JPET Fast Forward. Published on April 14, 2004 as DOI: 10.1124/jpet.104.067819

CYP2C44, A New Murine CYP2C that metabolizes Arachidonic Acid to Unique Stereospecific Products

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Running Title: Cloning and Characterization of Mouse CYP2C44

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Number of text pages 34

Number of Tables 4

Number of Figures 5

Number of references: 47

Number of words in Abstract: 245

Number of Words in Introduction: 664

Number of words in Discussion: 1247

ABBREVIATIONS: CYP, cytochrome P450; POR, NADPH-cytochrome P450 oxido-reductase; DHET, *vic*-dihydroxyeicosatrienoic acid; EET, *cis*-epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; ORF, open reading frame; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; SRSs, substrate recognition sites.

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Abstract

The human CYP2Cs have been studied extensively with respect to the metabolism of clinically important drugs and endogenous chemicals such as arachidonic acid (AA). Five members of the mouse CYP2C family have previously been described which metabolize arachidonic acid into regio- and stereospecific epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), which have many important physiological roles. Herein, we describe the cloning and characterization of a new mouse P450, CYP2C44, which has the lowest homology with other known mouse CYP2Cs. Western blotting and real-time PCR detected CYP2C44 mRNA and protein in liver>> kidney > adrenals. Kidney contained approximately 10% of the CYP2C44 mRNA content of liver. CYP2C44 metabolized AA to unique stereospecific products, 11*R*,12*S*-EET and 8*R*, 9*S*-EET, which are similar to those produced by rat CYP2C23. CY2C23 is highly expressed in rat kidney and has been suggested to be important in producing compensatory renal artery vasodilation in response to salt-loading in this species. Immunohistochemistry showed the presence of CYP2C44 in hepatocytes, biliary cells of the liver, and the proximal tubules of the kidney. Unlike mouse CYP2C29, CYP2C38 and CYP2C39, CYP2C44 did not metabolize the common CYP2C substrate tolbutamide. CYP2C44 was not induced by phenobarbital or pregnenolone-16α-carbonitrile (PCN), two prototypical inducers of hepatic P450s. The presence of CYP2C44 in mouse liver, kidney and adrenals and the unique stereospecificity of its arachidonic acid metabolites are consistent with the possibility that it may have unique physiological roles within these tissues, such as modulation of electrolyte transport or vascular tone.

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The human CYP2C subfamily is well characterized and known to metabolize clinically important pharmaceuticals such as the hypoglycemic drug tolbutamide (Veronese et al., 1991; de Morais et al., 1994), the anticoagulant warfarin (Rettie et al., 1992) and nonsteroidal anti-inflammatory drugs such as diclofenac, ibuprofen and acetylsalicylic acid (Leemann et al., 1993). Members of the human CYP2C subfamily also metabolize arachidonic acid (Daikh et al., 1994). CYP2Cs have been identified in the chicken and mammalian species with four members in humans (Goldstein and de Morais, 1994), seven in rats (Legraverend et al., 1994; Strom et al., 1994; Nelson et al., 1996) and nine members in rabbits (Nelson et al., 1996). The mouse CYP2C family appears to be the most complex with five members published to date (Matsunaga et al., 1994; Luo et al., 1998) with at least ten unpublished new members and four pseudogenes identified (Wang et al., 2004 and Zhao, Goldstein, Zeldin, and unpublished data) (for update see http://drnelson.utmem.edu/CytochromeP450.html). With increasing attention being given to the mouse as a model to study the physiological relevance of enzymes *in vivo*, identification and characterization of the individual members of the CYP2C subfamily is an important first step in identifying their physiological and pathological roles.

Most members of the CYP families 1 through 4 are expressed predominately in liver but are also found in extrahepatic tissues of both humans and rats. These include the CYP1A, CYP2C, CYP2D, CYP2E, CYP2J and CYP3A subfamilies (Murray et al., 1988; Peters and Kremers, 1989; Rich et al., 1989; de Waziers et al., 1990; Shimizu et al., 1990; Fasco et al., 1993; Zeldin et al., 1997; Zhang et al., 1998; Dey et al., 1999). Interestingly, many of these CYPs are not only capable of metabolizing foreign compounds, but they

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are also able to metabolize endogenous compounds such as arachidonic acid to physiologically important metabolites.

Arachidonic acid is known to be biotransformed by three types of enzymes: lipoxygenases, cyclooxygenases and CYP monooxygenases (Zeldin, 2001). The CYP2B, CYP2C and CYP2J subfamily biotransform AA to four epoxyeicosatrienoic acids (EETs): 14,15-, 11,12-, 8,9-, and 5,6-EETs. These products are stereospecific and exist as either the *R,S* or the *S,R* enantiomers (Zeldin, 2001). P450-mediated metabolism of arachidonic acid can also produce ω-terminal HETEs, (16-, 17-, 18-, 19-, and 20-HETE), as well as lipoxygenase-like metabolites (5-, 8-, 9-,11,- and 12-, and 15-HETEs) (Zeldin, 2001).

Substantial evidence has accumulated showing that regiospecific metabolites formed from the P450 arachidonic acid pathway are involved in regulating many physiological effects including kidney transport, gluconeogenesis, cellular proliferation and vascular tone. Biological effects are also frequently stereospecific (Campbell et al., 1996; Imig et al., 1996a). Previous reports have shown a physiological role of 11,12-EET and 14,15-EET as vasodilators of renal arterioles (Imig et al., 1996b). In the rat, dietary salt has been shown to upregulate CYP2C23 in the kidney (Holla et al., 1999). This enzyme produces a unique stereospecific product 11*R*,12*S*-EET. Importantly, when renal arteries were preconstricted with epinephrine, 11,12-EET increased the diameter of the renal arteries, and this effect was specific to the 11*R*,12*S*-enantiomer (Imig et al., 1996a). Thus the increase in rat CYP2C23 appears to be part of a compensatory pathway to protect against salt-induced hypertension in the rat, and the production of 11*R*,12*S*-EET may be involved in controlling renal vasodilation.

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In this study, ESTs from a mouse kidney library were identified as belonging to a previously unknown member of the CYP2C subfamily and the sequence information was used to clone a novel CYP2C which is stereospecific for biosynthesis of 11*R*,12*S*-EET and 8*R*,9*S*-EET. The stereospecificity of these metabolites is unique from those produced by other previously reported mouse CYP2Cs (Luo et al., 1998). This new isoform is expressed primarily in liver, but also found in kidney and adrenal. Immunohistochemistry studies demonstrated specific staining for CYP2C44 in the hepatic bile duct epithelial cells and hepatocytes of the liver as well as the proximal tubules of the kidney. Understanding the function of this P450 and its products may reveal unique physiological roles in these tissues.

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Materials and Methods

Materials. Female and Male C57/BL6 mice, approximately 60 days old, were obtained from Charles River (Raleigh, NC). Recombinant human NADPH-P450 oxido-reductase was purchased from Oxford Biomedical Research Inc. (Oxford, MI). Restriction endonucleases were purchased from New England BioLabs, Inc. (Beverly, MA) [α-^{32}P]dCTP was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ) and [1-^{14}C]arachidonic acid was purchased from DuPont-NEN (Boston, MA).

Cloning of the mouse CYP2C44 cDNA. A new CYP2C subfamily sequence was discovered from searching an EST mouse database (http://drnelson.utmem.edu/UNIGENE.mouse.html). Total RNA was prepared from C57/BL6 mouse kidneys using Qiagen RNeasy Mini Kit (Valencia, CA). Reverse transcribed-PCR was performed with the SuperScript™ II Reverse Transcriptase kit from Invitrogen (Carlsbad, CA). Briefly, 500 ng of total RNA was used to synthesize cDNA utilizing the oligo (dT) primer. For PCR, primers utilized for CYP2C44 amplification were: ATGGAGCTGGCTGGGTCTCCCTACG (sense primer) and AGATTTCAGGTTAAAGTTCTG (antisense primer). The PCR reaction contained 1 X PCR buffer, 1.5 μM $MgCl_2$, 0.2 μM dNTPs, 0.25 μM each primer and 2 U Taq DNA polymerase from Life Technologies, (Gaithersburg, MD) in a final volume of 50μl. PCR was performed on a Perkin-Elmer 4800 thermal cycler (PE Applied Biosystems, Foster City, CA). The cycling conditions consisted of an initial denaturation of 94^{o}C for 3 min followed by 35 cycles of 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 1 min and 30 sec with

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a final extension at 72°C for 10 min. The resulting product was isolated on a 1.5% ethidium bromide-stained agarose gel, gel purified using QIAquick Gel Extraction Kit from Qiagen, (Valencia, CA) and cloned into PCR II vectors using a TA cloning kit from Invitrogen (Carlsbad, CA). The subsequently cloned DNA was sequenced. Based on amino acid sequence homology with other mouse CYP2Cs, the new mouse hemoprotein encoded by the cDNA was designated CYP2C44 by the Committee on Standardized Cytochrome P450 Nomenclature. Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK005218. Analysis of the cDNA and protein sequences was performed by the Genetic Computer Group (GCG) program (Madison, WI).

