0026-895X/90/020207-07$02.00/0

MOLECULAR PHARMACOLOGY, 38:207-213

Studies on the Expression and Metabolic Capabilities of Human Liver Cytochrome P450IIIA5 (HLp3)

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Received March 27, 1990; Accepted May 11, 1990

SUMMARY

The human P450III family has been shown to be composed of at least four members, P450IIIA3 (HLp), P450IIIA4 (P450NF), P450IIIA5 (HLp3), and P450IIIA6 (HLp2). Due to the lack of probes that specifically recognize the individual members of this family, little is known about their relative expression. We prepared a form-specific antibody to P450IIIA5 by immunoabsorption of anti-P450IIIA5 IgG against Sepharose 4B upon which microsomes that did not contain P450IIIA5 or purified P450IIIA3 had been bound. Immunoblot analyses demonstrated that P450IIIA5 was expressed at detectable levels in only 19 of 66 (29%) human livers. The expression of P450IIIA5 was not influenced by the gender or medical history of the patients. When the expression of P450IIIA5 in different age groups was examined, it was observed that P450IIIA5 was detected in a statistically significantly higher percentage of children and adolescents (19 years old and under), as compared with the remaining population (8 of 17, 47%, versus 11 of 46, 24%, respectively). Furthermore, P450IIIA5 was detected in 1 of 10 human fetal livers. Of the large number of compounds identified as substrates of P450III family members, P450IIIA5 was found to actively metabolize nifedipine, testosterone, estradiol, dehydroepiandrosterone 3-sulfate, and cortisol, whereas it metabolized poorly or did not metabolize erythromycin, quinidine, 17α-ethynylestradiol, and aflatoxins. The acetylenic steroid gestodene was found to be an effective mechanism-based inhibitor of both P450IIIA4 and P450IIIA5. Immunoblots of microsomes isolated from untreated and dexamethasone-, phenobarbital-, or 3-methylcholanthrene-treated HepG2 cells that were developed with an antibody that recognizes all the P450III family members demonstrated that no proteins in the P450III family were expressed by the HepG2 cells. In conclusion, our studies indicate that P450IIIA5 is polymorphically expressed at all stages of human development and is more limited in its metabolic capabilities than is P450IIIA4.

The human P450III family has been demonstrated to be composed of at least four genes (1–6). These genes encode four highly related proteins referred to as P450IIIA4 (P450NF), P450IIIA3 (HLp), P450IIIA6 (HLp2), and P450IIIA5 (HLp3)[1] (6–10). Several recent reports have attempted to characterize the expression of the various members of the human P450III family (1, 3, 9–12). From this work it appears that P450IIIA3 and P450IIIA4 are encoded by different genes (1, 3, 11, 12). However, these genes are so highly related that their relative expression at the mRNA level as determined by oligonucleotide hybridization is in dispute (11, 12) and at the protein level is unknown (10). Immunochemical studies using antibodies that recognize both P450IIIA4 and P450IIIA3[2] have demonstrated that one or both of these proteins is one of the major forms of P450 expressed in the adult human liver and is inducible in humans by a number of clinically useful drugs including dexamethasone (5), macrolide antibiotics (5), and phenobarbital (10). In addition, these forms of P450 appear to be responsible for the majority of the oxidative metabolism of an amazingly large number of structurally unrelated agents including erythromycin, cyclosporine, aflatoxins, quinidine, nifedipine, testosterone, androstenedione, progesterone, cortisol, 17β-estradiol, 17α-ethynylestradiol, midazolam, benzphetamine, and aldrin (13–15).

This work was supported in part by Grants CA44353 and ES00267 (F.P.G.) and AA08139 (J.L.R.) from the National Institutes of Health.

[1] The genes corresponding to P450IIIA4, P450IIIA3, and P450IIIA5 have been named, respectively, CYP3A4, CYP3A3, and CYP3A5 (6). The product of the CYP3A5 gene here and elsewhere (10) has been referred to as HLp3 and has also been referred to as hPCN3 by Aoyama *et al.* (1) and P450HLp2 by Schuetz *et al.* (12). The fetal P450IIIA6 has also been termed HLp2 (9) and appears to be equivalent to HFLa (4).

[2] As previously indicated (1, 3, 10), it is not currently possible to separate P450IIIA3 from P450IIIA4. In addition, antibodies prepared against either of these P450s would appear to react with the other. Therefore, the results of the immunoquantifications reported here are expressed as the total of both proteins and are indicated as P450IIIA3/4 levels.

