Cytochrome P450/NADPH-dependent biosynthesis of 5,6-*trans*-epoxyeicosatrienoic acid from 5,6-*trans*-arachidonic acid

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5,6-*trans*-AA (5,6-TAA, where TAA stands for *trans*-arachidonic acid) is a recently identified *trans* fatty acid that originates from the *cis*–*trans* isomerization of AA initiated by the NO₂ radical. This *trans* fatty acid has been detected in blood circulation and we suggested that it functions as a lipid mediator of the toxic effects of NO₂. To understand its role as a lipid mediator, we studied the metabolism of 5,6-TAA by liver microsomes stimulated with NADPH. Profiling of metabolites by liquid chromatography/MS revealed a complex mixture of oxidized products among which were four epoxides, their respective hydrolysis products (dihydroxyeicosatrienoic acids), and several HETEs (hydroxyeicosatetraenoic acids) resulting from allylic, bis-allylic and ($\omega - 1$)/($\omega - 2$) hydroxylations. We found that the C5–C6 *trans* bond competed with the three *cis* bonds for oxidative metabolism mediated by CYP (cytochrome P450) epoxygenase and hydroxylase. This was evidenced by the detection of 5,6-*trans*-EET (where EET stands for epoxyeicosatrienoic acid), 5,6-*erythro*-dihydroxyeicosatrienoic acid and an isomer of 5-HETE. A standard of 5,6-*trans*-EET obtained by iodolactonization of 5,6-TAA was used for the unequivocal identification of the unique

microsomal epoxide in which the oxirane ring was of *trans* configuration. Additional lipid products originated from the metabolism involving the *cis* bonds and thus these metabolites had the *trans* C5–C6 bond. The 5,6-*trans*-isomers of 18- and 19-HETE were likely to be products of the CYP2E1, because a neutralizing antibody partially inhibited their formation without having an effect on the formation of the epoxides. Our study revealed a novel pathway of microsomal oxidative metabolism of a *trans* fatty acid in which both *cis* and *trans* bonds participated. Of particular significance is the detection of the *trans*-epoxide of AA, which may be involved in the metabolic activation of such *trans* fatty acids and probably contribute to their biological activity. Unlike its *cis*-isomer, 5,6-*trans*-EET was significantly more stable and resisted microsomal hydrolysis and conjugation with glutathione catalysed by hepatic glutathione S-transferase.

Key words: arachidonic acid, biosynthesis, cytochrome P450, free radical, microsomal epoxidation and hydroxylation, *trans* fatty acid.

INTRODUCTION

In addition to functioning as a precursor of prostaglandins and leukotrienes, AA (arachidonic acid) is also a target for free radical-mediated transformations, many of which have been detected *in vivo*. For example, a peroxidation mechanism initiated by ROS (reactive oxygen species) contributes to the formation of prostaglandin-like products (*F*₂-isoprostanes) in many pathological settings [1,2]. Our study has established that a major product of the AA reaction with an NO₂ radical is a mixture of four AA isomers containing three *cis* and one non-conjugated *trans* bond called TAAs (*trans*-AAs) [3–5]. Together with other novel lipids containing a nitro group, such as vicinal nitrohydroxyeicosatrienoic acids [6] and nitrolinoleic acids [7,8], these products have revealed that the nitration of unsaturated fatty acids occurs *in vivo* [9]. We have found that TAA isomers occur in the blood plasma of humans [4] and rats [5], and, because the levels of circulating TAA increase in inflammation [5] and in the plasma of cigarette smokers (M. Balazy, unpublished work), we suggested that the TAA isomers function as specific mediators/markers of NO₂ radical-dependent modifications of AA and cell membrane lipids [9]. The present study was inspired by the observation that TAAs are not readily metabolized by enzymes that metabolize AA such as platelet cyclo-oxygenase and lipoxygenase, thus we have reasoned that perhaps TAA are more amenable for hepatic microsomal metabolism. In particular,

we have addressed a more general question of whether the *trans* bond can compete with the three *cis* bonds in the same molecule for oxidative metabolism by microsomal CYP (cytochrome P450) mono-oxygenases. Our preliminary study, which detected four vicinal diol lipids from microsomal oxidation of a radiolabelled TAA isomer (14,15-TAA), suggested that formation of an epoxide having a *trans* configuration was possible [10]. Because unequivocal identification and isolation of such an epoxide would require synthesis of a specific standard, in the present study, we used *trans*-epoxy AA standards and compared their properties with microsomal lipid products. Profiling of 5,6-TAA metabolites by LC/MS (where LC stands for liquid chromatography) revealed that all four bonds participated in microsomal epoxidation and we were able to isolate and characterize a unique 5,6-*trans*-epoxide (5,6-*trans*-EET, where EET stands for epoxyeicosatrienoic acid). The microsomal metabolic profile of 5,6-TAA was different from that of AA and suggested that TAA are *trans* fatty acids that appear to be metabolically similar to xenobiotics.

