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Occurrence and Inducibility of Cytochrome P450IIIA in Maternal and Fetal Rats during Prenatal Development†

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ABSTRACT: The purpose of this study was to quantify cytochrome P450IIIA1 in fetal and maternal livers of uninduced and pregnenolone-16α-carbonitrile (PCN) induced rats during the course of prenatal development. The activities and levels of P450IIIA in hepatic microsomes from maternal rats and fetuses at 15-21 days of gestation were measured by triacetyloleandomycin (TAO) inhibited debenzylation of (benzyloxy)phenoxazone and by immunoassay with defined antiserum specific for P450IIIA. P450IIIA was not detectable (<10 pmol/mg for maternal microsomes and <2 pmol/mg for fetal microsomes) by immunoassay in uninduced maternal or fetal livers. In hepatic microsomes from PCN-induced dams, values ranged from 59.3 to 116 μg P450IIIA1/mg of protein during the same gestational period. Changes in debenzylase activity of 15.9-46.5 pmol of resorufin (mg of protein)$^{-1}$ min^{-1} were consistent with these findings as were the changes in TAO-inhibitable debenzylase activity. In the transplacentally induced fetal liver, debenzylase activity increased steadily from 0.19 pmol of resorufin mg^{-1} min^{-1} at day 15 to 9.34 pmol of resorufin mg^{-1} min^{-1} at day 21 and was paralleled by the TAO-inhibitable activity that ranged from 0.09 pmol of resorufin mg^{-1} min^{-1} at day 15 to 3.33 pmol of resorufin mg^{-1} min^{-1} at day 21. The amount of immunoreactive P450IIIA1 also increased from 0.5 to 28.7 μg/mg of microsomal protein. When the dose of PCN used to induce the maternal animals was increased from 50 mg/kg, once daily for 3 days, to 40 mg/kg, twice daily for 4 days, fetal debenzylase activity was decreased by approximately 10-20% over the gestational period as was the TAO-inhibited debenzylase activity. Analogously, immunoreactive P450IIIA1 was also slightly decreased. The data indicate that P450IIIA1 in fetal rat liver is PCN-inducible as early as day 15 of gestation and that inducibility progressively increases as a function of fetal age.

The cytochromes P450 play a major role in the metabolism of lipophilic exogenous and endogenous substances including drugs, fatty acids, aromatic hydrocarbons, vitamins, pesticides, eicosanoids, and steroids. While P450-dependent metabolism most often serves to detoxify and eliminate active compounds, reactive intermediates may arise. The generation of reactive metabolites and the potential of such metabolites to combine with cellular macromolecules provide an inherent mechanism for profound biologic consequences including carcinogenesis, teratogenesis, and genotoxicity.

This paper focuses on an enzyme of the steroid-inducible subfamily of the P450 cytochromes. Characteristics that distinguish this P450 cytochrome include its inducibility by selective agents and its substrate specificities. P450IIIA1 is

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induced by glucocorticoids such as dexamethasone (DEX),[1] by catatoxic anti-glucocorticoids such as pregnenolone-16α-carbonitrile (PCN),[2] by macrolide antibiotics such as triacetyloleandomycin (TAO), by phenobarbital (PB), and by "PB-like" inducing agents such as chlordane (Wrighton et al., 1985a; Guzelian et al., 1984; Schuetz & Guzelian, 1984; Schuetz et al., 1984, 1986; Hueman et al., 1982; Bonfils et al., 1985; Delaforge et al., 1983). Among the many substances metabolized by P450IIIA1 are testosterone (Waxman, 1984), warfarin (Porter et al., 1981; Kaminsky et al., 1984), aldrin (Wolff & Guengerich, 1987), erythromycin (Delaforge et al., 1983; Sartori et al., 1985), TAO (Delaforge et al., 1983; Simmons et al., 1987), 2-(acetylamino)fluorene (Astrom & DePierre, 1985; McManus et al., 1987), and (benzyloxy)-phenoxazone (Namkung et al., 1988).

Recently, investigators using human liver tissues have reported that a P450IIIA-like enzyme has been positively correlated with 6β-testosterone hydroxylation (Guengerich et al., 1986; Kitada et al., 1987a,b; Waxman, 1987; Waxman et al., 1988). Additionally, the 6β-hydroxylated metabolite was found to be the major hydroxylation product of testosterone in humans (Waxman et al., 1988). It was also recently reported that a P450IIIA-immunoreactive protein, which catalyzes 6β-testosterone hydroxylation, is a major component of the total, reduced, carbon monoxide binding heme protein occurring in human fetal liver (Kitada et al., 1985, 1987a,b). However, Cresteil et al. (1986) found that P450IIIA does not occur in uninduced or PB-induced fetal rat livers near term. To date, there have been no reported studies that demonstrate the inducibility or quantitate the specific immunological and catalytic activities of P450IIIA in prenatal liver of experimental animals. In this paper, we report that induction can be detected in fetal rat liver as early as day 15 of gestation. We have performed both immunoquantitative and functional analyses of the induced enzyme from the stage of late organogenesis to parturition. Also reported are the data for the occurrence and inducibility of P450IIIA in hepatic microsomes of maternal animals at the corresponding stages of gestation.

Experimental Procedures

Chemicals. Dexamethasone, 3-methylcholanthrene (MC), NADPH, *o*-phenylethylenediamine dihydrochloride, 4-chloro-1-naphthol, and protein A–Sepharose CL-4B were obtained from Sigma Chemical Co., St. Louis, MO. Phenobarbital was obtained as the sodium salt from the University Hospital Pharmacy, Seattle, WA. Triacetyloleandomycin was the generous gift of Dr. Salvatore Giorgianni of Roerig, a division of Pfizer, Inc. PCN was either purchased from Upjohn Co., Kalamazoo, MI, or synthesized in our own laboratory. The PCN obtained from Upjohn was recrystallized three times to purify it, as assessed by the sharpness of its melting point (231–232 °C). A similar melting point was determined for PCN synthesized according to methods described by Mazur and Cella (1959). Methoxy-, ethoxy-, pentoxy-, and (benzyloxy)phenoxazones were synthesized and purified in our laboratory according to the previously published methods (Mayer et al., 1977; Koltz et al., 1984). All other chemicals and reagents were of the highest purity commerically available.

