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Human hepatic CYP2B6 developmental expression: The impact of age and genotype

Edward L. Croom [a], Jeffrey C. Stevens [b], Ronald N. Hines [c], Andrew D. Wallace [a], Ernest Hodgson [a,*]

[a] Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC, United States
[b] Pfizer, Pharmacokinetics, Dynamics, and Metabolism, St. Louis, MO, United States
[c] Department of Pediatrics, Medical College of Wisconsin, and Children's Research Institute, Children's Hospital and Health Systems, Milwaukee, WI, United States

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This paper is dedicated to the late Dr. Randy Rose who initiated this study.

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ABSTRACT

Although CYP2B6 is known to metabolize numerous pharmaceuticals and toxicants in adults, little is known regarding CYP2B6 ontogeny or its possible role in pediatric drug/toxicant metabolism. To address this knowledge gap, hepatic CYP2B6 protein levels were characterized in microsomal protein preparations isolated from a pediatric liver bank ($N = 217$). Donor ages ranged from 10 weeks gestation to 17 years of age with a median age of 1.9 months. CYP2B6 levels were measured by semi-quantitative western blotting. Overall, CYP2B6 expression was detected in 75% of samples. However, the percentage of samples with detectable CYP2B6 protein increased with age from 64% in fetal samples to 95% in samples from donors >10 years of age. There was a significant, but only 2-fold increase in median CYP2B6 expression after the neonatal period (birth to 30 days postnatal) although protein levels varied over 25-fold in both age groups. The median CYP2B6 level in samples over 30 postnatal days to 17 years of age (1.3 pmol/mg microsomal protein) was lower than previously reported adult levels (2.2–22 pmol/mg microsomal protein), however, this likely relates to the median age of these samples, *i.e.*, 10.3 months. CYP2B6 expression did not vary significantly by gender. Furthermore, CYP2B6 levels did not correlate with CYP3A4, CYP3A5.1 or CYP3A7 activity, consistent with different mechanisms controlling the ontogeny and constitutive expression of these enzymes and the lack of significant induction in the pediatric samples.

1. Introduction

CYP2B6 is the only functional member of the human *CYP2B* family and was originally thought to be absent in most individuals. Improved antibody preparations have now demonstrated the presence of immunoreactive CYP2B6 protein in the livers of most adults tested [1], although interindividual differences are among the largest of the cytochromes P450 that have been studied. Thus, a recent meta-analysis reported that CYP2B6 and CYP3A5 had the lowest minimum expression levels (1.0 pmol/mg microsomal protein). However, CYP2B6 exhibited maximum expression levels (45 pmol/mg microsomal protein) comparable to CYP1A2, 2A6, 2C8, and 2E1 (52–68 pmol/mg microsomal protein) and higher than either CYP2C19 (20 pmol/mg microsomal protein) or CYP2D6 (11 pmol/mg microsomal protein) [2]. This conclusion also is consistent with relative abundance levels. The relative abundance of CYP2B6 (0.4–8.4%, 21-fold range) and 3A5 (0.4–22%, 55-fold range) exhibited a much larger range than any of the other cytochromes P450 (all less than 3-fold range). Similar to the CYP2C and CYP3A family members, a portion of the interindividual variability in CYP2B6 expression may be explained by the ability of both the constitutive androstane (CAR, NR1I3) and pregnane X (PXR, NR1I2) receptors to induce CYP2B6 expression several fold in a ligand-dependent manner [3]. Although CYP2B6 is considered primarily a hepatic enzyme, it has been detected at lower levels in several other organs, including the brain [4], kidney and lung [5].

CYP2B6 participates in the oxidative metabolism of numerous pharmaceuticals, including the anti-depressant bupropion [6], the anesthetics propofol [7] and lidocaine [8], the chemotherapeutic agents cyclophosphamide, ifosfamide [9] and tamoxifen [10], the anti-retroviral agent efavirenz [11] and the anti-malarial drug artemesinin [12]. CYP2B6 also plays a role in the metabolism of methadone [13] and the drugs of abuse, nicotine [14] and ecstasy [15]. Environmental contaminants such as styrene [16] and several pesticides, including chlorpyrifos [17] and endosulfan [18] are also excellent substrates. CYP2B6 also plays a role in the metabolism of

* Corresponding author at: Department of Environmental and Molecular Toxicology, Box 7633, NC State University, Raleigh, NC 27695-7633, United States. Tel.: +1 919 515 5295; fax: +1 919 515 7169.
E-mail address: ernest_hodgson@ncsu.edu (E. Hodgson).