MATERIALS AND METHODS

Chemicals

AA (purity > 99 %), *m*-chloroperoxybenzoic acid, dicyclohexyl urea and acetic acid were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Hexane and acetonitrile of HPLC grade

Abbreviations used: AA, arachidonic acid; CYP, cytochrome P450; DiHETE, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; GST, glutathione S-transferase; HETE, hydroxyeicosatetraenoic acid; LC/MS, liquid chromatography/MS; PFB, pentafluorobenzyl; TAA, *trans*-AA; TMS, trimethylsilyl.

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were from Fisher Scientific (Suwanee, GA, U.S.A.). Eicosanoid standards were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). 5,6-TAA was prepared as described previously [11] and was free from its *cis*-isomer, i.e. AA. 5,6-TAA was generously given by Dr J. R. Falck (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). 5,6-*trans*-EET and 5,6-*cis*-EET were prepared according to Corey procedure via iodolactonization [12] with modifications as described by us [13,14]. The quality and purity of this epoxide was established by MS and NMR [13]. Rabbit polyclonal anti-CYP 2E1 antibody (ab4239; 5 mg/ml) was obtained from Abcam (Cambridge, MA, U.S.A.). This antibody cross-reacts with rat CYP2E1 [15,16].

Isomerization of 19-HETE (hydroxyeicosatetraenoic acid)

19-HETE (1 μ g) was dissolved in hexane (100 μ l) and mixed with 100 μ l of a 1% solution of NO₂ in hexane. The reaction was carried out for 1–10 min. The solvent was evaporated under nitrogen, and the lipids were subjected to HPLC purification on a Phenomenex Luna C18 column (150 mm $\times$ 2 mm, particle size 5 μ m). The fraction at 6–8 min was collected and analysed by LC/MS and GC/MS. The isomerization produced four isomers of 19-HETE after treatment with NO₂ for 5 min. Other HETE molecules (20-, 18- and 16-HETE) were also isomerized by NO₂ and the *trans*-isomers were isolated by HPLC and characterized by GC/MS.

Microsomal metabolism of 5,6-TAA

A suspension of rat liver microsomes (CellzDirect, Tucson, AZ, U.S.A.) in phosphate buffer (200 μ l, 1 mg/ml) was preincubated at 37°C for 15 min and mixed with 5,6-TAA (final concentration of 73–736 μ M). Control experiments were performed with AA (73–736 μ M). An NADPH-regeneration system solution was mixed with the tetrasodium salt of reduced β -NAD phosphate (0.3 mg) and added to the microsomal mixture containing fatty acid substrate to stimulate the CYP-dependent metabolism. The NADPH-regeneration solution (CellzDirect) was composed of NADP⁺ and glucose-6-phosphate (solution A, 100 μ l) and glucose-6-phosphate dehydrogenase (solution B, 20 μ l), diluted 20- and 100-fold respectively. The final concentrations for the components of the NADPH-regeneration system were as follows: 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 unit/ml glucose-6-phosphate dehydrogenase and 3.3 mM MgCl₂. The incubations were carried out for 10 and 20 min at 37°C and were terminated by the addition of ethyl acetate (0.5 ml) and water (0.5 ml). The solution was thoroughly mixed using a vortex and then centrifuged at 2862 g for 10 min. The supernatant was decanted and the remaining solution was re-extracted with ethyl acetate two more times, and the extracts were combined. The solvent was evaporated under a stream of nitrogen. The lipid residue was dissolved in 100 μ l of acetonitrile/water (1:1) and subjected to analysis by LC/MS. Acid was not used in the extraction to minimize hydrolysis of the epoxides to diols. Control experiments were performed without microsomes and/or NADPH-generating system and did not show metabolite formation. In some experiments, the microsomes were preincubated with anti-CYP2E1 IgG protein (5 and 10 μ g) for 20 min at 37°C. The effect of microsomal epoxide hydrolase was tested by pretreatment of microsomes with a solution of dicyclohexyl urea (1 μ l in DMSO, final concentration of 10 mM) [17].