Animals. Adult (200–250 g) male and female Sprague-Dawley rats were obtained from Tyler, Redmond, WA. Pregnant animals were time-mated such that the morning following copulation was defined as the beginning of day 0. All animals were housed two or three per cage and allowed Purina rodent chow and water ad libitum. Food was removed the night previous to sacrifice by cervical dislocation. Livers from 3 or more adult animals were pooled and 30–50 fetal livers from 3 or more pregnant females were pooled to obtain the microsomal samples.

Induction. Two different dosage schedules were used for the induction of pregnant females at various stages of gestation with PCN. The lower dosage consisted of 50 mg of PCN/kg, suspended in corn oil and injected intraperitoneally (IP), once daily for 3 days. For the higher dose, 40 mg/kg was injected IP twice daily for 4 days. Animals were sacrificed 24 h after the final dose, and all enzymic analyses were performed with fresh tissues. The days of gestation designated are given as the day of sacrifice. Nonpregnant females received IP injections of 80, 100, or 20 mg/kg of PCN, DEX, or MC, respectively, daily for 4 days. All three agents were suspended in corn oil. PB was dissolved in saline with one drop of Tween 80 and injected IP for 3 days at 80 mg kg^{-1} day^{-1}. Again, final injections were administered 24 h prior to sacrifice. TAO was mixed with ground Purina Rat Chow (1.5 g/100 g) and fed for 11 days. The feed was removed 16 h prior to sacrificing the animals.

Microsomal liver fractions were prepared according to previously published procedures (Guengerich, 1982) with minor modifications. Because fragments of endoplasmic reticulum of fetal livers sediment at low centrifugation speeds, fetal liver homogenates were centrifuged at 9000*g* for only 5 min before the subsequent 104000*g* centrifugation. In an effort to preserve the minute quantities of fetal microsomes, these

[1] Abbreviations: PCN, pregnenolone-16α-carbonitrile; TAO, triacetyloleandomycin; DEX, dexamethasone; PB, phenobarbital; MC, 3-methylcholanthrene; IP, intraperitoneally; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PBST, phosphate-buffered saline with Tween 20; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween 20; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G fraction.

[2] Pregnenolone-16α-carbonitrile (PCN) inducible P450s have been referred to as P450PCN-E (Guengerich et al., 1982), P450p (Schuetz & Guzelian, 1984), $P450_{TAO}$ (Wrighton et al., 1985a), P450PCN1 (Gonzales et al., 1986, 1987), P450PB2a (Waxman et al., 1984, 1987), and P450PCNa and P450PCNb (Graves et al., 1987). On the basis of N-terminal amino acid sequences (Wrighton et al., 1985a; Gonzales et al., 1986; Hostetler et al., 1987; Graves et al., 1987), it has been suggested that these PCN-inducible P450s can be separated into two distinct groups (Halpert, 1988; Waxman, 1988). P450PCN1, P450PCNa, P450p, and $P450_{TAO}$ exhibit a degree of amino acid sequence homology distinct from homology shared by P450PB2a, P450PCN-E, and P450PCNb. A third group has been suggested (Hostetler et al., 1987; Waxman et al., 1988) for the immunologically related but noninducible enzymes. The proteins previously referred to as P450PCN2 (Gonzales et al., 1986) P4502a (Waxman et al., 1984), and P450PCNc (Halpert, 1988) can be included in this third group. The steroid-inducible P450 enzymes have been newly classified, with a criterion of at least 77% amino acid homology, as members of a subfamily of P450 cytochromes referred to as P450IIIA (Nebert et al., 1987). According to this recommended system of classification, the subfamily, P450IIIA, is further divisible. By definition (Nebert et al., 1987), P450IIIA1 is distinguished from P450IIIA2 in that IIIA1 appears not to be constitutive in either adult male or female rats but is inducible in both sexes. P450IIIA2 is constitutive only in adult male rats (Gonzales et al., 1985; Wrighton et al., 1985a,b; Waxman et al., 1985). The recommended notation, P450IIIA1, should not be taken to represent any single enzyme since there is evidence of more than one inducible protein of the IIIA subfamily (Hostetler et al., 1987). Additionally, the recommended nomenclature designates the human orthologue of the P450IIIA family, P450IIIA3 (Nebert et al., 1987). As with IIIA1, IIIA3 is now thought to incorporate at least two distinct gene products (Wrighton et al., 1988; Bork & Guengerich, 1988). In addition to these findings in rats and humans, there is some evidence for multiple P450IIIA species in mice (Hietanen et al., 1986), and rabbits (Wrighton et al. 1985b; Bonfils et al., 1986). Here we refer to PCN-inducible cytochrome P450(s) as P450IIIA1. We use this notation (Nebert et al., 1987) to indicate that a steroid-inducible form is under investigation. Although members of subfamilies IIIA1 and IIIA2 are known to cross-react immunologically, the enzyme referred to as IIIA2 is constitutive and not steroid-inducible. For cases in which a more general designation is appropriate, the term P450IIIA is utilized.

samples were not washed after resuspension. All microsomal samples used for enzymatic determinations were suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and analyzed immediately. All protein assays employed the Bradford method (Bradford, 1976), using bovine immunoglobulin as the standard.

Isolation of P450IIIA. P450IIIA was isolated from male rats as a TAO complex (P450IIIA–TAO) according to a previously published method (Wrighton et al., 1985a). Identity of the isozyme was confirmed by the absorbance maximum of the TAO-complexed form at 457 nm, with subsequent shift to 450 nm upon addition of 50 μM ferricyanide and its migration as a single band on sodium dodecyl sulfate (SDS) polyacrylamide gel consistent with a molecular weight of 51 000 as reported (Wrighton et al., 1985a).

Antibody Preparation. Antibodies were prepared by applying 125 μg of the purified P450IIIA–TAO to an acrylamide density gradient SDS gel, 10–20%, and excising the only evident band after staining with Coomassie Blue. The gel, containing the isozyme, was homogenized in 0.5 mL of a solution containing 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.68 mM KCl at pH 7.4 (PBS) and sonicated with an equal volume of Freund's complete adjuvant. The preparation was injected subcutaneously into a young adult female New Zealand White rabbit. After 6 weeks, the antibody titer was boosted by administering a subcutaneous injection of 80 μg of P450IIIA–TAO suspended in Freund's incomplete adjuvant. Blood was drawn 2 weeks after the titer was boosted.

