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Developmental regulation of flavin-containing monooxygenase form 1 in the liver and kidney of fetal and neonatal rabbits

Shelley A. Larsen-Su[a], Sharon K. Krueger[a], Mei-Fei Yueh[a,1], Clifford B. Pereira[b], David E. Williams[a,*]

[a]*Department of Environmental and Molecular Toxicology, The Linus Pauling Institute, Oregon State University, 1007 ALS, Corvallis, OR 97331, USA*
[b]*Department of Statistics, Oregon State University, Corvallis, OR 97331, USA*

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Abstract

Flavin-containing monooxygenases (FMOs) comprise a multi-gene family and catalyze the oxygenation of soft nucleophilic sulfur, nitrogen, phosphorus, and selenium in xenobiotics. Previous studies have demonstrated that FMO is regulated developmentally and by the administration of certain steroid hormones. This study examined the expression of FMO form 1 in the livers and kidneys of fetal and neonatal rabbits, from day 25 of gestation through 3 weeks of age, by assaying FMO1 mRNA and protein levels, as well as catalytic activity. FMO1 mRNA and protein expression and FMO catalytic activity were present in fetal livers at the earliest time point measured (day 25 of gestation), although at levels approximately 10% of that found in adult livers. Hepatic FMO1 mRNA levels increased during and after gestation; levels were not significantly different from those measured in adult male livers. FMO1 protein content and activity rose rapidly after birth to reach 70–80% of adult levels by 3 weeks of age. The expression of FMO1 in fetal and neonatal kidneys was markedly lower than in liver. FMO1 mRNA levels never averaged more than 3.4% of adult male liver levels, but did not differ from adult kidney levels at any of the points measured. Protein levels and enzyme activity rose significantly after birth to approximately 30% of the level in adult kidneys by 3 weeks of age. The early developmental appearance of FMO1 suggests a possible role in the metabolism of xenobiotics through transplacental or lactational exposures.

Keywords: Flavin-containing monooxygenase; FMO1; Developmental regulation; Drug-metabolizing enzymes; Fetal/neonatal development

1. Introduction

FMOs are located in the endoplasmic reticulum of most mammalian cells, and catalyze the NADPH-dependent oxidation of a wide variety of compounds containing nitrogen, sulfur, phosphorus, and selenium [1–3]. This gene family [4–6] has six members (FMOs 1–6) and, as is the case with the CYP-dependent monooxygenases, they display a broad substrate specificity. Oxygenation occurs at soft nucleophilic heteroatoms contained in numerous drugs, pesticides, and other xenobiotics [1]. Although FMO isoforms are distributed widely in mammalian tissues, relatively little is known of the importance of FMOs in human drug metabolism compared with the much-studied CYP monooxygenase system [7]. FMOs are known to metabolize several endogenous substrates, including cysteamine, methionine, and trimethylamine, and it has been hypothesized that FMOs evolved to metabolize alkaloids and other plant materials in the mammalian diet [3]. In most cases, FMO oxygenation of drugs and xenobiotics represents a detoxication pathway; however, a few instances of bioactivation have been described [8].

One characteristic that distinguishes the flavin and CYP monooxygenases is that the FMOs are refractory to dietary or chemical induction. FMOs do appear to be developmentally regulated, and are responsive to the action of certain steroid hormones [9–18]. FMO1 in maternal rabbit liver is induced during pregnancy or by the administration of dexamethasone or progesterone [16]. There are other examples of FMO induction by exogenous glucocorticoids

* Corresponding author. Tel.: +1-541-737-3277; fax: +1-541-737-5077.
E-mail address: david.williams@orst.edu (D.E. Williams).
[1] Present address: Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093, USA.

Abbreviations: FMO, flavin-containing monooxygenase; CYP, cytochrome P-450; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; AcTC, acetylthiocholine; DTNB, 5,5-dithiobis-[2-nitrobenzoate]; DPTU, diphenylthiourea; TC, thiocholine.

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and sex steroids and sex-dependent expression of different isoforms [19–26]. In contrast to induction, our laboratory has characterized a marked down-regulation of FMO1 expression in the livers and small intestines of male rats fed the phytochemical indole-3-carbinol from cruciferous vegetables [27].

