0026-895X/96/020260-09$3.00/0

MOLECULAR PHARMACOLOGY, 49:260–268 (1996).

Expression, Induction, and Catalytic Activity of the Ethanol-Inducible Cytochrome P450 (CYP2E1) in Human Fetal Liver and Hepatocytes

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Received July 25, 1995; Accepted October 26, 1995

SUMMARY

The mechanisms responsible for ethanol-mediated teratogenesis have not been resolved. However, possible etiologies include the local formation of the teratogen acetaldehyde or oxygen radicals by fetal ethanol-oxidizing enzymes. As alcohol dehydrogenases are expressed at very low concentrations in human embryonic tissues, the ethanol-inducible P450 enzyme, CYP2E1, could be the sole catalyst of fetal ethanol oxidation. With this in mind, we examined the expression of this P450 in liver samples from fetuses ranging in gestational age from 16 to 24 weeks. Immunoblot analysis of fetal liver microsomes revealed the presence of a protein immunoreactive with CYP2E1 antibodies that exhibited a slightly lower molecular weight than that found in adult liver samples. Embryonic CYP2E1 expression was further confirmed by the reverse transcriptase reaction with RNA from a 19-week gestational fetal liver used as template. Catalytic capabilities of human fetal microsomes were assessed by measurement of the rate of ethanol oxidation to acetaldehyde, which were 12–27% of those exhibited by adult liver microsomes. Immunoinhibition studies with CYP2E1 antibodies revealed that the corresponding antigen was the major catalyst of this reaction in both fetal and adult tissues. We then assessed whether embryonic CYP2E1 was, like the adult enzyme, inducible by xenobiotics. Treatment of primary fetal hepatocyte cultures with either ethanol or clofibrate demonstrated a 2-fold increase in CYP2E1 levels compared with untreated cells. Collectively, our results indicate that CYP2E1 is present in human fetal liver, that the enzyme is functionally similar to CYP2E1 from adults, and that fetal hepatocyte CYP2E1 is inducible in culture by xenobiotics, including ethanol. Because fetal CYP2E1 mediates ethanol metabolism, the enzyme may play a pivotal role in the local production of acetaldehyde and free radicals, both of which have potential deleterious effects on the developing fetus.

The human fetus is susceptible to teratogenesis from maternal exposure to a variety of foreign chemicals. Many drugs are known to produce morphological and/or neurological abnormalities in the fetus. Examples of some well known drug teratogens include phenytoin (1), thalidomide (2), and valproic acid (3). Among the most prevalent of teratogenic disorders are those associated with the maternal consumption of ethanol, which results in a broad spectrum of embryonic abnormalities called FAE and FAS (4). Resulting harmful effects range from gross morphological defects to more subtle cognitive/behavioral dysfunctions. Also, central nervous system abnormalities may occur, such as mental deficiency or developmental delay. Prenatal alcohol exposure is one of the leading known causes of mental retardation in developed countries (5).

A great deal of research has focused on characterizing the adverse effects of *in utero* alcohol exposure, with the major emphasis being on morphological and neurological damage. Now that the teratogenic actions of alcohol are firmly established, the major focus of investigations has been on the underlying mechanisms of FAE/FAS (4). An understanding of these mechanisms is essential for treatment and holds promise for devising effective preventive and/or intervention measures for FAE/FAS. One mechanism underlying alcohol- and chemical-promoted teratogenesis may involve the transfer of xenobiotics across the placenta and their subsequent

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This work was supported by Department of Health and Human Services Grants AA08990 (J.L.R.) and AA07842 (J.M.L.) and Liver Transplant Procurement and Distribution System, National Institutes of Health Grant N01-DK62274.

This work was presented in partial fulfillment of the requirements for a doctoral degree in toxicology at the University of New Mexico (S.P.C.).

ABBREVIATIONS: CYP or P450, cytochrome P450; AcA, acetaldehyde; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; bp, base pair(s); FAE, fetal alcohol effects; FAS, fetal alcohol syndrome; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/tween-20; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G.

metabolic activation by the fetus. Fetal P450 enzymes could promote conversion of alcohol and other agents to reactive metabolites that can bind to tissue macromolecules and produce cytotoxicity. Although P450-mediated bioactivation can also occur in the mother, the extensive reactivity and lack of stability of most reactive intermediates preclude their transport across the placental barrier into the fetus. Thus, the presence of P450 enzymes in fetal tissues may prove to be detrimental under conditions where mothers are exposed to drugs, alcohol, or other chemicals.

A limited number of drug-metabolizing P450 enzymes have been identified in the human fetus (6), with the major constituents being members of the CYP3A subfamily, CYP3A5 and CYP3A7 (7, 8). The adult counterpart, mainly CYP3A4, is considered to be a primary catalyst in the oxidation of numerous therapeutic agents. Because of structural and catalytic similarities between adult and fetal CYP3A enzymes, the fetal forms may also be responsible for a number of drug oxidations. Convincing evidence for the presence of other P450 enzymes in the fetus has been somewhat limited, especially when one considers that numerous and diverse compounds are metabolized by human fetal hepatic microsomes (9).

CYP2E1 is considered to be toxicologically important because of its ability to convert a variety of agents, including drugs, [acetaminophen (10)], solvents [carbon tetrachloride (11)], and environmental procarcinogens [*N*-nitrosodimethylamine (12)] to reactive intermediates that can elicit organ damage and/or tumorigenesis (13). As all of these compounds are metabolized by human fetal liver microsomes (6), CYP2E1 may indeed be expressed in the fetal liver. In addition, CYP2E1 has proved to be the most effective catalyst of ethanol oxidation among the human P450 enzymes examined to date (14). The capacity of fetal CYP2E1 to also metabolize ethanol could have serious consequences for the developing fetus. First, the primary metabolite produced during ethanol oxidation is AcA, which can elicit tissue damage via protein adduct formation (15) and may cause chromosomal damage, as it has in *Saccharomyces cerevisiae* (16). AcA is considered to be one of the possible etiologic agents in FAE/FAS. CYP2E1 is also responsible for the formation of reactive oxygen species generated during metabolism of alcohol (17). These activated forms of oxygen may play an important role in the peroxidation of cell membrane lipids (18).

