Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5893–5897, August 1990
Biochemistry

Endogenous biosynthesis of arachidonic acid epoxides in humans: Increased formation in pregnancy-induced hypertension

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Communicated by K. Frank Austen, May 14, 1990

ABSTRACT **Arachidonic acid is metabolized by means of P450 isoenzyme(s) to form epoxyeicosatrienoic acids (EETs) and their corresponding dihydroxy derivatives (DHETs). In the present study, we established the presence in human urine of 8,9-, 11,12-, and 14,15-EETs and their corresponding DHETs by developing quantitative assays and using negative ion, chemical ionization GC/MS and octadeuterated internal standards. Urinary excretion of 8,9- and 11,12-DHET increased in healthy pregnant women compared with nonpregnant female volunteers. By contrast, excretion of 11,12-DHET and 14,15-DHET, but not the 8,9-DHET regioisomer, increased even further in patients with pregnancy-induced hypertension. Intravenous administration of [^{3}H]14,15-EET to three dogs markedly increased its DHET in plasma. The terminal half-life ranged from 7.9–12.3 min and the volume of distribution (3.5–5.3 liters) suggested limited distribution outside the plasma compartment. Negligible radioactivity was detected in urine; this fact infers that under physiological circumstances, urinary DHETs largely derive from the kidney. That P450 metabolites of arachidonic acid are formed in humans supports the hypothesis that these metabolites contribute to the physiological response to normal pregnancy and the pathophysiology of pregnancy-induced hypertension.**

Studies *in vitro* have demonstrated that arachidonic acid is metabolized by P450 monooxygenases (1–3). Indeed, a P450 isoenzyme with substrate specificity for arachidonate acid has recently been purified from human liver (4). The products of this reaction include hydroxyeicosatetraenoic acids, ω and ω-1 alcohols, and epoxyeicosatrienoic acids (EETs) and their corresponding diols (5).

Among the biological properties of cytochrome P450-dependent arachidonic acid metabolites *in vitro* are the modulation of vascular tone (6–8) and the regulation of ion transport (9, 10) and water handling (11) in the renal tubule and toad bladder (12). These metabolites inhibit Na^+/K^+-transporting ATPase in the cornea (13), depress glomerular filtration (14) and inhibit platelet function (15). In addition, the vascular capacity to form these compounds has recently been shown (16, 17) to be exaggerated in several animal models of hypertension.

Despite these observations and the regionally selective formation of endogenous EETs in rat liver (18), skepticism has persisted about the role of EETs in human disease *in vivo*. This uncertainty stems from a paucity of information on the actual synthesis of these compounds *in vivo* (19) and caution in extrapolating tissue capacity for forming EETs in the *in vivo* situation. For example, for other arachidonic acid metabolites, tissue capacity exceeds *in vivo* formation rates by orders of magnitude and has been a misleading guide to alterations in prostaglandin biosynthesis (20).

To study whether disordered EET formation might contribute to the pathophysiology of human disease, we assessed the *in vivo* synthesis of EETs in a setting where their properties might be of pathophysiological relevance, that of pregnancy-induced hypertension (PIH) (21–24).

