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SIGNIFICANCE OF CYTOCHROME P-450 (P-450 HFLa) OF HUMAN FETAL LIVERS IN THE STEROID AND DRUG OXIDATIONS

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Abstract—The purpose of this study was to clarify the pharmacological and physiological significance of P-450 HFLa. Thus, correlations between cytochrome P-450 (P-450 HFLa) level and different monooxygenase activities were investigated in liver homogenates from human fetuses. Poor correlation was seen between P-450 HFLa level and the activity of benzphetamine *N*-demethylation or aniline hydroxylation. In contrast, the content of P-450 HFLa was highly correlated with the activity of benzo(*a*)pyrene hydroxylation, 7-ethoxycoumarin *O*-deethylation or testosterone 6β-hydroxylation. In microsomes from human adult livers, a moderate relationship was also observed between testosterone 6β-hydroxylation and P-450 HFLa level. Furthermore, antibodies to P-450 HFLa inhibited testosterone 6β-hydroxylase activity in fetal and adult livers to similar extents.

We conclude that P-450 HFLa is a form of cytochrome P-450 which catalyzes testosterone 6β-hydroxylation and limited drug oxidations in human fetal and adult livers.

Cytochrome P-450 is the major component involved in metabolism of xenobiotics and endogenous substrates such as testosterone and laurate. Multiple forms of cytochrome P-450 have been shown to be present in the liver of laboratory animals and are characterized by an overlapping substrate specificity [1, 2]. Moreover, these isozymes of cytochrome P-450 can participate differently in the bioactivation and detoxication of a wide variety of xenobiotics. Unlike widely used experimental animals, the activities of monooxygenases are detectable at a significant rate even at early stages of gestation in the human and subhuman primate fetuses [3–5]. Therefore, the characterization of cytochrome P-450 in human fetal tissues seems to be important in terms of prenatal pharmacology and toxicology. Recently, we succeeded in the purification and partial characterization of one of the major forms of cytochrome P-450 (P-450 HFLa) in human fetal livers [6]. The P-450 HFLa has been shown to catalyze different monooxygenase reactions in the presence of NADPH-cytochrome P-450 reductase purified from phenobarbital-treated rats and dilauroyl-L-3-phosphatidylcholine. The purpose of the present study was to clarify the pharmacological and physiological significance of P-450 HFLa.

MATERIALS AND METHODS

NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co. Protein A-Sepharose CL-4B was from Pharmacia and nitrocellulose membrane from Bio-Rad. 6β-Hydroxytestosterone was a gift from Dr D. N. Kirk, Queen Mary College, Unviersity of London. Emulgen 913 and benzphetamine were kindly provided by Kao Atlas Co., and Upjohn Company, respectively. Other reagents were of the highest grade commercially available.

Livers were obtained from fetuses, stillborn or prematurely delivered for medical reasons and were stored at −70° until use. Adult liver samples excised in judicial or administrative dissections of individuals with no history of drug poisoning or liver diseases were used within about 20 hr after death, and were stored at −70° until use. No information was available about smoking and drinking habits.

A typical incubation mixture contained 0.1 M K-phosphate (pH 7.4), 0.1 mM EDTA, a NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase, 6 mM $MgCl_2$), a substrate and liver homogenates of human fetuses or liver microsomes of human adults in a final volume of 1.0 ml. In some cases, liver homogenates of human fetuses were washed to remove contaminated hemoglobin by ultracentrifugation at 105,000 *g* for 30 min.

Activity of benzphetamine *N*-demethylation was estimated by determining formaldehyde by the method of Nash [7]. Aniline hydroxylation, 7-ethoxycoumarin *O*-deethylation and benzo(*a*)pyrene hydroxylation were assayed according to the methods of Imai *et al.* [8], Greenlee and Poland [9] and Kuntzman *et al.* [10], respectively. For testosterone hydroxylation, liver homogenates from fetuses or liver microsomes from adults were incubated for 15 min at 37° with 0.1 mM testosterone. After extraction with benzene and evaporation to dryness under nitrogen, residues were dissolved in isopropanol. 6β-Hydroxylated metabolite was separated on HPLC equipped with Senshu pak Silica-1251-N (250 × 4.6 mm) according to the procedure of Hayashi and Okuda [11]. Protein concentrations

