QUANTITATIVE ANALYSIS OF FMO GENE mRNA LEVELS IN HUMAN TISSUES

Jun Zhang and John R. Cashman

Human BioMolecular Research Institute, San Diego, California

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ABSTRACT:

The developmentally and tissue-specific expression of flavin-containing monooxygenase (FMO) enzymes has been previously characterized in a number of animal species, including humans, mice, rats, and rabbits. In this study, we used sensitive real-time reverse transcription-polymerase chain reaction methodology to systematically quantify the steady-state mRNA levels of FMO1, 2, 3, 4, and 5 in human tissues. We examined the developmental regulation of these enzymes in brain tissue. FMO1 was found to be down-regulated in human adult brain. The amount of other FMO mRNAs in human brains in different age groups was not significantly different. The study also provided a systematic quantitative comparison of the steady-state mRNA levels of FMO1 to 5 in several major human organs (i.e., liver, lung, kidney, small intestine, and brain).

Flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) are a family of NADPH- and FAD-dependent enzymes that catalyze the oxygenation of a wide variety of compounds containing nucleophilic nitrogen, sulfur, and phosphorus heteroatoms (Ziegler, 1988; Cashman, 1995). The enzyme is recognized as a broad spectrum monooxygenase, and functional diversity of this family is determined by the expression of five FMO genes, named FMO1 to FMO5, and their variants. There are also a large number of FMO splice variants detected in human tissue (Lattard et al., 2004). A number of other FMO pseudogenes have been described, but apparently no other functional proteins are expressed (Hines et al., 2002; Hernandez et al., 2004).

The developmentally and tissue-specific expression of FMOs has been characterized in a number of animal species, including humans, mice, rats, and rabbits (Hines et al., 1994). Methods that have been used to characterize FMO expression include RNase protection assays for FMOs in human kidney and liver (Dolphin et al., 1996) and mouse (Jannmohamed et al., 2004) to quantify mRNA. Western blot analysis in human liver to quantify protein level (Koukouritaki et al., 2002), and microsomal activity measurements for human liver FMO (Overby et al., 1997) and rabbit FMOs (Shein-Johnson et al., 1995). The functional characterization of FMO previously reported has mainly focused on hepatic- and kidney-mediated metabolism, where the majority of the metabolism of drugs and exogenous chemicals occur. Limited animal studies, mostly using rodents, show that FMO activity exists in brain (Kawaji et al., 1995; Blake et al., 1996; Lattard et al., 2003). To further our understanding of the contribution of FMO function to chemical metabolism in brain, a systematic quantitative analysis of expression of brain FMO is essential. A gap in our knowledge of brain FMOs may be related to the significantly lower amounts of FMOs in brain tissue compared with that of liver or kidney. In addition, as discussed above, the developmental and tissue-specific expression profiles of FMOs in tissues examined thus far (i.e., liver and kidney) are fairly distinct among different animal species. Studies of FMO in brain of rodents are not easily interpretable for predicting the expression and regulation of FMO in human brain.

In this study, we used highly selective real-time RT-PCR methodology to systematically categorize the mRNA of FMO1 to FMO5 in human brain tissues to quantify the steady-state mRNA levels of FMO1 to 5. We examined the developmental regulation and gender-dependent mRNA level in brain for these genes. In addition, the study provided a quantitative systematic comparison of the mRNAs of FMO1 to FMO5 in the major human organs (i.e., liver, lung, kidney, small intestine, and brain). The nature of the quantitative analysis allowed a comparison between samples as well as among FMO genes, and also allowed quantification of low amounts of transcripts in tissues such as brain.

The nature of the quantitative analysis allowed a comprehensive comparison of each FMO mRNA in different tissues as well as among FMO isoforms in the same tissue. A comparison between fetal liver and adult liver showed that FMO1 was the only FMO that was down-regulated; all other FMOs had greater amounts of mRNA in adult liver. FMO5 was the most prominent FMO form detected in fetal liver. The FMO5 mRNA level was nearly as abundant as FMO3 in adult liver. Whereas other FMOs displayed a significant, dominant tissue-specific mRNA profile (i.e., FMO1 in kidney, FMO2 in lung, FMO3 and FMO5 in adult liver), FMO4 mRNA was observed more broadly at relatively comparable levels in liver, kidney, lung, and small intestine.

