Characterization of Four New Mouse Cytochrome P450 Enzymes of the CYP2J Subfamily

Joan P. Graves, Matthew L. Edin, J. Alyce Bradbury, Artiom Gruzdev, Jennifer Cheng, Fred B. Lih, Tiwanda A. Masinde, Wei Qu, Natasha P. Clayton, James P. Morrison, Kenneth B. Tomer, and Darryl C. Zeldin

Laboratory of Respiratory Biology (J.P.G., M.L.E., J.A.B., A.G., J.C., D.C.Z.), Laboratory of Structural Biology (F.B.L., K.B.T.), and the Cellular and Molecular Pathology Branch (T.A.M., N.P.C.), National Toxicology Program Laboratory (W.Q.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; and Charles River Laboratories–Pathology Associates, Durham, North Carolina (J.P.M.)

Received October 22, 2012; accepted January 11, 2013

ABSTRACT

The cytochrome P450 superfamily encompasses a diverse group of enzymes that catalyze the oxidation of various substrates. The mouse CYP2J subfamily includes members that have wide tissue distribution and are active in the metabolism of arachidonic acid (AA), linoleic acid (LA), and other lipids and xenobiotics. The mouse Cyp2j locus contains seven genes and three pseudogenes located in a contiguous 0.62 megabase cluster on chromosome 4. We describe four new mouse CYP2J isoforms (designated CYP2J8, CYP2J11, CYP2J12, and CYP2J13). The four cDNAs contain open reading frames that encode polypeptides with 62–84% identity with the three previously identified mouse CYP2J. All four new CYP2J proteins were expressed in S21 insect cells. Each recombinant protein metabolized AA and LA to epoxyeicosatrienoic and hydroxy eicosatetraenoic acids. Specific antibodies, mRNA probes, and polymerase chain reaction primer sets were developed for each mouse CYP2J to examine their tissue distribution. CYP2J8 transcripts were found in the kidney, liver, and brain, and protein expression was confirmed in the kidney and brain (neuropil). CYP2J11 transcripts were most abundant in the kidney and heart, with protein detected primarily in the kidney (proximal convoluted tubules), liver, and heart (cardiomyocytes). CYP2J12 transcripts were prominently present in the brain, and CYP2J13 transcripts were detected in multiple tissues, with the highest expression in the kidney. CYP2J12 and CYP2J13 protein expression could not be determined because the antibodies developed were not immunospecific. We conclude that the four new CYP2J isoforms might be involved in the metabolism of AA and LA to bioactive lipids in mouse hepatic and extrahepatic tissues.

Introduction

Cytochromes P450 (P450s) are a large gene superfamily of over 500 distinct isoforms that encode heme-thiolate proteins. P450s catalyze the metabolism of a wide range of xenobiotics, including drugs, carcinogens, and environmental pollutants (Nelson et al., 1996; Nebert and Russell, 2002). Certain P450s are also active in the metabolism of endogenous compounds such as arachidonic acid (AA) to bioactive eicosanoids (Nelson et al., 1996; Kroetz and Zeldin, 2002). AA, a polyunsaturated fatty acid present in mammalian cell membranes, is metabolized by multiple P450s into epoxyeicosatrienoic acids (EETs), midchain hydroxyeicosatetraenoic acids (HETEs), and ω-terminal HETEs (Capdevila et al., 2000; Kroetz and Zeldin, 2002; Nebert and Russell, 2002). EETs have substantial vasodilatory effects both in vitro and in vivo, and provide protection from ischemia in brain, heart, and lung models (Seubert et al., 2007; Zhang et al., 2008; Townsley et al., 2010). EET levels are regulated by both synthesis and breakdown. Epoxide hydrolases such as EPHX2 hydrolyze EETs to their dihydroxyeicosatetraenoic acids, which are physiologically less active (Fleming, 2001).

CYP2J subfamily members have been identified in many species, including rabbits, rats, mice, and humans (Kikuta et al., 1991; Zhang et al., 1997; Nelson, 2009). The majority of P450s are primarily expressed in the liver, but the mouse CYP2J subfamily includes members that have a wide tissue distribution. For example, mouse CYP2J5 transcripts are detected most prominently in the kidney and liver (Ma et al., 1999), mouse CYP2J6 is most abundantly expressed in small intestine but is present at lower levels in other tissues (Ma et al., 2002), and mouse CYP2J9 is predominantly expressed in the brain but also is present in the kidney (Qu et al., 2001). Human CYP2J2 is highly expressed in the heart, liver, kidney, and other tissues (Wu et al., 1996). Interestingly, there is a single human CYP2J subfamily gene (CYP2J2), but there are seven murine CYP2J genes (Cyp2j5, Cyp2j6, Cyp2j8, Cyp2j9, Cyp2j11, Cyp2j12, and Cyp2j13)

This work was supported by the Intramural Research Program of the National Institutes of Health National Institute of Environmental Health Sciences [Grant Z01 ES025034].

dx.doi.org/10.1124/dmd.112.049429.

