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Cytochrome P450/NADPH-dependent formation of *trans* epoxides from *trans*-arachidonic acids

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Abstract—*Trans*-arachidonic acids (*trans*-AA) are products of *cis*-*trans* isomerization of arachidonic acid by nitrogen dioxide radical (NO_2), and occur in vivo, but their metabolism is unknown. We found that hepatic microsomes oxidized *trans*-AA via cytochrome P450/NADPH system to epoxides, which were hydrolyzed by epoxide hydrolase to diols (DiHETEs). 14,15-*trans*-AA produced one *erythro* diol and three *threo* diols each having one *trans* double bond.

1. Introduction

Most of what has been known in the area of polyunsaturated fatty acid metabolism by hepatic microsomes involves studies of cytochrome P (CYP) 450/NADPH-dependent monooxygenation of the *cis* double bonds into epoxides of *cis* configuration. Such a process generates four epoxides of arachidonic acid (AA), epoxyeicosatrienoic acids (EETs).[1,2] Further metabolism of the EETs involves hydrolysis to vicinal diols, dihydroxyeicosatrienoic acids (DiHETEs), by cytosolic or microsomal epoxide hydrolase.[3] In contrast, microsomal metabolism of fatty acids that have both *cis* and *trans* bonds has not been well characterized. Studies of *trans* fatty acid (*t*FA) metabolism could be important in providing a better understanding of their biological effects[4,5] as these fatty acids have been suggested to contribute to cardiovascular disorders, cancer and other pathologies.[6,7] It has been proposed that oxygenated, and therefore more active metabolites could mediate biological effects of *t*FA.[8] We recently described a group of four isomers of arachidonic acid containing 1 *trans* and 3 *cis* bonds (*trans*-AA), which originate from *cis*-*trans* isomerization induced by NO_2 radical.[5,9] The *trans*-AA are found in vivo, and their formation increases in inflammation and possibly in other conditions that involve pathobiochemistry of NO_2 radical.[9,10] Since both endogenous and dietary *t*FA enter hepatic circulation, a study of their metabolism into highly activated forms such as epoxides is important and timely. While *cis* EETs have unique biological properties,[2] such properties of *trans* EETs that originate from *trans*-AAs are unknown. We have been interested in characterization of origins, metabolism and biological effects of *trans*-AA, and in development of methods for their analysis and synthesis. In this study we address a more general question of how hepatic microsomes oxidatively metabolize a fatty acid having both *cis* and *trans* double bonds as in *trans*-AA. We describe here that microsomes show a much higher and unique selectivity for a fatty acid isomer with a *trans* bond as compared to the all *cis* isomer.

2. Generation, isolation, purification and analysis of microsomal *trans*-AA metabolites

Rat liver microsomes (from CellzDirect, Tuscon, AZ) were suspended in phosphate buffer (1 mg/mL) and added to *trans*-AA isomers (final concentration 15 μM) and incubated for 30–180 min at 37 °C. The metabolism was stimulated by addition of NADPH (1 mM) and its regenerating system. The *trans*-AA were synthesized by a stereospecific deoxidation of EETs as previously described.[11] In experiments involving 14,15-*trans*-AA, its radiolabeled analogue, [1-^{14}C]-14,15-*trans*-AA (specific activity of 50 mCi/mmol, radiochemical purity >99%),[12] was used (0.25 μCi of [1-^{14}C]14,15-*trans*-AA per incubation). Some experiments involved co-incubation of [1-^{14}C]-14,15-*trans*-AA with equimolar amounts of [1-^{14}C] AA (New England Nuclear, Boston,

