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HUMAN EMBRYONIC CYTOCHROME P450S: PHENOXAZONE ETHERS AS PROBES FOR EXPRESSION OF FUNCTIONAL ISOFORMS DURING ORGANOGENESIS

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Abstract—Human embryonic tissues were investigated during the period of organogenesis with a combination of substrate probes, selective inhibitors and immunoprobes in terms of their capacity to express functional P450 isoforms. A series of phenoxazone ethers utilized as substrate probes revealed that human embryonic hepatic, pulmonary, renal, adrenal and cardiac tissues each contained a complement of functional P450 isoforms when analyzed between days 50 and 60 of gestation. Preparations of each of these tissues contained isoforms capable of catalyzing O-demethylation, O-deethylation, O-dependentylation and O-debenzylation of the respective phenoxazone ethers. Investigations with chemical inhibitors and inhibitory antibodies as well as comparisons with vector-expressed, human P450 isoforms suggested that isoforms of P450 subfamilies 1A, 2B, 2C or 3A were not major contributors to any of the observed reactions. The P450-dependent reactions studied exhibited several unexpected and unusual characteristics including a preference for NADH over NADPH as the initial electron donor. Results were consistent with the concept that conceptal-specific P450 isoforms participate in the human embryonic O-dealkylation/debenzylation probe reactions investigated.

Isoforms of the cytochrome P450 superfamily catalyze oxidative biotransformation of endogenous substrates such as steroids, fatty acids, eicosanoids and retinoids as well as of a wide variety of exogenous organic chemicals of low molecular weight [1–3]. In general, P450 cytochromes play important roles in the detoxication of such xenobiotics. However, it is also well recognized that these enzymes will catalyze bioactivation reactions and that the reactive intermediates generated can produce a variety of toxic effects including cytotoxicity, carcinogenesis, mutagenesis and dysmorphogenesis [4–7].

The expression of P450 isoforms and P450-dependent enzymic activities in relatively high quantities has been reported for tissues of prenatal humans and chicks [8–10]. Functional P450s in much lesser quantities have also been reported to occur in prenatal tissues of rodents and rabbits [11 –14]. In rat conceptuses, studies [13] with probe substrates have suggested that at least three P450 cytochromes are expressed constitutively during the period of organogenesis (days 10–14 of gestation). In those studies, there were also clear indications that 3-methylcholanthrene and other polycyclic aromatic hydrocarbons could induce rat conceptal P450 during organogenesis, and an inducible isoform was tentatively identified by immunochemical and biochemical analyses as CYP1A1§ or a very closely related isoform [15]. In more recent studies in mice, inducible Cyp1a1 mRNA was detected in various murine conceptal tissues but not in the embryo *per se*. Lack of detection of embryonic Cyp1a1 mRNA led the authors to conclude that Cyp1a1 may not be an active participant in the early development and differentiation of embryos [16]. Nevertheless, Cyp1a1 mRNA was detected in other murine conceptal tissues in a developmental/stage-dependent manner and was also reported recently to have been detected with the polymerase chain reaction (PCR) in various prenatal tissues of humans as early as day 45 of gestation [17]. At later stages of human gestation, CYP3A7 and CYP3A5 have been identified in fetal livers and, thus far, CYP3A7 has been detected only in human fetal livers and cultured hepatoma cells [18–21].

Currently, our knowledge of the nature, function and significance of P450 cytochromes expressed during the period of organogenesis is extremely limited. There is some evidence to suggest that at least some P450 isoforms expressed during prenatal life may be absent from older animals and/or replaced by different isoforms postnatally [22]. Our own studies with rat conceptal P450 isoforms also suggested that constitutive P450s in organogenesis-stage conceptal tissues were distinct from adult hepatic isoforms [15, 23]. Clearly, it is of importance to understand the dynamics of functional "embryonic" or "fetal" P450 isoforms not only in terms of their physiologic/developmental roles but also in terms of their roles as determinants of the embryotoxic/fetotoxic effects of xenobiotic chemicals to which the conceptus may be exposed during the course of pregnancy.