This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: AA, arachidonic acid; bp, base pair; CYPOR, cytochrome P450 oxidoreductase; DHOME, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecamonoenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecenoic acid; LA, linoleic acid; Mb, megabase; ps, pseudogene; P450, cytochrome P450; RT-PCR, reverse-transcription polymerase chain reaction.
and three pseudogenes (Cyp2j7-ps, Cyp2j14-ps, and Cyp2j15-ps) (Wu et al., 1996; Nelson et al., 2004). CYP2J members have been shown to be active in the metabolism of AA and linoleic acid (LA) as well as various xenobiotics (Ma et al., 1999; Qu et al., 2001).

Mouse models are increasingly important in understanding the physiologic function of genes; however, using the mouse as a model of human physiology is more challenging when mouse and human genes lack clear orthologous relationships. Characterization of all the mouse CYP2J subfamily isoforms is an important initial step to understanding the functional significance of human CYP2J. Based on the exonic sequences of known mouse, rat, and human CYP2Js and with the use of the Celera Discovery System and the National Center for Biotechnology Information databases, we identified all the mouse Cyp2j genes and pseudogenes. We then cloned the cDNAs for five new subfamily members designated CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13. CYP2J lacked an open reading frame, and, based on sequence analysis, would be expected to produce a non-functional protein, so it was designated a pseudogene. The remaining CYP2J isoforms were expressed in S2/1 insect cells. Each of the new isoforms was shown to be active in the metabolism of AA and LA, albeit with different catalytic efficiencies and product profiles. We also determined the tissue distribution of each new CYP2J isoform at both the mRNA and protein levels using isof orm-specific probes.

Materials and Methods

Reagents. AA and LA were purchased from Cayman Chemical (Ann Arbor, MI). NADPH tetrasodium salt hydate, isocitrate dehydrogenase, and isocitric acid were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides were synthesized by BioServe Biotechnologies (Laurel, MD). Restriction enzymes were purchased from New England BioLabs (Beverly, MA). All other chemicals, reagents and kits were purchased from Sigma-Aldrich unless otherwise specified.

In Silico Gene Identification. Basic Local Alignment Search Tool (BLAST) searching and physical map assembly were accomplished using the Celera Discovery System (assembly R26). Alignments of known mouse, rat, and human CYP2J cDNAs to the mouse genomic sequence were used to identify putative exons of new members of this P450 subfamily. An identity and human CYP2J cDNAs to the mouse genomic sequence were used to BLAST searching and physical map assembly were accomplished using the Celera Discovery System and the National Center for Biotechnology Information databases, we identified all the mouse Cyp2j genes and pseudogenes. We then cloned the cDNAs for five new subfamily members designated CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13. CYP2J lacked an open reading frame and, based on sequence analysis, would be expected to produce a non-functional protein, so it was designated a pseudogene. The remaining CYP2J isoforms were expressed in S2/1 insect cells. Each of the new isoforms was shown to be active in the metabolism of AA and LA, albeit with different catalytic efficiencies and product profiles. We also determined the tissue distribution of each new CYP2J isoform at both the mRNA and protein levels using isof orm-specific probes.

Cloning of cDNAs for the Novel CYP2J Subfamily Members. Total RNA was prepared from C57BL/6 mouse tissues using the RNeasy Midi Kit from Qiagen (Valencia, CA) following the manufacturer’s instructions. Based on the RNA sequences derived from the Celera Discovery System analysis, primer pairs (Table 1) were designed to amplify the coding regions of the CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13 cDNAs. For CYP2J11, CYP2J12, and CYP2J13, the ProSTAR Ultra HF RT-PCR (reverse-transcription polymerase chain reaction) System from Stratagene (La Jolla, CA) was used for RT-PCR cloning. Briefly, first-strand cDNA was synthesized from 200 ng of tissue total RNA using StrataScript reverse transcriptase with oligo(dT) priming. The PCR amplifications were performed with 1.0 µl of cDNA template, 0.4 µM of each primer, 0.2 µM dNTPs, and 2.5 U of Pfu Turbo DNA polymerase in a total volume of 50 µl. The PCR conditions were as follows: 95°C for 1 minute; 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 68°C for 5 minutes; the final extension was at 68°C for 10 minutes. For the RT-PCR cloning of CYP2J7 and CYP2J8, the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity from Invitrogen (Carlsbad, CA) was used. We mixed 400 ng of template RNA with 25 µl 2X Reaction Mix, 0.2 µM concentration of each primer, and 1 µl of the SuperScript III RT/Ptanium Taq High Fidelity Enzyme Mix in a total volume of 50 µl. The conditions were as follows: 1 cycle of 50°C for 30 minutes; 94°C for 2 minutes; and 40 cycles of 94°C for 15 seconds, 52°C for 30 seconds, and 68°C for 2.5 minutes; the final extension was at 68°C for 5 minutes. The PCR products were TA-cloned into the pCR2.1 vector from Invitrogen. Positive clones were identified by blue-white selection and were sequenced using the ABI Prism Big Dye DNA Sequencing Kit from Applied Biosystems (Foster City, CA).

