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 to commercially available TaqMan primer/probe assays for specificity towards mouse Cyp2j cDNA, analysis of tissue distribution and regulation of Cyp2j genes. Each TaqMan® primer/probe and SYBR® Green primer sets 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 kidney, while Cyp2j11 was highly expressed in both male and female kidney. Cyp2j6 was expressed in multiple tissues with the highest expression in small intestine and duodenum. Cyp2j8 was detected in various tissues, with highest expression found in skin. Cyp2j9 was highly expressed in the brain, liver, and lung. Cyp2j12 was predominately expressed in brain. We also determined Cyp2j isoform expression in Cyp2j5 knockout (KO) mice to determine if there was compensatory regulation of other Cyp2j isoforms, and assessed Cyp2j isoform regulation during various inflammatory models including influenza A, bacterial lipopolysaccharide (LPS), house dust mite (HDM) 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 (Kroetz & Zeldin, 2002; Nebert & Russell, 2002; Nelson et al, 1996). Arachidonic acid (AA) is a polyunsaturated fatty acid present in mammalian cell membranes that can be epoxygenated to bioactive epoxyeicosatrienoic acids (EETs) or hydroxylated to hydroxyeicosatraenoic acids (HETEs) by various P450s (Capdevila et al, 2000; Kroetz & Zeldin, 2002; Nebert & Russell, 2002). EETs exhibit potent vasodilatory (Campbell et al, 1996; Larsen et al, 2006), anti-inflammatory (Node et al, 1999), cardioprotective (Seubert et al, 2004; Seubert et al, 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 (Graves et al, 2013; Ma et al, 2002; Ma et al, 1999; Qu et al, 2001). While the majority of P450s are abundantly expressed in liver, the mouse Cyp2j subfamily members are predominantly found in extrahepatic tissues. Previous studies indicated that Cyp2j5, Cyp2j8, Cyp2j11 and Cyp2j13 are predominantly expressed in kidney (Graves et al, 2013; Ma et al, 1999), Cyp2j6 transcripts are primarily detected in intestine (Scarborough et al, 1999), Cyp2j8, Cyp2j9 and Cyp2j12 transcripts are highly expressed in brain (Graves et al, 2013; Qu et al, 2001). These previous studies have all used non-quantitative, reverse transcriptase-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 quantification of transcripts 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. SYBR® Green based assays report abundance of double stranded DNA, which is potentially confounded by homology between related transcripts 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 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
biological 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).

In this study, primer sets were developed 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 to 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 7 Cyp2j isoforms. In addition, both qPCR methods were used to quantify the expression of the Cyp2j subfamily members in kidney from 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 NIH 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 3 female and 3 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 4 female and 4 male Cyp2j5 KO and WT kidneys. For the influenza exposure study, WT male and female C57BL/6 mice were infected with 200 PFU of influenza A/Hong Kong/8/68 (H3N2) in a total volume of 50 μl by intranasal instillation. Lungs were collected four days after infection and total RNA was isolated. For the LPS studies, WT male C57BL/6 mice were given a single dose of 50 μg of LPS intranasally. After 4 hours, the mice were euthanized, the lungs were collected, and RNA was isolated. For the HDM experiments, male C57BL/6 mice were sensitized with 10 μg HDM allergen in 50 μl saline via oropharyngeal aspiration (OPA) on days 1 and 8, followed by challenge on days 15, 16 and 17 with 2 μg HDM in 50 μl saline via OPA. Lungs were collected on day 18 and total RNA isolated. For acute corn pollen exposure experiments, female BALB/C mice were given a single dose of 4 mg of corn pollen in 75 μl PBS by aspiration. Twenty four hours after dosing, the mice were euthanized, lungs were collected, and RNA was isolated. For chronic corn pollen exposure experiments, female BALB/C mice were given 1 mg of corn pollen in 75 μl PBS by aspiration for 7 consecutive days. The mice were euthanized 24 hours after the last dose of corn pollen, lungs were collected and
total RNA was isolated. Saline controls were included in both acute and chronic corn pollen exposure experiments.