Hydrolysis of 5,6-*trans*-EET and 5,6-*cis*-EET by microsomal epoxide hydrolase

5,6-*trans*-EET, 5,6-*cis*-EET and their mixture (100 ng each) were incubated with rat microsomes (100 μ g in 100 μ l) for 10, 20,

60, 180 and 360 min. The incubations were terminated by the addition of 100 μ l of methanol. The mixture was vortex-mixed, centrifuged and aliquots (20 μ l) were analysed by LC/MS (see below).

Conjugation of 5,6-*trans*-EET and 5,6-*cis*-EET with microsomal GST (glutathione S-transferase)

GST from rat liver (5 mg, 18 units/mg of protein; Sigma) was suspended in 500 μ l of distilled water. The GST solution contained 18 units/100 μ l along with 1 μ mol of GSH (pH 6.5). A mixture of 5,6-EET isomers (100 ng each, final concentration of 3.12 μ M) were incubated with 100 μ l of GST solution for 1, 6 and 12 h at 37°C with shaking. The incubations were terminated by centrifugation and aliquots (20 μ l) were analysed by LC/MS.

MS

LC/MS analysis was performed on a triple quadrupole mass spectrometer model API 2000 (AB Sciex Instruments, Foster City, CA, U.S.A.) interfaced with an HP1100 HPLC system (Agilent Technologies, Wilmington, DE, U.S.A.). Negative ions were generated with a turbo ion spray source having a temperature of 450°C. Typical parameters of the mass spectrometer system were as follows: –4200 V ion spray potential, 6 V CAD potential, –6 V deconvolution potential and –16.5 V capillary exit potential. The MS/MS (tandem MS) spectra were acquired by using collision energy from –27 to –40 V with nitrogen in quadrupole 2, which resulted in a decrease of the precursor ion intensity by approx. 50%. The lipid solutions were prepared in microvials and injected using an autoinjector. Lipids were analysed on a Phenomenex Luna C18 column (150 mm $\times$ 2 mm, 5 μ m) having a 10 mm precolumn. The samples were eluted with a solution composed of water/acetonitrile/acetic acid (37.5:62.5:0.01, by vol.) at a rate of 300 μ l/min. UV spectra were acquired by an on-line diode-array detector (HP1100) set to scan a wavelength range of 200–400 nm/s. In some experiments, the effluent from the HPLC column was fractionated using a Gilson fraction collector to collect 1 min fractions for additional analysis by GC/MS.

GC/MS analysis was performed on an HP5973 system (Agilent) operating in a negative-ion chemical ionization mode (methane, 40 p.s.i.; 1 p.s.i. = 6.9 kPa). The source and transfer line temperatures were 250 and 280°C respectively. The samples were analysed on an HP-5MS column (30 m, 0.25 mm inner diameter and 0.25 μ m film thickness). The initial temperature of the column (150°C) was maintained for 1 min and then increased to 280°C at a rate of 40°C/min. The injector was used in the splitless mode at a temperature of 280°C. Helium was used as the carrier gas at a pressure of 29.57 p.s.i., delivering a total flow of 30.1 ml/min and resulting in a column flow of 1.3 ml/min. Samples for GC/MS analysis were derived as PFB (pentafluorobenzyl) esters and TMS (trimethylsilyl) ethers as described in [14] and were injected in iso-octane (1–2 μ l).

RESULTS

Microsomal metabolism of 5,6-TAA

Hepatic microsomes metabolized 5,6-TAA into a complex mixture of oxidized lipid products (Figure 1). The metabolism required oxygen and NADPH and appeared to involve several microsomal CYP isoenzymes. The rate of 5,6-TAA metabolism appeared to be higher than that described for 14,15-TAA [10], and the maximal accumulation of metabolites was observed within the first 10 min of the incubation. We applied an MS analysis based on an LC/MS with negative-ion detection to establish the

Figure 1 LC/MS analysis of eicosanoids extracted from a solution of hepatic microsomes incubated with 5,6-TAA

