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

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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 CYP2Js. All four new CYP2J proteins were expressed in Sf21 insect cells. Each recombinant protein metabolized AA and LA to epoxides and hydroxy derivatives. 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 (Campbell et al., 1996; Larsen et al., 2006), anti-inflammatory (Node et al., 1999), fibrinolytic (Node et al., 2001), cardioprotective (Seubert et al., 2004; Seubert et al., 2006), and angiogenic (Wang et al., 2005) 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 dihydroxyeicosatrienoic 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)
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 CYP2J2. 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. CYP2J7 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 S21 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 isoform-specific probes.

Materials and Methods

**Reagents.** AA and LA were purchased from Cayman Chemical (Ann Arbor, MI). NADPH tetrasodium salt hydrate, 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 cutoff of 55% was used in this analysis (Nelson et al., 1996). The nine exons for each of the three known mouse Cyp2j genes (Cyp2j3, Cyp2j6, and Cyp2j9) were found on two continuous contigs on chromosome 4 (GA_x6K02T2- QEQQ:17000001.17500000 and GA_X6K02T2QEQQ:17500001.18000000). We identified four new Cyp2j2 genes and three new Cyp2j pseudogenes on these contigs by combining the putative exonic sequences. A physical map of the mouse Cyp2j locus was then assembled using this information (Fig. 1). Each of the nine new Cyp2j genes and pseudogenes was given a formal name by the Committee on Standardized P450 Nomenclature (see http://drnelson.uthsc.edu/CytochromeP450.html).

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/Platinum 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 coexpression the human CYPOR, which is necessary for enzymatic activity of most P450 proteins. Takara LA Taq 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. S21 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

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**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 mM pyruvate kinase, and 100 mM AA or LA was equilibrated to 37°C. Preceding the addition of NADPH, the reaction mixture was incubated at 37°C for 1 hour with gentle shaking. Control reactions with microsomes from vector-infected cells, without lipid addition, with boiled microsomes, or without NADPH addition were performed by liquid chromatography/mass spectrometry. Briefly, a reaction mixture was equilibrated to 37°C without substrate or enzyme. The incubation was initiated by the addition of NADPH. After 30 minutes, aliquots of the reaction mixtures were extracted with ethyl acetate and analyzed for AA and LA by reverse-phase liquid chromatography/mass spectrometry. The AA and LA were converted to their corresponding CoA derivatives by adenosine triphosphate-dependent CoA ligase and analyzed by reverse-phase liquid chromatography/mass spectrometry.

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**RT-PCR Analysis.** Specific primers to the new CYP2J cDNAs (Tables 2 and 3) were used for RT-PCR analysis. Qiagen’s OneStep RT-PCR Kit was used to determine the tissue distribution at the mRNA level. The primer pairs were designed based on the cDNA sequences derived from the Celera Discovery System and National Center for Biotechnology Information database analysis. The primer pairs were designed to amplify the coding regions of the five new CYP2Js. Corresponding positions are relative to the ATG initiating codon designated as +1.

**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 was equilibrated to 37°C without substrate or enzyme. The incubation was initiated by the addition of NADPH. After 30 minutes, aliquots of the reaction mixtures were extracted with ethyl acetate and analyzed for AA and LA by reverse-phase liquid chromatography/mass spectrometry. The AA and LA were converted to their corresponding CoA derivatives by adenosine triphosphate-dependent CoA ligase and analyzed by reverse-phase liquid chromatography/mass spectrometry.

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**Table 1**

<table>
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<th>cDNA and Direction</th>
<th>Primer Sequence 5'→3'</th>
<th>Corresponding Position</th>
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<td>CYP2J7</td>
<td>GGCGCAAAGAATGGGATGGCC</td>
<td>+164 to +176</td>
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<tr>
<td>Reverse</td>
<td>GCTTATGCTCTCTCTACGTTG</td>
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</tr>
<tr>
<td>CYP2J9</td>
<td>AAAGGATCTCCATGTGAC</td>
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</tr>
<tr>
<td>Reverse</td>
<td>TTTTAGGATCTCTTTTCAG</td>
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</tr>
<tr>
<td>CYP2J11</td>
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</tr>
<tr>
<td>Reverse</td>
<td>+1628 +1648</td>
<td></td>
</tr>
<tr>
<td>CYP2J12</td>
<td>GTGTCGACTCCTTTACGAGGC</td>
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</tr>
<tr>
<td>Reverse</td>
<td>+1975 +1993</td>
<td></td>
</tr>
<tr>
<td>CYP2J13</td>
<td>GTGTCGACTCCTTTACGAGGC</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>+2115 +2135</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

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<thead>
<tr>
<th>Transcript</th>
<th>Primer (5'-3')</th>
</tr>
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<tr>
<td>CYP2J8</td>
<td>AGTCAGGCATTGTCCTCTAGCCAGTCTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGACGCCCTTCTCTACGTTG</td>
</tr>
<tr>
<td>CYP2J9</td>
<td>AGTCAGGCATTGTCCTCTAGCCAGTCTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGACGCCCTTCTCTACGTTG</td>
</tr>
<tr>
<td>CYP2J11</td>
<td>AGTCAGGCATTGTCCTCTAGCCAGTCTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGACGCCCTTCTCTACGTTG</td>
</tr>
<tr>
<td>CYP2J12</td>
<td>AGTCAGGCATTGTCCTCTAGCCAGTCTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGACGCCCTTCTCTACGTTG</td>
</tr>
<tr>
<td>CYP2J13</td>
<td>AGTCAGGCATTGTCCTCTAGCCAGTCTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGACGCCCTTCTCTACGTTG</td>
</tr>
</tbody>
</table>

