

# Differential Localization of Flavin-Containing Monooxygenase (FMO) Isoforms 1, 3, and 4 in Rat Liver and Kidney and Evidence for Expression of FMO4 in Mouse, Rat, and Human Liver and Kidney Microsomes

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## ABSTRACT

Flavin-containing monooxygenases (FMOs) play significant roles in the metabolism of drugs and endogenous or foreign compounds. In this study, the regional distribution of FMO isoforms 1, 3, and 4 was investigated in male Sprague-Dawley rat liver and kidney using immunohistochemistry (IHC). Rabbit polyclonal antibodies to rat FMO1 and FMO4, developed using anti-peptide technology, and commercial anti-human FMO3 antibody were used; specificities of the antibodies were verified using Western blotting, immunoprecipitation, and IHC. In liver, the highest immunoreactivity for FMO1 and FMO3 was detected in the perivenous region, and immunoreactivity decreased in intensity toward the periportal region. In contrast, FMO4 immunoreactivity was detected with the opposite lobular distribution. In the kidney, the highest immunoreactivity for

FMO1, -3, and -4 was detected in the distal tubules. FMO1 and FMO4 immunoreactivity was also detected in the proximal tubules with strong staining in the brush borders, whereas less FMO3 immunoreactivity was detected in the proximal tubules. Immunoreactivity for FMO3 and FMO4 was detected in the collecting tubules in the renal medulla and the glomerulus, whereas little FMO1 immunoreactivity was detected in these regions. The FMO1 antibody did not react with human liver or kidney microsomes. However, the FMO4 antibody reacted with male and female mouse and human tissues. These data provided a compelling visual demonstration of the isoform-specific localization patterns of FMO1, -3, and -4 in the rat liver and kidney and the first evidence for expression of FMO4 at the protein level in mouse and human liver and kidney microsomes.

Flavin-containing monooxygenases (FMOs) are microsomal enzymes that catalyze oxidation of pharmaceutical drugs, pesticides, and endogenous compounds. In general, FMO oxidation increases the polarity of substrates, aiding in excretion and detoxification; however, some substrates are bioactivated to reactive or toxic metabolites. Five expressed FMO isoforms (FMO1–5) have been detected in humans (Lawton et al., 1994). FMO isoforms have different substrate

selectivity and exhibit distinct sex-, tissue-, age-, and species-dependent expression profiles (Hines et al., 1994).

The liver typically contains the largest concentrations of xenobiotic-metabolizing enzymes. Adult human liver mRNA exhibits high FMO3 expression, moderate FMO4 expression, and extremely low FMO1 expression (Dolphin et al., 1996; Cashman and Zhang, 2006). In contrast, rat or mouse liver has moderate FMO1 protein expression (Falls et al., 1995; Lattard et al., 2002). To our knowledge, FMO localization in liver has been elucidated only in mice by *in situ* hybridization (Janmohamed et al., 2004). FMO1 and FMO5 mRNAs were detected across the acinus, with a concentration gradient decreasing from the perivenous (PV) to the periportal (PP) hepatocytes. FMO2, -3, and -4 mRNAs were localized heavily in the PP region (Janmohamed et al., 2004). The heterogeneous expression patterns of FMO isoforms within the liver may suggest distinct roles in metabolism of drugs, xenobiotics, and endogenous compounds.

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**ABBREVIATIONS:** FMO, flavin-containing monooxygenase; PV, perivenous; PP, periportal; PT proximal tubules; DT, distal tubules; IHC, immunohistochemistry; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; P450, cytochrome P450; DCVC, S-(1,2-dichlorovinyl)-L-cysteine.

The kidney plays a large role in extrahepatic metabolism because of high exposure via perfusion and potential concentration of substrates within the tissue. Regions of the kidney, such as the proximal tubules (PT), may have a high density of xenobiotic-metabolizing enzymes (Lock and Reed, 1998). In human kidney, mRNA expression levels are high for FMO1, moderate for FMO4, and low for FMO3 (Dolphin et al., 1996; Zhang and Cashman, 2006). In the rat, FMO3 mRNA expression is greater in the kidney than the liver (Burnett et al., 1994; Lattard et al., 2001). In male and female mouse kidney, FMO1, -2, -3, -4, and -5 mRNAs were localized to the PT and distal tubules (DT) of the cortex and to the collecting tubules of the renal medulla, whereas only FMO1 mRNA was detected in the glomeruli (Janmohamed et al., 2004). In male rat kidneys, immunoreactivity with antibodies to rabbit lung FMO2 was localized in the PT and DT of the renal cortex and the collecting ducts of the renal medulla, but it was not detected in the glomeruli (Bhamre et al., 1993).

In previous studies, FMO3 and FMO5 mRNA levels did not correlate with FMO isoform protein levels in human liver samples (Overby et al., 1997). mRNA levels are not always well correlated to protein expression because of transcript instability and post-transcriptional regulation. Thus, in the present study, we used antibodies to FMO1, FMO3, and FMO4 to assess protein expression levels. We focused on these isoforms because of their known or suspected greater metabolic activities in comparison with FMO2 and FMO5.

