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ω-Hydroxylation of 15-Hydroxyeicosatetraenoic Acid by Lung Microsomes from Pregnant Rabbits

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SUMMARY

15-Hydroxyeicosatetraenoic acid (15-HETE) was converted by lung microsomes from pregnant rabbits to a polar metabolite that was identified by mass spectrometry as the 15,20-dihydroxyeicosatetraenoic acid. The formation of the 20- or ω-hydroxylated product was NADPH dependent, with a specific activity of 1.87 ± 0.53 nmol/min/mg of microsomal protein. Other hydroxylated derivatives of eicosatetraenoic acid that possessed hydroxy groups at the 5- and 12-carbon atoms were not metabolized by the lung microsomes. This hydroxylation of 15-HETE was observed in lung microsomes of pregnant rabbits and only minor amounts were formed by nonpregnant rabbits. The specific activity for 15-HETE ω-hydroxylation was similar to the value obtained for prostaglandin E_1 (1.48 ± 0.33 nmol/min/mg). It is known that rabbit lungs possess a cytochrome P-450 that is induced during pregnancy and catalyzes the 20-hydroxylation of prostaglandins. The addition of the antibody to cytochrome P-450 prostaglandin ω-hydroxylase or prostaglandin E_1, a substrate of this enzyme, resulted in potent inhibition of 15-HETE ω-hydroxylation, providing strong evidence that a common cytochrome P-450 catalyzes the ω-hydroxylation of both prostaglandins and 15-HETE.

Lung microsomes of pregnant or progesterone-treated rabbits exhibit a dramatic increase in ω- or 20-hydroxylase activity of various prostaglandin derivatives (1–3) as demonstrated by a 127-fold increase in the prostaglandin $F_{2\alpha}$- 20-hydroxylase activity in 25-day-old pregnant rabbits (2). This activity is mediated by a cytochrome P-450, and a unique species of this family of hemeproteins was purified and characterized by Williams *et al.* (4) from pregnant rabbits and by Yamamoto *et al.* (5) from rabbits treated with progesterone. Masters *et al.* (6) reported that the increase in prostaglandin 20-hydroxylase activity correlated with the induction of this specific form of cytochrome P-450 during the gestational period.

In addition to this lung cytochrome P-450-dependent prostaglandin 20-hydroxylase, cytochromes P-450 in the adrenal gland, liver, and kidney may hydroxylate prostaglandins at either carbon atoms 19 or 20 (7). Other eicosanoids also were hydroxylated at carbon atom 20. Capdevila *et al.* (8) reported that liver microsomes of rats treated with ciprofibrate catalyze the 20-hydroxylation of arachidonic acid. Marcus *et al.* (9, 10) and Wong *et al.* (11) observed the production of 12,20-diHETE after incubating 12-HETE with human polymorphonuclear leukocytes. Soberman *et al.* (12) and Shak and Goldstein (13, 14) reported that LTB_4 is hydroxylated to its 20-hydroxy derivative by human polymorphonuclear leukocytes and Harper *et al.* (15) observed the ω-oxidation of LTB_4 in isolated rat hepatocytes.

In this study, lung microsomes from pregnant rabbits were examined for their ability to catalyze the 20-hydroxylation of products of the lipoxygenase pathway. The hydroxylation of 5-HETE, 12-HETE, 15-HETE, and LTB_4 was studied, and only 15-HETE was observed to be metabolized at a specific activity comparable to that of PGE_1. The results of this study indicate that a single cytochrome P-450 catalyzes the ω-hydroxylation of PGE_1 and 15-HETE, and that products of the 15-lipoxygenase pathway may share a common route of metabolism with prostaglandins.

Experimental Procedures

Materials. Nonpregnant and pregnant rabbits were purchased from Smith's Rabbitry (Seymour, WI). Sodium isocitrate was obtained from

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ABBREVIATIONS: 12,20-DiHETE, 12(*S*),20-dihydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoicacid; 5-HETE, 5(*S*)-hydroxy-6-*trans*,8,11,14-*cis*-eicosatetraenoic acid; 12-HETE, 12(*S*)-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; 15-HETE, 15 (*S*)-hydroxy-5,8,11-*cis*,13-*trans*-eicosatetraenoic acid; 15,20-diHETE, 15(*S*),20-dihydroxy-5,8,11-*cis*,13-*trans*-eicosatetraenoic acid; LTB_4, 5(*S*),12(*R*)-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-eicosatetraenoic acid; PGE_1, prostaglandin E_1; HETE, hydroxyeicosatetraenoic acid; GC-MS, gas chromatography-mass spectroscopy.

