Quantitative Polymerase Chain Reaction Analysis of the Mouse *Cyp2j* Subfamily: Tissue Distribution and Regulation[§]

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ABSTRACT

Members of the cytochrome P450 CYP2J subfamily are expressed in multiple tissues in mice and humans. These enzymes are active in the metabolism of fatty acids to generate bioactive compounds. Herein we report new methods and results for quantitative polymerase chain reaction (qPCR) analysis for the seven genes (*Cyp2j5*, *Cyp2j6*, *Cyp2j8*, *Cyp2j9*, *Cyp2j11*, *Cyp2j12*, and *Cyp2j13*) of the mouse *Cyp2j* subfamily. SYBR Green primer sets were developed and compared with commercially available TaqMan primer/probe assays for specificity toward mouse *Cyp2j* cDNA, and analysis of tissue distribution and regulation of *Cyp2j* genes. Each TaqMan primer/probe set and SYBR Green primer set were shown to be specific for their intended mouse *Cyp2j* cDNA. Tissue distribution of the mouse *Cyp2j* isoforms confirmed similar patterns of expression between the two qPCR methods. *Cyp2j5* and *Cyp2j13*

were highly expressed in male kidneys, and *Cyp2j11* was highly expressed in both male and female kidneys. *Cyp2j6* was expressed in multiple tissues, with the highest expression in the small intestine and duodenum. *Cyp2j8* was detected in various tissues, with highest expression found in the skin. *Cyp2j9* was highly expressed in the brain, liver, and lung. *Cyp2j12* was predominately expressed in the brain. We also determined the *Cyp2j* isoform expression in *Cyp2j5* knockout mice to determine whether there was compensatory regulation of other *Cyp2j* isoforms, and we assessed *Cyp2j* isoform regulation during various inflammatory models, including influenza A, bacterial lipopolysaccharide, house dust mite allergen, and corn pollen. Both qPCR methods detected similar suppression of *Cyp2j6* and *Cyp2j9* during inflammation in the lung.

Introduction

The cytochrome P450 (P450) gene superfamily includes enzymes that catalyze the metabolism of a wide range of xenobiotics and endogenous compounds (Nelson et al., 1996; Nebert and Russell, 2002; Kroetz and Zeldin, 2002). Arachidonic acid (AA) is a polyunsaturated fatty acid present in mammalian cell membranes that can be epoxidized to bioactive epoxyeicosatrienoic acids (EETs) or hydroxylated to hydroxyeicosatrienoic acids by various P450 enzymes (Capdevila et al., 2000; Nebert and Russell, 2002; Kroetz and Zeldin, 2002). EETs exhibit potent vasodilatory (Campbell et al., 1996; Larsen et al., 2006), anti-inflammatory (Node et al., 1999), cardioprotective (Seubert et al., 2004, 2006), fibrinolytic (Node et al., 2001), and angiogenic (Wang et al., 2005) effects both in vitro and in vivo.

Mouse *Cyp2j* subfamily members are active in the metabolism of AA and other substrates (Ma et al., 1999; Qu et al., 2001; Ma et al., 2002; Graves et al., 2013). The majority of P450 enzymes are abundantly expressed in the liver, but the mouse *Cyp2j* subfamily members are predominantly found in extrahepatic tissues. Previous

studies indicated that *Cyp2j5*, *Cyp2j8*, *Cyp2j11*, and *Cyp2j13* are predominantly expressed in the kidney (Ma et al., 1999; Graves et al., 2013), *Cyp2j6* transcripts are primarily detected in the intestine (Scarborough et al., 1999), and *Cyp2j8*, *Cyp2j9*, and *Cyp2j12* transcripts are highly expressed in the brain (Qu et al., 2001; Graves et al., 2013). These previous studies have all used nonquantitative reverse-transcription polymerase chain reaction (RT-PCR) to detect the presence or absence of mouse *Cyp2j* transcripts in tissue.

Quantitative polymerase chain reaction (qPCR) is a powerful and accurate tool to measure gene expression. While RT-PCR allows for the detection of a transcript, the ability of qPCR to monitor the amplification of target sequences in real time allows for the quantification of transcript levels. All qPCR assays rely on cDNA-specific primer pairs to amplify a unique target sequence from transcripts of interest. The real-time detection of the PCR reaction is typically quantified in one of two ways. One method relies on a double-stranded DNA-binding dye (e.g., SYBR Green) to serve as a reporter (Ponchel et al., 2003). The other common method of quantifying the PCR reaction in real time is the use of a fluorescently labeled transcript-specific oligo/probe (e.g., TaqMan and Quantifast assays). These primer/probe assays rely on the endonuclease activity of Taq polymerase to cleave the fluorophore from the probe, which has a covalently attached fluorescent quencher (Heid et al., 1996).

Both methods of detection have advantages and disadvantages. One drawback of both methods becomes evident in the quantification of evolutionarily related genes such as the *Cyp2j* subfamily members.

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ABBREVIATIONS: AA, arachidonic acid; EET, epoxyeicosatrienoic acid; HDM, house dust mite; KO, knockout; LPS, lipopolysaccharide; P450, cytochrome P450; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; WT, wild type.