Moderate FMO4 mRNA levels were detected in the liver and kidney in many mammalian species, including rat and human (Burnett et al., 1994; Cashman and Zhang, 2006; Nishimura and Naito, 2006). The expression of truncated or mutated human FMO4 in a heterologous system resulted in a protein that is metabolically active with methimazole, *S*-allyl-L-cysteine, and L-methionine (Itagaki et al., 1996; Ripp et al., 1999; Krause et al., 2003). Because of the potential biological activity of FMO4 and its moderate expression, we included this isoform in our IHC studies. In previous studies, tissue-specific expression of two FMO4 variants was characterized in rat brain and kidney by Western blot using two different antibodies to rat FMO4 peptides (residues 126–140 and 409–423 of the deduced FMO4 long form; Lattard et al., 2003). The detection of FMO4 in rat tissue is the only protein level examination of FMO4. Thus, we used the FMO4 anti-peptide antibody to examine the protein expression of FMO4 in mouse and human liver and kidney microsomes.

## Materials and Methods

**Materials.** Human cDNA-expressed FMO1, -3, and -5 and anti-human FMO3 antibody were purchased from BD Biosciences (Woburn, MA). Protein A Plus and disuccinimidyl suberate cross-linker were purchased from Thermo Fisher Scientific (Waltham, MA). For IHC studies, adult male Sprague-Dawley rats (300 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) or Harlan (Madison, WI). Adult male and female B6C3F<sub>1</sub> mice were purchased from The Jackson Laboratory. Animals were maintained on a 12-h light/dark cycle with free access to food and water. For Western blots and immunoprecipitation experiments, male Sprague-Dawley rat liver and kidney were purchased from Pel-Freez Biologicals (Rogers, AR), human liver samples from white adults were purchased from SRI International (Menlo Park, CA), and human kidney samples from white adults were procured from International Bioresearch

Solutions (Pasadena, CA). All tissues were made into “washed” microsomes by standard methods (Sausen and Elfarra, 1990). Protein concentrations were determined using the bicinchoninic acid assay (Thermo Fisher Scientific) using bovine serum albumin as the standard.

**Antibodies.** Antibodies were produced against synthetic peptides derived from the predicted sequence of rat FMO1 and FMO4. Full-length amino acid sequences were obtained from the National Center for Biotechnology Information (Bethesda, MD) and subjected to computer analysis using GeneRunner (Hastings Software Inc., Hastings on Hudson, NY). A peptide from each protein was selected for antibody production based on indices of antigenicity, surface probability, and structural complexity. Database homology analysis using BLASTp (National Center for Biotechnology Information) confirmed the specificity of these candidate antigens for each rat FMO isoform. Chosen peptides were rat FMO1 (403–423) and rat FMO4 (long form Cys-415–434, corresponding to Cys-352–371 of the short form). Cysteine was used as a cross-linking site. For peptides without Cys in their sequence Cys was added to the N terminus, and all peptides were acetylated at the N terminus and amidated at the C terminus. The immunogen consisted of synthesized peptides cross-linked to keyhole limpet hemocyanin (Open Biosystems, Huntsville, AL). For each peptide immunogen, two New Zealand White rabbits were subcutaneously injected with 0.50 mg of antigen at 10 sites. Subcutaneous booster injections of 0.25 mg of antigen at four sites were performed on days 14, 28, and 42. Antisera (Open Biosystems) were harvested on days 56 and 72 and throughout a 3-month period.

Antisera were purified by column affinity chromatography against the synthetic peptides. Affinity matrices were prepared using Sulfo-Link media (Thermo Fisher Scientific) according to the manufacturer's protocol. To purify the antisera, samples were diluted with equal amounts of 10 mM Tris, pH 7.5, and pumped through the 1-ml column with the same buffer, resulting in the elution of a large protein peak. Next, the column was washed to elute loosely adhering protein with 10 mM Tris and 0.5 M sodium chloride, pH 7.5. Purified antibody was eluted with 100 mM glycine, pH 2.5, or 100 mM acetic acid, pH 4.0, into tubes containing an equal volume of 1 M Tris, pH 8.0. Protein fractions were monitored at 280 nm (ISCO, Lincoln, NE). Antibody-containing fractions were pooled and dialyzed against 0.05 M phosphate-buffered saline (PBS), pH 7.4; concentrated by centrifugal filtration; and stored with 1% bovine serum albumin and 0.1% sodium azide. Antisera were characterized by immunoblotting, immunoprecipitation, and immunohistochemistry as described below.

**Immunoblotting.** Antibodies were characterized by the immunoreactivity to proteins separated by SDS-PAGE (12.5% Tris Criterion precast gel; Bio-Rad Laboratories, Hercules, CA). Protein samples were diluted in activated sample buffer containing 2-mercaptoethanol. Immunoblots used for characterization of antibodies included prestained molecular weight standards (Bio-Rad Laboratories); human cDNA-expressed FMO1, FMO3, and FMO5 (1.5 µg; BD Biosciences); and rat liver and kidney microsomes (20 or 10 µg). Immunoblots for immunoprecipitation used rat kidney microsomes (10 µg) as the positive control and immunoprecipitated samples (15 µl) to determine reactivity with the retained protein. Immunoblots used to determine species cross-reactivity of the FMO4 antibody contained rat kidney (15-µg), rat liver (30-µg), human liver and kidney (15-µg), and mouse liver and kidney (30-µg) microsomal samples. The gels were run for 80 min with 200 V and then transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) at 70 mA for 70 min. The nitrocellulose was incubated in blotting reagent (5% powdered milk and 0.06 M NaCl in 25 mM Tris, pH 7.5) for 1 h and washed in Tris-buffered saline with 0.1% Tween 20 multiple times. The nitrocellulose was exposed overnight to primary antibody. For immunoblots with rat tissue, dilutions of 1:250 or 1:500 were used; for immunoblots with human or mouse tissue, dilutions of 1:250 or 1:100 were used. Blots were washed in Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit anti-

body (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). After washing, blots were treated with West Pico Super Signal chemiluminescent substrate (Thermo Fisher Scientific).