Sigma Chemical Co. (St. Louis, MO). Isocitrate dehydrogenase and NADPH were supplied by Boehringer-Mannheim (Indianapolis, IN). Unlabeled HETEs and PGE_1 were obtained from Cayman Chemicals (Ann Arbor, MI). Tritiated eicosanoids were supplied by New England Nuclear (Boston, MA). Acetonitrile, benzene, and ethyl acetate were purchased from American Burdick and Jackson (Muskegon, MI). RPI 3a70B (Mount Prospect, IL) was used as scintillation cocktail for both the Radiomatic Flo-One detector system and the LKB 1718 Rackbeta counter.

Methods. Nonpregnant female and 25- to 28-day-old pregnant rabbits were sacrificed by an injection of sodium pentobarbital in the marginal ear vein, and lung microsomes were prepared by the method of Williams *et al.* (4). The ω-hydroxylase activity of PGE_1 or HETEs was assayed in 55 mM potassium phosphate buffer (pH 7.4), containing 10 mM $MgCl_2$, 100 mM sodium isocitrate, 0.1 mg of isocitrate dehydrogenase, 0.5–1.5 mg of lung microsomal protein, 1 mM NADPH, and 0.032 mM 5-HETE, 12-HETE, 15-HETE, LTB_4, or PGE_1 containing 0.2 μCi of the respective tritiated eicosanoid. The reaction volume was 1 ml. The reactions were performed in a shaking water bath thermostatted at 37° and were terminated at 15 min for incubations containing 15-HETE, PGE_1, and microsomes from pregnant rabbits or 60 min for nonpregnant rabbits. Reactions that contained 5-HETE, 12-HETE, or LTB_4 were allowed to incubate for 60 min. The reactions were terminated by the addition of HCl to decrease the pH to 3.5 and the samples were extracted with ethyl acetate as described by Williams *et al.* (4).

The 15,20-diHETE was separated on a Varian 5020 HPLC apparatus (Sunnyvale, CA) using a Bio-Rad (Richmond, CA) ODS-5S column (150 × 4 mm) equipped with an ODS-5S microguard column (40 × 4.6 mm). An isocratic solvent system of 60% acetonitrile, 39.6% water, 0.2% benzene, and 0.2% acetic acid was used to elute the 15,20-diHETE (4.5 min) and the 15-HETE (13.9 min). The separation of 20-OH-PGE_1 was performed on the same column, but using a solvent system of 40% acetonitrile, 59.6% water, 0.2% benzene, and 0.2% acetic acid. The elution profile was monitored in a Radiomatic (Tampa, FL) CU scintillation detector system using RPI scintillation cocktail 3a70B. The isolation of the 15,20-diHETE for GC-MS identification was performed by repeated chromatographic separations using the solvent system described above. The 15-HETE product was converted to its methyl ester derivative with diazomethane and then silylated by the method of VanRollins and Murphy (16) or catalytically hydrogenated and then silylated for GC-MS analysis. The catalytic hydrogenation was performed by suspending 5 mg of 5% rhodium on alumina in 0.5 ml of methanol and bubbling hydrogen through the suspension for 20 min.

The mass spectra were obtained on a Hewlett Packard 5790 quadrapole mass selective detector, operated under electron impact (70 eV) conditions. The samples were separated in a Hewlett Packard 5790A gas chromatograph equipped with a 10-meter DB-1 capillary column (0.25 mm, J & W Scientific, Rancho Cardona, CA) with helium as carrier gas. The column was temperature programmed from 80–200° at 20°/min and then from 200–280° at 5°/min following injection into a split/splitless injector (280°).

The antibody inhibition experiments were performed by incubating the lung microsomes with the goat-IgG fraction prepared against the purified rabbit lung cytochrome P-450 which catalyzed the 20-hydroxylation of prostaglandins as described by Masters *et al.* (6). The antibody or nonimmune fraction was incubated with the microsomes for 10 min at 37° before starting the reaction.