Expression of New Mouse CYP2J Recombinant Proteins. The open reading frames of the four new mouse CYP2Js were subcloned downstream of pRC201, the polyhedron promoter of the pAUw51-CYPOR (cytochrome P450 oxidoreductase) baculovirus expression vector (Ma et al., 1999). The pRC201 vector coexpresses the human CYPOR, which is necessary for enzymatic activity of most P450 proteins. Takara LA Tag Polymerase (Takara Mirus Bio, Madison, WI) was used to amplify the open reading frame of the new CYP2Js, and the final vectors were verified by sequencing. The expression vectors were cotransfected with BaculoGold DNA into S9 insect cells using the BD BaculoGold Transfection Kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer’s instructions and were maintained as high-titer viral stocks. S2/1 insect cells were infected at a multiplicity of infection of 6, 6, 10, and 8, respectively, with the stocks of CYP2J8, CYP2J11, CYP2J12, and CYP2J13 along with 100 µM 5-aminolevulinic acid and 100 µM iron citrate. Infected cells were harvested 72 hours after infection and lysed in a buffer of 0.25 M sucrose, 10 mM Tris (pH 7.5), 0.1 µg/ml of leupeptin, 0.04 U/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 µg/ml of pepstatin. Differential centrifugation was used to collect the microsomal fraction, which was resuspended in 50 mM Tris (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA (pH 7.5), and 20% glycerol. P450 concentrations were determined by carbon monoxide (CO) difference spectra (Fig. 3) using a Beckman DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA) (Omura and Sato, 1964). Microsomal P450 concentrations were determined (and expressed as nmol

Fig. 1. Organization of the mouse Cyp2j subfamily on chromosome 4. Exon sequences of known mouse, rat, and human Cyp2j subfamily members were used to BLAST search the mouse genome in the Celera Discovery System and the National Center for Biotechnology Information databases. Seven Cyp2j genes (black arrows) and three pseudogenes (gray arrows) were mapped to the negative strand in a 0.62-Mb cluster on chromosome 4.
P450/mg total microsomal protein) so that metabolic studies could be performed with equal P450 concentrations.

**Arachidonic and Linoleic Acid Metabolism by Recombinant CYP2Js.** Recombinant CYP2J proteins were incubated with either AA or LA as previously described elsewhere (Ma et al., 1999) with the modifications that nonradioactive AA and LA were used and that the sample analyses were performed by liquid chromatography/mass spectrometry. Briefly, a reaction mixture of 0.05 M Tris-Cl (pH 7.5), 0.15 M KCl, 0.01 M MgCl2, 8 mM sodium isocitrate, 0.5 U/ml isocitrate dehydrogenase, 0.25 μM recombinant protein, and 100 μM AA or LA was equilibrated to 37°C. Previously we had determined that the Km for CYP2J enzymes for AA is ~80 μM (Ma et al., 1999). We used a higher amount of substrate to ensure saturation of the enzyme (i.e., the substrate was not limiting). Buffers, lipid concentrations, and incubation conditions were performed with equal P450 concentrations. The secondary antibody used was goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and blots were visualized by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti-CYP2J8pep1 was used at a dilution of 1:8000 in 5% milk/phosphate-buffered saline containing 0.1% Tween 20 (Bio-Rad Laboratories) overnight at 4°C, and the secondary goat anti-rabbit antibody at a 1:10,000 dilution for 30 minutes at room temperature.

**RT-PCR Analysis.** Specific primers to the new CYP2J cDNAs (Tables 2 and 3) were designed for RT-PCR analysis. Qiagen OneStep RT-PCR Kit; Life Technologies was used as a control for cDNA quantity. PCR products were analyzed on 1% agarose gels stained with ethidium bromide.

**Immunoblotting.** The polypeptides specific to the deduced amino acid sequences of CYP2J8, CYP2J11, CYP2J12, and CYP2J13 were designed. The specific peptides were synthesized, purified, and then coupled to keyhole limpet hemocyanin via a C-terminal cysteine by Research Genetics (Huntsville, AL). These specific peptides were then used to raise polyclonal antibodies (α-CYP2J8pep1, α-CYP2J11pep1, α-CYP2J12pep1, and α-CYP2J13pep1) in New Zealand white rabbits at Covance Research Products, Inc. (Denver, PA). C57BL/6 mouse tissue microsomes were prepared by differential centrifugation as previously described elsewhere (Ma et al., 1999). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Tissue microsomes (40 μg/lane) were loaded on 12% Tris-glycine gels from Invitrogen, separated by electrophoresis on 12% Tris-glycine gels from Invitrogen, transferred onto nitrocellulose membranes, and immunoblotted using the CYP2J antibodies. The secondary antibody used was goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and blots were visualized by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti-CYP2J8pep1 was used at a dilution of 1:8000 in 5% milk/phosphate-buffered saline containing 0.1% Tween 20 (Bio-Rad Laboratories) overnight at 4°C, and the secondary goat anti-rabbit antibody at a 1:10,000 dilution for 30 minutes at room temperature. We purified α-CYP2J11pep1, α-CYP2J12pep1, and α-CYP2J13pep1 with the Protein A IgG Purification and the AminoLink Plus Immobilization Kits from Pierce in an attempt to increase specificity.