**RT-PCR Analysis.** Specific primers to the new CYP2J cDNAs (Tables 2 and 3) were used for RT-PCR analysis. Qiagen’s OneStep RT-PCR Kit was used to determine the tissue distribution at the mRNA level. The primer pairs were designed based on the cDNA sequences derived from the Celera Discovery System and National Center for Biotechnology Information database analysis. The primer pairs were designed to amplify the coding regions of the five new CYP2Js. Corresponding positions are relative to the ATG initiating codon designated as +1.
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 175000001 . . . 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 within this region.

**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. The sequenced CYP2J8, CYP2J11, 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
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). CYP2J19 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, CYP2J13 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 CYP2Js produced two patterns of HETE regioisomers. CYP2J5, CYP2J6, CYP2J8, CYP2J9, and 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.
amino acid sequences (Table 4). The 62 light of the proteins (unpublished data). This cross-reactivity is not surprising in not useful to determine the tissue distribution of CYP2J13 at the tissue distribution at the protein level. The cross-reactivity with multiple CYP2C recombinant proteins (unpublished data) all the recombinant mouse CYP2J proteins and also had cross-reactivity with recombinant proteins (Fig. 6). In contrast, the a-CYP2J12pep1 antibody (Fig. 6). The a-CYP2J12pep1 recognized 84% amino acid identity –CYP2J13pep1 was also 84% amino acid identity between the mouse CYP2J recombinant proteins to examine the specificity of the new polyclonal antibodies revealed that a-CYP2J8pep1 and a-CYP2J11pep1 are highly immunospecific for their respective recombinant proteins (Fig. 6). In contrast, a-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 CYP2J12 tissue distribution at the protein level. The a-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 a-CYP2J8pep1 antibody (Fig. 6). The a-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 a-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 a-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 a-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>
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<tr>
<th>Protein</th>
<th>Distribution (%) Total EET</th>
<th>Distribution (%) Total EpOME</th>
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<tbody>
<tr>
<td>CYP2J2</td>
<td>71 18 6 5</td>
<td>58 42</td>
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<tr>
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Data for human CYP2J2 are shown for comparison.
been thought to be a mouse Cyp2j gene (Nelson et al., 2004), is a pseudogene.

The mouse Cyp2j genes have been mapped to chromosome 4 and are located in a 0.62 Mb region, in close proximity to the mouse Cyp4a gene cluster (Nelson et al., 1996; Ma et al., 1998). Members of the Cyp4a subfamily have been shown to be active in the metabolism of AA to 20-HETE, and may be involved in regulation of renal vascular tone and ion transport (Capdevila, 2007; Fidelis et al., 2010). This is similar to the human Cyp2j2 gene, which is in close proximity to the Cyp4a gene cluster on chromosome 1 (Nelson et al., 1996; Ma et al., 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 Cyp2j enzymes 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 Cyp2j enzymes that also show a preference for 14,15-EET production. In contrast, many Cyp2c epoxygenases 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 Cyp2j enzymes, 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 regioselective 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 isoforms (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 (Zelink et al., 1995; Bylund et al., 1998), the function of DiHOMEs (dihydroxyoctadecanonoenoic acids) in the kidney, brain, and heart is less clear. Increased DiHOMEs are associated with reduced recovery from ischemic/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-CYP2j8pep1 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

### TABLE 7
Regiochemical distribution of hydroxyeicosatetraenoic acid (HETE) products formed during incubation of recombinant CYP2j enzymes with arachidonic acid.

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### Fig. 5
Tissue distribution of the new CYP2j transcripts by RT-PCR. Total RNA extracted from multiple mouse tissues was analyzed by a one-step RT-PCR with sequence-specific primer pairs (Table 3). All tissues, unless specified or female-specific, were from males. Primer specificity was determined using the mouse CYP2j cDNAs as templates. A mouse β-actin primer pair was used as a control for RNA quantity. The results are representative of three independent experiments.
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...
Like human CYP2J2, these four new CYP2J enzymes might be murine CYP2Js show similar tissue expression patterns to CYP2J2. Although none of the individual mouse (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 results in improved postischemic recovery of contractile function (Seubert et al., 2004). We found CYP2J11 to be present in cardiomyocytes. Like CYP2J2, CYP2J11 metabolizes AA with a preference for 14,15-EET. This suggests that CYP2J11 to be present in cardiomyocytes. Like CYP2J2, CYP2J11 derived AA metabolites may play a role in cardiac function after ischemia. In summary, all the mouse Cyp2j1 genes are located in a −0.62 Mb cluster on chromosome 4. We report that CYP2J 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 CYP2J11-derived AA metabolites may play a role in cardiac function after ischemia.

References


Supplemental Figure 1. Schematic representation of isolated CYP2J7 cDNA variants. CYP2J7_ps1 was isolated from the kidney and contains a 146 bp insert between exons 1 and 2. CYP2J7_ps2 was isolated from the testes and contains a 62 bp insert between exons 1 and 2. CYP2J7_ps3 was isolated from the testes and contains a 16 bp insert between exons 3 and 4.