ABBREVIATIONS: P450, cytochrome P450; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHEA, dehydroepiandrosterone 3-sulfate; DLPC, dilauroylphosphatidylcholine.

Studies on the expression of P450IIIA6 indicate that this member of the human P450III family is the major form of P450 present in the liver of the fetus (9). In addition, P450IIIA6 appears to be responsible for the metabolism of benzo(*a*)pyrene, 7-ethoxycoumarin, testosterone, and DHEA in the fetus (16, 17). Finally, a protein with the same amino-terminal amino acid sequence as P450IIIA6 has been isolated from one adult human liver, thus indicating that P450IIIA6 may also be expressed at low levels in the adult (18).

Immunochemical and molecular studies have demonstrated that P450IIIA5 is polymorphically expressed (1, 10, 12). Specifically, only 25 to 30% of the human liver specimens examined have been found to contain detectable levels of P450IIIA5 (1, 10). The metabolic capabilities of P450IIIA5 appear to overlap somewhat with those of P450IIIA4, although P450IIIA5 appears to have slower turnover rates than P450IIIA4 in some cases (1). Comparisons of the deduced amino acid sequences of P450IIIA4 and P450IIIA5 indicate that they are 84% similar (1). The small apparent molecular weight difference between P450IIIA5 and P450IIIA6 (10) has made it difficult to determine their relative distribution in liver specimens.

The studies on the regulation of expression of the human P450III family have been severely hampered by the lack of probes that specifically recognize a single member of this family. In addition, studies using either oligonucleotide or partial cDNA probes are capable of determining only the presence of mRNA encoding a protein and not whether the protein itself is being expressed. Even studies with purified proteins are often questionable because the true sequence may not be known. Here we report the characterization of the first probe specific for a single member of the human P450III family, that being an antibody that specifically recognizes P450IIIA5. Furthermore, we employed this antibody in studies that examined the expression of P450IIIA5 in samples of human liver and in the human hepatoma cell line HepG2. Finally, using purified P450IIIA5 and an expressed P450IIIA4 in reconstitution systems, we found that P450IIIA5 contributes relatively little to the metabolism of the vast majority of the compounds known to be metabolized by the P450IIIA family.

Experimental Procedures

Materials. Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Piscataway NJ). Bodman (Doraville, GA) was the source of Whatman DE-52 DEAE-cellulose. Nitrocellulose, secondary antibodies, electrophoresis materials, and immunoblot reagents were obtained from Bio-Rad (Richmond, CA). Gestodene (13-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one) was provided by Dr. H. Kuhl, University of Frankfurt (FRG). HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). Sprague-Dawley rats were purchased from Harlan Laboratories (Madison, WI). All other chemicals were of regent grade or better.

Liver specimens. The human liver samples were obtained at surgery, under protocols approved by the Committee for the Conduct of Human Research at the institution at which they were received (Medical College of Wisconsin and Vanderbilt University), or from the Liver Transplant Procurement and Distribution System or National Disease Research Interchange. Liver specimens from the Medical College of Wisconsin are denoted by the letters of the alphabet, those from Vanderbilt are denoted by code numbers preceded by the letter V, and those from the Liver Transplant Procurement and Distribution System or the National Disease Research Interchange are denoted by six-digit code numbers. Fetal livers were obtained at the Medical College of Virginia and have been used in previous studies (9, 19).

Female Sprague-Dawley rats (200 g) were treated with β-napthoflavone, dexamethasone, phenobarbital, or saline, as previously described (20).

Enzyme sources. P450IIIA3 was purified from human liver specimen 11 (21) to a specific content of 17.5 nmol of P450/mg of protein, by the method of Watkins *et al.* (8). Human liver P450IIIA5 was purified from specimen E, by the method of Wrighton *et al.* (10), to a specific content of 8.1 nmol of P450/mg of protein. P450IIIA4 was expressed in *Saccharomyces cerevisiae* transformed with the human liver cDNA clone NF 24 (2) and partially purified as described[3] from microsomes isolated from the yeast. NADPH-cytochrome P450 reductase and human cytochrome b_5 were purified as previously reported (22).