Table 1. Comparison of monooxygenase activities in fetal and adult human livers

	Human fetus	Human adult
Benzo(*a*)pyrene hydroxylation (pmole/min/g of liver)	6.8 ± 1.9 (11)	882.7 ± 440.3 (13)
7-Ethoxycoumarin *O*-deethylation (nmole/min/g of liver)	0.03 ± 0.01 (13)	8.70 ± 2.33 (14)
Aniline hydroxylation (nmole/min/g of liver)	1.3 ± 0.1 (13)	11.0 ± 1.8 (13)
Benzphetamine *N*-demethylation (nmole/min/g of liver)	18.4 ± 1.6 (13)	75.0 ± 33.3 (6)
Testosterone 6β-hydroxylation (nmole/min/g of liver)	3.4 ± 0.4 (13)	24.7 ± 14.4 (14)

Each value represents mean ± SE. Figures in parentheses indicate number of livers studied.

were measured by the method of Lowry *et al.* [12] using bovine serum albumin as a standard.

Electrophoresis and staining (Western blot-PAP staining) were carried out as described by Laemmli [13] and Geungerich *et al.* [14], respectively.

The purification of P-450 HFLa from liver homogenates of human fetuses and the preparation of antibodies in a rabbit have been described elsewhere [6]. The immunoglobulin fractions were prepared with protein A-Sepharose CL-4B column chromatography.

RESULTS

The activities of oxidative metabolism of aniline, benzphetamine, 7-ethoxycoumarin, benzo(*a*)pyrene and testosterone in fetal and adult human livers are shown in Table 1. Monooxygenase activities in fetal livers, expressed as a percentage of the level in adult livers, varied dependent on the substrate studied. The activities of benzo(*a*)pyrene hydroxylation and 7-ethoxycoumarin *O*-deethylation in fetal livers were only 0.8% and 0.3%, respectively, of corresponding activities in adult livers. In contrast, the activities of aniline hydroxylation and benzphetamine *N*-demethylation in fetal livers were found to be about 12% and 25%, respectively, of the corresponding values in adult livers. Moreover, testosterone 6β-hydroxylation in fetal livers showed about 14% of the adult values although both activities obtained with fetal and adult livers were lower than those reported by Cresteil *et al.* [15]. These percentages based on average values were in accord with the findings reported previously [15–17].

The amounts of P-450 HFLa immunochemically determined in liver homogenates from individual human fetus were plotted separately against different monooxygenase activities (Figs 1 and 2). The P-450 HFLa levels in liver homogenates varied among the human fetuses studied. In addition, the correlations between monooxygenase activities and the P-450 HFLa level were dependent on the substrate used. As shown in Fig. 1, no significant correlation was seen between the P-450 HFLa level and aniline hydroxylase ($r = 0.241$) or benzphetamine *N*-demethylase ($r = 0.481$, not shown) activity. Based on milligrams of protein, activities of aniline hydroxylase and benzphetamine *N*-demethylase in homogenates of fetal livers increased by about only 18% (mean of four determinations) and 15% (mean

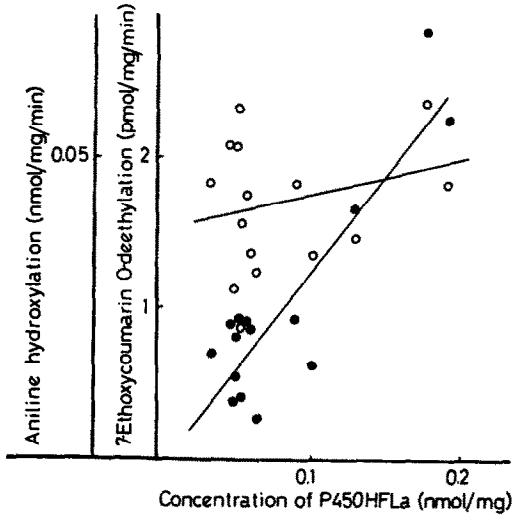

Fig. 1. Correlation between P-450 HFLa content and aniline hydroxylase activity or 7-ethoxycoumarin *O*-deethylase activity in human fetal livers. The concentrations of aniline and 7-ethoxycoumarin used were 2 mM and 0.4 mM, respectively. The activities of aniline hydroxylase (○) and 7-ethoxycoumarin *O*-deethylase (●) are expressed as nmole/min/mg protein and pmole/min/mg protein, respectively, and immunochemically determined P-450 HFLa content as nmole/mg protein.