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MA). Standards of EETs and DiHETEs were obtained from Cayman Chemical Co. (Ann Arbor, MI). Standards of *trans*-EET, and deuterium-labeled EET were prepared by reaction of *m*-chloroperoxybenzoic acid (MCPBA) with *trans*-AA and AA-d_8, respectively as previously described.[13] Standards of *threo*- and *erythro*-DiHETEs were prepared by a mild acidic hydrolysis of *cis*- and *trans*-EETs, respectively[13] or by incubation with microsomes. The epoxides obtained from the reaction of 14,15-*trans*-AA with MCPBA were fractionated and incubated with microsomes. This procedure yielded 14,15-*erythro*-DiHETE. The microsomal incubations were terminated by addition of 5 mL of cold methanol, vortexed, and centrifuged for 5 min at 1100 *g* to precipitate microsomal debris and proteins. The supernatant, which contained >90% of radioactivity was transferred to a glass tube and the solvent was removed under vacuum. The residue was dissolved in 1 mL of ethyl acetate and mixed with 1 mL of acidified water (pH was adjusted to 4.0 with 10% acetic acid). The organic phase was separated by centrifugation and transferred to a glass tube and evaporated to dryness. The residue was dissolved in methanol and an aliquot (20 μL) was analyzed by HPLC (HP1050, Hewlett-Packard) using a C18 Ultrasphere ODS column (250×4.6 mm, Beckman Instruments, CA). Compounds were eluted with a gradient of acetonitrile in water (12.5% to 100% in 60 min, pH 4, flow 1 mL/min). An on-line radio-chromatography detector (Radiomatic Flo-One/Beta Series A-100) detected radioactivity with a Sigma-Fluor scintillation fluid. Gas chromatography was performed on a HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) using an Agilent 5973 GC–MS instrument. The samples were eluted with a temperature program from 150 to 300 °C at a rate of 20 °C/min. The temperatures of the injector, transfer line and the ion source were 280, 280, 230 °C, respectively. Methane was used for negative ion chemical ionization. The pentafluorobenzyl esters (PFB), ditrimethylsilyl ethers (diTMS) were prepared as described.[14]

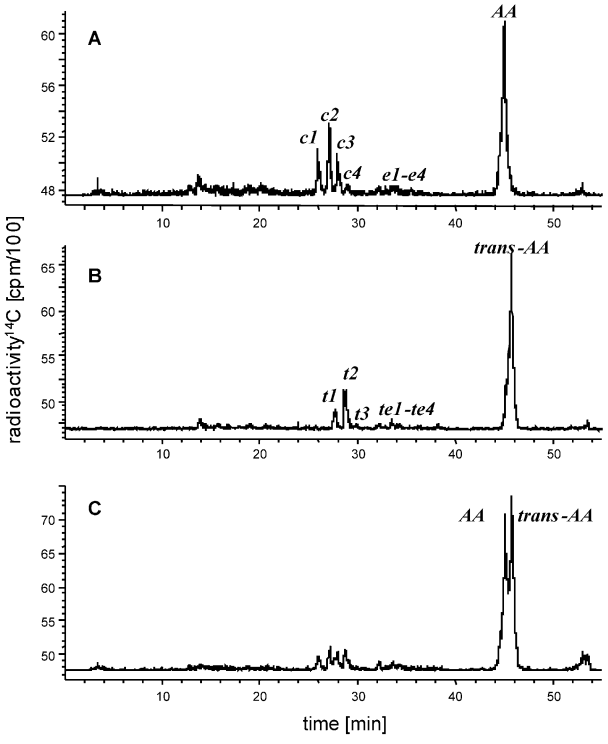

Figure 1. Comparison of radiochromatograms obtained by HPLC analysis of products extracted from incubation of rat hepatic microsomes (180 min, 37 °C) with ^{14}C-radiolabeled forms of: AA (A), 14,15-*trans*-AA (B) and AA plus 14,15-*trans*-AA (C). Metabolites ***c1–c4*** and ***t1–t3*** were identified as diols (DiHETEs) whereas metabolites ***e1–e4*** and ***te1–te4*** as epoxides (EETs).

3. Characterization of microsomal *trans*-AA metabolites

Metabolism of AA and *trans*-AA required oxygen and reduced NADP indicating that these fatty acids were oxidized by a CYP450 epoxygenase, a monooxygenase enzyme. At incubation time of 90 min or longer, vicinal diols (DiHETEs) were the major products (Fig. 1). They originated from hydrolysis of the initially formed epoxides (EETs) of *trans*-AA and AA by microsomal epoxide hydrolase.

However, the metabolic profile of 14,15-*trans*-AA was much different than that of AA (Fig. 1A and B). While AA produced four major metabolites (***c1–c4***), 14,15-*trans*-AA produced three metabolites (***t1–t3***) at comparable rate of metabolism. The retention times of ***t1–t3*** were longer than ***c1–c4*** by about 1 min suggesting that they were likely to be *trans* isomers of ***c1–c4***, because a similar difference was seen between *trans*-AA and AA. Incubations of equimolar amounts of AA and *trans*-AA generated a complex mixture of metabolites and indicated that 14,15-*trans*-AA modulated the metabolism of AA (Fig. 1C).

