Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 455-459

12(R)-HYDROXYEICOSATETRAENOIC ACID SYNTHESIS BY 3-METHYLCHOLANTHRENE- AND CLOFIBRATE-INDUCIBLE CYTOCHROME P450 IN PORCINE CILIARY EPITHELIUM

Tadashi Asakura and Hitoshi Shichi

Department of Ophthalmology, Wayne State University School of Medicine Detroit, MI 48201

Received July 10, 1992

SUMMARY: Porcine ciliary epithelial microsomes synthesized 12[S]-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12[S]-HETE) from arachidonic acid by a membrane-bound lipoxygenase and 12[R]-isomer by the cytochrome P450-dependent monooxygenase system. The activity to form 12(R)-isomer was markedly enhanced by 3-methylcholanthrene and clofibrate. Both basal and induced levels of 12(R)-HETE synthesizing activity were considerably higher in nonpigmented epithelial cells than in pigmented cells of the ciliary processes. The induced activity was suppressed by polyclonal antibodies raised against purified cytochrome P450 IA1 and NADPH-P450 reductase but not by substrates for clofibrate-inducible ω/ω-1 hydroxylases (P450 IVA-mediated). These results suggest that 12(R)-HETE synthesis by porcine ciliary microsomes may be mediated by a cytochrome P450 of the IA family.

12-Hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) is synthesized from arachidonic acid by 12-lipoxygenase as well as the microsomal cytochrome P450-dependent monooxygenase system. The formation of 12-HETE is stereospecific; 12-lipoxygenase produces 12(S)-HETE, while the P450 enzyme synthesizes 12(R)-isomer (1). The P450 enzyme involved in the formation of 12(R)-HETE has not been characterized in any tissue.

The major eicosatetraenoic acid formed by porcine ciliary epithelium is 12-HETE (2). In the eye, the epithelium demonstrates highest P450-mediated activities such as aryl hydrocarbon hydroxylase (3), alkoxyresorufin O-dealkylases (4) and ω/ω-1 prostaglandin hydroxylases (5). In this work we have therefore investigated P450-dependent synthesis of 12-HETE in this tissue and report here that the microsomal activity to produce 12(R)-HETE is enhanced or induced markedly by 3-methylcholanthrene (3-MC) and clofibrate and the induced activities are inhibited by antibodies against purified cytochrome P450 IA-1.

MATERIALS AND METHODS

Isolation of porcine ciliary epithelial cells for preparation of microsomes . Nonpigmented epithelial (NPE) cells and pigmented epithelial (PE) cells were isolated from fresh porcine ciliary processes as described previously (6). For induction of enzyme activity, NPE cells and PE cells (still attached to the stroma) were collected in Dulbecco's Modified Eagle Medium (Gibco) containing 17% fetal bovine serum, gentamycin and fungizone and cultured for 48 hours with or without inducer (1 μM 3-MC or 2 mM clofibrate) as described (6). At the end of culture, PE cells were separated from the stroma and centrifuged. NPE and PE cells were then

0006-291X/92 $4.00

homogenized separately in 0.25 M sucrose/10 mM Tris buffer, pH 8.0 and centrifuged at 10,000 x g, 15 min. The supernatant was then centrifuged at 100,000 x g for 60 min. to prepare the microsomal fraction. Anti-P450 antibody used was guinea pig serum raised against purified rabbit liver cytochrome P450 IA1. The antibody was used previously for immunoprecipitation of P450 IA1 mutant rats (7), inhibition of 3-MC induced ethoxyresorufin O-dealkylase in porcine ciliary NPE cells (6) and immunocytochemical localization of P450 IA1 in the ciliary epithelium (8). Anti-NADPH-cytochrome P450 reductase (rat liver) antibody raised in rabbit was purchased from Oxygene (Dallas, Texas).