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endogenous substrates including the steroid testosterone [19]. Like other members of the cytochrome P450 family of proteins, CYP2B6 can detoxify and facilitate the elimination of toxicants and drugs, however, depending on the chemical properties of the substrate, CYP2B6-dependent oxidation also can increase the toxicity of several compounds, *e.g.*, tamoxifen, aflatoxin B1 [10] and chlorpyrifos [17]. Most important for the subject of this study, purposeful or accidental childhood exposures to all of these compounds have been documented.

CYP2B6 functional polymorphisms have been identified but null alleles are rare. The most clinically relevant polymorphism is the *CYP2B6*6* allele (g.15631G>T, rs3745274; g.18053A>G, rs2279343). The g.15631G>T transversion in the *CYP2B6*6* allele is predicted to result in the loss of a splice enhancer and is linked to the formation of transcript variant predicted to encode a non-functional protein [20]. However, because the *CYP2B6*6* allele exhibits incomplete penetrance, it is associated with reduced protein expression and reduced in vivo metabolism, rather than a complete loss of function [21].

Little is known about CYP2B6 expression during development. An early study of age-dependent CYP2B6 expression found lower levels in infant liver samples when compared to adults [22], but this study included just two fetal samples and eight infant samples. Samples from individuals ranging from 2 to 72 years of age were not sufficient in number to permit a determination of any further temporal changes in CYP2B6 expression.

The objective of this study was to characterize CYP2B6 developmental expression and determine the possible impact of genetic variation on this process. The absence of an in vivo probe for CYP2B6 activity suitable for use in children, the difficulty in collecting pharmacokinetic data from infants, and the ethical issues preventing the study of in vivo fetal CYP2B6 metabolism necessitated the use of post-mortem human liver microsomal samples. Comparing CYP2B6 expression with previously characterized CYP3A levels in the same samples also was performed in an attempt to gain some information regarding possible regulatory mechanisms.

2. Material and methods

2.1. Materials

Polyclonal antibodies against CYP2B6 and lymphoblast-expressed CYP2B6 were purchased from BD Biosciences (San Jose, CA). IR800 dye-labeled goat anti-rabbit IgG antibodies were purchased from LI-COR (Lincoln, NE). Nitrocellulose membranes were supplied by Bio-Rad (Hercules, CA). EZ-Run pre-stained recombinant protein molecular weight markers, methanol and Tris–glycine buffer were obtained from Thermo Fisher Scientific (Waltham, MA). *CYP2B6* single nucleotide polymorphism (SNP) genotyping assays; C_25986767_70 (g.19154G>A, rs4244285), C_7817765_60 (g.15631G>T, rs3745274), C_26201809_30 (g.6986A>G, rs776746), a previously designed custom assay to detect A785G (g.18053A>G, rs2279343) [23] and Taqman Genotyping Master Mix were supplied by Applied Biosystems (Foster City, CA). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Human liver samples

Individual human liver microsomal samples ($N = 217$) were obtained from donors ranging in age from 10 weeks gestation to 17 years of age. The median donor age was 1.9 months. There were 56 prenatal, 39 neonatal (birth to 30 days postnatal age) and 122 samples over 30 days postnatal age. Of the samples over 30 days postnatal age, 63 were less than 1 year of age. The source of pediatric liver samples and the preparation of fetal and postnatal microsomal samples were described previously [24]. DNA samples were prepared as previously described [25]. Individual adult human liver microsomes (HK23) were purchased from BD Biosciences (San Jose, CA).

