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Expression of *CYP2E1* during Embryogenesis and Fetogenesis in Human Cephalic Tissues: Implications for the Fetal Alcohol Syndrome

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Reverse transcription and the polymerase chain reaction (RT-PCR) with oligonucleotide primers designed to target cDNA nucleotides 1241-1357 corresponding to exons 8 (3′ end) and 9 (5′ end) in human genomic *CYP2E1* detected consistently strong signals in 9 of 10 prenatal human brains. Cephalic tissues analyzed were between 54 and 78 days of gestation. RT-PCR signals for expression of *CYP2E1* in corresponding human hepatic or adrenal tissues were weaker or, with only 2 exceptions, undetectable. Attempts to approximate levels of P4502E1 mRNA with Northern blots and RNase protection assays indicated that levels in human prenatal whole brain tissues tended to increase as a function of gestational age but, at the early stages investigated, were far lower than the constitutive levels in hepatic tissues of adult humans or male rats. Localized, P4502E1-dependent cephalic bioactivation of ethanol, with associated generation of several reactive chemical species, could contribute significantly to the etiology of neuroembryotoxic effects of prenatal ethanol exposure. © 1997 Academic Press

The biosynthesis and function of xenobiotic-biotransforming P450 heme-thiolate proteins (Families 1-3) in cephalic tissues has been a topic of considerable interest during the past few years. Numerous investigators have reported significant levels of several P450 isoforms of Families 1-3 in adult brains of rodents and primates and the topic has been the subject of several recent reviews (1-4). As of this writing, however, no reports of expression of such P450 isoforms in *prenatal* cephalic tissues of vertebrate species have appeared in the literature to our knowledge. This gap in information is particularly notable in view of the high sensitivity of the developing prenatal brain to chemical insults and of the capacity of P450 heme-thiolate proteins to modulate the embryotoxic effects of chemicals (5-8). Insult of the developing human brain by recreational usage of ethanol represents a societal problem of especially grave concern in view of the capacity of ethanol to elicit persistent birth defects often referred to as the fetal alcohol syndrome (FAS), fetal alcohol effects (FAE) and alcohol-related birth defects (ARBDs). FAS/FAE/ARBDs are strongly associated with mental and behavioral deficits of varying degrees of severity and prenatal exposure to ethanol is now regarded as a leading known cause of childhood mental retardation in developed countries (9-11).

It is now recognized that cytochrome P4502E1 (CYP2E1) is an important enzymatic catalyst of ethanol biotransformation in adults and that this heme-thiolate protein assumes an increasingly important role in ethanol disposition as plasma/tissue levels of ethanol increase (3, 12). In adults, class I alcohol dehydrogenases, present at high levels in hepatic tissues, appear to be the most important catalysts for oxidative biotransformation of ethanol when plasma/tissue levels of ethanol are low. The alcohol dehydrogenase enzymes represent a high affinity/low capacity ethanol-biotransforming system whereas the P4502E1 enzymes represent a low affinity/high capacity system. At high blood alcohol concentrations and in chronic users, P4502E1 appears to play a substantial role in ethanol biotransformation in adults due to saturation of the dehydrogenase system at relatively low ethanol concentrations as well as to induction of P4502E1 by chronic ethanol exposure (3, 13). During prenatal life, when class I alcohol dehydrogenases are expressed at extremely low levels (11, 14), expression of P4502E1 in prenatal tissues would assume a high degree of added significance. This would be particularly true in the case of prenatal cephalic tissues which represent prime targets for the local deleterious effects of ethanol and its associated, CYP2E1-generated reactive intermediates on the de-

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veloping conceptal brain. The purpose of these investigations, therefore, was to ascertain whether and the extent to which the gene (*CYP2E1*) coding for cytochrome P4502E1 would be expressed in prenatal human cephalic tissues. Principal focus is upon the earlier stages of gestation (7-17 weeks).