METHODS

GC/MS Analysis of Urinary EETs. *Synthesis of the deuterated analogs.* Octadeuterated epoxide standards were prepared by nonselective epoxidation of [$^2H_8$]arachidonic acid by *m*-chloroperoxybenzoic acid.Thus, 100 μg of 5,6,-8,9,11,12,14,15-[^{2}H]arachidonic acid (0.32 μmol) was placed in a 1.0-ml reaction vial and concentrated to dryness under a nitrogen stream. The arachidonic acid was then dissolved in 31 μl of dry methylene chloride and the vial was cooled at 0°C in an ice-water slurry. *m*-Chloroperoxybenzoic acid was then added in 69 μl of dry methylene chloride and the solution was placed under dry argon, mixed on a Vortex mixer and warmed to room temperature for >1 hr. The solvent was next removed with a stream of dry nitrogen, and the reactants were redissolved in methanol/water/acetic acid 80:20:0.01 (vol/vol/vol). The products were chromatographed over a 5-μm reversed-phase column (4.6 × 250 mm; Altex) using methanol/water/acetic acid, 80:20:0.01 (vol/vol/vol) as the mobile phase at a flow rate of 1.0 ml per min. Absorbance was monitored at 210 nm and the epoxides were manually collected. The 14,15-epoxide appeared at 16.1 min as a single peak. The 11,12-epoxide appeared at 18.4 min and was collected as a mixture with the 8,9-epoxide, which appeared at 19.1 min. The 14,15-epoxide was used without further purification, whereas the 11,12-epoxide was separated from the 8,9-epoxide by straight-phase chromatography. Thus, the 8,9- and 11,12-epoxides were concentrated to dryness and taken up in 100 μl of hexane. They were then chromatographed over a straight-phase chromatography column (5 μm reversed-phase 4.6 × 250 mm; Altex) using hexane/isopropanol/acetic acid, 100:0.4:0.1 (vol/vol/vol) as the mobile phase at a flow rate of 2.0 ml/min. Absorbance was monitored at 210 nm and the epoxides were again manually collected. The 11,12-epoxide appeared at 17.2 min and the 8.9-epoxide appeared at 22.9 min. The epoxides were then used without further purification after quantitation by comparison with a known amount of undeuterated synthetic material.

Extraction and purification. The approach taken was similar to that used by Turk *et al.* (25). Ten microliters of methanol were added to 10 ml of urine containing a mixture of the octadeuterated analogs of the 8,9-, 11,12-, and 14,15-EETs. After equilibrating for ≈15 min at room temperature, 1 ml of 70% (vol/vol) perchloric acid was added and the samples were allowed to stand at room temperature for 6 hr. Acidification quantitatively converted the epoxides to their respective diols. The urine was passed through a C_{18} cartridge (PrepSep, Fisher) conditioned with 2 ml of methanol

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Abbreviations: EET, epoxyeicosatrienoic acid; DHET, dihydroxy derivative of EET; PIH, pregnancy-induced hypertension.

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and 2 ml of water. The cartridge was then washed with 10 ml of 60% (vol/vol) methanol/water and dried by applying a vacuum. Diols were eluted with 1.8 ml of ethyl acetate, dried under a gentle stream of nitrogen, dissolved in 30 μl of methanol, and applied to the preadsorbent zone of a silica gel TLC plate (LK6D, Whatman, Inc.). The plate was developed twice in a solvent of heptane/ethyl acetate/acetic acid (25:15:0.3). Two separate zones were scraped; the lowest contained the 8,9-isomer and the highest contained the 11,12- and 14,15-isomers. This latter pair of isomers separate on the GC column. Zones of interest were identified by simultaneously developing a plate loaded with ≈2 μg each of the three diols. This plate was visualized by spraying with 10% (wt/vol) phosphomolybdic acid/ethanol and heating on a hot plate. When a single isomer was being purified, the extraction procedure allowed more complete recovery. However, when multiple isomers were analyzed, the zones scraped from the TLC were more narrow to ensure isomer purity; this technique reduced recovery to ≈85%. However, accuracy of the stable isotope-dilution assay is independent of recovery at this step. After being scraped, the silica gel was placed into a microcentrifuge tube and 1 ml of ethyl acetate was added with enough 1% formic acid to cover the silica gel. After mixing and centrifuging, the upper phase was transferred to another tube and dried. The pentafluorobenzyl ester was formed by adding 10 μl of diisopropylethanolamine and 20 μg of 12.5% pentafluorobenzyl bromide in acetonitrile and holding the samples at room temperature for 10 min. After drying the reagents, samples were dissolved in 30 μl of methanol and applied to a TLC plate as above. The plate was developed with a mobile phase consisting of heptane/ethyl acetate (24:16), and the samples were visualized and scraped as above, substituting distilled water for 1% formic acid. The dried samples were converted to the trimethylsilyl ether derivatives by adding 10 μl of both pyridine and *N,O*-bistrimethylsilyl trifluoroacetamide and holding samples at room temperature for 30 min. Reagents were then dried, and samples were dissolved in 20 μl of dodecane for GC/MS analysis.