Results and Discussion

Lung microsomes from 25- to 28-day-old pregnant rabbits catalyzed the formation of a polar metabolite of 15-HETE that eluted with a retention time of 4.5 min (Fig. 1). The reaction was NADPH dependent, and only trace amounts of the product were formed by lung microsomes of nonpregnant rabbits (data not shown). The metabolite was identified as the 15,20-diHETE

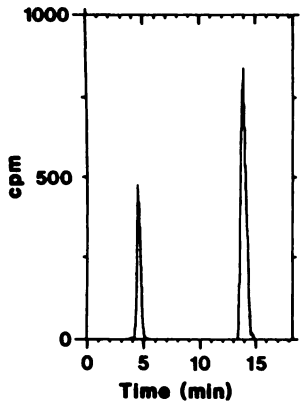

Fig. 1. The high pressure chromatographic elution pattern of the 15-HETE metabolite that was produced by lung microsomes prepared from pregnant rabbits. The incubation and chromatographic conditions are described under Experimental Procedures.

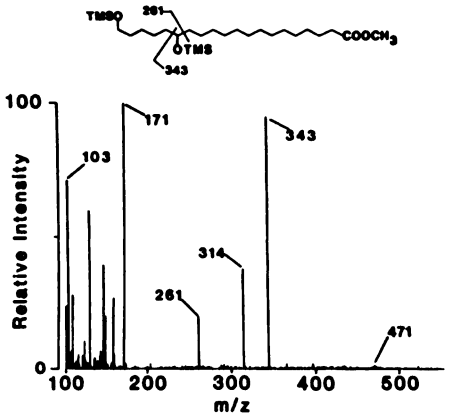

Fig. 2. The mass spectrum of the hydrogenated methyl ester, trimethylsilylated derivative of the 15-HETE metabolite.

derivative by GC-MS. The mass spectrum of the methyl ester, trimethylsilylated ether derivative demonstrated ions at m/z 313 ($M^+ - 181$), loss of CH_2—CH═CH—CH_2—CH═CH—$(CH_2)_3$—$COOCH_3$); 335 ($M^+ - 159$), loss of $\cdot CH_2$—$(CH_2)_3$—$COSiMe_3$; and 261 for the α-cleavage fragment between carbon atoms 14 and 15. The mass spectrum of the hydrogenated methyl ester, trimethylsilylated derivative (Fig. 2) produced ions at m/z 343 ($M^+ - 159$), the α-cleavage product at carbon atom 15 with loss of $\cdot CH_2$—$(CH_2)_4$—$COSiMe_3$, and at m/z 261 ($M^+ - 241$), the α-cleavage product at carbon atom 15 with loss of $\cdot CH_2$—$(CH_2)_{12}$—$COOCH_3$. The ion at m/z 103 was indicative of a primary alcohol group and provided further evidence that this second alcohol group was at carbon atom 20.

The specific activities of 15-HETE and PGE_1 20-hydroxylase were 1.87 ± 0.53 and 1.42 ± 0.33 nmol/min/mg of microsomal protein, respectively (Table 1). Only trace amounts of polar metabolites were observed when 5-HETE, 12-HETE, or LTB_4 was incubated with lung microsomes of pregnant rabbits, and these metabolites were not identified.

The similar specific activities for the 15-HETE and PGE_1 20-hydroxylases and the observation that the reaction occurred in lung microsomes from pregnant but not nonpregnant rabbits suggested that a single cytochrome P-450 may catalyze both the 15-HETE and PGE_1 20-hydroxylation reactions. To determine whether a common cytochrome P-450 was involved, PGE_1 was added to the reaction system to inhibit the 15-HETE 20-

TABLE 1

The metabolism of various eicosanoid derivatives by lung microsomes of pregnant (25- to 28-day gestation) or nonpregnant rabbits

The results are the mean and standard deviation of three experiments.

Substrate	Pregnant	Nonpregnant
	nmol/min/mg	
PGE_1	1.43 ± 0.33	Trace
15-HETE	1.87 ± 0.53	Trace
5-HETE	Trace[a]	ND[b]
12-HETE	Trace	ND
LTB_4	Trace	ND

[a] Trace, less than 0.02 nmol of product/min/mg.
[b] ND, not detected.