Antiserum Titer Determination. To determine the titer of the antiserum, microsomes from either TAO-induced males or uninduced females were suspended in 10 mM sodium carbonate buffer (pH 9.6) containing 0.2% sodium cholate (coating buffer) to a final concentration of 100 ng of microsomal protein/mL. ELISA (enzyme-linked immunosorbent assay) plates were coated in triplicate (125 μL/well) with the suspensions of microsomes. The plates were allowed to stand overnight at 4 °C. Plates were washed, blocked and incubated, and the color was developed as described below. In addition to preimmune and immune sera, the immunoglobulin G fractions (IgG) of each were assayed for antibody titer. Each IgG fraction was isolated on a protein A-Sepharose column. It was found that when compared to uninduced adult female rat liver microsomes, the TAO-induced male microsomes produced an absorbance significantly above that detected with the uninduced female microsomes to a final concentration of 400 ng of IgG/mL, which corresponds to an approximate antiserum dilution of 1:10 000.

Dealkylation/Debenzylation Assays. Rates of conversions of the methoxy-, ethoxy-, pentoxy-, and (benzyloxy)phenoxazone ethers to resorufin were quantified according to methods described by Rettie et al. (1985). For inhibition of dealkylation and debenzylation with TAO we employed a final concentration of 120 μM, which is approximately 10 times the reported K_s of TAO for P450IIIA (Pessayre et al., 1982; Wrighton et al., 1985a; Bonfils et al., 1985). All determinations are the average of three or more measurements obtained with freshly prepared microsomes. Our limit for fluorometric detection of resorufin was 0.10 pmol/min.

Immunoblotting. Microsomal samples were applied to 7.5% acrylamide SDS gels and electrophoresed according to the method of Laemmli (1970). Utilizing slight modifications of the method of Towbin et al. (1979), we transferred protein to nitrocellulose sheets (pore size of 0.2 μm) in 49.6 mM Tris buffer (pH 8.3), containing 0.1% SDS, 384 mM glycine, and 20% methanol. We found that best results were obtained by allowing the nitrocellulose to dry prior to rinsing and by blocking overnight with 1% gelatin. Visualization of the reactive bands was accomplished with the addition of adsorbed immune serum and goat anti-rabbit IgG-conjugated horseradish peroxidase as described for the ELISA plate assays except that 20 mM Tris buffer (pH 7.5), containing 500 mM NaCl (TBS) and 0.05% Tween 20 (TBST), was used. 4-Chloro-1-naphthol (2.8 mM) with 0.015% H_2O_2 in TBS was used as an insoluble substrate for horseradish peroxidase. The amount of microsomal protein applied to the SDS gel was carefully adjusted (between 0.03 and 500 μg) such that complete transfer (of those proteins that migrated to a location consistent with a molecular weight of 51 000) was achieved without subsequent loss through the nitrocellulose sheet. Quantitative transfer was monitored by posttransfer staining of the gel, including a second sheet of nitrocellulose, and P450IIIA–TAO standards with each immunoblot. Blots were quantitated by scanning the entire immunoreactive band using an LKB Ultroscan XL laser densitometer and the two-dimensional analysis capability of 2400 GelScan XL software. Our limit of detection using this procedure was 1.0 pmol of purified P450IIIA–TAO.

Enzyme-Linked Immunosorbent Assay. To provide semiquantitative determinations of P450IIIA1 in fetal and adult liver microsomes, duplicate samples were serially diluted (2-fold) in coating buffer. The plates were allowed to stand at 4 °C overnight (16 h). The plates were then emptied and allowed to dry. They were washed, allowing 3 min of contact with PBS containing 0.05% Tween 20 (PBST). The wash was repeated twice. Gelatin (200 μL/well), 0.5% in PBST, was used to block unoccupied sites on the plate. The plates were incubated at 37 °C for 2 h. They were then washed as above and incubated for 3 h at 37 °C with 125 μL/well of serial dilutions (2-fold, in PBST) of either preimmune or immune serum. Plates were again washed and incubated 1 h at 37 °C with goat anti-rabbit IgG-conjugated horseradish peroxidase, diluted 1:2000 in PBST. The plates were washed, and 100 μL/well of *o*-phenylethylenediamine dihydrochloride, 2.2 mM in citrate–phosphate buffer, pH 7.0, containing 0.012% H_2O_2, was added to each well. After 30 min, the reaction was stopped with 50 μL/well of 4 M H_2SO_4. Absorbance was read at 490 nm by using a Dynatech MR650 microplate reader, and the absorbances from wells receiving preimmune serum were subtracted from the corresponding absorbances from wells that received immune serum. Plate-to-plate reproducibility was assured by including a reference sample with each plate. Those plates with a reference sample absorbance falling outside a 95% confidence interval about the mean absorbance were excluded. Absorbance versus dilution was graphed for each microsomal sample, and the absorbance readings from those wells showing maximum values over three dilutions were averaged for each sample. Whereas the pH optimum of horseradish peroxidase is approximately 5, we used a pH of 7.0 to widen the range of detectability. At pH 5.0, samples having high specific content reached the maximum detectable absorbance of 2.0 and were not distinguishable. At pH 7.0 the peroxidase reaction was sufficiently slowed to allow differentiation of fetal and adult samples during a 30-min incubation period.

Immune Adsorption of Sera. Immune or preimmune serum was diluted 1:500 in TBST (for use in protein blotting) or PBST (for use in ELISA). Hepatic microsomes from uninduced adult female rats (100 mg of microsomal protein/55 mL) were added to diluted serum and incubated at 37 °C for