In the present report, we demonstrate that human fetal liver contains CYP2E1 and that the P450 enzyme is inducible by treatment of hepatocytes with alcohol and other xenobiotics. Furthermore, considerable ethanol metabolism occurs in fetal liver microsomes, suggesting that CYP2E1 plays a role in xenobiotic activation in the human fetus. Our findings may have an impact on the understanding of mechanisms involved in alcohol-mediated teratogenesis.

Materials and Methods

Tissue acquisition. Human fetal liver samples were obtained from either the Anatomic Gift Foundation (Baltimore, MD) or the Center Laboratory for Human Embryology (Seattle, WA) and were shipped overnight on wet or dry ice. Adult liver samples (>15 years of age) were obtained from Liver Transplant Procurement and Distribution System. Those samples were shipped on dry ice and stored at −70° until use, whereas those shipped on wet ice were immediately used for the isolation of hepatocytes.

Immunoblot analysis. Microsomes were prepared from liver samples thawed before use according to a previously described method (19). Protein concentrations were determined as described (20), with bovine albumin used as a standard. Microsomal proteins were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose filters (10). Filters were subsequently blocked in 5% nonfat dry milk/TBST (20 mM Tris buffer, pH 7.6, 137 mm NaCl, and 0.1% Tween-20) for 1 hr at 37° and allowed to react overnight with a previously characterized anti-human CYP2E1 IgG (1 μg/ml) (Ref. 21 and references therein) or anti-human CYP3A4 (2 μg/ml) in TBST at room temperature. Filters were then incubated for 60 min with biotinylated goat anti-rabbit IgG (1:2000 dilution in TBST), followed by a 60-min incubation with streptavidin-conjugated horseradish peroxidase (1:2000 in TBST) at room temperature. Immunochemical staining was performed by reacting the filters with 10 ml of ECL detection reagents (Amersham, Arlington Heights, IL) for 1 min at room temperature and exposure to Amersham Hyperfilm for 10–30 sec. Immunoreactive CYP2E1 content was determined from the developed film by scanning laser densitometry. In preliminary experiments, various amounts of microsomal protein (0.5–20 μg) were applied to SDS-PAGE to determine the linearity of immunochemical staining. The concentration of microsomal protein (1–10 μg) used for all subsequent immunoblot analyses was within the linear portion of that curve.

Reverse transcriptase/polymerase chain reaction. Total RNA from fetal and adult liver samples was isolated by a previously described procedure (22) and quantified by measuring its absorbance at 260 nm; purity was assessed by determining the 260 nm/280 nm ratio. First-strand cDNA synthesis was performed on RNA samples isolated from a fetal liver at 19 weeks' gestation and from an adult liver sample. Briefly, the reaction mixture contained total RNA (5 μg), transcription buffer (20 mM Tris·HCl, pH 8.4, 50 mM KCl), 0.5 μg oligo$(dT)_{12-18}$ primer, 0.5 mM dNTPs, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, and 200 units M-MLV reverse transcriptase (Invitrogen, San Diego, CA). After incubation at 42° for 60 min, the cDNA was amplified using oligonucleotide primers that were 21 bp in length and flanked *CYP2E1* exons 4 (between bp 501–523) and 6 (between bp 954–976) (21). The amplification reactions consisted of both forward and reverse primers (1 μM each), 2 μl of previously synthesized first-strand cDNA, 200 μM dNTPs, polymerase chain reaction buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$), and 2.5 U AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT). Amplification conditions consisted of 5 min at 94° (1 cycle), 1 min at 94°, 1 min at 37°, 1 min at 72° (30 cycles), and 5 min at 72° (1 cycle). The result of amplification with these primers was a cDNA of 475 bp, which was verified by agarose gel electrophoresis. The adult and fetal amplimers were then ligated into the pCRII vector (Invitrogen, San Diego, CA) and transformed into competent (INVaF′) *Escherichia coli* cells. Ultrapure plasmid DNA was isolated from the *E. coli* transformants and sequenced using the dideoxy-chain termination method (23) and Sequenase (US Biochemicals, Cleveland, OH). Sequences were determined to have 100% identity with the same portion of adult *CYP2E1* cDNA (24).

RNase protection assays. The human fetal *CYP2E1* cDNA was used to generate a specific RNA probe as follows. The DNA was incubated at 37° for 60 min with the restriction endonuclease *Hin*dIII, which linearized the DNA but left intact the T7 promoter and the cDNA insert. T7 DNA polymerase (10 U) was then used to produce a radiolabeled RNA probe in a reaction (20 μl final volume) containing transcription buffer (Ambion, Austin, TX), 10 mM dithiothreitol, 1.5 mM each dNTPs, 1 μl RNase inhibitor (Ambion), 1 μg linearized template, and 3 μM [α-^{32}P]CTP. The incubation was allowed to proceed for 60 min at 37°. For use as an internal standard, a human β-actin cDNA (301 bp) was transcribed in the same manner as the *CYP2E1* cDNA. DNA template remaining after RNA transcription reactions was digested by incubation for 15 min at 37° with RNase-free DNase. The RNA probe was then gel-purified by 8 M urea/5% polyacrylamide gel. The band was excised from the gel and

eluted in 500 μl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS) overnight at 37°.

After synthesis of the radiolabeled RNA, 25 μg of total RNA from fetal livers ranging in gestational age from 10 to 24 weeks and 5 μg of RNA from adult liver were incubated with both the CYP2E1 (9×10^4 dpm) and β-actin probe (9×10^2 dpm) overnight at 42° in hybridization buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA). Nonhybridized material was then digested using 200 μl of a 1:100 RNase A/RNaseT1 (Ambion) mixture for 15 min at 37°. The protected fragments were then precipitated in ethanol at −20°, suspended in loading buffer, and subjected to electrophoresis on an 8 M urea/5% polyacrylamide gel. After electrophoresis, the gel was dried onto paper and exposed overnight to X-OMAT autoradiography film with intensifying screens at −70°. The protected sequence signal intensities were quantified using scanning laser densitometry.