2.3. Electrophoresis and immunoblotting

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [26] using a Tris–glycine buffer, pH 8.3. Human liver microsomal proteins (10–40 μg per lane) were separated on 10% precast Novex gels and transferred to nitrocellulose membranes using a Transblot apparatus (Bio-Rad). Efficient transfer was verified with Ponceau S staining of membranes. Membranes were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS)/Tween 20 (0.1%, v/v) for 90 min at room temperature and incubated with primary antibodies overnight at 4 °C. Anti-CYP2B6 antibodies (BD Gentest) and anti-actin antibodies (Sigma) were used at 1:500 and 1:1000 dilutions, respectively. After four washes in PBS/Tween 20 (0.1%, v/v), the membranes were incubated with the secondary antibody for 1 h at room temperature in the dark. The secondary antibody, IR 800 labeled goat anti-rabbit IgG (LI-COR), was used at a 1:1250 dilution. Images were captured using the Odyssey Infrared Imaging System (LI-COR), software version 1.2. Lymphoblast-expressed CYP2B6 containing microsomes (BD Biosciences) were used to derive a standard curve using 31, 63, 125, 250 and 500 fmol of CYP2B6 protein. The pre-stained molecular weight markers served as a negative control. An adult human liver microsomal sample, HK23 (BD Biosciences), containing 7 pmol CYP2B6/mg microsomal protein was used as a positive control. The limit of detection was defined as 3-times the background intensity.

2.4. Genotyping

DNA samples were amplified using Taqman SNP genotyping assays and SNP Genotyping Master Mix (Applied Biosystems). Using 96-well optical plates and following manufacturer instructions, DNA and reagents were combined in a hood dedicated to polymerase chair reaction (PCR) set-up. Following an initial denaturation step of 95 °C for 10 min, amplification was performed for 50 cycles of 92 °C for 15 s and 60 °C for 90 s. Reactions were run on a Mastercycler (Eppendorf,Westbury, NY) and after amplification, results were read on a 7300 RT PCR system (Applied Biosystems). DNA free negative controls were used for each experiment. Positive controls were DNA samples from donors previously genotyped using PCR-restriction fragment length polymorphism and chosen to represent variant, referent and heterozygous genotypes respectively.

2.5. Statistical analysis

Linear regression was used to assess the dynamic range of response for the standard curve, and subsequently, to quantitate CYP2B6 levels in microsomal samples. Coefficients of determination (r^2) of 0.90 or above were accepted. Statistical analysis of correlations between CYP2B6 and CYP3A protein levels were performed using JMP version 7.0 (SAS, Cary, NC). Data was log transformed before partitioning analysis was used to search for age groups differing in CYP2B6 protein levels. Logworth values >1.3 and p-values <0.05 were considered significant. Groups were compared with the Mann–Whitney Rank Sum test or Kruskal–Wallis nonparametric ANOVA with a Dunn's post hoc test using SigmaPlot version 11.0 (Systat, San Jose, CA). Two-way ANOVA with a Holm–Sidak post hoc test were used to examine interactions. Box and Whisker plots were created using SigmaPlot.

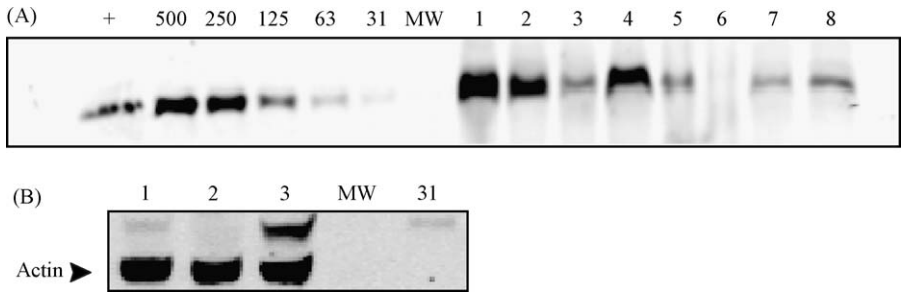

Fig. 1. Detection of CYP2B6 by western blot. (A) A representative western blot showing the relative amounts of CYP2B6 protein from 40 μg microsomal protein in a random set of individual pediatric-human liver microsome preparations. Expressed recombinant CYP2B6 (31–500 fmol/lane) was used to establish a standard curve. A previously characterized adult microsomal protein sample (+) and the molecular weight markers were used as positive and negative controls, respectively. While sample 6 had no detectable CYP2B6, samples 1, 2 and 4 exceeded the limits of the standard curve and were re-run with only 10 μg protein. (B) A comparison of actin and CYP2B6 levels. The arrow indicates the actin protein. CYP2B6 levels ranged from 5 pmol/mg microsomal protein in sample 3 to below the limits of detection (0.25 pmol/mg microsomal protein) in sample 2.