**Immunoprecipitation.** Purified anti-peptide FMO1 and FMO4 antibodies or FMO3 antibody (BD Biosciences) were bound to Protein A Plus (Thermo Fisher Scientific) by incubating together overnight at 4°C. The bound antibodies were washed and cross-linked according to manufacturer's instructions. Rat kidney microsomes were dialyzed against PBS, solubilized with 1% Triton X-100, and precleared by incubating (1–3 h) with Protein A Plus. The microsomes were incubated overnight at 4°C with the antibody cross-linked to Protein A Plus. The unbound protein was washed off with PBS, and the bound protein was eluted with ImmunoPure IgG elution buffer (150  $\mu$ l, pH 2.8; Thermo Fisher Scientific). The eluted protein was immediately neutralized with 1 M Tris, pH 9.5. Eluted samples were then run by SDS-PAGE and immunoblotted as described above.

**Immunohistochemistry.** Male Sprague-Dawley rats were deeply anesthetized with isoflurane and then sodium pentobarbital (60 mg/kg i.p.), thoracotomized, and the right atrium was opened and the left ventricle was cannulated. Rats were then perfused intracardially with a flush solution (Tyrode's solution containing 0.1% heparin and 0.2% procaine) followed by a histological fixative (4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5). Liver and kidney blocks were removed and fixed overnight in the same fixative at 4°C. Tissues were dehydrated in ascending concentrations of ethanol and xylene and finally embedded in paraffin. Tissue sections (10  $\mu$ m) were cut on a rotary microtome, and sections were mounted on gelatin-subbed slides.

Sections were deparaffinized with xylene and rehydrated with descending concentrations of ethanol to PBS, to prepare the tissue for IHC processing. Sections were incubated in 0.5% hydrogen peroxide in methanol (30 min) to quench endogenous peroxidase activity and then washed. Finally, sections were subjected to an antigen retrieval protocol in which sections were incubated in 10 mM citric acid (85°C for 10 min), cooled, and washed in PBS. This procedure results in intensified staining. Sections were immunohistochemically stained using a proprietary polymer-based peroxidase staining method (Biocare Medical, Concord, CA). Sections were first subjected to a blocking step for 5 min in Background Sniper (Biocare Medical), washed in IHC buffer (0.05 M PBS, pH 7.4, with 0.1% gelatin, 0.002% neomycin, and 5% normal goat serum), and incubated in diluted 1:25 to 1:200 anti-peptide FMO antibodies in IHC buffer overnight in the refrigerator. Slides were drained and washed in IHC buffer and processed for bound antibody detection by incubation in full-strength MACH2 Polymer-horseradish peroxidase conjugate (Biocare Medical) for 30 min; washed in 0.05 M PBS and 0.1 M Tris, pH 7.5; developed with diaminobenzidine (30 mg), hydrogen peroxide (0.01%), and nickel ammonium sulfate (0.02%) for 7 to 10 min; washed in 0.1 M Tris, pH 7.5, and PBS; dehydrated in ethanol; cleared in xylene; and mounted with a coverslip with Permount (Thermo Fisher Scientific).

Control IHC studies included processing of tissue with omitted, soluble, or solid matrix immunoabsorbed anti-FMO antibodies. Control tissues were run in parallel with antibody-incubated tissues. Sections were viewed using an Eclipse-600 photomicroscope (Nikon, Tokyo, Japan) equipped with a Spot digital camera (Instruments, Inc., Sterling Heights, MI). Photographs were quality-enhanced using Paint Shop Pro (Corel, Ottawa, ON, Canada).

## Results

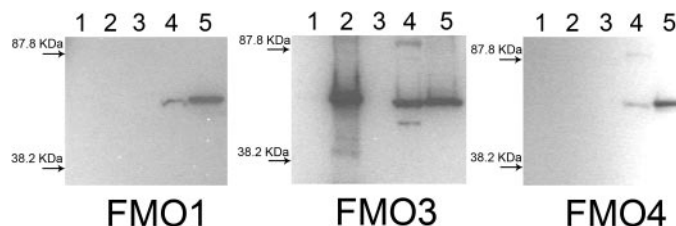
**Characterization of the Specificity of FMO1, FMO3, and FMO4 Antibodies.** To our knowledge, no commercial antibodies are available for rat FMO1, -3, or -4. We produced rabbit polyclonal antibodies to rat FMO1 and FMO4 by selecting rat FMO1 and FMO4 peptides based upon their high

degree of antigenicity, surface probability, and structural complexity. The selected peptides underwent a BLASTp search and were unique to the FMO isoform. Before use, rabbit antisera were purified by column affinity chromatography against the synthetic peptides to increase specificity and purity of the antibodies. The anti-peptide FMO1 antibody reacted strongly with rat liver and kidney microsomes at the approximate molecular mass of FMO1 (~60 kDa) but not with cDNA-expressed human FMO1, -3, and -5 (Fig. 1). The anti-peptide FMO4 antibody reacted with rat liver and kidney microsomes but not with cDNA-expressed human FMO1, -3, and -5 (Fig. 1). In some immunoblots, rat liver microsomal protein exhibited two bands at slightly different electrophoretic mobilities (56 and 63 kDa; see Fig. 7). Commercial anti-human FMO3 detected the orthologous rat protein and was used in this study (Fig. 1). Anti-human FMO3 antibody (BD Biosciences) reacted with cDNA-expressed human FMO3 and rat liver and kidney microsomes at the approximate molecular mass of FMO3 (~60 kDa) but not with cDNA-expressed human FMO1 and FMO5 (Fig. 1).