Ethanol oxidation. Ethanol oxidase assays were performed as described elsewhere (25). Reaction mixtures contained 0.75 mg of microsomal protein, 50 mM ethanol, 100 mM potassium phosphate buffer, pH 7.4, 1 mM azide, 0.5 mM desferrioxamine, and 0.5 mM NADPH in a final volume of 1.0 ml. For immunoinhibition assays, incubation mixtures also contained 0.5 mg total IgG, and microsomes were preincubated with the various anti-P450 IgGs for 3 min at 37° before initiation of the reaction. Rates of microsomal ethanol oxidation were determined from standard curves constructed using known amounts of acetaldehyde (25). Under these conditions, rates of acetaldehyde formation by fetal and adult liver microsomes were linear with regard to time and protein concentration.

Isolation and culturing of fetal hepatocytes. Fetal liver samples that had been shipped overnight on wet ice were used to isolate hepatocytes (26). Briefly, fetal livers were minced and washed by incubation in PBS, pH 7.4, three times for 10 min each at 37° in a shaking water bath. The tissue was then washed two more times in the same manner using PBS containing 0.5 mM EGTA. After the final wash, livers were digested by incubation for 20–30 min during shaking at 37° in Hanks' buffered salt solution containing 5 mM $CaCl_2$, 0.05% (w/v) collagenase H/dispase, 0.1% (w/v) hyaluronidase, and 0.03% DNase I. After incubation, any remaining undigested tissue was disrupted by mechanical dispersion through a large-bore pipet. The dispersed cells were then filtered through a sterile 100-μm mesh screen and isolated by centrifugation for 4 min at $50 \times g$. The cell pellet was washed once with heat-inactivated FBS and twice with Williams' E Media containing 5 mM HEPES, 5 mg/l hydrocortisone, 2 mg/l insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. A 10-μl aliquot of cells was suspended in 90 μl of Trypan blue solution for assessment of cell number and viability.

After isolation, hepatocytes were suspended in Williams' E Media, pH 7.5, and 10% FBS at a concentration of $\sim 2.5 \times 10^5$ cells/ml, and 10-ml aliquots were placed into 150-mm culture dishes and incubated at 37° in an atmosphere of 95% air/5% CO_2. Cells used to perform immunocytochemical analysis were suspended in Williams' E Media/10% FBS and plated at a density of 50,000 cells/well on two-well chamber slides. After 15 hr, the medium was changed to remove cellular debris, and xenobiotic exposure was initiated. Cells were treated with either 100 mM ethanol, 1 μM rifampicin dissolved in DMSO, 1 mM clofibrate dissolved in DMSO, or DMSO alone. DMSO concentrations in the culture media did not exceed 0.1%. Cells plated onto 150-mm dishes were harvested by scraping at 0, 6, 12, and 24 hr after treatment, and microsomes were prepared as described above except for the addition of sonication (3×20 sec) before the initial centrifugation. Protein concentrations were assessed (20), and P450 reductase activity was determined on microsomes prepared from cells through monitoring of the rate of cytochrome *c* reduction at 550 nm in 0.3 M potassium phosphate, pH 7.7, buffer at 30°. The 1.0-ml incubation mixtures contained 20 μg of microsomal protein and 5 mg/ml cytochrome *c*. Microsomes were then used for immunoblot analyses to assess CYP2E1 or CYP3A7 content.

Immunocytochemistry. Cells plated for immunocytochemical analysis were fixed 6 hr after xenobiotic treatment by rinsing the chamber slides 2×5 min in PBS, followed by exposure to 3.7% formaldehyde/PBS for 10 min at 25°. After fixation, slides were rinsed 3×5 min at 25°, permeabilized in acetone for 5 min at −20°, and allowed to air dry. The fixed cells were then blocked in 2.0% nonfat dry milk/PBS for 1 hr at 37°, followed by incubation overnight at 4° with 2 μg/ml anti-human CYP2E1 IgG/0.2% nonfat dry milk/PBS. Goat anti-rabbit IgG fluorescein isothiocyanate conjugate (Sigma Chemical Co., St. Louis, MO) at a 1:32 dilution in 0.2% nonfat dry milk, 10% normal human serum, and PBS was then added for 30 min at 37°. Slides were counterstained with 20 μl of a 1:2000 dilution of propidium iodide in Vectashield mounting media (Vector Laboratories, Burlingame, CA), and coverslips were applied. Vectabond reagent (Vector Laboratories) was added to secure the tissue to the slide. Visualization of slides for fluorescence was by microscopy at 40× magnification with an Olympus microscope interfaced to an Insight-IQ image processor (Meridian Instruments, Okemos, MI). Micrographs were taken using an Insight Plus Automatic Photomicrographic System and Kodak 1600 ASA slide film.

Statistical analysis. Data were statistically analyzed using linear regression analysis, analysis of variance, or Student's *t* test. Statistical significance was set at $p < 0.05$.

Results

CYP2E1 expression in human fetal liver. Human fetal liver samples were obtained from fetuses ranging in gestational ages from 10 to 24 weeks. Fetuses included in these studies were from mothers who did not have a history of alcohol abuse. Hepatic microsomes prepared from fetuses at later gestational ages, i.e., 16–24 weeks, were subjected to SDS-PAGE followed by immunoblot analysis. Anti-human CYP2E1 IgG recognized a single protein in microsomes from adult (>15 years of age) and fetal liver (Fig. 1). This immunoreactive fetal protein exhibited a slightly lower molecular weight than that of the corresponding protein from an adult liver sample. Densitometric quantification revealed a 5-fold greater staining (per μg of microsomal protein) of microsomes from adult liver compared with that of fetal liver microsomes. Furthermore, CYP2E1 was expressed at various levels among the liver samples. Interestingly, at the ages examined here, gestation period did not appear to influence the content of CYP2E1 in fetal liver.