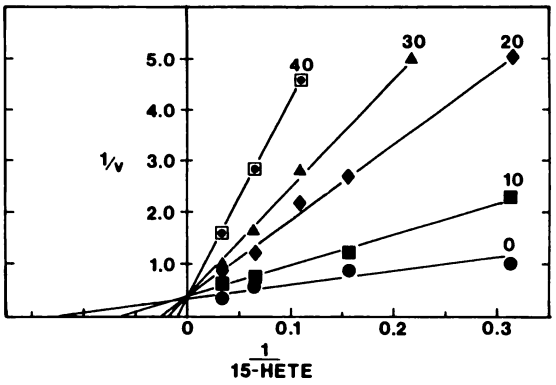

Fig. 3. Inhibition of 15,20-diHETE formation when unlabeled PGE_1 was incubated in the reaction system. The concentrations of PGE_1 were 10, 20, 30, and 40 μM. The results indicate that PGE_1 is a competitive inhibitor of 15-HETE hydroxylation.

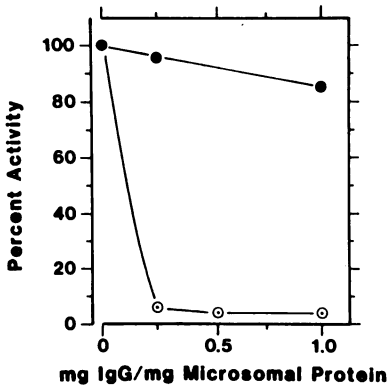

Fig. 4. Inhibition of the formation of 15,20-diHETE by the antibody to lung cytochrome P-450 that hydroxylates prostaglandins. The *open circles* represent activities observed in the presence of anti-lung cytochrome P-450 and the *solid circles* represent the effects of nonimmune IgG on the 15-HETE 20-hydroxylase activity. The results are the average of duplicate experiments.

hydroxylase activity. The hydroxylation of 15-HETE was inhibited competitively by PGE_1 (Fig. 3), which was determined to have a K_i value of 11 μM. Further evidence that a single cytochrome P-450 catalyzed both reactions was obtained by the inhibition of 15,20-diHETE formation by the antibody prepared against the cytochrome P-450 that was purified from the lungs of pregnant rabbits and characterized as the prostaglandin 20-hydroxylase (4, 6). As shown in Fig. 4, this antibody was a potent inhibitor of the hydroxylation of 15-HETE as the addition of 0.25 mg of the IgG fraction/mg of microsomal protein produced a total inhibition of 15,20-diHETE formation.

These data indicate that 15-HETE, in addition to PGE_1, is a substrate for lung microsomes and that a common cytochrome P-450 is responsible for this 20-hydroxylation reaction. The apparent K_m for the 15-HETE in the lung microsomes is 8 μM (Fig. 3), which is similar to the K_m value (3 μM) reported for PGE_1 in the reconstituted systems (4, 5).

The similar specific activities and K_m values for 15-HETE and PGE_1 indicate that the cyclopentane ring of prostaglandin molecules is not essential for enzyme recognition. The lack of activity toward 5-HETE, 12-HETE, and LTB_4 by this lung cytochrome P-450 indicates the importance of the 15-hydroxy group of 15-HETE and prostaglandin molecules for enzyme recognition. These results suggest that the substrate specificity of other cytochromes P-450 from liver and kidney that are reported to hydroxylate prostaglandins should also be examined with 15-HETE as substrate.

It has been reported that 15-HETE can be produced from isolated human tracheal epithelial cells incubated with exogenous arachidonic acid (17) and that 15-HETE is recovered in the bronchiolar lavage fluid of antigen-challenged asthmatics (18). It has been suggested that, in the airway, 15-HETE may serve as an irritant and stimulator of mucus secretion (19). Further studies are necessary to determine whether 15-HETE can be further metabolized to 15,20-diHETE by isolated cells or *in situ* in the lung, and whether the ω-hydroxylation of 15-HETE represents an inactivation pathway similar to the metabolism of LTB_4.