MATERIALS AND METHODS

Tissue sources. Fresh or snap-frozen tissues from human embryos and fetuses were provided by the Birth Defects Research Laboratory of the University of Washington (Dept. of Pediatrics), Seattle, WA. Handling of these tissues was in accordance with the guidelines of the Human Subjects Review Committee at the same institution. After surgical procedures, (dilatation and curettage), tissues were either placed immediately into a denaturing solution (Solution D, reference 15) or snap-frozen in liquid nitrogen and were delivered to the laboratory within 3-4 hrs. Gestational ages ranged between 52-117 days as estimated from measurements of foot lengths. Tissues were then either analyzed immediately or stored under liquid nitrogen until analyzed. Rodent tissues were obtained under aseptic conditions after ether anesthesia from adult male Wistar rats and stored similarly.

Isolation/analyses of total RNA and mRNA. Total RNA was isolated from the respective tissues by the guanidinium/phenol/chloroform extraction procedure described by Chomczynski and Sacchi (15). mRNA was isolated using the Micro Fast-Track procedure described by Invitrogen (San Diego, CA) with very minor variations. Quantities of mRNA were estimated by spectrophotometry using the absorbance ratio A_{260}/A_{280}. Integrity was determined by visualization of the 18S and 28S ribosomal RNA subunits at 254 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Samples of total RNA were carefully treated with RQ1 DNase (Promega, Madison, WI) in accordance with Promega Protocols (16). Reverse transcription was performed according to described methods after mixing 4-10 μg of total RNA with oligo $dT_{12\text{-}18}$ (0.068 μg/μg total RNA), 17 units of AMV-RT (Life Sciences, Inc., St. Petersburg, FL) and DEPC-treated distilled water to a final volume of 20 μl. For the PCR reaction, sense and antisense oligonucleotide primers utilized (5′-AGCCAGAACACCTTCCTGAATGA-3′ and 5′-GAAACAACTCCATGCGAGCC-3′, respectively) were identical to those described by Farin et al. (17). The reaction was conducted with minor modifications of the described procedures (17). Thirty PCR cycles were performed with 93°C/45 s. for denaturation, 53°C/1 min for annealing and 72°C/1.5 min for elongation. Post-PCR was at 72°C/5 min, then at 4°C until collection of samples. Southern blotting was performed as described by Schatz (18).

Northern blotting. Analyses were carried out essentially as described (19, 20). Hybridizations utilized a 1.6 kb *CYP2E1* cDNA and a 1.1 kb β-actin cDNA fragment (ATCC, Rockville, MD). The plasmid pBacBAK8 containing the full length human cDNA to *CYP2E1* was generously provided by Dr. S. Nelson, University of Washington. Both probes were radiolabeled using the Ready-To-Go DNA Labeling procedure (Pharmacia Biotech, Uppsala, Sweden). Hybridization was overnight at 42°C. Membranes were successively washed with 2 × SSC/0.05% SDS for 60 min. at room temperature and with 0.1 × SSC/0.1% SDS for 20 min. at 50°C.

Ribonuclease protection assays. For usage as a specific probe in RNase protection assays, a 475 bp amplimer was synthesized, amplified, subcloned and radiolabeled as described by Carpenter et al. (21). 20-40 μg of human conceptal total RNA or 40 μg of human adult hepatic total RNA (Clontech, Palo Alto, CA) were incubated with both human CYP2E1 (250,000 cpm) and human β-actin (250,000 cpm) probes for hybridization for 16-24 hr as described in the RPA II Ribonuclease Protection Assay Kit (Ambion, Austin, TX). Hybridized fragments were concentrated by ethanol precipitation and prepared for 5% polyacrylamide/8M urea gel electrophoresis by addition of gel loading buffer and heat denaturation at 90-95°C. Radiolabeled RNA Century Marker Plus (Ambion) was coelectrophoresed at 200 V with the various samples and yeast RNA control until disappearance of the front dye. The gel was then transferred to filter paper and dried before exposure to Kodak XAR film with intensifiying screens (Fisher Scientific) for 2-10 days at −70°C.