**Immunohistochemistry.** Immunohistochemical staining was performed for CYP2J8 in mouse brain and for CYP2J11 in mouse kidney and heart tissues using the standard avidin-biotin peroxidase technique, using methods described previously (Zeldin et al., 1996). Formalin-fixed, paraffin-embedded tissues were deparaffinized and unmasked with heat-induced epitope retrieval in 0.01 M citrate buffer, pH 6.0 (Biocare Medical, Concord, CA), performed in
a microwave. The sections were incubated for 1 hour at room temperature in rabbit α-CYP2J8pep1 at 1:1000 or rabbit α-CYP2J11pep1 at 1:25. The OmniMap anti-rabbit HRP Kit on the Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) was used for detection of CYP2J8. The Vectastain Rabbit IgG Elite Kit (Vector Laboratories, Burlingame, CA) was used for the detection of CYP2J11. Staining was visualized with 3,3′-diaminobenzidine chromagen (DakoCytomation California, Carpinteria, CA) then counterstained with hematoxylin. For negative controls, the primary antibody was replaced with normal rabbit serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA).

Results

Organization of the CYP2J Subfamily Locus on Mouse Chromosome 4. Using the exon sequences from the known mouse CYP2J subfamily members as well as the exons from human and rat CYP2J isoforms, the Celera Discovery System database was searched using the BLAST program. The three known mouse Cyp2j genes were mapped to two continuous Celera contigs of mouse chromosome 4 (GA_x6Ko2TQEEQ:17000001 . . . 17500000 and 17500001 . . . 18000000) (Fig. 1). We also identified four potential new Cyp2j genes (designated Cyp2j8, Cyp2j11, Cyp2j12, and Cyp2j13) as well as three new pseudogenes (designated Cyp2j7-ps, Cyp2j14-ps, and Cyp2j15-ps) in this region. All the mouse CYP2J subfamily members, including the pseudogenes, are located on the minus strand of chromosome 4 in a 0.62-megabase (Mb) cluster (Fig. 1).

Cloning of the CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13 cDNAs. The cDNAs of CYP2J7, CYP2J8, CYP2J11, CYP2J12, and CYP2J13 were cloned from RNAs from various mouse tissues. CYP2J7, CYP2J11, and CYP2J13 cDNAs were cloned from mouse kidney RNA, whereas CYP2J8 and CYP2J12 cDNAs were cloned from mouse brain RNA. The sequenced CYP2J7, CYP2J11, CYP2J12, and CYP2J13 cDNAs were 99 to 100% identical to the predicted mouse genomic database sequences. For 58 independent CYP2J7 clones from the kidney, there was a 146 base pair (bp) sequence between the predicted exons 1 and 2 that was spliced into the final CYP2J7 transcript. Of the 19 independent CYP2J7 clones from

Fig. 2. Amino acid sequences for the mouse CYP2J subfamily members. Conserved regions present in the deduced amino acid sequences of the CYP2Js are denoted as follows: the proline-rich region is underlined; the six substrate recognition sites (SRS) are bound in solid lines; the heme-binding region is bound in a hatched line with the invariant cysteine in bold and highlighted; and the location of the peptides used to raise polyclonal antibodies is highlighted. Stars indicate conserved amino acids among the seven CYP2J isoforms.
testis, 12 had a 16-bp sequence from a splice junction in intron 3 inserted between exons 3 and 4, and the remaining 7 clones had a 62-bp insert from a splice junction from intron 1 between exons 1 and 2 (Supplemental Fig. 1). All three splice variants of CYP2J7 result in early truncation of the predicted protein. Each of the cDNAs for CYP2J8, CYP2J11, CYP2J12, and CYP2J13 contains an open reading frame that encodes polypeptides of 503, 504, 502, and 503 amino acids, respectively. Domains present in all P450s are conserved in these new CYP2J isoforms, including a putative heme-binding peptide with an invariant cysteine at position 451, a proline-rich region between residues 40 to 51, and six putative substrate recognition sites (Gotoh, 1992) (Fig. 2). The four new and three previously identified mouse CYP2J isoforms, all encoding full-length proteins, are 62–84% identical at the amino acid level (Table 4).

**Heterologous Expression and Enzymatic Characterization of the New CYP2Js.** CYP2J8, CYP2J11, CYP2J12, and CYP2J13 were coexpressed with CYPOR in Sf21 insect cells using the baculovirus expression system with the modified pRC201 vector, as described previously elsewhere (Ma et al., 1999). CYP2J isoform expression levels varied between 0.05 and 0.2 nmol P450/mg of microsomal protein. All four new CYP2Js displayed typical b-type cytochrome P450-reduced CO difference spectra with Soret maxima at ~450 nm (Fig. 3). In the presence of NADPH and CYPOR, the four novel CYP2J enzymes metabolized both AA and LA, albeit with different catalytic efficiencies and product profiles (Fig. 4; Tables 5-7). For comparison, a similar analysis was performed on CYP2J2, CYP2J5, CYP2J6, and CYP2J9. These enzymes showed similar epoxygenase and hydroxylase activities as previously published, and they metabolized AA and LA with similar regiochemical specificity (Wu et al., 1996; Ma et al., 1999, 2002; Qu et al., 2001).