Construction of a CYP2C44 expression plasmid. Recombinant mouse CYP2C44 was expressed in *E. coli* using the pCW vector (Barnes, 1996). To produce high levels of expression of the P450 in bacteria, the N-terminus of CYP2C44 was modified to that of bovine CYP17, MALLLAVF (Barnes et al., 1991). The modification was accomplished by PCR amplification of the open reading frame of CYP2C44 using *Pfu* DNA polymerase from Stratagene (La Jolla, CA). The forward primer, GGGGATGGCTCTGTTATTAGCAGTTTTTCTTACAAAAATGCGCACTGG, contains an *NdeI* restriction site followed by the bovine CYP17 N-terminus, and the antisense primer, GGGGCTAATGGCTACAAAAATGCGCACTGGG, contains a *HindIII* restriction site. The PCR products were cloned into the pCW vector using the *NdeI* and *HindIII* restriction sites and the resulting plasmid was sequenced and confirmed to be without PCR errors.

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Expression and partial purification of CYP2C44. An overnight bacterial culture was grown at 37°C in LB broth in the presence of ampicillin (100 μg/ml). A 50 ml aliquot was diluted 10-fold with TB broth and cultured at 25°C for 48 h to 72 h in the presence of ampicillin (100 μg/ml) until the OD_{600} was approximately 0.4-0.6. Isopropyl-B-$_D$-thiogalactoside (0.5 mM final concentration) and δ-aminolevulinic acid (0.5 mM final concentration) were then added to the culture at log phase. Samples were taken at 24, 48 or 72 h intervals, and the P450 spectrum analyzed using a DW-2000 spectrophotometer (Omura and Sato, 1964). The cultured cells were harvested after 72 h and P450 proteins were partially purified as described previously (Luo et al., 1998).

Pregnenolone-16α-carbonitrile (PCN) and phenobarbital (PB) induction studies. C57/BL6 female and male mice were fed a standard solid diet and tap water for 5 days. For PCN induction studies, mice received either vehicle (corn oil) or 50 mg/kg PCN by intraperitoneal injection for 4 days. For phenobarbital induction studies, mice received vehicle (corn oil), or PB (80 mg/kg) via oral gavage at a volume of 10 ml/kg for 4 days. Mice were then sacrificed on the 5th day, and livers were collected for both total RNA and protein studies.

Salt Treatment. Salt loading was done by giving C57/BL6 female and male mice either salt water (2% NaCl by weight) for 2 weeks or regular tap water (control mice). Mice were given free access to the drinking water. After the two week period, the mice were sacrificed and the kidneys and livers were harvested for Western blotting.

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Isolation of total RNA and Real-Time PCR Analysis. Normal C57/BL6 male and female mouse tissues were excised, placed in RNA*later*™ (Ambion, Austin, Texas) and stored at -20^{o}C until use. Total RNA was extracted from animal tissues using RNeasy® from Qiagen (Valencia, CA) following the manufacturer's protocol. RNA (200 ng) was transcribed to cDNA using 200 units of Superscript™ II reverse transcriptase from Invitrogen (Carlsbad, CA), 100 ng Random Hexamers and 10 mM each of dGTP, dATP, dTTP and dCTP in 1X buffer (supplied) in a total volume of 20 µl following the manufacturer's protocol. The reaction was initially incubated at 42^{o}C for 2 minutes and then at 25^{o}C for 10 min before a final incubation at 42^{o}C for 50 min. Real-time PCR was performed in the presence of 1X SYBR Green Master Mix, 10 pmol of gene specific primers and 1 µl of reverse-transcribed cDNA. PCR mixtures contained 17µl SYBR Green Buffer, 10 pmol of gene specific primers and 1 µl of diluted (3-fold dilution) reverse transcriptase product in a total volume of 20 µl. Reactions were run in an ABI Prism™ 7700 Sequence Detector from Applied Biosystems (Foster City, CA). The samples were subjected to the following conditions: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 40 cycles of 95^{o}C for 15 sec, 62^{o}C for 30 sec and 72^{o}C for 30 sec, with a ramping of 19:59 and a final cycle of 95^{o}C for 15 sec. The resultant PCR products were electrophoresed on a 3% agarose gel containing ethidium bromide. A standard curve was generated for each primer pair developed from a dilution of the cDNA products. Primer pairs are shown in Table 2. PCR products were quantified by comparison to the linear range of the standard curve. β-actin was used as an internal control to normalize all unknown sample values. Melting curves produced a single prominent product, which was

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further verified by agarose gel electrophoresis. This band was not detected in the blank sample, which contained no RNA.

Protein Immunoblotting. A CYP2C44-specific peptide CRGPLPIIEDSQK was synthesized by ResGen (division of Invitrogen, Carlsbad, CA) and coupled to Keyhole Limpet Hemocyanin (KLH) through the terminal cysteine. Custom polyclonal antibodies specific for CYP2C44 were then produced by Covance Research Products Inc. (Denver, PA) as follows: At the start of production, a 1 mg/ml suspension of conjugated peptide was diluted 1:1 with Freund's Complete Adjuvant and each of two rabbits received 500 µg (1 ml) following pre-bleed. After 4 weeks, rabbits were each boosted with 250 µg of peptide diluted 1:1 with Freund's Incomplete Adjuvant, followed with a test bleed after 2 weeks. Rabbits received 4 additional boosts at 4-week intervals, with production bleeds beginning after the third boost. Rabbits were sacrificed by exsanguination at completion of production. Antibodies used in this study were from the second production bleed.

Microsomes were prepared from frozen mouse tissues by differential centrifugation at 4^{o}C. Microsomes and partially purified recombinant proteins were electrophoresed in SDS-10% (w/v) polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were immunoblotted with rabbit anti-CYP2C44 peptide-specific antibody (1:500) and donkey anti-rabbit IgG conjugated to horseradish peroxidase from Amersham Pharmacia Biotech Inc., (Piscataway, NJ). Specific bands were visualized using SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford, IL) and a SynGene GeneGnome chemiluminescence detection system from Synoptics (Cambridge, UK).

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Tolbutamide Hydroxylation Assays [ring-U-^{14}C]. Tolbutamide stock was evaporated to dryness in a Speed Vac Model SC100 Concentrator (Savant Instruments, Inc., Holbrook, NY), resuspended in a small volume of methanol and combined with unlabeled tolbutamide to obtain the desired concentration and specific activity. The reconstitution mixture consisted of the CYP2C44 partially purified recombinant protein with 0.3 μg/pmol P450 of 1,2-didodecanoyl-sn-glycero-3-phosphocholine, 4 pmol/pmol P450 of recombinant human NADPH-P450 oxidoreductase (POR), and 2 pmol/pmol P450 of human cytochrome b_5. This mixture was incubated at 37^{o}C for 5 minutes and then placed on ice. For single point assays, a reaction volume of 250 μl which contained 20 pmol of reconstituted enzyme, was combined with 1 mM [ring-U-^{14}C] tolbutamide (8 mCi/mmol) containing 20 mM HEPES, (pH 7.4), 0.1 mM EDTA and 1.25 mM $MgCl_2$. NADPH at a final concentration of 4 mM was added to initiate metabolism after the reaction mixtures were prewarmed for 3 minutes at 37^{o}C. This reaction proceeded for 45 minutes until the addition of methanol in a volume equal to reaction volume terminated the reactions. Reactions were centrifuged at 10,000 x g for 10 minutes and aliquots of supernatant removed for assay of total radioactivity by liquid scintillation (Beckman LS6500 Multi-Purpose Scintillation Counter, Beckman Coulter Inc, Fullerton, CA). Metabolites were examined by HPLC as previously described (Blaisdell et al., 2002) with the following modifications: the mobile phase consisted of acetonitrile/0.05% trifluoroacetic acid (40:60) and the standard was nonradioactive tolbutamide. The elution times of the unlabeled hydroxytolbutamide standard (Gentest Corp., MA USA) was used to identify the radiolabeled metabolites.