Plasma samples, after being treated with the octadeuterated analogs, were acidified with 1% formic acid and applied on the preconditioned C_{18} cartridge and eluted with ethyl acetate that was subsequently dried under nitrogen. Samples were then redissolved in 100 μl of 7% (vol/vol) perchloric acid left at room temperature for 6 hr and extracted on a C_{18} cartridge. The subsequent steps were as for the urinary samples.

Measurement. A Nermag 10-10C mass spectrometer (Nermag, Paris) operated in the electron-capture negative-ion/chemical ionization mode (methane), interfaced to a Nermag SIDAR data system and a Varian Vista 6000 gas chromatograph (Varian), was used for quantitation. A 15-m DB-1 column (i.d. 0.25 mm) was heated at 20°C per min from 190 to 300°C. The injector and interface were maintained at 260°C; carrier gas was helium. The ions monitored were mass/charge (*m/z*) 481 for the endogenous diols and *m/z* 489 for the octadeuterated analogs. The full negative-ion/chemical ionization mass spectra of the pentafluorobenzyl derivatives of DHETs show that fragment ions other than that corresponding to the loss of the *m*-pentafluorobenzyl ester moiety are irrelevant for quantitative purposes. Peak height ratios were used for measurement. Linear correlation ($r = 0.89$; $P < 0.001$) of the $^2H_0$:$^2H_8$ species was observed over the range of biological quantitation. Water blanks, samples in which deionized water was substituted for urine or plasma, were prepared simultaneously and measured similarly. The ratio of $^2H_0$ to $^2H_8$ species was <0.01. These values were subtracted from the observed sample ratios. Limits of detection for urinary DHETs in these assays approximated 10 pg/ml.

Study population. The patients' clinical parameters are displayed in Table 1. All patients had hypertension and proteinuria and the majority had low platelet counts. Control groups consisted of normotensive pregnant women and of age-matched female volunteers who had a negative pregnancy test.

Assessment of the origin of urinary EETs. Because of a recent report (26) identifying a product of human reproductive tissues that comigrates with authentic 14,15-EET, we selected this isomer as the focus of studies designed to assess the potential extrarenal contribution to urinary DHETs.

[^{3}H]14,15-EET was synthesized by the same procedure used for the deuterated analog. Thirty microcuries (1 Ci = 37 GBq) was mixed with 1.5 mg of unlabeled material to give a specific activity of 6.4 μCi/μmol. The sodium salt was attained by dissolving equivalent nmol of 14,15-EET in 50 mM Na_2CO_3. This mixture was then diluted to 10 ml with saline and injected as a bolus into the antecubital vein of two mongrel dogs anesthetized with pentobarbitone 20 mg/kg i.v. supplemented with 1–2 mg/kg as required. A third anesthetized dog was given only the unlabeled material.

Ten-milliliter blood samples were collected from the contralateral vein before injection, and 0.5, 1, 2, 5, 15, and 30 min and 1, 2, 4, and 8 hr afterward into syringes containing EDTA and β-hydroxytoluene at 5 mM and 0.002% final concentrations, respectively. Urine was collected through a bladder catheter 1 hr before injection and at 1-hr intervals for 8 hr in the two dogs that received radioactive material. Plasma and urine samples were immediately treated and stored frozen until assayed.

Statistical and pharmacokinetic analysis. Comparison between the groups was based on nonparametric analysis of variance followed by pairwise comparisons as appropriate (27). Pharmacokinetic analysis was based on the plasma concentrations of 14,15-DHET (as measured by GC/MS)—which fell below the limit of detection of the method 1 hr after injection—and were fitted to a two-compartment model (28). Systemic clearance was calculated as the ratio of the dose of [^{3}H]14,15-EET infused to the area under the plasma concentration time curve with values >1 hr extrapolated to infinity. Areas under the plasma concentration time curves were calculated by using the logarithmic trapezoidal rule for descending data (29).