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The functional characterization of FMO previously reported has mainly focused on hepatic- and kidney-mediated metabolism, where the majority of the metabolism of drugs and exogenous chemicals occur. Limited animal studies, mostly using rodents, show that FMO activity exists in brain. To further our understanding of the contribution of FMO function to chemical metabolism in brain, a systematic quantitative analysis of expression of brain FMO is essential.

In this study, we used highly selective real-time RT-PCR methodology to systematically categorize the mRNA of FMO1 to FMO5 in human brain tissues to quantify the steady-state mRNA levels of FMO1 to 5. We examined the developmental regulation and gender-dependent mRNA level in brain for these genes. In addition, the study provided a quantitative systematic comparison of the mRNAs of FMO1 to FMO5 in the major human organs, and also allowed quantification of low amounts of transcripts in tissues such as brain.

ABBREVIATIONS: FMO, flavin-containing monooxygenase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; q-PCR, quantitative polymerase chain reaction; TBP, TATA-Box binding protein; HPRT, hypoxanthine phosphoribosyltransferase; ANOVA1, analysis of variance 1.
Materials and Methods

Chemicals and Reagents. cDNA synthesis components were purchased from Invitrogen (Carlsbad, CA). q-PCR reagents were purchased from Bio-Rad (Hercules, CA). All chemicals and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) in the highest purity commercially available. Other reagents, buffers, and solvents were obtained from VWR Scientiﬁc (West Chester, PA).

RNA and Tissue Samples. Standard human tissue RNA samples were purchased from BD Biosciences Clontech (Palo Alto, CA). Adult human brain RNA was obtained from whole brain from three healthy male or female white adults, ages 21 to 45 years, after sudden death from a fatal accident. Human fetal brain RNA was isolated from normal fetal brain tissue pooled from 21 spontaneously aborted male or female white fetuses, ages 26 to 40 weeks. Adult human liver RNA was pooled from healthy liver from seven male or female white adults, ages 22 to 40 years, after sudden death from a fatal accident. Human fetal liver RNA was isolated from healthy fetal livers from a pool of 63 spontaneously aborted male or female white fetuses, ages 22 to 40 weeks. Human kidney RNA was from a pool of six male or female white adults, ages 20 to 52 years, after sudden death from a fatal accident. Human lung RNA was from a pool of healthy lung tissue from three male or female white people ages 15 to 45 who died from sudden death. Human small intestine RNA was isolated from a pool of small intestine tissue from five male or female white adults, ages 22 to 60 years, after sudden death from a fatal accident. Human kidney RNA was from a pool of six male or female white adults, ages 20 to 52 years, after sudden death from a fatal accident. Human small intestine RNA was isolated from a pool of small intestine tissue from five male or female white adults, ages 22 to 60 years, after sudden death from a fatal accident. Human brain tissues were obtained from the National Institutes of Health-supported Brain and Tissue Bank for Development and Disorder at the University of Miami (Miami, FL) and the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). The ethics committee of Independent Review Consulting, Inc. (San Anselmo, CA) approved this study. Tissue samples were collected using protocols approved by the Tissue Banks. The midbrain sections were requested based on our earlier studies which showed that the substantia nigra region of human brain was selectively labeled with anti-human FMO3 antibody (Cashman and Zhang, 2002). However, due to the small size of the embryonic brain, the portion of the section that represented the whole brain could be different compared to that of the adult brain. Human brain RNA samples were collected using a protocol from the manufacturer (Invitrogen). RNA samples were prepared and stored at −80°C until analysis.

