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Flavin-Containing Monooxygenase-3 and 5: Tissue Distribution, Age-Related Expression and Regulation by Endoplasmic Reticulum Stress

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FLAVIN-CONTAINING MONOOXYGENASE-3 AND 5: TISSUE DISTRIBUTION, AGE-RELATED EXPRESSION AND REGULATION BY ENDOPLASMIC RETICULUM STRESS

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ABSTRACT

Background Flavin-containing monooxygenases (FMO) constitute a class of oxidative enzymes. FMO3 and FMO5 are two major FMOs that are highly expressed in the liver. The study was performed to determine regulated expression of both enzymes by age, fructose, therapeutic agents and endoplasmic reticulum (ER) stressors. The expression of both enzymes in 33 extrahepatic tissues was determined as well.

Methods The tissue distribution of FMO3 and FMO5 was determined by immunohistochemistry. For age-related expression, livers were collected and divided into 5 age groups: I (< 31 days), II (35-70 days), III (89-119 days), IV (123-198 days), and V (>18 years of age). These samples were analyzed for the expression of FMO3 and FMO5 by RT-qPCR and Western blotting. For the regulated expression by other factors, human primary hepatocytes were treated with a chemical and the expression was determined. The reporter activity was determined in response to these chemicals.

Results FMO3 and FMO5 were strongly stained in the liver but exhibited overlapping and distinct expression patterns in extrahepatic tissues. Both enzymes showed a neonatal surge in mRNA expression and were correlated well with age during the first 7 months after birth. The mRNA expression of both enzymes was suppressed by ER stressors and induced by the steatotic agent valporic acid. Similar changes of the reporter activities were observed with an exception of fructose, a sugar associated with

metabolic diseases. This carbohydrate induced FMO5 mRNA but did not activate the FMO5 reporter.

Conclusions The expression of FMO3 and FMO5 varies depending on a tissue, age, ER stress and nutritional status. Of significance are the abundant presence of FMO3 and FMO5 in some endocrine cells and regulated expression by ER-stressors and fructose. These observations conclude that FMO3 and FMO5, in addition to xenobiotic metabolism, are involved in pathogenesis, particularly related to metabolic diseases.

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PREFACE

This thesis was prepared in manuscript format according to University of Rhode Island Graduate School guidelines. This thesis consists of one manuscript that meets the requirements of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

TABLE OF CONTENTS

ABSTRACT ..... **ii**

ACKNOWLEDGMENTS ..... **iv**

PREFACE ..... **vi**

TABLE OF CONTENTS ..... **viii**

LIST OF TABLES ..... **viii**

LIST OF FIGURES ..... **ix**

MANUSCRIPT **1**

INTRODUCTION **4**

MATERIALS AND METHODS **9**

RESULT **12**

DISCUSSION **18**

REFERENCE **24**

LIST OF TBALES

TABLE PAGE

Table 1. Summary of immunohistochemistry on FMO3 and FMO5..... 30

LIST OF FIGURES

FIGURE PAGE

Figure 1. Immunohistochemical analysis..... 33

Figure 2. Expression of FMO3 and FMO5 as a function of age..... 34

Figure 3. Regulated expression of FMO3 and FMO5 and determination of reporter activity..... 35

MANUSCRIPT

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FLAVIN-CONTAINING MONOOXYGENASE-3 AND 5: TISSUE DISTRIBUTION, AGE-RELATED EXPRESSION AND REGULATION BY ENDOPLASMIC RETICULUM STRESS[1]

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Methods: The tissue distribution of FMO3 and FMO5 was determined by immunohistochemistry. For age-related expression, livers were collected and divided into 5 age groups: I (< 31 days), II (35-70 days), III (89-119 days), IV (123-198 days), and V (>18 years of age). These samples were analyzed for the expression of FMO3 and FMO5 by RT-qPCR and Western blotting. For the regulated expression by other factors, human primary hepatocytes were treated with a chemical and the expression was determined. The reporter activity was determined in response to these chemicals.

Results: FMO3 and FMO5 were strongly stained in the liver but exhibited overlapping and distinct expression patterns in extrahepatic tissues. Both enzymes showed a neonatal surge in mRNA expression and were correlated well with age during the first 7 months after birth. The mRNA expression of both enzymes was suppressed by ER stressors and induced by the steatotic agent valporic acid. Similar changes of the

reporter activities were observed with an exception of fructose, a sugar associated with metabolic diseases. This carbohydrate induced FMO5 mRNA but did not activate the FMO5 reporter.

Conclusions: The expression of FMO3 and FMO5 varies depending on a tissue, age, ER stress and nutritional status. Of significance are the abundant presence of FMO3 and FMO5 in some endocrine cells and regulated expression by ER-stressors and fructose. These observations conclude that FMO3 and FMO5, in addition to xenobiotic metabolism, are involved in pathogenesis, particularly related to metabolic diseases.