SYBR Green–based assays report the abundance of double-stranded DNA, which is potentially confounded by homology between related transcripts and thus resulting in multiple off-target amplicons being produced. Conducting an end-point melting curve assay after a SYBR Green assay is critical to confirm that the fluorescence measured is due to a unique target sequence amplicon rather than multiple products being produced from unintended transcripts.

Primer/probe assays have increased sensitivity due to the target-specific fluorescent probe, but they lack the end-point confirmation that the measured fluorescence is due to amplification of the desired amplicon. This is especially problematic when quantitating highly conserved paralogs such as the *Cyp2j* subfamily members. Accurate qPCR of the mouse *Cyp2j* subfamily members will allow for assessment of their tissue distribution and aid in elucidating their biologic function and regulation. Developing specific primer sets for use with the SYBR Green reporter can be challenging due to nucleotide coding sequence homology (75%–88% identity) between the subfamily members (Graves et al., 2013).

This study developed primer sets for qPCR use with the SYBR Green dye based on the published and cloned cDNA sequence of the mouse *Cyp2j* subfamily members. These were compared with commercially available TaqMan primer/probe sets for qPCR. Each of the SYBR Green primer sets and the TaqMan primer/probes were found to be specific for their intended transcript. Similar tissue distributions were observed for both qPCR methods for all seven *Cyp2j* isoforms. In addition, both qPCR methods were used to quantify the expression of the *Cyp2j* subfamily members in the kidney of *Cyp2j5* knockout (KO) mice to determine whether compensatory changes in *Cyp2j* expression exist. Lastly, the levels of *Cyp2js* were determined in several inflammatory models, including influenza A, lipopolysaccharide (LPS), house dust mite (HDM), and corn pollen.

Materials and Methods

Reagents and Supplies. SYBR Green primer set oligonucleotides were synthesized by BioServe BioTechnologies (Laurel, MD) and Integrated DNA Technologies (Coralville, IA). TaqMan primer/probe assays, MicroAmp Optical 384-Well Reaction Plates, and an ABI Prism 7700 Sequence Detection System were from Life Technologies (Carlsbad, CA).

Animal Treatment. All animal experiments were performed according to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

For tissue distribution assessment, total RNA was prepared from tissues collected from three female and three male wild-type (WT) C57BL/6 mice using RNeasy Midi or Mini Kits from Qiagen (Valencia, CA) following the manufacturer's instructions. For the experiments using *Cyp2j5* KO mice, RNA was prepared from four female and four male *Cyp2j5* KO and WT kidneys.

For the influenza exposure study, WT male and female C57BL/6 mice were infected with 200 plaque-forming units of influenza A/Hong Kong/8/68 (H3N2) in a total volume of 50 μ l by intranasal instillation. Lungs were collected 4 days after infection, and the total RNA was isolated.

For the LPS studies, WT male C57BL/6 mice were given a single dose of 50 μ g of LPS intranasally. After 4 hours, the mice were euthanized, the lungs were collected, and the RNA was isolated.

For the HDM experiments, male C57BL/6 mice were sensitized with 10 μ g of HDM allergen in 50 μ l of saline via oropharyngeal aspiration on days 1 and 8, followed by challenge on days 15, 16, and 17 with 2 μ g of HDM in 50 μ l of saline via oropharyngeal aspiration. Lungs were collected on day 18, and the total RNA was isolated.

For acute corn pollen exposure experiments, female BALB/C mice were given a single dose of 4 mg of corn pollen in 75 μ l of phosphate-buffered saline by aspiration. At 24 hours after dosing, the mice were euthanized, the lungs

were collected, and the RNA was isolated. For chronic corn pollen exposure experiments, female BALB/C mice were given 1 mg of corn pollen in 75 μ l of phosphate-buffered saline by aspiration for 7 consecutive days. The mice were euthanized 24 hours after the last dose of corn pollen, the lungs were collected, and the total RNA was isolated. Saline controls were included in both acute and chronic corn pollen exposure experiments.

cDNA Synthesis. For each experimental animal, 1 μ g of total RNA was treated with 1 unit of DNase I (Life Technologies, Grand Island, NY) for 15 minutes. DNase I was inactivated by addition of 1 μ l of 25 mM EDTA and heat treatment at 65°C for 10 minutes. RNase-free water was added to bring each sample to a total volume of 50 μ l. The High Capacity cDNA Reverse Transcription Kit from Life Technologies was used according to the manufacturer's protocol to produce cDNA from the DNase I-treated RNA. The final concentration of the cDNA was 10 ng/ μ l. The cDNA was diluted to a working concentration of 1 ng/ μ l with RNase-free water.

TaqMan qPCR. TaqMan primer/probes for mouse *Cyp2j* subfamily members were purchased from Life Technologies and are listed in Table 1. For all qPCR reactions, 0.5 μ l of the 20X primer/probes were used in a 10 μ l total reaction volume. Maxima Probe/ROX qPCR Master Mix (2X) from Thermo Scientific (Pittsburgh, PA) was used in the qPCR master mix. The PCR conditions used were as follows: 50°C for 2 minutes; 95°C for 10 minutes, followed by 40 repeat cycles of 95°C for 15 seconds, and 60°C for 1 minute. Samples were run in triplicate.