3. Results

3.1. CYP2B6 immunoquantitation

A single immunoreactive protein was detected by western blot in the pediatric microsomal samples that comigrated with lymphoblast-expressed CYP2B6 and the adult positive control sample (Fig. 1A). CYP2B6 protein levels were below the limits of detection (10 fmol/lane, 0.25 pmol/mg protein) in 25% of the microsomal samples, even though Ponceau S staining of the western blot showed even transfer and similar protein levels for all samples. To verify the presence of microsomal protein in the absence of CYP2B6 expression, a small number of blots were incubated with anti-actin antibodies and equivalent amounts of actin were confirmed for each sample (Fig. 1B). Detectable CYP2B6 protein was observed in most samples from donors with ages throughout gestation and the postnatal period examined, although there was considerable interindividual variation (Fig. 2).

To determine whether or not there was any significant change in CYP2B6 expression as a function of age, a partitioning analysis was performed and a break-point identified at the end of the neonatal period, *i.e.* 30 days after birth (log worth value 3.8). Samples from donors with a postnatal age greater than 30 days had median CYP2B6 levels (1.3 pmol/mg microsomal protein, range = 0.0–23.9 pmol/mg microsomal protein) approximately 2-fold higher than samples from younger donors (0.6 pmol/mg microsomal protein, range = 0.0–36.7 pmol/mg microsomal protein) [Mann–Whitney ($p < 0.001$)] (Fig. 3). The percentage of individuals with detectable levels of CYP2B6 protein also increased with age such that CYP2B6 protein was detectable in 64% of the fetal samples ($N = 56$) while after 6 months postnatal age ($N = 67$), 90% of the samples expressed protein levels above the limit of detection. This trend continued with age, as 95% of the samples from donors 11–17 ($N = 20$) years of age had detectable CYP2B6 protein levels (Fig. 4). Both the percentage of samples with detectable CYP2B6 and the median CYP2B6 levels for these samples are significantly lower than reported adult median levels (Table 1). Only a small percentage of samples had CYP2B6 levels above the median adult levels (Fig. 5).

3.2. Genotyping

Somewhat surprisingly, there was no difference in CYP2B6 protein levels between individuals genotyped as *CYP2B6*6/*6* ($N = 16$) versus *CYP2B6*1/*1* ($N = 104$) in either of the age groups (fetal to 30 days postnatal age versus older than 30 days) (Fisher's Exact Test).

3.3. Correlation of CYP2B6 and CYP3A levels

Because of some shared regulatory mechanisms reported in adults [27], CYP2B6 levels were compared to the levels of CYP3A7,

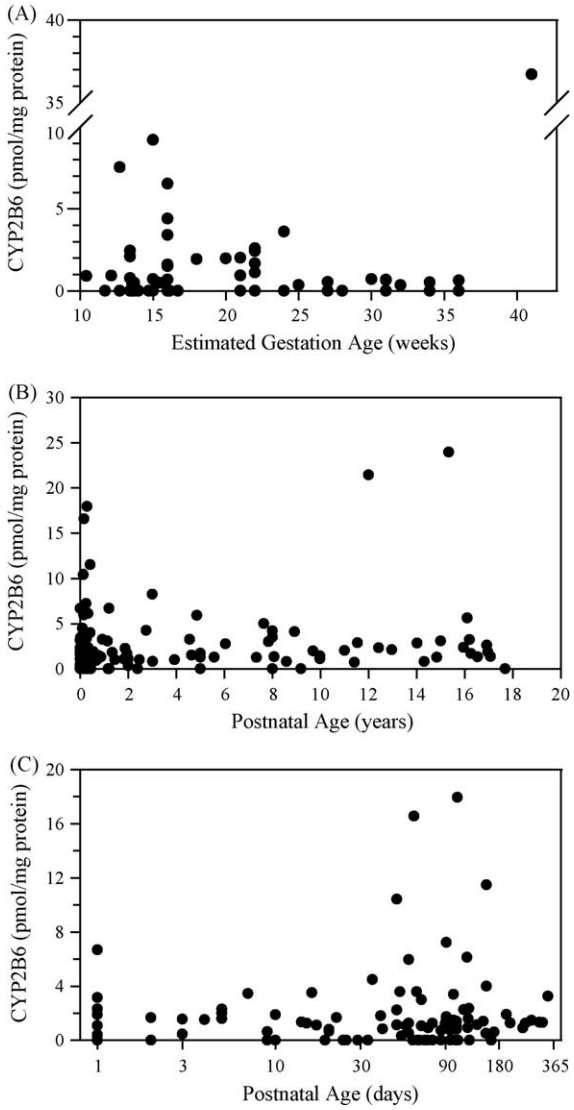

Fig. 2. CYP2B6 expression in individual tissue samples is shown for (A) prenatal samples, (B) postnatal samples and (C) samples from birth to 1 year of age. Note that the postnatal age for graph (C) is on a log scale to better display the changes in CYP2B6 expression after 30 days of age.