INTRODUCTION

Flavin-containing monooxygenases (FMOs, EC 1.14.13.8) constitute a class of major oxidative enzymes (Petriello *et al*., 2017; Phillips and Shephard, 2017; Rossner *et al*., 2017). Traditionally, these enzymes are established to play important roles in the metabolism and detoxification of drugs, pesticides and dietary compounds (Phillips and Shephard, 2017). Emerging evidence, nonetheless, has linked their functionality to many pathophysiologic processes including metabolic diseases and aging (Petriello *et al*., 2017; Rossner *et al*., 2017). Without exceptions, all mammalian species examined express multiple forms (Hines et al., 2002; Phillips et al., 1995; Hernandez et al., 2004; Lattard *et al*., 2004; Hao *et al*., 2009). The human genome encodes a total of 11 FMO genes but only 5 of them produce catalytic proteins, which are commonly referred to as FMO1, 2, 3, 4 and 5, respectively (Hernandez *et al*., 2004). The first 4 genes are closely clustered in the chromosome 1 (Hernandez *et al*., 2004; Hao *et al*., 2009). While the FMO5 gene is also located in chromosome 1, but it is 20 Mb away from others. Interestingly, other species have similar genomic arrangement among these FMO genes. For example, the mouse genome has 4 Fmo genes (1 to 4) closely clustered in chromosome 1, whereas the Fmo5 gene is located in chromosome 3 (Hernandez *et al*., 2004). In addition, many FMO genes produce alternative splicing transcripts, further diversifying the expression species (Lattard *et al*., 2004; Rossner *et al*., 2017).

FMO enzymes, like many other drug-metabolizing enzymes, have a broad tissue distribution. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) transcripts for all 5 FMOs among 23 different tissues with few exceptions (Nishimura and Naito, 2006). No FMO1 transcript is present in leukocytes, nor is FMO2 mRNA in leukocytes and placenta. Among tissues that expression FMO mRNA, the relative abundance on FMO1 mRNA varies by as much as 6000-fold between the liver and bone marrow (Nishimura and Naito, 2006). While the liver generally expresses the highest abundant drug-metabolizing enzymes, other organs but not the liver express the highest mRNA of FMO1, FMO2 and FMO4. The kidney has the most abundant mRNA expression of FMO1 and FMO4; the lung has the most abundant mRNA expression of FMO2. The tissue expression patterns are largely similar cross species but there are exceptions. For example, Western blotting detects higher expression of FMO3 in the kidney than liver (Novick et al., 2009). The cellular localization shows species-differences as well. For example, rat FMO3 is predominately expressed in the perivenous region whereas mouse Fmo3 in the periportal region (Janmohamed et al., 2004; Novick et al., 2009). The cellular localization of human FMOs remains largely unknown.

The expression of FMOs is regulated by xenobiotics and inflammatory stimuli (Chung et al., 1997; Zhang et al., 2009; Celius et al., 2010; Rudraiah et al., 2014). Rifampicin, a prototypical activator of the pregnane X receptor in human, has been shown to induce FMO4 and FMO5 (Rae et al., 2001). Extracts from phellinus baumii, a widely

used medicinal plant in Asia, have been shown to induce mouse Fmo2, Fmo3 and Fmo4 but not Fmo1 and Fmo5 (Sainkhuu et al., 2016). Interestingly, the induction of FMO mRNA may not lead to significant induction of FMO protein (Celius et al., 2010; Rudraiah et al., 2014). The environmental chemical 3-methylcholanthrene, for example, induces Fmo3 mRNA by as much as 30-fold but only modest increase occurs at Fmo3 protein (Celius et al., 2010). Furthermore, inflammatory stimuli regulate the expression of FMOs but the regulatory effect varies depending on an inflammatory model and an FMO gene as well as a strain (Zhang et al., 2009). For example, the immunostimulant lipopolysaccharide downregulates fmo1 but not fmo4. The downregulation in HeoU mice is greater than that in HeJ mice. Consistent with an established role of FMOs in detoxification, overexpression of FMOs protects against endoplasmic reticulum (ER) stress, a common initiator for many pathological processes (Liao et al., 2016).

In addition to xenobiotics and inflammatory stimuli, many other factors have been shown to regulate the expression of FMOs including age, sex and hormones (Dolphin et al., 1996; Cherrington et al., 1998; Ripp et al., 1998; Koukouritaki et al., 2002; Larsen-Su et al., 2002; Zhang and Cashman, 2004; Hines, 2006;). In humans, FMO1 is considered to be a fetal liver enzyme and the expression of FMO1 mRNA and protein decreases with gestational days (Dolphin et al., 1996; Koukouritaki et al., 2002). In contrast, FMO3 is expressed higher in adult livers at both mRNA and protein levels (Dolphin et al., 1996; Koukouritaki et al., 2002). In mice, high levels of

FMO1 and FMO5 are present in postnatal and adult animals (Janmohamed et al., 2004). In contrast, FMO2, 3 and 4 exhibit age-dependent increases with FMO3 being expressed the highest. Several FMOs exhibit sex-preferable expression (Janmohamed et al., 2004). For example, female mice express higher liver Fmo1 than male, but the opposite is true in the kidney. Consistent with sex-preferable expression, sex hormones have been shown to regulate the expression of FMOs. The female sex hormone 17β-estradiol, for example, decreases FMO activity by 56% in cultured rat hepatocytes (Coecke et al., 1998). In male mice, castration dramatically increases FMO3 expression, and testosterone replacement to castrated mice results in ablation of FMO3 expression (Falls et al., 1997).

