Ethanol-Mediated Transplacental Induction of CYP2E1 in Fetal Rat Liver

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ABSTRACT

We examined the potential for the widely consumed xenobiotic ethanol to transplacently induce fetal rat CYP2E1. Throughout gestation, rat dams were fed a liquid diet containing 5% ethanol or two separate control diets. At 2 days before term, the dams were killed, and maternal and embryonic tissues were collected. Immunoblot analysis of microsomes from fetal liver, placenta and maternal brain revealed a band that comigrated with adult liver CYP2E1. The identity of the immunoreactive protein in placenta, brain and fetal liver was substantiated as CYP2E1 through restriction enzyme digestion of a reverse transcription-polymerase chain reaction product. Quantification of immunoblots containing microsomes from maternal and fetal liver of ethanol-treated dams displayed a 1.4- and 2.4-fold increase in CYP2E1, respectively, compared with microsomes with pair-fed controls. Chlorzoxazone and low substrate concentrations of N-nitrosodimethylamine were used as metabolic probes for CYP2E1. The rate of chlorzoxazone metabolism by maternal hepatic microsomes from dams fed the 5% ethanol diet was 2.6-fold greater than that of controls. Conversely, a negligible increase was observed in the rate of metabolism by hepatic microsomes from ethanol-exposed fetuses compared with pair-fed animals. When N-nitrosodimethylamine demethylation was examined, these same fetal samples exhibited greater rates of activity (1.5-fold) compared with microsomes from control animals. However, this increase was not as great as expected considering the 2.4-fold increase in CYP2E1 protein. Collectively, fetuses exposed to a 5% ethanol diet throughout gestation exhibited transplacental induction of an hepatic CYP2E1 that may possess different catalytic properties from the analogous adult enzyme.

Embryotoxicity may result from a variety of environmental factors, including in utero exposure of the fetus to drugs, alcohol or environmental contaminants. The mechanisms for chemical-initiated teratogenesis are unknown at present; however, recent evidence suggests that several toxicities result from reactive intermediates formed during xenobiotic oxidation by cytochrome P450 enzymes (Wells and Winn, 1996). One of the most significant P450 enzymes involved in producing toxic intermediates from a number of xenobiotics is CYP2E1 (Raucy et al., 1993). Transplacental chemical exposure coupled with the presence of this P450 in the fetus provides a mechanism for fetal abnormalities associated with certain xenobiotics.

One such xenobiotic, ethanol, has the capacity to produce teratogenesis. Teratogenic effects associated with alcohol consumption during pregnancy have been described in humans as FAS and FAE (Hanson et al., 1976; Jones et al., 1974; Jones and Smith, 1973; Ouellette et al., 1977). Laboratory animals, including rats, mice, guinea pigs and chickens, exhibit similar abnormalities (Kronick, 1976; Papara-Nicholson and Telford, 1957; Sandor and Amels, 1971; Sandor and Elias, 1968), making these animals good models for the study of ethanol-mediated teratogenesis. The mechanisms by which ethanol produces FAS are highly speculative but may involve alcohol as well as its metabolic products. Of these oxidation products, the most likely candidates for producing the pathologies common to this disorder are the primary metabolite AcA and oxygen intermediates. Oxygen radical formation is primarily associated with the CYP2E1-mediated oxidation of ethanol (Albano et al., 1991). Therefore, if this enzyme is present in fetal tissues, it would most likely be involved in the in utero oxidation of ethanol with subsequent generation of AcA and oxygen intermediates, including

ABBREVIATIONS: AcA, acetaldehyde; BAC, blood alcohol concentration; bp, base pair; CYP, cytochrome P450; CZX, chlorzoxazone; DTT, dithiothreitol; FAE, fetal alcohol effects; FAS, fetal alcohol syndrome; FITC, fluorescein-5-isothiocyanate; HCHO, formaldehyde; HPLC, high-pressure liquid chromatography; NDMA, N-nitrosodimethylamine; OD, optical density; PCR, polymerase chain reaction; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween; TCA, trichloroacetic acid; PBS, phosphate-buffered saline.
peroxide, lipid hydroperoxides, hydrogen peroxide and hydroxyl radicals (Dai et al., 1993). Formation of these oxygen radicals within the fetus could result in local cellular toxicities, including lipid peroxidation (Nanjí et al., 1994). Conversely, the production of oxygen radicals by maternal tissues would most likely result in reactivity of intermediates with cellular components at the site of formation. Alternatively, oxygen radicals produced during maternal ethanol oxidation may also be metabolized by hepatic or circulatory superoxide dismutase or catalase, thereby precluding exposure of the fetus to these reactive molecules.