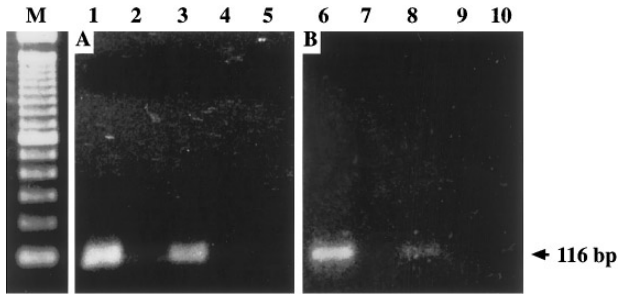

FIG. 1. RT-PCR analyses. Amplimers were generated by RT-PCR treatment of total cellular RNA obtained from human conceptal brain and liver homogenates (see Materials and Methods). 10 μl of each PCR mixture was loaded onto the gel. (A) Lane M, DNA size marker (100 bp); (1) human embryonic brain (54 days); (2) same as lane 1 but without reverse transcriptase; (3) human embryonic liver (54 days); (4) same as lane 3 but without reverse transcriptase; (5) same as lane 1 but without first strand cDNA. (B) (6) Human fetal brain (78 days); (7) same as lane 6 but without reverse transcriptase; (8) human fetal liver (78 days); (9) same as lane 8 but without reverse transcriptase; (10) same as lane 6 but without first strand cDNA. The arrow indicates the expected size (116 bp) of the amplimer.

RESULTS

Samples of ten brains, six livers and four adrenal glands from human embryos/fetuses were investigated by RT-PCR analyses for evidence of expression of *CYP2E1.* For these initial investigations, gestational ages ranged between 54 and 78 days (7 and 11 weeks) of gestation. PCR amplification of nine of the ten brain samples resulted in bands clearly visible on agarose gels. Representative samples are presented in Figure 1. In contrast, similar amplification performed on six liver samples resulted in detectable bands for only two of the livers analyzed. The two visible bands were also clearly fainter than any of the bands observed with brain tissues. RT-PCR analyses of adrenal gland tissues yielded only negative results with no detectable evidence in any sample. Further analyses thus focused on cephalic preparations. Southern blot analyses (Fig. 2) revealed signals at the expected 116 bp position and provided confirmatory evidence that *CYP2E1* is significantly expressed in human conceptal cephalic tissues as early as day 54 in gestation. In addition, a band corresponding to a coelectrophoresed full length human *CYP2E1* cDNA (1649 bp) appeared on the same blots.

Northern blotting analyses were then performed on seven different human conceptal cephalic samples. One sample consisted of three pooled brains of days 54-57

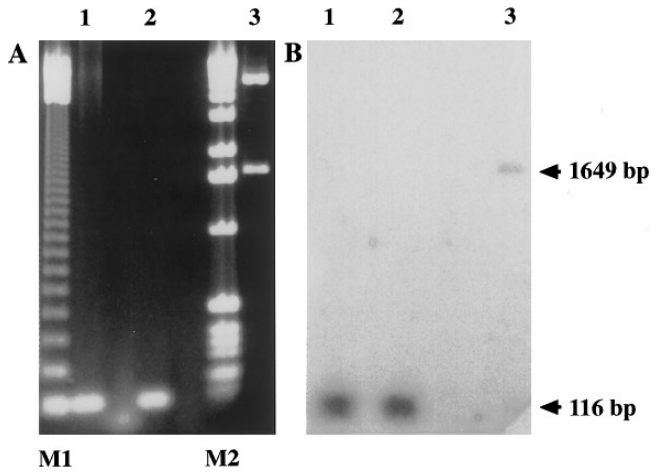

FIG. 2. RT-PCR amplimers generated by total cellular RNA from human conceptal brain homogenates and corresponding Southern blotting. (A) Lane M1, DNA size marker (100 bp); (1) human embryonic brain (54 days); (2) human fetal brain (67 days); M2, DNA size marker (1 kb); (3) full-length human *CYP2E1* cDNA (1649 bp) used as internal control. (B) Southern blotting with lanes 1, 2, and 3 corresponding to lanes 1, 2, and 3 in A. The arrows indicate the expected sizes of the amplimers and the full-length cDNA.