CYP2J5, CYP2J8, CYP2J11, and CYP2J12 showed little substrate preference for AA compared with LA. In contrast, CYP2J13 metabolized LA at a rate that was 2.5-fold higher than that for AA. Of these enzymes, CYP2J8 had the highest catalytic turnover, metabolizing AA and LA at 167 and 137 pmol/nmol/min, respectively. CYP2J6, which had previously been shown to be an unstable P450 (Ma et al., 2002), had the lowest turnover of the CYP2J enzymes for AA at 22 pmol/nmol/min, which was approximately 7.5 times lower than that for CYP2J8. CYP2J9 had the lowest turnover of the CYP2J enzymes for LA at 10 pmol/nmol/min, which was approximately 14 times lower than that for CYP2J8. CYP2J5, CYP2J6, and CYP2J9 were primarily AA epoxygenases, with 74, 81, and 66% of total products being EETs, respectively. In contrast, CYP2J12 was primarily an AA hydroxylase, with 67% of total products being HETEs. CYP2J8, CYP2J11, and CYP2J13 showed comparable AA epoxygenase and AA hydroxylase activity, producing nearly equal mixtures of EETs (53, 48, and 43%, respectively) and HETEs (47, 52, and 57%, respectively). The seven murine CYP2J enzymes were also active in the metabolism of LA to epoxyoctadecamonoenoic acids (EpOMEs) and hydroxyoctadecanoic acids (HODEs) (Table 5). CYP2J6, CYP2J8, and CYP2J12 primarily metabolized LA to EpOMEs (93, 70, and 80% of total products, respectively), while CYP2J9 exclusively metabolized LA to HODEs. CYP2J5, CYP2J11, and CYP2J13 metabolized LA to mixtures of EpOMEs (45, 46, and 43%, respectively) and HODEs (55, 54, and 57%, respectively).

Regiochemical analysis of EETs formed during incubations with the recombinant CYP2Js and AA revealed a preference for the formation of 14,15-EET (43–72% of total EETs) for all the CYP2J isoforms (Table 6). Formation of 11,12-EET was lower (9–20% of total EETs), except for CYP2J8 (34% of total EETs). All CYP2J6 produced lower amounts of 8,9-EET (6–14%) and 5,6-EET (5–13%). The regioisomer distribution of EpOME products formed during incubations of recombinant CYP2J5, CYP2J6, CYP2J9, and CYP2J13 with LA showed a mixture of 12,13-EpOME (52–59%) and 9,10-EpOME (41–48%) (Table 6). Incubation of CYP2J8 with LA revealed a preference for 9,10-EpOME (71%), whereas CYP2J11 showed a slightly higher preference for 12,13-EpOME (61%) over 9,10-EpOME (39%).

The seven murine CYP2Js produced two patterns of HETE regioisomers. The CYP2J5, CYP2J6, CYP2J8, CYP2J9, CYP2J11, and CYP2J12 generated predominantly 15-, 8-, and 5-HETE. In contrast, CYP2J8 and CYP2J13 generated 19-HETE (28% of hydroxylated compounds for both) as their major hydroxylated metabolite. In addition, CYP2J8 and CYP2J13 produced detectable amounts of 20-HETE (7 and 22%, respectively), which was similar to that observed for human CYP2J2 (Table 7).

**Expression Pattern of the New CYP2J mRNAs.** To determine the relative abundance of the new CYP2J transcripts, RT-PCR (Fig. 5) using CYP2J isoform-specific primer pairs (Table 3) was performed with RNA extracted from various C57BL/6 mouse tissues. There were prominent CYP2J8 transcripts detected in the kidney, liver, small intestine, and brain by RT-PCR. The RT-PCR analysis of CYP2J11 showed prominent transcripts in the kidney and heart, with fainter
transcripts seen for liver, ovary, and other tissues. CYP2J12 RT-PCR analysis revealed a prominent transcript in the brain, with fainter transcripts for all of the other tissues examined including the liver. CYP2J13 transcripts were present in all the mouse tissues examined but were more prominently expressed in the kidney. The CYP2J13 transcript was also seen to be more highly expressed in the male

Fig. 4. Liquid chromatography–tandem spectrometry (LC-MS/MS) profiles for the metabolism of AA and LA by the recombinant CYPJs. A representative chromatogram of the incubation of CYP2J8 with AA and with (top) or without (bottom) NADPH is shown. Detection of individual AA metabolites is indicated. Experiments were performed in triplicate for each P450 enzyme.
kidney as compared with the female kidney. The CYP2J13 transcript expression pattern in the kidney is similar to that of CYP2J5, which is higher in male versus female kidneys (Ma et al., 1999). The majority of P450s are expressed in the liver, so it is not surprising that transcripts for the four new CYP2J2s were present in the liver, albeit at low levels. It is not clear whether these liver transcripts reflect expression of the new CYP2Js or whether they represent cross-reactivity to other P450s in mouse liver.