That CYP2E1 is a major culprit in producing oxygen radicals via ethanol metabolism is not unique to any one species but is common among most, including humans (Nordmann et al., 1992; Rashba-Step and Cederbaum et al., 1993). Furthermore, unlike several other P450 enzymes, expression of CYP2E1 is governed in a similar manner across species and exhibits analogous functionality (Gonzalez, 1989; Gonzalez and Nebert, 1990; Guengerich, 1990; Raucy et al., 1993). Although fetuses of experimental animals differ in their drug-metabolizing capacity from human fetuses (for a review, see Raucy and Carpenter, 1993), the similarities between rodent and human CYP2E1 suggests that examination into the inductive and catalytic properties of this P450 in the rat will provide insights into similar characteristics of the human enzyme. The present study was designed to test the hypothesis that maternal ingestion of ethanol throughout pregnancy results in increased levels of fetal hepatic CYP2E1. The consequences associated with elevation of this P450 in the fetus include a greater risk for abnormalities due to enhanced fetal metabolism of several xenobiotics with subsequent local formation of toxic intermediates. Accumulation of these toxins within the fetus may be highly significant to chemically mediated teratogenesis.

Materials and Methods

Animal treatment. Breeding of rat dams was performed as previously described (Farr et al., 1989), with visualization of spermatozoa on vaginal smears designated as day 1 of gestation. At conception, 24 pregnant female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were separated into three groups, with each group consisting of eight rats, and placed on one of three diets. Two of the three diets consisted of BioServ liquid diets (Frenchtown, NJ), a modification of the Lieber-DeCarli formulation (Lieber and DeCarli, 1968), and one of the liquid diets contained 5% ethanol [(v/v), 26.1% ethanol-derived calories] while the other was made isocaloric to the ethanol diet by the addition of a maltose-dextrin mixture. The latter liquid was given to one group of eight rat dams that served as pair-fed controls for the ethanol-treatment group. Food consumption in this pair-fed group was matched to that of the ethanol-fed rats. A third group of eight dams received Purina breeder block chow ad libitum and served as a control for the paired feeding paradigm. To avoid undue stress, animals were weighed only once a day at 5:30 p.m. and 9:30 a.m. daily throughout pregnancy.

Because the stress of taking blood samples for alcohol determinations is detrimental to the course of pregnancy, a separate group of pregnant females were exposed to the 5% alcohol liquid and used solely for the determination of blood alcohol levels. Food intake in these additional dams was matched to the daily volume of food ingested by the dams that produced the offspring. At 7 hr after introduction of food, on gestational days 15, 17 and 19, 100-μl samples of whole blood were collected from the tail vein. The blood samples were immediately mixed with 0.2 ml of 6.6% perchloric acid, frozen and stored at −20°C until assayed. Blood ethanol standards were created by mixing whole blood from untreated rodents with known amounts of ethanol ranging from 0 to 240 mg/dl and then mixing 100-μl aliquots of each standard with perchloric acid and storing the standards frozen with the samples. Blood ethanol samples were assayed in triplicate by a modified method (Lundquist, 1979). Standards and samples were thawed and centrifuged at 10,000 × g for 5 min. The supernatant (0.1 ml) was added to 3 ml of a reaction mixture containing 75 mM pyrophosphate buffer, pH 8.8, 1.8 μmol of NAD, 75 mM semicarbazide, 22 mM glycine and 150 units of yeast-derived alcohol dehydrogenase (Sigma Chemical Co., St. Louis, MO). The mixture was reacted at 22°C for 30 min, and the OD was measured at 340 nm by spectrophotometry. A curve was constructed by plotting the blood ethanol concentration of the standards against the OD. The standard curve was linear over the 0 to 240 mg of ethanol/dl range. Sample blood ethanol values were determined from the standard curve.

On gestational day 20, two of the three dietary groups contained eight pregnant animals. Those receiving the pair-fed diet contained seven dams; one animal failed to conceive. The gravid rats were anesthetized with ether and decapitated, and prenatal animals were dissected from the uterus. Livers and brains were excised from the fetuses and weighed, and tissues from each litter were pooled. Placentas, livers and brains were also removed from the dams and weighed. Maternal and fetal tissue samples were divided into three groups: the first group of tissues were either non-frozen in dry ice/methanol-cooled isopentane to be used for immunohistochemical analysis. The other two groups of tissues were either mixed with microsomal preparation buffer (0.1 M Tris base 0.1 M KCl, 1 mM EDTA, pH 7.4) for microscopic isolation or placed in TRIZol Reagent (GIBCO, Grand Island, NY) for RNA preparation. Both samples were then quick-frozen in liquid nitrogen and stored at −80°C until use.

Microsomal preparation. Microsomes were prepared from tissue samples according to a previously described method (Raucy and Lasker, 1991), except that the final microsomal pellets were suspended in sucrose buffer (10 mM KPO4, pH 7.4, 0.25 M sucrose, 0.1 mM EDTA and 0.1 mM DTT). Microsomal protein concentrations using bovine serum albumin as a standard, and NADPH-P450 reductase activity were measured as previously described (Bensadoun and Weinstein, 1976; Carpenter et al., 1996).