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References

1. Powell, W. S., and S. Solomon. Formation of 20-hydroxyprostaglandins by lungs of pregnant rabbits. *J. Biol. Chem.* **253**:4609–4616 (1978).
2. Powell, W. S. ω-Oxidation of prostaglandins by lung and liver microsomes. Changes in enzyme activity induced by pregnancy, pseudopregnancy, and progesterone treatment. *J. Biol. Chem.* **253**:6711–6716 (1978).
3. Powell, W. S. Metabolism of prostaglandins, prostaglandin analogs, and thromboxane B_2 by lung and liver microsomes from pregnant rabbits. *Biochim. Biophys. Acta* **575**:335–349 (1979).
4. Williams, D. E., S. E. Hale, R. T. Okita, and B. S. S. Masters. A prostaglandin ω-hydroxylase cytochrome P-450 (P-$450_{PG-\omega}$) purified from lungs of pregnant rabbits. *J. Biol. Chem.* **259**:14600–14608 (1984).
5. Yamamoto, S., E. Kusunose, K. Ogita, M. Kaku, K. Ichihara, and M. Kusunose. Isolation of cytochrome P-450 highly active in prostaglandin ω-hydroxylation from lung microsomes of rabbits treated with progesterone. *J. Biochem.* **96**:593–603 (1984).
6. Masters, B. S. S., R. T. Okita, D. E. Williams, S. E. Hale, A. S. Muerhoff, and R. F. Hevner. Prostaglandin and fatty acid ω- and (ω-1)-hydroxylation by cytochromes P-450 in extrahepatic tissue, in *Microsomes and Drug Oxidations* (A. R. Boobis, J. Caldwell, F. DeMatteis, and C. R. Elcombe, eds.). Taylor and Francis, Philadelphia, 74–83 (1985).
7. Kupfer, D. Endogenous substrates of monoxygenases: fatty acids and prostaglandins. *Pharmacol. Ther.* **11**:469–496 (1980).
8. Capdevila, J., Y. R. Kim, C. Martin-Wixtrom, J. R. Falck, S. Manna, and R. W. Estabrook. Influence of a fibric acid type of hypolipidemic agent on the oxidative metabolism of arachidonic acid by liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* **243**:8–19 (1985).
9. Marcus, A. J., L. B. Safier H. L. Ullman, M. J. Broekman, N. Islam, T. D. Oglesby, and R. R. Gorman. 12*S*,20-Dihydroxyeicosatetraenoic acid: a new eicosanoid synthesized by neutrophils from 12*S*-hydroxyeicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. *Proc. Natl. Acad. Sci. USA* **81**:903–907 (1984).
10. Marcus, A. J., L. B. Safier, H. L. Ullman, N. Islam, M. J. Broekman, and C. von Schacky. Studies on the mechanism of ω-hydroxylation of platelet 12-hydroxyeicosatetraenoic acid (12-HETE) by unstimulated neutrophils. **79**:179–187 (1987).
11. Wong, P. Y.-K., P. Westlund, M. Hamberg, E. Granstrom, P. H.-W. Chao, and B. Samuelsson. ω-Hydroxylation of 12-L-hydroxy-5,8,11,14-eicosanoid acid in human polymorphonuclear leukocytes. *J. Biol. Chem.* **259**:2683–2686 (1984).
12. Soberman, R. J., T. W. Harper, R. C. Murphy, and K. F. Austen. Identification and functional characterization of leukotriene B_4 20-hydroxylase of

human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA* **82**:2292–2295 (1985).
13. Shak, S., and I. M. Goldstein. Leukotriene B_4 ω-hydroxylase in human polymorphonuclear leukocytes. Partial purification and identification as a cytochrome P-450. *J. Clin. Invest.* **76**:1218–1228 (1985).
14. Shak, S., and I. M. Goldstein. ω-Oxidation is the major pathway for the catabolism of leukotriene B_4 in human polymorphonuclear leukocytes. *J. Biol. Chem.* **259**:10181–10187 (1984).
15. Harper, T. W., M. J. Garrity, and R. C. Murphy. Metabolism of leukotriene B_4 in isolated rat hepatocytes. Identification of a novel 18-carboxy-1,20-dinor leukotriene B_4 metabolite. *J. Biol. Chem.* **261**:5414–5418 (1986).
16. VanRollins, M., and R. C. Murphy. Autooxidation of docosahexaenoic acid: analysis of ten isomers of hydroxydocosahexaenoate. *J. Lipid Res.* **25**:507–517 (1984).
17. Hunter, J. A., W. E. Finkbeiner, J. A. Nadel, E. J. Goetzl, and M. J. Holtzman. Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. *Proc. Natl. Acad. Sci. USA* **82**:4633–4637 (1985).
18. Murray, J. J., A. B. Tonnel, A. R. Brash, L. J. Roberts II, P. Gosset, R. Workman, A. Capron, and J. A. Oates. Release of prostaglandin D_2 into human airways during acute antigen challenge. *N. Engl. J. Med.* **315**: 800–804 (1986).
19. Marom, Z., J. H. Shelhamer, F. Sun, and M. Kaliner. Human airway monohydroxyeicosatetraenoic acid generation and mucus release. *J. Clin. Invest.* **71**:122–127 (1983).

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