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Incubation of recombinant CYP2C44 with arachidonic acid and linoleic acid production characterization. Purification of the [1-14C]AA and LA was performed by passing it over a 0.5cm x 2cm silica gel column (230-400 mesh, average pore diameter 60 Angstroms, Sigma-Aldrich) using hexane/0.5% acetic acid as the mobile phase. The fraction containing the radiolabeled AA or LA (0-9ml) is dried under a nitrogen stream and used within 30 min. Following a previous protocol for regiochemical analysis of the metabolites of AA that were produced by recombinant murine CYP2Cs (Luo et al., 1998), partially purified recombinant CYP2C44 protein was preincubated with NADPH-P450 oxido-reductase (POR/CYP ratio, 4:1) and dilauroylphosphatidylcholine (50 μg/ml, final concentration) on ice for 30 min. The enzyme mixtures were then added to reaction mixtures containing 0.05 M Tris-HCl buffer (pH 7.5), 0.15 M KCl, 0.01 M $MgCl_2$, 8 mM sodium isocitrate, 0.5 IU/ml isocitrate dehydrogenase and either arachidonic acid or linoleic acid (55-56 μCi/μmol; 100 μM final concentration) and constantly stirred at 37^{o}C. 1 mM final concentration of NADPH was added to initiate the reaction and incubation continued for 30 min. Control experiments without NADPH, AA or LA were also included which yielded no product. The reaction products were then extracted and analyzed by reverse-phase HPLC for regiochemical distribution of EETs, HETEs, HODEs and EOAs as described previously (Luo et al., 1998). All products were identified by comparing reverse-and normal phase HPLC properties with those of authentic EET, HETE, HODEs and EOAs standards. For chiral analysis, batchwise collections of EETs were derivatized, purified, and resolved into cooresponding antipodes by chiral phase HPLC as previously described (Hammonds et al., 1989).

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Immunohistochemistry. Immununohistochemistry was performed on mouse liver and kidney sections using the CYP2C44-specific peptide. Briefly, a 1:500 dilution of the primary CYP2C44 peptide antibody was applied to the sections for 30 min, after the sections had been microwave-treated, cooled and blocked with normal rabbit serum. Negative controls contained preimmune sera. The bound antibody was visualized by avidin-biotin-peroxidase detection, using a Vectastain Rabbit Elite Kit (Vector Laboratories, Burlingame, CA) following manufacturer's protocol. Harris hemotoxylin was used as the counterstain and sections were coverslipped with Permount (Fisher, Springfield, NJ). To validate the specificity of the antibody, the specific CYP2C44 peptide (reconstituted to 100 μg/ml), was diluted to 50- or 100-fold and incubated with the antibody overnight at 4^{o} for maximum binding. The following day, the immunohistochemistry procedure was conducted as described above.

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Results

Cloning of Mouse CYP2C44 cDNA. A new full-length member of the mouse CYP2C subfamily was first identified from a murine EST database. The sequences were assembled and a full length cDNA was cloned by PCR from reverse-transcribed RNA from a C57/BL6 mouse kidney. The amino acid sequence of the new form aligned with the previously published mouse CYP2Cs is shown in Figure 1. The amino acid sequence is a 493 residue sequence that contains a putative heme-binding domain with conserved residues between 432-441 and an invariant cysteine at position 438. This polypeptide contains structural features associated with other CYPs, including a hydrophobic N-terminal leader and a proline rich region between residues 33-40. Like CYP2C23 (Holla et al., 1999), CYP2C44 contains a few extra amino acids at the N-terminus (MELL) with a glutamic acid next to the N-terminal methionine. A comparison of the deduced amino acid sequence of this new cDNA with that of known mouse CYP2Cs and rat CYP2C23 is shown in Figure 1. CYP2C44 has the lowest sequence homology of all the mouse CYP2Cs, with the closest identity to CYP2C29 (59.7%) and CYP2C37 (59.5%).

Distribution of CYP2C44 by Protein Immunoblotting. Western blotting with a peptide antibody that differentiates CYP2C44 from the known mouse CYP2Cs (Luo et al., 1998) as well as several new CYP2Cs (Wang et al., 2004;Zhao, Goldstein, Zeldin, unpublished data) is shown in Figure 2A. This antibody detects a single protein band at 55kDa with recombinant CYP2C44, but does not cross-react with recombinant CYP2C29, CYP2C38, CYP2C39, CYP2C40, CYP2C50, CYP2C54 and CYP2C70. In Figure 2B, the immunoblot detects an ~ 52 kDa band in male and female liver

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microsomes (lane 1 and lane 2). A slight difference in the mobility of the recombinant CYP2C44 and the protein in liver microsomes is due to the alteration in the N-terminus of the recombinant protein. Similar effects of the N-terminus on mobility have been noted for other recombinant CYP2Cs in our laboratory (Tsao et al., 2000). In extrahepatic tissues, this 52 kDa band was detected in female kidney as well as male and female adrenal, although levels were considerably lower than those observed in liver. Female kidneys and adrenals contained higher levels of CYP2C44 than those of males. CYP2C44 could not be detected in a number of other extrahepatic tissues examined including: lung, heart, brain, aorta, skin, eye, colon, testis, epididymis, seminal vesicles, ovary, uterus and cervix (data not shown). Hepatic content of CYP2C44 protein was not increased by treatment with prototypical cytochrome P450 inducers PB or PCN (data not shown). Moreover, administration of salt in the drinking water did not affect levels of CYP2C44 protein in liver or kidney under the regimen tested (data not shown).

Real-Time PCR. Real-time PCR was used to determine the relative mRNA levels of CYP2C44 in both male and female C57/BL6 mice. The mRNA content of CYP2C44 PCR products was normalized to β-actin and then normalized to female small intestine, for which CYP2C44 mRNA was in low abundance. Male and female liver CYP2C44 mRNA content was 2.5 X 10^5 higher than female small intestine mRNA content (Figure 3). CYP2C44 mRNA content in female kidney was 0.18 X 10^5 higher than female small intestine, and approximately 10-fold lower than liver. CYP2C44 mRNA content of male kidney was lower than that in female kidney (0.008 X 10^3 fold over female small intestine). CYP2C44 mRNA content in female adrenals was higher than that of male adrenals with values that were 12 X 10^3 and 3 X 10^3 greater than that of

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female small intestine. All other tissues (lung, heart, brain, aorta, skin, eye, colon, testis, epididymis, seminal vesicle, ovary, uterus and cervix) showed undetectable levels of CYP2C44 mRNA.

Metabolism of AA and LA by CYP2C44. The CYP2Cs have previously been shown to produce specific arachidonic acid metabolites (Luo et al., 1998; Tsao et al., 2000; Tsao et al., 2001; Wang et al., 2004). In this study, recombinant CYP2C44 cDNA was expressed in *E.coli* at levels of 1.7-3.3 nmol/P450 per liter of bacterial culture and partially purified as described in the Methods and Materials. After reconstitution with POR, recombinant CYP2C44 metabolized arachidonic acid primarily to EETs with lesser amounts of ω-terminal HETEs and mid-chain HETEs (Figure 4). Specifically, CYP2C44 produced 11,12-EET (45% of total) as well as 8,9-EET (23% of total) and 14,15-EET (14% of total) (Table 3). EETs were produced in a highly stereospecific fashion with 11*R*,12*S*-EET (94% optical purity) and 8R,9S-EET (95% optical purity) being the predominant enantiomers. The catalytic turnover number for arachidonic acid was 0.92 nmol/min/nmol P450. Linoleic acid was also metabolized by CYP2C44 to 39% E0As and 61% HODES. The catalytic number for linoleic acid was 0.15 nmol/nmol/P450.