FMO1 is the major liver isoform of most adult mammals, the exception being primates and females of certain strains of mice [25,28,29]. Primate and mouse fetal livers express FMO1, but at some point after birth hepatic FMO1 expression is repressed and FMO3 becomes the dominant isoform expressed. FMO1 remains the dominant isoform expressed in the kidneys of adult primates [30]. To date, the mechanisms for the control of this isoform switching are unknown. Although gender-dependent expression of hepatic FMO1 occurs in mouse and rat, no apparent sex difference has been observed in adult rabbit liver; however, female adult rabbits do express slightly higher levels of FMO1 in the kidneys than do male rabbits [31]. In humans, there does not appear to be any sex difference in FMO1 expression.

In this study, we examined the developmental expression of FMO1 mRNA, protein, and catalytic activity in the livers and kidneys of fetal and neonatal rabbits. FMO1 expression in the liver was readily apparent at the earliest time point measured (day 25 of gestation). Following parturition, FMO1 levels continued to rise until the last time point measured, 21 days of age at which point the kits were weaned. The expression of hepatic microsomal FMO1 at 21 days of age was 70–80% that of the adults. FMO1 levels in the fetal kidney followed a similar pattern of expression. At 21 days of age, the kidney microsomal FMO1 level in the kits was 50% that of the adult kidney and 35% that of the adult liver. RNA measurements from livers and kidneys, which were characterized by a high degree of inter-individual variability, did not differ significantly from adult levels by the time the kits were 21 days of age.

The early developmental appearance of FMO1 in rabbit liver and kidneys suggests a potential role in the metabolism of xenobiotic compounds through transplacental or lactational exposure.

2. Materials and methods

2.1. Chemicals

G6P, G6PDH, $NADP^+$, catalase, AcTC, thiourea, benzylimidazole, and DTNB were purchased from the Sigma–Aldrich Chemical Co. Highly purified DPTU was a gift from Dr. Daniel M. Ziegler (University of Texas at Austin). TC was prepared by the method of Guo and Ziegler [32].

2.2. Animals

Timed-pregnant New Zealand white rabbits were obtained from the Rabbit Research Institute at Oregon State University, and housed individually in birthing cages in the Laboratory Animal Resource Center. The rabbits were maintained on a 12-hr day–night cycle with rabbit chow and water provided *ad lib*. Three rabbits were killed by CO_2 asphyxiation on days 25, 28, and 31 of gestation and postpartum days 1, 8, and 21. The livers and kidneys were removed, quick-frozen in liquid nitrogen, and stored at -80° until analysis. Due to the small tissue-size, fetal tissues were pooled from each litter, as were the tissues from 1-day-old kits. The liver and kidney tissues from 8–21-day-old kits were analyzed separately, but results were averaged over individuals within each mother for statistical analysis. The Oregon State University Animal Care and Use Committee approved the protocols used in this study.

2.3. Microsome preparation

Fetal and neonatal liver tissues were thawed rapidly on ice in 4 vols . of homogenization buffer [0.1 M potassium phosphate (pH 7.25), containing 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 20 μM butylated hydroxytoluene] and homogenized with a polytron (Brinkmann). Microsomes were prepared by differential centrifugation as described by Guengerich [33]. Microsomal protein was assayed by the method of Lowry *et al*. [34] with bovine serum albumin as the standard.

2.4. FMO catalytic activity

FMO1 activity in microsomes was assayed at 37° by a modification of the method of Guo and Ziegler [32], which followed the DPTU-dependent oxidation of TC, utilizing the reaction conditions previously described by Larsen-Su and Williams [27]. Initial reaction conditions were as follows: 0.1 M potassium phosphate (pH 7.5), 0.4 mM EDTA, 0.25 mM $NADP^+$, 2.5 mM G6P, 1 U/mL of G6PDH, 100 μM DPTU, 100 U catalase, and 2 mM benzylimidazole. The total assay volume was 2.5 mL.

2.5. Western blots

Proteins were resolved by SDS–PAGE [35] and transferred electrophoretically to nitrocellulose [36]. The reagents and equipment employed were purchased from Bio-Rad. Western blotting was performed utilizing a semi-dry transfer cell. FMO1 protein was visualized by staining with primary antibody to the porcine FMO1 raised in rabbits followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Purified porcine FMO1 was used as the standard. Both the primary antibody and purified FMO1 were gifts from Dr. Daniel M. Ziegler. A standard curve of purified FMO1 was utilized for quantification on each individual blot. Quantification was performed by visualization with an ECL chemiluminescence kit from the Amersham

Corp. Densitometry of autoradiograms was accomplished using an HP ScanJet IIcx/T flatbed scanner, employing NIH Image, version 1.54 software (public domain, available from NIH at rsbweb.nih.gov).