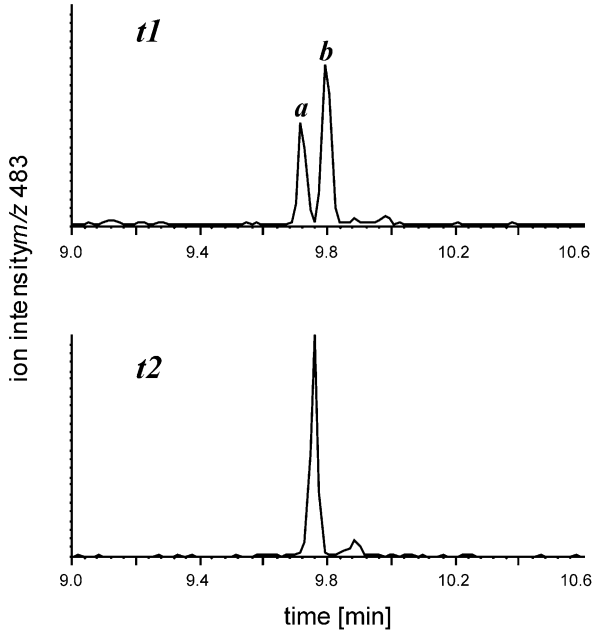

Figure 2. Chromatograms of ion at *m*/*z* 483 obtained from analysis by GC–MS (negative ions) of products ***t1*** and ***t2*** isolated as described in Figure 1B. Ion *m*/*z* 483 corresponds to a diTMS derivative of a ^{14}C-labeled DiHETE carboxylic anion.

GC–MS analysis confirmed that metabolites ***c1–c4*** were DiHETE molecules. Each fraction produced a single chromatographic peak and a spectrum with an abundant ion at *m*/*z* 483, which corresponded to a diTMS ^{14}C-labeled DiHETE carboxyl anion. Comparison with synthetic standards allowed structural identification of ***c1–c4*** (Table 1). Thus metabolites ***c1–c4*** corresponded to four DiHETEs, which were the hydrolysis products of the respective EETs (***e1–e4***).[1,15] GC–MS analysis of ***t1–t3*** also revealed ions at *m*/*z* 483 (Fig. 2). Fraction **t1** contained two closely eluting peaks ***t1a*** and ***t1b*** (Fig. 2) whereas ***t3*** was a relatively minor product. This suggested that ***t1–t3*** were DiHETEs and thus 14,15-*trans*-AA, like AA, was metabolized primarily by epoxygenase and epoxide hydrolase system. Comparison of retention times (Table 1) revealed that metabolite ***t1b*** had similar retention time as 14,15-DiHETE and thus was likely to be 14,15-erythro-DiHETE resulting from hydrolysis of 14,15-trans-EET. Consistent with another study,[16] the erythro isomer ***t1b*** eluted before the threo isomer ***c1***. Further confirmation of this observation was obtained by analysis of products of the reaction of 14,15-*trans*-AA with MCPBA, which produced a mixture of epoxides. These epoxides were separated and hydrolyzed by incubation with microsomes. The most polar and thus earlier eluting epoxide by HPLC was isolated and following hydrolysis produced a DiHETE that had identical retention time as the microsomal product ***t1b*** (Fig. 2). This procedure provided fairly pure standards of 14,15-*trans*-EET and 14,15-*erythro*-DiHETE for comparison with microsomal products. Other 14,15-*trans*-AA-derived microsomal metabolites had retention times similar to those of other standard DiHETEs (Table 1). Thus, because three *cis* bonds in 14,15-*trans*-AA were available for epoxidation, our work suggests that 14,15-*trans*-AA was metabolized via a CYP450/NADPH system to four epoxides, which were hydrolyzed by epoxide hydrolase to one *erythro* diol and three *threo* diols each having a *trans* double bond (Fig. 3). Hydrolysis of epoxides by microsomal and cytosolic epoxide hydrolases is highly stereoselective. Thus, *cis*-EETs generate exclusively *threo*-DiHETEs by an S_N2 mechanism,[15] and while *trans*-EETs have not been observed before, other *trans* epoxides such as a *trans*-epoxide of stearic acid are hydrolyzed exlusively into *erythro* diols.[17] Our work for the first time identified a mechanism by which *trans* epoxides of arachidonic acid and their hydrolysis products could be formed in vivo.

