases generates several eicosanoids which play significant roles in renal function [1,2]. More recently, in vitro studies have shown that subcellular fractions or isolated cell preparations obtained from rat, rabbit or human kidneys actively metabolize arachidonic acid via a cytochrome P-450 dependent epoxygenase activity to 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids [2–4]. The potential significance of this reaction has been highlighted by: (i) the documentation of the EETs as endogenous constituents of human urine and of rat and rabbit kidney [5–7], and (ii) by their potent in vitro biological activities as inhibitors of ion transport in isolated cortical collecting tubules [8] and of vasopressin stimulated water transport in isolated cortical collecting tubules and toad bladder [9,10]. Furthermore, in vivo studies in rat showed that renal arterial administration of 5,6-EET-Me induces vasoconstriction concomitant with a reduction in glomerular filtration rate [6].

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Abbreviations: EETs, *cis*-epoxyeicosatrienoic acids; EET-Me, methyl-*cis*-epoxyeicosatrienoate; EET-PFB, pentafluorobenzyl-*cis*-epoxyeicosatrienoate; HPLC, high-pressure liquid chromatography; GC, gas-liquid chromatography; NICI/MS, electron capture negative ion chemical ionization mass spectrometry

Whereas biochemical studies of the epoxygenase reaction have been indispensable in the enzymatic characterization of this metabolic pathway, they provide only limited information regarding its participation in the metabolism of endogenous arachidonic acid, the in vivo formation of its metabolites and their organ steady state concentrations. We document herein the presence of EETs in human kidney and demonstrate the enantioselective formation of 14,15- and 11,12-EET by the human kidney cortex.

2. MATERIALS AND METHODS

Samples of human kidney cortex from adult males were obtained within 12 h of accidental death and maintained in liquid N_2 until use. Prior to freezing, they were maintained in iced Euro-Collins solution as part of our routine protocol of maintaining tissue for human transplantation. Only organs excluded for human use for technical reasons were studied. The protocol utilized for EET extraction and purification is that of [11]. Briefly, the frozen tissue was homogenized in CH_3OH/H_2O (2:3 v/v; 20 ml/g wet tissue) containing 0.1 mM triphenylphosphine. Aliquots of the homogenates were transferred to test tubes containing an equivalent volume of a 2.0 mM solution of triphenylphosphine in $CHCl_3$ and an equimolar mixture of [1-^{14}C]EET internal standards (55 mCi/mmol) (0.02–0.03 μCi, 0.15–0.13 μg, each of 14,15-, 11,12- and 8,9-EET). After mixing and centrifugation, the organic phase was collected and evaporated under argon. To the residue, 1.0 ml of 0.2 N KOH/CH_3OH (2:8 v/v) was added. After 1 h at 50°C, the suspension pH was adjusted to 4.0, the organic material extracted into ethyl ether, concentrated under argon, dissolved in HOAc/hexane (0.5:99.5 v/v) and purified by SiO_2 column (200–400 mesh, 5 × 25 mm) as described [11]. The radiolabeled EETs were resolved into 14,15-EET and a coeluting mix-

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ture of 8,9- and 11,12-EET by reversed phase HPLC [11]. The methyl ester of 14,15-EET was prepared as in [11]. The mixture of 8,9- and 11,12-EET was converted to the corresponding pentafluorobenzyl esters by reaction with pentafluorobenzyl bromide [11]. The mixture of 8,9- and 11,12-EET-PFB was resolved by normal phase HPLC on a 5-μm Dynamax Microsorb Si column (4.6 × 250 mm, Rainin Instruments Co.) utilizing a solvent mixture of 2-propanol/hexane (0.17:99.83 v/v), isocratically at 2 ml/min (R_t: 13.7 and 16.5 min for 8,9- and 11,12-EET-PFB, respectively). The optical isomers of 14,15-EET-Me, 8,9- and 11,12-EET-PFB were resolved utilizing either a Chiralcel OB (14,15-EET-Me) or a Chiralcel OD column (8,9- and 11,12-EET-PFB) (4.6 × 250 mm, J.T. Baker Chemical Co.) as described [12]. Absolute configurations were assigned as reported [12]. The purified 8,9- and 11,12-EET-PFB enantiomers were analyzed and quantified by GC/NICI/MS. The resolved 14,15-EET-Me enantiomers were converted to their corresponding PFB ester derivatives as in [11].

