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COMPARISON OF HUMAN FETAL HEPATIC AND ADRENAL CYTOCHROME *P*450 ACTIVITIES WITH SOME MAJOR GESTATIONAL STEROIDS AND ETHYLMORPHINE AS SUBSTRATES

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Summary—The immunoidentified human fetal liver and adrenal microsomal contents of cytochromes *P*450IIIA and *P*450XVIIA1 were compared to the metabolism of steroids and ethylmorphine. In *fetal liver microsomes*, 16α-hydroxylation of dehydroepiandrosterone (DHA) was catalyzed at a high rate in almost all investigated specimens and accompanied by a high ethylmorphine *N*-demethylase activity. Progesterone 16α- and 17α-hydroxylation was found only in the livers with the highest DHA 16α-hydroxylation activities, while 21-hydroxylation of progesterone was catalyzed only occasionally in these samples. In *fetal adrenal microsomes*, 21-hydroxylation of progesterone to 11-desoxycorticosterone (DOC) and 11-desoxycortisol (DOCOL) was catalyzed. In contrast to fetal liver, the adrenals also catalyzed the 17α-hydroxylation of pregnenolone and the formation of DHA from 17α-OH-pregnenolone. 16α-hydroxylation of DHA and ethylmorphine *N*-demethylation were modest in the adrenals. *P*450IIIA/HLp was immunoidentified in all investigated liver specimens except two (18/20) in which no ethylmorphine *N*-demethylation or 16α-hydroxylation of DHA was found. *P*450XVIIA1 bands were observed in 8/20 blots of liver specimens, but there was no correlation between the density of these bands and the 17α-hydroxylation of progesterone. All 11 fetal adrenal samples catalyzed DHA 16α-hydroxylation, although only 8 were positive for *P*450IIIA/HLp. All investigated adrenals were positive in regard of the *P*450XVIIA1 band, except one (8/9) with a low 17α-hydroxylation of progesterone. All adrenal specimens catalyzed 21-hydroxylation of progesterone and contained *P*450C21 bands in immunoblots and all samples catalyzed the formation of DOC and DOCOL from progesterone.

Our findings in the fetal livers show a correlation between the DHA 16α-hydroxylation and immunoidentified *P*450IIIA/HLp bands. In adrenals, there was a correlation between the immunoidentified *P*450XVIIA1 bands and the 17α-hydroxylation of progesterone.

INTRODUCTION

In contrast to fetuses of several experimental animals, the human fetus is capable of metabolizing many foreign substrates at substantial rates, even at early stages of gestation [1–4].

Recently it has been shown that the human fetal liver contains high concentrations of an isozyme of cytochrome *P*450, denoted as human fetal liver "a" (HFLa) [5, 6] or *P*450IIIA6 [7]. We have identified a fetal *P*450 form in immunoblotting experiments using a monoclonal antibody (MAb PCN 2-13-1/C2) against a pregnenolone-16α-carbonitrile induced rat liver cytochrome *P*450 [8]. Its level was closely related to the ethylmorphine *N*-demethylation activity in human fetal livers. Surprisingly, this enzyme activity was comparable to that in the human adult liver [9]. It was also demonstrated that some of the steroids abundant in the fetal circulation inhibit this fetal *N*-demethylase. Therefore, our data suggested an essential role of the enzyme in fetal life, probably in the intermediary metabolism of steroids of fetal adrenal and placental–maternal origin [9].

Several pieces of information indicate that the actual fetal enzyme is a member of the cytochrome *P*450III family. In addition to the *P*450IIIA6, there are at least three human adult cytochrome *P*450 forms that have been identified as members of the *P*450III family: IIIA3 [10], IIIA4 [11, 12], and IIIA5 [13]. The latter two forms have an 84% similarity in amino acid sequence, slightly higher than the similarity between IIIA5 and IIIA3 (82%) [13].

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They are both catalyzing the 6β-hydroxylation of testosterone, progesterone and androstenedione, the IIIA4 being consistently more active than the IIIA5 form. Other steroid hydroxylations are also catalyzed.