The opposing effects on FMO expression by male and female hormones are also observed with other pairs of counterbalancing hormones such as glucagon and insulin. In mice, the glucose-elevating hormone glucagon induces all Fmos with Fmo3 being induced by as many as 14-fold (Miao et al., 2015). In contrast, insulin suppresses the expression of these enzymes by 30-69%. In streptozotocin-induced diabetic rats, insulin treatment reverses diabetic-induced elevation of FMO1 activity (Borbás et al., 2006). An early microarray study reports a 59% decrease of FMO5 mRNA in Type 2 diabetic patients (Takamura et al., 2004). In support of critical roles of Fmos in metabolic homeostasis, mice deficient in one of Fmos through knockout or knockdown exhibit a lean phenotype with decreased body weight and/or reduced adiposity (Shephard and Phillips, 2010; Veeravalli et al., 2014; Schugar et al., 2017;

Scott et al., 2017). Consistent with the lean phenotype, these mice show favorable metabolic profiles including lower levels of plasma glucose, cholesterol and triglycerides.

In this study, we used a comprehensive approach and investigated human FMO3 and FMO5 for their subcellular localization, age-related expression and regulated expression by ER stressors, fructose and xenobiotics. Both FMO3 and FMO5 were strongly present in the liver but exhibited overlapping and distinct expression patterns in extrahepatic tissues. Both enzymes showed a neonatal surge in mRNA expression and were correlated well with age during the first 7 months after birth. Both enzymes were suppressed by ER stressors and induced by the steatotic agent valporic acid. These observations conclude that FMO3 and FMO5, in addition to xenobiotic metabolism, are involved in pathogenesis, particularly related to metabolic diseases.

MATERIALS AND METHODS

Chemicals and reagents: Brefeldin A (BFA) and Hanks balanced salt solution were from Sigma (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) and *Taqman* probes were from Life Technology (Carlsbad, CA). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, IL). Thapsigargin (THAP) was from R&D Systems (Minneapolis, MN). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Expression constructs and the FMO5 reporter were from OriGene Technologies Inc (Rockville, MD). Unless otherwise specified, all other reagents were purchased from ThermoFisher Scientific (Fair Lawn, NJ).

Immunohistochemistry: Formalin-fixed paraffin-embedded (FFPE) array slides with a total of 33 tissues were purchased from Pantomics Inc (Richmond, CA). The slides were warmed up in 60°C oven for 60 min followed by deparaffinization and rehydration, essentially as described previously (Yan et al., 1995). The slides were then incubated in Dako Target Retrieval Solution (Agilent, Santa Clara, CA) in pressure cooker in microwave for 15 min and then cool for 30 min. The slides were washed 3 times (3 min/each) in distilled water and then incubated in Dako quenching reagent for 10 min at room temperature. The quenched slides were washed 3 times again and incubated at 4C with primary antibody (FMO3 or FMO5) or pre-immunized rabbit serum at a dilution of 1:1000 overnight. After 3 washings, the slides were incubated with Dako EnVision + Dual Link System-HRP secondary antibodies (anti-rabbit) followed by 3 washings. The washed slides were incubated with substrate-chromogen for 10 min. The reactions were stopped in distilled water and the slides

were once again washed 3 times. Thereafter, the slides were dehydrated and mounted in Cytosealtm 60 media.

Liver RNA and S9 fractions: A total of 59 tissue samples were used in this study as described previously. Briefly, liver tissues were acquired primarily from the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). The samples were divided into several groups: I (1-31 days, n=12, Male/female= 6/6), II (35-70 days, n=13, M/F= 5/8), III (89- 119 days, n=10, M/F= 7/3), IV (123-198 days, n=10, M/F=8/2) and IV (adult, n=14, M/F= 7/7). Isolation of total RNA from the liver tissues was described previously (Shi et al., 2011), and the quality was determined by electrophoresis. S9 fractions were prepared by differential centrifugation as described previously (Shi et al., 2011). The use of the human samples was approved by the Institutional Review Board.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western analysis: The mRNA levels were determined by RT-qPCR with *TaqMa*n Gene Expression Assay (Applied Biosystems, Foster City, CA). The *TaqMan* assay identification numbers were: FMO3, Hs00199368_m1 (NM_001002294.2), FMO5: Hs00356233_m1 (NM_001461.3), and polymerase (RNA) II, Hs01108291_m1 (NM_000937). All samples were analyzed in triplicate and the signals were normalized to polymerase (RNA) II and then expressed as relative levels of

mRNA. For Western analysis, S9 fractions (0.25 g) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After nonspecific binding sites were blocked with 5 % non-fat milk, the blots were incubated with an antibody against FMO3, FMO5 or CES1. The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase, and horseradish peroxidase activity was detected with a chemiluminescent kit (Super Signal West Pico). The chemiluminescent signals were captured by an ECL Imager (Thermo Fisher Scientific, Fair Lawn, NJ) and the relative intensities were quantified by the ECL Imager Analysis Software.

Culture and treatment of primary hepatocytes: Plated human primary hepatocytes were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota). Fresh medium (free serum) was added to each well, and the cultures were returned to the humidified chamber. Hepatocytes were cultured in the same medium for overnight and treated with a chemical at clinically relevant concentrations for 24 h. Treated hepatocytes were collected and total RNA was isolated. The expression of FMO3 and FMO5 was determined by RT-qPCR.

Reporter constructs and co-transfection assays: The FMO3 promoter reporter (-4940 FMO3Luc) was a gift of Ronald N. Hines of the National Health and Environmental Effects Research Laboratory, and the FMO5 promoter reporter (-1082FMO5Luc) was purchased from OriGene Technologies Inc. To determine the reporter activities, co-transfection of Huh7 cells was performed. Transfection mixtures contained 100 ng of a

reporter plasmid and 0.2 ng of CMV Renilla luciferase plasmid. Cells were transfected for 12 h and the medium was replaced with fresh medium supplemented with 1% fetal bovine serum. The treatment lasted for 24 h and the cells were washed once with phosphate buffered saline and collected by scraping. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System as described previously (Yang et al., 2011).