Full scan data were acquired (negative ion) and an extracted ion profile is shown for m/z 319 (a) for the carboxylic anions from HETEs and EETs. Structural assignment of metabolites h1–h8 and e1–e4 is summarized in Table 1. For comparison, the standards derived from microsomal metabolism of AA (b, c) analysed under the same conditions are shown. The ion profile for m/z 337 was used to detect the epoxide hydrolysis products (DiHETrE; Figure 4b). Lipids were analysed on a Phenomenex C18 column using a solution composed of water/acetonitrile/acetic acid (37.5:62.5:0.01, by vol.) at a rate of 300 μ l/min.

complete microsomal metabolic profile of 5,6-TAA [18]. The initial LC/MS analysis of the lipid extracts performed with selected monitoring of ions at m/z 319 and 337 detected mono- and di-oxygenated lipids (Figure 1a, Table 1). The MS/MS mass spectra revealed that 5,6-TAA was metabolized into three classes of lipid products: epoxides (EET), vicinal dihydroxy products [DiHETrE (dihydroxyeicosatrienoic acid)] and HETEs. Allylic, bis-allylic and $\omega - 1$ plus $\omega - 2$ alcohols were the three forms of HETE molecules derived from 5,6-TAA. The structural characterization of metabolites indicated that the CYP isoenzymes oxidized both *cis* and the *trans* bonds by at least two mechanisms (epoxidation and hydroxylation) leading to a mixture of oxidized lipids.

trans-EETs

Among the lipid metabolites were epoxides, suggesting that the microsomes contained a specific CYP epoxigenase, a mono-

Table 1 Identification of microsomal 5,6-TAA metabolites

The retention time and assignment of metabolites corresponds to the labelling in Figure 1. x, a compound showing a parent ion at m/z 319 that did not produce identifiable fragments after collisional activation and thus remained unidentified.

Metabolite	Retention time (min)	5,6- <i>trans</i> -Product
h1	5.8	19-HETE
h2	6.7	13-HETE
h3	7.3	10- and 7-HETE
h4	8.1	15-HETE
h5	8.8	11-HETE
h6	9.3	12-HETE
h7	10.2	8-HETE
h8	11.0	5-HETE
Unknown	13.2	x
e1	14.5	14,15-EET
e2	16.9	11,12-EET
e3	17.8	8,9-EET
e4	19.9	5,6-EET

oxygenase that was capable of epoxidation of the 5,6-TAA double bonds (Figure 1a). The mass spectral analysis revealed that four epoxides were formed (products e1–e4 in Figure 1a, Figures 2a–2d, Table 1). Each of these products eluted from the Microbore HPLC column shortly after the elution of the corresponding synthetic standard (Figure 1b), which was consistent with the presence of a single *trans* bond in the microsomal epoxides [3]. The mass spectra of metabolites e1–e4 contained prominent ions at m/z 319, which upon collisional activation generated characteristic fragments that allowed structural identification of four epoxides (Figures 2a and 2d). These spectra were qualitatively similar to those that detected the *cis*-EETs [19], yet subtle differences were noted. The identification of these epoxides also involved comparison of chromatographic and mass spectrometric properties with synthetic standards obtained by the stereospecific epoxidation using iodolactonization of 5,6-TAA [13] and by the reaction of 5,6-TAA with *m*-chloroperoxybenzoic acid [20]. Of particular interest was a relatively minor product that showed identical chromatographic and mass spectrometric properties as the synthetic 5,6-*trans*-EET (Figure 1a, product e4). A spectrum containing a characteristic ion at m/z 191 and a fragmentation pattern similar to that of the 5,6-*trans*-EET standard [13] was observed for product e4 that eluted at 19.9 min (Figure 2a). Additional MS analysis involved a reaction monitoring technique to reveal only the compounds that contained a precursor ion at m/z 319 and a fragment ion at m/z 191 (transition m/z 319 $\rightarrow$ 191) during the analysis of the microsomal lipid extract (Figure 3). This analysis showed a peak at 20.7 min (Figure 3b), which had a retention time identical with the peak generated from the 5,6-*trans*-EET standard (Figure 3a). Thus this experiment confirmed that liver microsomes oxidized 5,6-TAA to an epoxide having the oxirane ring of *trans* configuration. The experiment shown in Figure 3(b) also revealed a peak at 18.7 min, which probably originated from metabolite e3 identified as 5,6-*trans*-8,9-*cis*-EET. The mass spectrum of metabolite e3 showed a minor fragment ion at m/z 191 (Figure 2b). However, the retention time of the epoxide e3 was shorter than the retention time of 5,6-*cis*-EET standard by 0.3 min (Figure 3a). This excluded the possibility that the microsomal extract contained 5,6-*cis*-EET.