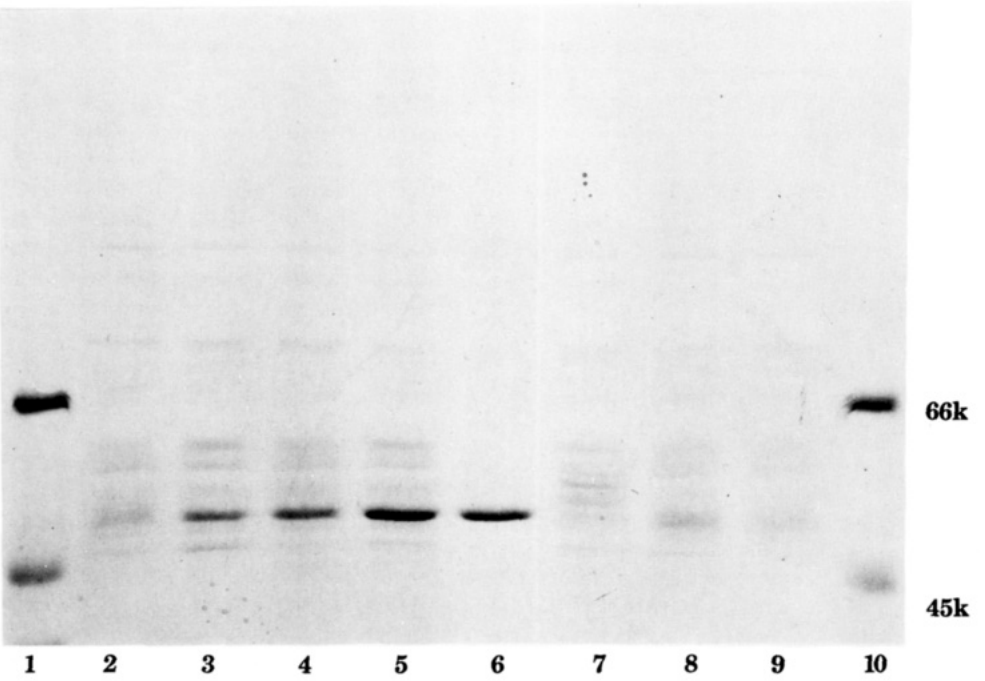

FIGURE 1: Coomassie Blue stained SDS gel of adult uninduced and induced hepatic microsomes and purified P450IIIA–TAO. (Lane 1) Molecular weight markers; (lane 2) 20 μg of microsomal protein from adult females induced with PB; (lane 3) 20 μg of microsomal protein from adult females induced with PCN; (lane 4) 20 μg of microsomal protein from females induced with DEX; (lane 5) 20 μg of microsomal protein from adult females induced with TAO; (lane 6) 5 μg of purified P450IIIA–TAO; (lane 7) 20 μg of microsomal protein from adult females induced with MC; (lane 8) 20 μg of microsomal protein from uninduced adult males; (lane 9) 20 μg of microsomal protein from uninduced adult females; (lane 10) molecular weight markers.

1 h. The microsomes were removed by centrifugation at 104000*g* for 1 h. The adsorbed sera were stored at −70 °C in 5 mg/mL bovine serum albumin.

Results

Immunoquantitative Determination. Since the relative levels of P450IIIA in hepatic microsomes from adult male and female rats have been established (Waxman et al., 1985), we used induced and uninduced adult tissues as positive and negative controls for quantitation of P450IIIA1 in fetal samples (Figure 1). By use of protein immunoblot densitometry, 259, 155, 147, 27.7, 20.7, 6.8, and 0.6 μg of P450IIIA/mg of microsomal protein were determined for TAO-, PCN-, DEX-, and PB-induced adult female, uninduced male, MC-induced adult female, and uninduced adult female rats, respectively (Figure 2).

Fetal hepatic microsomes were prepared by pooling livers from 24–64 fetuses from 3–8 dams for each gestational age ranging from day 15 to day 21. Using uninduced fetal microsomes, no immunological activity was detected even when 500 μg of microsomal protein was loaded onto the SDS gel for protein immunoblot analysis. P450IIIA levels in uninduced fetal hepatic microsomes were, therefore, less than 2.0 pmol/mg of protein. Analyses of fetal microsomes from rats that received the higher dose (40 mg/kg, twice daily for 4 days) of PCN revealed that levels of immunoreactive protein ranged from 4.7 to 20.6 μg P450IIIA1/mg of protein and exhibited a general tendency to gradually increase over the gestational period investigated. Fetal microsomal samples from rats treated with the lower dose (50 mg/kg, once daily for 3 days) exhibited a broader range of activity, 0.5–28.7 μg/mg of protein, also increasing gradually as a function of gestational age and fetal weight (Figure 3).

In hepatic microsomes from uninduced maternal rats, no significant immunological activity could be detected, even when as much as 100 μg of microsomal protein was loaded onto the gel. Therefore, P450IIIA specific content in the uninduced dams were determined to be less than 10 pmol/mg of microsomal protein. Hepatic microsomes from maternal animals that received the lesser amount of PCN exhibited levels of immunoreactivity that, percentagewise, varied only minimally when determined by quantitative protein immunoblot densi-

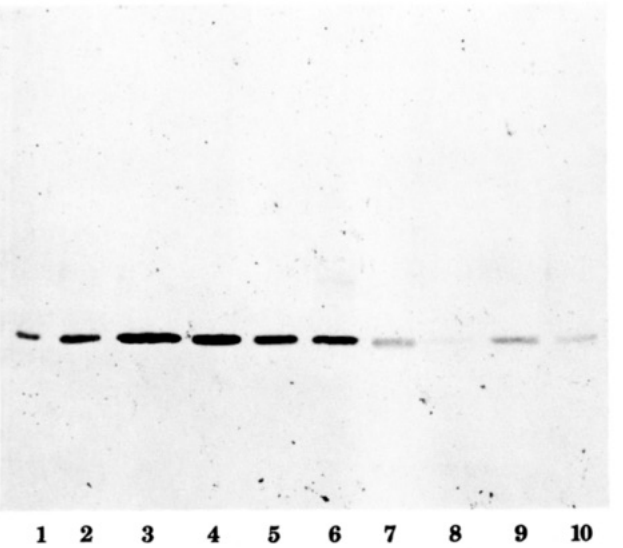

FIGURE 2: Protein immunoblot of adult hepatic microsomes from induced and uninduced adult rats and purified P450IIIA–TAO. (Lane 1) 0.25 μg of purified P450IIIA–TAO; (lane 2) 0.50 μg of purified P450IIIA–TAO; (lane 3) 0.75 μg of purified P450IIIA–TAO; (lane 4) 5 μg of microsomal protein from adult females induced with TAO; (lane 5) 5 μg of microsomal protein from adult females induced with DEX; (lane 6) 5 μg of microsomal protein from adult females induced with PCH; (lane 7) 50 μg of microsomal protein from uninduced males; (lane 8) 100 μg of microsomal protein from uninduced females; (lane 9) 50 μg of microsomal protein from adult females induced with PB; (lane 10) 100 μg of microsomal protein from adult females induced with MC.