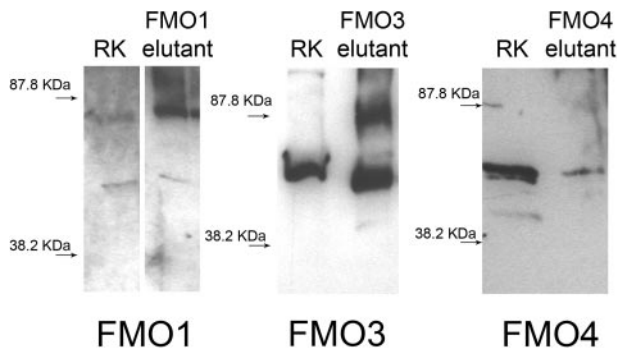
Immunoprecipitation of FMO1, -3, and -4 was done by cross-linking the antibodies to Protein A and incubating with 1% Triton X-100-solubilized rat kidney microsomes. The immunoprecipitated sample for each antibody was run by SDS-PAGE and immunoblotted with the antibody used in immunoprecipitation. A band at the approximate molecular mass of FMOs was detected, indicating the respective FMO was retained by the antibody (Fig. 2). Denaturation by SDS was not necessary because the antibodies were able to react with the native FMO protein as indicated by the immunoprecipitation and IHC results (discussed below).

During IHC, titration studies of anti-FMO3, FMO1, and FMO4 anti-peptide antibodies yielded staining patterns in liver and kidney that were diminished by dilution. For FMO1 and FMO4 anti-peptide antibodies, the three parallel control experiments with omitted, soluble, or solid matrix-absorbed antibody resulted in a complete loss of staining (Figs. 3, 4C, and 6D). In addition, there seemed to be no difference between staining results within the pairs of antibodies produced in individual rabbits for each FMO isoform.

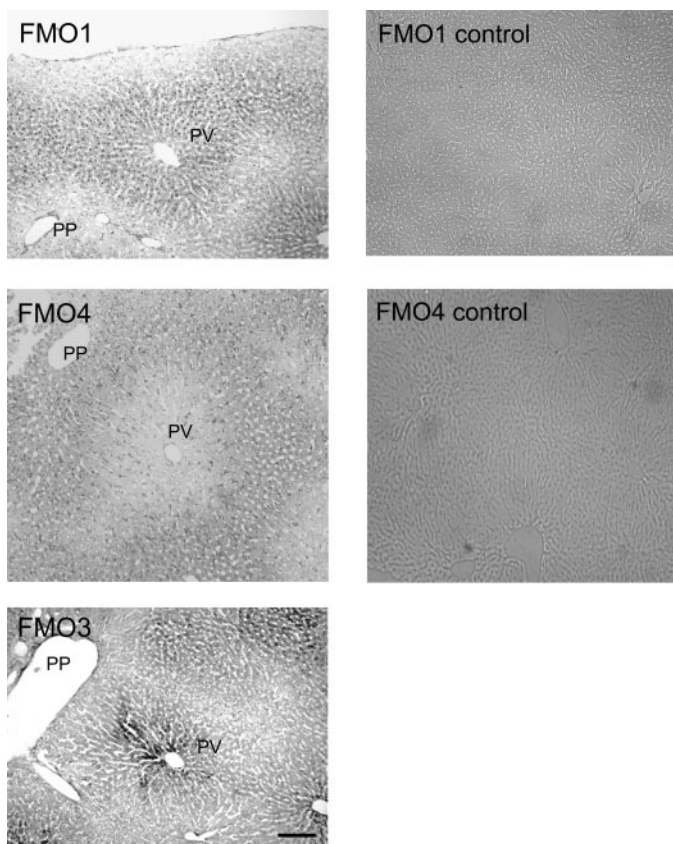
**Localization of FMO1, FMO3, and FMO4.** Liver immunoreactivity showed a reverse intralobular distribution for FMO1 and FMO3 versus FMO4 (Fig. 3). FMO1 and FMO3 immunoreactivity was centered around the PV region at the interior of the liver lobules, gradually diminishing radially from this site. The distribution of immunoreactivity to FMO4 anti-peptide antibody was the reverse, with higher concen-



**Fig. 1.** Immunoblotting to characterize FMO antibodies. Immunoblotting was performed as described under *Materials and Methods* and probed with rabbit polyclonal anti-peptide FMO1 and FMO4 antibodies or anti-human FMO3 antibody. For all antibodies, lanes 1 to 3 are cDNA-expressed human FMO1, FMO3, and FMO5, respectively (1.5  $\mu$ g); lane 4 is rat liver microsomal protein (20  $\mu$ g); and lane 5 is rat kidney microsomal protein (10  $\mu$ g). Positions of molecular mass markers are shown.