Reverse Transcription. cDNA was synthesized from human RNA samples using SuperScript III (Invitrogen) and analyzed for housekeeping gene transcription. Based on results of housekeeping gene quantification (i.e., TATA-Box binding protein (TBP) and hypoxanthine phosphoribosyltransferase (HPRT)); samples that displayed less than 10% of the median-level housekeeping gene expression were considered to possess poor RNA quality and were eliminated from further analysis. Sixty cDNA samples were selected for FMO mRNA quantification. The cDNAs were stored at −20°C until further evaluation.

Validation of q-PCR Conditions. Gene-specific primers were designed for human FMO1 to 5 and housekeeping genes TBP and HPRT (Table 1). All q-PCR reactions were run in 96-well PCR plates using an iCycler Thermal Cycler (Bio-Rad). The PCRs were prepared in 20-μl volumes for each sample using the following components: Bio-Rad SYBR Super mix (50 nM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each deoxynucleoside-5′-triphosphate, 3 mM MgCl2, 25 U/ml iTaq DNA polymerase, SYBR Green I, 10 mM fluorescein, and stabilizers), gene-specific primer, MgSO4, H2O, and cDNA. The q-PCR conditions, including annealing temperature, primer concentration, and Mg2+ concentration, were optimized using plasmid DNA as a template for each gene in Table 1. The q-PCR was run at 95°C for 5 min followed with 50 cycles at 95°C for 15 s, 55°C (or as indicated for each specific gene listed in Table 1) for 30 s, and 72°C for 30 s. At the end of the PCR cycling steps, melting curve data collection and analysis was enabled. For each plate, samples were set up in duplicate. For each standard series, duplicate no-template controls were included for each gene analyzed on the same plate.

Data Normalization and Gene Transcription Quantification by a Standard Curve Method. To normalize the intersample variation in quality inherently associated with RNA preparation from human tissues, the transcription level of two housekeeping genes, TBP and HPRT, were quantified for all samples to obtain the normalization factors. The two housekeeping genes were selected because their low amount of transcription was comparable to the low amount of transcription of FMO in tissues such as human brain. Fetal brain RNA (BD Biosciences Clontech) was used to generate a standard curve. Fetal brain RNA was diluted in the presence of 100 ng/μl yeast tRNA to obtain 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 ng of fetal brain RNA/μl to create a standard series. RNA standards were reverse-transcribed to generate the cDNA samples together with other tissue RNA samples. The standards and sample cDNAs were then subjected to q-PCR analysis for both HPRT and TBP transcription. A normalization factor for each individual sample was calculated based on the standard curve generated from the fetal brain cDNA standards. The normalization factors calculated from the standard curves were used for normalization of all FMO transcription data obtained from q-PCR analyses.

External calibration curves of recombinant DNA were generated from human FMO plasmid DNA and used for quantification calculations. Human FMO1, 2, 3, 4, and 5 gene fragments were amplified through RT-PCR and directly cloned into pCR2.1 using TA cloning methods as described by the manufacturer (Invitrogen). The sequence of selected clones was verified by re-sequencing and analysis. Plasmid DNA containing the specific FMO genes were prepared with Midi-Prep kits (QIAGEN, Valencia, CA) and quantified based on UV absorbance at 260 nm. The plasmid DNA was then serially diluted to 106, 105, 104, and 103 ag/μl to generate standard calibration curves for q-PCR analysis. The gene copy number for each gene was then calculated based on the molecular weight of each plasmid. Transcription levels of FMO1, 2, 3, 4, and 5 were analyzed with q-PCR for cDNA derived from human tissues.

TABLE 1

q-PCR primers and conditions

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Data Processing and Statistical Analysis. Statistical analysis was done with GraphPad Prism programs (GraphPad Software Inc., San Diego, CA). For analyzing age-dependent changes of FMO transcripts in brain, the 60 brain samples were placed into five groups based on their age distribution. The data were first analyzed with the Kolmogorov-Smirnov test for data distribution normality. The data were then analyzed with Bartlett’s test for equal variance or homogeneity. The confidence interval was set to be at least 95%. For data that passed Bartlett’s test, the primary data were analyzed with an ANOVA1 test followed by Tukey’s multiple comparison test to compare the significant difference between the different age groups. For data that did not pass Bartlett’s test, primary data were transformed into ln Y to reduce sample variability. The transformed data passed Bartlett’s test and were then analyzed with an ANOVA1 test followed by Tukey’s multiple comparison test to compare the significant difference between different age groups. For the analysis of transcription of each FMO gene in different tissues, data were analyzed with an ANOVA1 test followed by Tukey’s multiple comparison test.