Preparation of the antibodies. Rabbits were given subcutaneous injections at multiple sites along the flanks of 100 μg of P450IIIA5 in 0.75 ml of sterile phosphate-buffered saline in an emulsion made with 1.25 ml of Freund's complete adjuvant. Four weeks later, 50 μg of P450IIIA5 in 0.75 ml of sterile phosphate-buffered saline in an emulsion made with 1.25 ml of Freund's complete adjuvant were injected subcutaneously into the flanks of the rabbit. Eight weeks from the initial immunization, 50 μg of P450IIIA3 in 300 μl of sterile phosphate-buffered saline were injected intravenously into the ear vein of the rabbit. Antiserum to P450IIIA5 was isolated 10 weeks after the initial immunization and weekly thereafter. The IgG fraction was then isolated from high titer antisera (23). Antibodies to P450IIIA3 were prepared in a similar fashion, except the initial immunization was with 300 μg of P450IIIA3.

Anti-P450IIIA5 was immunoabsorbed as previously described (23, 24), first against a solid support column prepared by binding solubilized microsomes isolated from human liver A (10) to cyanogen bromide-activated Sepharose 4B (15 mg of solubilized microsomes/ml of Sepharose). This procedure was repeated once. Then anti-P450IIIA5 was immunoabsorbed against a solid support column prepared by binding purified P450IIIA3 to cyanogen bromide-activated Sepharose 4B (15 nmol of P450IIIA3/ml of Sepharose). The column was regenerated (24) and the procedure was repeated. The final anti-P450IIIA5 antibody preparation that resulted from this immunoabsorption procedure was demonstrated by immunoblot analysis to react exclusively with P450IIIA5 (see below).

HepG2 cultures. HepG2 cells were seeded in 60-mm-diameter culture dishes and grown to approximately 85% confluency or postconfluency in Eagle's minimal essential medium containing 10% (v/v) fetal bovine serum, in a humidified chamber with CO_2/air (1:19). The medium was then removed and the cells were washed three times with cold 100 mM phosphate-buffered saline and harvested in cold 100 mM potassium phosphate buffer (pH 7.4). The cells were recovered by centrifugation at 3000 × *g* for 10 min and lysed by sonication. Microsomes were isolated by differential centrifugation.

Enzyme assays. The catalytic specificity of P450IIIA5 was determined using the general assay conditions previously described (10). For comparison, the enzymatic activities of partially purified P450IIIA4 from yeast were also determined. Briefly, purified P450IIIA5 or P450IIIA4 (100 pmol) was reconstituted with 200 pmol of rabbit NADPH-cytochrome P450 reductase, 50 pmol of human cytochrome b_5, 165 nmol of CHAPS, and either 25 μg of a human liver microsomal lipid extract or 15 nmol of DLPC in 0.1 M potassium phosphate buffer (pH 7.4 or 7.7). The human liver extract was obtained by extracting human liver microsomes with $CHCl_3$/CH_3OH (2:1, v/v) drying the lipid fraction under nitrogen, and then resuspending the lipid in water by sonication. The lipid was prepared fresh using nitrogen-purged solutions. Additions of cytochrome b_5 to the reaction mixtures increased enzymatic activities for P450IIIA5 but not P450IIIA4, so it was omitted from the P450IIIA4 reaction mixtures in these comparisons.

The inhibition of nifedipine oxidation by gestodene was determined

[3] W. R. Brian, M. A. Sari, M. Iwasaki, T. Shimada, L. S. Kaminsky, R. S. Lloyd, and F. P. Guengerich. Catalytic activities of human liver cytochrome P-450IIIA4 expressed in *Saccharomyces cerevisiae*. Submitted for publication.

using human liver microsomes (sample V110), partially purified P450IIIA4 (from yeast), and P450IIIA5 (from human liver). Reaction mixtures were prepared using 100 pmol of P450 (and NADPH-cytochrome P450 reductase and lipid in the case of the reconstituted systems), the NADPH-generating system, and gestodene (20 μM). After incubation at 37° for 30 min, nifedipine was added (200 μM) and the amount of oxidized nifedipine was determined after 10 min (14).

General procedures. Protein concentrations were determined colorimetrically using bovine serum albumin as the standard (25). Microsomes were prepared from the human and rat liver samples as previously described (26). Quantitative immunoblot analyses were performed as described elsewhere (8).

Results

Preparation of monospecific anti-P450IIIA5. As shown in Fig. 1A, when anti-P450IIIA5 was used to develop an immunoblot of human liver microsomes, fetal liver microsomes, and purified P450IIIA3 and P450IIIA5 before immunoabsorption, it preferentially recognized P450IIIA5 with respect to P450IIIA3/4[2] and P450IIIA6. On the other hand, anti-P450IIIA3/4 appears to strongly react on immunoblots with all the P450III family members (Fig. 1B). In order to remove the antibodies in anti-P450IIIA5 that also recognize P450IIIA3/4 and P450IIIA6, anti-P450IIIA5 was immunoabsorbed first against a solid support column upon which microsomes from human liver A were bound and then against a column upon which purified P450IIIA3 was bound. Fig. 1C demonstrates that anti-P450IIIA5 after immunoabsorption recognized only purified P450IIIA5 and P450IIIA5 in microsomes and was free of antibodies that react with P450IIIA3/4 or P450IIIA6. Thus, this preparation of anti-P450IIIA5 was rendered apparently monospecific for P450IIIA5.