Assay of 12-HETE synthesizing activity . A mixture (1.0 ml) of an NADPH generating system (40 μmol glucose-6-phosphate, 1.5 u glucose-6-phosphate dehydrogenase and 1.2 μmol NADP, all from Sigma), 100 μmol Tris-Cl (pH 8.0) and microsomes (ca. 0.5 mg protein) was preincubated at 37°C for 10 min. with or without inhibitor [100 μM lauric acid (Sigma), 100 μmol prostaglandin E2 (Cayman Chemicals), or antibody]. [^{3}H]Arachidonic acid (1 nmol, NEN) in ethanol was added to the mixture to start the incubation at 37°C. After 15 min., the reaction was stopped with 20 μl 6N HCl and [^{3}H]metabolites were extracted with ethylacetate and analyzed by HPLC [Ultrasphere ODS column (5 μ, 4.6 x 150 mm, Altex), elution with 50% acetonitrile in 0.1% phosphoric acid at 1.0 ml per min., detection at 235 nm]. The retention time for 12-HETE was 18.5 min. The concentration of product was estimated from radioactivity and the activity was expressed as pmol 12-HETE formed per mg protein in 15 min.

Analysis of 12-HETE stereoisomers. 12-HETE from the above HPLC column was transferred to chloroform, dried in vacuo and reacted with ethereal diazomethane for 1 hour at room temperature. 12-HETE methyl ester thus prepared was dried, dissolved in hexane and anaylzed by HPLC [Bakerbond DNBPG (ionic) chiral column (4.6 x 250 mm, J.T. Baker), elution with hexane/isopropyl alcohol (100/0.5) at 0.7 ml per min., detection at 235 nm]. The retention time for 12(S)- and 12(R)-isomer was 29 and 30 min., respectively. Ethereal diazomethane was prepared by shaking Diazald (Sigma) in diethyl ether (200 μl) with 20 N KOH (50 μl) and absorbing the product in ether (5 ml).

RESULTS

Induction of 12-HETE synthesizing activities of porcine ciliary epithelial microsomes. As shown in Fig. 1, the 12-HETE synthesizing activity of NPE cells was twice as high as that of PE cells. The basal activity of NPE cells was enhanced about 2.4 fold by treatment of primary cultures of NPE cells with 3-MC or clofibrate. Similar increases in the basal activities of PE

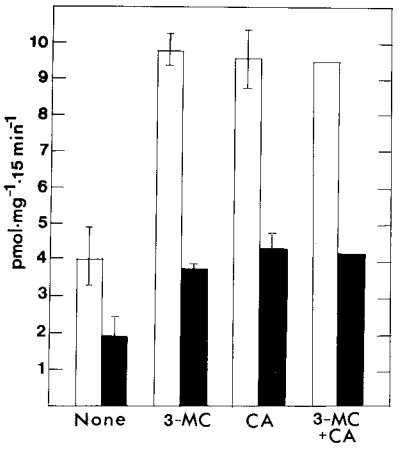

Fig. 1. Induction of 12(R)-HETE synthesizing activity. Open bars and filled bars represent activities (Mean ± SEM, n=3) of NPE cells and PE cells, respectively. CA: Clofibrate.

Table 1. Induction of 12-HETE synthesizing activities in porcine ciliary epithelium

Inducer	Stereoisomer	NPE (pmol · mg^{-1} · 15 min^{-1})		PE (pmol · mg^{-1} · 15 min^{-1})	
None	R	2.71	(100)*	1.14	(100)*
	S	2.15		0.86	
3-MC	R	6.42	(236)	2.56	(226)
	S	2.81		1.11	
CA	R	5.84	(215)	2.84	(250)
	S	2.62		1.78	
3-MC + CA	R	6.60	(243)	2.95	(259)
	S	2.74		1.17	

* Arbitrarily taken as 100% for comparison. CA, clofibrate

cells by 3-MC and clofibrate were also observed. Incubation of NPE cells and PE cells with a combination of 3-MC and clofibrate did not enhance the activities more than the levels induced by either 3-MC or clofibrate alone. The induced activities of both NPE and PE cells by treatment with 3-MC and clofibrate were inhibited almost completely by cycloheximide or actinomycin D (data not shown).