2.6. Total RNA isolation, detection and quantification

Total RNA was isolated by a single-step procedure [37] using TriReagent (Molecular Research Center) and was stored at -80° until analysis. RNA concentrations were estimated from the A_{260}. RNA was separated electrophorectically on formaldehyde–agarose gels, and 18S and 28S rRNA bands were examined for lack of degradation to confirm RNA integrity.

DNA from a full-length (1641 bp) rabbit FMO1 cDNA clone was double-digested with *Nco*I/*Eco*RI yielding 689 and 952 bp fragments. A 952-bp fragment (containing 915 bp FMO1 encoding AA 231–535 of the cDNA) was isolated as previously described [38]. The purified cDNA was labeled [39] with digoxigenin-11-dUTP using random primers and was quantified according to the instructions of the manufacturer (Roche). Before probing RNA blots, lack of cross-hybridization with FMO2 was confirmed by hybridization of the FMO1 probe with dot blots containing 1 ng of FMO2 cDNA and FMO1 cDNA at concentrations ranging from 0.0001 to 1 ng. Although we did not perform an exhaustive analysis, given that the sequence identity between all rabbit FMO isoforms is similar (52–57% identity [40]), this lack of cross-reactivity with FMO2 suggests that it would be unlikely that the FMO1 probe would cross-react significantly with any FMO isoform.

RNA concentrations were normalized based on amounts of 16S rRNA. A 15-mer antisense oligonucleotide (obtained from Dr. Steven Giovannoni, Oregon State University) universally complementary to 16S rRNA isolated from eubacteria, archaebacteria, and eukaryotes [41] was 3′-end labeled with digoxigenin-11-dUTP and quantified according to the instructions of the manufacturer (Roche). The probe, designated 1406R, was used at a concentration of 5 pmol labeled:100 pmol unlabeled oligonucleotide per millilitre of hybridization buffer.

Total RNA was blotted onto Hybond N^+ Nylon membranes (Amersham Pharmacia) and fixed by UV cross-linking and baking. Hybridization and detection of mRNA levels were performed by the method of Engler-Blum *et al.* [42], with modifications [31]. Chemiluminescence emissions were measured by densitometry of Hyperfilm MP (Amersham Pharmacia) exposed to the blots, using the same equipment and software as for the western blots. Total RNA from liver tissue of an adult male rabbit was blotted at a range of concentrations on each membrane as a standard and was used in linear regression analysis to fit density to RNA concentrations, and in combination with 1406R to normalize the density for variations in RNA sample loading.

2.7. Statistical analysis

For all analyses it was assumed that the use of common standards and calibration curves removed any potential membrane effects (western blots and RNA), so that one-way ANOVA and one-way rank tests could be used. With one exception, for each response (RNA, protein, and enzyme activity level) and for each organ (liver and kidney) gestation times were compared using ANOVA followed by *t*-tests to compare adjacent time points. The exception was the enzyme response for the kidney where an overall test of gestation time differences was conducted by a non-parametric test (exact Kruskal–Wallis test) because responses were below detection limits for all but two developmental times. For the RNA response, three adult livers could be compared to the liver and kidney data from the developing rabbits by using Dunnett's procedure for all pair-wise comparisons to a control. ANOVA-based analyses were conducted on the log transformed data because graphs and diagnostics indicated a constant coefficient of variation (standard deviation divided by mean) and skewness on the original scale. The relationships between the FMO1 RNA, protein, and enzyme activity level were explored with graphs and linear regression within each organ. All analyses were done with the SAS System for Windows, version 8.01 [43].

3. Results

3.1. Fetal and neonatal liver FMO1 levels

Results of the overall ANOVA indicated that the FMO1 content measured at different gestation times changed significantly during development, by all methods used to measure FMO ($P < 0.002$ by each response). FMO1 mRNA was readily detected at the earliest time point measured, day 25 of gestation, where it was 12% of the mean level observed in adult male rabbit liver (Fig. 1). By day 28 of gestation, FMO1 mRNA levels had risen approximately 3.4-fold and remained at this level through 8 days following birth. At 21 days after birth, the average FMO1 mRNA level had increased to 135% of the mean adult male liver level. The increase in FMO1 mRNA between days 25 and 28 of gestation was significant ($P = 0.004$), as was the increase between days 8 and 21 post-partum ($P = 0.012$). Only the FMO1 mRNA levels measured on day 25 of gestation were significantly lower ($P = 0.005$) than in the adult male liver.