Results

Validation of the q-PCR Assay. In this study, we designed, optimized, and validated real-time RT-PCR methods for the quantification of human FMO1, 2, 3, 4, and 5 in liver, lung, kidney, small intestine, and brain. The amplification of FMO (reverse-transcribed cDNAs and external calibration standard plasmids) was linear over a wide range of input copies, and great sensitivity was achieved. As few as 10 molecules of FMO could be detected with the quantification methods established in this study. The large range of linearity of the assay (i.e., 10–10^5 copies/reaction) permitted the quantification of FMO transcripts in both high transcription tissue and low transcription tissue simultaneously and allowed direct quantitative comparison between the samples examined that possessed large differences. The quantification calculations were based on standard curves that determined the absolute amount of each FMO transcript, and it was therefore possible to cross-compare FMO transcript levels in different tissues, as well as among different genes.

Amplification of genomic DNA was avoided by using primer pairs that were located on different exons, and consequently, a DNase treatment of total tissue RNA samples was omitted. A two-step q-PCR approach was used, so that a single cDNA sample was generated from each tissue RNA. Random hexamers and oligo(dTs) were used for the analysis of all genes examined (i.e., FMO genes and housekeeping genes, TBP and HPRT). This approach minimized data variation generated from multiple sample handling and multiple reverse transcription. Two housekeeping genes, TBP and HPRT, were quantified for normalization of the cDNA input in each q-PCR reaction. These two genes were selected because their low level of mRNA was comparable to the low level of FMO transcripts in human brain. However, during the age-dependent analysis of brain, the HPRT mRNA level was found to be down-regulated during later stages of gestation (i.e., 22–39 weeks) and in adult, whereas the TBP mRNA level remained constant (data not shown). Based on this finding, all analyses for FMO mRNA level presented below were normalized with TBP mRNA level.

Age-Dependent mRNA Regulation of FMO Isoforms in Brain. Steady-state mRNA levels of FMO1, 2, 3, 4, and 5 in brain were examined in 60 samples. Based on the age of the donor, the samples were separated into five age groups (i.e., prenatal 18–21 weeks, prenatal 22–39 weeks, 0–8 months, 15–40 years, and 41–61 years) as shown in Fig. 1. Although the age groups were selected as above, correlation analysis was done between each age group using a GraphPad Prism program and showed no significant difference of FMO mRNA level within each age group. Similarly, regression tree analysis broke the samples into four groups and also did not show a difference between FMO mRNA levels within each age group. When 25 and 75 percentile values were analyzed for each group (GraphPad Prism software), variation within each group was less than 10-fold (data not shown), except for the age 15 to 40 years group for FMO1 (21-fold difference between the 25% and the 75% value). The scatter plot of data from all 60 samples is presented in Fig. 1. Data from all age groups were analyzed for Gaussian distribution using the Kolmogorov-Smirnov test (GraphPad Prism software) to evaluate normal distribution. All data presented in Fig. 1 passed the normality test (data not shown).

FMO1. FMO1 mRNA level in brain was much lower than that in other tissues examined and represented less than 1% of FMO1 in a tissue rich in FMO1 such as the kidney (see below). Changes in mRNA level of brain FMO1 as a function of age were studied. Adult brain contained less FMO1 than prenatal brain (Fig. 1). An ANOVA1 test followed by Tukey’s multiple comparison test showed a significant difference between prenatal 18- to 21-week samples and the three postnatal groups (i.e., 0–8 months, p < 0.05; 15–40 years, p < 0.05; and 41–61 years, p < 0.001). The results indicated that the steady state of FMO1 mRNA in brain was down-regulated after birth.