DiHETrEs

Another group of microsomal 5,6-TAA metabolites was DiHETrE, which probably originated from hydrolysis of the

Figure 2 Mass spectra of four epoxides (metabolites e1–e4) obtained by collisional activation of carboxylate anion at m/z 319

The mass spectra correspond to the following structures of the *trans*-isomers of epoxides: 5,6-EET (a), 8,9-EET (b), 11,12-EET (c) and 14,15-EET (d).

corresponding epoxides (d1–d4, Figure 4). The mass spectra revealed that four such compounds were detected at retention times slightly longer than those for respective all *cis*-EET standards (Figure 1b). The structures were assigned based on MS/MS spectra obtained by collisional activation of carboxylate anion at m/z 337. This result suggested that because one bond of the precursor substrate fatty acid was of *trans* configuration and one of the precursor epoxide had *trans* configuration, the resulting 5,6-DiHETrE eluting at 6.1 min (Figure 4, product d4) should have the *erythro* configuration. It co-eluted with a 5,6-DiHETrE standard prepared by the acid hydrolysis of 5,6-*trans*-EET (results not shown). In the three other DiHETrE metabolites (Figure 4), the configuration of the 1,2-diol was *threo* because it originated from the hydrolysis of the *cis*-epoxides. Relative abundance of DiHETrEs and EETs obtained from the ratio of intensity for ions at m/z 337 and 319 revealed that there was approx. 7-fold more of 5,6-DiHETrE in the microsomal extract compared with 5,6-*trans*-EET, whereas the proportion was 1:3 for other epoxides (Figures 1 and 4). This suggested that the 5,6-*trans*-EET was hydrolysed significantly faster than the three other *cis*-epoxides (having a *trans* bond) and thus was relatively unstable in the microsomal milieu that contains a stereoselective epoxide hydrolase [21]. To

explain this observation, the microsomes were preincubated with dicyclohexyl urea, an inhibitor of microsomal and soluble epoxide hydrolases [17]. The resulting metabolic profile of 5,6-TAA was nearly identical with that from untreated microsomes (results not shown). In particular, the abundance of EET did not increase relative to DiHETrE, suggesting that these epoxides were not readily hydrolysed by a microsomal epoxide hydrolase.

HETEs

The MS/MS spectra also revealed that 5,6-TAA was metabolized to a group of products (h1–h8 in Figure 1a, Table 1) identified as isomers of HETEs. These compounds originated from CYP-dependent oxygenation by three mechanisms, namely allylic, bis-allylic and subterminal hydroxylations. Among these lipids, metabolite h8 (Figure 1a) showed a mass spectrum having fragment ions at m/z 203 and 115 that was very similar to the spectrum of 5-HETE standard obtained from AA metabolism by 5-lipoxygenase (Figure 5). However, although metabolite h8 showed a UV spectrum typical for a conjugated diene with an α -hydroxyl, its maximum absorbance was at 236 nm, i.e. 2 nm more than in the UV spectrum of the 5-HETE standard (Figure 5).

Figure 3 Identification of 5,6-*trans*-EET in the microsomal extract

Multiple reaction monitoring (LC/MS/MS) was used to detect specific products based on mass transition and retention time. The chromatograms show the relative intensity of the peaks for the equimolar mixture of *cis*- and *trans*-isomers of 5,6-EET (m/z 319 → 191) (a) and the microsomal extract (b). A metabolite at 20.7 min has the same transition and retention times as the 5,6-*trans*-EET standard. The LC/MS conditions were as in Figure 1, except that the mobile-phase flow rate was 250 $\mu\text{L}/\text{min}$.

Figure 4 MS identification of metabolites d1–d4 as diols (DiHETEs) resulting from hydrolysis of the corresponding epoxides

Full scan spectra were acquired and the extracted ion profile is shown for m/z 337.