### Tissue Distribution of the New CYP2J Proteins

Unique peptides, chosen by aligning the amino acid sequences of all members of the CYP2J subfamily (Fig. 2), were used to raise polyclonal antibodies for the new CYP2Js (α-CYP2J8pep1, α-CYP2J11pep1, α-CYP2J12pep1, and α-CYP2J13pep1). Immunoblotting with the mouse CYP2J recombinant proteins to examine the specificity of the new polyclonal antibodies revealed that α-CYP2J8pep1 and α-CYP2J11pep1 are highly immunospecific for their respective recombinant proteins (Fig. 6). In contrast, α-CYP2J12pep1 recognized all the recombinant mouse CYP2J proteins and also had cross-reactivity with multiple CYP2C recombinant proteins (unpublished data); therefore, this antibody was not useful to determine the CYP2J2 tissue distribution at the protein level. The α-CYP2J13pep1 was also not useful to determine the tissue distribution of CYP2J13 at the protein level, as it recognized both the CYP2J13 and CYP2J5 proteins (unpublished data). This cross-reactivity is not surprising in light of the ~80% identity of the deduced CYP2J5 and CYP2J13 amino acid sequences (Table 4). The 62–84% amino acid identity between the mouse CYP2J subfamily members makes it difficult to identify unique peptide stretches to raise isoform-specific polyclonal antibodies.

CYP2J8 protein expression was analyzed by immunoblotting of whole tissue lysates using the α-CYP2J8pep1 antibody (Fig. 6). The α-CYP2J8pep1 antibody detected two proteins in brain: one at 58 kDa corresponding to CYP2J8 and another higher molecular weight protein. We did not detect an immunoreactive protein band in the kidney with α-CYP2J8pep1, despite the presence of a CYP2J8 transcript in the kidney (Fig. 5). CYP2J11 protein expression was analyzed by immunoblotting of microsomal fractions of various mouse tissues using α-CYP2J11pep1 (Fig. 6). A prominent ~57 kDa band was observed in the kidney, liver, and stomach, with a slightly higher molecular weight protein in the heart. The difference in electrophoretic mobility between the recombinant CYP2J11 protein and endogenous CYP2J11 in the heart may possibly be explained by the endogenous protein being posttranslationally modified, or having cross-reactivity to a related, slightly higher molecular weight protein that is highly expressed in the heart. A slightly lower molecular weight protein was detected in the testis and lung, and it likely represents cross-reactivity with an unknown protein given the lack of CYP2J11 transcripts in the testis or lung (Fig. 5). Further investigation will be needed to account for these minor differences in electrophoretic mobility between the CYP2J11 recombinant protein and endogenous CYP2J11.

### Localization of CYP2J8 and CYP2J11 Protein Expression in Mouse Tissues

Formalin-fixed, paraffin-embedded mouse brain sections were stained with either α-CYP2J8pep1 or normal rabbit serum (negative control) to determine the cellular distribution of CYP2J8 protein in the brain (Fig. 7, A and B). Diffuse staining was present in the neuropil of the gray matter throughout the brain, including the forebrain and the globus pallidus; this staining may represent expression of CYP2J8 protein in neuronal processes such as dendrites and unmyelinated axons. White matter tracts were devoid of staining. Staining of large neurons was nonspecific; similar staining patterns were observed in the negative control sections.

---

**TABLE 5**

Metabolism of arachidonic acid (AA) and linoleic acid (LA) by recombinant mouse CYP2J enzymes

<table>
<thead>
<tr>
<th>Protein</th>
<th>AA Rates (pmol/mg/min)</th>
<th>LA Rates (pmol/mg/min)</th>
<th>AA Epoxy</th>
<th>AA Hydroxyl</th>
<th>LA Epoxy</th>
<th>LA Hydroxyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2J2</td>
<td>254</td>
<td>399</td>
<td>72</td>
<td>28</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>CYP2J5</td>
<td>76</td>
<td>85</td>
<td>74</td>
<td>26</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>CYP2J6</td>
<td>22</td>
<td>29</td>
<td>81</td>
<td>19</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>CYP2J8</td>
<td>167</td>
<td>137</td>
<td>53</td>
<td>47</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>CYP2J9</td>
<td>37</td>
<td>10</td>
<td>66</td>
<td>34</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CYP2J11</td>
<td>37</td>
<td>62</td>
<td>48</td>
<td>52</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>CYP2J12</td>
<td>35</td>
<td>42</td>
<td>33</td>
<td>67</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>CYP2J13</td>
<td>78</td>
<td>193</td>
<td>43</td>
<td>57</td>
<td>43</td>
<td>57</td>
</tr>
</tbody>
</table>

**TABLE 6**

Regiochemical distribution of epoxyeicosatrienoic acid (EET) and epoxyoctadecamonoenoic acid (EpOME) products formed during incubation of recombinant mouse CYP2Js with arachidonic acid and linoleic acid

<table>
<thead>
<tr>
<th>Protein</th>
<th>14,15-EET (%)</th>
<th>11,12-EET (%)</th>
<th>8,9-EET (%)</th>
<th>5,6-EET (%)</th>
<th>12,13-EpOME (%)</th>
<th>9,10-EpOME (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2J2</td>
<td>71</td>
<td>18</td>
<td>6</td>
<td>5</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>CYP2J5</td>
<td>53</td>
<td>20</td>
<td>14</td>
<td>12</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>CYP2J6</td>
<td>78</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>CYP2J8</td>
<td>43</td>
<td>34</td>
<td>13</td>
<td>10</td>
<td>29</td>
<td>71</td>
</tr>
<tr>
<td>CYP2J9</td>
<td>75</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>CYP2J11</td>
<td>73</td>
<td>14</td>
<td>7</td>
<td>7</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>CYP2J12</td>
<td>63</td>
<td>15</td>
<td>9</td>
<td>13</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>CYP2J13</td>
<td>72</td>
<td>14</td>
<td>6</td>
<td>7</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>
TABLE 7
Regiochemical distribution of hydroxyeicosatetraenoic acid (HETE) products formed during incubation of recombinant CYP2J enzymes with arachidonic acid.