To verify that CYP2E1 was present in fetal liver and that anti-CYP2E1 IgG was not cross-reacting with another micro-

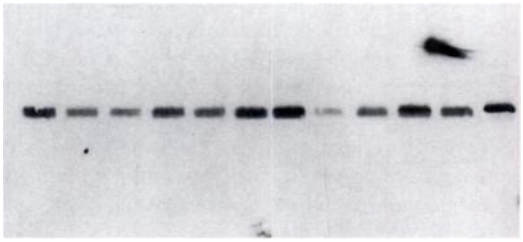


Fig. 1. Immunoblot analysis of CYP2E1 content in microsomes from human fetal liver. Microsomal proteins (5 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose filter. CYP2E1 was localized on the filter using the immunochemical staining procedure described in Materials and Methods. The filter was subsequently reacted with anti-CYP2E1 IgG, and reactivity was detected by chemiluminescence. *Lanes 1–11*, microsomes from liver samples of fetuses at 16, 19, 19, 20, 20.5, 21, 22, 22, 23, 24, and 24 weeks' gestation, respectively. *Lane 12*, microsomes from a representative adult liver sample (1 μg).

somal protein, we performed reverse transcription/polymerase chain reaction. Total liver RNA isolated from a fetus at 19 weeks' gestation and from an adult liver sample were used as templates in a reverse transcriptase reaction to generate cDNA. These liver cDNAs were then mixed with specific primers and amplified 30 cycles by polymerase chain reaction. Amplification yielded a 475-bp fragment from fetal liver RNA that was similar in size to that fragment amplified from adult hepatic RNA (Fig. 2). Sequence analysis revealed that both amplimers were 100% homologous to the corresponding region of *CYP2E1* cDNA previously described by Song *et al.* (24). We then used the fetal *CYP2E1* cDNA as a template to generate a RNA probe for ribonuclease protection assays. Total RNA samples prepared from fetal liver of 19, 23, and 24 weeks' gestation and those at 10 weeks' gestation were examined for CYP2E1 mRNA expression. CYP2E1 transcripts were found in the former samples and in adult liver but were not detectable in 10-week gestation liver samples and were absent from yeast RNA (Fig. 3). β-Actin transcripts were found in all of the liver samples, indicating that the RNA from the 10-week gestation samples was intact.

Catalytic activity of human fetal liver microsomes. We examined the ability of fetal liver microsomes to metabolize a known CYP2E1 substrate, i.e., ethanol (Table 1). For comparison, we also assessed ethanol oxidation by microsomes from adult liver. Adult hepatic microsomes catalyzed the conversion of ethanol to AcA at rates averaging 0.80 nmol AcA/min/mg microsomal protein. These rates were similar to those reported previously (27), where rates ranged from 1.03 to 2.87 nmol AcA/min/mg microsomal protein for seven separate hepatic microsomal samples. In contrast, fetal liver microsomes metabolized ethanol to AcA at rates ranging from 0.10 to 0.22 nmol product/min/mg microsomal protein, rates that were 12–27% of those observed for the adult liver samples examined here.

The role of CYP2E1 in ethanol oxidation by fetal liver was assessed in immunoinhibition studies (Table 2). In adult liver microsomes, anti-CYP2E1, at a concentration of 10 mg IgG/nmol P450, inhibited this reaction by 90%. Similarly, anti-CYP2E1 IgG at the same concentration inhibited ethanol oxidation by fetal liver microsomes by >70%. In contrast, anti-CYP2C9 and anti-CYP3A4 antibodies had little effect on total microsomal ethanol oxidation. These results suggest

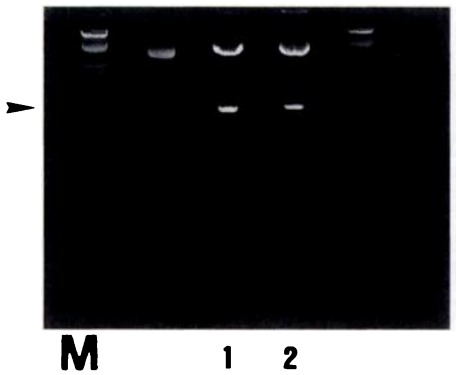


Fig. 2. Comparison of CYP2E1 cDNAs prepared from human fetal and adult liver. First-strand cDNA was synthesized by reverse transcription from total RNA isolated from a fetal liver sample (19 weeks' gestation) and from an adult specimen. Specific oligonucleotide primers were used to amplify by polymerase chain reaction a 475-bp region from the fetal and adult cDNAs as described in Materials and Methods. The amplimers from both adult and fetal liver were cloned into the pCRII vector, isolated by restriction endonuclease digestion, and subjected to agarose gel electrophoresis. *Lane 1*, CYP2E1 polymerase chain reaction product from adult liver. *Lane 2*, 475-bp product from fetal liver.

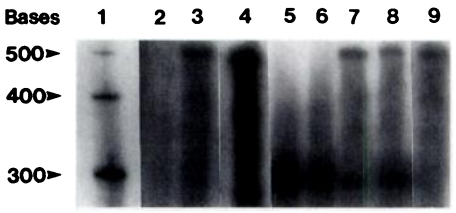


Fig. 3. Ribonuclease protection assay. Human fetal CYP2E1 and β-actin RNA probes, prepared as described under Materials and Methods, were hybridized to 5 and 25 μg of total RNA derived from adult and fetal liver samples, respectively. The protected RNA fragments were resolved on a 5% denaturing polyacrylamide gel and exposed to autoradiography film with intensifying screens for 48 hr at −70°. Bands migrating at 500 bp represent CYP2E1 mRNA sequences, whereas those migrating at 300 bp represent β-actin mRNA sequences. *Lane 1*, RNA standards of 300, 400, and 500 bp (*bottom* to *top*); *lane 2*, yeast RNA; *lane 3*, CYP2E1 RNA probe alone; *lane 4*, RNA from adult liver; *lanes 5 and 6*, RNA from fetal livers (10 weeks' gestation); and *lanes 7–9*, RNA from fetal livers at 19, 23, and 24 weeks' gestation, respectively.

TABLE 1

Ethanol oxidation by adult and fetal human liver microsomes

Reaction mixtures (1.0 ml) contained 0.75 mg liver microsomes, 50 mM ethanol, 0.5 mM NADPH, 100 mM potassium phosphate buffer, (pH 7.4, 1 mM azide, and 0.5 mM desferrioxamine. Reactions were initiated with NADPH and stopped after 45 min at 37°, and acetaldehyde formation was then assessed by head-space gas chromatography. Values are the average of at least three determinations.