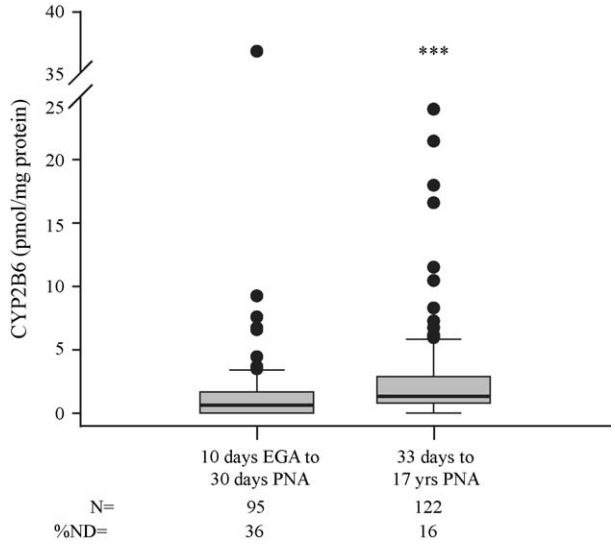

Fig. 3. Age brackets were chosen based on partitioning analysis using 217 samples. Boxes represent the interquartile values. The line represents the median value and the whiskers represent the 10th and 90th percentile values. Individual data points represent outliers (1.5-times the interquartile values). EGA = estimated gestational age, PNA = postnatal age.

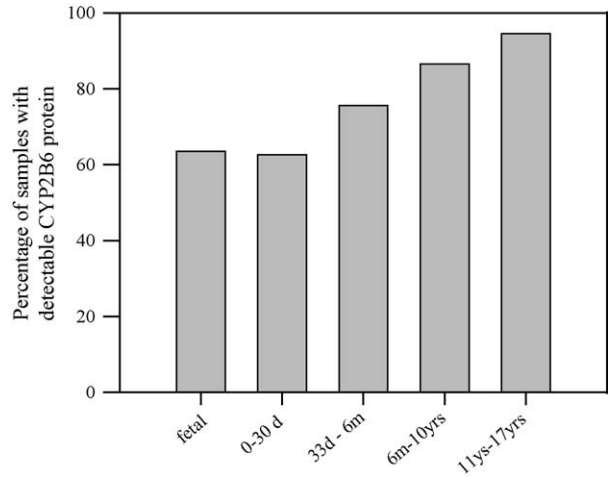

Fig. 4. Percentage of samples with detectable CYP2B6 protein. The percentage of samples in the indicated age groups with detectable levels of CYP2B6 is shown. Fetal ($N = 56$), 0–30 days postnatal ($N = 39$), 31 days to 6 months ($N = 55$), 6 months to 10 years ($N = 47$), 11–17years ($N = 20$).

CYP3A5 and CYP3A4 determined in a previous study using these same tissue samples [28]. No correlation was observed between CYP3A4, CYP3A5 or CYP3A7 and CYP2B6 expression levels (Fig. 6).

3.4. Correlation of CYP2B6 levels with ethnicity and sex

CYP2B6 expression was compared among African American ($N = 82$), European American ($N = 93$) and Hispanic American samples ($N = 18$). Other ethnic groups were represented by too few samples for analysis. Samples from African American donors were found to have lower levels of CYP2B6 than European American samples [Kruskal–Wallis, with Dunn's post hoc ($p < 0.05$)]. However, this may be explained by the observation that African American donors had a significantly lower median age (1.7 months) than European American donors (3.4 months) [Mann–Whitney ($p < 0.05$)]. CYP2B6 expression did not differ significantly between females ($N = 77$) and males ($N = 132$) [Mann–Whitney ($p < 0.90$)]. Finally, CYP2B6 levels did not correlate with post-mortem intervals (time between death and freezing of tissue samples), consistent with previous reports on other enzymes using these same samples [24,25,28,29].