RESULTS

Definitive confirmation of the presence of EETs in human urine was based on a synthetic compound characterized by electron impact mass spectra of the methyl ester bistrimethylsilyl ether derivatives of the diols. A representative spectrum of the 14,15-diol is depicted in Fig 1.

Urinary excretion of 14,15-DHET (Fig. 2 *Top*) in nonpregnant women ranged from below limits of detection (≈3 pg/mg of creatinine) to 24 pg/mg of creatinine. In healthy pregnant women, there was a trend toward an increase in 14,15-DHET (range, <10–118) with a median of 85 pg/mg of creatinine;

Table 1. Preeclamptic patients

Patient	Gestational age, weeks	Blood pressure, mmHg	Proteinuria	Platelet count, $\times 10^3/\mu l$
1	40	176/109	2+	200
2	30	232/112	4+	108
3	24	172/108	3+	50
4	33	154/110	2+	25
5	31	190/120	4+	313
6	31	190/118	4+	62
7	29	162/104	4+	38
Mean ± SE	31 ± 1.8	182 ± 10/112 ± 3	3 ± 0.4	114 ± 40

however, the difference was not statistically significant. Urinary excretion in patients with severe PIH ranged between 679 and 22,000 pg/mg of creatinine (median, 2781) and was significantly elevated ($P < 0.05$) compared with the levels excreted in healthy pregnant women.

Similarly, urinary excretion of 11,12-DHET (Fig. 2 *Middle*) was increased in healthy pregnancy as compared with nonpregnant women (range, 23–142.3 versus <3–17 pg/mg of creatinine; median, 43 versus 3; $P < 0.01$) and a further marked increase (range, 104–5500 pg/mg of creatinine; median, 364; $P < 0.01$) was seen in patients with pregnancy-induced hypertension. By contrast, urinary excretion of the 8,9-isomer (Fig. 2 *Bottom*) was increased in normal pregnancy (range, 58.9–394 versus 5.1–110 pg/mg of creatinine; median, 121 versus 27.1 pg/mg of creatinine; $P < 0.001$) with no further increase in women affected with severe pregnancy-induced hypertension (range, 85–643; median, 200; P = not significant).

After the bolus injection of [^{3}H]14,15-EET, the concentration of 14,15-DHET (as detected by GC/MS) in dog plasma went up from a baseline of 0.33 ± 0.6 ng/ml to 781 ± 82.5 ng/ml after 30 sec. When a two-compartment pharmacokinetic analysis was used, 14,15-DHET disappeared from the circulation with a median terminal half-life of 11.9 min (Table 2; Fig. 3). However, only a negligible percentage of the radioactivity injected appeared in the urine of the two dogs in which it was measured during the 8 hr after injection (Table 3). The low (median 4.8 liters) volume of distribution was consistent with the compound being largely confined to the plasma compartment (Table 2). Noncompartmental analysis of the data (30) provided a similar, low estimate of volume of distribution (range, 4.12–7.43 liters).

DISCUSSION

Despite their striking properties *in vitro*, there has been little evidence that EETs are formed *in vivo*. Bioassays for these compounds have not been used to quantitate their formation, and RIAs have yet to be developed. Toto *et al.* (19) reported detection of EETs in human urine. However, a labeled internal standard was not incorporated in the assay, precluding reliable measurement. We have developed a stable isotope-dilution GC/MS assay using octadeuterated epoxides as internal standards. This method permits us to account for the conversion of EETs to the corresponding DHETs due to chemical hydrolysis during the isolation and purification procedure. This conversion is important, as chemical or enzymatic hydrolysis to DHETs may partially account for the apparent biological actions of EETs *in vitro*.

In the present study, we provide evidence for endogenous formation of P450-derived metabolites of arachidonic acid in humans. Consistent with this finding reflecting formation of the compounds *in vivo*, rather than autoxidation of arachidonic acid in urine *ex vivo*, was the observation that quantitation was not influenced by adding the antioxidant triphenylphosphine to urine or by delaying analysis of the sample at room temperature for 24 hr.