The goal of the research reported in this paper was to initiate investigations into the possibility that functional P450 isoforms capable of biotransforming

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§ Abbreviations: P450 isoforms are designated in accordance with recommendations outlined in Ref. 3. Other abbreviations are: G6PDH, glucose-6-phosphate dehydrogenase: G6P, glucose-6-phosphate, ANF, 7,8-benzoflavone; DMSO, dimethyl sulfoxide; TAO, triacetyloleandomycin; and PCR polymerase chain reaction.

xenobiotic substrates might be expressed in various human tissues during the period of organogenesis (normally regarded as the most sensitive to chemical dysmorphogenesis), extending approximately from day 18 to day 60 of gestation [6, 8]. We have utilized a combination of highly sensitive and relatively selective initial probes, including probe substrates, chemical inhibitors and inhibitory antibodies in order to gain preliminary insights into the expressions, identities, activities and quantities of organogenesis-stage, human conceptal P450 cytochromes. The results obtained support the following concepts: a large multiplicity of functionally active P450 isoforms is expressed in human tissues during organogenesis; many of these isoforms will utilize xenobiotics as substrates; and several organogenesis-stage human organs and tissues are capable of expressing a wide variety of P450 isoforms.

MATERIALS AND METHODS

Chemicals. Glucose-6-phosphate dehydrogenase (G6PDH), protein A-Sepharose CL-4B, nonimmune rabbit IgG, retinol, NADH and NADPH were purchased from the Sigma Chemical Co. (St. Louis, MO). Glucose-6-phosphate (G6P) was purchased from Boehringer Mannheim (Indianapolis, IN). Resorufin, 7,8-benzoflavone (ANF), dicoumarol and orphenadrine hydrochloride were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Resorufin was purified (>99.5%) according to methods described by Klotz *et al.* [24]. Spectrophotochemical grade dimethyl sulfoxide (DMSO) was purchased from the J.T. Baker Chemical Co. (Phillipsburg, NJ). Metyrapone was received as a gift from Searle Laboratories (Chicago, IL); triacetyloleandomycin (TAO) as a gift from the Pfizer Laboratories (New York, NY); and benzphetamine as a gift from Upjohn Laboratories (Kalamazoo, MI). HepG2 hepatoma cell-expressed adult human P450 cytochromes [1] were provided by Dr. Frank J. Gonzalez of the National Institutes of Health. Phenoxazone ethers were synthesized in our laboratories according to methods described by Mayer *et al.* [25] and were purified (>99.5%) by the methods described by Klotz *et al.* [24]. These included 7-pentoxyphenoxazone, 7-benzyloxyphenoxazone, 7-ethoxyphenoxazone and 7-methoxyphenoxazone. All other chemicals used were of the highest purity commercially available.

O-Dealkylation/O-debenzylation measurements. Rates of O-dealkylation and O-debenzylation of phenoxazone ethers were assessed fluorimetrically according to slightly modified methods originally described by Burke *et al.* [26]. Unless otherwise specified, the incubation mixtures (1.0 mL) contained 0.2 to 0.5 mg of 600 *g* (5 min) or 2000 *g* (10 min) supernatant protein in 0.1 M potassium phosphate buffer (pH 7.4), phenoxazone ethers (10 μM) dissolved in 10 μL of DMSO, 1.0 mM NADPH or 1.0 mM NADH and an NADPH-regenerating system consisting of 5.0 mM G6P and 1 unit of G6PDH. Reactions were initiated by additions of NADPH and temperatures of the reaction mixtures were maintained at 37° during measurements of resorufin generation. Reactions were monitored continuously as a function of time with a Farrand recording fluorometer with respective excitation and emission wavelengths of 530 and 585 nm. Generation of resorufin was measured for at least 10 min and the slopes of the line for the first 3 min were used to calculate the rates of the reactions. The extent of fluorescence increase was calibrated against a known concentration of resorufin in each assay. Preliminary experiments indicated that additions of dicoumarol (0.01 mM) did not increase significantly the rates of reactions in which human embryonic tissues were employed as enzyme source and, unless otherwise indicated, dicoumarol was not a component of the reaction mixtures. The limit of detection was 0.1 pmol/min for each of the phenoxazone ethers.

Human embryonic tissues. All human embryonic tissues were procured through the Central Embryology Laboratory of the University of Washington (Department of Pediatrics), Seattle, WA. All procedures involving handling and assay of these tissues were in accordance with the guidelines of the Human Subjects Review Committee at the same institution. The tissues were obtained immediately following surgical procedures (dilatation and curettage), placed on ice and delivered to the laboratory within 3–4 hr. For these investigations, the gestational ages of the specimens obtained were 50–60 days as estimated by measurements of the foot lengths. Tissue specimens were either analyzed immediately upon arrival in the laboratory or were immediately frozen at −75°, stored at the same temperature, and assayed within 4 weeks. The tissues were homogenized by hand in 2 vol. of ice-cold, 0.1 M potassium phosphate buffer (pH 7.4) in a glass homogenizing vessel with a glass pestle. The resultant homogenate was then centrifuged at 600 *g* for 5 min and the supernatant fraction was used for all subsequent analyses. Protein concentrations were assayed according to the method of Lowry *et al.* [27].