4. Discussion

In an earlier report, CYP2B6 protein was detected in only 2 of 10 liver microsomal samples from donors >37 weeks gestation but <10 months of age (mean ± SD = 2.7 ± 5.9 pmol/mg microsomal protein), but was found in 7 of 10 samples from donors ranging in age from 2 to 72 years (mean ± SD = 19.4 ± 23.9 pmol/mg microsomal protein) [22]. Although this same trend was observed in the current study, CYP2B6 levels above the limit of detection were observed in 64% of all fetal ($N = 56$) and samples from birth to 30 days postnatal age ($N = 39$) and increased to nearly 95% of samples with detectable CYP2B6 protein in samples from donors between 11 and 17 years of age ($N = 20$) (Fig. 4). The more frequent detection of CYP2B6 in the current study probably is due to a much larger sample size and a lower limit of quantification based on the availability of improved antibody preparations.

Among those samples with detectable CYP2B6 levels, expression varied approximately 75-fold in the samples over 30 days postnatal age and 25-fold in the samples younger than 30 days postnatal age once a fetal outlier with 4-fold greater expression than any other sample in that age group was removed from the analysis. This fold variation is considerably less than that reported in an earlier meta-analysis [2]. Also, studies using adult samples have reported a maximum CYP2B6 level 2–4-times higher than the maximum CYP2B6 level observed in the current study of pediatric liver samples (Table 1). The lower levels of CYP2B6 observed in these pediatric samples is likely due to the purposeful weighting of the sample set to the period from birth to 1 year of age (median age of all postnatal samples = 3.5 months, median age of samples older than 30 days postnatal = 10.3 months), although fewer exposures to agents known to induce CYP2B6 expression cannot be ruled out as contributing to this observation. Consistent with the latter premise, several earlier studies included in the meta-analysis described in Table 1 incorporated data from donor samples known to be exposed to CYP2B6 inducing agents, such as phenytoin and alcohol [1,30]. Also consistent with the latter premise, the lack of correlation between CYP2B6 and CYP3A4 expression in the postnatal samples and the lower degree of interindividual variation would be consistent with the observed CYP2B6 levels representing constitutive rather than induced expression.

CYP2B6 levels increased with age with partitioning analysis revealing a significant approximate 2-fold difference between

Table 1
Comparisons of CYP2B6 hepatic expression.

Detection (%)	pmol/mg protein median (range)	Age in years median (range)	Variability	References
107/112 (96)	4 (0.3–82)[a]	41 (2.5–71)	273-fold	Meta-analysis[b]
101/122 (83)	1.6 (0.3–23.9)[a]	0.85 (0.09–17.7)	74-fold	Current study
60/95 (63)	0.6 (0.3–9.2)[a,c]	Fetal (fetal–0.08)	28-fold	Current study

[a] Values for samples with detectable levels of CYP2B6 protein.
[b] Code et al. [10], Ekins et al. [19], Stresser and Kupfer [1], Gervot et al. [32].
[c] Without a 36 pmol/mg outlier.