This suggested that compound h8 had a different double-bond configuration compared with the 5-HETE standard, possibly such that the C6–C7 bond was of *cis* configuration. Available UV absorbance results of fatty acid isomers containing hydroxypentadiene fragments in various configurations [22] and isomers of parinaric acids [23] suggest that a change of the conjugated diene configuration from *cis*–*trans* to *cis*–*cis* usually causes a bathochromic shift. Therefore the likely structure of metabolite h8 was such that double bonds were all of *cis* configuration (all *cis*-5-HETE) as shown in Figure 5. The mass spectra also revealed other HETE metabolites (Table 1) having structures of

Figure 5 Identification of 5-HETE isomer (metabolite h8)

Comparison of UV spectra of metabolite h8 and 5-HETE standard (upper panel). MS/MS spectrum of metabolite h8 shows characteristic fragments for 5-HETE (lower panel).

the allylic (lipoxygenase-derived) HETEs with the C5–C6 double bond in the *trans* configuration.

Subterminal hydroxylation

A HETE metabolite eluting at 6.7 min (h1, Figure 1a) showed a mass spectrum (Figure 6a) that was nearly identical with the spectrum of 19-HETE, a known product of AA metabolism by microsomal ω –1 hydroxylase [24]. The ions at m/z 59 and 231 were characteristic for metabolite h1 and 19-HETE and corresponded to fragments indicated in Figure 6(a). Metabolite h1 was further analysed by GC/MS as a PFB, TMS derivative and revealed two components (Figures 7a and 7b). We compared the retention times of these two components with the retention time of the all *cis*-HETE standards (Figure 7a) and the products obtained by isomerization of 19-, 18- and 20-HETE induced by the reaction with the NO_2 radical (Figure 7c). This experiment demonstrated that a component eluting at 8.12 min (Figure 7b) matched precisely one of the four isomers of 19-HETE generated from the NO_2 -mediated isomerization (Figure 7c). A minor component eluting at 7.92 min (Figure 7b) was tentatively characterized as 5,6-*trans*-18-HETE because it co-eluted with a product of 18-HETE isomerization (results not shown). Because this metabolic pattern of HETEs resembled the one that we observed previously with CYP2E1-mediated metabolism of AA [25], we tested whether this isoform of CYP was involved in the microsomal metabolism of 5,6-TAA. Preincubation of microsomes with a CYP2E1 antibody reduced the formation of metabolite

Figure 6 MS/MS spectra of metabolites h1 and h2 identified as *trans*-isomers of 19-HETE (a) and 13-HETE (b)

h1 (5,6-*trans* forms of 19- and 18-HETE) by 57% (Figure 8). Biosynthesis of allylic HETEs was reduced by 28–36% and bis-allylic HETEs (see below) by 40–48%. The CYP2E1 antibody had minimal effect on biosynthesis of epoxides. The results provided evidence that hepatic CYP2E1 isoenzyme participated in the oxidation of 5,6-TAA at carbon atoms C-19 and C-18 by a mechanism similar to the one previously reported by us for AA [25].

Bis-allylic hydroxylation

Mass spectra of metabolites h2 and h3 showed close similarity to the spectra obtained by Bylund et al. [24] for the three bis-allylic HETEs formed by liver microsomes [26,27]. Metabolite h2 showed a prominent ion at m/z 193 and 149 ($193-\text{CO}_2$) and a fragmentation pattern similar to that in the spectrum of 13-HETE (Figure 6b) [24]. Metabolite h3 showed characteristic ions at m/z 137, 153, 181, 193 and 203, suggesting that this metabolite could be a mixture of 5,6-*trans*-isomers of 10-HETE, 7-HETE and perhaps some 13-HETEs [24]. Consistent with previous observations, the bis-allylic HETEs eluted before allylic HETEs on the reverse-phase HPLC and did not show UV absorbance above 205 nm [28]. It is worth noting that whereas 13- and 10-HETE metabolites of 5,6-TAA ought to have retained the *cis,cis* configuration of the double bonds at the hydroxyl carbon, the 7-HETE metabolite should have the structure of a *trans,cis* bis-allylic alcohol.

Figure 7 GC/MS analysis of metabolite h1

Chromatograms were obtained by monitoring an ion at m/z 391 generated from the PFB, TMS derivatives and represent analysis of (a) a mixture of five all-*cis*-HETE standards; (b) metabolite h1; and (c) products of 19-HETE treatment with NO_2 showing co-elution (.....) of one of the isomers with the microsomal metabolite.