FMO2. FMO2 mRNA level in brain was among the lowest observed for FMO2 in the tissues examined and represented less than 1% of FMO2 in lung (see Fig. 2). No significant difference for FMO2 was observed among the different age groups examined through prenatal and postnatal samples (p > 0.05) (Fig. 1).

FMO3. FMO3 mRNA level in brain was among the lowest observed for FMO3 in the tissues examined and represented less than 1% of FMO3 in adult liver (see Fig. 2). No significant statistical difference was observed for FMO3 among the different age groups examined through prenatal and postnatal samples (p > 0.05) (Fig. 1).

FMO4. FMO4 mRNA level in brain was among the lowest in the tissues examined and represented less than 1% of FMO4 in tissues rich in FMO4 such as adult liver or kidney (see below). No significant statistical difference was observed for FMO4 among the different age groups examined from prenatal to postnatal samples (p > 0.05) (Fig. 1).

FMO5. FMO5 mRNA level in brain was among the lowest in the tissues examined and represented less than 1% of FMO5 in a tissue rich in FMO5 such as adult liver (see below). No significant difference was observed for FMO5 among the different age groups examined from prenatal to postnatal samples (p > 0.05) (Fig. 1).

Gender-Selective Regulation of mRNA Levels of FMO Isoforms in Brain. q-PCR data were also analyzed based on the gender of the sample donor. Because adult female samples were somewhat limited, we analyzed two age groups, prenatal samples (n = 11 female and 13 male) and 0 to 4 months postnatal samples (n = 5 female and 5 male). No significant differences were found for all FMO isoforms (i.e., FMO1-5), analyzed between the male and female groups at the indicated age (data not shown). Therefore, based on the limited number of samples examined, gender-specific regulation did not appear to affect FMO isoform mRNA steady-state level during prenatal and early postnatal age in human brain.

Steady-State mRNA Levels of FMO Isoforms in Different Tissues. Total human RNA from human liver, lung, kidney, and small intestine was also quantified with the same standards used in the studies using brain samples described above. The data were plotted in Fig. 2 for specific FMO isoforms and in Fig. 3 for specific tissues. FMO1 mRNA was mainly detected in kidney that contained FMO1 in significantly greater amounts compared with all other tissues examined (p < 0.001). FMO1 transcripts in fetal liver and small intestine were 10- to 14-fold less than that observed in kidney, but there was no significant difference between fetal liver and small intestine (p > 0.05). Transcripts of FMO1 in lung were at the level of...
about 2.8% of FMO1 in kidney. FMO1 transcripts in brain and adult liver represented less than 1% of FMO1 quantified in kidney. Adult liver contained significantly less FMO1 than that of fetal liver (p < 0.001).

FMO2 mRNA was mainly detected in lung tissue and contained FMO2 in significantly greater amounts compared with all other tissues examined (p < 0.001). FMO2 transcripts in kidney were about 7-fold less than that observed in lung. The mRNA of adult liver and small intestine FMO2 was approximately 2% of the amount of FMO2 detected in the lung. Brain and fetal liver showed less than 1% of FMO2 mRNA in the lung. Adult liver contained significantly more FMO2 than that observed in fetal liver (p < 0.001).

FMO3 mRNA was mainly detected in adult liver, which contained FMO3 in significantly larger amounts compared with all other tissues examined (p < 0.01). Lung, kidney, and fetal liver contained similar amounts of FMO3 mRNA that were approximately 4.5%, 3.7%, and 2.1% of the amount in adult liver, respectively. Small intestine and
both fetal and adult brain tissues contained FMO3 mRNA in amounts less than 1% of the amount in adult liver.

FMO4 mRNA was mainly detected in adult liver and kidney, which contained FMO4 in significantly greater amounts compared with other tissues examined \((p < 0.01)\). Fetal liver, small intestine, and lung contained approximately 10.9%, 10.8%, and 7.0%, respectively, of the amount of FMO4 in adult liver. Both fetal and adult brain contained FMO4 in amounts less than 1% of that of FMO4 observed in adult liver.