SYBR Green qPCR. Specific primer sets of oligonucleotides were designed, spanning exon/exon boundaries if possible, for the mouse *Cyp2j* subfamily members (Table 2). Some primer sets were those that had previously been published for use in nonquantitative RT-PCR (Ma et al., 1999; Graves et al., 2013). All primers were diluted to a concentration of 10 μ M, and 0.5 μ l was used in a total volume of 10 μ l for the qPCR reactions. Power SYBR Green PCR Master Mix from Applied Biosystems by Life Technologies was used for all the SYBR Green qPCR reactions. The PCR conditions used were as follows: 50°C for 2 minutes; 95°C for 10 minutes, followed by 40 repeat cycles of 95°C for 15 seconds, then 60°C for 1 minute. A dissociation stage of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds was added. For the *Cyp2j6*, *Cyp2j9*, *Cyp2j12*, and *Cyp2j13* primer sets an annealing/extension temperature of 64°C was used. All samples were run in triplicate.

qPCR Analysis. Data generated from the qPCR reactions were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). All graphs were generated using the GraphPad Prism 6.0C program (GraphPad Software, San Diego, CA). Most mouse tissue expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The cloned cDNAs for SYBR Green were normalized to plasmid control β -lactamase levels. There were significant increases in the relative expression of *Gapdh*, β_2 -microglobulin (*B2m*), and β -actin in the influenza A-treated mice compared with saline controls; therefore, *18s* rRNA was used as the housekeeping gene for this experiment (Supplemental Fig. 1).

For the HDM and corn pollen studies, the relative expression of *Gapdh*, *B2m*, *actin*, and *18s* housekeeping genes were significantly decreased in the treated versus saline control (Supplemental Figs. 2 and 3). For these two studies all the C_T values for the experiment were averaged and used to calculate individual sample ΔC_T . The average ΔC_T values of the control group were used

TABLE 1
TaqMan primer/probes for the mouse *Cyp2j* subfamily

Subfamily Member	TaqMan Primer/Probe
<i>Cyp2j5</i>	Mm00487292_m1
<i>Cyp2j6</i>	Mm01268197_m1
<i>Cyp2j8</i>	Mm03412388_m1
<i>Cyp2j9</i>	Mm00466426_m1
<i>Cyp2j11</i>	Mm01268190_m1
<i>Cyp2j12</i>	Mm01262103_m1
<i>Cyp2j13</i>	Mm00523816_m1
<i>Gapdh</i>	Mm99999915_g1
<i>18s</i>	Mm03928990_g1

TABLE 2
SYBR Green primer set sequences for the mouse *Cyp2j* subfamily

Transcript	Forward (5'-3')	Reverse (5'-3')	Reference
<i>Cyp2j5</i>	ATCAGAGAAGCGAAAAGAATGTAG	CCATTTCCTCTGATTCTGACTCAT	Ma et al., 1999
<i>Cyp2j6</i>	GGACCTCTTCTTTGCTGGAACAG	GCAAGTCTTGCTGCCCTCTTCT	
<i>Cyp2j8</i>	TTGGGGATCCAGCCGTTTGT	TGAGCCTCATCCTGTATGCGT	
<i>Cyp2j9</i>	AACCGTTTCCCCAGTCAGTC	ATTGCACGCACTCTCTGTCA	
<i>Cyp2j11</i>	ATTGGAGTATGCCCTGAAGAT	GTGATGGCCCATTAATTGAGG	
<i>Cyp2j12</i>	AAGGAGGCTGACTGTCTTGTGG	GACTGTCCTCATACTCAAAGCGC	Graves et al., 2013
<i>Cyp2j13</i>	GTCAGTGAGCCACTGCCCCATA	GTCTCATCTTGGGCACGAACC	
<i>Gapdh</i>	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT	
<i>18s</i>	TCGTATTGCGCCGCTAGAGGT	GGGTCATGGGAATAACGCCGC	
<i>β-lactamases</i>	CGCAGAAGTGGTCTCTGCAAC	TCTGCTATGTGGCGCGGTAT	

to calculate the $\Delta\Delta C_T$ values for individual samples. Samples below the limit of detection ($C_T > 38$) were set to $C_T = 38$. The reference/control group was set to 1 for all five experimental studies.

For the HDM and corn pollen studies, transcripts of several *Cyp2j* isoforms were below the limit of detection for all samples ($C_T > 38$). In the figures, these data points are shown without error bars. Outliers were detected and removed using the Grubbs Outlier test (Grubbs, 1950). Significance was calculated by the Student's *t* test. Multiple test comparisons are not reported because the primary focus of this study was the qPCR analysis of the seven *Cyp2j* subfamily members.