Sample	Ethanol oxidation
	nmol AcA formed/min/mg protein
Adult	
UC8926	0.624
021389/2	0.984
Fetal	
34385 (19 weeks)	0.210
33954 (20 weeks)	0.177
35468 (22 weeks)	0.195
25544 (23 weeks)	0.100
34326 (24 weeks)	0.216
35417 (24 weeks)	0.121

TABLE 2

Effect of human P450 antibodies on ethanol oxidation by human fetal and adult liver microsomes

Incubation mixtures contained 1.0 mg liver microsomes, 100 mM potassium phosphate buffer, pH 7.4, 50 mM ethanol, 1 mM azide, 0.5 mM desferrioxamine, 0.5 mM NADPH, and 0.5 mg of total IgG protein in a final volume of 1.0 ml. Microsomes were preincubated with the various IgGs for 3 min at 37° and then 10 min at ambient temperature. After the addition of remaining components, reactions were initiated with NADPH and were terminated after 45 min at 37°c. Acetaldehyde formation was then assessed by head-space gas chromatography.

Sample	Antibody[a]	Ethanol oxidation [b]	Inhibiton
		nmol AcA formed/min/mg protein	%
Adult: UC8926	Preimmune	0.948	
	Anti-CYP2E1	0.093[c]	90
Fetal: 32385	Preimmune	0.261	
	Anti-CYP2C9	0.206	21.1
	Anti-CYP3A4	0.260	0
	Anti-CYP2E1	<0.08[c]	>70

[a] Ratio of IgG/P450 used was 10 mg/nmol P450.
[b] Values are expressed as nmol AcA formed/min/mg microsomal protein and are the average of at least three separate determinations.
[c] Limit of detection. A small AcA peak (greater than blank) was present on the chromatogram; however, the area could not be integrated.

that similar to the adult, CYP2E1 in fetal liver is the predominant ethanol-metabolizing P450 enzyme.

Regulation of CYP2E1 expression by xenobiotics. The induction of human fetal CYP2E1 by xenobiotics was assessed *in vitro* in a cell culture system. Fetal livers were perfused with collagenase and hepatocytes isolated. Typically, cell yields were 5×10^8 cells/liver, and viability, assessed by Trypan blue exclusion, was >90%.

Before examining CYP2E1 inducibility in culture, we assessed the stability of the enzyme by determining microsomal CYP2E1 content in hepatocytes collected at various times in culture. On plating, hepatocytes were allowed to attach for 15 hr, and the medium was changed. Cells were harvested at various times thereafter, including 0, 3, 6, 12, and 24 hr. Microsomes were then prepared from the cell homogenates and subjected to Western blot analysis with anti-CYP2E1. Immunoreactive CYP2E1 was found to be stable up to 12 hr (Fig. 4). At 24 hr in culture, there was a slight decrease in enzyme content, but this decrease was not statistically significant, indicating that CYP2E1 levels were stable for up to 24 hr in culture after an initial 15-hr attachment period. It is important to note that CYP2E1 in cultured hepatocytes did not differ significantly from that of freshly isolated unplated cells at any of the time points examined.

Xenobiotic induction of CYP2E1 in cultured hepatocytes was examined by treating the cells from 24-week-gestation fetuses with a known CYP2E1 inducer, ethanol. The dose required to produce maximal CYP2E1 induction was determined by treating hepatocytes with ethanol, 25, 50, 100, and 200 mM. The concentration of ethanol that elicited optimal induction (2-fold) was found to be 100 mM (data not shown). In addition, the length of ethanol exposure required to induce hepatocyte CYP2E1 was determined to be 6 hr. After the 15-hr initial plating period, cells were exposed to ethanol for 0, 3, 6, 12, or 24 hr. Maximal induction (2.2-fold) occurred when cells were exposed to ethanol for 6 hr (Fig. 5). The lack

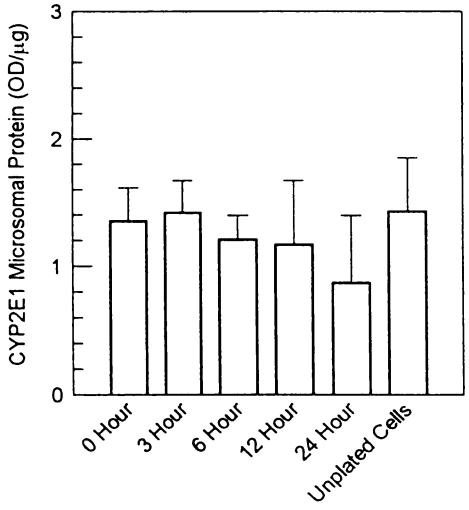


Fig. 4. Stability of CYP2E1 in cultured human fetal hepatocytes. Hepatocytes were isolated from fetal liver samples and cultured in Williams' E Media fortified with 10% FBS. The medium was replaced 15 hr after plating, and cells were harvested at 0, 3, 6, 12, and 24 hr thereafter. Microsomes were prepared from cellular homogenates and subjected to anti-CYP2E1 immunoblot analysis. *Bars,* mean ± standard error of hepatocytes obtained from three separate liver samples of 24 weeks' gestation fetuses.