Preparation of antibodies. Polyclonal antibodies were raised against adult rat hepatic CYP1A1, CYP2B1 and CYP2C11 by intradermally injecting 0.25 mg of the corresponding purified cytochrome P450 in Freund's adjuvant into adult, female New Zealand White rabbits according to slight modifications [15] of methods described by Thomas *et al.* [28]. These antibodies are known to cross-react with other members of the same subfamily [1, 3, 29]. For antibody inhibition studies, embryonic enzyme sources were preincubated for 15 min at 25° with IgG fractions (0.5 to 5.0 mg IgG protein per incubation vessel) prepared from the antisera by passage through a protein A-Sepharose CL-4B column [30]. For reconstituted P450 systems [31] in which purified isoforms were the enzyme sources, 4.2 mg of IgG/nmol of P450 were used as described earlier. Preparation and usage of vector-expressed human P450s donated by Dr. Gonzalez are described by Aoyama *et al.* [32].

RESULTS

Analyses with phenoxazone ether probes. Rates of

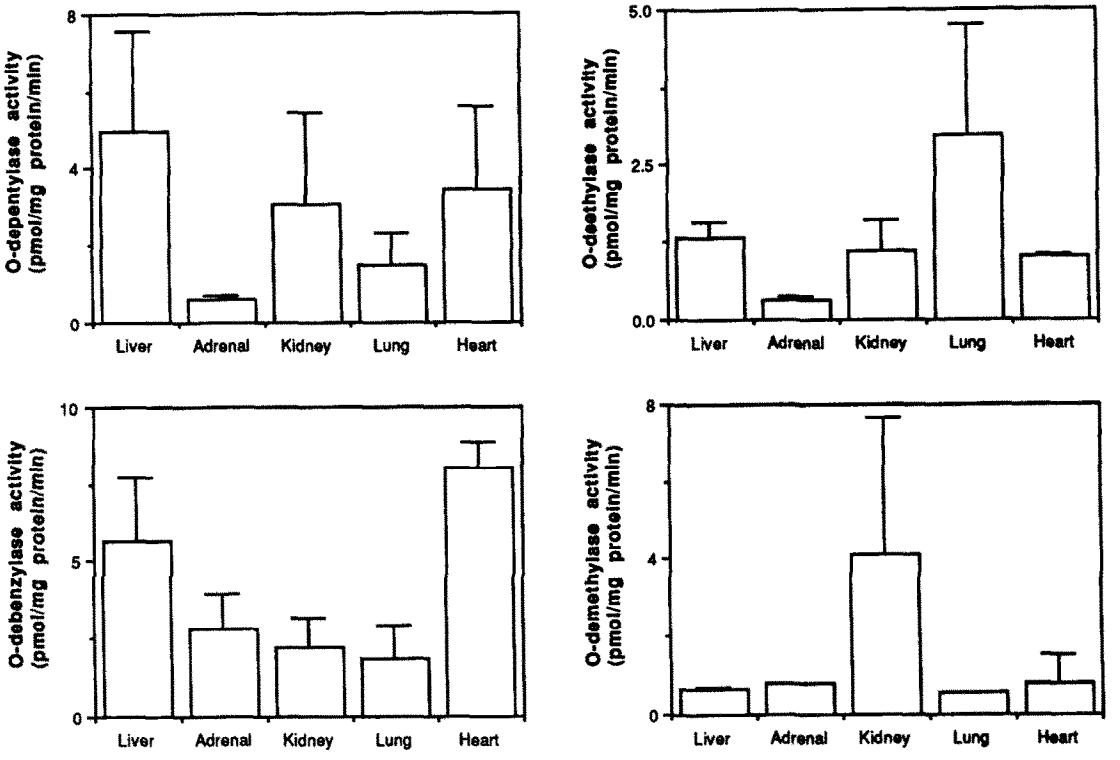

Fig. 1. Catalysis of the O-dealkylation/O-debenzylation of four phenoxazone ethers (methoxy, ethoxy, pentoxy and benzyloxy) by enzymes present in 600 *g* (5 min) supernatant fractions of human embryonic livers, adrenal glands, kidneys, lungs and hearts. All tissues were obtained between days 50 and 60 of gestation. Each bar represents the mean ± SD of assays on non-pooled tissues from 3 to 6 separate embryos.