While the IIIA4 form seems to be expressed in all human adult livers, the IIIA5 form is found only in 10–20% of the cases [13]. Analyses of partial amino terminal sequences were suggestive of a close relation between the IIIA5 form and the HFLa which has also been termed HLp2 [14]. The complete sequence of HFLa was published recently [15]. This *P*450 was shown to be distinct from *P*450IIIA3, IIIA4 [16] and IIIA5 [13] but displayed an 82–88% amino acid similarity with these three forms [13]. It is not clear if the fetal liver cytochrome *P*450 isozyme identified by MAb PCN 2-13-1/C2 is identical to the human adult IIIA5 form. Recent data have demonstrated differences in the metabolism of midazolam and cyclosporin A in fetal and adult human liver [17], results which suggest that some developmental change takes place during ontogenesis. The recent discovery of three other human fetal *P*450 forms [18] emphasizes the complexity of the ontogenic development of *P*450 isozymes.

In the present study, our aim was to study the relation between the immunoidentified contents of cytochrome *P*450IIIA and *P*450XVIIA1 in microsomes from human fetal liver and adrenal specimens and their potential to catalyze various steps in the steroid metabolism or the ethylmorphine *N*-demethylation. For this purpose, the following polyclonal antibodies were used: anti(a)-*P*450IIIA/HLp, a-*P*450XVIIA1 and a-*P*450 c21.

EXPERIMENTAL

Chemicals

Reagents for electrophoresis, transfer and immunodetection were purchased from Bio-Rad (Richmond, CA). Progesterone, 16α-OH- and 17α-OH-progesterone, dehydroepiandrosterone (DHA), 16α-OH-DHA, pregnenolone and 17α-OH-pregnenolone, desoxycorticosterone (DOC) and desoxycortisol (DOCOL) were purchased from Sigma (St Louis, MO).

Antibodies

The following polyclonal antibodies were purchased from OxyGene Comp. (Houston, TX): anti-porcine *P*450XVIIA1, anti-human *P*450IIIA/HLp and anti-bovine *P*450C21.

Biological material

Human fetal tissue specimens from 28 fetuses between 13 and 24 weeks of gestation were obtained at legal abortions made for socio-medical reasons. The abortions were performed by prostaglandin induction and the fetuses kept at +4°C until tissues were excised. The fetal tissues (liver, adrenals) were usually excised within 120 min of the abortion and frozen at −70°C until assay.

Our study was approved by the Ethics committee of the University Hospital.

Microsomal preparation and enzyme activities

Microsomes from human and rat tissues were prepared by ultra-centrifugation and incubation with steroid and drug substrates was performed as described previously [19]. The microsomal pellets were resuspended in 50 mM Tris–HCl, pH 7.4, 0.25 M sucrose buffer, and stored at −70°C until assay. Analyses of the metabolic products were performed by high performance liquid chromatography [19].

The following reactions were studied: 16α-hydroxylation of DHA; 16α-hydroxylation of progesterone; 17α-hydroxylation of progesterone; 21-hydroxylation of progesterone to DOC and DOCOL; 17α-hydroxylation of pregnenolone (only in adrenals); DHA formation from 17-OH-pregnenolone; progesterone formation from pregnenolone; norethylmorphine formation from ethylmorphine; androstenedione formation from 17-OH progesterone (only in liver); and androstenedione formation from DHA (only in liver).

Protein was measured according to Lowry *et al.* [20] using bovine serum albumin as standard.

Electrophoresis and immunoblot techniques

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a discontinuous buffer according to Laemmli [21]. 10% of polyacrylamide in 0.75 mm gels used in a Mini-protean II dual slab cell Bio-Rad equipment. Protein samples were treated with a mixture containing 10 mM dithiothreitol (DTT), 1.8% SDS, 45 mM Tris–HCl pH 8.8, 24% glycerol, 0.08 μg/ml bromophenol blue and boiled for 5–10 min. α-Iodo-acetamide—an alkylating agent—was then added and allowed to stand for 25–30 min in order to complete the cleavage reaction of disulfide bridges. 10 μl samples containing 10 or 20 μg protein were applied to each well.