Stereospecificity of induced activities to synthesize 12-HETE. Both NPE microsomes and PE microsomes synthesized 12(R)-isomer as well as 12(S)-isomer (Table 1). However, only the formation of 12(R)-HETE was NADPH-dependent and induced by 3-MC and clofibrate. A combination of the two compounds did not cause a cummulative increase in the induced activity. The activity to synthesize the (S)-isomer did not require NADPH and was probably attributed to a microsome-bound 12-lipoxygenase similar to the enzyme reported recently (9).

Inhibition of induced activities. Since 12(R)-HETE synthesizing activity was inducible by 3-MC, the effect of rabbit antibody against 3-MC-inducible cytochrome P450 IA1 was examined. The antibody inhibited the activities of NPE as well as PE cells induced by both 3-MC and clofibrate (Table 2). These activities were also suppressed significantly by anti-P450 reductase antibody, a result confirming that 12(R) HETE formation was P450-mediated. Commercial nonimmune sera used for control showed no effect on the activities. Lauric acid and prostaglandin E2, which are substrates for P450-dependent ω/ω-1 hydroxylase activities of porcine ciliary epithelium (5), were not competitive inhibitors of 12(R)-HETE synthesizing activity.

DISCUSSION

This study demonstrated that both 12(R)-HETE and 12(S)-HETE are synthesized by porcine ciliary microsomes. The microsomal activity to synthesize 12(R)-HETE requires NADPH and is enhanced or induced by 3-MC and clofibrate. The induced activity is inhibited by antibodies against NADPH-cytochrome P450 reductase and purified P450. From these findings it is concluded that synthesis of 12(R)-HETE in this tissue is P450-mediated.

Table 2. Inhibition by antibodies of 12-HETE synthesizing activities in porcine ciliary epithelium

Inhibitors	Stereoisomer	NPE (pmol · mg^{-1} · 15 min^{-1}) total act.	NPE (pmol · mg^{-1} · 15 min^{-1}) total act. minus basal act.	PE (pmol · mg^{-1} · 15 min^{-1}) total act.	PE (pmol · mg^{-1} · 15 min^{-1}) total act. minus basal act.
1. MC-treated microsomes					
None	R	6.87	4.16 (100)*	2.44	1.30 (100)*
	S	2.90		0.97	
Anti-MC inducible P450 IA1	R	4.16	1.44 (35)	1.61	0.47 (36)
	S	2.46		0.83	
Anti-P450 reductase	R	4.97	2.26 (54)	1.90	0.76 (59)
	S	2.16		0.90	
Lauric acid	R	7.56	4.85 (117)	2.68	1.54 (118)
	S	3.08		1.02	
PGE_2	R	7.21	4.50 (108)	2.56	1.42 (109)
	S	2.96		0.99	
2. CA-treated microsomes					
None	R	5.70	2.99 (100)*	2.78	1.64 (100)*
	S	2.84		1.12	
Anti-MC inducible P450 IA1	R	4.00	1.29 (43)	2.00	0.86 (52)
	S	2.50		0.96	
Anti-P450 reductase	R	4.62	1.19 (64)	2.23	1.09 (66)
	S	2.55		1.01	
Lauric acid	R	6.27	3.56 (119)	3.06	1.92 (117)
	S	3.00		1.37	
PGE_2	R	5.99	3.28 (110)	2.92	1.78 (109)
	S	2.90		1.14	

*Arbitrarily taken as 100% for comparison.