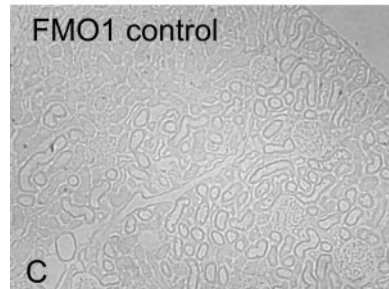
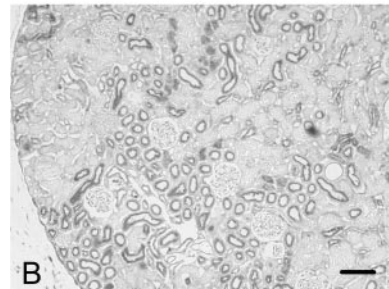
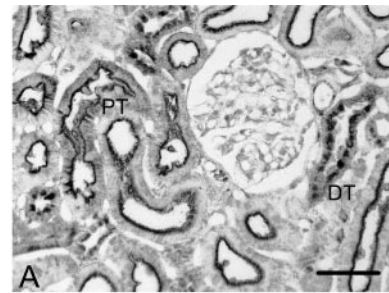
**Fig. 2.** Immunoprecipitation of FMOs from solubilized rat kidneys. FMOs were immunoprecipitated from rat kidney microsomal protein as described under *Materials and Methods*. Rat kidney microsomal protein (RK; 10  $\mu$ g) and the eluted proteins (15  $\mu$ l) were run by SDS-PAGE and immunoblotted by the antibody used to immunoprecipitate them. Positions of molecular weight markers are shown.



**Fig. 3.** Immunoreactive FMO1, FMO3, and FMO4 in the liver lobule. The PV region at the center of the liver lobule and PP sites at the periphery are labeled. Controls using immunoabsorbed anti-FMO antibodies are shown. Scale bar, 100  $\mu$ m.

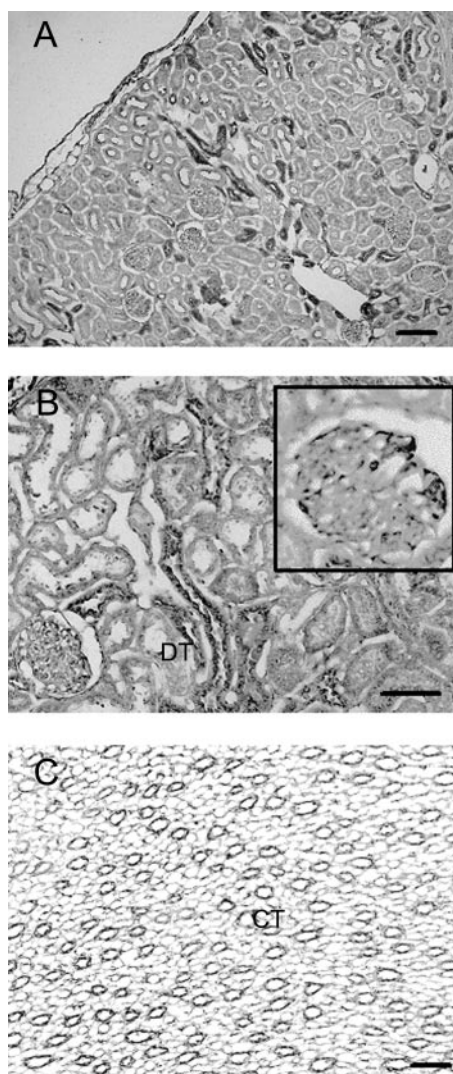
tration near the periportal regions at the corners of the lobules and gradually diminishing toward the central PV region. FMO1 and FMO4 antibodies that were exposed to the affinity column matrix containing the synthesized peptide did not exhibit any staining patterns in the liver (Fig. 3).

Sections of the kidney cortex showed a large number of stained renal tubules among other unstained tubules for FMO1 (Fig. 4), FMO3 (Fig. 5), and FMO4 (Fig. 6). Cortical staining was equally immunopositive for all three FMO isoforms in the DT. In this site, intense staining filled the cytoplasm of the cells and close perinuclear region, sugges-



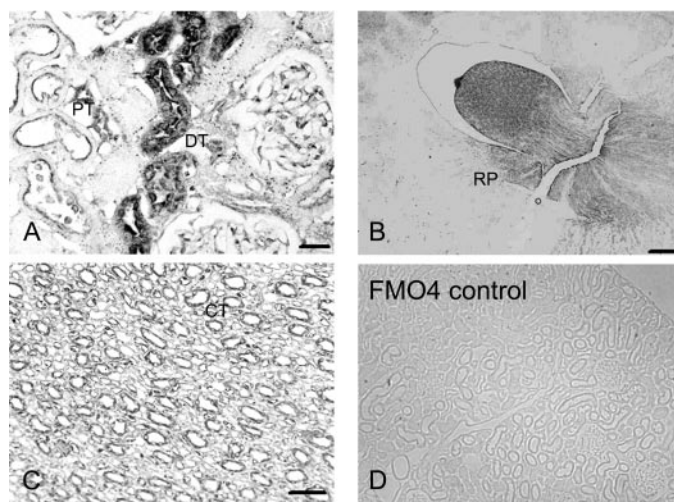
**Fig. 4.** Immunoreactive FMO1 in rat kidney. PT and distal DT within a matrix of unstained glomeruli and tubular elements are labeled. C, a control using immunoabsorbed FMO1 antibody is shown. Scale bars, 50  $\mu$ m (A) and 200  $\mu$ m (B and C).