of gestation; the remainder of the samples consisted of single brains ranging from days 54 to 95 of gestation. Adult rat liver was utilized as a positive control in these experiments (Fig. 3). Cross-hybridization of the human and rodent forms is well established. The results indicated lack of a positive signal for any of the human conceptal brain tissues investigated with a concomitant very strong signal for the simultaneous positive control (rat liver). These results indicated that, although *CYP2E1* is expressed in human prenatal cephalic tissues, levels are sufficiently low as to be undetectable with this less sensitive method of detection.

A total of 12 prenatal human brains were analyzed for P4502E1 mRNA expression with RNase protection assays (Table 1) and representative results are illustrated in Figure 4. Signals were relatively weak during the embryonic period (days 52-58 of gestation) but increased in intensity during the fetal period and particularly at the latest stages of gestation investigated (108-117 days). Even the strongest prenatal cephalic signals, however, were considerably weaker than those observed with adult human liver samples. At 108 days of gestation, the human fetal liver exhibited an extremely faint band. β-Actin transcripts were clearly observable in all samples indicating that all samples were intact.

A summary of all results obtained with each assay system, RT-PCR, Northern blotting and RNase protection assays, is presented in Table 1. Together, the results from all modes of analysis were in good general agreement and provided good evidence for a low but consistent and probably significant level of expression of *CYP2E1* in human prenatal brain (but not liver and adrenal) tissues during late embryogenesis and early fetogenesis.

DISCUSSION

These initial investigations provide definitive evidence for the low but significant expression of *CYP2E1* in human prenatal cephalic tissues as early as 52 days (7 weeks) in gestation. A total of 32 human conceptuses were analyzed with 3 separate assay systems (RT-PCR, Northern blotting and RNase protection assays) in terms of cephalic mRNA. With RT-PCR, the assay of highest sensitivity, strong signals were readily detectable in 9 of 10 human prenatal cephalic samples. Lack of detection in one sample remains unexplained but may be due to genetic or environmental factors. This