because of

O-dealkylation of methoxy-, ethoxy- and pentoxyphenoxazone ethers and of O-debenzylation of the benzyloxyphenoxazone ether were assessed utilizing 600 *g* supernatant fractions of human embryonic livers, kidneys, lungs, adrenal glands and hearts as enzyme sources. The results of these experiments are summarized in Fig. 1. The O-debenzylation reaction was readily measurable in preparations of each of the five tissues studied and, surprisingly, embryonic cardiac tissues exhibited the highest *O*-debenzylase activities. For the O-dependentylation reaction, liver, kidney and heart tissues exhibited the highest activities while enzymes present in adrenal gland and lung catalyzed the reaction at slower, although readily measurable rates. Both *O*-deethylase and *O*-demethylase activities were generally lower than O-debenzylation and O-dependentylation except that one kidney preparation (fresh, unfrozen tissue) exhibited an exceptionally high *O*-demethylase activity. Liver, kidney and heart tissues exhibited relatively high activities for each of the four probe substrates. The lung exhibited the highest (although variable) *O*-deethylase activity but only relatively low activity for each of the other three probes. Surprisingly, the adrenal gland exhibited low (although measurable) activity with all probes except the benzyloxy ether, for which adrenal activities were higher than those of the kidney or lung but lower than those of the liver or heart. It is noteworthy that all five embryonic organs exhibited marked activity with each of the four probe substrates.

Comparisons of activities of individual human P450 isoforms with the same four probe substrates (Fig. 2) were informative but did not permit final conclusions with respect to the identities of the human embryonic P450s.

Studies with chemical inhibitors. For subsequent studies, focus was largely upon the dependentylation reaction although other reactions were also studied but less intensively. (Reasons for this focus are noted in the Discussion.) Because the O-dependentylation reaction is selectively inhibited by metyrapone and orphenadrine [33, 34] in adult hepatic tissues, we investigated the effects of these two inhibitors on the human embryonic O-dependentylation reaction. Experiments with metyrapone (Table 1) indicated that this compound was a very ineffective dependentylase inhibitor in each of the five human embryonic tissues studied. Only when inhibitor concentrations were raised to 0.5 mM and substrate concentrations were decreased to 1.0 μM (routinely used substrate concentrations were 10 μM) could statistically significant inhibition be achieved. Even then, observed inhibition was only 25–35%. Studies of higher inhibitor concentrations were impractical due to solubility considerations. In separate control experiments,

