Human Hepatic CYP2E1 Expression during Development

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ABSTRACT

Human hepatic CYP2E1 expression developmental changes likely have an impact on the effects of xenobiotics metabolized by the encoded enzyme. To resolve previous conflicting results, CYP2E1 content was determined in human hepatic microsomes from samples spanning fetal (n = 73, 8–37 weeks) and postnatal (n = 165, 1 day–18 years) ages. Measurable immunodetectable CYP2E1 was seen in 12 of 15 third-trimester (93–186 gestational days) and 12 of 15 third-trimester (>186 days) fetal samples (medians = 0.35 and 6.7 pmol/mg microsomal protein, respectively). CYP2E1 in neonatal samples was low and less than that of infants 31 to 90 days of age, which was less than that of older infants, children, and young adults [median (range) = 8.8 (0–70); 23.8 (10–43); 41.4 (18–95) pmol/mg microsomal protein, respectively; each P < 0.001, analysis of variance, post hoc]. Among those older than 90 days of age, CYP2E1 content was similar. A 4-fold or greater intersubject variation was observed among samples from each age group, with the greatest variation, 80-fold, seen among neonatal samples. Among subjects of known gestational and postnatal age (n = 29) increasing protein content was associated with increasing postnatal age (P < 0.001, linear regression), but only equivocally with increasing gestational age (P = 0.07). Individuals from the third trimester through 90 days postnatal age with one or more CYP2E1*1D alleles had lower CYP2E1 protein content than similar-aged subjects who were homozygous CYP2E1*1C. In summary, CYP2E1 was clearly expressed in human fetal liver. Furthermore, the postnatal data suggest that infants less than 90 days old would have decreased clearance of CYP2E1 substrates compared with older infants, children, and adults.

CYP2E1 (EC: 1.14.14.1) is a member of the cytochrome P450-dependent monooxygenase superfamily and is important for the oxidative metabolism of several therapeutics (e.g., acetaminophen, halothane, and chloroxazone), as well as the bioactivation of multiple, small-molecular-weight toxicants (e.g., ethanol, benzene, toluene, and nitrosamines) (Tanaka et al., 2000). Regulated at multiple levels that remain poorly understood (Novak and Woodcroft, 2000), CYP2E1 expression is known to be elevated by ethanol consumption (Takahashi et al., 1993), by obesity (O’Shea et al., 1994), and in type II diabetes (Wang et al., 2003). Genetic variation also appears to contribute to interindividual variation in CYP2E1 expression and disease susceptibility (McCarver et al., 1998; Itoga et al., 2002). Similarly, changes in CYP2E1 protein levels during development also would likely have an impact on drug efficacy and toxicity as well as susceptibility to environmental toxicants.

Human hepatic CYP2E1 developmental expression is controversial. Wrighton et al. (1988) and Shimada et al. (1996) were unable to detect immunoreactive CYP2E1 in five fetal liver samples less than 12 weeks’ gestational age or three fetal liver samples between 11 and 13 weeks’ gestational age, respectively. In a study of 16 fetal livers ranging in age from 11 to 24 weeks, Hakkola et al. (1994) were unable to detect CYP2E1 mRNA using reverse transcriptase coupled polymerase chain reaction DNA amplification (RT-PCR), consistent with earlier reports using Northern blot analysis from Komori et al. (1990) (unspecified sample number and age) and from Jones et al. (1992) (12 fetal livers ranging from 10 to 17 weeks’ gestational age). Finally, Vieira et al. (1996) reported an inability to detect CYP2E1 mRNA, protein, or catalytic activity in 66 fetal liver samples less than 30 weeks’ gestational age (mean = 23.2 ± 3.6 weeks). These studies are in contrast to three reports demonstrating early fetal CYP2E1 expression and catalytic activity. Carpenter et al. (1996) described immunodetectable CYP2E1 in 11 fetal liver samples ranging from 16 to 24 weeks’ gestational age, anti-CYP2E1 sensitive and NADPH-dependent ethanol oxidation in six of these same samples, and CYP2E1 mRNA (detected by RT-PCR) in three fetal livers at 19, 23, and 25 weeks’ gestational age. However, consistent with earlier studies, they were not found.