Expression of P450IIIA5 protein. Previous work demonstrated that P450IIIA5 was expressed at detectable levels in only 11 of 46 human liver specimens (10). However, due to the lack of an antibody that specifically recognized P450IIIA5 and the poor separation of P450IIIA3, P450IIIA4, P450IIIA6, and P450IIIA5 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the level of P450IIIA5 protein was not determined.

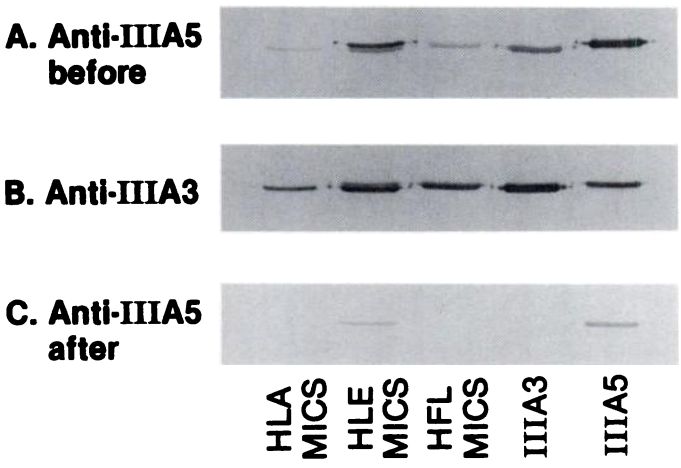

Fig. 1. Immunoblots of human liver microsomes (*MICS*) and purified P450IIIA3 and P450IIIA5. Immunoblot analyses of microsomes isolated from human livers A (*HLA*) (20 μg) or E (*HLE*) (20 μg) or five combined fetal livers (*HFL*) (35 μg) and purified P450IIIA3 (1 μg) or P450IIIA5 (1 μg) were performed as described in Experimental Procedures and developed with anti-P450IIIA5 before immunoabsorption (A), anti-P450IIIA3 (B), or anti-P450IIIA5 after immunoabsorption (C).

The preparation and characterization of form-specific anti-P450IIIA5 allowed the quantification of the levels of P450IIIA5 in the various microsomal samples. Therefore, by using the form-specific anti-P450IIIA5 antibody and purified P450IIIA5 as the standard, the level of P450IIIA5 was determined not only in those samples previously demonstrated to contain P450IIIA5 but also in 20 new samples (Table 1). With the addition of the new samples, P450IIIA5 has now been detected in 19 of 66 (29%) human liver specimens (Table 1) (10).

Our previous work (10) indicated that, unlike P450IIIA3/4, the expression of P450IIIA5 did not appear to be influenced by the administration of inducers of the P450III family to the patients or by the age, gender, or smoking habits of the patients. In addition, after examining a very limited number of specimens, we suggested that P450IIIA5 may be expressed in children and adolescents. However, after a total of 17 liver specimens obtained from patients 2 months to 19 years old were examined, only 8 (47%) were found to contain P450IIIA5. Thus, P450IIIA5 is not universally expressed in children and adolescents, although it is expressed in a statistically significantly ($p \leq 0.08$) higher percentage in this age group, compared with the remaining population of patients (11 of 46, 24%) for which their ages were known. Although P450IIIA5 appears to be expressed in a higher percentage of females (10 of 27, 37%) than males (9 of 37, 24%), gender does not seem to influence the expression of P450IIIA5, because this difference does not reach statistical significance ($p \leq 0.27$).

The expression of P450IIIA5 protein in the fetal liver has not been reported, due to the technical difficulties in separating P450IIIA6 from P450IIIA5 (10). However, Schuetz *et al.* (12) have reported that an oligonucleotide that was synthesized to specifically hybridize to the mRNA encoding P450IIIA5 does recognize RNA species related to P450IIIA5 in at least one sample of fetal RNA. Fig. 2A demonstrates that anti-P450IIIA5

TABLE 1

Patient information and immunoquantification of P450IIIA5 and P450IIIA3/4

The immunoquantifications were performed as described in Experimental Procedures. Because the antibody used to detect P450IIIA3 appears to also react with P450IIIA4, the value for this determination is reported as P450IIIA3/4. All values represent means of at least duplicate determinations.