Other analyses: Protein concentrations were determined with BCA assay (Pierce) based on albumin standard. The preparation of antibody against CES1 was described elsewhere (Zhu et al., 2000). Data are presented as mean SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Statistical analyses were performed with SPSS-PASW Statistics 18. Significant differences were tested according to Spearman for correlation or One-way ANOVA followed by a DUNCAN's test for comparison of means. In all cases, significant differences were observed when *p* values were less than 0.05.

RESULTS

Tissue distribution and cellular localization The focus of this study is on FMO3 and FMO5, because they are major FMO enzymes in human liver (Phillips and Shephard, 2017). An early study detected the presence of FMO3 and FMO5 transcripts in a wide range of tissues (Nishimura and Naito, 2006). In this study, we initially tested whether FMO3 and FMO5 proteins have a broad tissue distribution and

whether they are localized in certain cell populations in a tissue.
Immunohistochemical staining was performed with tissue arrays (33 tissues). As shown Fig. 1, FOM3 was strongly stained in the liver and adrenal; moderately in the lung, brain cortex, spinal cord, thyroid, urinary bladder and testis; and slightly in the small intestine, pituitary, kidney, and ureter. In the liver, FMO3 was stained the most between central vein and portal triad. All staining was detected in the cytoplasm of hepatocytes. Hepatocytes surrounding the central vein was less stained. No staining was detected in the periportal area including bile ducts. In the adrenal gland, strong staining was restricted to the medullar with no staining of the cortex. In addition, moderate staining was detected in some of the pneumocytes of the lung, the colloid and follicular cells of the thyroid and spermatocytes of the testis. Slight staining was detected in some of the proximal tubes of the kidney. Interestingly,
no staining was detected in the duodenum, colon and parathyroid gland. Table I summarizes the immunohistochemical staining of FMO3.

FMO5, like FMO3, was stained strongly in the liver. Strong FMO5 staining was also detected in the parathyroid gland. In addition, FMO5 was stained moderately in the stomach and colon; and slightly in the small intestine, lung, pituitary, brain cortex, kidney, bladder, heart, testis, skeletal muscle and ureter. In contrast to FMO3 in the liver, FMO5 was stained across the entire hepatic acinus. In the parathyroid gland, strong staining was detected in oxyphil cells and moderate staining in chief cells. In the duodenum and colon, slight or moderate staining of FMO5 was detected in the epithelium, contrasting strikingly to no FMO3 staining in the digestive tract (Fig. 1,

Table I). In the adrenal, FMO5 was stained in the cortex but not medullar as seen with FMO3. In the kidney, FMO5 was stained in both proximal and distal tubules. Once again, Table I shows the relative staining intensity and cellular localization of immunohistochemistry.

A surge in FMO3 and FMO5 mRNA expression during the neonatal period It has been reported that the level of FMO3 mRNA is relatively low in pediatric livers compared with adult counterparts and the level of FMO3 protein is low in the liver during the first month of life and increases sharply thereafter (Koukouritaki et al., 2002). The level of FMO5 mRNA, on the other hand, is slightly higher in pediatric liver, but the ontogenic expression of FMO5 protein remains to be determined. We previously reported that the expression of CES1 exhibited a neonatal surge in mRNA and protein expression between the first and second month after birth (Shi et al., 2011). In this study, we tested whether the expression of FMO3 and FMO5 (mRNA and protein) has a neonatal surge as seen with CES1. Specifically, a large number of individual liver tissues were tested from donors at an age of birth to 198 days. Based on the age distribution among the donors, samples were divided into several groups: I (< 31 days), II (35-70 days), III (89-119 days), and IV (123-198 days). For comparison, liver tissues from adults were analyzed.

The expression was determined by RT-qPCR and Western blotting. As shown in Fig. 2A, group I (1-31 days of age) expressed the lowest level of FMO3 mRNA, whereas

group V, the adult group, expressed the highest level of FMO3 mRNA. Group I expressed ~10% of the adult level. Groups II to IV, the other three pediatric groups, expressed much higher FMO3 mRNA levels and represented 66-76% of the adult level. More importantly, a 7-fold increase of FMO3 mRNA was detected between group I and II. Overall, the level of FMO3 mRNA was correlated significantly with age in the pediatric samples. With an exception of group 1, the protein expression of FMO3 was consistent with the level of the mRNA in other groups. Interestingly, two samples in group 1 had extremely higher levels of FMO3 protein, which contributed to the high abundance in the pooled samples (i.e., group 1). The level of FMO5 mRNA (Fig. 2B), likewise, showed a surge between group 1 and 2. Group 2, 3 and 4 expressed comparable levels of mRNA, and the adult group expressed slightly lower levels of FMO5 mRNA. As seen with FMO3, FMO5 mRNA was correlated significantly with age. In addition, FMO5 protein expression was consistent with the corresponding mRNA level with an exception of group 1. Once again, the very two samples in this group expressed a much higher FMO5 protein level than other samples in this group.