Table 1. Comparison of retention times of standards and metabolites from experiments illustrated by Figures 1 and 2[a]

Standards	AA metabolites	14,15-*trans*-AA metabolites
14,15-DiHETE 9.84	***c1*** 9.83	***t1b*** 9.80
11,12-DiHETE 9.74	***c2*** 9.73	***t2*** 9.76
8,9-DiHETE 9.72	***c3*** 9.70	***t1a*** 9.72
5,6-DiHETE 9.73	***c4*** 9.69	***t3*** 9.68

[a] Values are retention times in min obtained by GC–MS analysis on a HP-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Ions at *m*/*z* 481 and 483 were recorded for standards and metabolites, respectively.

4. Significance

Recent years have witnessed a growing interest in the understanding of pathobiochemical effects of NO_2, a potent oxidant and nitrating radical originating from oxidation of NO, nitrite and other sources.[18] NO_2 causes AA peroxidation,[19] *cis-trans* isomerization[5] and nitration.[20] We hypothesized that *trans*-AA could function as specific lipid markers or mediators of the damage to biological membrane by NO_2.[8] The *trans*-AA isomers have been detected in vivo in the rat and in humans,[5,9,21] and their levels increase in inflammation,[9]

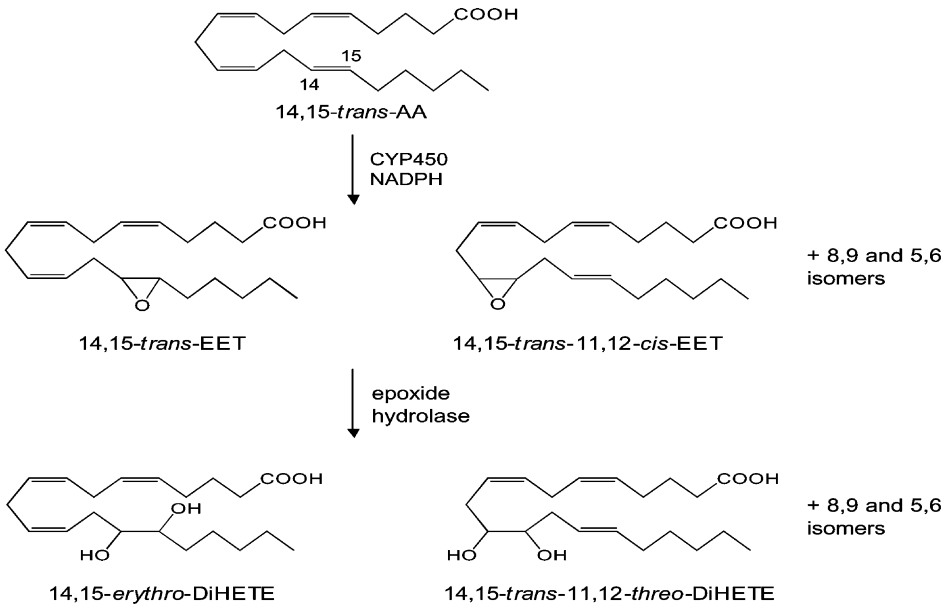

Figure 3. Microsomal epoxidation of 14,15-*trans*-AA produced 4 epoxides one of which was a *trans* epoxide and 3 other were *cis* epoxides. Microsomal epoxide hydrolase converted the epoxides into diols, one of which was an *erythro* diol and 3 other were *threo* diols with one *trans* double bond.

reaching 0.5 μM level in blood plasma.[9] Our current work shows that *trans*-AA can be metabolized by CYP450 into unique molecules that may serve as markers of the *trans*-AA hepatic metabolism. Preliminary results also show that three other *trans*-AA isomers are metabolized to *trans* epoxides.

14,15-*trans*-AA may originate from two sources: NO_2-induced isomerization of AA and diet. It appears that 12,13-*trans* linoleic acid (LA), a rather minor component of dietary *t*FA, can be converted to 14,15-*trans*-AA via steps of elongation and desaturation in the liver.[22] Whether or not the other LA isomer (9,10-*trans*-LA) can be converted to 11,12-*trans*-AA remains to be confirmed.[22] However 5,6-*trans*-AA and 8,9-*trans*-AA can only be derived from NO_2-mediated isomerization and not from diet. Thus hepatic microsomal metabolism of the *trans*-AA could be an important aspect of the activation of both the dietary *trans*-LA and NO_2-derived *trans*-AAs.