FMO5 mRNA was mainly detected in adult liver, which contained FMO5 in significantly greater amounts compared with other tissues examined \((p < 0.001)\). Steady-state mRNA levels of FMO5 in fetal liver, small intestine, and kidney were at 18.1%, 12.8%, and 9.8%, respectively, of the amount in adult liver, and there was no statistically significant difference between these tissues \((p > 0.05)\). FMO5 mRNA levels in lung were 4% of the amount detected in adult liver. Both fetal and adult brain contained FMO5 in an amount less than 1% of the FMO5 present in adult liver.

Steady-State mRNA of Different FMO Isoforms in the Same Tissue. In adult liver, the mRNA levels of FMO3 and FMO5 were similar \((p > 0.05)\), even though FMO3 was the FMO isoform commonly reported to be prominently expressed in adult liver (Cashman, 1995). Although FMO5 represented approximately 46% or more of the total FMO transcripts in adult human liver, the enzyme activity contribution of FMO5 to hepatic metabolism has not been clearly established. FMO5 does not apparently catalyze the oxygenation of
common FMO substrates (i.e., methimazole, ranitidine, cimetidine) (Overby et al., 1997). The question whether FMO5 has a selective substrate specificity different from that of other FMOs remains to be determined. FMO4 mRNA levels in adult liver were found to be about 18% of those of FMO3 and FMO5. FMO1 and FMO2 mRNA levels were 0.4% and 4%, respectively, of that of FMO5.

In fetal liver, FMO5 mRNA was the prominent FMO form and was present at the highest amounts measured. Other FMOs showed moderate mRNA amounts with FMO1 at 23%, and FMO3 and FMO4 at 11% of the amount of FMO5 mRNA levels in fetal liver. FMO2 mRNA in fetal liver was about 2.3% of the amount of FMO5.

Comparison of the mRNA level of FMO between fetal and adult liver showed that FMO1 was down-regulated about 10-fold, and FMO3 was up-regulated about 50-fold from fetal liver to adult liver. We also observed that FMO1 was the only FMO isoform that was down-regulated in adult liver, and all other FMOs were detected with greater amounts in adult liver compared with fetal liver.

In kidney, FMO1 was the prominent isoform observed. FMO2 was observed at approximately 76% of the amount of FMO. Transcripts of FMO3, FMO4, and FMO5 were 11.7%, 2.8%, and 4.1-fold less, respectively, than that of FMO1.

In lung, results showed that the mRNA level of FMO2 was more than 150-fold higher than that of FMO1 and FMO4 and about 50-fold higher than that of FMO3 and FMO5. FMO2 has been reported to be the dominant form of FMO transcribed in lung in rabbit (Larsen-Su et al., 1999). Because a functional allele encoding full-length functional FMO2 is lacking in white people and is at a frequency of 13 to 20% in African Americans (Whetstone et al., 2000), the functional significance of high amounts of FMO2 transcripts remains to be established.

In small intestine, FMO3 was the dominant form detected. FMO1, FMO2, and FMO4 mRNAs were found at levels of 22%, 39%, and 15%, respectively, similar to that of FMO5. Of all the FMOs examined, FMO3 was at the lowest amount in small intestine.