Regulated expression of FMO3 and FMO5 by ER stressors Both FMO3 and FMO5 have been implicated in metabolic homeostasis (Gonzalez Malagon et al., 2015; Miao et al., 2015). ER stress is a common pathogenic theme for many diseases including metabolic disorders (Hetz and Saxena, 2017; Sozen and Ozer, 2017; Urra et al., 2017). On the other hand, it was reported that overexpression of FMO3 protected against ER-stress (Liao et al., 2016). We next tested whether ER-stress alters the

expression of FMO3 and FMO5. To have a broader implication, valproic acid and fructose were included. Valproic acid is a widely used antiepileptic agents with strong steatotic activity (Bai et al., 2017), whereas fructose consumption is closely associated with the development of metabolic disorders (Bidwell, 2017). Human primary hepatocytes were treated with these chemicals at clinically relevant concentrations and the expression of FMO3 and FMO5 was determined. As shown in Fig. 3A, THAP and BFA, profoundly downregulated FMO3 and FMO5 mRNA expression by as much as 67%. Z-Guggulsterone, a lipid-lowering agent, suppressed FMO3 and FMO5 mRNA expression with FMO3 mRNA decreased to a greater extent (62 and 18%). Valproic acid (Val) induced the expression of FMO3 and FMO5 by 1.3 and 2.6-fold, respectively. Fructose (Fruct) differentially regulated the expression of FMO3 and FMO5. This dietary component suppressed FMO3 mRNA expression by 27%, whereas induced FMO5 mRNA expression by 127% (Fig. 3A).

The altered mRNA expression of FMO3 and FMO5 pointed to two levels of regulation: mRNA stability, transcription or both. We therefore tested whether transcriptional regulation is involved. Promoter reporters were tested for their response to these chemicals. As shown in Fig. 3B, valproic acid (Val) markedly increased the activities of both reporters with the FMO5 reporter being increased 25% higher. In contrast, BFA repressed the activities of both reporters with the FMO5 reporter being repressed to a greater extent. THAP, on the other hand, repressed the FMO5 reporter

by as much as 85%. However, this ER-stressor showed little repressive activity toward the FMO3 reporter.

DISCUSSION

Endogenous and xenobiotic compounds undergo phase I, phase II and phase III biotransformation (Almazroo et al., 2016). While phase III reactions are mediated by transporters, Phase I and phase II reactions are achieved by enzymes. Phase I reactions add or expose functional groups such as hydroxyl moieties, which serve the sites for phase II reactions. Nevertheless, oxidative metabolism represents the major phase I reactions, primarily catalyzed by P450s and FMOs (Strolin Benedetti, 2011). The P450 system has been well characterized. In contrast, the FMO system remains to be fully characterized. In this study, we have shown that FMO3 and FMO5, two major FMOs, have overlapping and tissue-differential expression. We have also shown that the mRNA expression of both FMO3 and FMO5 exhibited a neonatal surge between the first and second month after birth. The expression of FMO3 and FMO5 was regulated by ER stress and dietary component and xenobiotics. The regulated expression varied depending on an enzyme (i.e., FMO3 versus FMO5) and a compound.

The cellular localization of FMO3 and FMO5 in the liver may have special implications in pharmacological interactions and zone-specific tissue toxicity. In this study, we have shown that FMO3 was stained the most between central vein and portal triad. Hepatocytes surrounding the central vein was less stained (Fig. 1, Table I). No staining was detected in the periportal area including bile ducts. This

localization differs from its rodent counterparts. Rat FMO3 is present around the perivenous region (centrilobular) gradually decreasing toward the periportal area, but the opposite is true with mouse Fmo3 (Janmohamed et al., 2004; Novick et al., 2009). FMO5, on the other hand, was stained across the entire hepatic acinus. Cellular localization of FMOs may alter therapeutic efficacy. For example, tamoxifen, a widely used anticancer agent, undergoes metabolism by FMO3 and CYP enzymes such as CYP3A4 (Gjerde et al., 2010). Importantly, metabolites produced by CYP3A4 but not FMO3 are therapeutically active. Based on the cellular localization, FMO3 is exposed to higher concentrations of tamoxifen upon oral administration than CYP3A4. It is conceivable that such competition leads to reduced efficacy, although the relative affinity toward tamoxifen is a critical factor.

While FMOs are generally involved in xenobiotic metabolism, the cellular localization of FMO3 and FMO5 also points to physiological roles, particularly related to endocrinology. In this study, we have shown that FMO3 and FMO5 were strongly or moderately stained in the adrenal gland, parathyroid gland and/or thyroid gland (Fig. 1). In the adrenal gland, FMO3 was strongly stained in the medullar, the main region that produces the catecholamines adrenaline and noradrenaline (Ehrhart-Bornstein and Bornstein, 2008). It remains to be determined whether FMO3 is involved in the biosynthesis and secretion of these hormones. FMO3 is nevertheless established to metabolize the catecholamine releasing agent tyramine (Lin and Cashman, 1997). The substantial staining of FMO3 in the follicular cells of the thyroid gland linked the functionality of this enzyme to the synthesis and secretion of thyroid hormones.

Interestingly, FMO5 was strongly stained in chief and oxyphil cells, two unique cell populations in the parathyroid gland. Chief cells are the source for parathyroid hormone, whereas the functionality of oxyphil cells remain to be elucidated (Howson et al., 2015).