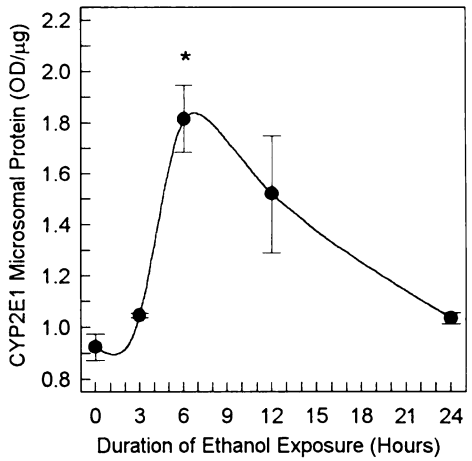
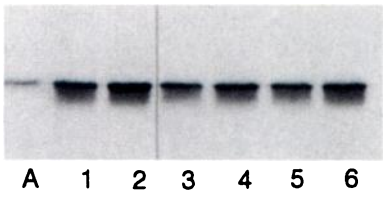


Fig. 5. Effects of ethanol exposure time on CYP2E1 content in cultured human fetal hepatocytes. Cultures of fetal hepatocytes were exposed to 100 mM ethanol for 0, 3, 6, 12, or 24 hr. Microsomes were then prepared from the cells, and CYP2E1 content was assessed by Western blotting. ●, Average enzyme content (expressed as optical density units/μg microsomal protein) ± standard error for three different preparations of hepatocytes derived from liver specimens of 24 weeks' gestation fetuses.

of induction at 24 hr and the decline at 12 hr are most likely due to evaporation of ethanol from the medium as ethanol was not replaced after its initial addition. In all subsequent experiments, 100 mM ethanol was used for a 6-hr exposure period.

To ensure that there was no generalized loss of monooxygenase activity during the culture period, which included the 15 hr subsequent to initial plating, or by ethanol treatment, expression of a microsomal enzyme other than CYP2E1 was assessed. The activity of NADPH:P458, an enzyme closely associated with P450 enzymes, was determined in fetal hepatocytes that were untreated or treated with 100 mM ethanol. Although fetal hepatocyte microsomes displayed slightly less P450 reductase activity (98 ± 26 units/mg protein) than fetal liver microsomes (125 ± 7 units/mg protein), there was no difference in enzyme activity between untreated hepatocytes cultured for 6 hr (98 ± 26 units/mg protein) and cells treated with ethanol for 6 hr (94 ± 13 units/mg protein).

The morphological effects of incubating fetal hepatocytes with 100 mM ethanol for 6 hr was assessed by phase contrast microscopy. Cultured fetal hepatocytes exhibited the typical structural features of parenchymal cells with no obvious deleterious effects due to the isolation process. When ethanol-treated cells were compared with untreated hepatocytes, nuclear or cytoplasmic changes due to ethanol exposure were not evident. That the induction of CYP2E1 in fetal hepatocytes was due to ethanol and not to the membrane-solubilizing properties of this agent was confirmed by treating the cells with another P450 enzyme inducer. Clofibrate, a CYP4A inducer (28), was found to induce fetal hepatocyte CYP2E1 without producing any adverse affects on the cells. Immunoblot analysis of fetal hepatocyte microsomes cultured with either 100 mM ethanol or 1 mM clofibrate revealed that both agents produced a ~2-fold increase in hepatocyte CYP2E1 after 6 hr of exposure (Fig. 6). Conversely, rifampicin, a CYP3A inducer, failed to elicit an increase in CYP2E1 in fetal hepatocyte microsomes. To further illustrate the responsiveness of fetal hepatocytes to P450 enzyme inducers, micro-



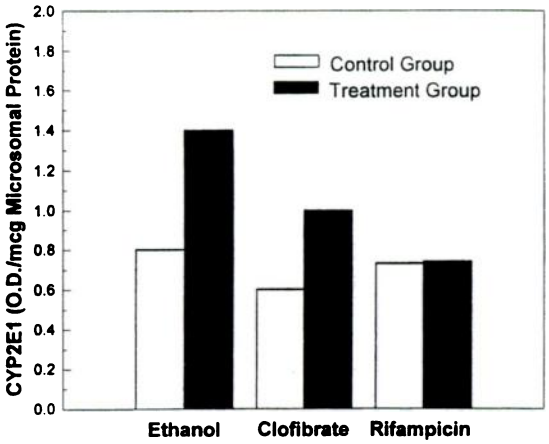


Fig. 6. CYP2E1 expression in human fetal hepatocytes treated with ethanol, clofibrate, and rifampicin. *Top*, Cultured fetal hepatocytes prepared from liver samples of 20 weeks' gestation were treated with 100 mM ethanol, 1 mM clofibrate in DMSO, or 1 μM rifampicin in DMSO, or DMSO alone. The cells were harvested 6 hr later, microsomes were prepared, and CYP2E1 levels were assessed by immunoblot analysis. *Lane A*, microsomes (1 μg) from adult human liver; *lane 1*, microsomes (5 μg) from untreated cultures; *lane 2*, microsomes (5μ) from ethanol-treated cultures; *lane 3*, microsomes (5μg) from DMSO-treated cultures; *lane 4*, microsomes (5 μg) from clofibrate-treated cultures; *lane 5*, microsomes (5μg) from DMSO-treated cultures; and *lane 6*, microsomes (5 μg) from rifampicin-treated cultures. *Bottom*, Densitometric analysis of immunoblot shown in A. CYP2E1 content is expressed as optical density units/μg microsomal protein. *Bars*, results obtained from a single preparation of cultured cells.

somes from cell cultures treated with rifampicin were subjected to anti-CYP3A4 immunoblot analysis. Reaction of these hepatocyte microsomes with anti-human CYP3A IgG revealed a 4-fold increase in staining compared with untreated cells (data not shown). DMSO treatment did not increase CYP2E1 content over that of untreated hepatocytes.

To corroborate results obtained in microsomal CYP2E1 Western blot analyses, we used immunofluorescent microscopy. This technique allows visualization of the effects of ethanol on CYP2E1 in individual cells. Control and ethanol-treated fetal hepatocytes were reacted with anti-CYP2E1 IgG and then examined microscopically. An increase in antibody-mediated CYP2E1 fluorescence staining was observed in cells from ethanol-treated cultures compared with untreated cells as well as with those reacted with a preimmune IgG (Fig. 7). Clofibrate-treated fetal hepatocytes also exhibited an increase in fluorescence staining compared with untreated hepatocytes (data not shown).