Patient code	P450IIIA5	P450IIIA3/4	Age	Gender
	pmol/mg	*pmol/mg*	*years*	
K		54	23	M[a]
L		38	58	F
M		50	18	M
X	60	260	0.2	M
V30	54	201	16	F
V37		142	15	F
V91		131	14	M
V99		175	19	M
V105		212	16	M
V107		128	18	M
V119	42	157	12	F
V120	5	73	20	M
V123		57	10	F
V126		35	16	M
V132	2	6	16	F
V133	11	44	19	M
860630	19	9	5	M
860805	D	58	16	M
870126		132	21	M
860624		15	15	M

[a] M, male; F, female; D, detected but at a level below the limit of quantification.

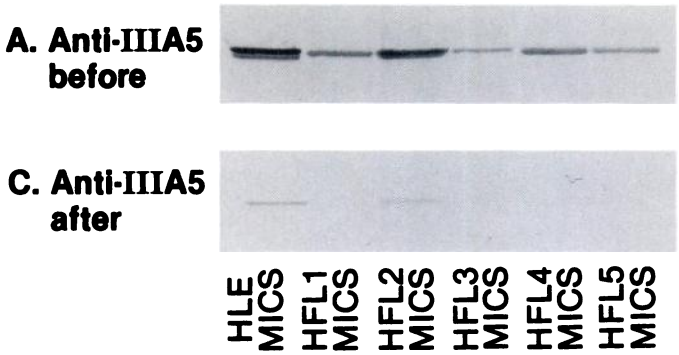

Fig. 2. Immunoblots of human liver microsomes (*MICS*) developed with anti-P450IIIA5 antibodies. Immunoblot analyses of microsomes isolated from human liver E (*HLE*) (20 μg) and five individual fetal livers (HFL 1–5) (35 μg each) were performed as described in Experimental Procedures and developed with anti-P450IIIA5 before (A) or after (C) immunoabsorption.

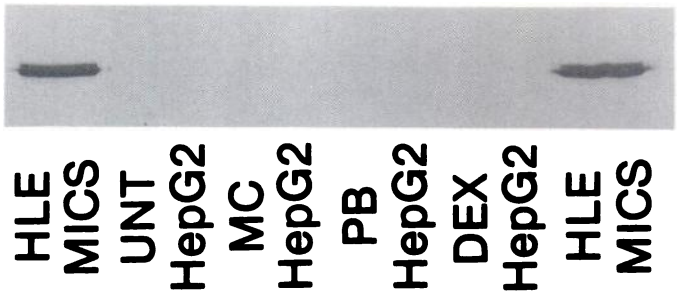

Fig. 3. Immunoblots of microsomes isolated from cultures of HepG2 cells. Immunoblot analyses of microsomes (*MICS*) isolated from human liver E (*HLE*) (20 μg) and untreated (*UNT*), phenobarbital-treated (*PB*), dexamethasone-treated (DEX), or 3-methylcholanthrene-treated cultures of HepG2 cells (50 μg each) were performed as described in Experimental Procedures and developed with polyclonal anti-P450IIIA3.

before immunoabsorption recognizes a protein (P450IIIA6) in each of the five fetal livers that migrates at an apparent molecular weight between that of P450IIIA3 (Fig. 2A, *lower band* in the *lane with HL-E*) and P450IIIA5 (Fig. 2A, *upper band* in the *lane with HL-E*), thus confirming what we have previously reported for the expression of P450IIIA6 in fetal liver (9, 19). Upon close examination of the reaction of anti-P450IIIA5 before immunoabsorption with the proteins of fetal liver microsomal sample 2, the reaction appeared to be a fused doublet, suggesting the presence of both P450IIIA6 and P450IIIA5 in this sample. When an identical immunoblot was developed with the form-specific anti-P450IIIA5 antibody (Fig. 2C), P450IIIA5 (or a highly related protein with a similar apparent molecular weight) was detected in microsomes isolated from human liver E and in microsomes from only one of five fetal livers, that being fetal liver 2. Thus, it appears that P450IIIA5 is expressed at the protein level in a limited number of fetal livers.