of three determinations), respectively, by washing of homogenates by suspension and ultracentrifugation. The content of P-450 HFLa significantly correlated with the activities of 7-ethoxycoumarin *O*-deethylase ($r = 0.878$, $P < 0.01$, Fig. 1) and benzo(*a*)pyrene hydroxylase ($r = 0.814$, $P < 0.01$, not shown). 6β-Hydroxytestosterone is a major metabolite formed during incubations of testosterone with fetal livers [18]. As can be seen in Fig. 2, a high correlation ($r = 0.921$, $P < 0.01$) was found between P-450 HFLa level and the 6β-hydroxylation of testosterone. As reported elsewhere [6], a form of cytochrome P-450 which is immunochemically and electrophoretically similar to P-450 HFLa purified from fetal livers is present in liver microsomes from human adults. Therefore, in each of the adult livers, the activity of testosterone 6β-hydroxylase was plotted against the content of cytochrome P-450 immunochemically detectable with anti-P-450 HFLa antibodies (Fig. 2). A relatively good correlation was observed between

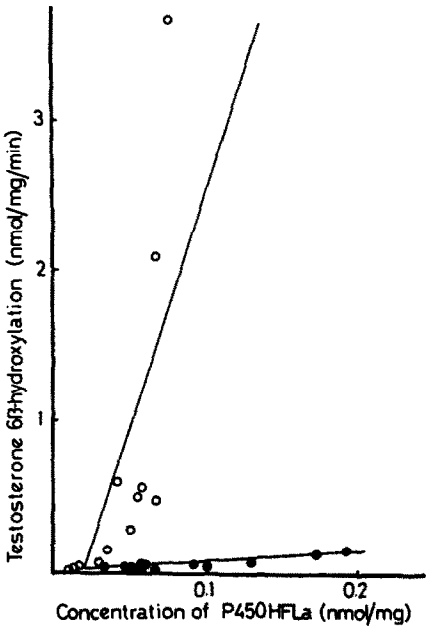

Fig. 2. Correlation between P-450 HFLa content and testosterone 6β-hydroxylase activity in human fetal and adult livers. The concentration of testosterone used was 0.1 mM. Testosterone 6β-hydroxylase activity in fetal liver (●) or in adult liver (○) is expressed as nmole/mg protein, and immunochemically determined P-450 HFLa content in human fetal and adult livers as nmole/mg protein.