Discussion

Quantitative RT-PCR was used to determine the tissue-specific steady-state mRNA levels for FMOs 1, 2, 3, 4, and 5, and set the stage for future investigations of tissue-specific regulation of FMO isoforms in humans. Steady-state mRNA levels in tissue are generally controlled by transcription regulation, alternative splicing, and mRNA degradation. Information regarding the regulation of FMO transcription is beginning to emerge. Characterization of the promoter region and identification of regulatory elements for FMO have been reported recently (Shehin-Johnson et al., 1996; Hines et al., 2003; Koukouritaki et al., 2005). Dominant mRNA levels of FMO1 in adult kidney, FMO2 in lung, and FMO3 and FMO5 in adult liver suggest that the promoter regions of these FMOs likely contain regulatory elements responsive to transcription factors specifically present in corresponding tissues. Identification of such regulatory elements at the genetic level and tissue-specific transcription factors at the protein level will be essential for further understanding the regulatory mechanism for FMO isoforms. Alternative splicing of FMOs has been reported in human tissues including liver, kidney, and brain (Lattard et al., 2004). With the exception of FMO4 in human brain, alternative splicing does not appear to be the major factor contributing to the tissue-specific regulation of FMOs. To minimize the complexity of the current study, the q-PCR primers were carefully designed to avoid FMO alternative spliced regions and allow quantification of total transcripts. There is little information available regarding the RNA stability regulation for FMO isoforms. The RNA stability regulation is especially interesting for FMO2 because human FMO2 mRNA is present at high levels in lung, even though there is generally no functionally active enzyme expressed (i.e., whites, Asians), due to a common polymorphism resulting in a premature termination of the protein open reading frame, with the exception of a small percentage of the population carrying the functional form (Krueger et al., 2004). Full-length FMO2 is expressed

![Figure 3. Tissue-dependent mRNA levels of FMO in humans. Column bar graphs (GraphPad Prism software) were used to show the mRNA levels of FMO isoforms in the different human tissues examined. The mRNA levels displayed represent copies of the respective FMO transcripts per µg RNA normalized with the mRNA level of TBP.](image-url)
in rabbit, monkey, and wild rat. Apparently, humans retain the genetic regulatory elements present in other small animals (i.e., rabbit and rat) for high level transcription of FMO2 in lung despite the nonfunctional common polymorphism. Generally, mammals are protected from accumulation of transcripts containing a premature termination codon like FMO2 by nonsense-mediated decay, a mRNA surveillance mechanism that leads to selective degradation of premature termination codon-containing transcripts (Holbrook et al., 2004). However, rare mutations sometimes give rise to truncated proteins that overwhelm the proteolytic system of the cell and cause toxic precipitation of insoluble protein chains (Thein et al., 1990). Abnormal transcripts not degraded could also result in synthesis of truncated, dominant oncoproteins. To date, neither of these situations has been observed for FMO2.

Age-dependent regulation of mRNA levels are not statistically significant for FMO2, 3, 4, and 5 in human brain. FMO1 mRNA levels in the brain are down-regulated in the adult relative to the neonate. Overall, FMO mRNA levels in brain are much lower than those in most other tissues examined, and at the level of less than 1% as compared to the most abundant tissues observed (i.e., FMO1 in kidney, FMO2 in lung, and FMO3 and 5 in liver). Although FMO functional activity has been reported in animal (Bhamre et al., 1993, 1995; Kawaji et al., 1995) and human brain (Bhamre et al., 1995; Bhagwat et al., 1996), based on the data herein, it is not clear whether FMOs make a significant contribution to functional chemical metabolism in human brain. This study focuses on brain tissue from the substantia nigra where FMO has been reported (Cashman and Zhang, 2002); other regions of the brain may be selectively enriched in FMO. For example, in mouse brain, FMO1 and 5 were localized to the cerebrum of newborns (Jamnoahmed et al., 2004), and FMO1 was detected in the choroid plexus, a region rich in blood vessels. Age-dependent mRNA levels of FMOs were also evaluated using pooled fetal liver and adult liver. With the exception of FMO1, whose mRNA level is higher in fetal liver, all other FMOs (i.e., FMO2, 3, 4, and 5) show substantially higher mRNA levels in adult liver. Future studies will be required to more fully define the transcription regulation and functional consequences of FMO expression, and examine whether exposure during the neonate time period is different from that of adults for chemicals and drugs detoxicated by FMOs.