Immunoblot analysis. Microsomal proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose filters and stained with anti-CYP2E1 (Carpenter et al., 1996). The only procedural modification in this study was substitution of anti-human CYP2E1 IgG with anti-hamster antibodies (5 μg/ml) previously shown to be monospecific (Raucy et al., 1991). Immunochromatographic staining was performed by reacting the filters for 1 min at room temperature with 5 ml of ECL detection reagents (Amersham, Arlington Heights, IL). The filter was then exposed to Amersham Hyperfilm for 1 to 5 sec. Immunoreactive CYP2E1 content was quantified with a Microtek Scanner containing NIH software and Quant software. Microsomal protein was applied at various concentrations (0.1–50 μg) to determine the linear range of signal intensity of immunoblots. The concentration of microsomal protein (0.25–15 μg) used for all subsequent immunoblot analyses was within the linear portion of that curve.

Isolation of RNA and RT-PCR analyses. Total RNA from fetal and adult tissue was isolated using TRIzol Reagent. The RNA was quantified by measuring its absorbance at 260 nm; purity was assessed by determining the 260/280 nm ratio, which was typically >1.8. First-strand cDNA synthesis was performed as previously described (Carpenter et al., 1996). The cDNA from each tissue was then subjected to amplification using oligonucleotide primers that were 21 bp in length and flanked CYP2E1 exons 4 (bp 501–523) and 6 (bp 954–976). The amplification reactions used the following conditions: 5 min at 94°C for 1 cycle and 30 cycles at 94°C for 1 min, 1
min at 50°C and 2 min at 72°C. The final cycle consisted of 7 min at 72°C. The result of amplification with the above primers was a cDNA of 475 bp, which was verified by agarose gel electrophoresis. The PCR-produced amplimers were purified using Ultrafree-MC filters (Millipore, Bedford, MA) and digested with either the restriction enzyme Smal or Bsp1286 I (New England Biolabs, Beverly, MA) for 1 hr at 37°C. The restriction fragments were then separated by electrophoresis on 2% agarose gels for visualization. The bands generated from these enzyme digests were 298 and 177 bp or 330 and 145 bp for Smal and Bsp1286 I, respectively.

Catalytic activities. The conversion of CZX to 6-hydroxyCZX by microsomes from various tissues of rat dams and their fetuses was performed according to a modified procedure of Peter et al. (1990). Briefly, 250 μg of microsomal protein was added to a 1-ml reaction volume containing 100 mM KPO4 buffer, pH 7.4, and 500 μM CZX. The reaction was initiated by the addition of 1 mM NADPH, and the incubation was allowed to proceed for 30 min at 37°C. After incubation, the reactions were terminated with 50 μl of 43% phosphoric acid. At this point, theophylline (final concentration, 16.53 μM) was added as an internal standard. The mixture was extracted with 3 ml of ethyl acetate and dried under nitrogen. The residue was resuspended in 100 μl of mobile phase (37% H3PO4 and 26% acetonitrile). The metabolite was separated from substrate by HPLC and detected at 287 nm. Product formation was then quantified from a standard curve constructed with 6-hydroxyCZX.

N-Demethylation of NDMA at a substrate concentration of 1.0 mM (Levin et al., 1986; Tu and Yang, 1985) was determined as previously described (Anderson et al., 1986; Nash, 1953). The reaction components consisted of 1 mM NDMA (Aldrich Chemical Co., Milwaukee, WI), 10 mM semicarbazide, 0.5 ml of 50 mM KPO4, pH 6.7 buffer, and 1 mg of microsomal protein from adult liver or 2 mg of protein from fetal liver. Reactions were started by the addition of 1 mM NADPH and terminated after 30 min at 37°C with 0.5 ml of cold trichloroacetic acid (w/v). After removal of precipitated protein by centrifugation, the supernatant was mixed with 1 ml of Nash reagent and incubated for 8 min at 60°C. The amount of HCHO produced was measured spectrophotometrically at 412 nm and determined from a standard curve constructed with various amounts of HCHO ranging from 2.5 to 112.7 nmol. Product formation was linear over incubation times of 5 to 60 min and protein concentrations of 0.5 to 4.5 mg of microsomal protein.

Immunohistochemical analysis. Rat tissues that had been quick-frozen in dry ice/methanol-cooled isopentane were sectioned with a cryostat equipped with a microtome. Tissue sections (8 μm thick) were placed on Superfrost/Plus (Fisher, Pittsburgh, PA)-charged slides and fixed in acetone for 10 min at 4°C. Before being blocked, the slices were rinsed at room temperature three times for 5 min in PBS. The tissue slices were subsequently blocked in 2.0% nonfat dry milk in PBS for 1 hr at 37°C followed by incubation with anti-hamster CYP2E1 IgG (2 μg/ml in 0.2% milk in PBS) overnight at 4°C. Slides were rinsed at room temperature three times for 5 min in PBS. Sections were then incubated in goat anti-rabbit IgG conjugated to FITC (Sigma Chemical) at a 1:32 dilution in 0.2% milk in PBS at room temperature for 2 hr. Slides were coveredslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and sealed with DPX mountant (BDH Laboratory Supplies, Poole, UK).