tive of reactivity in the Golgi apparatus. Immunoreactivity was also seen in the PT, but its intensity and distribution varied between the three isoforms. The brush border was most intensely stained for FMO1 (Fig. 4A), slightly less for FMO4 (Fig. 6A), and very faintly or not at all for FMO3 (Fig. 5B). FMO3 staining of the PT showed regional differences, with the S1 segment staining lightly or not at all, the S2 segment staining more densely, and the S3 segment staining most densely. PT cells reacting with FMO1 had strong intracellular body immunoreactivity but lightly to moderately stained cytoplasm (Fig. 4A). For FMO4, there were densely stained basal bodies, but the cytoplasmic staining was weak (Fig. 6A). Staining for FMO1 was absent in the kidney medulla but strong in kidneys reacted with antisera to FMO3 (Fig. 5, A and C) and FMO4 (Fig. 6C). This staining was attributed to strong collecting tubule immunoreactivity (Figs. 5C and 6C). Glomeruli showed immunoreactivity for FMO3 (Fig. 5B) and FMO4 (Fig. 6A) but not FMO1 (Fig. 4A). FMO3 glomerular immunoreactivity was strongest (Fig. 5B, inset). FMO4 immunoreactivity was strong in the renal papilla showing striations of the tubules feeding into it from the outer zone of the medulla (Fig. 6B). The immunoabsorbed controls for FMO1 and FMO4 exhibited a complete loss of staining (Figs. 4C and 6D). The immunohistochemical findings are summarized in Table 1.



**Fig. 5.** Immunoreactive FMO3 in rat kidney. A, low-power micrograph shows distribution of stained tubular elements in the cortex. B, at mid-magnification, these are shown to be primarily distal tubules. B, inset, the glomerular immunoreactivity is shown. C, collecting tubules were also labeled. Scale bars, 200  $\mu\text{m}$  (A and C) and 50  $\mu\text{m}$  (B).

**Antibody Cross-Reactivity in Human and Mouse Tissues.** The FMO1 and FMO4 anti-peptide antibodies were tested for immunoreactivity in male and female mouse and human liver and kidney microsomes. The anti-peptide FMO4 antibody reacted with mouse and human liver and kidney microsomes (Fig. 7). The bands of immunoreactivity in mouse and human tissues exhibited the same electrophoretic mobility as the band characterized in rat kidney. Male and female rat liver exhibited an immunoreactive band at a slightly lower molecular mass ( $\sim 56$  kDa) than the band in rat kidney ( $\sim 63$  kDa), corresponding to the predicted molecular mass of the short- and long-splicing variants of FMO4 (Fig. 7). Low antibody dilutions were used to detect mouse FMO4 that may have resulted in detection of protein degradation or aggregation products exhibited in this immunoblot (Fig. 7). FMO1 anti-peptide antibody did not react with human liver and kidney microsomes, consistent with the lack of reactivity of the anti-peptide FMO1 antibody with human cDNA-expressed FMO1 (Fig. 1). When we immunoblotted the FMO1 antibody against microsomal protein from male and female



**Fig. 6.** Immunoreactive FMO4 in rat kidney. Renal papilla (RP) are labeled as well as stained DT and PT and collecting tubules (CT). D, a control using immunoabsorbed FMO4 antibody is shown. Scale bars, 40  $\mu\text{m}$  (A), 500  $\mu\text{m}$  (B), and 250  $\mu\text{m}$  (C and D).

mouse liver and kidney (15  $\mu\text{g}$ ), no reaction was observed. However, weak cross-reaction of rat anti-peptide FMO1 was observed in male mouse liver and kidney at high protein loads (40  $\mu\text{g}$ ) and low antibody dilution (1:50; data not shown).

## Discussion

The localization of FMO1, FMO3, and FMO4 in male rat liver and kidney was characterized to assess the potential role of different FMO isoforms in drug and toxicant metabolism and toxicity. FMO1 and FMO4 antibodies were produced against a synthetic peptide with unique homology to each FMO isoform. The immune sera were affinity-purified with the peptide to increase specificity. Immunoblotting showed reactivity at the correct molecular mass and lack of reactivity against alternate human cDNA-expressed FMO isoforms. Characterization of the antibodies by immunoprecipitation and by IHC controls provided further evidence that the exhibited immunoreactivity is isoform-specific.

The localization of FMO1 and FMO3 in the PV region in male rat liver is similar to the localization of P450s (Lindros, 1997). In a previous study, FMO3 mRNA in male mouse liver was reported to have the opposite intralobular distribution using *in situ* hybridization methods (Janmohamed et al., 2004). Although differences between the methods used in these studies complicate the comparison, the difference in FMO3 localization between rats and mice is supportive of the hypothesis that FMO3 expression is regulated differently between the two rodent species, as indicated by the difference in testosterone regulation (Falls et al., 1997; Lattard et al., 2002). The FMO4 localization pattern in the PP region is similar to phase II enzymes, such as glutathione peroxidase (Lindros, 1997). FMO4 localization in the PP region may cause the liver acinus to be selectively exposed to FMO4 metabolites, which could protect the liver or increase exposure to reactive metabolites.