Aliquots of the purified EET-PFB enantiomers were dissolved in dodecane and analyzed by GC/NICI/MS on a Nermag R1010C quadrupole instrument utilizing a 30 m SPB-30 fused silica column (0.32 mm inner diameter, 0.25-μm coating thickness, Supelco Inc., Bellefonte, PA) [11]. Quantifications were done by GC/NICI/selected ion monitoring at m/z 319 (loss of PFB from the endogenous EET-PFB) and 321 (loss of PFB from the [1-^{14}C]EET-PFB internal standard). The EET-PFB/[1-^{14}C]EET-PFB sample ratios were calculated from the integrated values of the corresponding ion current intensities. The [1-^{14}C]EET-PFB standards (55 mCi/mmol) were analyzed by GC/NICI/MS. All three standards showed an approximately 89 atom% enrichment in ^{14}C, with the expected base peak at m/z 321 (M-PFB). Enantiomerically pure 8,9-, 11,12- and 14,15-EET were prepared by total asymmetric synthesis according to published procedures [13,14]. Racemic standards were prepared as in [15,16]. [1-^{14}C]EET standards were synthesized from [1-^{14}C]arachidonic acid (55 mCi/mmol, Amersham Co., Arlington Heights, IL) as described [11].

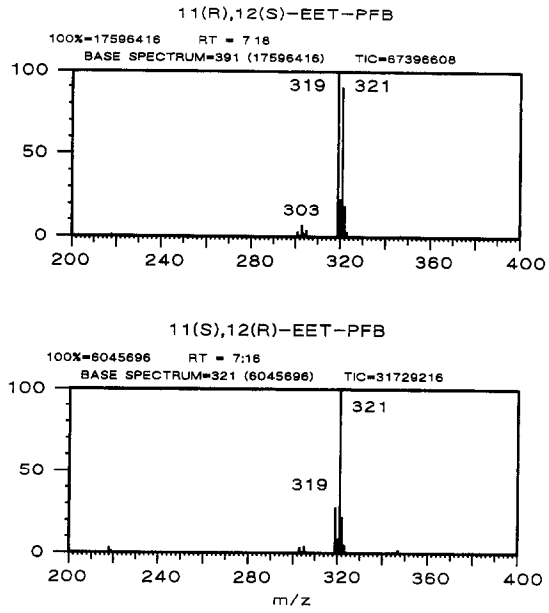

Fig. 1. Mass spectral fragmentation properties of the 12,12-EET-PFB enantiomers isolated from human kidney cortex. The 11(R),12(S)- (top) and 11(S),12(R)-EET-PFB enantiomers (bottom) purified from human kidney cortex were dissolved in a small volume of dodecane and analyzed by NICI/GC/MS as described in section 2. Shown are the fragmentation patterns generated by samples containing approximately 2 ng of each enantiomer. Abscissa, abundance as % of the base peak; ordinate, m/z.

3. RESULTS AND DISCUSSION

Synthetic [1-^{14}C]EETs (55 mCi/mmol) were utilized as internal standards for the following reasons: (i) they are readily synthesized from [1-^{14}C]arachidonic acid available in high isotopic purity, (ii) they coelute with the corresponding endogenous [$^{12}C_{20}$]EETs during all chromatographic procedures utilized, including chiral phase HPLC [11], (iii) they are easily quantified by liquid scintillation techniques, and (iv) the molecular mass difference (2 amu) between the [1-^{14}C]-labeled standards and the endogenous [$^{12}C_{20}$]EETs permits quantification by NICI/GC/MS. The validity of this experimental approach has been demonstrated [11].

Arachidonic acid autooxidation leads to the formation of reactive hydroperoxides which can then serve as precursors for the artifactual recovery of several icosanoids [17,18]. To minimize artifactual EET generation during sample extraction and purification, the peroxide reducing agent triphenylphosphine was used in all purification steps, prior to the removal of endogenous arachidonic acid by reversed phase HPLC. Control experiments demonstrated that under these experimental conditions, autooxidation leading to EET is negligible and below our detection sensitivity [11]. Since the labile 5,6-EET suffers extensive decomposition during the extraction and purification procedures utilized it was not further investigated.