Based on the size of the kidney and the FMO transcript copy number, we estimate that the FMO1 transcripts in adult kidney are approximately 33-fold less than FMO3 transcripts in the adult human liver. Since FMO1 accepts larger substrates than FMO3 does (Kim and Ziegler, 2000), it is interesting to investigate whether larger substrates are oxygenated by kidney FMO1 in preference to hepatic FMO3. Supporting previous work, FMO3 transcripts are present in kidney, lung, and small intestine in low abundance, and are up-regulated in the adult liver of humans. In male mice, FMO3 transcripts were shown to be down-regulated in liver, but female mice contained 80-fold more FMO3 mRNA than male mice (Cherrington et al., 1998; Jamnoahmed et al., 2004). The gender-specific transcription is not found in human brain and is not determined in human liver in this study. Although FMO4 transcripts are present in adult liver and kidney to a similar extent, the functional significance of human FMO4 is not clearly understood. Contributing to the uncertainty of FMO4 functional activity is the difficulty of expression and purification of recombinant FMO4 and the lack of stability of the isolated protein (Lattard et al., 2003). The paucity of human FMO4 functional studies has hampered establishment of substrate specificity and an estimate of the contribution of FMO4 to FMO-mediated metabolism. FMO5 transcripts are found to be equally prevalent as FMO3 in human adult liver. FMO5 is also the most abundant FMO transcript present in human small intestine and may contribute to intestinal first-pass metabolism. The functional significance of FMO5 is not clear because FMO5 does not oxygenate typical FMO substrates (i.e., methimazole, ranitidine, or cimetidine) (Rettie et al., 1994; Overby et al., 1995; Cherrington et al., 1998). FMO5 has been reported to S-oxygenate theihother with a proximal carboxylic acid, a somewhat unique FMO substrate activity (Ohmi et al., 2003). It has been difficult to establish a substrate specificity for FMO5 due to the relatively low contribution of enzyme functional activity associated with the majority of FMO-mediated hepatic metabolism. We hypothesize that, as new specific substrates are discovered, FMO5 may show selective functional activity.

The quantitative nature of this study provides information on an absolute level for FMO transcripts and makes it possible to compare amounts of FMO mRNA from different tissues, at different ages, and at different stages of development. However, this study is not focused on the amount of protein expression or the activity of the FMO isozymes. A comparison of the mRNA levels of different FMOs with previously reported FMO protein levels in various tissues and during development indicates that, in most situations examined, the level of mRNA detected in this study reflects the FMO protein expression profile qualitatively, indicating that regulation at this level is presumably one of the major regulatory mechanisms for FMO tissue-specific expression. For example, human hepatic FMO1 expression is restricted to the fetus, whereas FMO3 and FMO5 are expressed in adult liver. However, the relationship between steady-state mRNA level and the FMO enzyme level does not always prove to be quantitatively translated. Overby et al. (1997) reported human adult liver FMO3 and FMO5 levels that ranged from 12.5 to 117 and 3.5 to 34 pmol/mg microsomal protein, respectively. In the previous study, the concentration of FMO3 was greater than that of FMO5 in all samples examined, but the ratio of FMO3 to FMO5 varied from 2:1 to 10:1. The mRNA levels of FMO3 and FMO5 we observed in human liver show no significant differences. For FMO1, the maximum protein level in the fetal liver during the first trimester was reported with a median value of 7 pmol/mg microsomal protein, although values ranged as high as nearly 20 pmol/mg microsomal protein (Koukouri-taki et al., 2002). This was in agreement with another report of fetal liver FMO1 levels of 14.4 ± 3.5 pmol/mg microsomal protein (Yeung et al., 2000). In contrast, adult FMO1 kidney levels were reported to be 47.0 ± 9.0 pmol/mg microsomal protein. The fold difference in FMO1 protein levels between fetal liver and kidney previously reported was considerably less than the 10.7-fold difference in FMO1 transcript levels identified in the study reported herein. The high levels of FMO2 transcripts in lung does not translate to functional FMO2 protein in most of the population as discussed earlier. Therefore, the systematic study herein provides a general picture for FMO transcripts in human liver, kidney, lung, small intestine, and brain. How does such information translate to the functional aspects at the protein level is controlled by various other aspects of regulation and remains to be evaluated empirically.

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**Address correspondence to:** John R. Cashman, Human BioMolecular Research Institute, 5310 Eastgate Mall, San Diego CA 92121. E-mail: jcashman@hbri.org