The rat P450III family has been shown to contain at least three members (23). In order to determine whether one of these forms is orthologous to P450IIIA5, we developed an immunoblot of microsomes isolated from the livers of β-naphthoflavone-, phenobarbital-, or dexamethasone-treated female and untreated male and female rats with form-specific anti-P450IIIA5. Despite recognizing P450IIIA5 in a positive control (microsomes from human liver E), the form-specific antibody did not recognize a protein in any of the samples of rat liver microsomes (data not shown).

Aoyoma *et al.* (1) have shown that the human hepatoma cell line HepG2 does not appear to express a protein that is recognized by a monoclonal antibody that recognizes all the P450III family members when the cells are cultured under standard conditions. However, in response to a dexamethasone challenge, HepG2 cells have been shown to produce RNA species that hybridize to a P450IIIA3 cDNA (5). Whether a protein in the P450III family is expressed in these cells has not been reported. Thus, in order to examine the expression of the P450III family in HepG2 cells, immunoblots of microsomes isolated from untreated and dexamethasone-, 3-methylcholanthrene-, or phenobarbital-treated cultures of HepG2 cells were developed with a polyclonal anti-P450IIIA3 antibody that recognizes all of the human P450III family members identified to date (Fig. 1B). As indicated in Fig. 3, untreated HepG2 cells do not appear to express a protein related to P450IIIA3 and such a protein was not induced in these cells by treatment with the above agents. In addition, when microsomes isolated from preconfluent cultures of HepG2 cells were examined by immunoblots developed with the polyclonal anti-P450IIIA3 antibody, no members of the P450III family were detected.[4] Finally, no proteins were recognized in immunoblots of the HepG2 microsomes developed with the same monoclonal used by Aoyoma *et al.* (1) or with the monospecific anti-P450IIIA5 antibody (not shown). Thus, these data indicate that none of the currently identified proteins in the P450III family are expressed in HepG2 cells under normal conditions or after treatment of the cultures with compounds known to induce members of this family *in vivo* and known to induce in HepG2 cells RNA species that hybridize with a P450IIIA3 cDNA.

Catalytic specificity of P450IIIA5. Two classic metabolic activities associated with the P450III family are erythromycin *N*-demethylation and nifedipine oxidation (7, 8). P450IIIA4, partially purified from yeast and reconstituted, catalyzed both these reactions and the 3-hydroxylation and *N*-oxidation of quinidine (Table 2). When P450IIIA5 from human liver was reconstituted and the same reaction conditions were used as with P450IIIA4 (except cytochrome b_5 was included), P450IIIA5 was found to catalyze nifedipine oxidation (Table 2). However, in contrast to P450IIIA4, P450IIIA5 was found to have no measurable catalytic activity for erythromycin *N*-demethylation or quinidine *N*-oxidation and 3-hydroxylation (Table 2).

P450IIIA4 and P450IIIA5 isolated from human liver (10) or produced in transformed HepG2 cells (1) were previously shown to catalyze testosterone 6β-hydroxylation, although the rates for P450IIIA5 were lower than for P450IIIA4. These results were confirmed here using human lipid extract, although the rate of testosterone 6β-hydroxylation by P450IIIA5 with DLPC was below the limit of detection, due to an unusually high background (Table 2). P450IIIA5 was also found to catalyze 6β-hydroxylation of cortisol at about 50% of the rate of P450IIIA4. In addition, P450IIIA4 and P450IIIA5 catalyzed the 16α-hydroxylation of DHEA, a reaction previously shown to be catalyzed by P450IIIA6 (17).

The P450IIIA family in humans appears to catalyze the 2- and 4-hydroxylations of 17β-estradiol and the 2-hydroxylation of 17α-ethynylestradiol (13). P450IIIA4 was found to catalyze

[4] S. Wrighton, unpublished observations.

TABLE 2

Catalytic activities of P450IIIA4 and P450IIIA5

Activities expressed as nmol of product formed/min/nmol of P450, except for the hydroxylation of DHEA, which is expressed as pmol of product/min/nmol of P450. The catalytic activities are corrected for the background of each assay. Some of the activities of P450IIIA4 have appeared elsewhere.