Data for human CYP2J2 are shown for comparison.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Regiochemical Distribution (% Total HETEs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-HETE 19-HETE 15-HETE 12-HETE 11-HETE 8-HETE 5-HETE</td>
</tr>
<tr>
<td>CYP2J2</td>
<td>17 19 14 7 10 20 13</td>
</tr>
<tr>
<td>CYP2J5</td>
<td>1 1 18 0 10 23 47</td>
</tr>
<tr>
<td>CYP2J6</td>
<td>2 1 34 8 11 25 20</td>
</tr>
<tr>
<td>CYP2J8</td>
<td>7 28 9 18 8 21 9</td>
</tr>
<tr>
<td>CYP2J9</td>
<td>3 1 33 11 12 25 14</td>
</tr>
<tr>
<td>CYP2J11</td>
<td>11 1 28 2 11 24 23</td>
</tr>
<tr>
<td>CYP2J12</td>
<td>2 1 35 4 13 21 26</td>
</tr>
<tr>
<td>CYP2J13</td>
<td>22 28 13 3 7 13 14</td>
</tr>
</tbody>
</table>

To determine the cellular distribution of CYP2J11, formalin-fixed, paraffin-embedded mouse heart and kidney sections were stained with either α-CYP2J11 pep1 or the normal rabbit serum (Fig. 7, C–F). Diffuse staining was observed in cardiomyocytes and renal proximal tubules; glomeruli and distal tubules did not stain. There was little or no background staining with normal rabbit serum. Fixed liver sections were also stained with α-CYP2J11 pep1 or normal rabbit serum (unpublished data). No differences were detected between the α-CYP2J11 pep1 and normal rabbit serum with respect to staining in the liver.

Discussion

P450 enzymes have many physiologically relevant functions including regulating vascular tone in the heart (Campbell and Harder, 1999; Fisslthaler et al., 1999), mediating ion transport in the kidney (Zou et al., 1996), anti-inflammatory properties in the vasculature (Node et al., 2001), and controlling the secretion of pancreatic peptide hormones (Falck et al., 1983). CYP2J2, the lone human CYP2J subfamily member, plays an active role in the metabolism of endogenous fatty acids (Wu et al., 1996). Previously cloned and characterized mouse CYP2J isoforms (CYP2J5, CYP2J6, and CYP2J9) have been shown to actively metabolize AA and LA (Ma et al., 1999, 1998). Mouse CYP2J subfamily members are highly homologous at the amino acid level, with 62–84% identity. Individual mouse CYP2J isoforms likely arose by gene duplication events (Nelson et al., 1996). Alignment of the mouse CYP2J isoforms reveals that the sequence is most divergent within the six putative substrate recognition sites. These alterations are likely responsible for the varied substrate specificity and catalytic properties of the mouse CYP2J proteins.

Each of the new CYP2Js is active in the metabolism of AA, with CYP2J8 having the highest rate; however, the rates are lower than the AA metabolism rate of human CYP2J2. CYP2J11, CYP2J12, and CYP2J13 showed a 4- to 5-fold preference for metabolism of AA to 14,15-EET compared with 11,12-, 8,9-, or 5,6-EETs. This preference for 14,15-EET production is similar to human CYP2J2 and the previously reported mouse CYP2J1 enzymes that also show a preference for 14,15-EET production. In contrast, many CYP2C epoxidegenses show a preference for 11,12-EET production (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). CYP2J8 showed nearly equal preference for both 11,12-EET and 14,15-EET.

All of the new mouse CYP2J enzymes are also active in the metabolism of LA. The distribution of LA epoxides and alcohols among the new CYP2Js differs, with both CYP2J8 and CYP2J12 having epoxides as the principle products while CYP2J11 and CYP2J13 have similar amounts of epoxide and hydroxyl products. Notably, CYP2J9 has a strong preference for LA hydroxylation, which has not been reported previously. The regioisomeric distribution of EpOME products from LA also varies between the CYP2J enzymes. It is interesting to note that although the CYP2J8 and CYP2J11 isoforms have the highest amino acid identity among the mouse CYP2J (Table 4), they show different metabolic rates and preferences for LA metabolites and EET regiosomers. Although the metabolism of LA by the CYP2J subfamily members into biologically active products has been previously reported (Zeldin et al., 1995; Bylund et al., 1998), the function of DiHOMEs (dihydroxyoctadecamonoenoic acids) in the kidney, brain, and heart is less clear. Increased DiHOMEs are associated with reduced recovery from ischemia/reperfusion injury in the heart (Edin et al., 2011). In addition, soluble epoxide hydrolase (EPHX2) inhibitors improve recovery from ischemic injury in the kidney, which is associated with increased EpOME to DiHOME ratios (Lee et al., 2012). These toxic effects of LA metabolites may be mediated through induction mitochondrial dysfunction (Moran et al., 2000). This suggests a possible role for CYP2J-derived LA metabolites in the pathogenesis of ischemia/reperfusion injury in kidney and other tissues.