Transfer to nitrocellulose membranes was carried out for 4 h at 4°C in Mini-Transblot cell (Bio-Rad equipment), according to Towbin *et al.* [22]. After transfer, the nitrocellulose blots were placed in plastic bags with phosphate-buffered saline pH 7.5 (PBS) with 10% newborn calf serum overnight. The following day immunodetection was carried out as follows: after two washes with PBS the nitrocellulose sheet was incubated for 1.5 h with the antibody in PBS containing 10% serum. Upon several washes with PBS the blots were then incubated for 2 h with the secondary antibody (goat anti-mouse alkaline phosphatase conjugated antibody). After several washes the color was developed by 5-bromo-4-chloroindoxyl phosphate substrate and nitroblue tetrazolium according to Blake *et al.* [23].

Gels and Western blots were scanned with a laser light Ultroscan XL densitometer (LKB, Sweden) and the protein amount was estimated using a purified rat liver cytochrome *P*450 (PCN) as control. Previous experience showed a good correlation between the density values of the immunoblots and the protein amount. Molecular weights of the bands shown on SDS–PAGE were estimated by linear regression of the log standard molecular weight as a dependent variable versus the log migration rate according to Poduslo and Rodbard [24].

RESULTS

The major operating pathways of steroid hormone synthesis in the human maternal–placental–fetal unit [25] are depicted in Fig. 1.

Liver metabolism

Only DHA and, to some extent, progesterone served as substrates of the fetal liver microsomal *P*450. Hydroxylation of DHA at the 16α position was vividly catalyzed in almost all (22/28) investigated liver specimens. High 16α-hydroxylation rate was accompanied by high *N*-demethylation of ethylmorphine ($r = 0.626$, Table 1, Fig. 2).

Progesterone 16α- and 17α-hydroxylation was catalyzed (at low and about equal rates) only in the liver specimens with the highest DHA 16α-hydroxylation activity (Table 1). There was no 21-hydroxylation of progesterone except occasionally in specimens with high DHA 16α-hydroxylation.

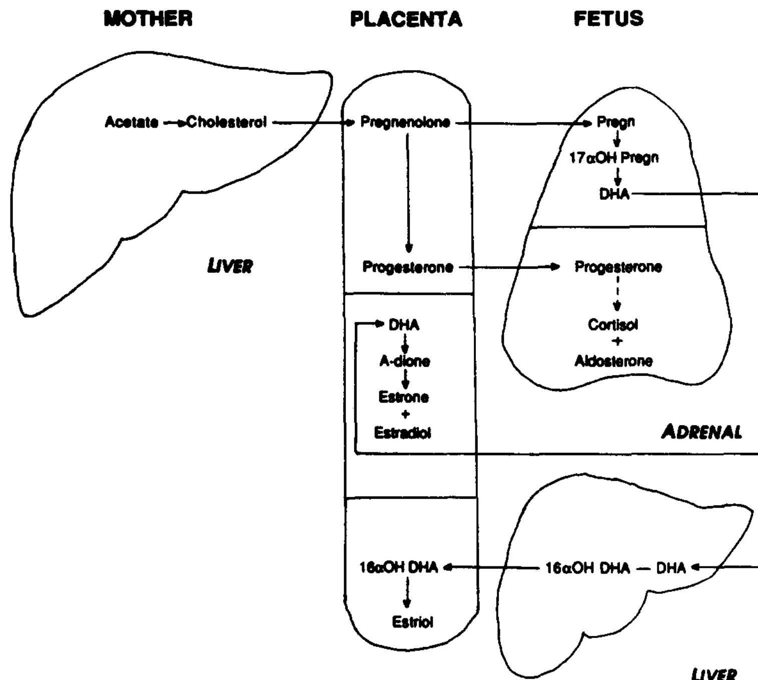

Fig. 1. The maternal–fetal–placental unit in steroid hormone synthesis. Progesterone in the maternal and fetal circulations is synthesized in the placenta from maternal cholesterol. Cortisol and aldosterone in the fetus are synthesized from progesterone derived from the placenta. Maternal and fetal estradiol and estrone are synthesized in the placenta from DHA derived mainly from the fetus. Estriol in the maternal circulation is synthesized in the placenta from 16α-OH-DHA. This precursor must be provided by the combined action of the fetal adrenal gland and liver on pregnenolone supplied by the placenta. A-dione, androstenedione; Pregn, pregnenolone.