Discussion

This investigation provides evidence that CYP2E1 protein and the corresponding mRNA are expressed in human embryonic liver between the gestational ages of 19–24 weeks. Moreover, CYP2E1 protein was also found in fetal liver as young as 16 weeks of gestation. These results are significant because the presence of CYP2E1 in human prenatal liver indicates that the fetus is capable of metabolizing those compounds known to be substrates for this P450 enzyme, including alcohol. Before this report, evidence for CYP2E1 expression in human fetal liver was limited to metabolic studies, which showed that fetal hepatic microsomes are capable of catalyzing aniline hydroxylation, *N*-nitrosodimethylamine demethylation, halothane oxidation, and acetaminophen activation (6). As these drug-metabolizing reactions are mediated primarily by CYP2E1 (10–12, 29), it was proposed that this enzyme was present in the fetus. However, in recent studies in which CYP2E1 protein and mRNA levels were examined in liver samples from fetuses of <16 weeks' gestation, CYP2E1 was not detected (30, 31). Furthermore, a separate report (32) demonstrated that CYP2E1 mRNA was undetectable in fetal samples of gestational ages similar to those in the present study, i.e., 16–18 weeks. Although the results of the two former studies (30, 31) differ from the results presented here, it should be noted that the liver samples used in our investigation were from fetuses of 16 weeks and older. Therefore, detection of the enzyme in prenatal liver may be a function of gestational age, and at earlier stages of ontogeny, CYP2E1 may not be expressed. In fact, we provide evidence here indicating that hepatic CYP2E1 mRNA is not present at 10 weeks of gestation (Fig. 3), suggesting that CYP2E1 expression may indeed be related to specific stages of ontogeny. However, the study by Jones *et al.* (32), in which CYP2E1 mRNA could not be detected in fetal samples of 16–18 weeks' gestation, suggests that embryonic age may not be the only factor influencing CYP2E1 expression. Interindividual differences may also play a role in the expression of CYP2E1 in fetal liver, so that not all second trimester samples contain the enzyme. The marked variation in hepatic CYP2E1 mRNA expression occurring among adult subjects is illustrated in this same report (32).

Interestingly, we found that fetal CYP2E1 had a molecular weight slightly less than that of the adult enzyme (Fig. 1). This lower molecular weight of the fetal protein may represent a fetal variant form of CYP2E1. Such a protein may be synthesized by the fetus if variations in CYP2E1 mRNA splicing occur during development. The CYP2E1 structural gene is known to be highly methylated in the human fetus (32), and methylation of specific nucleotides could result in mRNA splicing sites different than those used in the adult. A second possible mechanism for a variant protein includes the inability of fetal liver to post-translationally modify CYP2E1. Post-translational processing, such as phosphorylation, occurs for several P450 enzymes, including CYP2E1. However, phosphorylation of CYP2E1 results in a denatured enzyme that is more rapidly degraded rather than in a protein with electrophoretic features distinct from the native form (33). P450 enzymes that undergo glycosylation do, however, exhibit different molecular weights than their nonglycosylated counterparts (34). For example, aromatase from human placenta is an *N*-glycosylated P450 that exhibits a molecular mass that is 2000 Da less on SDS-PAGE after endoglycosidase treatment to remove the carbohydrate moieties (35). Whether CYP2E1 is glycosylated is unknown at present, but when one considers the difference in molecular mass between