The precise roles of the abundant presence of FMO3 and/or FMO5 in the endocrine cells remain to be determined. One of the functional features shared by these cells is their high secretory capacity. FMO enzymes are abundant in the ER and present in the ER/Golgi transport vesicles (Hay et al., 1997). It is conceivable that FMO enzymes function as trafficking proteins for facilitated secretion of hormones (e.g., parathyroid hormone). These hormones, potentially regulated by FMOs for secretion, are all established to maintain energy balance and metabolic homeostasis (Hay et al., 1997; Lenders and Eisenhofer, 2014; Hannoush and Weiss, 2017). Interestingly, it has been increasingly recognized that FMO enzymes have broad impact on glucose, lipid metabolism and atherosclerosis. Indeed, FMO5 knockout mice show a lean phenotype with less weight gain and lower plasma glucose and cholesterol concentrations (Shephard and Phillips, 2010; Veeravalli et al., 2014; Schugar et al., 2017; Scott et al., 2017). The metabolic effect of FMO5 is more evident in aged animals (Gonzalez Malagon et al., 2015). Likewise, knockdown of Fmo3 decreases plasma lipids, glucose and insulin (Miao et al., 2015; Shih et al., 2015). Conversely, overexpression of Fmo3 delivers the opposite effect. It has been shown that the overexpression of this enzyme increases the conversion of trimethylamine to trimethylamine-N-oxide, a metabolite

linked to metabolic diseases (Oellgaard et al., 2017; Subramaniam and Fletcher, 2017).

The involvement of FMOs in metabolic homeostasis is confounded by metabolic conditions that regulate the expression of these enzymes. It has been reported that diabetic condition induced FMOs and insulin suppressed them (Takamura et al., 2004; Borbás et al., 2006; Miao et al., 2015). It remains to be determined how elevation of blood glucose (i.e., diabetic condition) interplays with insulin resistance (elevated insulin) in terms of regulated FMO expression. In this study, we have shown that valproic acid induced both FMO3 and FMO5 and activated their promoters (Fig. 3). Interestingly, this antiepileptic is known to induce liver steatosis (Bai et al., 2017) and surprisingly reduce insulin resistance in rats (Kan et al., 2016). Mice deficient in Fmo3 or Fmo5 showed lean phenotypes with favorable lipid and sugar profiles, and transgenic expression of Fmo3 had the opposite effect (Shephard and Phillips, 2010; Veeravalli et al., 2014; Shih et al., 2015; Schugar et al., 2017; Scott et al., 2017). It appears that induction of FMO3 and FMO5 by valproic acid is a likely contributing to hepatic steatosis. Interestingly, fructose induced FMO5 mRNA but did not activate the FMO5 reporter (Fig. 3), suggesting that increased stability of FMO5 mRNA is involved in the mRNA induction. Given the fact that valproic acid induced FMO5 mRNA and activated the FMO5 reporter, induced expression of FMO5 by fructose and valproic acid was achieved through distinct mechanisms.

It has been reported that the expression of FMOs is ontogenically regulated and our age-related expression study provides additional important findings regarding developmental regulation (Dolphin et al., 1996; Janmohamed et al., 2002; Koukouritaki et al., 2002). The mRNA levels of both FMO3 an FMO5 showed a postnatal surge (Fig. 2), as seen in many other drug-metabolizing enzymes. The adult group, compared with the pediatric groups (groups 2 to 4), expressed higher levels of FMO3 mRNA but surprisingly lower levels of FMO5 mRNA, although the changes did not reach the level of statistical significance. For the levels of protein expression, both FMO3 and FMO5 exhibited age-related increases with an exception of the postnatal group (group 1). As matter of fact, this group expressed slightly higher FMO3 protein than and comparable FMO5 protein to the adult group. Western blotting with individual samples detected unusually high protein expression of two samples. These two donors were premature babies with congenital heart defect. These two samples, nonetheless, had comparable levels of RNA. These findings collectively suggest that the expression of FMO3 and FMO5 is regulated through multiple mechanisms. It should be noted that an early study reported a postnatal surge of FMO3 protein (Koukouritaki et al., 2002).

In summary, our work points to several important conclusions. First, both FMO3 and FMO5 are abundant proteins in hepatocytes, pointing to their roles in xenobiotic metabolism and maintenance of energy balance. Second, endocrine cells producing metabolically related hormones have a strong presence of FMO3, FMO5 or both. FMO enzymes are localized in the secretory pathway from the ER to Golgi complex,

suggesting that FMO3 and FMO5 are involved in hormonal secretion. Third, the expression of both FMOs is regulated by age, xenobiotics and nutritional components, and the regulation is achieved through multiple mechanisms. In addition, the catalytic action of FMO3 produces reactive oxygen species, which likely cause ER stress. It is conceivable that these enzymes likely regulate their own expression by products they produce.

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Table I. Summary of immunohistochemistry on FMO3 and FMO5

Tissue	FMO3	FMO5
Duodenum	No	Epithelium (1*)
Lung	Pneumocytes (1, 30%**)	Pneumocytes (1, 30%)
Pituitary	(1, 30%)	(1, 30%)
Cerebral cortex	(2, 70%)	(1, 50%)
Stomach	No	(1-2)
Liver	Hepatocytes (3)	Hepatocytes (2-3)
Parathyroid	No	Chief cell (1-2, 50%), oxyphil (3, 100%)
Spinal cord	Dendritic process (2) No	
Thyroid	Colloid/follicular cells (2)	No
Kidney	Proximal tubules (1, 5%)	Proximal and distal tubules (1)
Bladder	Stromal tissues (2)	(1, 70%)
Heart		(1, 70%)
Testis	Spermatocytes (2, 10%)	Spermatocytes (1, 10%)
Adrenal	Medullar (3)	Cortex (1, 50%)
Colon		Epithelium (1-2)
Skeletal muscle		(1, 40%)
Ureter	(1, 5%)	(1, 70%)

* Indicating the intensity of staining: 1 = slight staining; 2 = moderate staining; and 3 = strong staining.