Tolbutamide Metabolism. Tolbutamide is a prototypical drug substrate which is metabolized by all of the human CYP2Cs (Goldstein and de Morais, 1994). Therefore, we examined the ability of the mouse CYP2Cs to metabolize this substrate. Partially purified recombinant CYP2C29, CYP2C37, CYP2C38, CYP2C39, CYP2C40 and CYP2C44 were reconstituted with POR and incubated with 1 mM [ring-U-^{14}C] tolbutamide (8 mCi/mmol) as described in materials and methods, and turnover numbers

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are shown in Table 4. CYP2C44 demonstrated no activity towards tolbutamide. In contrast, mouse CYP2C29, CYP2C38 and CYP2C39 exhibited tolbutamide hydroxylase activity with turnover numbers of 1.25 ± 0.02, 0.95 ± 0.13 and 0.74 ± 0.05 nmol/min/nmol respectively, compared to that of human CYP2C9 which had a turnover number of 4.39 ± 0.02 nmol/min/nmol.

Immunohistochemistry Sections of formalin-fixed, paraffin embedded liver, kidney and adrenals from C57BL/6 mice were immunostained using a specific CYP2C44 peptide antibody. Figure 5A shows a representative liver section detecting cytoplasmic staining of hepatocytes, as well as staining in bile duct epithelial cells. The inset shows a representative negative control. In peptide inhibition studies, (Figure 5B), the staining in the liver is completely blocked with the addition of the CYP2C44 specific peptide, indicating that the staining is specific for CYP2C44. In a representative kidney section (Figure 5C), staining is seen in the proximal tubules at the corticomedullary junction which was appreciably blocked by the addition of the CYP2C44 peptide, indicating that most of this staining represents CYP2C44 (Figure 5D). Staining was also observed in the arterial walls of the kidney which disappears when the antibody is preincubated with the specific peptide. Much less staining was observed in the collecting ducts. Bowman's capsule, endothelial cells and the glomeruli of the kidney were negative.

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Discussion

In the present study, we isolated and characterized a cDNA for a new mouse P450, CYP2C44, from mouse kidney. CYP2C44 is the least homologous to other known mouse CYP2C proteins, but highly (84%) homologous to rat kidney CYP2C23. Like CYP2C23 (Holla et al., 1999), CYP2C44 contains a few extra amino acids at the N-terminus (MELL) with a glutamic acid next to the N-terminal methionine. In contrast, the amino acid identity of the newly cloned CYP2C44 to mouse CYP2Cs ranged from 60% (for CYP2C29) to 52% (for CYP2C40). From the mouse genomic database in the Celera Discovery System, we determined that the five known mouse *Cyp2c* genes, 10 new *Cyp2c* genes and four *Cyp2c* pseudogenes are all located in a 1.5 Mb cluster on chromosome 19, except for Cyp2c44 which is located ~ 3.8 Mb downstream on this chromosome (Nelson et al., 2004). The remote location of the *Cyp2c44* from the man cluster of *Cyp2c* genes would result in a decreased chance of crossover which presumably accounts for the lower homology of this gene to the other CYP2C genes. Four of the new CYP enzymes have been recently cloned and characterized in our laboratories (Wang et al., 2004).

CYP2C44 metabolized arachidonic acid primarily to EETs (77%) and a smaller proportion of HETEs (23%) with a turnover number of 0.92 nmol/min/nmol P450. This compares to other murine CYP2Cs, with the turnover numbers for CYP2C29, CYP2C37, CYP2C38, CYP2C39, CYP2C40, CYP2C50, CYP2C54 and CYP2C55 at 0.34, 1.1, 5.2, 0.15, 0.7, 1.0, and 1.2 nmol/min/nmol P450, respectively (Luo et al., 1998; Wang et al., 2004). The principal arachidonic metabolite produced by CYP2C44 was 11*R*,12*S*-EET. Rat CYP2C23, which is highly homologous to CYP2C44, has been found to be the

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predominant CYP2C in rat kidney and is also active in the metabolism of AA to 11*R*,12*S*-EET (Katoh et al., 1991; Karara et al., 1993; Holla et al., 1999) as well as 14*S*,15*R*-EET (Capdevila et al., 1991). The 11*R*,12*S* metabolites produced by CYP2C23 were found to be the predominant EET enantiomers produced by rat kidney microsomes (Capdevila et al., 1991).

Imig *et al* found that 11,12-EET increased the diameters of interlobular and afferent arterioles preconstricted with epinephrine in *in vitro* blood-perfused justamedullary nephron preparations (Imig et al., 1996a). This response was found to be highly stereoselective for the 11*R*,12*S*-EETs; indeed, 11*S*,12*R*-EETs did not increase vessel size. 14,15-EET was found to have a lesser effect and 8,9-EET did not increase the diameter of these vessels. This effect appears to involve activation of Ca^{2+}-activated K+ channels. Renal CYP2C23 is upregulated by dietary salt intake in some strains of rat (Holla et al., 1999). This upregulation is thought to be an important compensatory mechanism in response to dietary salt intake, producing vasodilation and inhibiting the reabsorption of sodium by the proximal tubules. Importantly, inhibition of P450 leads to salt-induced hypertension in rats (Makita et al., 1994; Holla et al., 1999).

Immunostaining studies showed that CYP2C44 is found in proximal tubules of mouse kidney. Immunochemical localization indicated immunostaining of the proximal tubules and arterial walls of the kidney, but not Bowman's capsule with much less staining of the collecting ducts. This immunostaining was significantly inhibited by a CYP2C44 specific peptide indicating the specificity of this method. Although CYP2C44 was not upregulated by dietary salt under the limited set of conditions used in the current

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study, its localization in mouse proximal tubules and arterial walls is consistent with a possible role of CYP2C44 in vasodilation and sodium transport in the kidney.

Other CYP subfamilies are also found in rodent kidney. The CYP4A family is expressed in kidney and is found in the renal vasculature (Marji et al., 2002). The CYP4A family (e.g. CYP4A2, CYP4A2, CYPA3, and CYP4A4) produces predominantly 20-HETE, although some members (CYP4A2, and CYP4A3) also produce smaller amounts of 11,12-EET (Nguyen et al., 1999). 20-HETE has been found to be the major AA metabolite in mouse kidney (Honeck et al., 2000). Nguyen have suggested that 11,12-EET and 20-HETE have important biologically opposing roles in vasodilation and constriction in the kidney (Nguyen et al., 1999). CYP2J5 is another murine P450 which is also found in renal proximal tubules and collecting ducts of the mouse, and it metabolizes AA to 8,9-, 11,12-,and 1 4,15-EET as ell as 11-HETE (Ma et al., 1999).

The extrahepatic distribution of the CYP2C44 enzyme differs from that of other CYP2Cs in the mouse. CYP2C40 was found to be abundant in colon and intestine (Tsao et al., 2000). CYP2C29 mRNA was found in a variety of extrahepatic tissues including lung (Luo et al., 1998; Tsao et al., 2001). CYP2C50, originally identified from a partial clone from heart (Tsao et al., 2001), has recently been cloned and the protein detected in the heart as well as the liver (Wang et al., 2004). In contrast, CYP2C44 was not detected in colon, small intestine, lung or heart. There was a sex difference in the extrahepatic distribution of CYP2C44. We have previously reported an abundance of CYP2Cs in female adrenals, particularly in the X-zone which is present seen in females but disappears after at approximately 9 weeks of age. However, this staining in the X-zone

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apparently does not represent CYP2C44, since histochemical analysis of the adrenals for CYP2C44 showed no in the X-zone (data not shown).

Immunoblotting and Real-time PCR indicated that CYP2C44 was more abundant in liver than in extrahepatic tissues. EETs are known to be endogenous constituents of rat liver (Yoshida et al., 1990). EETs have been shown to be important in glycogenolysis in the liver, probably as a consequence of increasing cytosolic calcium and activation of phosphorylase A. Although 14,15-EET was the most active, 11,12-EET also activated phosphorylase A. The stereoselectivity of this effect was not examined. The intense immunostaining of CYP2C44 in the epithelial cells of the bile duct is noteworthy. Ion transport is regulated by specific transporters found in tissues such as kidney and liver. Although effects of EETs on hepatic transport have not been investigated, EETs might conceivably affect transporters in the bile duct, such as the bile acid export pump (BSEP) which transports bile acids (Kullack-Ublick, 2003).

The rate of turnover for CYP2C44 for linoleic acid was low (0.15 nmol/min/nmol P450) compared to that of arachidonic acid (0.92 nmol/min/nmol P450). CYP2C44 metabolized linoleic acid principally to HODEs as major metabolites (61%) and EOAs as minor metabolites (39%). Although the action of HODEs and E0As have received little study, 9,10- EOA is a hepatic toxin (Ozawa et al., 1986) and 9,10-EOA and 12,13-EOA have been associated with death due to severe burns (Kosaka et al., 1994).