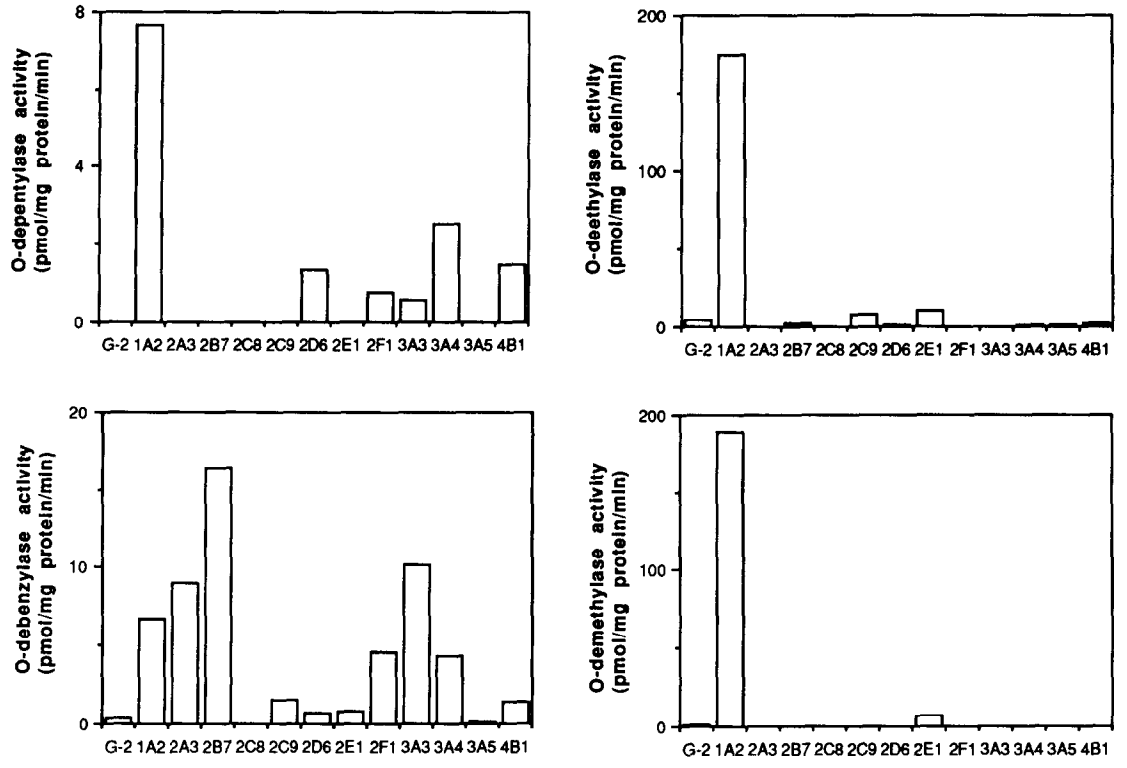

Fig. 2. Catalytic activities of individual adult human P450 isoforms expressed in HepG2 hepatoma cells (see Materials and Methods) for O-dealkylation/O-debenzylation of four phenoxazone ethers (methoxy, ethoxy, pentoxy and benzyloxy). Assays were performed using the 2000 *g* (10 min) supernatant fractions of hepatoma cell homogenates as enzyme source. Each bar is the mean of duplicate assays.

Table 1. Inhibition of human embryonic *O*-dependentylase by metyrapone and orphenadrine

Pentoxyresorufin (μM)	Inhibitor (500 μM)	*O*-Dependentylase activity* (pmol/mg protein/min)				
		Liver	Kidney	Lung	Adrenal	Heart
1.00	Control	0.27 ± 0.06	—	0.60 ± 0.06	0.24 ± 0.03	—
1.00	metyrapone	0.18 ± 0.04	—	0.39 ± 0.07	0.17	—
		(67%)†	—	(65%)	(75%)	—
2.50	Control	0.32 ± 0.03	0.33 ± 0.01	1.67 ± 0.18	0.56 ± 0.16	1.16 ± 0.15
2.50	metyrapone	0.34	0.56 ± 0.45	1.40 ± 0.06	0.59 ± 0.14	0.89 ± 0.07
		(105%)	(170%)	(84%)	(107%)	(77%)
2.50	Control	0.32 ± 0.03	0.33 ± 0.01	0.78 ± 0.04	0.56 ± 0.16	4.00 ± 0.61
2.50	Orphenadrine‡	0.33 ± 0.02	0.22 ± 0.04	0.90 ± 0.03	0.51 ± 0.01	0.34 ± 0.11
		(100%)	(67%)	(115%)	(92%)	(9%)

* Values are means ± SD, N = 3—6. All tissues were between 50 and 60 days gestational age. Enzyme sources were the 600 *g* (5 min) supernatant fractions of the homogenates of each tissue.

† Numbers in parentheses are percentages of control values.

‡ Orphenadrine was preincubated for 8 min as described in Materials and Methods.

concentrations of metyrapone as low as 1.0 μM produced 90% inhibition of dependentylase activity in phenobarbital-induced adult rat hepatic microsomes.

The inhibitory effects of orphenadrine, selective for isoforms of the CYP2B and 2C subfamilies [33, 34], are reportedly the result of "suicide" conversion of this compound to a metabolite(s) that forms an inhibitory complex with the same P450 isoform(s) that catalyzes the reaction. Thus, orphenadrine was preincubated with the enzyme source, NADPH and NADPH-regenerating system for 8 min prior to initiation of the reaction with substrate. Rates of dependentylation in experiments with hepatic, adrenal or pulmonary preparations from human embryos were not affected significantly (Table 1). However reactions catalyzed by renal preparations

Table 2. Inhibition of human embryonic *O*-depentylase or *O*-debenzylase by 7, 8-benzoflavone and carbon monoxide

Enzyme preparation*	Concentration of inhibitors (μM)		O-Debenzylation (pmol/mg protein/min)	O-Dependentylation inhibition by carbon monoxide† (%)	
Liver	Control	—	5.52	Control	—
	ANF	(10)	5.65	CO	100
Adrenal	Control	—	4.39	Control	—
	ANF	(10)	3.59	CO	100
Kidney	Control	—	1.95	Control	—
	ANF	(10)	1.55	CO	100
Lung	Control	—	1.94	Control	—
	ANF	(10)	2.21	CO	100
Heart	Control	—	7.43	Control	—
	ANF	(10)	7.98	CO	100
Adrenal	Control	—	4.03		
	TAO	(600)	6.35		
Liver	Control	—	6.18		
	TAO	(600)	6.18		

* All tissues were between 50 and 60 days gestational age. Enzyme sources were the 600 *g* (5 min) supernatant fractions of the homogenates of each tissue.

† Gas phase in control flasks contained N_2:O_2 (80:20); gas phase in experimental flasks contained CO:O_2 (80:20).

were inhibited by approximately 30–35% and reactions catalyzed by cardiac preparations exhibited 80–95% inhibition. These effects were observed at very high inhibitor concentrations (0.5 mM) and low substrate concentrations (2.5 μM). Additions of benzphetamine (0.5 mM, preincubated 8 min), retinol (0.03 mM) or ethanol (171 mM) did not inhibit significantly the rates of dependentylation in human embryonic hepatic preparations (data not shown).