Table 1. Steroid metabolism in human fetal liver microsomes in relation to the N-demethylation of ethylmorphine. The numbers give the formation rates of metabolites from progesterone, dehydroepiandrosterone and ethylmorphine, respectively, and the rates are expressed as pmol × mg microsomal protein^{-1} × min^{-1}

Fetus No.	Gestational age (weeks)	16α-OH Progesterone	17α-OH Progesterone	16α-OH dehydro-epiandrosterone	Norethyl-morphine
F1	23	0	0	81	267
F2	13	0	0	76	0
F3	24	0	0	0	73
F4	13	0	0	86	303
F5	15	0	0	440	0
F6	15	10	0	2358	854
F7	15	6	0	0	1028
F8	22	0	0	0	54
F9	21	0	0	0	0
F11	14	0	0	86	96
F12	19	87	80	3570	4215
F13	13	0	0	188	199
F14	16	0	0	139	0
F15	15			951	244
F16	15			799	219
F17	16			395	33
F18	13			44	0
F19	17			0	0
F20	15			0	0
F21	15	35	3	3901	839
F22	17	46	27	2580	149
F24	15	0	0	120	74
F25	15	6	6	1064	36
F26	15	6	0	1064	108
F27	18	2	4	309	40
F28	22	1	0	407	86
F29	21	6	9	562	117
F30	13	32	52	1740	220

No formation of DHA from pregnenolone or 17-OH-pregnenolone was observed in any liver microsomes. Similarly, there was no formation of androstenedione, either with 17-OH-progesterone or DHA as substrates (data not shown).

Adrenal metabolism

In most fetuses, it was possible to assay and compare the hepatic enzyme activities with the adrenal activities in the same individuals. The adrenals catalyzed a broader spectrum of steroid reactions including the 21-hydroxylation of progesterone to DOC and DOCOL, compounds which are further metabolized to cortisol and aldosterone, respectively. The rate of formation of DOC and DOCOL was 553–3730 and 73–2024 pmol/mg microsomal protein/min, respectively, in 13/16 investigated samples. One sample had very low and two had no measurable activities.

The rate of 17α-hydroxylation of progesterone varied betwen 966 and 9400 pmol/mg microsomal protein/min in the same 13/16 adrenal samples, two of which had no activity and one only 109 pmol/mg/min. P450XVIIA1 identified bands were detected in all active samples except the one with the lowest activity. In addition, pregnenolone and 17α-OH-pregnenolone were biotransformed to 17α-OH-pregnenolone and DHA, respectively, which was not the case in the liver.

In contrast to the liver, 16α-hydroxylation of DHA and ethylmorphine N-demethylation were modest in the adrenals. The DHA 16α-hydroxylation was studied in the same 16 samples as above which, without exception, were found to be active. The range of activities was 76–570 pmol/mg microsomal protein/min, and the average was 34% of the corresponding value in the same individual liver specimens.

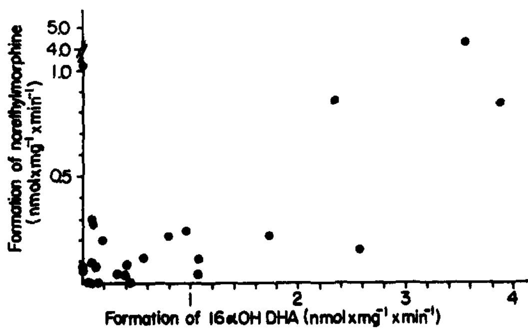

Fig. 2. Correlation between 16α-hydroxylation of DHA and ethylmorphine N-demethylation in human fetal liver microsomes.