ABBREVIATIONS: RT-PCR, reverse transcriptase-polymerase chain reaction; AFLP, amplified fragment-length polymorphism; ANOVA, analysis of variance; MANOVA, multiple analysis of variance.
able to detect CYP2E1 mRNA in two fetal livers at 10 weeks’ gestational age. Using only RT-PCR, Boutelet-Bochan et al. (1997) reported detectable CYP2E1 mRNA in two of six fetal livers ranging from 7 to 11 weeks’ gestational age. In particular, fractions from three pooled liver samples, ranging from 53 to 59 days’ gestational age, Khalighi et al. (1999) demonstrated low but measurable oxidation of the CYP2E1-substrate chloroxazone that was inhibited by diethyldithiocarbamate. Thus, the literature is conflicting about whether CYP2E1 is expressed during early and midgestation and silent about expression in later gestation and early childhood.

To help resolve this controversy and gain a more complete understanding of CYP2E1 temporal-specific expression, a study was undertaken to examine CYP2E1 protein levels in fetal liver samples representing most of the gestational period and postnatal development. The results definitively demonstrate fetal hepatic CYP2E1 protein expression beginning in the second trimester (93–186 days of gestation) with most individuals exhibiting readily detectable levels in the third trimester (>186 days). Adult protein levels were attained at about 90 days’ postnatal age. Among samples representing the time period of dramatic age-dependent increases in CYP2E1 protein content, CYP2E1*1D presence was associated with lower CYP2E1 content.

Materials and Methods

Materials. Polyclonal rabbit antibody raised against human CYP2E1 was obtained from Oxford Biomedical Research (Oxford, MI). Purified, human recombinant CYP2E1 was purchased from PanVera Corp. (Madison, WI). Horseradish peroxidase-conjugated goat anti-rabbit IgG, nitrocellulose membranes, and enhanced chemiluminescence Western blotting kits were obtained from Amersham Biosciences Inc. (Arlington Heights, IL). Protein molecular weight standards (Bench Mark prestained protein ladder), DNAzol reagent, and Taq polymerase were from Invitrogen (Carlsbad, CA). The micro bicinchoninic acid protein assay reagent kit was from Pierce (Rockford, IL). All other reagents were obtained from common commercial sources at the purest grade available.

Tissue Samples. Frozen specimens of human liver were obtained from the Brain and Tissue Banks at the University of Baltimore and University of Miami (National Institute for Child Health and Human Development, NOI-HD-8-3283 and NOI-HD-8-3284, respectively). Additional fetal and embryonic tissues were procured from the Central Laboratory for Human Embryology at the University of Washington (National Institute of Child Health and Human Development, HD-00836). Samples from individuals with disease processes that potentially would involve liver damage were excluded from procurement. A total of 238 liver samples were obtained representing ages from 8 weeks’ gestation to 18 postnatal years. Gender information was provided for 219 samples, 136 being male and 83 female. Ethnicity was provided for 201 samples: 88, 93, and 20 were African-American, Northern European-American, and Hispanic-American, respectively. Other than major diseases, cause of death, and age, no other personal identifiers were available. Tissue was stored at −80°C until used for the preparation of microsomal suspensions. This study was approved by the Children’s Hospital of Wisconsin and the Medical College of Wisconsin Institutional Review Boards.

Preparation of Microsomes, Electrophoresis, and Immunoblotting. Liver microsomes were prepared by differential centrifugation and analyzed for CYP2E1 specific content essentially as described previously (Koukouritaki et al., 2002). Protein concentrations were determined by the micro bicinchoninic acid protein assay (Smith et al., 1985) using bovine serum albumin as a protein standard. Five to 20 micrograms of microsomal protein from each liver sample were fractionated by SDS-PAGE along with 25, 50, 100, 250, and 500 fmol of recombinant human CYP2E1. Both the CYP2E1 primary antibody and horseradish-conjugated goat anti-rabbit IgG secondary antibody were diluted 1:4000 in phosphate-buffered saline containing 0.5% nonfat dry milk for immunodetection. After determining the integrated optical densities of the digitized, immunoreactive bands (Kodak Digital Science ID Software), the CYP2E1 specific content of the patient samples was determined by linear regression based on a standard curve determined from the recombinant human CYP2E1 included on each blot (GraphPad Instat version 3.00, GraphPad Software Inc., San Diego, CA). In all blots, bands corresponding to CYP2E1 were identified by reference to the recombinant CYP2E1 and molecular weight standards. The limit of detection of this assay was 10.0 fmol. The coefficient of determination (r²) values for the standard curves were all greater than 0.90 and the coefficient of variation for independent experiments (n = 6) was 6.1%.