FMO1 protein content, as determined by western blotting with polyclonal antibody to porcine FMO1, also increased during development (Fig. 1). The FMO1 protein content on day 25 of gestation was approximately 7% of the adult level. The FMO1 content increased significantly ($P \leq 0.003$) between days 28 and 31, days 1 and 8 post-partum (pp), and days 8 and 21 pp. Upon weaning at

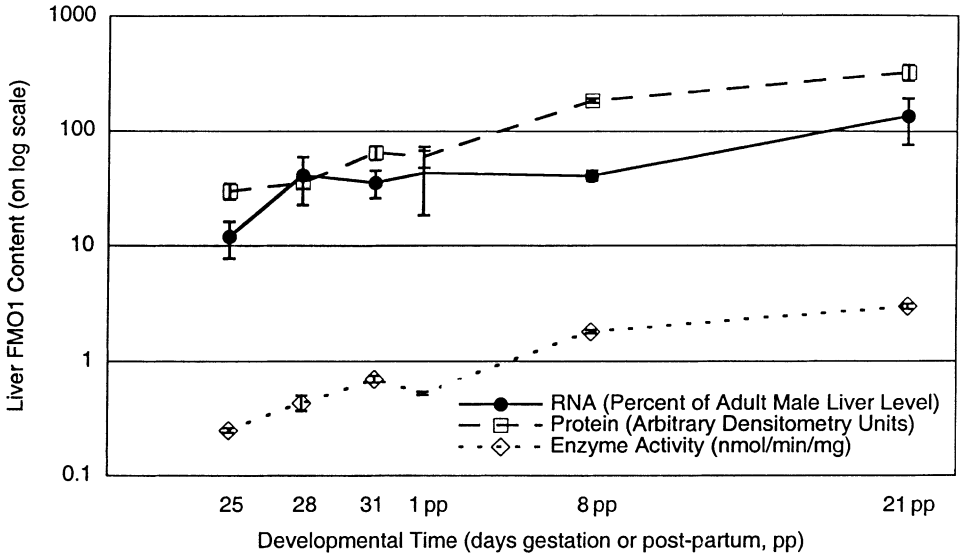

Fig. 1. FMO1 levels in fetal and neonatal rabbit livers. Fetal and neonatal FMO1 levels were determined for each developmental time point by mother, from either pooled liver samples (fetal and 1-day-old kits from three mothers per day) or the average response from individuals within each mother (8- and 21-day-old kits from two mothers per day; 7 and 11 kits, respectively). FMO1 levels shown by day are the mean and standard deviation of the pooled and averaged individuals. FMO1 mRNA levels were determined from 10 μg total RNA. Results are reported as the percent of the mean level quantified from liver RNA of three adult male rabbits. FMO1 protein levels were determined by densitometry of western blots probed with antibody to porcine FMO1. Results shown were measured as arbitrary densitometry units. The catalytic activity was determined as DPTU *S*-oxygenation. Each sample was assayed in triplicate, and results determined as nmol min^{-1} mg^{-1}.

21 days of age, the hepatic FMO1 protein levels were 70% that found in adult liver.

The catalytic activity of fetal and neonatal liver FMO1 was determined by measurement of the *S*-oxygenation of DPTU. Low but detectable activity was measured at 25 days of gestation. The FMO1 level was significantly different at each successive developmental time examined ($P \leq 0.002$). This reflected a steady increase in FMO1 content between all successive time points except between day 31 of gestation and 1 pp when levels declined (Fig. 1). At the onset of weaning at 21 days of age, the FMO catalytic activity had also reached 70% of the adult liver level.

All three measurements of FMO1 content demonstrated that significant increases occur during development in liver tissue. While the correlation between each of these measurements was significant ($P \leq 0.002$), the correlation coefficient of RNA content with either protein content or enzyme activity was not high ($R^2 = 0.53$ and 0.58, respectively). By contrast, protein content and enzyme activity were highly correlated ($R^2 = 0.99$).