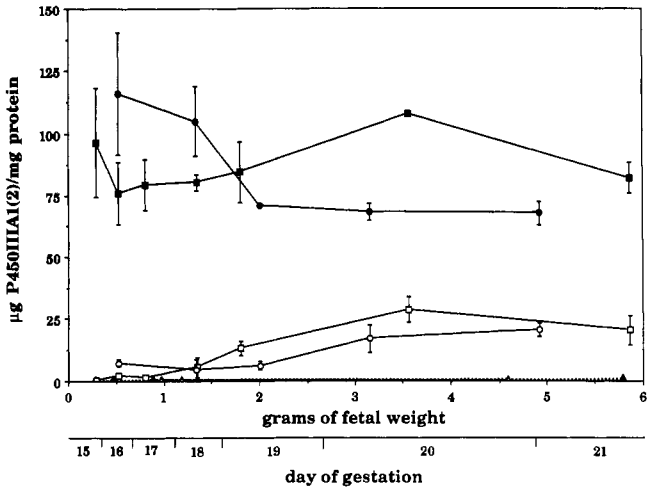

FIGURE 3: Quantitation of P450IIIA1 in maternal and fetal rat liver microsomes by protein immunoblot densitometry. Solid symbols represent maternal microsomes. Open symbols represent fetal microsomes. Circles correspond to microsomes from animals that received the high PCN dose (40 mg/kg, twice daily for 4 days). Squares correspond to microsomes from animals that received the low PCN dose (50 mg/kg, once daily for 3 days). Triangles correspond to microsomes from uninduced animals. Error bars represent standard deviations of the mean of three determinations.

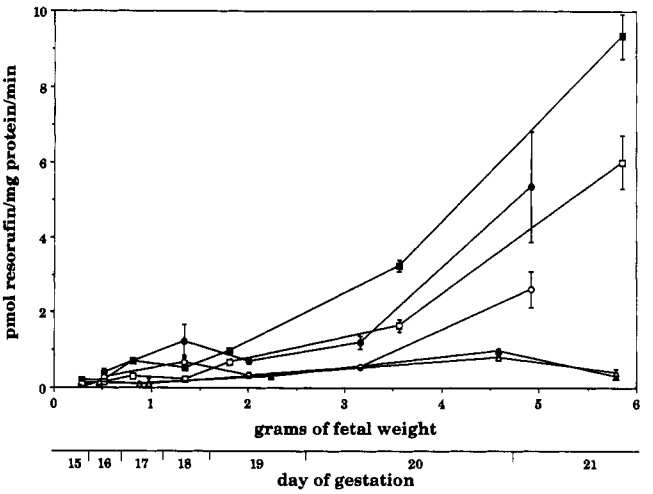

FIGURE 4: Debenzylation and TAO inhibition of debenzylation of (benzyloxy)phenoxazone catalyzed by freshly isolated hepatic microsomes from uninduced and transplacentally induced fetal rats. Solid symbols correspond to uninhibited debenzylase activity. Open symbols correspond to activity measured after a 10-min preincubation with 120 μM TAO. Squares correspond to activity catalyzed by fetal liver microsomes from dams injected with the low dose of PCN (50 mg/kg, once daily for 3 days). Circles correspond to fetal microsomes from dams that received the high dose of PCN (40 mg/kg, twice daily for 4 days). Triangles correspond to uninduced fetal microsomes. Error bars represent standard deviations of the mean of three determinations.

tometry. The range measured was from 76.0 to 108 μg P450IIIA/mg of protein, whereas the hepatic microsomes from the higher dosed dams, immunoreactivity varied somewhat more widely during late gestation, ranging from 61.0 to 116 μg/mg (Figure 3).

Enzymatic Determinations. To evaluate the potential of TAO for specific inhibition of the enzymic activity of P450IIIA1, debenzylating and dealkylating activities of hepatic microsomes from adult male and female rats induced with PCN, DEX, PB, or MC (see Experimental Procedures) were determined by using (benzyloxy)-, methoxy-, ethoxy-, and pentoxyphenoxazone ethers as substrates. All uninhibited activities found were in accordance with levels reported earlier (Namkung et al., 1988). Dealkylation of pentoxyphenoxazone (using microsomes from PB-induced animals as the source of enzyme) was inhibited by 6% with the addition of TAO, while there was no change in depentylase activity when TAO was added to microsomes from uninduced animals. TAO did not inhibit the deethylase activity of microsomes from uninduced or MC-induced rats nor did TAO have a measurable effect on demethylation catalyzed by microsomes from MC-induced animals. Debenzylase activity was 0%, 67%, 17%, 24%, and 70% inhibited with microsomes from uninduced and DEX-, MC-, PB-, and PCN-induced rats, respectively. These data support the contention that TAO is a specific inhibitor of P450IIIA1.

In fetal hepatic microsomes isolated from uninduced dams, minimal [0.11–0.39 pmol of resorufin (mg of protein)$^{-1}$ min^{-1} debenzylation of (benzyloxy)phenoxazone was detected over the gestational period (Figure 4). Fetal hepatic microsomes obtained from animals injected with the low dose (50 mg/kg, once daily for 3 days) of PCN showed slightly greater debenzylase activity than fetal samples obtained from dams receiving the higher dose (40 mg/kg, twice daily for 4 days) at almost every stage of gestation. Increasing fetal debenzylase activity as a function of gestational age was demonstrated in microsomes from PCN-induced animals with both the high and low PCN dosages. The lower dose produced a rise in activity from 0.19 pmol mg^{-1} min^{-1} at day 15 to 9.34 pmol mg^{-1} min^{-1} at day 21, while the higher dose resulted in a rise from 0.41 to 5.35 pmol mg^{-1} min^{-1} at days 16 and 21 of gestation, respectively. The extent of TAO inhibition of debenzylase activities was positively correlated (r = 0.96 for fetuses transplacentally induced with the high dose of PCN and r = 0.99 for those fetuses induced with the lower dose of PCN) with the rise in debenzylase activity associated with fetal weight gain in both sets of induced samples. TAO had no measurable effect on the activities assayed in uninduced samples. In none of the induced samples that exhibited measurable debenzylation was it possible to inhibit the activity to the level detected in the uninduced samples, even when the final concentration of TAO was increased from 120 to 600 μM.

Debenzylase activity of uninduced maternal liver microsomes varied from 1.73 to 4.82 pmol resorufin mg^{-1} min^{-1} with no distinct trend with respect to stage of gestation. Induced activities varied widely, 15.9–46.5 pmol mg^{-1} min^{-1}. TAO-inhibitable debenzylase activity correlated (r = 0.78 for animals induced with the high dose of PCN and r = 0.90 for those animals induced with the lower dose of PCN) the uninhibited levels with both the high and low PCN dosages (Figure 5). These data, along with the corresponding immunological determinations, suggest that the inducibility of P450IIIA1 in maternal liver does not vary as a simple function of gestational age.