The P450-dependency of the reaction was verified by demonstrating that carbon monoxide very effectively inhibited the reaction (Table 2). With gas mixtures of CO:O_2 (80:20), 100% inhibition of the *O*-dependentylation reaction was observed in each of the five tissues studied. [Control experiments were performed with N_2:O_2 (80:20)]. The inhibitory effects of ANF (selective for isoforms of the CYP1 A subfamily) and TAO (selective for isoforms of the CYP3A subfamily) on rates of O-debenzylation of benzyloxyphenoxazone were also investigated (Table 2). Members of both subfamilies will catalyze the O-debenzylation reaction [11, 13] and we reasoned that if conceptal isoforms belonging to either of those subfamilies were catalyzing the reaction, inhibitory effects should be observable. In addition, ANF is known to increase rates of reactions catalyzed by members of the 3A subfamily [35]. However, only minor effects were observed at substrate concentrations of 0.01 mM, ANF concentrations of 0.01 mM and TAO concentrations of 0.6 mM. Like orphenadrine, TAO is also a "suicide" inhibitor and experiments with TAO were conducted as described above for orphenadrine. The greatest inhibitory effects were seen with ANF on the adrenal- and renal-catalyzed reactions but were only 18 and 20%, respectively.

Studies with inhibitory antibodies. Three polyclonal antibodies were used as probes in attempts to identify potential P450 subfamilies involved in the observed oxidative biotransformation of phenoxazone ethers in human embryonic tissues (Table 3). Currently it is presumed that a polyclonal antibody raised against one P450 isoform would cross-react with other isoforms of the same subfamily. In preliminary experiments, each of the antibodies were shown to be highly effective inhibitors of P450-dependent reactions in adult rat hepatic tissues. Deethylation or demethylation of ethoxyphenoxazone and methoxyphenoxazone in microsomes from 3-methylcholanthrene-induced adult rats could be inhibited by 90–95% by anti-CYP1A1. This antibody inhibits enzymic activities of all known isoforms of the CYP1A subfamily and also inhibits methylcholanthrene-induced *O*-deethylase activity in rat conceptal tissues [15]. Dependentylation of pentoxyphenoxazone in microsomes from phenobarbital-induced adult rats could be inhibited by 85–95% by anti-CYP2B1. This antibody inhibits enzymic activities of isoforms of the CYP2B subfamily. Hydroxylation of testosterone at the 16α position in microsomes from untreated adult, male rats could be inhibited by 85-90% by anti-CYP2C11. This antibody inhibits enzymic activities of isoforms of the very large CYP2C subfamily. As indicated in Table 3, additions of these three antibodies to reaction cuvettes produced only minor, insignificant effects on the oxidative biotransformation of phenoxazone ethers in preparations containing human embryonic tissues as the enzyme source. For these experiments, embryonic organs were not separated and assays were run on pooled samples in order to obtain direct comparisons of the three antibodies.

Table 3. Antibody inhibition of O-dealkylation or O-debenzylation reactions in human embryonic tissue preparations*

Rat P450 antibodies or preimmune-IgG† (0.5–5.0 mg/mL)	Phenoxazone ether substrates (10 μM)	Enzyme activity (pmol/mg protein/min)
Control	Ethoxyphenoxazone	1.23
Preimmune-IgG	Ethoxyphenoxazone	1.13
Anti-P450IA1	Ethoxyphenoxazone	1.23
Control	Pentoxyphenoxazone	1.28
Preimmune-IgG	Pentoxyphenoxazone	1.11
Anti-P450IIB1	Pentoxyphenoxazone	1.16
Control	Pentoxyphenoxazone	1.28
Preimmune-IgG	Pentoxyphenoxazone	1.11
Anti-P450IIC11	Pentoxyphenoxazone	1.10
Control	Benzyloxyphenoxazone	1.65
Preimmune-IgG	Benzyloxyphenoxazone	1.05
Anti-P450IIC11	Benzyloxyphenoxazone	1.37

* See first footnote, Table 2.

† Antibodies and preimmune-IgG were incubated with 600 *g* supernatant fractions of combined embryonic tissue preparation for 10 min prior to initiation of reactions with NADPH.

Table 4. Effects of 7,8-benzoflavone and methanol on phenoxazone ether O-deethylation in human embryonic tissue*

		Concentration of inhibitor† (mM)	*O*-Deethylase activity (pmol/mg protein/min)
Liver	Control	—	1.15 ± 0.09
	ANF	(0.01)	1.48 ± 0.06
Adrenal	Control	—	4.47 ± 0.87
	ANF	(0.01)	6.31 ± 0.27
Kidney	Control	—	4.16 ± 0.52
	ANF	(0.01)	19.90 ± 0.72
	Methanol	(250)	14.30 ± 8.97

* See first footnote, Table 1.