To address the hypothesis that EETs might be important in human disease, we have searched for alterations in their biosynthesis in a setting where their biological properties might be relevant. Pregnancy-induced hypertension is a common complication of pregnancy and is associated with both fetal and maternal morbidity (22, 31, 32). Although the cause of this disease is unknown, it is characterized by elevated blood pressure, decreased plasma volume, and decreased placental blood flow. Renal abnormalities include decreased glomerular filtration rate and renal plasma flow (23).

Abnormalities of eicosanoid formation have been described in PIH. For example, the increase in prostacyclin biosynthesis, which characterizes normal pregnancy, is blunted in women destined to develop PIH (33). This may relate to the increase in vascular sensitivity to infused pressor agents (34, 35), which also precedes the rise in blood pressure in such patients (36). Platelets are activated in normal pregnancy (37) and this fact is reflected by an increase in the biosynthesis of the predominant cyclooxygenase product in platelets, thromboxane A_2 (38). We have recently observed a further increment in thromboxane formation in patients with moderate-to-severe PIH. This condition correlates with their mean arterial pressure, plasma lactate dehydrogenase, and the degree of thrombocytopenia (39). Several small studies, which suggest that pretreatment with aspirin may reduce the incidence of PIH in women at risk of developing the disease (40–43), also support the possibility that thromboxane formation is of functional importance, perhaps contributing to reduced placental blood flow (22, 24).

In the present study, we provide evidence that biosynthesis of the P450-derived metabolites of arachidonic acid is altered in healthy pregnant women compared with nonpregnant controls. Patients with established PIH had alterations in EET biosynthesis distinct from and additional to those seen in normal pregnancy. This result is most unlikely to reflect either nonspecific autoxidation of an increase in urinary arachidonic acid or a generalized induction of cytochrome P450 in pregnancy. In both cases, excretion of all four isomers would be increased to a similar extent. By contrast, we found that while biosynthesis of the 11,12- and 8,9-DHETs was increased in healthy pregnant women, excretion

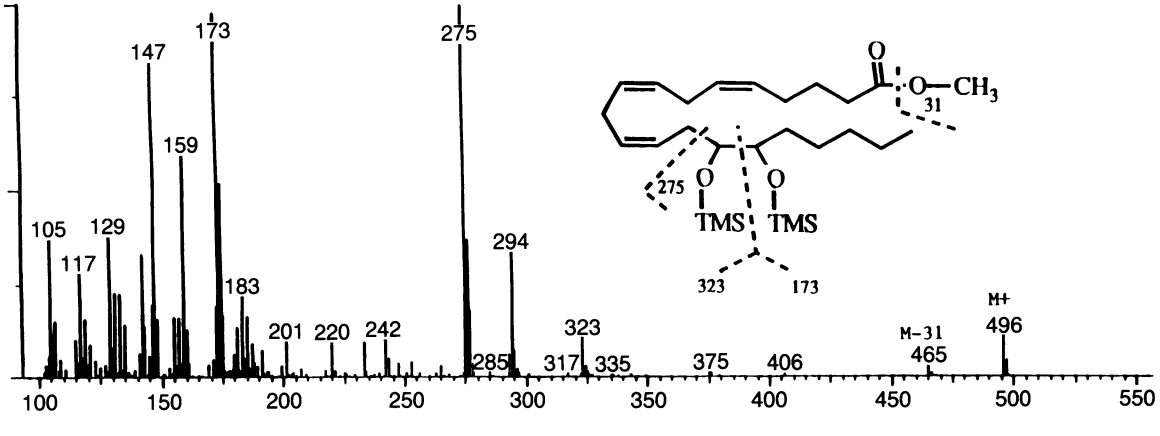

FIG. 1. Electron impact mass spectrum of the methyl ester, bistrimethylsilyl (TMS) derivative of 14,15-DHET. Major fragmentation sites are indicated by the dashed lines, and the corresponding ions are noted; these are apparent in the mass spectrum.