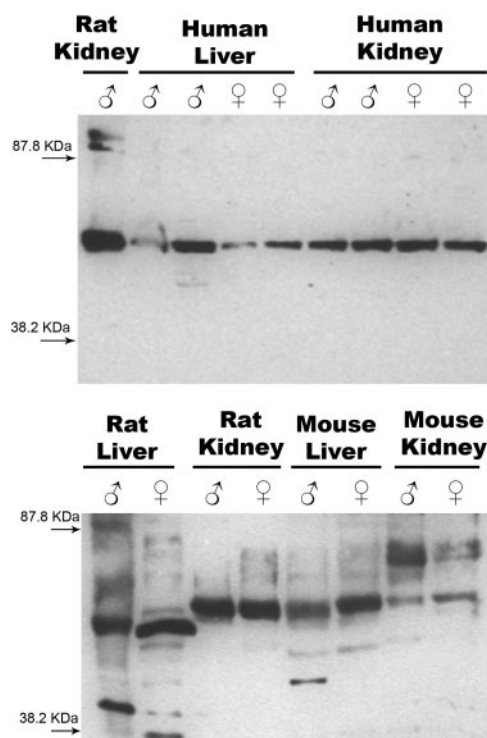
The differential patterns of FMO expression in the liver lobule are important for the toxicological evaluation of FMO-mediated substrates. In this regard, ketoconazole has been

TABLE 1

Immunohistochemical distribution of FMO isoforms in rat kidney and liver

Immunohistochemical staining for each antibody was performed as described under *Materials and Methods*. The degree of staining was assessed in PT, DT, collecting tubules (CT), glomeruli (Glom), PV, and PP. For the kidney sections, the 1+ represents staining that is slightly above background, and the 4+ represents the maximal density of staining. The 2+ and 3+ represent approximately 25% increments of increasing intensity between 1+ and 4+.

Isoform	Kidney				Liver	
	PT	DT	CT	Glom	PV	PP
FMO1	3+	4+	–	–	+	–
FMO3	1+	4+	3+	3+	+	–
FMO4	2+	4+	3+	1–2+	–	+



**Fig. 7.** Immunoblotting with the FMO4 anti-peptide antibody. Immunoblotting was performed as described under *Materials and Methods*, with 15  $\mu$ g of microsomal protein from male and female rat kidney and male and female human liver and kidney and 30  $\mu$ g of microsomal protein from male and female rat liver and male and female mouse liver and kidney. The additional bands in the lower panel are probably because of the low antibody dilutions used, which may have resulted in detection of protein degradation or aggregation products. Positions of molecular mass markers are shown.

documented to cause hepatotoxicity and liver necrosis in the PV region (Stricker et al., 1986). Because the hepatotoxicity of ketoconazole is caused, in part, by an FMO3 metabolite (Rodriguez and Acosta, 1997; Rodriguez and Miranda, 2000), our localization of FMO3 to the PV region suggests FMO3 may play a role in the zonal selectivity of ketoconazole necrosis.

Liver zonal heterogeneity between isoforms may be because of the microenvironment of hepatocytes that are exposed to different levels of hormones and xenobiotic inducers. Little is known about how FMO zonation is regulated and whether it is modified with endogenous or exogenous signals. FMO expression is affected by testosterone and estrogen exposure (Falls et al., 1997) and by foreign compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylchlorathane, and ranitidine (Chung et al., 1997; Rae et al., 2001; Tijet et al., 2006; Celius et al., 2008). It is noteworthy that individual P450s respond to inducers in the rat liver acinus in a region-dependent

manner, generally being induced in the same area that the isozyme is constitutively expressed (Bühler et al., 1992). Further investigation is needed to determine whether FMO localization patterns will change in response to endogenous or exogenous signals.

The IHC localization of FMO1, -3, and -4 immunoreactivity in the DT of renal cortex (Figs. 4A, 5B, and 6A) is consistent with the localization of other xenobiotic-metabolizing enzymes, including many isoenzymes in the P450 family (Cummins et al., 1999). In mice FMO1, -2, -3, -4, and -5 mRNA were all primarily localized to the distal tubules of the cortex (Janmohamed et al., 2004). Consistent with our results (Fig. 4A), *S*-benzyl-L-cysteine *S*-oxidase activity, primarily an FMO1-mediated reaction, was greater in isolated rat DT cells than PT cells (Lash et al., 1994).

The PT brush border is on the luminal surface of the epithelial cells and is involved in the reabsorption of the solutes in the filtrate through metabolically driven transport. This action can increase the concentration of many xenobiotics; thus, the PT plays a large role in chemical bioactivation and is highly susceptible to chemical insult. Many xenobiotic-metabolizing enzymes are localized in the PT cells, including P450s and glutathione transferase (Lock and Reed, 1998), and some have been localized to the PT epithelial cells (Murray et al., 1999; Muskhelishvili et al., 2001). This area stained most intensely for FMO1, followed by FMO4, and faintly for FMO3 (Figs. 4A, 5B, and 6A). This suggests the PT may be more sensitive to the substrates metabolized by FMO1, such as thiourea and hydrazines (for review, see Krueger and Williams, 2005).

The glomerulus is the entrance to the kidney, and FMO localization in this region would expose the rest of nephron to FMO metabolites. In our study, immunoreactivity to FMO3 and FMO4 was detected in the glomeruli and in the collecting tubules in the renal medulla. It is noteworthy that, in mice, all five FMO mRNAs were detected in the collecting ducts of the medulla, and only FMO1 mRNA was detected in the glomeruli (Janmohamed et al., 2004).