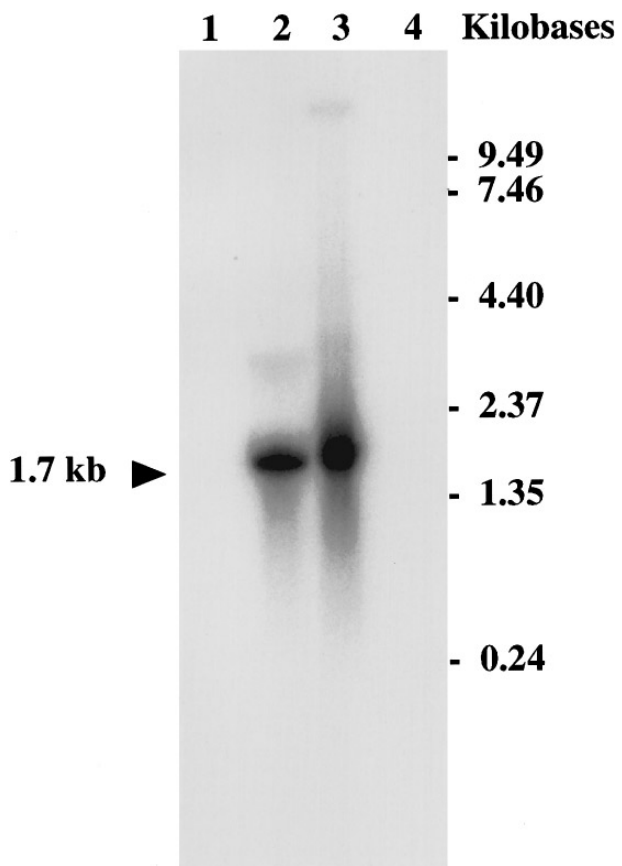

FIG. 3. Northern blot analyses of poly(A+) RNA from human conceptal brains and of total RNA from adult rat liver. Electrophoretic separation on 1.5% agarose gels containing 0.66M formaldehyde and transfer to Hybond-N+ membranes. Hybridization was with full length *CYP2E1* cDNA probe and autoradiographic exposure with intensifying screens was for 1 week at −70°C. (1) Human embryonic brain (54 days); (2) adult rat liver (rat 1); (3) adult rat liver (rat 2); (4) human fetal brain (95 days). Arrowhead indicates the expected position (1.7 kb) of the *CYP2E1* signal. RNA marker sizes (0.24-9.49 kb) are labeled.

TABLE 1

Detection of Hepatic and Cephalic Expression of CYP2E1 mRNA in Prenatal Human Tissues

Analyses	Developmental stage	Species	Tissue	
			Brain	Liver
RT-PCR	54 (GD)	Human	+++	++
	54 (GD)	Human	+++	−
	54 (GD)	Human	+++	ND
	59 (GD)	Human	+++	+/−
	59 (GD)	Human	+++	ND
	67 (GD)	Human	+++	ND
	67 (GD)	Human	+++	ND
	74 (GD)	Human	+++	−
	76 (GD)	Human	−	−
	78 (GD)	Human	+++	+
Northern blot	4 × 54 (GD)[a]	Human	−	ND
	54/57 (GD)[b]	Human	−	ND
	55 (GD)	Human	−	ND
	57 (GD)	Human	−	ND
	70 (GD)	Human	−	ND
	83 (GD)	Human	−	ND
	88 (GD)	Human	−	ND
	95 (GD)	Human	−	ND
	7 × adult[c]	Rat	ND	++++
RNase protection assay	52 (GD)	Human	+/−	ND
	52/53 (GD)[d]	Human	+	ND
	57 (GD)	Human	+	ND
	58 (GD)	Human	+/−	ND
	67 (GD)	Human	+	ND
	103 (GD)	Human	+	ND
	104 (GD)	Human	+	ND
	104 (GD)	Human	+	ND
	108 (GD)	Human	++	ND
	108 (GD)	Human	++	+/−
	117 (GD)	Human	++	ND
	Adult	Human	ND	++++

Note. Relative intensity of observed signals: ++++, very strong; +++, strong; ++, good; +, detectable; +/−, questionably detectable; −, not detectable; ND, not determined.

[a] 4 samples at day 54 of gestation were analyzed separately. Signals were not detectable (−) in each case.

[b] A pool of 3 samples at days 54-57 was analyzed.

[c] 7 adult rat samples (used as positive controls in each experiment) were analyzed separately. All exhibited very strong (++++) signals.

[d] A pool of 2 samples at days 52 and 53 was analyzed.

will require a more extensive investigation. With Northern blotting, the least sensitive of the three assay systems, signals were undetectable in all of the samples studied. The longest gestational age investigated was 95 days and, retrospectively, it seems possible that Northern signals might be detected at later gestational stages. This, too, will require additional investigation. With the RNase protection assay system (intermediate sensitivity), signals were detected definitively in 9 of 11 samples and were questionable in only two samples from the earliest stages investigated -- 52 and 57 days of embryonic gestation. Three samples from the later fetal stages of gestation (108-117 days) exhibited relatively strong signals. Together, the data left the impression that levels would increase as a function of increasing gestational age. More extensive investigations will be required to fully confirm this finding.