Discussion

We have used two immunoassays with an antiserum of defined selectivity in combination with P450IIIA1-specific induction (by PCN) and P450IIIA-specific inhibition (by TAO) to demonstrate the occurrence and activity of P450IIIA1 in maternal and fetal hepatic microsomes as a function of prenatal development. In addition to its extremely high sensitivity, TAO-inhibited debenzylation of (benzyloxy)phenoxazone was selected as the method of choice for catalytic determinations for several reasons. Many investigators have provided evidence that TAO is a specific inhibitor of P450IIIA (Wrighton et al., 1985a; Arlotto et al., 1986; Dansette et al., 1986). Our own investigations into the selectivity of TAO inhibition were consistent with its usefulness

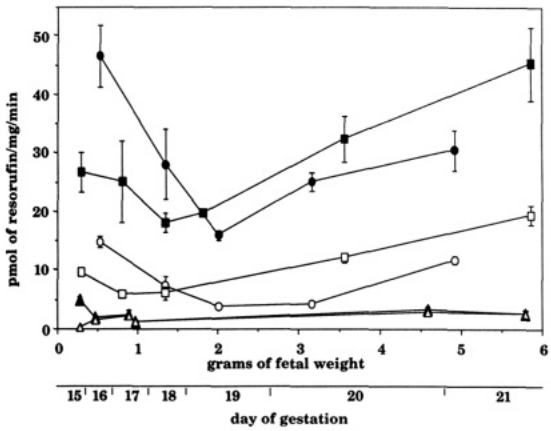

FIGURE 5: Catalysis of debenzylation and TAO inhibition of debenzylation of (benzyloxy)phenoxazone by freshly isolated hepatic microsomes from uninduced and induced maternal rats. Solid symbols correspond to uninhibited debenzylase activity. Open symbols correspond to activity measured after a 10-min preincubation with 120 μM TAO. Squares correspond to activity catalyzed by liver microsomes from dams injected with the low dose of PCN (50 mg/kg, once daily for 3 days). Circles correspond to microsomes from dams that received the high dose of PCN (40 mg/kg, twice daily for 4 days). Triangles correspond to microsomes from uninduced dams. The final injection was given 24 h prior to sacrifice on the day of gestation indicated on the abscissa. Error bars represent standard deviations of the mean of three determinations.

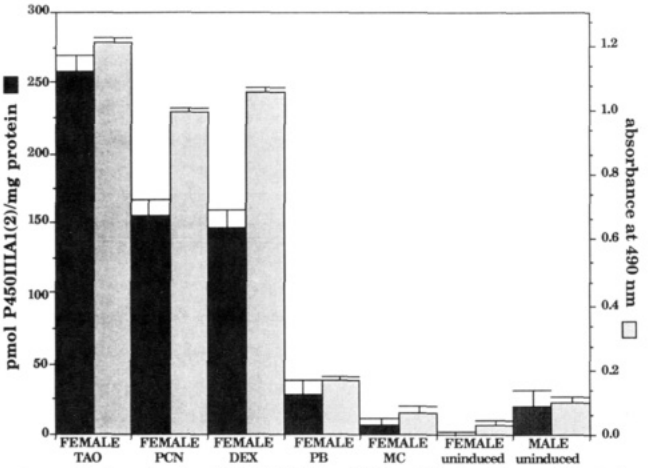

FIGURE 6: Levels of anti-P450IIIA activity. (Dark columns) Protein immunoblot densitometry; error bars represent the standard deviations from the mean of three determinations. (Light columns) ELISA; error bars represent standard deviations of the mean for triplicate determinations.

in subsequent determinations. The inability of TAO to inhibit P450IIB1, P450IA1, and P450IA2 was evident by the lack of significant reductions in dealkylation of pentoxyphenoxazone catalyzed by PB-induced liver microsomes or of ethoxy- and methoxyphenoxazones catalyzed by MC-induced microsomes. Further, the relative amounts of TAO-inhibitable debenzylation, with PCN-, DEX-, PB-, and MC-induced and female uninduced microsomes as enzyme source, correlated ($r = 0.95$) to the relative levels of P450IIIA1 immunoassayed in these samples. Additional support of our selection of TAO-inhibitable debenzylation of (benzyloxy) resorufin as a probe for functional P450IIIA1 was provided by experiments showing that, in uninduced fetal liver, minimal or no TAO-inhibitable debenzylation occurred. TAO-inhibitable catalytic activity was detected, however, in fetal livers from dams injected with PCN. PCN has been described as a specific inducer of P450IIIA1 (Wrighton et al., 1985a; Namkung et al., 1988). These findings indicate that, if the fetal response is similar to the adult response, activity detected in transplacentally induced fetal liver may be attributed solely to P450IIIA1. This assumption appears to be valid since the PCN-inducible debenzylase activities were closely paralleled by the TAO-inhibitable activities and since PCN-induced increases in catalytic activity were associated with corresponding increases in immunoreactive P450IIIA. The specificity of PCN induction and TAO inhibition, coupled with the very high sensitivity of a kinetic fluorometric assay (particularly useful for fetal tissue), render TAO-inhibited debenzylation of (benzyloxy)-phenoxazone an excellent choice for monitoring activity of P450IIIA1.

In an attempt to obtain a selective antiserum, the immunogen used was cut from a single band on a gradient SDS gel. Despite this approach, small amounts of anti-P450IIIA reactivity were evident in both immunoassays when uninduced female hepatic microsomes were used. This suggested the occurrence of cross-reactivity with P450 species constitutive in adult females. Therefore, to increase the P450IIIA specificity of the antiserum, it was preadsorbed against uninduced female liver microsomes. After adsorption, increased specificity resulted as a consequence of constitutive P450s binding to antibodies responsible for the cross-reactivity, and these complexes were removed by centrifugation. P450IIIA-specific epitopes are not present in adult females (Waxman et al., 1985; Wrighton et al., 1985a,b), and immunoglobulins specific for this enzyme remained in the serum. By use of antiserum adsorbed in this fashion, up to 100 μg (>200 times the protein necessary for detection of P450IIIA1 in TAO-induced microsomes) of uninduced female microsomal protein could be added to an SDS gel with only a trace band evident when blotted (Figure 2). Additionally, in the ELISA, the absorbance associated with uninduced female microsomes was reduced from 0.600 to less than 0.100 absorbance units when adsorbed preimmune and immune sera were used.