The FMO3 substrate *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) (Ripp et al., 1997; Krause et al., 2003), a metabolite of trichloroethylene, is a rat nephrotoxin. The S3 segment of the proximal tubules is the most sensitive area to DCVC and the more potent FMO-mediated metabolite DCVC sulfoxide (Jones et al., 1988; Lash et al., 1994). It is noteworthy that we found FMO3 to be more heavily localized in the S3 region of the PT cells than the S2 or S1 region, indicating FMO3 may play a role in DCVC-induced toxicity. The greater sensitivity of the S3 segment of the proximal tubules could also be because of selective accumulation of DCVC and DCVC sulfoxide in the PT (Zhang and Stevens, 1989). In vitro studies

TABLE 2

Sequence homology between species for the synthesized peptides  
 Bold amino acids differ from the rat FMO sequence used to make the synthesized peptides.

	Position	Sequence	Position
FMO1			
Rat	403	SVMMKEVNERKKNKHSGFGLC	423
Mouse	403	SVMM <b>E</b> VNERKKNKHSGFGLC	423
Human	403	SVMI <b>E</b> INARK <b>E</b> NK <b>P</b> SWFGLC	423
FMO4			
Rat	415	KRGVIKDTSDQKLDLFI SYMD	434
Mouse	415	KRGVIKDTSDQKLDLFI <b>T</b> YMD	434
Human	416	KRGV <b>F</b> KDT <b>S</b> KDK <b>F</b> DY <b>I</b> A YMD	435

have shown DCVC sulfoxide plays a role in DCVC toxicity in human PT cells (Lash et al., 2003).

The direct detection of FMO4 at the protein level in human and mouse tissue is novel (Fig. 7). These data confirm mRNA detection at moderate or low expression levels in human liver and kidney (Dolphin et al., 1996; Lattard et al., 2004; Cashman and Zhang, 2006; Nishimura and Naito, 2006). In a previous study, mRNA FMO4 transcripts were not detected in the liver of adult male CD-1 mice (Falls et al., 1995), but low levels of FMO4 mRNA were detected in male and female 129/SV mice (0–8 weeks). Our detection of FMO4 in adult male and female mouse liver shows that FMO4 is expressed in this species and that there may be strain- or age-related differences in expression. It is noteworthy that the immunoreactivity in rat liver was of different electrophoretic mobility than the immunoreactivity in rat kidney, mouse liver and kidney, and human liver and kidney (Fig. 7). This difference in molecular mass could be because of degradation of the sample, post-translation modifications, or because of tissue-specific splicing of the protein, as was observed with rat brain and kidney (Lattard et al., 2003). Our observed molecular masses of 56 kDa for rat liver and 63 kDa for rat kidney (Fig. 7) are consistent with the predicted molecular mass of 55,871 Da for the short form of rat FMO4 and 63,395 for the long form of rat FMO4 (Lattard et al., 2003). A tissue-specific splicing variant could be indicative of functional change and warrants further investigation.

It is noteworthy that the FMO4 antibody reacted with mouse and human tissue, whereas the FMO1 antibody did not react well with mouse or human tissue. A BLASTp search for the rat FMO1 peptide (residues 403–423) matched 20/21 amino acids in mouse FMO1 and 14/21 amino acids in human FMO1. A BLASTp for the rat FMO4 peptide (415–434 long form) matched 19/20 amino acids in mouse FMO4 and 15/20 amino acids in human FMO4 (Table 2). There is one amino acid difference between the synthesized peptide for FMO1 and FMO4 and the mouse amino acid sequences; yet, the FMO4 antibody has a stronger reaction with mouse tissue than the FMO1 antibody. The rat FMO4 synthesized peptide contains a serine residue in which there is a threonine residue in the mouse protein. Because both amino acids are polar with hydrophilic properties, the structure of the peptides may not be significantly different. In contrast, rat FMO1 peptide has lysine, a basic residue, switched to glutamic acid, an acidic residue, in the mouse protein. This change may alter the structure of the protein, affecting its reactivity with the antibody. Human FMO4 has five amino acid differences from the rat peptide but still reacts with the antibody. The changed residues include isoleucine and leucine to phenylal-

anine, which are all hydrophobic residues; glutamine to lysine, which have amphipathic or hydrophilic character; and phenylalanine to tyrosine, which have larger ring structures. There are seven amino acid differences between human and rat FMO1-synthesized peptide, and the anti-peptide antibody does not react with the human FMO1 protein. The residues that are different may cause structural changes such as glycine to tryptophan, which is a large change in the size of the side chain, and histidine to proline, because the ring structure of proline restricts conformational freedom. Ultimately, the determination of antibody reactivity remains empirical because it is difficult to predict how the antibody is reacting with the protein and which amino acid changes will affect reactivity.

This study characterized FMO isoform-specific antibodies, immunochemically localized FMO in rat liver and kidney, and provided the first detection of FMO4 protein in human and mouse liver and kidney. The localization patterns detected in rat tissues correspond to other xenobiotic-metabolizing enzymes, showing FMOs are optimally situated within the organ to act on foreign compounds. The potential species-specific difference between rats and mice are discussed and further investigation is needed to determine the role of FMO-mediated reactions in toxicity.

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