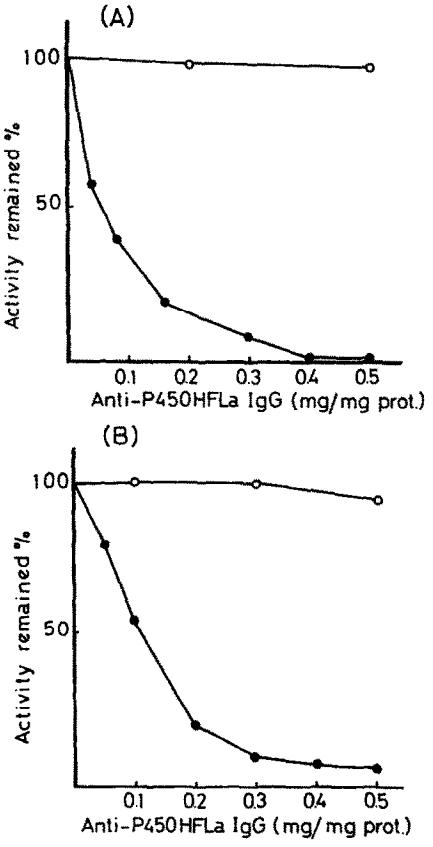

Fig. 3. Effects of anti-P-450 HFLa IgG on testosterone 6β-hydroxylase activities in human fetal and adult livers. Testosterone 6β-hydroxylase activity was assayed after preincubation with preimmun-IgG (○) or anti-P-450 HFLa IgG (●) for 10 min at 37°. The specific contents of P-450 HFLa in fetal and adult livers were 0.062 nmole/mg protein and 0.068 nmole/mg protein, respectively. (A) Testosterone 6β-hydroxylase activity in the absence of antibodies was 0.14 nmole/min/mg protein in fetal liver. (B) Testosterone 6β-hydroxylase activity in the absence of antibodies was 1.25 nmole/min/mg protein in adult liver.

P-450 HFLa level and the activity of testosterone 6β-hydroxylase in adult liver microsomes ($r = 0.703$). The effects of anti-P-450 HFLa IgG on testosterone 6β-hydroxylase in fetal and adult livers are shown in Fig. 3.

Testosterone 6β-hydroxylase in both fetal and adult livers were found to be sensitive to the antibodies. 6β-Hydroxylated metabolite of testosterone was not detectable when anti-P-450 HFLa IgG was added at a concentration of about 0.4 mg/mg of protein of fetal liver homogenates suggesting that P-450 HFLa may be almost exclusively responsible for the 6β-hydroxylation of testosterone in fetal livers. A similar result was observed in liver microsomes from human adults. Anti-P-450 HFLa inhibited the reaction by greater than 90% at a concentration of 0.3 mg of anti-P-450 HFLa IgG per microsomal protein. In addition, the extent of inhibition by the antibodies was independent of the amounts of the activity in the absence of antibodies (not shown).

DISCUSSION

As was expected from previous studies [19], in which anti-P-450 HFLa antibodies were inhibitory to benzo(*a*)pyrene hydroxylation and 7-ethoxycoumarin *O*-deethylation in fetal livers, a good correlation was observed between P-450 HFLa level and benzo(*a*)pyrene hydroxylation or 7-ethoxycoumarin *O*-deethylation. In addition, testosterone 6β-hydroxylase activity also highly correlated with P-450 HFLa contents. The intersection of the regression line at positive range on the abscissa may be due to the presence of apo-P-450 HFLa in fetal and adult livers. On the other hand, no significant correlation was observed between P-450 HFLa and aniline hydroxylation or benzphetamine *N*-demethylation in liver homogenates from fetuses indicating that P-450 HFLa contributes to a lesser extent in these reactions in fetal livers. From these results, it is suggested that monoxygenase activities are derived by multiple cytochrome P-450 species in fetal livers as has been suggested by Cresteil *et al.* [15].

More recently, cytochrome P-450 isozymes purified from microsomes of adult human livers have been shown to be present in fetal human livers [20, 21]. Based on substrate specificity and molecular weight, it seems that P-450 HFLa purified from liver homogenates of human fetuses may be different from isozymes 5, 8 and 9 purified from liver microsomes of adult human. However, the complete characterization of P-450 HFLa including NH_2-terminal amino acid sequencing is needed before any final

conclusions can be drawn on the similarity or identity of P-450 HFLa with previously described forms of human cytochrome P-450 [21–23].

It is interesting to note that although testosterone 6β-hydroxylation rate was higher in adult livers than in fetal livers, the reaction was also found to correlate positively with the content of P-450 HFLa in adult livers and was almost completely inhibited by the addition of anti-P-450 IgG to microsomes of adult human livers. Furthermore, the slope of the regression line obtained from the plots of testosterone 6β-hydroxylase activity versus the level of P-450 HFLa with adult livers (31.9) was quite different from that with fetal livers (0.72). In addition, it has been demonstrated that in some cases, NADPH-cytochrome P-450 reductase rather than cytochrome P-450 may be the rate limiting component in monoxygenase reactions [24–26]. However, no apparent increase in the activity of testosterone 6β-hydroxylase was observed when NADPH-cytochrome P-450 reductase purified from rat livers, which has been shown to reduce cytochrome P-450 of human fetal livers [27], was added to homogenates of fetal livers (not shown). It is therefore suggested that in spite of the immunochemical and electrophoretical similarity, P-450 HFLa from fetal and adult livers differ from each other in catalytic efficiency, although data available at present are not sufficient for any conclusion. The problem still remains unresolved whether catalytic properties of P-450 HFLa change postnatally.