Clofibrate is an effective inducer of fatty acid ω/ω-1 hydroxylases mediated by cytochrome P450 of the IVA family in porcine ciliary epithelium and enhances the basal activities 3-fold (5). The hydroxylase activities are also enhanced by 3-MC but no more than 1.3 fold of the basal level. In contrast, 3-MC and clofibrate are equally effective for induction of 12(R)-HETE synthesizing activity. Furthermore, the synthesis of 12(R)-HETE is not competed by lauric acid and prostaglandin E2 which serve as substrates for ω/ω-1 hydroxylases. These results do not seem to support that 12(R)-HETE synthesis is mediated by a P450 of the IVA family in this tissue. The inhibition of both 3-MC-inducible and clofibrate-inducible activities by antibody directed toward P450 IA1 suggests that P450 isozymes with similar, if not identical, epitopes to those of P450 IA1 are induced by these inducers.

It has been reported that 12(R)-HETE is synthesized by rabbit corneal epithelium and inhibits Na, K-ATPase activity in the cornea (10) and kidney (11). Recent papers report that the compound also inhibits the Na, K-ATPase activity of rabbit ciliary epithelium (12) and lowers intraocular pressure when administered topically (12, 13). Intraocular pressure is regulated by the kinetics of aqueous humor formation and absorption. Either increased formation of the aqueous by the ciliary epithelium or reduced absorption by the trabecular meshwork or both

leads to an increase in intraocular pressure. The Na, K-ATPase activity localized to the nonpigmented ciliary epithelial cells is believed to play a critical role in aqueous humor production (14). The present work is the first demonstration that 12(R)-HETE is synthesized by ciliary epithelial cells. It may be of physiological significance that the inducible activity to synthesize 12(R)-HETE is primarily associated with nonpigmented epithelial cells in which Na, K-ATPase activity is enriched.

ACKNOWLEDGMENTS

We thank Dr. Toshihiro Sugiyama for the gift of anti-cytochrome P450 antibody and Ms. Debra A. Jones for typing the manuscript. This work was supported by NIH grant EY04694 and a departmental grant from the Research to Prevent Blindness, Inc.

REFERENCES

1. Fitzpatrick, F.A. and Murphy, R.C. (1989) Pharmacol. Rev. 40, 229-241.
2. Sakamoto, S. and Shichi, H. (1991) J. Ocular Pharmacol. 7, 141-145.
3. Das, N.D. and Shichi, H. (1981) Exp. Eye Res. 33, 525-533.
4. Sakamoto, S. and Shichi, H. (1990) J. Ocular Pharmacol. 6, 211-217.
5. Asakura, T. and Shichi, H. (1992) Exp. Eye Res. in press.
6. Sakamoto, S. and Shichi, H. (1991) Biochem. Pharmacol. 41, 611-616.
7. Sugiyama, T., Takeichi, N., Kobayashi, H., Yoshida, M.C., Sasaki, M. and Taniguchi, N. (1988) Carcinogenesis 9, 1569-1572.
8. Shichi, H., Mahalak, S.M., Sakamoto, S. and Sugiyama, T. (1991) Curr. Eye Res. 10, 779-788.
9. Chang, W.C. Ning, C.C., Lin, M.T. and Huang, J.D. (1992) J. Biol. Chem. 267, 3657-3666.
10. Schwartzman, M.L., Balazy, M., Masferrer, J., Abraham, N.G., McGiff, J.C. and Murphy, R.C. (1987) Proc. Natl. Acad. Sci. USA 84, 8125-8129.
11. Escalante, B., Falck, J.R., Yadagiri, P., Sun. L. and Schwartzman, M.L. (1988) Biochem. Biphys. Res. Commun. 152, 1269-1274.
12. Delamere, N.A., Socci, R.R., King, K.L. and Bhattacherjee, P. (1991) Invest. Ophthalmol. Vis. Sci. 32, 2511-2514.
13. Masferrer, J.L., Dunn, M.W. and Schwartzman, M.L. (1990) Invest. Ophthalmol. Vis. Sci. 31, 535-539.
14. Davson, H. (1980) In Physiology of the Eye (H. Davson, Ed.) pp. 9-81. Academic Press, New York, NY.