3.2. Fetal and neonatal kidney FMO1 content

FMO1 mRNA was detected in all samples of fetal and neonatal kidneys and at all time points (Fig. 2); however, the levels were markedly lower than in the liver (the pooled fetal kidney samples from day 25 of gestation were lost prior to analysis). Levels ranged from 0.4 to 3.4% of adult male liver levels. The low mRNA levels required lengthy film exposures, and data from most time points were characterized by high inter-individual variation. Although on the average the FMO1 mRNA content in the kidney samples increased nearly 3-fold between day 28 of gestation and 21 days after birth, there was no evidence of a statistical difference between gestation times ($P = 0.098$). FMO1 levels in the developing kidney were not statistically different ($P > 0.11$) from either adult male or female kidney FMO1 levels (data not shown).

In fetal kidney, FMO1 protein levels were low but detectable on day 28 of gestation and increased significantly ($P < 0.001$) during development (Fig. 2). FMO1 protein levels remained consistently low until 1 day post-partum ($P > 0.116$). However, after birth, levels rose significantly ($P < 0.001$), with the mean level of FMO1, 8 days post-partum, nearly 5-fold higher than levels 1 day after birth. FMO1 levels increased ($P = 0.051$) nearly 2-fold between 8 and 21 days post-partum, at which point the FMO1 protein levels in the kidneys of the neonates were approximately 33% those of adult kidneys (data not shown).

The catalytic activity of FMO1 in fetal and neonatal kidneys was assayed by the *S*-oxygenation of DPTU. The activity was below the limit of detection (0.2 $nmol^{-1}$ min^{-1} mg) in fetal kidney and in 1-day-old kits (Fig. 2). The activity was readily apparent at 8 and 21 days post-partum and increased significantly during this interval ($P < 0.001$), with the specific activity reaching about 25% that of the adult kidney (data not shown).

While our measurements of FMO1 protein content and enzyme activity demonstrate that significant increases occur during development in kidney tissue, the test for a

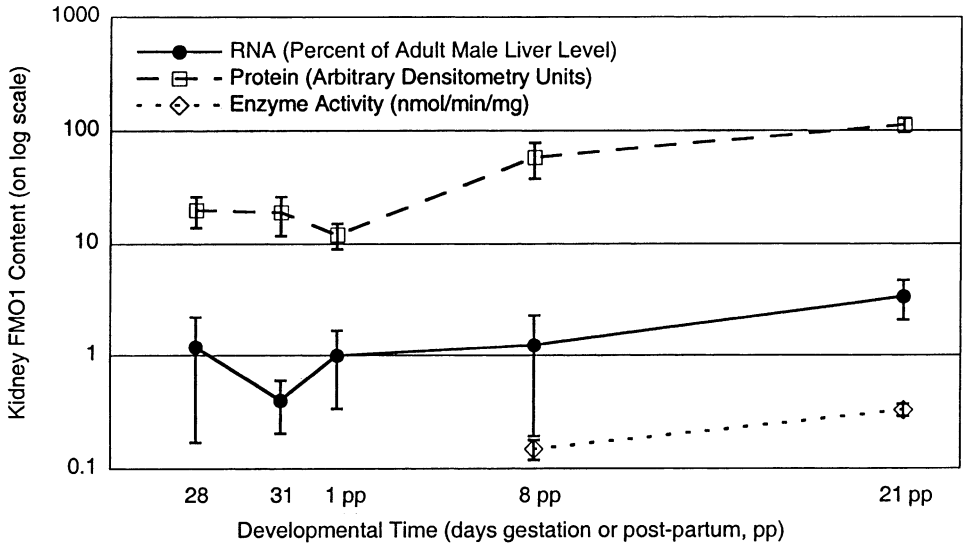

Fig. 2. FMO1 levels in fetal and neonatal rabbit kidneys. Fetal and neonatal FMO1 levels were determined for each developmental time point by mother, from either pooled kidney samples (fetal and 1-day-old kits from three mothers per day) or the average response from individuals within each mother (8- and 21-day-old kits from two mothers per day; 7 and 10 kits, respectively). FMO1 levels shown by day are the mean and standard deviation of the pooled and averaged individuals. FMO1 mRNA levels were determined from 10 μg total RNA. Since adult male kidney FMO1 mRNA levels were too low to be useful for normalization (not shown), results are reported as the percent of the mean level quantified from liver RNA of three adult male rabbits. FMO1 protein levels were determined by densitometry of western blots probed with antibody to porcine FMO1. Results shown were measured as arbitrary densitometry units. The catalytic activity was determined as DPTU *S*-oxygenation. Each sample was assayed in triplicate, and results determined as nmol min^{-1} mg^{-1}. No enzyme activity was detected until 8 days after birth.

significant rise in FMO1 RNA was not met. Although the correlation between each of these measurements was significant ($P \leq 0.006$), the correlation coefficient of RNA content with either protein content or enzyme activity was low ($R^2 = 0.55$ and 0.52, respectively). Protein content and enzyme activity, measured from the same microsomal protein preparations, once again, were highly correlated ($R^2 = 0.97$).