Genotyping. DNA was isolated from approximately 0.5 g of each tissue sample using DNAzol reagent (Chomczynski et al., 1997) following the protocol recommended by the manufacturer. Amplified fragment length polymorphism ( AFLP) was used to genotype for the CYP2E1*1C and CYP2E1*1D alleles (McCarver et al., 1998) using 5′-CAG CCA ACA GCA GAC GTG AT-3′ (forward) and 5′-GAT GCA GCT GTG TGA GT-3′ (reverse) primers, the latter being 5′-end labeled with the 3′-PA fluororescent WRed Dye (Invitrogen). Using a 96-well format with 100 ng of DNA template in a final volume of 10 µl, DNA amplification reactions contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl; 2.5 mM MgCl2; 50 nM each primer; 0.1 mM each dATP, dCTP, dGTP, and dTTP; and 0.25 U of Taq polymerase. Following an initial denaturation step at 94°C for 3 min, amplification was performed for 32 cycles of 1 min at 60°C, 2 min at 72°C, and 1 min at 94°C using an MJ Research (Watertown, MA) DNA Engine Tetrad thermal cycler. After a final extension at 72°C for 3 min, reactions were kept at 4°C until analyzed. Samples (1 µl), along with 0.2 µl of Size Standard 600 (Beckman Coulter, Inc., Fullerton, CA), were analyzed by capillary electrophoresis after diluting 1:40 in HiDi formamide (Applied Biosystems, Foster City, CA) using a Beckman-Coulter CEQ8000 Genetic Analysis System. After denaturation at 90°C for 2 min, samples were injected at 2.0 kV for 30 s and fractionated at a capillary temperature of 35°C for 60 min at 6.0 kV.

Data Analysis. The distribution of CYP2E1 specific content was normalized by log transformation. Stepwise multiple linear regression analysis and multiple and one-way analysis of variance (MANOVA) were used to evaluate factors contributing to the variation in CYP2E1 expression (SPSSPC, SPSS Science, Chicago, IL). Differences in CYP2E1 specific content among age and ethnic groups were assessed using ANOVA with a Dunnett’s T3 post hoc test. Distributions of discrete variables were tested using chi square analysis.

Results

Developmental Expression Pattern for Hepatic CYP2E1. Previous studies have provided conflicting data regarding the hepatic expression of CYP2E1 during human development. However, in many instances these studies were limited by the number and/or quality of the samples or the developmental time frame examined. In the present study the developmental protein expression pattern of human hepatic CYP2E1 was determined by SDS-PAGE and Western blot analysis of microsomal protein samples prepared from a bank of 238 human liver samples over a broad age range. As shown in a typical blot (Fig. 1), Western blotting revealed a single, major immunoreactive band with an apparent molecular mass near 57 kDa, corresponding to the intact CYP2E1 protein. Comigration also was observed with recombinant CYP2E1.
The overall temporal-specific CYP2E1 protein expression pattern is shown in Fig. 2, A and B. Although undetectable in the first trimester, measurable CYP2E1 was observed in 18 of 49 second trimester fetal livers (median 0.35 pmol/mg microsomal protein) and in 12 of 15 third trimester fetal livers (median 6.7 pmol/mg microsomal protein). However, all CYP2E1 specific content values were less than 15 pmol/mg microsomal protein. CYP2E1 specific content was markedly greater in postnatal samples, reaching adult values of 48.7 ± 22.4 pmol/mg microsomal protein within the first year of life.

To better assess the change in hepatic CYP2E1 protein content in the perinatal period, age relationships during the first 180 postnatal days were examined (Fig. 3). Immediately following birth, protein content was greater relative to fetal values in some, but not all, neonatal livers. The period of greatest increase in CYP2E1 protein content was during the first few postnatal months, with adult values reached by about 90 days' postnatal age. The differences observed were striking with only two samples from individuals with ages less than 90 postnatal days having a value >40 pmol/mg microsomal protein postnatal days, whereas about half of those older than 90 days age exceeded this value.

The data from Figs. 2 and 3 were summarized and further analyzed by grouping the postnatal data to minimize differences within an age group and maximize differences between age groups (Fig. 4). This analysis demonstrates that CYP2E1 protein content was significantly greater with each increasing age group (ANOVA, post hoc, \( P < 0.01 \), all comparisons) with the exception of the protein values between the third trimester fetal livers and infants less than 1 month of age (\( P = 0.1 \)). These data also demonstrate significant intersubject differences, with variation being at least 4-fold from the third trimester onward. However, an 80-fold variation was observed in the neonatal period.