As the fetus lacks significant 3β-hydroxysteroid dehydrogenase isomerase activity [26, 27], formation of progesterone from pregnenolone was not catalyzed in either fetal liver or adrenal microsomes.

Progesterone 16α- and 17α-hydroxylation was catalyzed in all but two specimens. These enzyme activities correlated highly with each other ($r = 0.98$).

Immunoblotting

Antibodies against the porcine *P*450XVIIA1 17α-hydroxylase isozyme, human *P*450IIIA/HLp and bovine *P*450C21 isozyme were used in the blotting experiments.

In *fetal liver*, polyclonal *P*450IIIA/HLp antibody-identified bands were observed in 18/20 investigated specimens (Table 2). The two liver specimens without visible bands in the Western blots were identical with the two (out of three) specimens not catalyzing either ethylmorphine *N*-demethylation or 16α-hydroxylation of DHA.

Bands identified by the *P*450XVIIA1 antibody were observed in 8/20 liver specimens. Fourteen of these samples were studied with respect to 17α-hydroxylation of progesterone. Only 2 samples catalyzed this reaction at appreciable rates, and 4 had very low rates. Among the active specimens, only the two most active were positive in the immunoblots (Table 3). *P*450C21-hydroxylase bands were not identified in any fetal liver specimens and there was no 21-hydroxylation activity either, except trace activity in one liver.

Table 2. Fetal liver microsomes: rates of 16β-hydroxylation of DHA in relation to findings min immunoblots (+: visible band; −: no band detected)

Enzyme activity (pmol/mg prot/min)	Immunoblotting	
	a-*P*450IIIA/HLp	a-*P*450XVIIA
3901	+	−
2580	+	+
1740	+	+
1064	+	−
1064	+	−
951	+	+
799	+	−
562	+	−
407	+	−
395	+	−
309	+	−
188	+	+
139	+	−
120	+	+
81	+	+
0	+	+
0	+	+
0	−	−
0	−	−

Table 3. Fetal liver microsomes: rates of 17-hydroxylation of progesterone in relation to findings in immunoblots (+: visible bands; −: no bands detected)

Enzyme activity (pmol/mg prot/min)	Immunoblotting	
	a-*P*450XVIIA1	a-*P*450IIIA/HLp
52	+	+
27	+	+
0	+	
0	+	
0	+	
0	+	
0	+	
9	−	+
6	−	+
4	−	+
3	−	+
0	−	
0	−	

In *adrenals*, 8/11 specimens were positive in regard of the *P*450IIIA/HLp protein. Both the "negative" and the "positive" samples catalyzed DHA 16α-hydroxylation at about the same rates.

Eight of nine investigated adrenal samples were positive in regard to the *P*450XVIIA1 band. All specimens catalyzed the 17α-hydroxylation of progesterone. However, the "negative" sample had a low (< 10% of average) enzyme activity. The intensity of the immunoidentified *P*450XVIIA1 bands, as measured by laser light densitometry, correlated with the activities of progesterone 17α-hydroxylase ($r = 0.83$).

The *P*450C21 enzyme was identified in the Western blots from all adrenal specimens. Without exception, these samples catalyzed the formation of DOC and DOCOL from progesterone.

Correlation between enzymes and between organs

In the adrenals, a correlation between enzyme activities was observed only for 16α- vs 17α-progesterone hydroxylation ($r = 0.98$). In the liver, there was also a correlation between 16α DHA-hydroxylation and *N*-demethylation of ethylmorphine ($r = 0.626$).

There was no correlation between the liver and adrenal activities of either progesterone 17α- or DHA 16α-hydroxylation.

DISCUSSION

The presence of human fetal liver cytochrome *P*450 [1] and its catalytic activity with many drug and endobiotic substrates has been known for many years. The biochemical features of the fetal enzymes were not studied until Kitada *et al.* [5, 28] purified one isozyme, cytochrome

*P*450 HFLa. Although it constituted about one-third of the total fetal liver *P*450 contents (as measured by immunoinhibition) it represented <5% of the human adult liver *P*450 contents [28].