Results

***Cyp2j* TaqMan Primer/Probes and SYBR Green Primer Sets Specificity.** The mouse *Cyp2j* subfamily cDNAs were previously cloned in our laboratory (Ma et al., 1999; Qu et al., 2001; Ma et al., 2002; Graves et al., 2013). Each cDNA was cloned into the pcDNA3.1 vector (Life Technologies) or pBS-SK+ vector (Agilent Technologies, Santa Clara, CA). All seven of the mouse *Cyp2j* TaqMan primer/probe assays and the seven SYBR Green primer sets were specific for their intended mouse *Cyp2j* cDNA (Figs. 1–7; A and B). In addition, the

Fig. 1. Specificity and tissue distribution of mouse *Cyp2j5* using TaqMan primer/probe and SYBR Green primer sets. The *Cyp2j5* TaqMan primer/probe (A) and the SYBR Green *Cyp2j5* primer set (B) are specific for the *Cyp2j5* cDNA. Both the *Cyp2j5* TaqMan primer/probe (C) and SYBR Green primer set (D) show the highest expression of *Cyp2j5* in male kidneys with lower expression in female kidneys and livers. Data shown are mean $\pm$ S.E., $n = 6$ per group except for sex-specific tissues where $n = 3$ per group; * $P < 0.05$, male versus female kidney.

Cyp2j TaqMan primer/probes and SYBR Green primer sets failed to amplify any of the 10 mouse *Cyp2c* cDNAs tested (data not shown). *Cyp2c* isoforms are a closely related gene subfamily with approximately 50% identity to the *Cyp2j* subfamily at the nucleotide level.

Tissue Distribution of Mouse *Cyp2j* mRNAs by qPCR. Using both the TaqMan primer/probe and SYBR Green primer sets, *Cyp2j5* was most abundant in male and female kidneys and in the liver (Fig. 1). The expression level of *Cyp2j5* in the male kidney was significantly higher than in the female kidney and liver, measured using either qPCR method. This finding is in agreement with previously reported, nonquantitative RT-PCR results (Ma et al., 1999).

For *Cyp2j6*, the highest expression levels using both the TaqMan primer/probe and SYBR Green primer set were in the small intestine, in particular in the duodenum (Fig. 2). *Cyp2j6* was also detected at low but detectable levels in the large intestine, stomach, liver, lung, ovary, uterus, skin, kidney, and visceral fat. This broad tissue distribution of *Cyp2j6* was also observed previously by nonquantitative RT-PCR methods (Scarborough et al., 1999).

Examination of qPCR results from the *Cyp2j8*-specific TaqMan primer/probe and SYBR Green primer set revealed that *Cyp2j8* expression was highest in skin and found at lower levels in a broad range of tissues including the liver, brain, and duodenum (Fig. 3). In

addition, we observed abundant expression in the kidney only with the SYBR Green *Cyp2j8* primer set. These findings are consistent with published results using nonquantitative RT-PCR methods (Graves et al., 2013), although skin was not included in the prior panel.

Cyp2j9 transcripts were detected in the brain, liver, and lung using either the TaqMan or SYBR Green qPCR methods (Fig. 4). Previous nonquantitative RT-PCR methods showed a similar pattern of expression in the brain and liver but not the lung (Qu et al., 2001). When using SYBR Green primers, high expression levels of *Cyp2j9* were detected in the visceral fat, ovary, skin, and small intestine that were not detected by the TaqMan primer/probe. To address the apparent discrepancy between the two assays, we compared the SYBR Green dissociation curve for brain, liver, and lung with that of fat, ovary, and small intestine (Supplemental Fig. 4). The single observed peak with a nearly identical melting temperature is highly suggestive that the measured fluorescence in all six tissues is due to an amplicon derived from the *Cyp2j9* transcript and not off-target amplification of unintended transcripts.

A prominent *Cyp2j11* transcript was detected in kidney samples using both TaqMan and SYBR Green qPCR methods (Fig. 5). Unlike *Cyp2j5* and *Cyp2j13*, which have male-selective kidney expression, both male and female kidneys abundantly expressed *Cyp2j11*

Fig. 2. Specificity and tissue distribution of mouse *Cyp2j6* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j6* TaqMan primer/probe (A) and SYBR Green primer set (B) are specific for the *Cyp2j6* cDNA. Similar tissue distribution profiles were observed using both the TaqMan primer/probe (C) and SYBR Green primer set (D), with the highest expression in the small intestine, duodenum, stomach, and large intestine. Data shown are mean $\pm$ S.E., $n = 6$ per group.

Fig. 3. Specificity and tissue distribution of mouse *Cyp2j8* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j8* TaqMan primer/probe (A) and SYBR Green primer set (B) are specific for *Cyp2j8* cDNA. Expression was highest in the skin using both the TaqMan primer/probe (C) and the SYBR Green primer set (D). Data shown are mean $\pm$ S.E., $n = 6$ per group.

transcripts. Previously published nonquantitative RT-PCR analysis detected abundant *Cyp2j11* expression in the kidney and heart (Graves et al., 2013); however, robust expression was not observed in the heart with either qPCR method.