† Control flasks contained no methanol. Flasks containing ANF also contained 10 μL of methanol (250 mM).

Table 5. NADH dependence of *O*-depentylase activity in human embryonic tissues*

	O-Depentylase activity (pmol/mg protein/min)		
	NADPH	NADH	NADH + CO†
Heart	3.34	4.27	1.40 ± 0.05
Kidney	2.38 ± 0.04	4.58 ± 0.33	—

* See first footnote, Table 1.

† See second footnote, Table 2.

Unusual characteristics observed. In addition to some of the striking features mentioned above, other unusual characteristics of the embryo-catalyzed O-dealkylation/debenzylation reactions appear worth noting at this point. In experiments with embryonic renal tissues (Table 4) it was noted that methanol (employed as a vehicle for ANF) produced consistent 3- to 4-fold increases in rates of O-deethylation of ethoxyphenoxazone. Only very slight increases were elicited in reactions containing preparations of other tissues as the enzyme source and no such increases were observed with ethanol on the O-debenzylation reaction. Finally, in experiments in which NADPH and NADH were directly compared as initial electron donors, it was found that NADH was slightly more effective than NADPH when cardiac or renal preparations were used as enzyme source and when pentoxyphenoxazone was used as probe substrate (Table 5). Rates of the NADH-dependent reactions were inhibited by 67% when CO was substituted for N_2 in the gas mixture as described above. This contrasted with 100% inhibition with NADPH as electron donor (Table 2).

DISCUSSION

Our results show that five prenatal human tissues (liver, heart, adrenal gland, kidney and lung) each contain P450 isoforms capable of catalyzing O-dealkylation/O-debenzylation of four phenoxazone ether probes as early as 50 days of gestational age. Use of these probes in conjunction with chemical and immunoinhibition permits preliminary ideas regarding the nature of the conceptal P450 isoforms that catalyze these reactions. It is currently believed that only four of the ten mammalian P450 families currently catalogued [3] contain isoforms capable of catalyzing xenobiotic biotransformation reactions. These are families 1, 2, 3 and 4. In family 4, no human isoforms are known to catalyze xenobiotic biotransformations. Within the other three families (families 1, 2 and 3), subfamilies 1A, 2A, 2B, 2C,

2D, 2E, 2F and 3A contain human isoforms capable of catalyzing xenobiotic biotransformation reactions. Isoforms of subfamily 1A (1A1 and 1A2) are frequently excellent catalysts for the O-deethylation of ethoxyphenoxazone and for the O-demethylation of methoxyphenoxazone and this generalization would also appear to apply to the human 1A isoforms as evidenced from data presented in these studies as well as from previous studies [36]. Lack of inhibition by ANF and anti-1A antibody, however, tends to suggest that these isoforms do not contribute significantly to the reactions observed. As indicated from Fig. 2, it would also appear unlikely that human adult isoforms from other subfamilies (subfamily 2E might be excepted) would be significant participants in the observed O-deethylation and O-demethylation reactions.

Subfamily CYP2B contains isoforms that are effective catalysts of the O-dependentylation reaction and O-dependentylation is often regarded as a diagnostic indicator of CYP2B activity. Data presented in Fig. 2 indicate that this does not appear to apply to isoform CYP2B7 (pseudogene product) even though 2B7 effectively catalyzed the O-debenzylation reaction. Neither reaction in human conceptal tissues was inhibited effectively by metyrapone or by inhibitory anti-2B antibodies, casting doubt on the participation of 2B isoforms in the observed conceptal O-dependentylation reactions. It was of interest, however, that human isoforms 1A2, 2D6, 2F1, 3A3, 3A4 and 4B1 each exhibited measurable catalysis of the O-dependentylation reaction. From the discussion above, it would seem unlikely that CYP1A isoforms participated significantly in human embryonic O-dependentylation and, from the discussion below, it would seem unlikely that isoforms of the CYP3A subfamily participated significantly in the observed O-dependentylation. Possible participation of isoforms CYP2D6, CYP2F1 [37] and CYP4B1 and/or perhaps other isoforms will require further investigation. It is of interest to note that the O-dependentylation reaction has also been observed in various tissues of the rat conceptus during early stages of gestation [15] and that, in addition to exhibiting many of the same characteristics observed with human embryonic tissues, it has been observed that cAMP is a positive regulator of the rat conceptal P450-dependent dependentylase reaction [23]. It was because of these observations that much of the focus of this study was on the embryonic dependentylation reaction.