Unlike mouse CYP2C29, CYP2C44 did not metabolize tolbutamide, a common substrate of the human CYP2Cs (Goldstein and de Morais, 1994). In addition, immunoblotting indicated that CYP2C44 was not induced by prototypical P450 inducers such as PB and PCN; although we have recently shown that CYP2C29 and CYP2B10

was induced by this dose of phenobarbital (Jackson et al., 2004). Thus, we hypothesize that CYP2C44 may not have a classical role in drug metabolism, but rather may have endogenous functions.

We report the cloning and characterization of a new mouse CYP2C that is found in liver as well as the extrahepatic tissues which metabolizes arachidonic acid to the stereospecific products 11*R*,12*S*-EETs and 8*R*,9*S*-EETs. The unique stereospecificity of this enzyme for 11*R*,12*S*-EET, its homology to rat CYP2C23, and its presence in liver and kidney suggests the possibility that it may have unique physiological roles in vasodilation and transport in these tissues. Additional studies will be required to establish its physiological role.

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Acknowledgments

We are grateful for the expert advice of Dr. Robert Maronpot of the Experimental Pathology Branch, NIEHS, in evaluating the immunohistochemical localization of CYP2C44 in sections of liver, kidney and other tissues.

JPET Fast Forward. Published on April 14, 2004 as DOI: 10.1124/jpet.104.067819
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JPET Fast Forward. Published on April 14, 2004 as DOI: 10.1124/jpet.104.067819
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Figure 1. Alignment of deduced murine CYP2C (mouse CYP29, 2C37, 2C38, 2C39, 2C40) and rat CYP2C23 cDNA sequences with the CYP2C44 sequence. Consensus amino acids in the corresponding column are indicated by a dark shading as well an asterick(*). If the amino acid sequence is not identical but similar residues form a consensus column, a light gray background is used. Closed circles mark termination codons of CYP2C sequences. The alignment of CYP2C44 amino acid sequences was performed with version 3.21 of BOXSHADE software (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

Figure 2. **Specificity of the CYP2C44 peptide antibody**. **A**. Partially purified proteins for ten known mouse CYP2Cs purified in our laboratories (Lou et al., 1998; Wang et al., 2004; Nelson et al., 2004) (0.1 pmol/lane) were electrophoresed on SDS-10% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with the anti-CYP2C44 antibody as described in Materials and Methods. The CYP2C44 peptide antibody is specific for CYP2C44. **B**. Microsomal fractions prepared from male and female mouse liver (10 μg/lane) and extrahepatic tissues (50 μg/lane): kidney, heart, brain, adrenal and aorta were electrophoresed on 10% SDS-polyacrylamide gel and resolved proteins were transferred to nitrocellulose membranes. The membrane was immunoblotted using mouse CYP2C44 peptide antibody as described in Materials Methods and visualized using SuperSignal West Pico Chemiluminescent Substrate and a SynGene GeneGnome chemiluminescence detection system. A specific CYP2C44 band

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with the appropriate mobility was detected in liver>>female kidney>>female adrenal>>male adrenal >> male kidney

Figure 3. Detection of CYP2C44 mRNA expression in mouse tissues by Real–time PCR. 200 ng of total RNA was used in the Real-time PCR assay reaction as described under Materials and Methods. A standard curve was made for each primer pair developed from a dilution of the cDNA products. The PCR products were quantified using the linear range of the standard curve. β-actin was used as an internal control to normalize all unknown sample values. Melting curves produced a single prominent product, further verified by agarose gel electrophoresis. The mRNA levels of both male and female CYP2C44 PCR products were normalized to β-actin and then normalized to female small intestine, mRNA showing low but detectable amounts of CYP2C44. Fold abundance indicates the amount of gene detected when comparing to small intestine. The inset shows female and male liver RNA for comparison. Values represent means $\pm$ SE.

Figure 4. Reverse-phase HPLC chromatograms of products formed from recombinant CYP2C44 with radiolabeled AA (top) and linoleic acid (bottom). Recombinant CYP2C44 was reconstituted with phospholipids and POR as described in Methods and Materials and incubated with radiolabelled AA or linoleic acid. EET and HETE metabolites were identified by coelution with authentic standards as indicated by the bars above the respective peaks. EOAs and HODEs were also identified by coelution with authentic standards as indicated by the bars above the respective peaks. Ordinate,

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radioactivity (cpm); abscissa, time (min). Results are representative of four different experiments.

Figure 5. Immunohistochemical staining of CYP2Cs. **A** Immunohistochemical analysis shows a representative mouse liver section showing strong cytoplasmic staining in the hepatocytes as well as cells lining the bile duct, (20X). *Inset demonstrates a representative negative liver control incubated with pre-immune sera* (20X). Arrow in A points to the hepatobiliary cells. Staining in the liver and bile acid secretions is completely blocked with peptide inhibition, thereby demonstrating high specificity of this antibody in the liver tissue **B** (20X). Immunohistochemical analysis of a representative mouse kidney section showing cytoplasmic staining in the proximal tubules at the corticomedullary junction **C** (20X). *The Inset demonstrates a representative negative kidney control incubated with pre-immune sera* (20X). The scale is shown by a dark line indicating 1micometer is 0.05 mm. Staining in the proximal tubules is significantly blocked by incubation with the CYP2C44 peptide indicating that CYP2C44 specific immunostaining occurs in these tissues. **D** (20X).

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TABLE 1

Amino acid identity among the mouse CYP2c subfamily members

	CYP2C29	CYP2C37	CYP2C38	CYP2C39	CYP2C40	CYP2C44
CYP2C29	**100**	**77**	**84**	**85**	**69**	**60**
CYP2C37		**100**	**74**	**75**	**71**	**60**
CYP2C38			**100**	**92**	**70**	**58**
CYP2C39				**100**	**70**	**59**
CYP2C40					**100**	**52**
CYP2C44						**100**

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TABLE 2

Sequence specific PCR primers for amplification of mouse 2C44 and β-actin for Real-time PCR

		Primer Sequence (5' –3')
CYP2C44	Forward	CTTTCCAACGAGCGATTCCC
CYP2C44	Reverse	TGTTTCTCCTCCTCGATCTTGC
B-actin	Forward	CCTAGAAGVATTTGCGGTGCACGATG
B-actin	Reverse	TCATGAAGTGTGACGTTGACATCCGT

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TABLE 3

Regiochemistry and stereochemistry of arachidonic acid metabolites produced by CYP2C44.

Metabolite	Distribution	Enantioselectivity	
	(%)	**R, S (%)**	**S, R (%)**
14,15-EET	14%	_	_
11,12-EET	45%	94	6
8,9-EET	18%	95	5
5,6-EET	0%		
HETEs	23%		

Recombinant partially purified CYP2C44 was reconstituted with cytochrome P450 reductase and phospholipids and incubated with [1-^{14}C]AA as described in Methods. The regiochemical distribution of EETs and HETEs were quantified by HPLC and liquid scintillation as described Methods. Values were representative of four separate experiments.