** Percentage of cells that were stained.

No staining for either FMO3 or FMO5: Cerebellum, spleen, umbilical cord, tonsil, bone marrow, fallopian, pancreas, skin, thymus, endometrium, breast, ovary, prostate, cervix, lymph node, placenta.

LEGENDS FOR FIGURES

Figure 1. Immunohistochemical analysis FFPE array slides were warmed up in 60° C oven for 60 min followed by deparaffinization and rehydration. The slides were then incubated in Dako Target Retrieval Solution in pressure cooker in microwave for 15 min and then cool for 30 min. The slides were washed 3 times and then incubated in Dako quenching reagent for 10 min at room temperature. The quenched slides were washed 3 times again and incubated at 4°C with primary antibody (FMO3 or FMO5) or pre-immunized rabbit serum at a dilution of 1:1000 overnight. After 3 washings, the slides were incubated with Dako EnVision + Dual Link System-HRP secondary antibodies (anti-rabbit) followed by 3 washings. The washed slides were incubated with substrate-chromogen for 10 min. The reactions were stopped and the slides were dehydrated and mounted in Cytosealtm 60 media.

Figure 2. Expression of FMO3 and FMO5 as a function of age

(A) Expression of FMO3 Total RNAs were subjected to RT-qPCR analysis for the level of FMO3 mRNA by a *Taqman* probe. The signals from each target were normalized based on the signal from Pol II and expressed as relative levels among all samples. The data are presented as mean SD, and the levels of FMO3 mRNA were correlated with age (days). An asterisk symbol indicates Statistical significance for the correlation ($p < 0.05$). To determine the expression of FMO3 protein, S9 fractions (0.25 g) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were incubated with an antibody against FMO3 and re-probed with an antibody to CES1. The signal was captured by MyCEL Imager.

The membrane was stained with 0.1% Ponceau S to verify the equal loading. *(B) Expression of FMO5* Once again, the expression of FMO5 was determined by RT-qPCR and Western blotting. The data are presented as mean SD, and the levels of FMO5 mRNA were correlated with age (days). An asterisk symbol indicates Statistical significance for the correlation ($p < 0.05$).

Figure 3. Regulated expression of FMO3 and FMO5 and determination of reporter activity

(A) Regulated expression of FMO3 and FMO5 Human primary hepatocytes (n=6) were cultured and treated with brefeldin A (BFA, 2 μ M), thapsigargin (THAP, 2 μ M) and, Z-guggulsterone (Gugg, 10 μ M), Valporic acid (0.5 mM), Fructose (Fruct, 25 mM) or vehicle for 24 h. The level of FMO3 or FMO5 mRNA was determined by RT-qPCR with *Taqman* probes. (B) Determination of reporter activity. Cells (Huh7) were transfected with a reporter construct (50 ng) and the pRL-null *Renilla* (5 ng) for 24 h and then treated with a chemical at the same concentrations as described for the regulated expression experiment. The transfected cells were cultured for another 24 h, collected with PBS and resuspended in passive lysis buffer. The reporter activities were assayed with a Dual-Luciferase Reporter Assay System. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the signals were expressed as percentages of the normalized luciferase activity of transfected cells treated with the vehicle.

Figure 1.

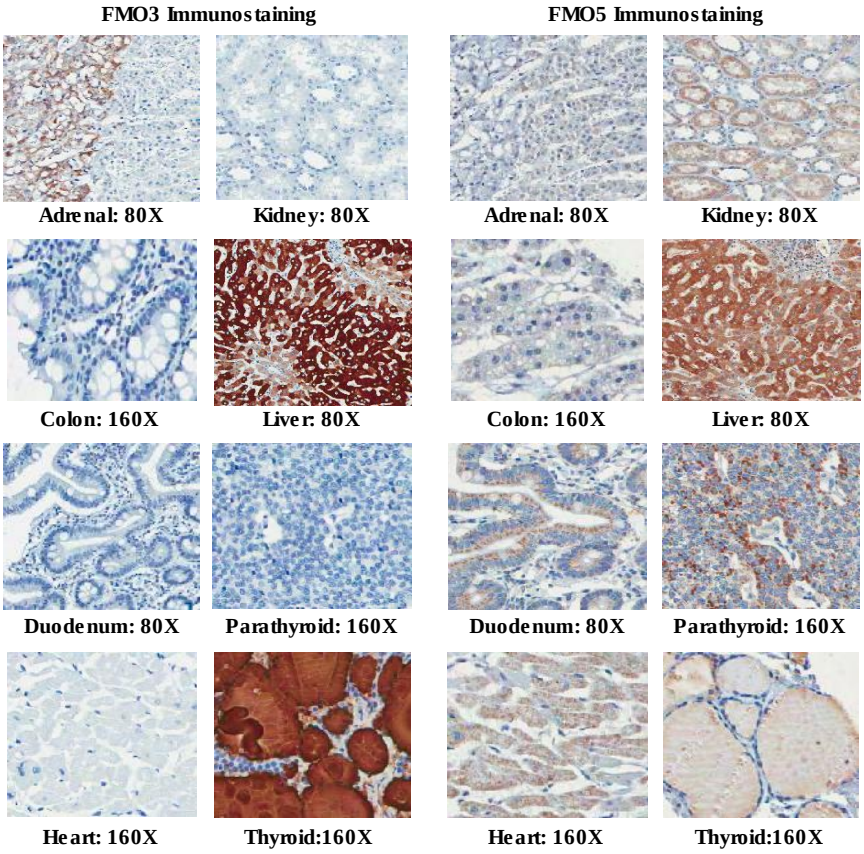
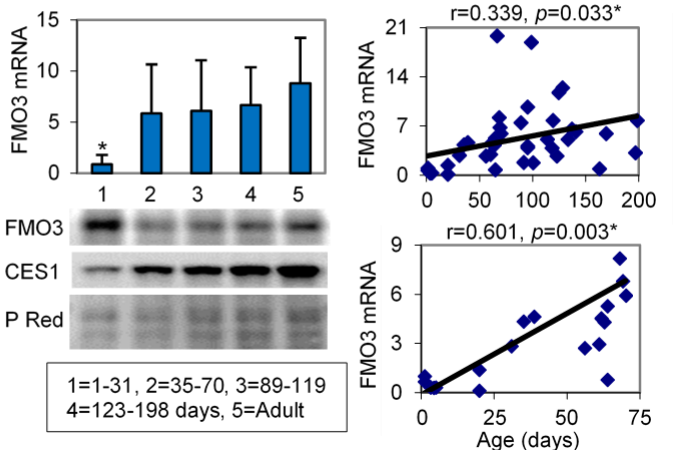