Figure 8 Effect of the CYP2E1 antibody on the microsomal metabolism of 5,6-TAA

The numbers in parentheses indicate the percentage of inhibition by antibody treatment for a metabolite represented by a chromatographic peak. The largest inhibitory effect is seen for the formation of *trans*-isomers of 19- and 18-HETE.

Figure 9 Comparison of hydrolysis rate for *cis* (c) and *trans* (t) isomers of 5,6-EET by hepatic microsomes

A quantitative LC/MS assay was used to determine the amount of each isomer (a) and the changes in their ratio (b) during incubation. After 60 min, 32 % of *trans*-epoxide and only 6.5 % of *cis*-epoxide remained in solution in an intact form (c), starting from initial equal amounts (100 ng) of both isomers (d).

Figure 10 Concentration of intact 5,6-EET *cis*- and *trans*-isomers (100 ng) remaining in solution after incubation with hepatic GST (18 units) and glutathione (1 μ M)

Comparison of 5,6-EET isomers hydrolysis and glutathione conjugation

Incubation of equal amounts of 5,6-*trans*-EET and 5,6-*cis*-EET with hepatic microsomes (Figure 9) and GST (Figure 10) revealed that the *trans*-epoxide was significantly more resistant to microsomal metabolism and glutathione conjugation than its *cis*-isomer. The initial rate of microsomal metabolism, a significant portion of which was hydrolysis, was approx. 2-fold faster for the *cis*-isomer. After incubation for 60 min, there was 3.6-fold more of the *trans*-isomer remaining in solution than of the *cis*-isomer. The 5,6-*trans*-EET was metabolized at a rate of 1.3 ng/100 μ g of protein/min. The LC/MS analyses showed the formation of vicinal diols and other minor products of the metabolism of EET isomers. After 360 min of incubation with hepatic GST, the *cis*-isomer was undetectable, whereas 39.2 % of the 5,6-*trans*-EET was detected in the intact form (Figure 10). A major product displaying an ion at m/z 627 was also detected in the incubation solution consistent with formation of a glutathione adduct with 5,6-EET isomers. Formation of a glutathione adduct with 5,6-*cis*-EET was reported previously [29].

DISCUSSION

TAA are products of the *cis*-*trans* isomerization of AA induced by NO_2 [3] and are found in the circulatory system of humans [4] and rodents [5]. The thiyl radical has been also shown to cause AA isomerization *in vitro* [30]. Endotoxemia [5], hyperoxia [31] and cigarette smoking (M. Balazy, unpublished work) are among conditions that increase the levels of TAA. We hypothesized that these TAA isomers could be considered specific lipid mediators that reveal modifications of AA induced by the NO_2 radical [9]. While some of the TAA isomers found in blood plasma may originate from elongation and desaturation of dietary *trans*-linoleic acids [4,32], 5,6-TAA is not likely to be of such origin but rather from free radical-mediated isomerization [4]. 5,6-TAA is a biologically active *trans* fatty acid that causes relaxation of the cerebral and retinal microvessels and inhibition of survival and viability of neuromicrovascular endothelial cells with an IC_{50} of 2 μ M [31]. We anticipated that microsomal metabolism could result in the oxidation of 5,6-TAA and other TAA isomers into activated forms such as *trans*-epoxides, which could be secondary mediators of the effects of the NO_2 radical attack on cellular lipids. The current study provided direct evidence that the microsomal epoxidation of the *trans* double bond in 5,6-TAA proceeded with a complete retention of olefin stereochemistry, which resulted in the biosynthesis of one *trans*- and three *cis*-epoxides. The key evidence for 5,6-*trans*-EET identification was obtained by a comparison of chromatographic and mass spectrometric properties with a standard synthesized by a stereospecific iodolactonization [13]. Epoxidation of the *trans* bond of 5,6-TAA did not involve epimerization, therefore, it was likely to proceed via a CYP-mediated epoxidation mechanism proposed for the *cis*-olefins [33,34].

Very little has been known about the epoxidation of *trans* bonds of *trans* fatty acids. While biomimetic experiments provide evidence of *trans*-epoxide formation from *trans*-olefins by CYP enzymes [33], the epoxidation of *trans*-linoleic acids and other *trans*-polyunsaturated fatty acids has not been studied in detail. In particular, it is not known whether *trans*-epoxides derived from *trans* fatty acids occur *in vivo*. Our current study suggests a mechanism by which such epoxides could be formed via hepatic microsomal epoxidation that leads not only to fatty acid *trans*-epoxides but also to fatty acid *cis*-epoxides that have a *trans* bond. Such complex mixtures of epoxides could originate from hepatic metabolism of dietary *trans*-linoleic acids and, possibly, from endogenously generated TAA.