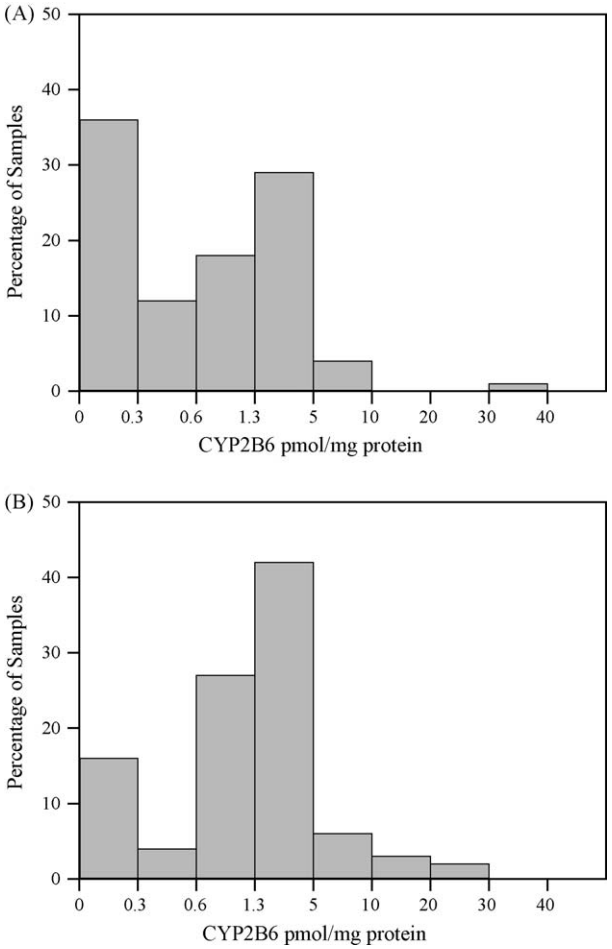

Fig. 5. Distribution of CYP2B6 expression levels. The percent of samples with various levels of CYP2B6 expression levels in fetal/neonatal samples (10 weeks gestation to 30 days postnatal age) (A) and samples older than 30 days postnatal age (B) is displayed in histograms. An expression level less than 0.3 pmol/mg microsomal protein was below the limit of detection. Samples with over 5 pmol CYP2B6/mg microsomal protein had levels above median adult CYP2B6 levels (Table 1).

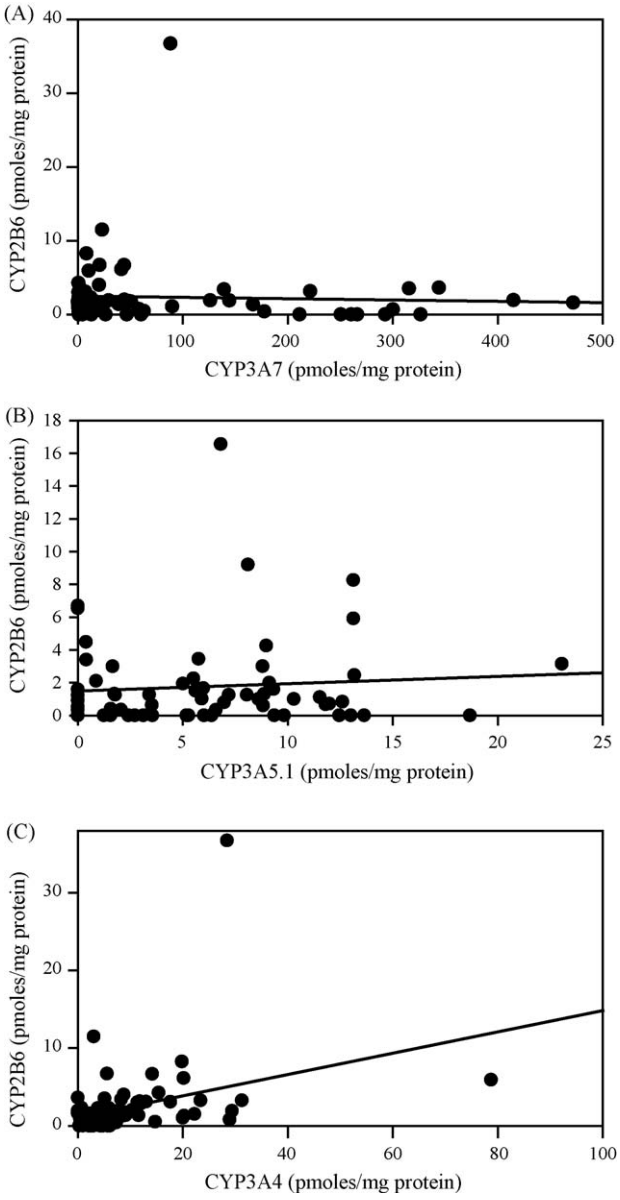

Fig. 6. Correlation between CYP2B6 and CYP3A expression. The correlation between CYP2B6 and CYP3A5*1 (A), CYP3A7 (B) or CYP3A4 (C) expression in individual samples is depicted.

CYP2B6 levels in the samples from younger donors (10 weeks gestation to 30 postnatal days) versus those from older donors (>30 days to 17 years). A similar increase around 1 month after birth has been reported for other enzymes (*e.g.*, CYP2E1), although the magnitude of this increase appears greater for many of the other enzymes studied [25].