Fluorescence was quantified with an Olympus microscope interfaced with Insight-IQ Image Processing and Quantitation Computer System (Meridian Instruments, Okemos, MI). Micrographs were photographed using the Insight Plus Automatic Photomicrographic System and Kodak 1600 ASA slide film. Prints were then reproduced from positive slides.

Statistical analysis. Data was analyzed using repeated-measures analysis of variance. Levels of significance were set at P ≤ .05.

Results

Daily monitoring of liquid diet consumption by rat dams throughout gestation ensured that animals in the two groups, ethanol and pair-fed controls, consumed similar quantities (table 1). Dietary ethanol exposure (5:30 p.m. to 9:30 a.m.), BACs were monitored, and peak BAC was determined to occur at 12:30 a.m., or 7 hr after the introduction of food. At that time, maternal BAC was 100 ± 3 mg/dl (table 1). Weight gain of dams during pregnancy was not significantly different among the three dietary groups (data not shown), and both pair-fed and ethanol-treated animals received 92% to 94% of their daily caloric intake, ensuring that animals were not starved or malnourished. Furthermore, consumption of the pair-fed or ethanol diets had no effect on litter size (table 1).

Rat dams were terminated on day 20 of gestation, and liver, brain and placenta were removed and weighed. The prenatal animals were dissected from the uterus and counted, and livers and brains were excised and weighed. Liver weights from dams given the 5% ethanol diet (11.7 ± 0.2 g) were similar to those of rats fed the pair-fed (11.9 ± 0.5 g) and ad libitum diets (11.8 ± 0.6 g). Moreover, weights of fetal liver (ad libitum, 0.17 ± 0.02 g; pair-fed, 0.17 ± 0.03 g; 5% ethanol, 0.14 ± 0.02 g), fetal brain (ad libitum, 0.14 ± 0.011 g; pair-fed, 0.14 ± 0.01 g; 5% ethanol, 0.12 ± 0.01 g), maternal brain (ad libitum, 1.8 ± 0.04 g; pair-fed, 1.8 ± 0.03 g; 5% ethanol, 1.9 ± 0.03 g) and maternal placenta (ad libitum, 7.4 ± 0.34 g; pair-fed, 7.4 ± 0.40 g; 5% ethanol, 7.6 ± 0.05 g) were not significantly different among the three dietary groups.

Microsomes prepared from isolated maternal and fetal tissues were subjected to immunoblot analysis (fig. 1). Samples from fetal liver (fig. 1B), placenta (fig. 1C) and maternal brain (fig. 1D) exhibited a protein band that both reacted with anti-CYP2E1 IgG and comigrated with the maternal liver enzyme (fig. 1A). However, at this gestational age, an immunoreactive band in microsomes prepared from fetal brain, suggesting that in this tissue CYP2E1 was below the limits of detection by our system. Immunoblot analysis of microsomes from all 23 animals (maternal and fetal) were also performed to quantify CYP2E1 levels (fig. 2). Although maternal liver microsomes from rats fed the ethanol diet exhibited only a modest increase in CYP2E1 (1.4-fold), hepatic microsomes from fetuses exposed to the alcohol

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Food Consumeda</th>
<th>Ethanol Consumeda</th>
<th>Serum Ethanolc</th>
<th>Litter Sized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum control</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>12.8 ± 0.69</td>
</tr>
<tr>
<td>Pair-fed control</td>
<td>85.9</td>
<td>N.A.</td>
<td>12.5 ± 0.84</td>
<td></td>
</tr>
<tr>
<td>5% Ethanol</td>
<td>87.1</td>
<td>13.1</td>
<td>100 ± 3</td>
<td>12.5 ± 0.78</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M.

a ml of liquid diet consumed/day (1 ml of liquid diet = 1 kcal).
b g ethanol consumed/day/kg bwt.
c mg of ethanol/dl serum, 7 hr after introduction of the feeding tubes.
d Number of live births/litter.

N.A., not analyzed.

TABLE 1

Effects of prenatal ethanol consumption on rat dams and their fetuses

On day 1 of gestation, rat dams were separated into three dietary groups. The diets were administered between 5:30 p.m. and 9:30 a.m. throughout gestation. On day 20 of gestation, the 5% ethanol and ad libitum groups consisted of eight rats, whereas the pair-fed group contained seven animals. Rat dams were anesthetized and decapitated; fetuses were removed, and litter size was determined.
diet displayed a 2.4-fold elevation of this P450 compared
microsomes from ad libitum and pair-fed controls. Negligible
changes in CYP2E1 due to ethanol exposure were observed in
microsomes from either maternal brain or placenta (figs. 1
and 2).