The overall recovery of the [1-^{14}C]EET internal standards, prior to GC/MS analysis, was 20%, 22%, and 15% for 8,9-, 11,12- and 14,15-EET, respectively. Before quantification, each biological EET-PFB enantiomer was characterized by full scan NICI/GC/MS. For example, the material present in the biological samples with chromatographic properties identical to synthetic 11(R),12(S)-EET-PFB showed ion fragments derived from the [1-^{14}C]-labeled internal standard at m/z: 321 (90% abundance, loss of PFB) and 305 (4% abundance, loss of PFB and O) (Fig. 2A). Diagnostic ions for the endogenous 11(R),12(S)-EET-PFB were at m/z: 319 (base peak, loss of PFB) and 301 (3% abundance, loss of PFB and H_2O). The ion fragment at m/z 303 (7% abundance) was common to both the internal standard (loss of H_2O and PFB) and the endogenous EET (loss of O and PFB) (Fig. 2A). The fragment ions at m/z 322 and 320 (19% and 23% abundance, respectively) originate from the contribution of isotopic ^{13}C (1.108 atom% natural abundance) present in the endogenous and in the synthetic EET, after the loss of PFB (Fig. 2A). The mass spectra of the other enantiomer of 11,12-EET-PFB is shown in Fig. 2B. The fragmentation pattern of this sample shows the predominance of fragment ions derived from the [1-^{14}C]11(S),12(R)-EET-PFB internal standard at m/z

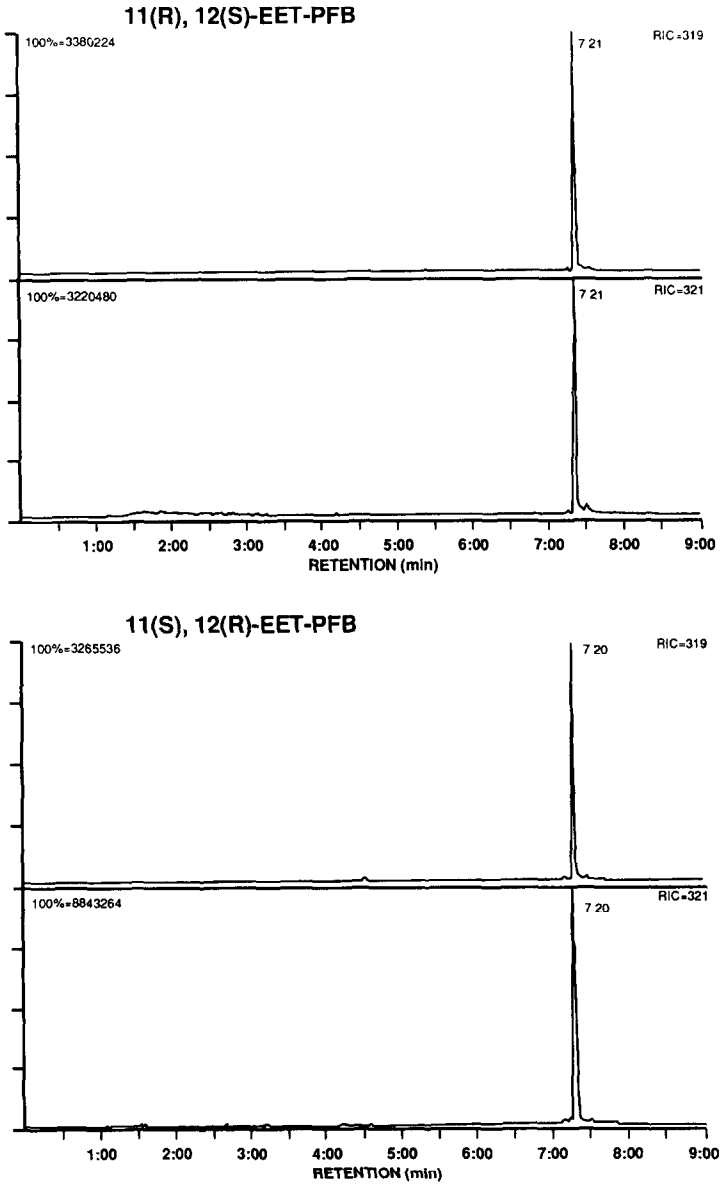

Fig. 2. Selected ion current profiles under NICI conditions for the 11,12-EET-PFB enantiomers present in human kidney cortex. The purified 11(R),12(S)- (top) and 11(S),12(R)-EET-PFB were individually analyzed by NICI/GC/selected ion monitoring at m/z 321 and 319 as described in section 2. The samples $^{14}C/^{12}C$ ratios were calculated from the integrated areas under the observed peaks. Abscissa, retention time in minutes; ordinate, abundance relative to the most intense peak.