Prominent *Cyp2j12* expression was detected in the brain with both the TaqMan primer/probe and SYBR Green primer sets (Fig. 6). Previously published nonquantitative RT-PCR methods also showed high transcript levels in the brain (Graves et al., 2013).

Cyp2j13 transcript expression is detected almost exclusively in male kidney by both the TaqMan primer/probe and SYBR Green primer sets (Fig. 7).

Expression of Mouse *Cyp2j* Isoforms in Kidneys of *Cyp2j5* KO Mice. Both TaqMan and SYBR Green qPCR were used to determine expression levels for the *Cyp2j* isoforms in *Cyp2j5* KO and WT kidneys (Fig. 8). Consistent with previous data (Ma et al., 1999), *Cyp2j5* transcript levels were below the level of detection ($C_T > 38$) in *CYP2J5* KO mice. Both qPCR methods showed that the expression levels of *Cyp2j6*, *Cyp2j8*, and *Cyp2j9* in the kidney were significantly increased in *Cyp2j5* KO mice relative to WT controls. In contrast, there were no compensatory changes in *Cyp2j11* or *Cyp2j13* expression in *Cyp2j5* KO mice.

Expression of the *Cyp2j* isoforms was also examined in *Cyp2j5* KO and WT livers (Supplemental Fig. 5). As in the kidney, *Cyp2j5* disruption leads to compensatory up-regulation of *Cyp2j8* and *Cyp2j9*.

Cyp2j6 expression was slightly reduced in livers from *Cyp2j5* KO mice relative to WT. This is in contrast to a modest induction of *Cyp2j6* in kidneys from *Cyp2j5* KO mice.

Expression of Mouse *Cyp2j* Isoforms in Lungs of Influenza A-Treated Mice. Treatment of mice with 200 plaque-forming units of influenza A is typically lethal beyond 4 days; therefore, mice were euthanized on day 4 to assess *Cyp2j* isoform expression. Relative expression of *Cyp2j* isoforms in mice treated with influenza A was calculated separately for female and male mice due to known sex differences in response to infection (Carey et al., 2007; Larcombe et al., 2011).

Compared with saline-treated controls, influenza A-treated lungs showed a significant decrease in expression of *Cyp2j9* (Fig. 9). This result was consistent using both qPCR methods and was similar in both males and females. In female mice there was also a trend toward reduction in levels of *Cyp2j5* ($P = 0.05$), *Cyp2j6* ($P = 0.06$), *Cyp2j8* ($P = 0.07$), and *Cyp2j11* ($P = 0.07$) isoforms with the TaqMan primer/probes. In contrast, with the SYBR Green primers only *Cyp2j6* tended to be reduced in influenza A-treated females relative to saline-treated controls ($P = 0.05$).

Expression of Mouse *Cyp2j* Isoforms in Lungs of LPS-Treated Mice. *Cyp2j* isoform expression was measured in mouse lungs 4 hours after LPS or saline treatment. Compared with saline-treated controls, LPS treated mice showed significantly reduced lung expression of

Fig. 4. Specificity and tissue distribution of mouse *Cyp2j9* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j9* TaqMan primer/probe (A) and SYBR Green primer set (B) are specific for the *Cyp2j9* cDNA. The highest expression was in the brain, liver, and lung using both the TaqMan primer/probe (C) and the SYBR Green primer set (D). Abundant expression was also observed in the ovary, skin, small intestine, and visceral fat for the *Cyp2j9* SYBR Green primer set. Data shown are mean $\pm$ S.E., $n = 6$ per group.

Cyp2j6, *Cyp2j9*, and *Cyp2j11* (Fig. 10). There were no differences in expression of the other *Cyp2j* isoforms. These findings were consistent in both TaqMan and SYBR Green assays.

Expression of Mouse *Cyp2j* Isoforms in Lungs of HDM-Treated Mice. To assess *Cyp2j* isoform expression in a model of allergic asthma, mice were sensitized and exposed to HDM in an 18-day protocol as described in *Materials and Methods*. Both the TaqMan primer/probes and SYBR Green primer sets detected significantly reduced expression of *Cyp2j6* and *Cyp2j9* in the lungs of HDM-treated mice relative to saline-treated controls (Fig. 11). In addition, when using the SYBR Green primer set, there was a small reduction in *Cyp2j11* expression in the HDM-treated lungs relative to saline-treated controls. None of the other isoforms exhibited differential expression patterns with either qPCR method.

Expression of Mouse *Cyp2j* Isoforms in Lungs after Acute and Chronic Exposure to Corn Pollen. Expression of *Cyp2j* isoforms was also assessed 24 hours after a single dose or 24 hours after the final consecutive daily dose of corn pollen. After acute exposure to corn pollen, *Cyp2j6* and *Cyp2j9* isoforms were significantly reduced compared with saline-treated controls (Fig. 12, A and B). Chronic corn pollen exposure also strongly reduced *Cyp2j6* and *Cyp2j9* transcript levels (Fig. 12, C and D). There was a smaller but statistically

significant reduction in *Cyp2j8* expression detected with the TaqMan primer/probe set (Fig. 12C). Because the corn pollen studies were conducted using BALB/C rather than C57BL/6 mice, we verified that the tissue expression profiles for the mouse *Cyp2j* isoforms were similar in both C57BL/6 and BALB/C mice. Examples of the BALB/C tissue profile expression for *Cyp2j6* and *Cyp2j9* are shown in Supplemental Fig. 6.