To verify that CYP2E1 was indeed present in maternal and
fetal tissues and that anti-CYP2E1 IgG was not cross-reacting
with a different protein immunohistochemically related to this
P450, RT-PCR was performed. Total RNA isolated from ma-
ternal liver, brain and placenta and fetal liver and brain was
used as a template in reverse transcriptase reactions to gen-
erate cDNA. The CDNA was mixed with specific primers to
rat CYP2E1 (described in Materials and Methods) and am-
plified by PCR. A 475-bp amplimer was identified in all
tissues except fetal brain, and restriction digestions were
performed to identify the 475-bp fragment. Figure 3 repre-
sents a gel containing digested cDNA amplified from liver
RNA of a fetus exposed to the pair-fed diet. The restriction
enzyme Sma I produced fragments of 298 and 177 bp,
whereas Bsp1286 I generated fragments of 330 and 145 bp.
Products of the restriction digests were analogous to those of
maternal liver CYP2E1 and that predicted for a partial
CYP2E1 cDNA in this coding region (Song et al., 1986).

CYP2E1 content in maternal and fetal liver were also
examined immunohistochemically. A photomicrograph of
liver sections (8 µm thick) obtained from a dam either pair-
fed or given the 5% ethanol diet is shown in figure 4. Liver
slices from both pair-fed (A and B) and ethanol-treated (C
and D) animals stained with rabbit anti-hamster CYP2E1
IgG (B and D) exhibited a 3-fold greater fluorescence com-
pared to those stained with preimmune IgG (A and C).
Indeed, sections from ad libitum and pair-fed animals had
average fluorescence values of 610 ± 35 and 859 ± 24 pixels,
respectively, whereas that from a rat dam given the 5%
ethanol diet displayed a mean fluorescence of 1816 ± 333
pixels. CYP2E1 staining was predominately localized to the
centrlobular region as previously reported (Tsutsumi et al.,
1989).

When liver sections were obtained from fetuses of dams
given the pair-fed (fig. 5, A and B) or ethanol diet (C and D)
and examined immunohistochemically, there was negligible
fluorescence in those stained with preimmune IgG (A and C).
Incubation with anti-hamster CYP2E1 antibody resulted in
detectable fluorescence, and in contrast to centrlobular
staining of maternal liver slices, localization of this P450 was

**Fig. 1.** A representative immunoblot of microsomal samples from ma-
ternal and fetal tissues. Tissue microsomes from dams fed the three
diets and their fetuses were subjected to immunoblot analysis. Micro-
somes were separated by SDS-PAGE and transferred electrophoreti-
cally to a nitrocellulose filter. The filter was then stained with anti-
hamster CYP2E1 IgG as described in Materials and Methods. Lanes
ML in A (1 µg of microsomal protein) and B–D (0.25 µg) represent liver
microsomes from an untreated dam and were used as a quantification
standard on all blots. Lanes 1–2, microsomes from dams, or their
fetuses, receiving the pair-fed diet. Lanes 5–6, microsomes from
fetuses, fed rat chow standard on all blots. Lanes 1–2, microsomes from dams, or their
fetuses, consuming the pair-fed diet. Lanes 3–4, microsomes from dams, or
their fetuses, consuming the pair-fed diet. Lanes 5–6, microsomes from
dams, or their fetuses, receiving the 5% ethanol diet. A, Maternal
hepatic microsomes (1 µg). B, Fetal hepatic microsomes (15 µg). C.
Microsomes from placenta (15 µg). D, Maternal brain microsomes (15
µg).

**Fig. 2.** Quantification of CYP2E1 in maternal and fetal tissues. Micro-
somes from fetal and gravid rat tissues were subjected to immunoblot
analysis, and anti-CYP2E1 immunoreactive protein was quantified
with a Microtek Scanmaker IIHR scanner interfaced to ImageQuant soft-
ware. Each bar represents the mean CYP2E1 content (OD/µg of micro-
somal protein applied to the original polyacrylamide gels) ± S.E.M.
of determinations from eight rat dams and their fetuses consuming the
ad libitum or 5% ethanol diets and seven dams and their fetuses
receiving the pair-fed diet. * Significant differences between animals
exposed to ethanol and those on either the pair-fed or ad libitum diets
(P < .05).