Reconstitution system	Nifedipine oxidation	Erythromycin *N*-demethylation	Quinidine		Testosterone 6β-hydroxylation	Cortisol 6β-hydroxylation	17β-Estradiol 2-hydroxylation	17α-Ethynyl estradiol 2-hydroxylation	DHEA 16α-hydroxylation
			3-Hydroxylation	*N*-Oxidation					
	nmol/min/nmol								*pmol/min/nmol*
P450IIIA5, DLPC	0.64	0	0	0	0	0.1	0.8	0	ND[a]
P450IIIA5, human lipid	0.68	0	0	0	0.3	0.1	1.0	0	0.21
P450IIIA4, DLPC	0.70	0.7	0.3	0.2	2.1	0.3	2.4	2.8	ND
P450IIIA4, human lipid	2.8	1.1	0.5	0.4	2.0	0.2	2.0	3.8	0.25

[a] ND, not determined.

the 2-hydroxylation of both compounds (Table 2). However, P450IIIA5 exhibited 2-hydroxylase activity toward only 17β-estradiol and not 17α-ethynylestradiol (Table 2).

Previous work has demonstrated that P450IIIA4 activates a number of procarcinogens in a transformation bioassay with *Salmonella typhimurium* (27). Reconstituted P450IIIA5 was found to have less than 15% of the activity of P450IIIA4 in this assay with aflatoxin B1, sterigmatocystin, and (−)-benzo(*a*)pyrene-7,8-diol and no activity with (+)-benzo(*a*)-pyrene-7,8-diol and 6-aminochrysene (data not shown).

Gestodene, a 17α-acetylenic steroid containing a Δ^{15} double bond, has been shown to be a potent mechanism-based inactivator of P450IIIA4, with a $k_{inactivation}$ of 0.4 min^{-1}, k_i of 46 μM, and partition ratio of 9 (28).[3] Incubation of P450IIIA4 (from yeast), P450IIIA5 (from human liver), or human liver microsomes with 20 μM gestodene for 30 min (at 37° in the presence of NADPH) reduced nifedipine oxidation activity in each case to about 30% (±10%). Thus, gestodene appears to be a selective mechanism-based inhibitor of the P450IIIA enzymes but does not discriminate between P450IIIA4 and P450IIIA5.

Discussion

Studies on the regulation of expression of the identified members of the human P450III family have been difficult to perform, due to the lack of probes that can distinguish between the different P450 proteins in this family. However, Fig. 1 clearly demonstrates that, by immunoabsorbing an anti-P450IIIA5 antibody first against microsomes that did not contain P450IIIA5 and then against purified P450IIIA3, we were able to prepare an anti-P450IIIA5 antibody that recognized only P450IIIA5 and not P450IIIA3 (and presumably P450IIIA4) and P450IIIA6. The preparation of this monospecific antibody allowed us for the first time to examine the expression of P450IIIA5 protein in various tissues and cells including human liver, human fetal liver, and HepG2 cells.

Previous reports have suggested that the expression of P450IIIA5 protein and mRNA was not influenced by the age, sex, or drug history of the patient (1, 10, 12). In addition, we previously suggested that P450IIIA5 may be expressed in children and adolescents (10). Therefore, we expanded our number of samples in this age group (19 and under) from 2 to 17 samples and found that only 47% of the specimens contained P450IIIA5 at detectable levels (Table 1). However, P450IIIA5 was expressed in a significantly higher percentage of the samples obtained from this age group, as compared with the adult population (47 versus 24%). Therefore, our data indicate that P450IIIA5 is expressed in a greater percentage of children, as compared with adults. Moreover, these data indicate that P450IIIA5 is not universally expressed in children.

Unlike what has been observed in experimental species, the human fetus is capable of metabolizing a large number of compounds (16, 17). Numerous reports have demonstrated that P450IIIA6 is the major form of P450 expressed in the fetal liver (9, 19). In addition, Schuetz *et al.* (12) found that RNA isolated from at least one human fetus contained two species of RNA that hybridized with an oligonucleotide probe that was synthesized to be specific for the CYP3A5 gene product (P450IIIA5). However, whether the message(s) related to P450IIIA5 was translated into a P450IIIA5-related protein in this fetal sample was not discussed. In our previous work on the expression of P450IIIA6, which used an antibody that recognizes all the P450III family members, we were not able to detect the presence of more than one member of the P450III family in fetal liver (9, 19). Because we have demonstrated that P450IIIA3 can be routinely separated from P450IIIA6 and P450IIIA5 by our gel system (10), P450IIIA3 does not appear to be expressed in at least the five fetal samples examined individually (Fig. 2) or in the additional five fetal liver samples that were pooled for the fetal microsome samples examined in Fig. 1. However, we have not been able to reproducibly separate P450IIIA6 from P450IIIA5 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, despite exhaustive efforts. Thus, the fetal samples previously reported to contain P450IIIA6 could also contain P450IIIA5. This problem was eliminated by the development of the monospecific anti-P450IIIA5 antibody. When immunoblots of microsomes prepared from the five individual (Fig. 2) and five pooled (Fig. 1) fetal livers were developed with the monospecific anti-P450IIIA5 antibody, only one liver (fetal liver 2) was found to express a P450IIIA5-related protein. Therefore, it would appear that P450IIIA5 protein can be expressed in the fetal liver at a frequency of about 1 out of 10. Thus, the gene product might well be overlooked in studies of a limited scope. How the expression of P450IIIA5 protein compares with the expression of P450IIIA5 mRNA is the subject of future work.