Factors Having an Impact on CYP2E1 Developmental Expression. Among the postnatal liver samples with complete data (\( n = 161 \)), increasing postnatal age and ethnicity were associated with greater CYP2E1 protein levels (\( P < 0.001 \) and 0.05, respectively; Table 1, stepwise linear regression), whereas gender was not significant. Importantly, the post-mortem interval (i.e., the time between death and the snap freezing of liver samples) also did not contribute significantly to differences in CYP2E1 specific content. Controlling for differences in subject age, greater CYP2E1 protein content was observed in Northern European-Americans than African-Americans (\( P < 0.03 \), ANOVA; Fig. 5), whereas a difference between Northern European-Americans and Hispanic-Americans was equivocal (\( P = 0.1 \)). In contrast, samples from African- and Hispanic-Americans were similar.
Among the subset of liver samples from individuals whose gestational age and postnatal age were known, increasing postnatal age was strongly associated with increasing CYP2E1 specific content, whereas increasing gestational age was equivocal (Table 2, \( P < 0.001, 0.07 \), respectively, step-wise linear regression). Gender, ethnicity, and the post-mortem interval were not associated with differences in CYP2E1 protein levels in this subset.

Of the multiple CYP2E1 polymorphisms identified to date, only the CYP2E1*1D has been linked to altered function, being associated with increased CYP2E1 metabolic capacity in obese individuals or those individuals consuming alcohol (McCarver et al., 1998) and more recently, occurring at a higher frequency in esophageal cancer patients than in healthy controls (Itoga et al., 2002). To assess whether this variant has any effect on CYP2E1 ontogeny, the genotype at this locus was determined for 188 of the liver samples that previously had been characterized for CYP2E1 protein content. An example of genotyping results using AFLP is shown in Fig. 6. The CYP2E1*1D allelic frequency was 6.9% with 87% of the entire sample being homozygous for the CYP2E1*1C allele, and about 13% having at least one CYP2E1*1D allele. About 8, 11, and 23% of individuals whose descent was described as Northern European, Hispanic, and African had at least one CYP2E1*1D allele (\( \chi^2 > 0.1 \)). Based on the relative absence of CYP2E1 in early fetal samples, the dramatic increase associated with birth, and the apparent attainment of mature CYP2E1 content by 90 days (Fig. 5), samples were grouped into early fetal (first and second trimester), developing (third trimester through 90 days), and mature samples (91 days through 18 years). The distribution of CYP2E1 genotype did not differ across these age groups (\( \chi^2, P > 0.1 \)). Controlling for the effect of age group, the interaction between age group and CYP2E1*1D presence was significantly associated with differences in CYP2E1 content (Table 3, \( P < 0.01, \) MANOVA). However, the relative effect size of the interaction was about one-tenth that of age alone. In contrast, the presence of the CYP2E1*1D allele alone was not significant. These three variables explained 74% of the variance in CYP2E1 content (Table 3).

The sample groups responsible for the significant age-genotype interaction term were identified using ANOVA with post hoc testing. Among developing liver samples (third trimester to 90 days), CYP2E1 content was lower among samples with at least one CYP2E1*1D allele compared with those who were homozygous CYP2E1*1C (Fig. 7, \( P < 0.01 \)). In contrast, there was no difference among early fetal samples (data not shown) or more mature samples (91 days through 18 years of age) based on genotype.

**Discussion**

CYP2E1 was clearly expressed in the developing human fetal liver as early as the beginning of the second trimester. Immunodetectable protein was measurable in nearly all third trimester tissue samples at mean levels that were 10%
of those seen in older postnatal samples (>90 days). The data also demonstrate that birth does not appear uniformly sufficient for the onset of human hepatic CYP2E1 expression. This variability in the onset of increased postnatal protein levels generated the greatest degree of intersubject variability, i.e., 80-fold, among tissue samples from the neonatal age group. This contrasts to the roughly 4-fold variation observed in other age groups which, in turn, is more consistent with the intersubject variation observed in adults (McCarver et al., 1998). Such differences also are consistent with the effect of variable protein stabilization by an endogenous substrate (e.g., ketones) or exogenous xenobiotics (e.g., ethanol), or genetic differences. However, the observation of ethnicity having an impact on intersubject variation would be more consistent with a genetic contribution and the significant interethnic differences in CYP2E1*1D allelic frequency (McCarver et al., 1998; Hu et al., 1999; Itoga et al., 2002).

Interestingly, after CYP2E1*1D genotype, age and their interaction were included as independent variables; ethnicity was no longer associated with difference in CYP2E1 protein content. Among infants of known gestational age, postnatal age was a stronger predictor of CYP2E1 specific content than gestational age, again consistent with peripartum or postnatal factors playing a dominant role in CYP2E1 ontogeny.