Discussion

Cytochromes P450 is a large gene superfamily of ubiquitous hemo-proteins that have physiologically relevant hepatic and extrahepatic functions (Wu et al., 1996; Zou et al., 1996; Campbell and Harder, 1999; Node et al., 2001). Members of the CYP2J subfamily metabolize AA and linoleic acid into bioactive lipids (Ma et al., 1999; Qu et al., 2001; Ma et al., 2002; Graves et al., 2013). Identifying tissue expression patterns of the mouse *Cyp2j* isoforms and quantifying their expression levels after various treatments is important for determining the physiologic role of these enzymes. The tissue distribution of the seven members of the mouse *Cyp2j* subfamily had previously been determined by RT-PCR and Western blot analysis (Ma et al., 1999; Qu et al., 2001; Ma et al., 2002; Graves et al., 2013). In a recent study, reverse-transcription, multiplex, ligation-dependent probe amplification was used

Fig. 5. Specificity and tissue distribution of mouse *Cyp2j11* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j11* TaqMan primer/probe (A) and SYBR Green primer set (B) are specific for the *Cyp2j11* cDNA. Expression was highest in the kidney using both the TaqMan primer/probe (C) and the SYBR Green primer set (D). Data shown are mean $\pm$ S.E., $n = 6$ per group.

to measure mouse *Cyp2j* gene expression (Zhou et al., 2013); however, qPCR is a more widely available quantitative assay and provides an accurate expression profile across tissues.

In this report, the TaqMan and SYBR Green qPCR showed excellent specificity toward each of the mouse *Cyp2j* subfamily members. Our results provide a catalog of *Cyp2j* tissue expression that is in general agreement with previously published data. Moreover, we used these two qPCR methods to document compensatory *Cyp2j* transcript expression in *Cyp2j5* KO mouse kidneys. We also quantified expression of *Cyp2j* genes in experimental models of lung inflammation induced by influenza A, LPS, HDM allergen, and corn pollen. The efficacy of the two qPCR methods to determine differences in expression of the various *Cyp2j* isoforms was comparable.

The *Cyp2j* TaqMan primer/probes and SYBR Green primer sets are highly specific to their respective *Cyp2j* isoform. The tissue expression profiles for all seven of the *Cyp2j* subfamily members were similar using both TaqMan and SYBR Green qPCR methods. Indeed, each *Cyp2j* tissue expression profile was consistent with previously reported nonquantitative RT-PCR results (Ma et al., 1999; Qu et al., 2001; Ma et al., 2002; Graves et al., 2013) with a few notable exceptions. For example, nonquantitative RT-PCR had detected *Cyp2j8* in the mouse kidney and heart, whereas both qPCR

methods confirmed abundant expression in the kidney but not the heart. In addition, we previously reported that *Cyp2j8* was abundantly expressed in the brain (Graves et al., 2013); however, qPCR revealed the highest expression in the skin, a tissue that was not included in the previous analysis.

CYP2J metabolites, the EETs, act as cerebral vasodilators and are neuroprotective (Zhang et al., 2008). The *Cyp2j9* and *Cyp2j12* transcripts are present in the brain by both qPCR methods. These results are in agreement with our prior nonquantitative RT-PCR results (Qu et al., 2001; Graves et al., 2013). *Cyp2j8* was previously reported by nonquantitative RT-PCR to be highly expressed in the brain (Graves et al., 2013); however, only low levels of *Cyp2j8* was detected in the brain using either of the qPCR methods in our current study. Notably, the SYBR Green *Cyp2j8* primer set used in this study is different than the ones used in the previous nonquantitative RT-PCR. Confirmation of the low expression levels of *Cyp2j8* in the brain by the TaqMan primer/probe set suggests that *Cyp2j8* is not highly expressed in the brain.

Cytochrome P450 enzymes located in the gastrointestinal tract may play a role in the biotransformation of dietary nutrients as well as xenochemicals (Kaminsky and Fasco, 1991). In the current study, *Cyp2j6* was shown by both TaqMan and SYBR Green qPCR methods

Fig. 6. Specificity and tissue distribution of mouse *Cyp2j12* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j12* TaqMan primer/probe (A) and SYBR Green primer set (B) are specific for the *Cyp2j12* cDNA. The highest expression was in the brain using both the TaqMan primer/probe (C) and the SYBR Green primer set (D). Data shown are mean $\pm$ S.E., $n = 6$ per group.

to have the highest expression in the small intestine, duodenum, stomach, and large intestine. This is in agreement with the published data on the expression of *Cyp2j6* in the small intestine as measured by nonquantitative RT-PCR (Scarborough et al., 1999; Ma et al., 2002). It is noteworthy that in previous studies the stomach and large intestine were not included in the tissue panel used for nonquantitative RT-PCR (Graves et al., 2013).