The addition of dexamethasone to the media of HepG2 cells has been shown to induce the production of mRNA species that hybridize with a P450IIIA3 cDNA (5). However, whether the induction of a P450IIIA3-related protein results from the dexamethasone treatment of HepG2 cells has not been reported. Fig. 3 demonstrates that HepG2 cells under basal conditions do not express proteins that are in the P450III family. This finding is in agreement with the observations of Aoyoma *et al* (1). Furthermore, after treatment of these cells with compounds known to induce members of this family *in vivo* (i.e., dexa-

methasone and phenobarbital), these cells still did not express proteins in the P450III family (Fig. 3). This family of P450s has been shown to play a crucial role in the oxidative metabolism of numerous clinically and toxicologically important agents (13). Therefore, the lack of expression at the enzyme level of the P450III family severely limits the usefulness of HepG2 cells as an in vitro model system for examining human drug and xenobiotic metabolism. It is also worth noting that our observations indicate that the rat, another widely used model system, apparently does not express a P450 orthologous to P450IIIA5, which compromises its usefulness in the study of the regulation of the expression of the P450III family.

Evidence now indicates that the human liver P450III family plays an important role in the oxidative metabolism of many drugs, steroids, and carcinogens (13). Determination of the roles of the individual members of this family in these processes is necessary for proper understanding of human health, drug metabolism, and carcinogenesis. Currently, P450IIIA4 has exhibited all the catalytic activities attributed to the P450III family (Table 2).[3]

The P450IIIA5 protein and cDNA clone have recently been isolated and characterized (1, 10, 12). P450IIIA5 is 84% structurally similar to P450IIIA4, is polymorphically expressed and accounts for 6 to 60% (Table 1) and in at least one case 100% (1) of the total P450III family in the livers in which it is expressed. Thus, it is important to determine the catalytic specificity of this enzyme, to aid in an understanding of its contribution to the reactions catalyzed by the P450IIIA family. Previously, we (10) and Aoyoma *et al.* (1) found that P450IIIA5 catalyzed testosterone 6β-hydroxylation at a rate slower than that of P450IIIA4. In the current study, P450IIIA5 purified from human liver and reconstituted with DLPC or human lipid extract catalyzed oxidation of nifedipine, 6β-hydroxylation of testosterone and cortisol, 16α-hydroxylation of DHEA, and 2-hydroxylation of 17β-estradiol. Reaction rates exhibited by P450IIIA5 were all lower than for P450IIIA4. P450IIIA5 did not catalyze erythromycin *N*-demethylation, quinidine *N*-oxidation or 3-hydroxylation, or 17α-ethynylestradiol 2-hydroxylation at significant rates. Apparently, P450IIIA5 does not contribute to these oxidative activities of the P450III family. Understanding of the significance of the role of P450IIIA5 is complex, due to its relatively limited catalytic activity, polymorphic expression, and low contribution to the overall P450III family pool in the liver.

It is intriguing how P450IIIA4 and P450IIIA5, being so structurally similar, can share some substrates but not others. For example, P450IIIA5 is a relatively good catalyst for 2-hydroxylation of 17β-estradiol, yet apparently does not catalyze the 2-hydroxylation of 17α-ethynylestradiol. These compounds differ by only an acetylenic group at position 17, whereas the P450-catalyzed hydroxylation occurs at the 2-position of the molecule. The lower number of substrates and catalytic rates of P450IIIA5, relative to P450IIIA4, suggest that the substrate binding sites of the two enzymes differ, although the differences between the two proteins appear to be distributed throughout the coding regions (1, 12). Ultimately, determination of the crystal structures will be necessary for an understanding of the structural differences of the two proteins. Site-directed mutagenesis studies will also assist in defining the differences in the metabolic activities of these P450s.

In conclusion, the data presented here indicate that, throughout the development of humans (i.e., from fetus to adult), P450IIIA5 appears to be polymorphically expressed and that P450IIIA5 metabolizes a limited number of the compounds known to be transformed by the P450III family.

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