FMO3 Immunostaining
FMO5 Immunostaining
Adrenal: 80X
Kidney: 80X
Adrenal: 80X
Kidney: 80X
Colon: 160X
Liver: 80X
Colon: 160X
Liver: 80X
Duodenum: 80X
Parathyroid: 160X
Duodenum: 80X
Parathyroid: 160X
Heart: 160X
Thyroid:160X
Heart: 160X
Thyroid:160X

Figure 2.

A


FMO3 mRNA
15
10
5
0
*
1 2 3 4 5
FMO3
CES1
P Red
1=1-31, 2=35-70, 3=89-119
4=123-198 days, 5=Adult
r=0.339, p=0.033*
21
14
7
0
0 50 100 150 200
FMO3 mRNA
r=0.601, p=0.003*
9
6
3
0
0 25 50 75
Age (days)
FMO3 mRNA

B

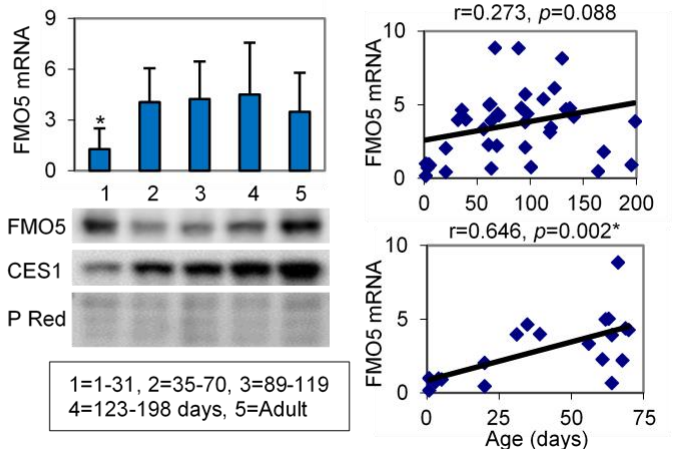
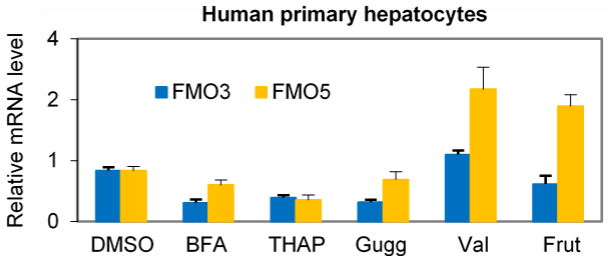
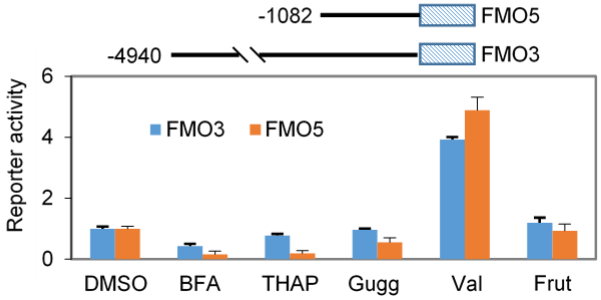

FMO5 mRNA
9
6
3
0
*
1 2 3 4 5
FMO5
CES1
P Red
1=1-31, 2=35-70, 3=89-119
4=123-198 days, 5=Adult
r=0.273, p=0.088
10
5
0
0 50 100 150 200
FMO5 mRNA
r=0.646, p=0.002*
10
5
0
0 25 50 75
Age (days)
FMO5 mRNA

Figure 3.

A


Human primary hepatocytes
Relative mRNA level
4
2
1
0
FMO3
FMO5
DMSO
BFA
THAP
Gugg
Val
Frut

B


-1082
FMO5
-4940
FMO3
Reporter activity
6
4
2
0
FMO3
FMO5
DMSO
BFA
THAP
Gugg
Val
Frut