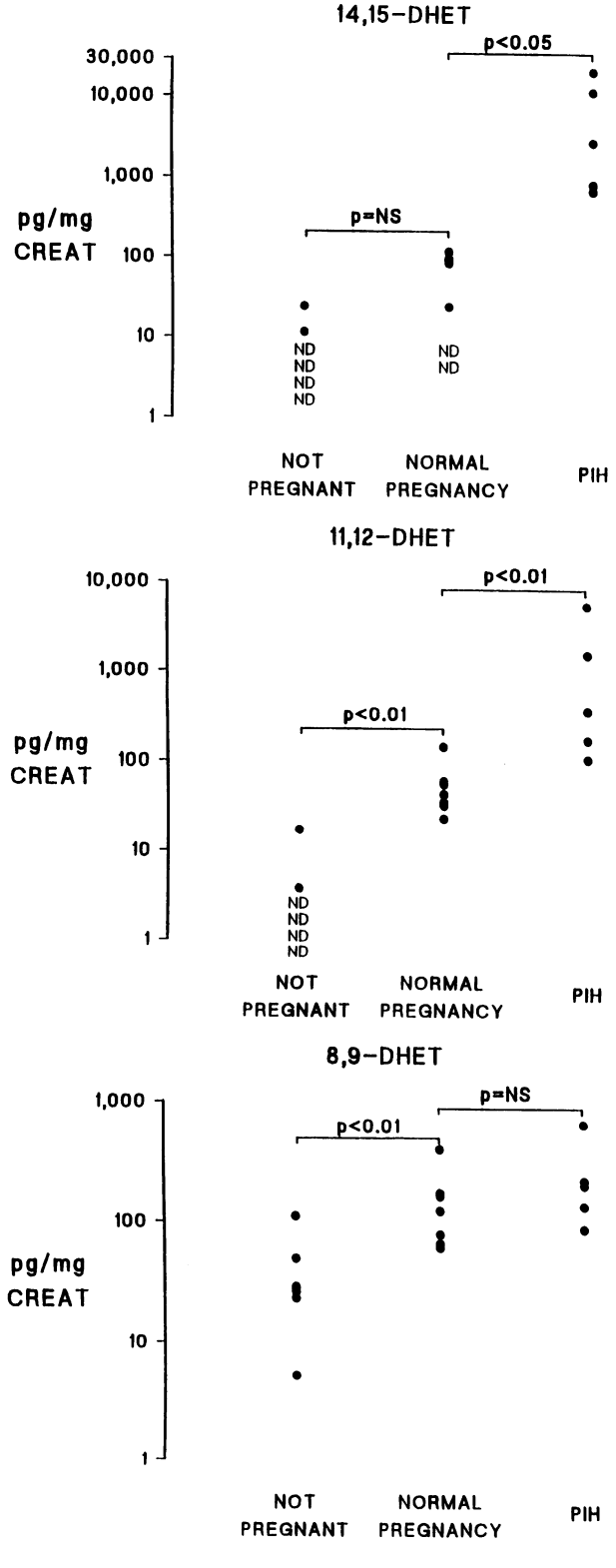

FIG. 2. Quantitation of 14,15-DHET (*Top*), 11,12-DHET (*Middle*), and 8,9-DHET (*Bottom*) in human urine. Values are depicted for women in whom pregnancy was excluded, women in the third trimester of normal pregnancy, and women with PIH. CREAT, creatinine; ND, not detectable; NS, not significant.

of the 11,12- and 14,15- isomers, but not of 8,9-DHET, was further augmented in patients with PIH. Specific inhibitors of EET biosynthesis or action are not available to elucidate further the functional importance of these changes in humans. Acetylenic fatty acid-based inhibitors (44) have been developed for a P450 with high affinity for arachidonic acid expressed in the lungs of pregnant rabbits (45).

Table 2. Pharmacokinetic parameters of 14,15-DHET

Dog	Systemic clearance, liters/hr	Terminal half-life, $t_{1/2\beta}$ in min	Volume of distribution, liters
1	21.098	11.94	3.45
2	36.698	7.86	4.798
3	36.499	12.3	5.255

These parameters were derived using a two-compartment model based on EDFAST (27). See text for details.