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TABLE 4

Tolbutamide Metabolism by the Recombinant Mouse CYP2Cs

Recombinant Protein	Mean Activity nmol/min/nmol
2C29	1.2 ± 0.1
2C37	0
2C38	1.0 ± 0.1
2C39	0.7 ± 0.1
2C40	0
2C44	0

Recombinant mouse CYP proteins were expressed in *Escheria coli*, and partially purified as described in Methods. The purified proteins were reconstituted with phospholipids and tolbutamide metabolism [ring-U-^{14}C] tolbutamide metabolism measured as described in Methods. Detection limit for the assay was <0.5nmol/min/nmol. Values represent the means ± SE of three samples. The turnover number for CYP2C9 is 4.39 ± 0.023 nmol/min/nmol.

```
CYP2c38      1 --[illegible]NFNQSLTNFSKTYGPVFT
CYP2c39      1 [illegible]SLTNFSKAYGPVFT
CYP2c29      1 ----MDLVVFLALTLSCLILLSLWRQSSGRGKLPPGPTPLPIIGNFLQIDVKNISQSFTNFSKAYGPVFT
CYP2c37      1 ----MDPILVLVLTLSCLFLLSLWRQSSERGKLPPGPTPLPIIGNILQIDVKDICQSFTNLSKVYGPVYT
CYP2c40      1 ----MDPFVVLVLCLSFLLVLSLWRQRSARGNLPPGPTPLPIIGNYHLIDMKDIGQCLTNFSKTYGPVFT
CYP2C44      1 MELLGLPTLALLVLVMSLSLLSVWTKMRTGGRLPPGPTPLPIIGNILQLDLKDIPASLSKLAKEYGPVYT
CYP2C23      1 MELLGFTTLALVVSVTCLSLLSVWTKLRTRGRLPPGPHPPSHYWESTATEPQGHPASLSKLAKEYGPVYT
consensus    1           * . .  * .**.*      * ***** *          .        .   * ****.*

CYP2c38     67 LYLGSRPIVVLHGYEAVKEALIDHGEEFSGRENIPMSEKINNGLGITFSNGNSWKETRRFTLMTLRNLGM
CYP2c39     67 LYLGSRPTVVLHGYEAVKEALIDHGEEFSDRGSIPMVEKINNGLGIVFSNGNRWKEIRRFTLTTLRNLGM
CYP2c29     67 LYLGSKPTVILHGYEAVKEALIDRGEEFAGRGSFPMAEKMIKGFGVVFSNGNRWKEMRRFTLMTLRNLGM
CYP2c37     67 LYLGRKPTVVLHGYEAVKEALVDHGEEFAGRGRFPVFDKATNGMGLAFSKGNVWKNTRRFSLMTLRNLGM
CYP2c40     67 LYFGSQPIVVLHGYEAIKEALIDHGEEFSGRGRIPVFDKVSTGKGIGFSHGNVWKATRVFTVNTLRNLGM
CYP2C44     71 LYFGSWPTVVLHGYDVVKEALLNQGDEFLGRGPLPIIEDSQKGHGIVFSEGERWKLLRRFSLMTLKNFGM
CYP2C23     71 LYFGTSPTVVLHGYDVVKELLQ-QGDEFLGRGPLPIIEDTHKGYGLIFSNGERWKVMRRFSLMTLRNFGM
consensus   71 ** *  * *.****. .** *   *.**  *   *. .    * *. ** *  **  * *.. **.* **