The selectivity of the adsorbed polyclonal antiserum was demonstrated by blotting microsomal protein of known P450IIIA content as shown in Figure 2. When 10 μg of uninduced and MC-induced female microsomal protein was blotted, no bands were detected. However, when amounts of protein were increased to 100 μg for these two samples, trace bands became evident as shown. The light band associated with uninduced female microsomes suggests that even after immune adsorption, some cross-reactivity remains. Waxman et al. (1985) also found residual P450IIIA immunoreactivity with uninduced female microsomes after immune adsorption of the antiserum. Other investigators have observed a similar increase in P450IIIA immunoreactivity when adult male rats were induced with MC (Cresteil et al., 1986). One might speculate that MC may induce, or that uninduced females contain, P450IIIA1(2). It seems more likely however, that immunological activity associated with female uninduced and MC-induced microsomes results from the occurrence of epitopes common to members of the superfamily.

When quantified, bands corresponding to TAO-, PCN-, DEX-, and PB-induced female and uninduced male exhibited the expected relative amounts of P450IIIA (Figure 6). Microsomes from animals induced with TAO contained the most immunological activity. The relative ranking of these samples is consistent with previously published levels, although absolute values varied (Guengerich, 1982; Wrighton et al., 1985b). This variation is most probably due to differences in immunoquantitation procedure. Our immunoquantitation of P450IIIA1 in TAO-induced female rat microsomes is, however, consistent with a specific content of approximately 5.0 nmol/mg as determined spectrophotometrically in our

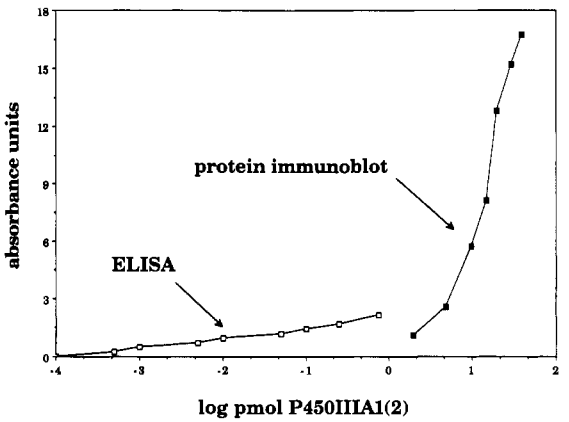

FIGURE 7: Standard curves for the ELISA and protein blot densitometry immunoassays. Assays utilized anti-P450IIIA immune and preimmune sera with purified P450IIIA–TAO as antigen. For the ELISA, the absorbance units represent the absorbance at 490 nm. For the blotting immunoassay, the absorbance units represent the absorbance at 633 nm times the area of the immunoreactive protein band.

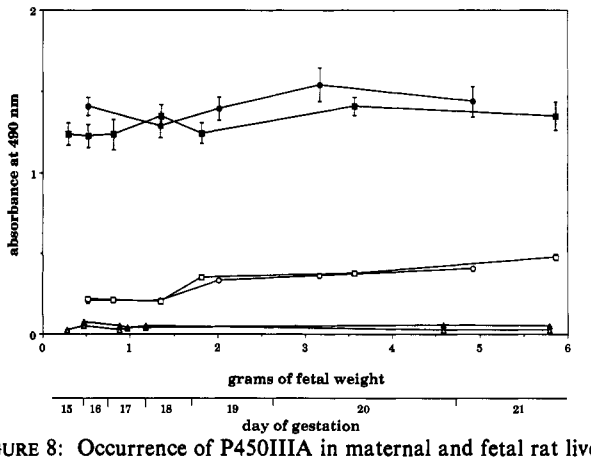

FIGURE 8: Occurrence of P450IIIA in maternal and fetal rat liver microsomes determined by ELISA. Symbols are as described for Figure 3. Error bars represent standard deviations of the mean of three assays, each in triplicate.

laboratory as well as with spectrophotometric results obtained by Wrighton et al. (1985a).

In addition to protein immunoblot densitometry, we developed an ELISA plate immunoassay by modifying the method of Paye et al. (1984). We used the samples shown in Figure 1 to demonstrate that relative levels of immunological activity determined with the ELISA were consistent with those determined by protein blotting of the same samples. While similar results were obtained with adult samples having high specific content (Figure 6), we found that each method had specific advantages and limitations for determinations using fetal tissues. For this reason, we compared the ELISA with protein immunoblot densitometry and evaluated the application of each in the detection of P450 isozymes in tissues (such as fetal livers) with low specific content.

We found that the two immunoassays complement one another, each contributing information not provided by the other. Isolated P450IIIA–TAO was utilized to generate standard curves for each immunoassay (Figure 7). From these data, it is evident that while the ELISA provides a lower limit of detection, it lacks the sensitivity (defined here as a large change in absorbance for change in quantity of P450IIIA–TAO) that immunoblot densitometry exhibited. The very low detection limit of the ELISA allowed us to clearly demonstrate the occurrence of immunoreactive protein in fetal livers as early as day 15 of gestation (Figure 8). The inducibility of P450IIIA1 at day 15 was less evident in the immunoblot determinations (Figure 4). Further, the range of detectability (0.10–750 fmol of P450IIIA–TAO) of the ELISA allowed us to compare the widely variable P450IIIA content of adult versus fetal livers using a single protocol. With the Ultroscan laser densitometer, however, we were able to more accurately quantitate P450IIIA1. The capability of this instrument to determine the absorbance over the entire area of the immunoreactive band allowed us to take advantage of the capacity of the nitrocellulose paper to immobilize a larger amount of antigen than can be adsorbed to the ELISA plate well. For this reason, we found protein immunoblot densitometry to be a more useful assay for the delineation of small differences in P450IIIA1 content such as occur during prenatal development and between the two PCN dosages used. However, blotting was found to have a narrower range of detectability, 1.0–40 pmol of P450IIIA–TAO. We found it necessary to carefully adjust the amount of protein loaded onto the SDS gel to fall within this range. Additionally, the amount of microsomal protein loaded must be balanced with the conditions for blotting to assure complete transfer without subsequent loss through the nitrocellulose sheet. Therefore, we stained all gels, posttransfer, and included a second sheet of nitrocellulose to assure the quality of each assay.

Both immunoassays clearly distinguished the specific content of P450IIIA in maternal microsomes from that of fetal microsomes (Figures 3 and 8). For this investigation, one of the advantages of protein blotting was that it distingushed the slight decreases in enzyme content associated with an increased dose of inducer in fetal liver (Figure 3). Although these differences were slight, corresponding decreases in enzymatic activity added confidence to our ability to distinguish small differences by using the blotting technique (Figure 4). The blotting method has the added advantage of a molecular weight criterion and also gives an indication of the presence or absence of cross-reactive proteins. In contrast, the microplate ELISA relies solely on the specificity of the polyclonal antibody. In the ELISA, all proteins are adsorbed onto the walls of the well and occurrence of cross-reactive proteins is not evident.