Should P450-derived metabolites of arachidonic acid have a role in pregnancy and/or its hypertensive complication, one possible site of action is the kidney. Studies *in vitro* (46, 47) have shown that these compounds are major products of arachidonic acid metabolism in the renal cortex and the cells of the thick ascending limb of the loop of Henle and are endogenous constituents of renal tissue (48). In rat kidney, EETs constitute ≈1 ng/g of wet tissue (14). Schwartzmann *et al.* (49) have partially identified the major arachidonic acid products of the cells of the thick ascending limb of Henle's loop as 11,12-DHET and 5,6-EET. The chromatographic fractions corresponding to these products were shown to inhibit Na^+/K^+-ATPase and to relax vascular smooth muscle, respectively. (*R*)-12-Hydroxyeicosatetraenoic acid, a possible derivative of 11,12-DHET, inhibits Na^+/K^+-ATPase in the cornea (10).

The tissue of origin of the compounds we measured in human urine was not addressed in this study. Given the tentative identification of 14,15-EET as a product of human reproductive tissues (26) and that the increment in 14,15-DHET excretion in PIH was so impressive, a fetoplacental origin of the increment in urinary epoxides seen in pregnant patients is possible. However, our experiments in the dog suggest that, at least in the absence of pregnancy, urinary DHETS probably derive predominantly from the kidney. Thus, whereas systemic administration of radiolabeled 14,15-EET resulted in readily detectable concentrations in plasma, only a negligible quantity of radioactivity was found in urine. DHETs could be subject to tubular uptake and secretion with the kidney. Changes in the erythrocyte electrolyte transport response to dietary sodium have been described (50) in human pregnancy. However, a discrete defect of tubular function, specific to PIH, has not been described and differential tubular handling of the various DHETs would be unexpected.

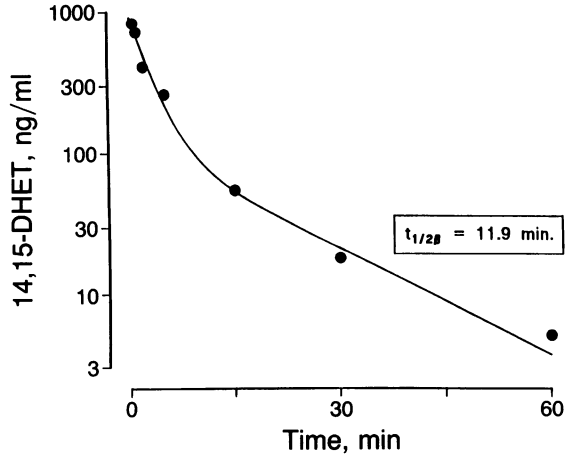

FIG. 3. Representative trace of 14,15-DHET in canine plasma after a bolus injection i.v. of 30 μCi of [^{3}H]14,15-EET mixed with 1.5 mg of the unlabeled material as described.

Table 3. Urinary recovery of 14,15-EET

	Radioactivity recovered after i.v. injection, %							
Dog	0–1 hr	0–2 hr	2–3 hr	3–4 hr	4–5 hr	5–6 hr	6–7 hr	7–8 hr
1	2	0.3	0.6	0.5	0.4	0.3	0.4	0.15
2	1.9	0.1	0.1	0.1	0.08	0.06	0.06	0.06

In summary, we have developed a noninvasive, specific and sensitive method for measuring EETs and their corresponding vicinyl diols that establishes that P450-derived metabolites of arachidonic acid are formed in humans. Experiments in the dog suggest that the turnover of these compounds in plasma is rapid and that the contribution of circulating levels to urinary EETs is probably minimal under physiological conditions. We have also demonstrated that EET biosynthesis is increased in human pregnancy and that a further increment in formation of the 11,12- and 14,15-isomers occurs in patients with PIH. These observations and the *in vitro* properties of these compounds are consistent with the hypothesis that P450-derived metabolites of arachidonic acid are of importance in the physiological response to human pregnancy and the pathophysiology of PIH.

This work was supported by grants from the National Institutes of Health (HL 30400 and HD 05797), the Pharmaceutical Manufacturers' Association Foundation and Daiichi Seiyaku. G.A.F. is an Established Investigator of the American Heart Association and the William Stokes Professor of Experimental Therapeutics.

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