**Fig. 3.** Restriction enzyme digests of a 475-bp truncated fetal liver
CYP2E1 amplimer isolated by RT-PCR. RT-PCR was performed on a
fetal liver RNA sample obtained from a fetus of a pair-fed dam to
generate a CYP2E1 fragment of 475 bp in length, as described in
Materials and Methods. DNA fragments resulting from enzyme digests
of the amplimer with Sma I and Bsp1286 I were subjected to electro-
phoresis on a 2% agarose gel. The restriction endonuclease Sma I
generated fragments of 298 and 177 bp, whereas Bsp1286 I produced
330- and 145-bp fragments.
diffuse in sections from either pair-fed or ethanol-exposed fetuses (B and D). Furthermore, liver sections from fetuses of pair-fed (317 ± 17 pixels) and *ad libitum* (294 ± 68 pixels) animals displayed a 1.5-fold lower staining intensity than did sections from ethanol-exposed animals (average fluorescence, 437 ± 19 pixels). It should be noted that unlike Western blots, which represent microsomes from the entire liver, immunohistochemistry allows examination of small sections; hence, different staining intensities representing changes in CYP2E1 among the dietary groups were expected for the two procedures.

The catalytic capability of CYP2E1 in hepatic microsomes from maternal and fetal rats was assessed by the oxidation of two well known substrates of this enzyme, namely, CZX and NDMA. In addition, NADPH P450-reductase activity was assessed and found to be an order of magnitude less in hepatic fetal (13.9 ± 2.8 nmol of cytochrome c reduced/min/mg of protein) compared to adult microsomes (177.7 ± 12.3 nmol of cytochrome c reduced/min/mg of protein). Initially, rates of CZX conversion to 6-hydroxyCZX were determined in microsomes from maternal liver, brain and placenta, as well as in fetal liver and brain. CZX hydroxylation was 2-to 2.6-fold higher in hepatic microsomes from dams given the 5% ethanol diet compared with microsomes from animals fed the pair-fed or *ad libitum* diets, respectively (table 2). Indeed, linear regression analysis of maternal liver samples revealed a correlation between CYP2E1 content, determined by immunoblot analysis, and CZX hydroxylation (*r = .67, P < .01*) (fig. 6A). When comparing CZX hydroxylation by microsomes from adult and fetal liver, much less activity (16–60-fold) was observed with fetal hepatic microsomes from animals in all dietary groups. However, based on CYP2E1 content, rates of CZX hydroxylation by adult and fetal liver microsomes from control animals were similar ([0.034 and 0.036 nmol of 6-hydroxyCZX formed/min/unit (OD/µg) of CYP2E1, respectively]. Despite elevated concentrations of CYP2E1 in hepatic microsomes from fetuses exposed to ethanol, CZX hydroxylation was not higher than that of microsomes from control animals (table 2). Indeed, when CZX hydroxylation was based on CYP2E1 content, rates were 6-fold lower in liver microsomes from ethanol-exposed fetuses. As a result, a lack of correlation (*r = .45*) was observed between CYP2E1 content and CZX metabolism when microsomes from fetal liver were analyzed (fig. 6B). In addition, maternal brain and placenta and fetal brain exhibited negligible rates of CZX hydroxylation in tissues obtained from all dietary groups (table 2).

The N-demethylation of low substrate concentrations (1 mM) of NDMA was assessed using hepatic microsomes from adult and fetal animals and found to be catalyzed by both
TABLE 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chlorozoxazone-6-hydroxylation</th>
<th>CYP2E1 Protein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ad Libitum</td>
<td>Pair-fed</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>0.473 ± 0.04</td>
<td>0.762 ± 0.21</td>
</tr>
<tr>
<td>Maternal brain</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Placenta</td>
<td>&lt;0.001</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>0.029 ± 0.004</td>
<td>0.023 ± 0.008</td>
</tr>
<tr>
<td>Fetal brain</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are expressed as nmol product formed/min/mg of microsomal protein and represent the mean ± S.E.M. of samples from eight animals or litters per treatment group, except the pair-fed group where the mean represents seven animals or litters. Tissue samples from individual litters were pooled to obtain an adequate amount of microsomal protein.

<sup>b</sup> Values are expressed as OD/μg microsomal protein × 10<sup>6</sup> and denote the mean ± S.E.M. of samples from dams or litters.

<sup>c</sup> Significantly different from ad libitum and pair-fed (P < .01).

<sup>d</sup> Significantly different from ad libitum and pair-fed (P < .05).

N.D., CYP2E1 content was below the limits of detection.

Discussion

Despite the importance of a link between P450 enzymes and teratogenesis, identification of individual P450s in fetal tissues of humans and experimental animals has largely been ignored. The low concentrations of these enzymes in fetuses have hindered their characterization. However, newer techniques are permitting the detection of low expressing proteins, allowing more rapid advances to be made. To date, several P450s have been identified in human fetal samples, including the highly expressed CYP3A7, as well as other P450 enzymes expressed at much lower levels, such as CYP1B1, CYP3A5 and CYP1A1 (Miller et al., 1996). More recently, CYP2E1 was identified in hepatic samples of human fetuses >17 weeks gestation (Carpenter et al., 1996). Although the recognition of these P450s in human fetal samples represents significant progress, detection of these toxicologically important enzymes in animals has not occurred at a similar pace. Unfortunately, this creates a situation in which few fetal animal models exist that can be used to mimic P450-mediated teratogenesis in humans. Here, we report on the expression and transplacental induction of one
P450, namely, CYP2E1 in rat. The occurrence of this enzyme in rodent fetal liver provides an experimental model for extrapolation to the human fetus regarding toxicological impact of locally produced reactive metabolites mediated by CYP2E1.