CYP2c38    137 GKRNIEDRVREEAQCLVEELRKTKGSPCDPTFILSCAPCNVICSIIFQDRFDYKDKDFLMLMKKLNENVK
CYP2c39    137 GKRNIEDRVQEEAQCLVEELRKTKGSPCDPTFILSCAPCNVICSIIFQDRFDYKDKDFLMLMEKLNENVK
CYP2c29    137 GKRNIEDRVQEEAQCLVEELRKTKGSPCDPTFILSCAPCNVICSIIFQNRFDYKDKEFLILMDKINENVK
CYP2c37    137 GKRSIEDRVQEEARCLVEELRKTNGSPCDPTFILGCAPCNVICSIIFQDRFDYKDRDFLNLMEKLNEITK
CYP2c40    137 GKRTIENKVQEEAQWLMKELKKTNGSPCDPQFIIGCAPCNVICSIVFQNRFDYKDKDFLSLIGKVNECTE
CYP2C44    141 GKRSLEERVQEEARCLVEELHKTEAQPFDPTFILACAPCNVICSILFNERFPYNDKTFLNLMDLLNKNFY
CYP2C23    140 GKRSLEERVQEEAWCLVEELQKTKAQPFDPTFILACAPCNVICSILFNDRFQYNDKTFLNLMDLLNKNFQ
consensus  141 *** .*  .****  *. ** ** . * ** **. **********.*. ** * *. ** *.  .*

CYP2c38    207 ILSSPWLQVCNNFPLLIDYCPGSHHKVLKNFKYIRSYLLEKVKEHQESLDATNPRDFIDYFLIKQKQANH
CYP2c39    207 ILSSPWLQVCNNFPLLIDYCPGSHHKVLKNVKYIRSYLLEKIKEHQESLDVTNPRDFIDYYLIKQKQANH
CYP2c29    207 ILSSPWLQVCNSFPSLIDYCPGSHHKIVKNFNYLKSYLLEKIKEHKESLDVTNPRDFIDYYLIKQKQVNH
CYP2c37    207 ILSSPWLQICNTYPALLDYCPGSHKQFFKNYACIKNFLLEKIKEHEESLDVTIPRDFIDYFLINGGQEDG
CYP2c40    207 ILSSPGCQIFNAVPILIDYCPGSHNKLFKNHTWIKSYLLGKIKEHEESLDVTNPRDFIDYFLIQRRQKNG
CYP2C44    211 QLNSIWIKMYNLWPTIMKYIPGKHREFSKRLGGVKNFILEKVKEHQESLDPANPRDYIDCFLSKIEEEKH
CYP2C23    210 QVNSVWCQMYNLWPTIIKYLPGKHIEFAKRIDDVKNFILEKVKEHQKSLDPANPRDYIDCFLSKIEEEKD
consensus  211  . *    . *  * .. * ** *    *    .. ..* *.***  ***   ***.** .*

CYP2c38    277 IEQAEYSLENLVCTINNLFAAGTETTSTTLRYALLLLMKYPDVTAKVQEEIDHVVGRHRSPCMQDRSRMP
CYP2c39    277 IQQAEFSLENLACTINNLFAAGTETTSTTLRYALLLLMKYPDVTAKVQEEIDHVIGRHRSPCMQDRNHMP
CYP2c29    277 IEQSEFSLENLASTINDLFGAGTETTSTTLRYALLLLLKYPDVTAKVQEEIDRVVGRHRSPCMQDRSHMP
CYP2c37    277 NQPLQNRLEHLAITVTDLFSAGTETTSTTLRYALLLLLKYPHVTAKVQKEIEHVIGKHRSPCMQDRSRMP
CYP2c40    277 IEHMDYTIEHLATLVTDLVFGGTETLSSTMRFALLLLMKHTHITAKVQEEIDNVIGRHRSPCMQDRNHMP
CYP2C44    281 NLKSDFNLENLAICGSNLFTAGTETTSTTLRFGLLLLVKHPEVQAKVHEELDRVIGRHQPPSMKDKMKLP
CYP2C23    280 NLKSEFHLENLAVCGSNLFTAGTETTSTTLRFGLLLLMKYPEVQAKVHEELDRVIGRHQPPSMKDKMKLP
consensus  281        .* *      *  .**** *.*.*..****.*   . ***  *.. *.*.*  * * *. ..*