Induced CYP2B6 expression in adult humans is controlled in part by one or more nuclear receptors, including the PXR (NR1I2), CAR (NR1I3) and glucocorticoid receptor (NR3C1) [3,27]. Regulation by these receptors and their activating ligands likely explains the observation that while some individuals have less than 1 pmol/mg microsomal protein of CYP2B6 present in their livers [19,20,30,31], CYP2B6 levels can be over 350-fold higher in other individuals [20,30]. These same nuclear receptors also can induce CYP3A4 expression, but the relative capacities of different ligands to induce CYP2B6 and CYP3A4 varies [27]. The expression of CAR or PXR proteins in human fetal liver is unknown, but it is possible as both CAR and PXR transcripts have been detected in both fetal and pediatric liver samples [32]. A single study has addressed possible age-dependent changes in CAR expression wherein CAR transcripts and protein were detected in neonatal (birth to 30 days postnatal age) samples at lower levels relative to adults [33]. However, this study was underpowered in that only three neonatal samples were used to make this comparison. Work with human hepatocytes has shown the ability of phenobarbitol and rifampicin to induce CYP2B6 expression in individuals as early as 2 and 3 years of age (Rose, unpublished results)[27], suggesting functional PXR and/or CAR nuclear receptors by at least this age. However, for the reasons discussed above, it would appear induction by these receptors contributed little or none to the differences in CYP2B6 expression reported in the current study. Only 10% of the older samples (>30 days to 17 years) and 5% of younger samples (10 weeks gestation to 30 days postnatal) had CYP2B6 levels above 5 pmol/mg (Fig. 5) providing further evidence that a change in constitutive CYP2B6 expression was involved in the age-related increase in median CYP2B6 levels.

CYP2B6 is highly polymorphic, but only rare alleles have been reported to eliminate expression. The *CYP2B6*6* allele has clear clinical relevance for substrates such as efavirenz and neverapine and has been shown to impact the metabolism of both compounds in children [34,35]. The explanation for a failure to observe an impact of the *CYP2B6*6* allele in the current data set is puzzling, but

may be explained by an age-dependent impact of the affected splicing enhancer on transcript processing.

CYP2B6 expression did not differ significantly by sex in the pediatric tissue samples. While sex differences in CYP2B expression have been well documented in laboratory animals [36], such sexual dimorphic patterns in gene expression are more common and quantitatively greater in rodent species relative to the human (*e.g.*, CYP2C, FMO3). Sex differences in CYP2B6 levels have been reported. However, these differences may be confounded by exposures to CYP2B6 inducers and the small sample sizes utilized. For example, it has been reported that Hispanic females have higher CYP2B6 expression, but only three female and four male donors were used to infer that conclusion [37]. Other studies are consistent with the observations in the current study and have failed to find significant differences between males and females [20]. However, the paucity of samples older than 11 years of age (*i.e.*, puberty) limits this conclusion.

Predicting effective doses for CYP2B6 therapeutic substrates can be difficult because of the highly inducible and polymorphic nature of the enzyme and potential complications resulting from both higher and lower CYP2B6 activity are a concern, including for the pediatric patient population [34]. For example, a pediatric patient receiving ifosfamide developed seizures after taking phenytoin and based upon a metabolic profile, drug-induced CYP2B6 induction was a suspected cause [38]. In another example, a reported childhood case of efavirenz-induced psychosis was suspected of being linked to the patient possessing a single copy of the *CYP2B6*6* allele [35]. Although a direct causal link to CYP2B6 was not made in either of these case-reports, these examples highlight the need to better understand and predict CYP2B6 metabolic ability. Such a need extends to a better understanding of CYP2B6 ontogeny, which is at least partially addressed in the current study. Thus, the ability to express CYP2B6 appears to exist throughout development for most individuals, although there appears to be considerable interindividual variability as to when the onset of expression is observed. Further, there is a modest, but significant increased level of CYP2B6 expression after the neonatal period (>30 days postnatal age). The large individual variability in expression observed in adults was less apparent among pediatric samples, although this was likely due to the lack of CYP2B6 induction in most of the samples studied. Importantly, while these data clearly indicate the ability of older children to metabolize CYP2B6 substrates, consistent with in vivo case-reports, (*e.g.*, [35]), these results also suggest that many infants, neonates and fetuses also may possess the ability to catalyze CYP2B6-dependent oxidation reactions.

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