A recent study (Wu and Cederbaum, 1996) described constitutive expression of CYP2E1 protein in livers of rat fetuses. In this study CYP2E1 was found not only in fetal rat liver but also in placenta. Interestingly, found that placental microsomes harbored two immunoreactive proteins. An additional band in placenta suggests that rats may express more than one member of the CYP2E subfamily in embryonic tissues. An extra member of this subfamily in rat would be analogous to CYP2E expression in rabbits, in which an additional protein (i.e., CYP2E2) was identified and found to be regulated ontogenically (Peng et al., 1991). Consequently, it would not be surprising to find a comparable fetal rat CYP2E enzyme under ontogenic control. That at least one of these protein bands in placental microsomes was CYP2E1 was confirmed by RT-PCR and restriction enzyme digestions. The immunoreactive band observed in fetal liver microsomes was also confirmed by the same technique (fig. 3), negating the possibility that the strong reactivity observed on the blots was due to an immunochemically related protein in either tissue (fig. 1). Previously, CYP2E1 was thought to be present only after parturition. This conjecture was based on the presence of very low levels of CYP2E1 mRNA in liver obtained from 6-hr postpartum rats (Umoto et al., 1988). It should be noted that in this study, CYP2E1 mRNA was not assessed in prenatal liver samples before birth. Thus, it is possible that CYP2E1 message was present, but the birthing process may add sufficient stress to down-regulate this P450. Indeed, a more recent report demonstrated CYP2E1 mRNA in 20-day-gestation fetal rat liver (Borlakoglu et al., 1993). In addition, hepatic microsomes from fetal rats, at the equivalent day of gestation, possessed the ability to metabolize carbon tetrachloride, a CYP2E1 substrate (Cambden-Gros et al., 1986). Collectively, from results presented here and those of others, it can be concluded that CYP2E1 is expressed in fetal rat liver, at least in the later gestational ages. Considering that this P450 plays a role in endobiotic metabolism of such substrates as acetone (Koop and Casaza, 1985), arachidonic acid (Laethem et al., 1993) and laurate (Amet et al., 1994), it is not surprising that CYP2E1 occurs prenatally.

We also showed, that there was an increase in CYP2E1 expression in fetal liver stemming from maternal ingestion of alcohol throughout gestation (fig. 2). In a previous report, ratsGiven liquid diets containing 6.7% alcohol failed to produce embryos with enhanced expression of hepatic CYP2E1 (Wu and Cederbaum, 1993). The cause for the discrepancy between our investigation and that of Wu and Cederbaum (1993) may reside in the feeding paradigms in which at least two differences were noted. First, a limited access feeding schedule, 16 hr, was used in this investigation, whereas the previous study allowed rats free access to the alcohol diet. We found that limiting exposure to food for only 16 hr resulted in higher maternal BACs (100 mg/dl) compared with the 24-hr unlimited access paradigm (80 mg/dl) (Queen et al., 1993). The higher BACs may be necessary to produce CYP2E1 induction in the fetus. Second, we used a 5% rather than a 6.7% ethanol-containing diet. The higher content of ethanol in the liquid diet is often accompanied by poor nutrition, which is reflected as higher fetal mortality rates and lower body weights at birth (Farr et al., 1989) and affect CYP2E1 expression. The nutritional status of an animal plays a significant role in the regulation of CYP2E1. Altered carbohydrate, fat, mineral and vitamin intake can cause changes in the expression of this P450 (Yang et al., 1992). A diet high in ethanol may interfere with adequate nourishment to the fetus and prevent induction of CYP2E1 in fetal tissues. In this report, nutritional status was not compromised with the 5% alcohol diet in either the dams or their fetuses as demonstrated by insignificant differences in maternal weight gain, litter size and fetal organ weights compared with those fed control diets. Thus, use of the 5% ethanol diet eliminates concerns regarding inadequate nutrition to the fetus. Despite sufficient nutrient supply to the embryo, one drawback of the 5% alcohol diet may be insufficient ethanol to cause induction of the maternal and, subsequently, fetal liver enzyme. This was clearly not the case here because we demonstrated that the 5% alcohol diet produced a 1.4-fold enhancement in CYP2E1 content in maternal liver over controls, which was similar to that determined for dams that received the 6.7% alcohol diet in which a 1.6-fold increase above controls was observed (Wu and Cederbaum, 1993). Thus, results presented here suggest that the 5% ethanol diet given by limited access produces sufficient CYP2E1 induction in adult liver, limits the risk of inadequate nutrition and causes higher BACs. Taken together, the 5% diet may be superior to the 6.7% alcohol-
containing liquid in producing transplacental induction of fetal liver CYP2E1.