CYP2c38    347 YTDAMIHEVQRFINLVPNNLPHAVTCDIKFRNYIIPKGTTVVTSLTSVLHDSKEFPNPEMFDPGHFLDAN
CYP2c39    347 YTDAMIHEVQRFINLVPNNIPRAVTCDIKFRNYLIPKGTTVVTSLTSVLHDSKEFPNPELFDPGHFLDAN
CYP2c29    347 YTDAMIHEVQRFIDLLPTSLPHAVTCDIKFRKYLIPKGTTVITSLSSVLHDSKEFPNPEMFDPGHFLNGN
CYP2c37    347 YTDAMIHEVQRFIDLIPNSLPHEVTCDIKFRNYFIPKGTTVITSLSSVLHDSTEFPNPEVFDPGHFLDGN
CYP2c40    347 YTNAMVHEVQRYIDLGPNGVVHEVTCDTKFRNYFILKGTQVMTSLTSVLHDSTEFPNPEVFDPGHFLDDN
CYP2C44    351 YTDAVLHEIQRYITLLPSSLPHAVVQDTKFRHYVIPKGTAVFPFLSSILLDQKEFPNPEKFDPGHFLDKN
CYP2C23    350 YTDAVLHEIQRYITLVGSSLPHAVVQDTKFRDYVIPKGTTVLPMLSSVMLDQKEFANPEKFDPGHFLDKN
consensus  351 ** *..**.**.* *    . . *  * *** * * *** *   *.*.. *  ** *** *******  *

CYP2c38    417 GNFKKSDYFMTFSAGKRVCAGEGLARMELFLILTTILQNFKLKSLVHPKDIDMIPFVNGLITLPPHYQLC
CYP2c39    417 GNFKKSDHFMPFSAGKRVCAGEGLARMELFLFLTTILQNFKLKPLVHPKDIDMIPFVNGLIALPPHYQVC
CYP2c29    417 GNFKKSDYFMPFSTGKRICAGEGLARMELFLILTTILQNFKLKSLVHPKEIDITPVMNGFASLPPPYQLC
CYP2c37    417 GKFKKSDYFMPFSTGKRICAGEGLARMELFLFLTSILQSFNLKPLVHPKDIDVTPMLIGLGSVPPAFQLC
CYP2c40    417 GNFKKSDYFVPFSAGKRICVGESLARMELFLFLTTILQNFKLKPLVDPKDIDMTPKHSGFSKIPPNFQMC
CYP2C44    421 GCFKKTDYFVPFSLGKRSCVGEGLARMELFLFFTTILQKFSLKALVEPKDLDIKPVTTGLFNLPPPYKLR
CYP2C23    420 GCFKKTDYFVPFSLGKRACVGESLARMELFLFFTTLLQKFSLKTLVEPKDLDIKPITTGIINLPPPYKLC
consensus  421 * ***.* *. ** *** * ** ********  *..** * ** ** **..*. *   *   .** . .

CYP2c38    487 FIPL-
CYP2c39    487 IIPR-
CYP2c29    487 FIPL-
CYP2c37    487 FIPS-
CYP2c40    487 FIPVE
CYP2C44    491 LVPR-
CYP2C23    490 LVPR-
consensus  491  .*
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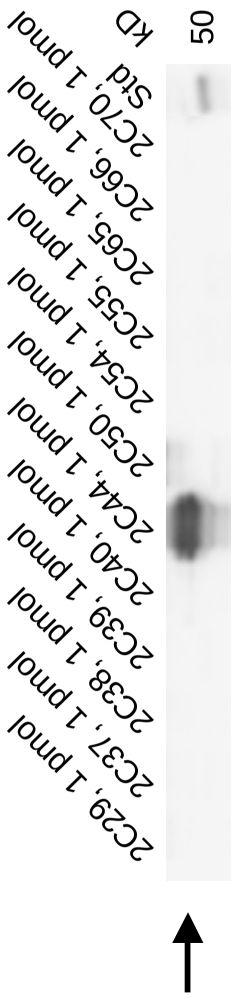
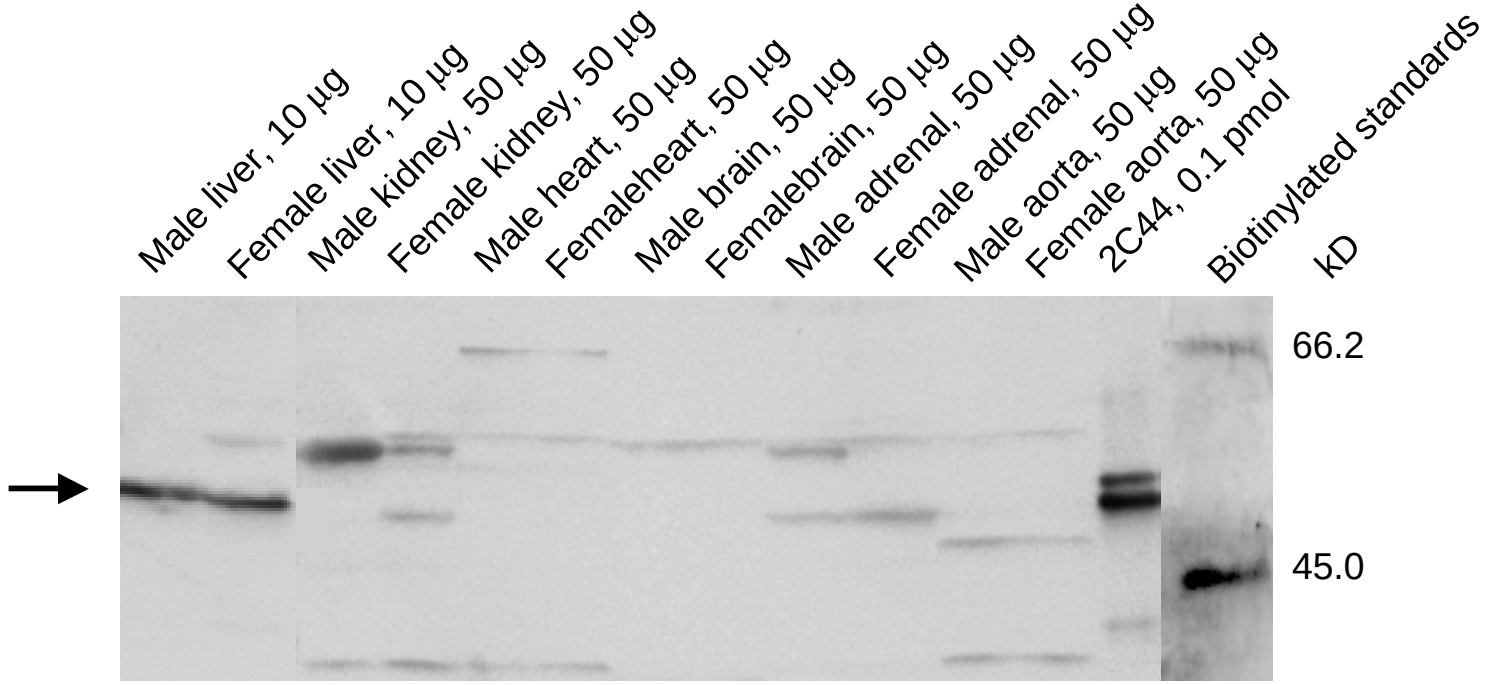


Figure 2A

Figure 2B



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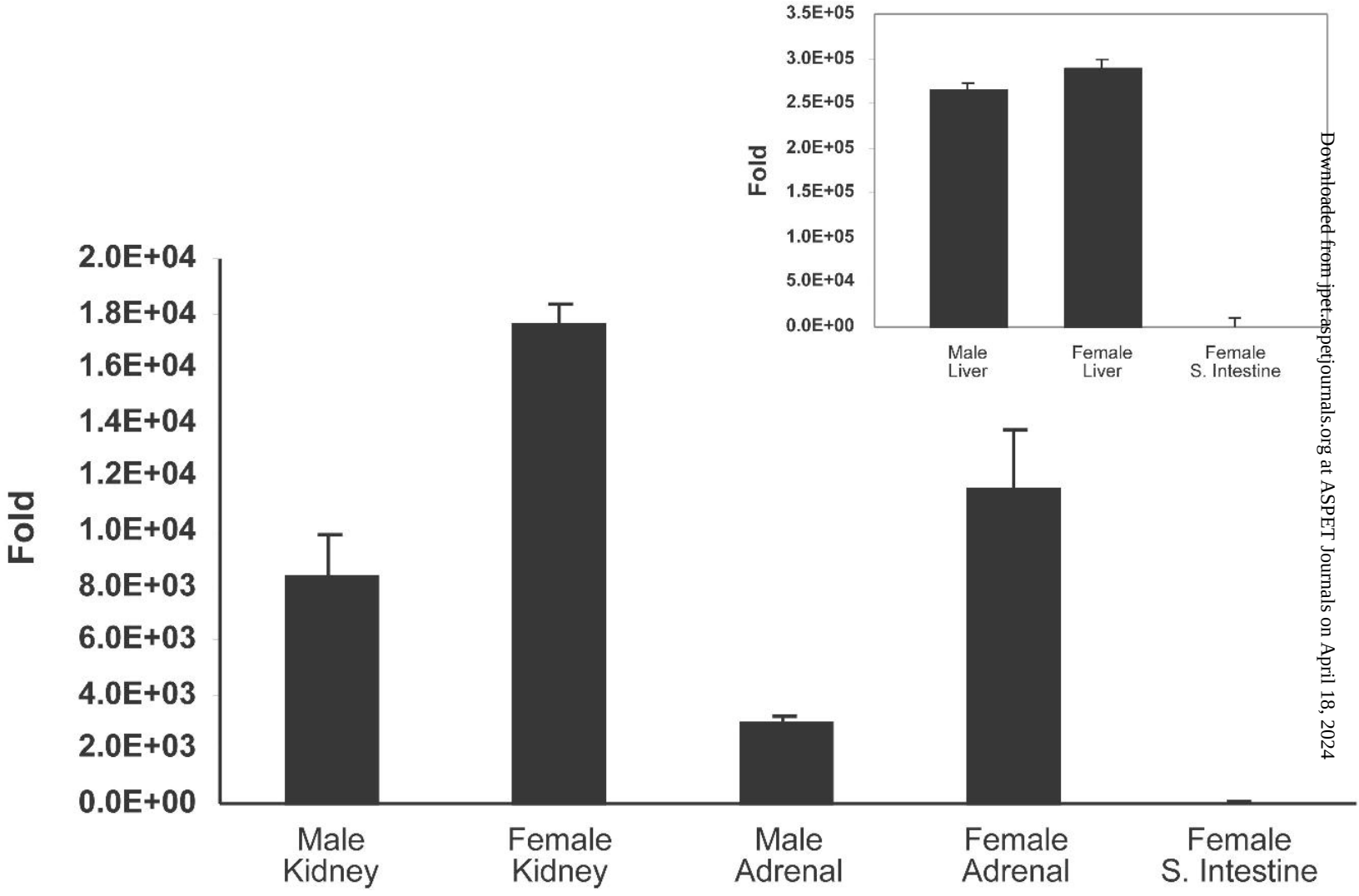

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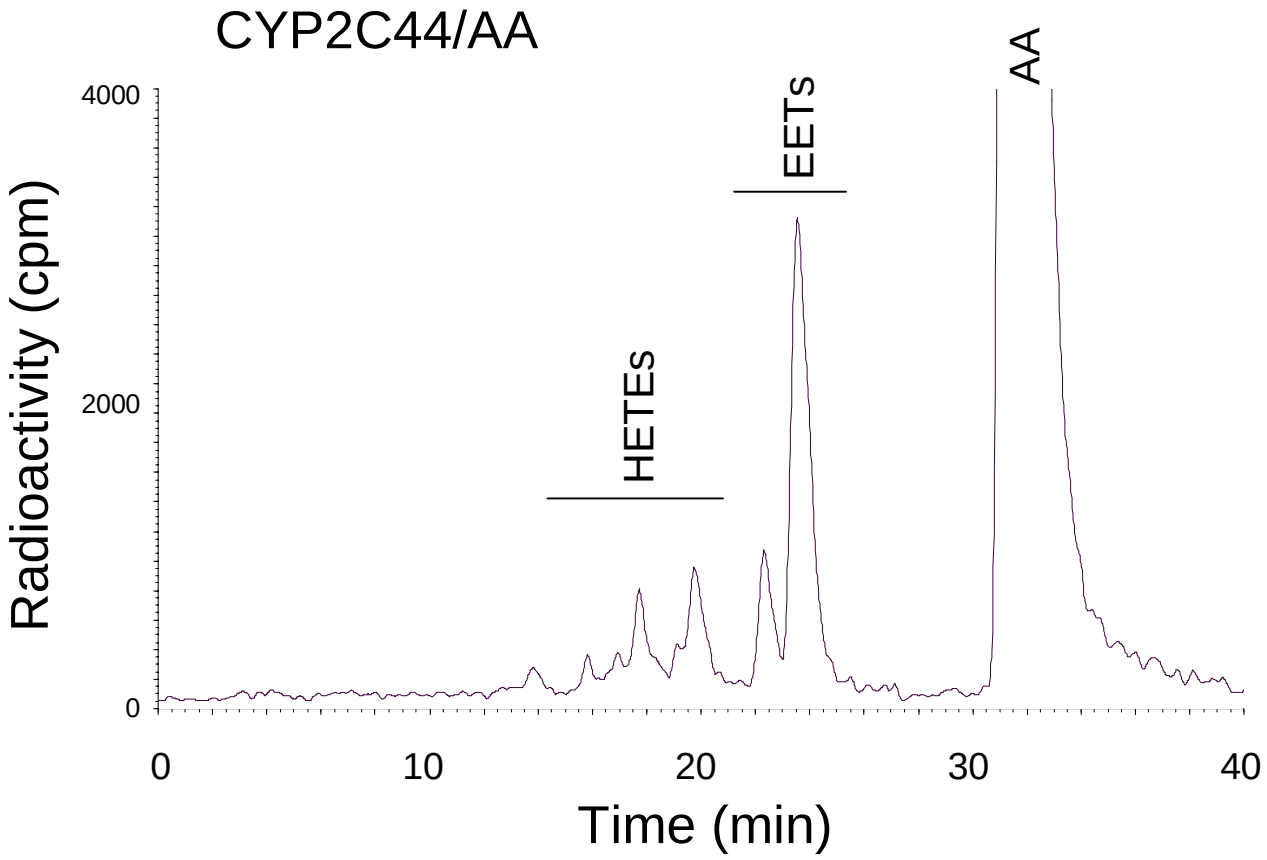

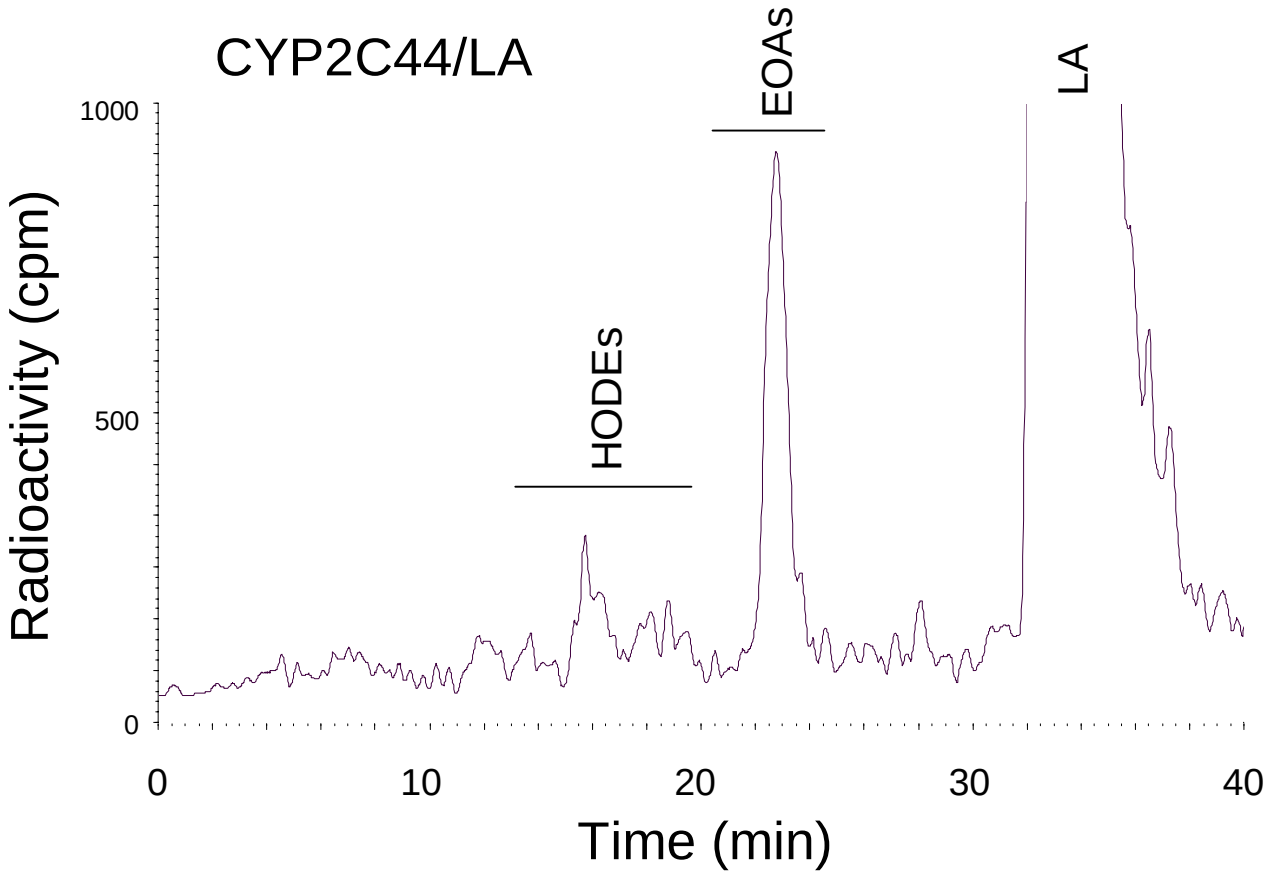

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