CYP2J metabolites, the EETs, play an important role in the regulation of blood pressure in both rodents and humans (Wu et al., 1996; Holla et al., 1999; Ma et al., 1999; Oyekan et al., 1999). Indeed, *Cyp2j5* KO mice develop spontaneous hypertension (Athirakul et al., 2008). Moreover, *Cyp2j* expression in the kidney is localized to the site of the nephron where EETs regulate the hormones that control blood pressure (Ma et al., 1999). Results from both qPCR methods indicated that all of the mouse *Cyp2j* subfamily members except *Cyp2j12* were expressed in the kidney. In fact, *Cyp2j5*, *Cyp2j11*, and *Cyp2j13* are most abundantly expressed in the kidney. *Cyp2j11* has comparable expression in both male and female kidneys, *Cyp2j5* has higher expression in male compared with female kidneys, and *Cyp2j13* is almost exclusively expressed in male kidneys.

Cyp2j6, *Cyp2j8*, and *Cyp2j9* also have detectable expression in the kidney by both qPCR methods. Regulation of kidney *Cyp2js* has not

been extensively studied. In this regard, we found that there was compensatory upregulation of *Cyp2j6*, *Cyp2j8*, and *Cyp2j9* in the kidneys of *Cyp2j5* KO mice relative to WT controls. Interestingly, *Cyp2j5* KO mice have increased blood pressure despite normal renal EET biosynthesis. Therefore, it is possible that either *Cyp2j5* does not significantly contribute to renal EET biosynthesis or alternatively that up-regulation of *Cyp2j6*, *Cyp2j8*, and *Cyp2j9* compensates for the loss of *Cyp2j5* and normalizes renal EET levels. Notably, there is no difference in expression of *Cyp2j11* in *Cyp2j5* KO versus WT kidneys using either qPCR method, despite the fact that both isoforms are abundantly expressed in renal proximal tubules (Ma et al., 1999; Graves et al., 2013).

Further studies will be needed to determine whether there is redundancy of function between the mouse renal *Cyp2j* isoforms, and whether this contributes to the hypertensive phenotype in *Cyp2j5* KO mice. In liver tissues, *Cyp2j5* disruption also induced compensatory changes in *Cyp2j8* and *Cyp2j9* expression; however, although *Cyp2j5* disruption induced *Cyp2j6* expression in the kidney, it suppressed *Cyp2j6* expression in the liver. Thus, compensatory changes in *Cyp2j6* expression in *Cyp2j5* KO mice appear to occur in a tissue-specific fashion.

EETs have been shown to possess significant anti-inflammatory properties (Campbell et al., 1996; Node et al., 1999; Larsen et al.,

Fig. 7. Specificity and tissue distribution of mouse *Cyp2j13* using TaqMan primer/probe and SYBR Green primer sets. Both the *Cyp2j13* TaqMan primer/probe (A) and the SYBR Green primer set (B) are specific for the *Cyp2j13* cDNA. Expression was present only in male kidney using both the TaqMan primer/probe (C) and the SYBR Green primer set (D). Data shown are mean $\pm$ S.E., $n = 6$ per group except for sex-specific tissues where $n = 3$ per group. * $P < 0.05$, male versus female kidney.

2006). Inflammation is known to affect the expression and metabolic capacity of hepatic and extrahepatic P450 enzymes (Morgan, 2001; Theken et al., 2011). P450 expression is usually suppressed in inflammatory states, but it may also be induced or unaffected (Morgan, 1997). In the current study, we examined expression of the *Cyp2j* isoforms in four lung inflammatory models. Influenza A infection induces inflammation in the lungs of mice (Virelizier et al., 1979; Dawson et al., 2000). LPS is part of the outer membrane of Gram-negative bacteria and induces airway inflammation and hyper-responsiveness (Zeldin et al., 2001). HDM allergen is a well-studied inducer of allergic airway inflammation (Gandhi et al., 2013). In addition, both acute and chronic corn pollen exposure induces allergic airway inflammation (Card, J.W., et al., unpublished data).

The two qPCR methods revealed similar patterns of suppressed *Cyp2j* expression in the lung during inflammation in these inflammatory models. As EETs are anti-inflammatory, the reduction of CYP2J epoxygenases during inflammation may serve to amplify inflammatory signals so that organisms may mount an efficient defense against pathogens. Influenza A-infected mice showed a modest reduction of most *Cyp2j* isoforms; only the reduction in *Cyp2j9* was statistically significant.

Sex differences in immune responses are well established (Carey et al., 2007). The reduction of *Cyp2j9* expression was similar in males

and females; however, we observed a trend for reduction of several other *Cyp2j* isoforms in female mice ($P = 0.05$ – 0.1). Interestingly, no significant change in any of the mouse *Cyp2j* isoforms was observed using a human respiratory syncytial virus (RSV) model (Supplemental Fig. 7). This suggests that changes in the expression of the mouse *Cyp2j* isoforms may be virus specific.