Metabolic profile shown in Figure 1C indicated that many metabolites of 14,15-*trans*-AA coeluted with metabolites of AA. An even more complex mixture was observed from microsomal metabolism of all four *trans*-AA isomers and AA. Such mixture might have contained up to 20 EETs, including 4 *trans*-EETs. It is thus possible that past measurements of endogenous CYP450-AA metabolites might have inadvertently detected EETs and DiHETEs derived from *trans*-AA. More advanced analytical methods will be needed to isolate and analyze these isomers. We also found that commercial AA, many batches of which are extracted from porcine liver, contains 1–5% of *trans*-AA. The *trans*-AA may originate from elongation-desaturation of dietary *trans*-LA and/or from isomerization of AA during extraction. Metabolism of commercial AA also revealed products of *trans*-AA oxidation.

Acknowledgements

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References and notes

1. Capdevila, J.; Chacos, N.; Werringloer, J.; Prough, R. A.; Estabrook, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5362.
2. Balazy, M.; McGiff, J. C. In *Eicosanoids, Aspirin, and Asthma*; Szczeklik, A., Gryglewski, R. J., Vane, J. R., Eds.; Marcel Dekker, Inc., 1998; p 45.
3. Chacos, N.; Capdevila, J.; Falck, J. R.; Manna, S.; Martin-Wixtrom, C.; Gill, S. S.; Hammock, B. D.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1983**, *223*, 639.
4. Balazy, M.; Lopez-Fernandez, J. *Adv. Exp. Med. Biol.* **2003**, *525*, 173.
5. Jiang, H.; Kruger, N.; Lahiri, D. R.; Wang, D.; Vatèle, J. M.; Balazy, M. *J. Biol. Chem.* **1999**, *274*, 16235.
6. Ascherio, A.; Hennekens, C. H.; Buring, J. E.; Master, C.; Stampfer, M. J.; Willett, W. C. *Circulation* **1994**, *89*, 94.
7. Hunter, J. E. *J. Natl. Cancer Inst.* **1982**, *69*, 319.
8. Balazy, M.; Poff, C. D. *Curr. Vasc. Pharm.* **2004**, *2*, 1.
9. Balazy, M. *J. Physiol Pharmacol.* **2000**, *51*, 597.
10. Balazy, M.; Nigam, S. *Ageing Res. Rev.* **2003**, *2*, 191.
11. Krishna, U. M.; Reddy, M. M.; Xia, J.; Falck, J. R.; Balazy, M. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2415.
12. Berdeaux, O.; Vatelè, J.-M.; Eynard, T.; Nour, M.; Poullain, D.; Noël, J.-P.; Sébédio, J.-L. *Chem. Phys. Lipids* **1995**, *78*, 71.
13. Balazy, M.; Nies, A. S. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 328.
14. Balazy, M. *J. Biol. Chem.* **1991**, *266*, 23561.
15. Zeldin, D. C.; Kobayashi, J.; Falck, J. R.; Winder, B. S.; Hammock, B. D.; Snapper, J. R.; Capdevila, J. H. *J. Biol. Chem.* **1993**, *268*, 6402.
16. Abalain, J. H.; Picart, D.; Berthou, F.; Ollivier, R.; Amet, Y.; Daniel, J. Y.; Floch, H. H. *J. Chromatogr.* **1983**, *274*, 305.
17. Gill, S. S.; Hammock, B. D. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 965.
18. Bian, K.; Gao, Z.; Weisbrodt, N.; Murad, F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**.
19. Pryor, W. A.; Lightsey, J. W. *Science* **1981**, *214*, 435.
20. Balazy, M.; Iesaki, T.; Park, J. L.; Jiang, H.; Kaminski, P. M.; Wolin, M. S. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 611.
21. Ratnayake, W. M.; Chen, Z. Y.; Pelletier, G.; Weber, D. *Lipids* **1994**, *29*, 707.
22. Sébédio, J. L.; Chardigny, J. M. In *Trans fatty acids in human nutrition*; Sébédio, J. L., Christie, W. W., Eds.; The Oily Press, 1998; p 191.