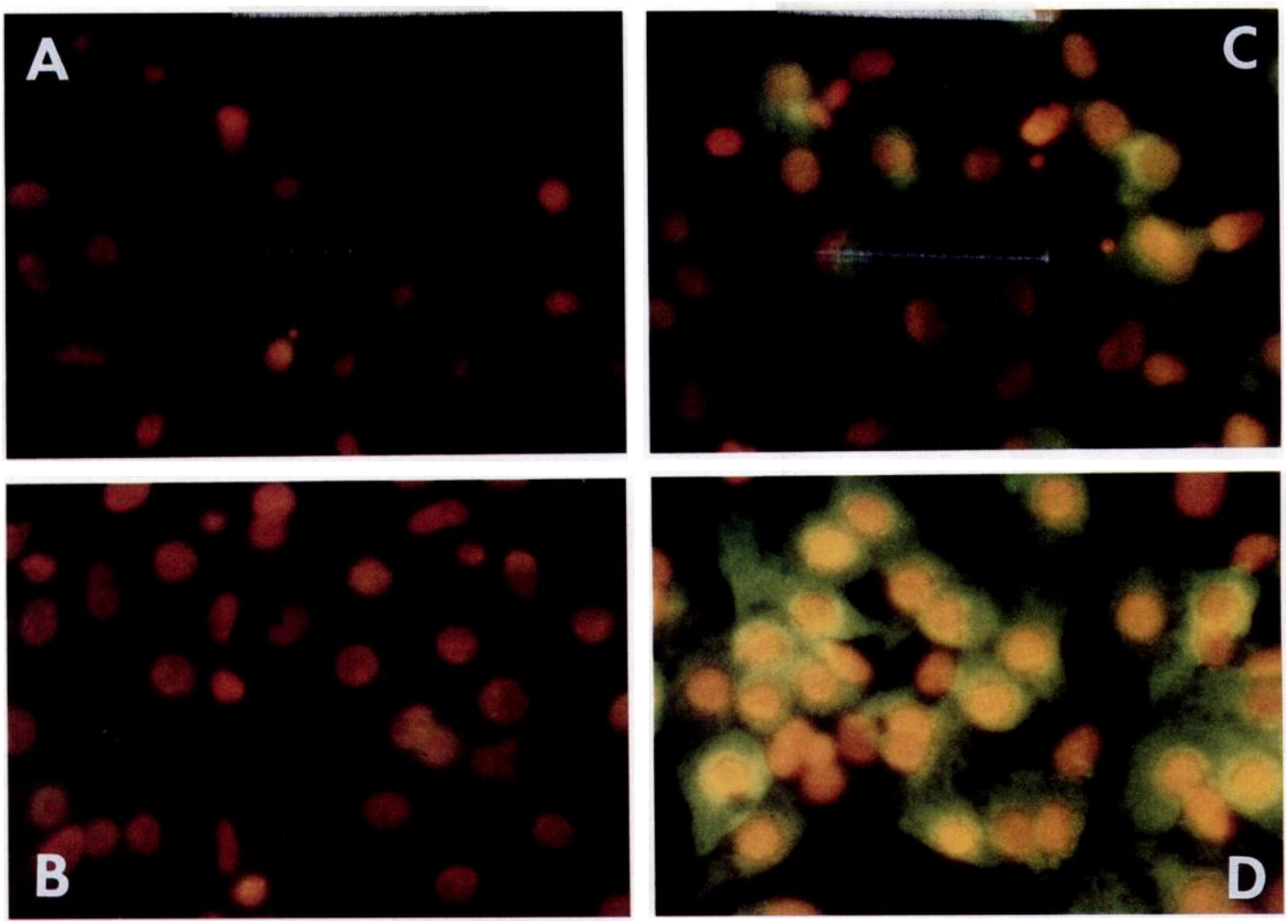


Fig. 7. Induction of CYP2E1 in cultured fetal hepatocytes by ethanol, with assessment by *in situ* immunohistochemistry. Fetal hepatocytes (derived from a liver specimen of 24 weeks' gestation) were cultured on chamber slides in the presence of 100 mM ethanol or medium alone for 6 hr. The cells were then subjected to *in situ* immunohistochemical staining with anti-CYP2E1 IgG as described in Materials and Methods. A, Untreated hepatocytes reacted with preimmune (control) IgG. B, Ethanol-treated hepatocytes reacted with preimmune (control) IgG. C, Untreated hepatocytes reacted with anti-CYP2E1 IgG. D, Ethanol-treated hepatocytes reacted with anti-CYP2E1 IgG. (Magnification 40×).

CYP2E1 in fetal and adult liver microsomes, it is possible that the former protein is carbohydrate deficient.

Despite any difference in molecular mass, CYP2E1 in fetal liver was catalytically active. We observed rates of ethanol oxidation by fetal liver microsomes that were 12–27% of those of adult hepatic microsomes (Table 1). Because alcohol dehydrogenase activity is reportedly very low in fetal liver, [0–5% of adult activity (Ref. 36 and references therein)], it had been believed that the human fetus was incapable of ethanol metabolism. It was therefore surprising to find substantial rates of ethanol oxidation by fetal liver microsomes. That antibodies to CYP2E1 markedly inhibited this activity suggests that CYP2E1 is the major catalyst involved in the oxidation of this substrate (Table 2). Indeed, the lack of inhibition of fetal microsomal ethanol oxidation by anti-CYP2C9 and anti-CYP3A4 IgG confirmed that CYP2C9, CYP3A5, and CYP3A7 did not catalyze this reaction. CYP3A enzymes in adult liver are responsible for metabolism of a broad variety of substrates (37), and CYP3A5 and CYP3A7 represent the major P450 enzymes identified so far in fetal liver microsomes (7, 8, 31). Although CYP3A5 and CYP3A7 could be candidates for metabolism of ethanol in fetal liver, our immunoinhibition results rule out this possibility.

We also presented evidence here that human fetal CYP2E1 is inducible by xenobiotics. Although these studies were performed in primary cultures of isolated hepatocytes, this *in vitro* system has proved to be most useful for examining xenobiotic regulation of P450 enzymes in humans. Furthermore, studies with cultured hepatocytes can serve to replicate events that may occur *in vivo*. With this system, we showed that treatment of fetal hepatocytes with ethanol caused a 2-fold enhancement in CYP2E1 levels (Figs. 5 and 6). Interestingly, clofibrate, a CYP4A-inducing agent (28), also increased CYP2E1 expression in fetal hepatocytes (Fig. 6). That this peroxisome proliferator exhibited this property in human fetal hepatocytes is in good agreement with a previous report that demonstrated enhanced expression of CYP2E1 in both liver and cultured hepatocytes of rats after treatment with clofibrate (38). The extent of CYP2E1 induction observed in human fetal hepatocytes with these two xenobiotics was similar to that previously noted in cultured rabbit hepatocytes treated with acetone (39) as well as that in 15-day gestation hamster liver after *in utero* exposure to ethanol (40). Taken together, our results suggest that transplacental induction of CYP2E1 in the human fetus is likely to occur during maternal ethanol consumption.

In summary, we have shown for the first time not only that CYP2E1 is present in human fetal liver but also that the fetal enzyme is capable of ethanol oxidation. Such findings augment our overall awareness that ethanol metabolism to reactive metabolites, i.e., AcA and reactive oxygen species, can indeed occur in the human embryo. Whether the formation of these toxicants results in any of the teratogenic responses observed with prenatal ethanol exposure is unknown. However, because P450-mediated ethanol metabolism has been linked to liver disease in alcohol abusers (41), it is likely that this same route of ethanol metabolism may be deleterious to the fetus. Because our studies have indicated that CYP2E1 appears during the second and third trimesters, teratogenic effects mediated via alcohol metabolism would most likely occur in neuronal tissues. Although the first trimester, a period when CYP2E1 is undetectable, may be important for morphological changes due to alcohol, e.g., low birth weight and facial anomalies, the brain may remain susceptible throughout gestation. Indeed, the brain is particularly sensitive to the adverse effects of alcohol during rapid growth, i.e., at 15–20 weeks' of gestation and again at 25 weeks' of gestation until 1 year after birth (42). As shown here, CYP2E1 is expressed by the fetus during one of these periods.

Of further impact is our finding of CYP2E1 inducibility in fetal hepatocytes by ethanol and clofibrate, which implies that *in utero* induction of this P450 enzyme may occur in mothers who are heavy drinkers or are being treated for hypercholesterolemia. Induction of fetal CYP2E1 may enhance the formation of toxic metabolites derived not only from ethanol but also from other CYP2E1 substrates (e.g., *N*-nitrosodimethylamine and acetaminophen) and could, in turn, increase fetal susceptibility to the teratogenic effects of these agents. Furthermore, as CYP2E1 is involved in arachidonic acid metabolism (43) and, possibly, prostaglandin disposition, induction of the fetal enzyme could perturb the balance of critical prostaglandins involved in fetal growth and development. Considering the many possible consequences of CYP2E1 induction in the fetus, our results may have a profound impact on the understanding of teratogenesis associated with ethanol consumption during pregnancy.

Acknowledgments

The authors thank Eileen Aramsombatdee for her excellent technical assistance in performing the ethanol oxidation reactions and immunoinhibition studies.

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