The discovery that significant levels of CYP2E1 mRNA can be detected in human prenatal cephalic tissues has important implications in terms of the prenatal exposure of human conceptuses to chemicals that are substrates for CYP2E1. Numerous (>70) low molecular weight xenobiotic chemicals (enumerated in reference 3) are now recognized CYP2E1 substrates and CYP2E1 will catalyze the conversion of many of these substrates to toxic reactive intermediary metabolites (1-4). Ethanol is a prime example because of the well-established and widely known prenatal toxicity resulting from prenatal human exposure to this heavily used/abused recreational chemical (9, 10). As a substrate for CYP2E1, ethanol is converted to acetaldehyde and to hydroxyethyl radical, both of which are highly toxic chemicals capable of covalent binding to tissue macromolecules and causing extensive tissue damage (3, 13, 22, 23). In addition, protein adducts of each will act as haptens involved in autoimmune reactions (3, 13, 24). Furthermore, substrate-bound, ferrous CYP2E1 is notorious for its capability to reduce molecular oxygen to superoxide anion (1-4, 25) with subsequent generation of several additional reactive oxygen species (ROS). ROS are capable of causing tissue damage via a variety of mechanisms, including initiation of lipid peroxidation with resultant cytotoxicity and generation of lipid hydroperoxides (13, 25). CYP2E1 also catalyzes the conversion of lipid hydroperoxides to highly toxic aldehydes including 4-hydroxynonenal and malondialdehyde (3, 13, 25). Thus, the

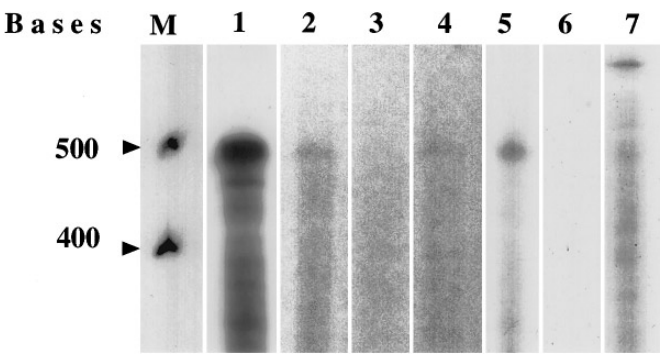

FIG. 4. RNase protection assays. Protected RNA fragments were electrophoretically resolved on denaturing polyacrylamide gels and exposed to autoradiographic film for 2-10 days. Expected band size for the *CYP2E1* hybrid is approximately 500 bp and for β-actin approximately 300 bp. Lane M, RNA markers; (1) human adult liver (20 μg loaded, 2 days exposure); (2) human embryonic brains (52 and 53 days gestation, pooled; 40 μg loaded, 10 days exposure); (3) human embryonic brain (57 days gestation, 20 μg RNA, 10 days exposure); (4) human fetal brain (67 days gestation, 40 μg RNA, 10 days exposure); (5) human fetal brain (108 days gestation, 40 μg loaded, 10 days exposure); (6) yeast with RNase treatment; (7) same as 6 but without RNase treatment.

possibilities for prenatal human cephalic CYP2E1 to mediate prenatal brain damage are numerous. The extent to which this hemoprotein actually participates in the elicitation of FAS/FAE/ARBDs, however, will require extensive future investigations.

These potentially highly important findings also leave open a large number of other questions that remain to be answered in subsequent investigations. These include questions of tissue/cellular localization of CYP2E1 in human prenatal cephalic tissues, the relative role of CYP2E1 vs. alcohol dehydrogenases and catalase (26, 27) in the biotransformation and bioactivation of ethanol in human prenatal brain tissues, rigorous quantitation of CYP2E1 protein and enzymic activity in human prenatal cephalic tissues, regulation (particularly with respect to inducibility) of prenatal cephalic CYP2E1 and the extent to which experimental animals may be used as models for studies of expression and regulation of expression of *CYP2E1* in the prenatal brain. Clearly, a huge amount of research remains to be accomplished in this area.

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