Different findings indicate that the fetal liver enzyme immunoidentified in our laboratory [8] belongs to the IIIA subfamily of the cytochromes *P*450 [29]. It is recognized in almost all liver specimens by antibodies raised against rat PCN-induced cytochrome *P*450 [8] and human cytochrome *P*450IIIA/HLp (present study).

Our results point to the fact that the fetal hepatic *P*450IIIA isozyme seems to have a specific role in the steroid metabolism, since 16α-hydroxylation of DHA was virtually the only reaction catalyzed. The fetal activity is higher than in human adult liver microsomes [30]. Our results are consistent with those of Kitada *et al.* [5] who found DHA-3-sulfate to be a good substrate of the fetal *P*450 HFLa.

In fetal liver specimens with high 16α-OH-DHA activity, progesterone hydroxylation was also observed, albeit at a very low rate. The adult hepatic progesterone hydroxylation is also very moderate.

The mechanisms triggering the development of the enzyme are not fully understood. Milewich *et al.* [31] ascribed this to estrogens, the concentrations of which rise in pregnancy. It seems difficult, however, to rule out the possible importance of other steroids such as progestagens that also rise considerably with gestational age in the feto–maternal circulation.

It is to be pointed out that cytochrome *P*450 HFLa has been shown to be a major enzyme involved in 6β-hydroxylation of testosterone [32], but this enzyme activity was not included in our study. The 16α-hydroxylation of DHA serves an important physiological role in human pregnancy. Our experiments showed that DHA is a preferred substrate of the 16α-hydroxylase compared to progesterone. Subsequent studies in our laboratory have also shown that 16α-hydroxylation of testosterone is low, or unmeasurable, in human fetal liver (Mäenpää, Pelkonen, Cresteil and Rane, unpublished).

As expected, the fetal adrenals were actively catalyzing the formation of 17α-OH-pregnenolone necessary for the important synthesis of DHA. Nevertheless, there was no intraindividual correlation between fetal adrenal 17α-hydroxylase and fetal hepatic 16α-hydroxylase activities. DHA is a precursor for placental production of estriol but also of estrone and estradiol. It is known that the fetal adrenals and liver are the major source of DHA and 16α-OH-DHA during pregnancy, since maternal estrogen levels become extremely low in the absence of normal fetal adrenal glands [25].

After birth, neonatal 16α-hydroxylation activity rapidly disappears [25], which is in accordance with low activities of DHA 16α-hydroxylation in human adult liver compared to fetal liver [present data; 33]. This is however difficult to reconcile with the correlation ($r = 0.626$) in fetal liver between on one hand 16α-OH-DHA formation and on the other ethylmorphine *N*-demethylation, which in turn is in the same range in adult and fetal liver [9]. In addition, the immunoidentified *P*450IIIA/HLp bands correlated moderately with DHA 16α-hydroxylation ($r = 0.59$), but not with ethylmorphine *N*-demethylation, which is a pathway catalyzed by the human purified *P*450 HFLa [28] and rat *P*450 PB-2a/PCN-E [34]. Although the HFLa form [28], subsequently sequenced by Komori *et al.* [15] and designated as CYP3A6 by Nebert *et al.* [7], differs from the human adult *P*450IIIA/HLp [15], the antibody against the latter form apparently reacts with our human fetal protein that probably corresponds to HFLa. Taken together, the present data do not support the contention that the major part of ethylmorphine *N*-demethylation and DHA 16α-hydroxylation are catalyzed by the same enzyme in fetal liver.

Our data demonstrate the utility of antibodies for the detection of fetal enzyme proteins, but they also point out some difficulties in the work with fetal tissues obtained at abortion. When attempting to correlate enzyme activities with immunoidentified protein bands, the results must be interpreted with caution. If a correlation is not found, it may be due to post mortem enzyme instability. Such post mortem changes of the enzyme activities are beyond our control. This may also explain the lack of intraindividual correlations between adrenal and hepatic activities, if they exist.

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