4. Discussion

FMO1 expression as determined by mRNA and protein levels, and DPTU *S*-oxygenation was readily detectable in the livers and kidneys of fetal rabbits. Mean FMO1 mRNA levels were as high as adult levels by day 28 of gestation, although there was a great deal of variability in the levels measured. Measurements of FMO1 protein levels and DPTU *S*-oxygenation were typically at levels appreciably lower than in adults. FMO1 expression increased gradually in fetal livers and kidneys during late gestation, but the most marked increase was between 1 and 3 weeks of age. This pattern of expression is markedly different from that of FMO2 in rabbit lungs [18] in which expression during late gestation approached adult levels. This was followed by a marked repression of expression immediately after birth with subsequent recovery of FMO2 to levels found in adult lungs by 3 weeks of age.

The data reported here are consistent with measurements of *N*-oxidation of *N*,*N*-dimethylaniline in neonatal rabbit liver microsomes in which activity at 30 days of age was approximately 80% that of the adult [11]. The rabbit resembles the human in not exhibiting a marked sex difference in FMO1 expression in liver, as is the case in rat and mouse. However, hepatic developmental expression of FMO1 in fetal and neonatal rabbits differs from humans in that the repression of FMO1 expression following birth and the induction of FMO3 (the human pattern [44]) do not occur.

Developmental control of tissue- and isoform-specific FMO expression is not well-understood. It appears that the control of FMO1 may be at the level of transcription, with higher estimates of mRNA levels preceding comparable estimates made from protein content. However, the alterations in protein and catalytic activity in fetal and neonatal rabbit livers were only weakly correlated with FMO1 mRNA expression levels, probably due in large part to, the high variation among mRNA estimates. The laboratory of Dr. Ronald Hines has documented that the rabbit *FMO1* gene has two promoters, a major promoter that initiates transcription from exon 0 and a minor one that initiates transcription at exon 1 [45,46]. The 5′ upstream region of the rabbit *FMO1* gene does not contain a TATA box or GC-rich regions, but at least three positive regulatory and two negative regulatory regions have been characterized. Consensus binding sequences include those for HNF-1, HNF-3, HNF-4, and C/EBP, but the relative role of these transcription factors in regulating *FMO1* gene expression is currently unknown.

Certainly, one of the most intriguing observations with respect to the developmental regulation of FMOs is the shift that occurs shortly after birth (in primates and females

of certain mouse strains) in the expression of FMO1 as the major hepatic FMO to FMO3. FMO1 remains the major FMO in human kidney. As FMO1 and FMO3 have a number of distinct properties with respect to substrate specificity, this isoform shift could have important implications in fetal versus adult drug metabolism. A similar shift occurs with respect to expression by members of the CYP3A sub-family. CYP3A7 is the major form expressed in human fetal liver, but begins to decline shortly after birth and is absent in adult liver. Conversely, CYP3A4, the major isoform in adult human liver, is barely detectable in the fetus, but begins to increase following birth [47].

In the rabbit, no repression of FMO1 expression in liver occurs. FMO1 expression and activity are relatively high, compared with CYP in these early life stages. In the rabbit, CYP1A1 and 1A2 are not expressed in fetal liver, but can be induced [48]. The expression of a major constitutive CYP, 3A6, occurs later (day 30 of gestation) than the onset of FMO1 expression in fetal liver. For this reason, compared to the adult, FMO may play a relatively more important role in drug and xenobiotic metabolism in the fetus and newborn. As is the case with CYP, the levels of liver and kidney FMO1 increase following birth with a marked increase coincident with weaning. This developmental pattern is similar to that observed with CYP1A1, 1A2, and 3A6 in rabbit liver [48]. One could postulate that such an enhanced expression of CYPs and FMOs would provide the animal with an advantage with respect to the metabolism of plant alkaloids and other new food constituents introduced upon weaning.

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