REFERENCES

1. F. P. Guengerich, G. A. Dannan, T. S. Wright, M. V. Martin and L. S. Kaminsky, *Xenobiotica* **12**, 701 (1982).
2. D. E. Ryan, P. E. Thomas, L. M. Reik and W. Levine, *Xenobiotica* **12**, 727 (1982).
3. S. J. Yaffe, A. Rane, F. Sjoqvist, L.-O. Boreus and S. Orrenius, *Life Sci.* **9**, 1189 (1970).
4. M. R. Juchau, M. G. Pedersen and K. G. Symms, *Biochem. Pharmac.* **21**, 2269 (1972).
5. M. R. Juchau, S. T. Chao and C. J. Omiecinski, *Clin. Pharmacokinet.* **5**, 320 (1980).
6. M. Kitada, T. Kamataki, K. Itahashi, T. Rikihisa, R. Kato and Y. Kanakubo, *Archs Biochem. Biophys.* **241**, 275 (1985).
7. T. Nash, *Biochem. J.* **55**, 416 (1953).
8. Y. Imai, A. Ito and R. Sato, *J. Biochem.* **60**, 417 (1966).
9. W. F. Greenlee and A. Poland, *J. Pharmac. exp. Ther.* **205**, 596 (1978).
10. R. Kuntzman, L. C. Mark, L. Brand, M. Jacobson, W. Levin and A. H. Conney, *J. Pharmac. exp. Ther.* **152**, 151 (1966).
11. S. Hayashi and K. Okuda, *J. Biochem.* in press.
12. O. H. Lowry, H. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
14. F. P. Guengerich, P. Wang and N. K. Davidson, *Biochemistry* **21**, 1698 (1982).
15. T. Cresteil, P. Beaune, P. Kremers, J.-P. Flinois and J.-P. Leroux, *Ped. Pharmac.* **2**, 199 (1982).
16. A. Rane and E. Ackerman, *Clin. Pharmac. Ther.* **13**, 663 (1972).
17. O. Pelkonen, E. H. Kaltiala, T. K. I. Larmi and N. T. Karki, *Clin. Pharmac. Ther.* **14**, 840 (1973).
18. M. Ingelman-Sundberg, A. Rane and J.-A. Gustafsson, *Biochemistry* **14**, 429 (1975).
19. M. Kitada, T. Kamataki, K. Itahashi, T. Rikihisa, R. Kato and Y. Kanakubo, *Biochem. biophys. Res. Commun.* **131**, 1154 (1985).
20. T. Cresteil, P. Beaune, P. Kremers, C. Celier, F. P. Guengerich and J.-P. Leroux, *Eur. J. Biochem.* **151**, 345 (1985).
21. P. Wang, P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larry and F. P. Guengerich. *Biochemistry* **22**, 5375 (1983).
22. P. Beaune, J.-P. Flinois, L. Kiffel, P. Kremers and J.-P. Leroux, *Biochim. biophys. Acta* **840**, 364 (1985).
23. P. B. Watkins, S. A. Wrighton, P. Maurel, E. G. Schuetz, G. Mendez-Picon, G. A. Parker and P. S. Guzelian, *Proc. natn. Acad. Sci., U.S.A.* **82**, 6310 (1985).
24. G. T. Miwa, S. B. West and A. Y. H. Lu, *J. biol. Chem.* **253**, 1921 (1978).
25. M. Kitada, H. Kitagawa and T. Kamataki, *Biochem. Pharmac.* **28**, 2670 (1979).
26. M. Kitada, K. Kubota, H. Kitagawa and T. Kamataki, *Jap. J. Pharmac.* **29**, 877 (1979).
27. M. Kitada and T. Kamataki, *Biochem. Pharmac.* **28**, 793 (1979).