Acute LPS treatment suppressed pulmonary expression of *Cyp2j6*, *Cyp2j8*, and *Cyp2j9*. This is very similar to previous reports that found no change in *Cyp2j5* but found reduced *Cyp2j9* expression after LPS exposure (Theken et al., 2011). We found that *Cyp2j5*, *Cyp2j11*, *Cyp2j12*, and *Cyp2j13* are expressed at very low levels in the lung, so a lack of inflammatory suppression of these genes is not surprising. While the bacterial LPS model suppressed *Cyp2j6*, *Cyp2j8*, and *Cyp2j9* expression, the influenza A virus only significantly reduced expression of *Cyp2j9*.

Different models of infection are known to affect different P450 enzymes in hepatic and extrahepatic tissues (Morgan, 1997). Further studies will be needed to determine the specific transcription factors responsible for suppression of the individual mouse *Cyp2j* isoforms in each of these models. The induction of allergy to HDM and the acute and chronic exposure of corn pollen yielded similar responses; both allergens induced a strong suppression of *Cyp2j6* and *Cyp2j9* expression in the lung. The other mouse *Cyp2js* are expressed at

Fig. 8. Detection of the mouse *Cyp2j* isoforms in *Cyp2j5* WT and KO kidney. Both the *Cyp2j5* TaqMan primer/probe (A) and the SYBR Green primer set (B) revealed significantly lower *Cyp2j5* expression in the KO kidney. In contrast, *Cyp2j6*, *Cyp2j8*, and *Cyp2j9* were increased in the *Cyp2j5* KO kidney relative to WT using both the TaqMan primer/probe (A) and the SYBR Green primer sets (B). Data shown are mean $\pm$ S.E., $n = 4$ per group; * $P < 0.05$ *Cyp2j5* KO versus WT.

lower levels in the lung and are not significantly altered in these models. To our knowledge, the effect of allergens on *Cyp2j* expression has not previously been reported. Whether suppression of the mouse *Cyp2js* is a factor in allergic lung response remains to be determined.

In summary, we compared the tissue expression profiles of the seven mouse *Cyp2j* isoforms using two different qPCR methods: one method using the TaqMan primer/probe assay and the other using SYBR Green primer sets that we developed in our laboratory. Overall, similar expression profiles were observed for all seven isoforms with the two qPCR methods. We found compensatory up-regulation of several mouse *Cyp2j* isoforms in the kidneys of *Cyp2j5* KO mice. In addition, the levels of several of the mouse *Cyp2j* isoforms were reduced in models of pulmonary inflammation. The SYBR Green qPCR primer sets we developed may be an accurate and less expensive alternative to TaqMan commercial primer/probe assays for the quantitative detection of expression of mouse *Cyp2j* subfamily members under normal and pathologic conditions.

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Authorship Contributions

Participated in research design: Graves, Gruzdev, Edin, Zeldin.
Conducted experiments: Graves.
Contributed new reagents or analytic tools: Bradbury, DeGraff, Li.
Performed data analysis: Graves, Gruzdev, Zeldin.
Wrote or contributed to the writing of the manuscript: Graves, Gruzdev, Hoopes, House, Edin, Zeldin.

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Fig. 9. Detection of the mouse *Cyp2j* isoforms in lungs of mice treated with influenza A virus. Similar expression profiles were observed in female mice with the use of both the *Cyp2j* TaqMan primer probes (A) and SYBR Green primer sets (B). For male mice the *Cyp2j* TaqMan primer/probe (C) and SYBR Green primer sets (D) also gave similar results. *Cyp2j9* was significantly reduced in the influenza A-treated mice for both female and male mice with both qPCR methods. Data shown are mean $\pm$ S.E., $n = 4$ per group; * $P < 0.05$, compared with saline control.

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Fig. 10. Detection of the mouse *Cyp2j* isoforms in lungs of LPS-treated mice. Similar profiles of expression were observed using the *Cyp2j* TaqMan primer/probes (A) and the SYBR Green primer sets (B). *Cyp2j6*, *Cyp2j9*, and *Cyp2j11* were decreased in the LPS-treated lung. Data shown are mean $\pm$ S.E., $n = 6$ per group; * $P < 0.05$, compared with saline control.

Fig. 11. Detection of the mouse *Cyp2j* isoforms in lungs from HDM-treated mice. Both the TaqMan primer/probes (A) and SYBR Green primer sets (B) showed decreased expression of *Cyp2j6* and *Cyp2j9* in the HDM-treated lungs. Data shown are mean $\pm$ S.E., $n = 6$ per group for HDM-treated lungs and $n = 5$ per group for saline-treated lungs; * $P < 0.05$, compared with saline controls.

Fig. 12. Detection of the mouse *Cyp2j* isoforms in lungs from corn pollen-treated mice. Using both qPCR methods, in acute (A and B) and chronic (C and D) exposure models, there was a significant decrease in expression of *Cyp2j6* and *Cyp2j9*. With chronic exposure to corn pollen, the TaqMan primer/probe (C), also showed decreased expression of *Cyp2j8*. Data shown are mean $\pm$ S.E., $n = 4$ per group; * $P < 0.05$, compared with saline controls.