Table I

Regioisomeric composition of the EETs isolated from human kidney cortex

EEG-regioisomer	ng/g wet tissue	% Distribution
8,9-EET	75 ± 14	23
11,12-EET	110 ± 4	33
14,15-EET	148 ± 12	44

The regioisomers of 8,9-, 11,12- and 14,15-EET were extracted, purified and quantified by NICI/GC/MS as described in section 2. Values are the averages ± SE calculated from 5 different experiments performed with samples extracted from two different human kidneys.

Table II

Stereochemical properties of the human kidney cortex endogenous EETs

EET-regioisomer	% R,S	% S,R
8,9-EET	51 ± 1	49 ± 1
11,12-EET	80 ± 1	20 ± 1
14,15-EET	66 ± 1	34 ± 1

The enantiomers of 8,9-, 11,12- and 14,15-EET were purified, resolved and quantified as described in section 2. Values are the averages ± SE calculated from 5 different experiments performed with samples extracted from two different human kidneys.

(% abundance) 321 (100), 322 (22), 305 (4) and 303 (4). Fragment ions for the corresponding $^{12}C_{20}$ endogenous enantiomer were at *m/z* (% abundance) 319 (28), 320 (8), 301 (2) (Fig. 2B).

For quantification, aliquots of the purified biological samples were analyzed by GC/NICI/selected ion monitoring at 321 and 319. For each sample, the [1-^{14}C]EET-PFB standard/endogenous EET-PFB ratio was calculated by integration of the areas under the corresponding ion current profiles (Fig. 2 for 11,12-EET-PFB). The concentration of each EET enantiomer in human kidney followed directly from the calculated ratio and the amount of internal standard added to the initial kidney homogenate.

The tissue extracts contained 14,15-EET as their major component (44% of the total) followed by 11,12- and 8,9-EET (33% and 23%, respectively) (Table I). Quantitative analysis showed that human kidney cortex contains approximately 333 ng of total EET/g of wet tissue (Table I). This value is within the same order of magnitude as reported for rat and rabbit kidney [5,6].

Chiral analysis of the endogenous EETs in human kidney showed that 11,12- and 14,15-EET are produced enantioselectively whereas the 8,9-regioisomer is racemic (Table II). Since stereoselective formation of eicosanoids is a sufficient criterion to establish their enzymatic origin we conclude that 11,12- and 14,15-EET are produced, in vivo, by the human kidney epoxygenase. Although the evidence for the 8,9-regioisomer at present is not conclusive we propose the enzymatic formation of both 8,9-EET antipodes by individual cytochrome P-450 forms with opposing enantiofacial selectivities. We have previously demonstrated that the enantiofacial selectivity of the epoxygenase reaction is under regulatory control and is dependent on the tissue cytochrome P-450 isoenzyme composition [11,19]. Additionally, the coexistence in the microsomal membrane of cytochrome P-450 epoxygenase forms with opposing enantioselectivities has been demonstrated [19].

The key role of the cytochrome P-450 protein catalyst in controlling the stereoselectivity of oxygenation is further evidenced by comparing the data in Table II with published values for rat liver EETs [11]. Thus, while the human renal and the rat liver epoxygenase(s) showed similar stereoselectivity towards the arachidonate 14,15-olefin, they epoxidized the 11,12-olefin with opposite enantiofacial selectivity, with the hepatic enzyme generating 11(S),12(R)-EET as the major antipode [11].

From the foregoing evidence, as well as published data [11,19], we conclude that the EETs are endogenous constituents of human kidney cortex generated, in vivo, by the tissue arachidonic acid epoxygenase. These results, in conjunction with the potent biological activities of the EETs, suggest a function for human renal cytochrome P-450(s) in the control of kidney function.

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