With the above considerations, we suggest that the ELISA is best for detecting cytochromes P450 occurring in tissues of low specific content and for semiquantitation in tissues with higher specific content. We found immunoblot densitometry to be the method of choice for the quantitation of P450IIIA1. Although we loaded a maximum of 500 μg of protein on SDS gels, the lower limit for detection with protein blotting is dependent only on the ability to electrophoretically resolve larger amounts of protein. It may be possible to quantitate P450 isozymes present in even smaller specific quantities than were measured here simply by loading larger amounts of protein on preparative gels and blotting.

Heuman et al. (1982) have shown that, in young adult female rats, levels of immunoquantifiable P450IIIA1 in liver microsomes continued to increase over a range of PCN doses from 30 to 300 mg/kg when administered daily by gastric gavage for 4 days. Our results from adult maternal rats dosed IP with 50 or 80 mg PCN/kg daily did not indicate a clear difference in the level of P450IIIA1 (Figure 3). Each dosage regimen produced very large increases in immunoreactive protein. Lack of increased levels with increased dose suggests that the maximal response in the maternal animals was achieved with the lower dosage. Additionally, 80 mg/kg, injected once daily for 4 days, resulted in some fetal death (data not shown). The difference in dose–response observed by Heuman et al. (1982) may reflect variations in efficiency of induction between the two routes used or the fact that our

animals were *pregnant* females.

Just prior to delivery, induced fetal microsomes exhibited approximately one-fourth the P450IIIA1 content and catalytic activity observed in the induced maternal liver. In uninduced fetal samples no significant catalytic activity or P450IIIA was detected. This is consistent with results reported by Cresteil et al. (1986), who were unable to detect P450IIIA immunological activity prenatally in uninduced animals. Our findings, however, appear to be at some variance with the suggestion of Waxman et al. (1985) that P450IIIA is present in uninduced male and female rats at birth. These investigators demonstrated the apparent occurrence of low quantities of P450IIIA in both male and female rats at 2 weeks of age but not earlier.

If, in the fetuses given the lower dosage of PCN, the amount of immunoreactive enzyme occurring at each stage, days 18–21, is averaged, the mean is significantly higher than the levels determined for uninduced fetuses, $p < 0.025$. In the corresponding fetal livers from the dams given the higher PCN dose, the mean level was also higher than in uninduced fetal livers but at a lower level of significance, $p < 0.05$ (Student's t test). These findings suggest that in fetuses the lower PCN dose may be slightly more effective than the higher PCN dose. Regarding this point we can only speculate that the higher dose may cause toxicity that interferes with induction. The possibility of toxicity from high dosage is reasonable considering the physiological stresses associated with pregnancy.

It was of interest that when all the fetal data for days 15–18 were averaged, the ratio of enzymatic activity/immune reactivity was 0.15. At day 21, the ratio of enzymatic activity/immune reactivity was 0.43. These determinations suggest that during days 15–18 the P450IIIA1 present is not as catalytically active as just prior to parturition. Disparities in immunological occurrence versus catalytic determinations have been reported by other [e.g., Waxman et al. (1985) and Wrighton et al. (1985b)]. To explain the greater increase in immunologically versus catalytically detectable enzyme observed upon induction, it has been suggested that a component of catalytic activity other than P450IIIA1, e.g., P450 reductase, becomes rate limiting (Waxman et al., 1985). Others have proposed the occurrence of immunoreactive protein, unresolved by electrophoresis, which does not have catalytic activity (Wrighton et al., 1985b). We suggest that the increase in catalytic activity associated with P450IIIA1 during late development may reflect one or both of these possibilities. It is also reasonable to suggest that the lower activity/enzyme ratio for the earlier time points could reflect the increased occurrence of apoprotein relative to active holoenzyme.

The general occurrence of IIIA immunoreactive proteins over a broad range of species (Watkins et al., 1985; Wrighton et al., 1985b) suggests an important physiological role for these enzymes. The prominent occurrence of a P450IIIA-like enzyme in human fetal as well as adult tissues further suggests that these enzymes may have an important role in human development. Additionally, the positive correlation of IIIA in humans to the 6β-hydroxylation of testosterone and the determination that the 6β-hydroxytestosterone was the major hydroxylation metabolite may be significant for the elucidation of the endogenous function of IIIA-like protein(s) in humans. Whether the IIIA-catalyzed metabolism of testosterone serves solely to eliminate the substrate or whether 6β-hydroxytestosterone (or a metabolite of it) is active physiologically has yet to be determined (Waxman et al., 1987).

In conclusion, our investigations have combined determinations of catalytic activity with quantitative immunoassays to demonstrate the inducibility of hepatic P450IIIA1 during rat prenatal development. Specific induction and specific inhibition of P450IIIA1-dependent monooxygenase activity and a carefully defined selectivity of anti-P450IIIA1 antibody were utilized to provide rigorous quantitative assessments. We have shown that this cytochrome P450 can be induced in rat fetal livers during the period of organogenesis and that inducibility increases as a function of gestational age. Other investigators have determined that the highest levels of P450IIIA detected in adult human liver are associated with drug therapies such as dexamethasone and macrolide antibiotics (Watkins et al., 1985; Guengerich et al., 1986; Waxman et al., 1988). Together these findings suggest to us a potential for induction of a P450IIIA cytochrome P450 in human fetal liver. The ramifications of human prenatal P450IIIA induction may be speculated to include developmental consequences since this enzyme is the major testosterone hydroxylase in humans (Waxman et al., 1988). Additionally, precocious occurrence of P450IIIA may increase the potential for the generation of reactive intermediates during a period of vulnerability prior to the development of conjugation and elimination capacity. Therefore, the consequence of prenatal induction of the steroid-inducible P450s in humans may also include an increased potential for carcinogenesis, teratogenesis, and genotoxicity. Such speculation concerning the inducibility of the human P450IIIA must be approached with caution, however, since it is apparent that important differences in the regulation of IIIA occur between humans and animal models (Wrighton et al., 1988).

Acknowledgments

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Registry No. PCN, 1434-54-4; TOA, 2751-09-9; DEX, 50-02-2; PB, 50-06-6; MC, 56-49-5; cytochrome P450, 9035-51-2; debenzylase, 107824-47-5.

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