Hepatic microsomes metabolized 5,6-TAA by several additional pathways that involved allylic, bis-allylic and subterminal hydroxylations. Compared with AA, the metabolic pattern of 5,6-TAA showed unique features. For example, while hepatic microsomal AA metabolism generates a series of five terminal-chain HETEs [35], 5,6-TAA was metabolized into only two such HETEs, possibly via the CYP2E1 isoenzyme, which metabolizes AA to similar products [25]. Among the allylic hydroxylation products was a unique isomer of 5-HETE. This observation suggested that hydroxylation must have occurred at the *trans* bond of the *cis*-*trans* pentadiene part of 5,6-TAA. Spectroscopic evidence suggested that the CYP caused a migration of the *trans* bond with the formation of a 1,3-*cis*-*cis* pentadiene at hydroxyl C-5. Such a mechanism appears to be similar to the mechanism of 5-lipoxygenase-mediated oxidation of AA, which generates a 1,3-*cis*-*trans* pentadiene configuration in the 5-HETE structure. The key difference is that the mono-oxygenase-dependent hydroxylation at a *trans* bond causes the formation of a hydroxyl conjugated to a *cis* bond. The stereochemistry of this new 5-HETE isomer was not determined because of the limited

Figure 11 Summary of the key steps involved in the hepatic microsomal metabolism of 5,6-TAA

amount of the material; however, previous work established that the microsomal oxidation of AA at carbon atom C-5 produces a nearly racemic mixture of 5-HETE enantiomers [36].

Our experiments demonstrate that 5,6-*trans*-EET was significantly more stable than its *cis*-isomer towards microsomal hydrolysis and GST-mediated conjugation when both epoxides were incubated. Thus 5,6-*trans*-EET was more resistant to metabolism by key enzymes active at the site of its formation in the liver. These two enzymes represent efficient pathways that inactivate exogenous and endogenous epoxides, many of which can cause toxicity by binding to DNA and proteins. While it appears that both 5,6-EET isomers can be formed by hepatic microsomes, the *trans*-isomer is more likely to escape hepatic metabolic transformations and perhaps enter circulation in the intact form. Our study has provided a first indication that such an epoxide may have a longer half-life than its opposite isomer. While many studies have detected the biological activity of the *cis*-epoxides of linoleic acid and AA [37–39], comparative biological data for fatty acid *trans*-epoxides are rare [13,40]. Our experiments do not exclude the possibility that the lower rates of hydrolysis and GST conjugation of the *cis*-epoxide were caused by the inhibitory effect of the *trans*-epoxide.

The lipids characterized in the present study constitute a novel class of lipids that could function as lipid markers of the NO₂ radical, originating from steps of AA isomerization followed by CYP-mediated mono-oxygenation. Many studies explored various aspects of microsomal hydroxylation and epoxidation of AA and other polyunsaturated fatty acids; however, considerably less is known about such a metabolism of *trans* fatty acids. Detection of oxygenated metabolites derived from TAA would require specific assays that would involve chromatographic separation of *cis*- and *trans*-isomers. 5,6-EET has been detected *in vivo* in the rat [41], in an esterified form in phospholipids of human platelets [42] and rat erythrocytes [40], and in other preparations

[43,44]. Because a special analytical approach needs be applied for the separation and simultaneous detection of the two 5,6-EET isomers, previous studies were likely to detect both of these isomers. Our findings could be further developed to detect 5,6-*trans*-EET stereospecifically *in vivo* along with other products of 5,6-TAA metabolism (summarized in Figure 11).

While many studies have addressed concerns regarding the adverse health effects of *trans* fatty acids [45–47], mechanisms of their activity are not well understood. Oxidative microsomal metabolism described in the present study could be an important aspect in the activation of TAA and possibly of other *trans* fatty acids into unique metabolites, among which the *trans*-epoxides could be a distinct and characteristic group, although it is unclear what cellular role they may play in the formation of biologically active metabolites. More research will be needed to uncover the potential role of such epoxides as mediators in the activity of *trans* fatty acids.

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