Results reported here as well as in previous studies [31] indicate that several P450 isoforms will effectively catalyze the O-debenzylation of benzyloxyphenoxazone. This includes several members of the CYP3A subfamily (3A1, 3A3, 3A4), but CYP3A5 did not catalyze the reaction in these experiments. The capacity of CYP3A7 to catalyze the reactions investigated in this study would be of interest because the 3A7 isoform is known to be expressed in human hepatic tissues at later stages of gestation [12, 20]. The fact that TAO is a highly effective inhibitor of CYP3A-catalyzed reactions and that ANF frequently increases rates of CYP3A-catalyzed reactions [35] is of significance because neither of these agents markedly affected O-debenzylation with human embryonic tissues as the enzyme source. These observations make it appear somewhat unlikely that human embryonic isoforms of the CYP3A subfamily were contributing significantly to human embryonic O-debenzylation. This, however, has not been ruled out entirely and further studies are indicated.

Taken together, the above studies appear to argue against the significant participation of CYP subfamilies 1A, 2B, 2C or 3A in the four reactions investigated. Subfamilies 2A, 2D, 2E, 2F and 4B will require further consideration but all other subfamilies seem unlikely at present. It must be emphasized, however, that the reported results represent initial investigations into this line of research and that further studies could possibly reverse the above-described hypothetical scenario. The unavoidable problems encountered in studies with limited quantities of human embryonic tissues prevent concrete conclusions at this point in the investigations. Characteristically, the human tissues studied exhibited a very high degree of variability. This variability also fosters a conservative attitude with respect to interpretation of the observed results. Nevertheless, the results are clear indicators of a number of highly important points: human embryonic tissues express several functionally active P450 cytochromes during the sensitive period of organogenesis; these cytochromes are expressed in a variety of tissues including liver, heart, lung, kidney and adrenal gland; and the P450s expressed appear to display properties unlike those of the more commonly studied adult isoforms.

A large number of unexpected and unusual results were encountered during the course of these investigations. These are summarized as follows: (1) We did not expect to observe rapid rates of O-dependentylation in human embryonic tissues. As stated above, this reaction is usually regarded as diagnostic for CYP2B isoforms and such isoforms are not expected to be present in embryonic tissues of any mammalian species. (2) We did not expect to observe high activities in cardiac tissues for any of the substrates investigated; cardiac tissue is characteristically relatively inactive in terms of P450-dependent xenobiotic biotransformation. (3) Lack of inhibition of human hepatic O-debenzylation of benzyloxyphenoxazone by TAO was unexpected. We felt that the most likely candidate for catalysis of human hepatic embryonic O-debenzylation would be a CYP3A isoform because members of this subfamily are known to be expressed in human fetal livers [12, 20] at later stages of gestation and because members of this subfamily are known to catalyze the O-debenzylation reaction [11]. (4) We did not expect to observe marked (3- to 4-fold) increases in rates of O-deethylation in preparations containing renal homogenate as enzyme source after additions of methanol. To our knowledge, this observation is unprecedented. (5) We did not expect that NADH would serve as a more effective electron donor than NADPH for a P450-dependent reaction in human tissues. Insofar as mammalian tissues are concerned, this observation also appears to be unprecedented although certain bacterial isoforms (e.g. CYP101) exhibit preferential NADH dependence [3]. (6) We did not expect to observe orphenadrine inhibition of

cardiac embryonic O-depentylation after both metyrapone and orphenadrine had failed to inhibit the same reaction in other human embryonic tissues. (7) We did not expect to see a complete lack of inhibition of the observed enzymic activity with all three inhibitory antibodies. Antibody cross-reactivity within P450 subfamilies would make it seem likely that at least one of the antibodies should have inhibited at least one of the observed reactions in at least one of the tissues studied. (8) We expected to observe higher activities in the embryonic adrenal gland because of very high monooxygenase activities previously observed in the human fetal adrenal gland at later stages of gestation [9]. Those activities, however, were with different substrates.

In conclusion, we have found readily measurable P450-dependent xenobiotic biotransformation of four phenoxazone ether probes in human embryonic liver, lung, adrenal gland, heart and kidney as early as day 50 of gestation. The data suggest that multiple P450 isoforms are expressed in several human embryonic tissues/organs and that this prenatal expression may be sufficient to effect bioactivation of xenobiotics to which organogenesis-stage human embryos might be exposed. Such bioactivation could lead to dysmorphogenic, carcinogenic, mutagenic or cytotoxic effects. We are currently engaged in studies to further understand monooxygenase systems in human embryonic tissues.

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