Increased fetal liver CYP2E1 levels verified that ethanol reached the fetus from the maternal circulation. Furthermore, greater induction of the fetal enzyme (2.4-fold) compared with that in dams (1.4-fold) (fig. 2) may be theorized as follows. First induction of fetal CYP2E1 occurred by a separate mechanism(s) from that in the adult. Second, hormones present in maternal rat, but not the fetus, attenuate induction of CYP2E1 by ethanol. Finally, more of the xenobiotic was concentrated in fetal than maternal tissues. For ethanol to concentrate in the fetus, it must pass through the placenta, which also expresses CYP2E1 (fig. 1). It is likely that placenta could metabolize ethanol, thereby causing a decrease rather than an increase in the amount presented to the fetus. Therefore, the greater ethanol-mediated induction in fetal liver compared with adult liver may be interpreted to mean that this P450 is governed by regulatory mechanisms distinct from those in adult liver. This is an intriguing supposition and warrants further investigation.

Morphological differences in the hepatic ultrastructure were noted among adult and 20-day-gestation fetal liver sections. In the rat fetus, the liver primordium appears at 11 days gestation and bile canaliculi become apparent at about day 17 of gestation (Kanamura et al., 1990). RER is prominent at early stages; however, the amount of RER is constant between periportal and perivenular hepatocytes until 1 day before birth (Kanamura et al., 1990). Interestingly, anti-CYP2E1 staining was diffuse and uniformly distributed throughout the lobule (fig. 5), which is consistent with the ultrastructure of 20-day-gestation liver. Furthermore, P450-reductase is uniformly distributed throughout the rat liver lobule until 4 days postnatal (Watanabe et al., 1993). Thus, distribution of CYP2E1 in 20-day-gestation liver sections closely mimicked that of P450-reductase. Other P450s, including CYP3A, CYP2C and CYP1A2, have been immunohistochemically localized in human fetal liver (Rastanavanh et al., 1991). These enzymes demonstrate a similar diffuse pattern of distribution to that observed here for CYP2E1 in rat.

One of the most significant observations of this investigation was that fetal liver CYP2E1 appeared to be catalytically distinct from that residing in adult liver. Despite immunohistochemical relatedness and similarity in nucleotide sequence between exons 4 and 6, rates of CZX hydroxylation did not change in fetuses from ethanol-treated dams compared with those of ad libitum or pair-fed controls (table 2). Conversely, there was a 1.5-fold increase in the rate of NDMA demethylation by hepatic microsomes from ethanol-exposed fetuses over those of microsomes from pair-fed and ad libitum control animals (fig. 7). These differences in metabolic activity mediated by microsomes from ethanol-exposed fetuses compared with controls may be subject to several interpretations. First, conversion of CZX to its metabolite may not be mediated by fetal CYP2E1 but rather by another highly expressed P450 (e.g., CYP3A7). Another explanation may be that ethanol exposure caused induction of a novel fetal CYP2E2 protein that was incapable of metabolism. This latter supposition is unlikely given that induced CYP2E2 catalyzed NDMA demethylation (fig. 7). A final reason may be that the fetal form of CYP2E1 is slightly altered from that of the adult and possesses distinct amino acids around the active site or substrate recognition sites causing a narrower range of substrate specificity. The active site might then be more constrained, allowing access to only aliphatic compounds and limiting accessibility of aromatic substrates, such as CZX. Nevertheless, further studies are warranted to establish such an hypothesis.

In summary, we have shown for the first time that CYP2E1 was transplacently induced by ethanol in fetal rat liver. A lack of correlation between fetal liver CYP2E1 content and CZX metabolism (fig. 6B) suggested that the fetal enzyme was unable to catalyze this substrate. Nevertheless, NDMA demethylation by hepatic microsomes from fetuses of ethanol-treated dams was enhanced over control values, but the higher rates did not reflect the extent of increase in CYP2E1 content. Considering the significance of this enzyme in bioactivation of numerous therapeutic agents, ethanol and procarcinogens, high levels of fetal CYP2E1 due to xenobiotic exposure may exacerbate chemically mediated teratogenesis. Taken together, this report demonstrates the importance of identifying P450 enzymes in fetal tissues, the mechanisms governing their expression and substrates metabolized by these P450s. Characterization of fetal P450 enzymes will have an enormous impact on assessment of xenobiotics for their potential as teratogens. Furthermore, understanding mechanisms by which known teratogens produce fetal abnormalities may become less complicated.

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