We also report the presence of CYP2J8 in the brain, similar to the previously reported expression of mouse CYP2J9 in the Purkinje cells (Qu et al., 2001). Immunohistochemistry with the α-CYP2J8 pep1 revealed abundant staining of the neuropil throughout the brain. The neuropil in the brain consists mainly of unmyelinated axons, dendrites, and glial cell processes. Because EETs are potent vasodilators and have been shown to be neuroprotective (Zhang et al., 2008), neural CYP2J8 expression may be involved in...
neuroprotective mechanisms. CYP2J12 transcripts have also been identified in the brain.

Regulation of kidney P450s is not fully understood, but some data suggest that renal EETs may play a pivotal role in the pathogenesis of hypertension in both humans and rodents. Renal P450 epoxygenases are under regulatory control by dietary salt intake, and their inhibition leads to salt-dependent hypertension (Holla et al., 1999; Oyekan et al., 1999). In women with pregnancy-induced hypertension, the urinary level of excreted epoxygenase metabolites is increased (Catella et al., 1990). There is also evidence that the CYP2J subfamily is involved in...

Fig. 6. Specificity and tissue distribution of the new CYP2Js by immunoblotting. (A) CYP2J recombinant proteins isolated from Sf21 insect cells infected with baculovirus stocks (0.5 pmol of P450/lane) were immunoblotted with α-CYP2J8pep1, α-CYP2J11pep1, α-CYP2J12pep1, or α-CYP2J13pep1. (B) Total tissue lysates (50 μg protein/lane) were prepared from various mouse tissues and immunoblotted with α-CYP2J8pep1. Microsomal fractions (40 μg protein/lane) prepared from various mouse tissues were immunoblotted with α-CYP2J11pep1. The results are representative of three independent experiments.

Fig. 7. Immunohistochemical staining of CYP2J8 protein in the brain and CYP2J11 protein in the heart and kidney. Sections of the brain were immunostained with (A) α-CYP2J8pep1 or (B) normal rabbit serum as described in Materials and Methods. Diffuse staining for CYP2J8 was present in the neuropil of the gray matter of the brain with no staining of the white matter. Sections of (C and D) heart and (E and F) kidney were immunostained with (C and E) α-CYP2J11pep1 or (D and F) normal rabbit serum. Staining for CYP2J11 protein was present in cardiomyocytes and renal proximal tubules.
the development of hypertension. Renal CYP2J expression has been localized to sites in the nephron where EETs regulate the actions of hormones that affect blood pressure (Ma et al., 1999). CYP2J2, the single human ortholog of the mouse CYP2J2 subfamily, is abundantly expressed in the kidney (Wu et al., 1996). Mouse CYP2J2 is found in the convoluted and straight portions of the proximal tubules in the kidney and is active in the metabolism of AA to EETs (Ma et al., 1999). CYP2J5 expression appears to be beneficial, as CYP2J5-deficient mice develop spontaneous hypertension (Athirakul et al., 2008). CYP2J11, like CYP2J5, is expressed in the kidney, is localized to the renal proximal convoluted tubules, and is active in the metabolism of AA to EETs, primarily 14,15-EET. Although none of the individual mouse CYP2J11 to be present in cardiomyocytes. Like CYP2J2, CYP2J11 might be a pseudogene, as all cloned cDNAs lacked open reading frames. CYP2J11, like CYP2J5, is expressed in the kidney, is localized to the renal proximal convoluted tubules, and is active in the metabolism of AA to EETs, primarily 14,15-EET. Although none of the individual mouse P450s are expressed in extrahepatic tissue. Although none of the individual mouse P450 genes to human chromosome 1 and mouse chromosome 4. We report that CYP2J1 is most likely a pseudogene, as all cloned cDNAs lacked open reading frames. We cloned the cDNAs of the four new CYP2J enzymes (CYP2J8, CYP2J11, CYP2J12, and CYP2J13) and demonstrated that the recombinant proteins are active in the metabolism of both AA and LA, albeit with different catalytic rates and product profiles. In addition, our data show each of the new P450s is expressed in extrahepatic tissues. Although none of the individual mouse CYP2J genes can be conclusively shown to be the homolog of human CYP2J2, together the murine CYP2Js show similar tissue expression patterns to CYP2J2. Like human CYP2J2, these four new CYP2J enzymes might be involved in fatty acid metabolism and may influence many cardiovascular and renal processes.

Acknowledgments

The authors are grateful to Dr. J. Todd Painter and Heather Jensen for immunohistochemical assistance, and to Lois Wyrick for graphic arts assistance.

Authorship Contributions

Participated in research design: Graves, Edin, Zeldin.
Conducted experiments: Graves, Edin, Bradbury, Lih, Masinde, Clayton, Tomer.
Contributed new reagents or analytic tools: Qa.
Performed data analysis: Graves, Edin, Clayton, Morrison, Zeldin.
Wrote or contributed to the writing of the manuscript: Graves, Edin, Gruzdev, Cheng, Zeldin.


Address correspondence to: Dr. Darryl C. Zeldin, NIH/NIEHS, 111 T.W. Alexander Drive, Building A214, Research Triangle Park, NC 27709. E-mail: zeldin@niehs.nih.gov.