The data demonstrating fetal liver CYP2E1 expression are in contrast to several previous reports that failed to detect fetal liver CYP2E1 protein, mRNA, or catalytic activity (Wrighton et al., 1988; Komori et al., 1990; Jones et al., 1992; Hakkola et al., 1994; Shimada et al., 1996; Vieira et al., 1996). However, the number of fetal tissues in most of these studies was limited (3–16). More importantly, most studies used samples from early gestation, with ages ranging from less than 8 to 24 gestational weeks, with the majority being less than 17 weeks. Comparing these results with those shown in Fig. 1, it is apparent that most of these earlier reports are, in fact, consistent with the data reported herein, as CYP2E1 is not detectable in most individuals' samples until the third trimester, i.e., about 27 weeks. For unknown reasons, the results remain inconsistent with those reported levels generated the greatest degree of intersubject variability, i.e., 80-fold, among tissue samples from the neonatal age group. This contrasts to the roughly 4-fold variation observed in other age groups which, in turn, is more consistent with the intersubject variation observed in adults (McCarver et al., 1998). Such differences also are consistent with the effect of variable protein stabilization by an endogenous substrate (e.g., ketones) or exogenous xenobiotics (e.g., ethanol), or genetic differences. However, the observation of ethnicity having an impact on intersubject variation would be more consistent with a genetic contribution and the significant interethnic differences in CYP2E1*1D allelic frequency (McCarver et al., 1998; Hu et al., 1999; Itoga et al., 2002).

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TABLE 3
Factors associated with greater log-transformed CYP2E1 protein content among 188 human hepatic microsomal samples genotyped for CYP2E1*1D

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age category</td>
<td>52.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(+)-CYP2E1*1D × age category</td>
<td>3.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(+)-CYP2E1*1D</td>
<td>1.9</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

* MANOVA, model r² = 0.74.
* Other variables tested included gender, ethnicity, and post-mortem interval.
by Vieira et al. (1996). Finally, it is somewhat surprising that Carpenter et al. (1996) were able to detect CYP2E1 in all of the 11 fetal livers examined between 16 and 24 weeks’ gestation. In contrast, the data in Fig. 1 show that only 18 of 49 samples within this same age range had detectable CYP2E1 protein levels. Both studies used the same range of microsomal protein concentrations for electrophoretic fractionation and both used the same chemiluminescent detection method. However, it is unclear from the report by Carpenter et al. (1996) whether the 11 samples with detectable CYP2E1 protein represented all of the samples examined, or only the subset that had detectable protein levels. If the latter, then the two reports would be consistent.

Previous studies provided evidence that the onset of CYP2E1 expression during development is largely or entirely controlled by cytokine demethylation at the transcription start site, within exon 1 and intron 1, with CYP2E1 mRNA levels correlating with the degree of demethylation (Vieira et al., 1996). The data presented in the current study indicate that birth does not appear sufficient for the onset of expression, and that expression during the neonatal period is highly variable. Thus, if the demethylation of key residues is critical for transcriptional activation, this would suggest the timing of demethylation varies among individuals. Studies by Carpenter et al. (1996) also suggested that CYP2E1 induction is possible, even in the fetal liver. Although the absence of complete clinical histories precludes a definitive confirmation of this, two of the neonatal samples with outlying CYP2E1 specific content values similar to the greatest adult values (Fig. 3) are consistent with this observation.

There was a small but statistically significant effect of the CYP2E1*1D genotype associated with decreased protein content in the developing liver, but not in liver samples from individuals older than 90 postnatal days. Previous studies have demonstrated an association of this same genotype with increased metabolic ability in obese or alcohol-consuming individuals (McCarver et al., 1998), consistent with an increased inducibility of the gene under these conditions. The mechanism(s) whereby this same variant might suppress CYP2E1 expression during development is unknown. However, it is possible that dietary factors that have an impact on CYP2E1 expression in infants and adults are absent during these earlier developmental stages and in their absence, the control elements encoded by the CYP2E1*1D allele have a suppressive affect on expression. A more definitive explanation and confirmation of biological significance must await a more complete functional analysis of the repeat sequences included in the CYP2E1*1D variant.

This study represents the most extensive analysis of CYP2E1 ontogeny, including a large number of liver samples that covers a broad range of ages from early gestational development to postpuberty. The findings resolve some of the earlier controversy surrounding CYP2E1 expression and provide new insight into the onset of protein expression during the first few months of life. The lower protein levels in this age group would suggest that infants of this age would have significantly lower CYP2E1-mediated fractional metabolic clearance of substrates such as acetaminophen and halothane. For the former, this may contribute to the decreased toxicity observed among young infants, consistent with what has been observed in CYP2E1-null mouse models (Wong et al., 1998; Zaher et al., 1998).

References

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