**cDNA Synthesis.** For each experimental animal, 1 μg of total RNA was treated with 1 unit of DNase I (Life Technologies, Grand Island, NY) for 15 min. DNase I was inactivated by addition of 1 μl 25 mM EDTA and heat treatment at 65°C for 10 min. RNase-free water was added to bring each sample to a total volume of 50 μl. 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/μl. The cDNA was diluted to a working concentration of 1 ng/μl 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 μl of the 20X primer/probes were used in a 10 μl 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 3). Some primer sets were those that had previously been published for use in non-quantitative RT-PCR (Ma et al., 1999; Graves et al., 2013). All primers were diluted to a concentration of 10 μM and 0.5 μl was used in a total volume of 10 μl 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; 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 $2^{-\Delta\Delta CT}$ method (Livak & 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, beta-2-microglobulin (B2m), and β-actin in the influenza A treated mice compared to 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 Fig. 2 & 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 to calculate the ΔΔC_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 paper is 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 (Graves et al, 2013; Ma et al, 2002; Ma et al, 1999; Qu et al, 2001). Each cDNA was cloned into the pcDNA3.1 vector (Life Technologies) or pBS-SK+ vector (Agilent Technologies, Santa Clara, CA). All 7 of the mouse Cyp2j TaqMan® primer/probe assays and the 7 SYBR® Green primer sets were specific for their intended mouse Cyp2j cDNA (Figs. 1-7; A and B). In addition, the 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 kidney, and in liver (Fig. 1). The expression level of Cyp2j5 in male kidney was significantly higher than in female kidney and liver using either qPCR method. This finding is in agreement with previously reported, non-quantitative 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 large intestine, stomach, liver, lung, ovary, uterus, skin, kidney, and visceral fat. This broad tissue distribution of Cyp2j6 was also observed previously by non-quantitative RT-PCR methods (Scarborough et al, 1999). Examination of qPCR results from 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 liver, brain, and duodenum (Fig. 3). In addition, we observed abundant expression in kidney only with the SYBR® Green Cyp2j8 primer set. These findings are consistent with published results using non-quantitative RT-PCR methods (Graves et al, 2013), although skin was not included in the prior panel. Cyp2j9 transcripts were detected in brain, liver, and lung using either the TaqMan® or SYBR® Green qPCR methods (Fig. 4). Previous non-quantitative RT-PCR methods showed a similar pattern of expression in brain and liver, but not in lung (Qu et al, 2001). When using SYBR® Green primers, high expression levels of Cyp2j9 were detected in 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 Figure 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 \textit{Cyp2j11} transcripts. Previously published non-quantitative RT-PCR analysis detected abundant \textit{Cyp2j11} expression in kidney and heart (Graves et al, 2013); however, robust expression was observed in heart with either qPCR method. Prominent \textit{Cyp2j12} expression was detected in brain with both the TaqMan® primer/probe and SYBR® Green primer set (Fig. 6). Previously published non-quantitative RT-PCR methods also showed high transcript levels in the brain (Graves et al, 2013). \textit{Cyp2j13} transcript expression is detected almost exclusively in male kidney by both the TaqMan® primer/probe and SYBR® Green primer set (Fig. 7).

**Expression of mouse \textit{Cyp2j} isoforms in kidneys of \textit{Cyp2j5} KO mice.** Both TaqMan® and SYBR® Green qPCR were used to determine expression levels for the \textit{Cyp2j} isoforms in \textit{Cyp2j5} KO and WT kidney (Fig. 8). Consistent with previous data (Ma et al, 1999), \textit{Cyp2j5} transcript levels were below level of detection ($C_T > 38$) in CYP2J5 KO mice. Both qPCR methods showed that the expression levels of \textit{Cyp2j6}, \textit{Cyp2j8} and \textit{Cyp2j9} in kidney were significantly increased in \textit{Cyp2j5} KO mice relative to WT controls. In contrast, there were no compensatory changes in \textit{Cyp2j11} or \textit{Cyp2j13} expression in \textit{Cyp2j5} KO mice. Expression of the \textit{Cyp2j} isoforms were also examined in \textit{Cyp2j5} knockout and wild type livers (Supplemental Figure 5). As in the kidney, \textit{Cyp2j5} disruption leads to compensatory upregulation of \textit{Cyp2j8} and \textit{Cyp2j9}. \textit{Cyp2j6} expression was slightly reduced in livers from \textit{Cyp2j5} knockout mice relative to wild type. This is in contrast to a modest induction of \textit{Cyp2j6} in kidneys from \textit{Cyp2j5} knockout mice.

**Expression of mouse \textit{Cyp2j} isoforms in lungs of influenza A-treated mice.** Treatment of mice with 200 pfu influenza A is typically lethal beyond four days;
therefore, mice were euthanized on day four to assess \textit{Cyp2j} isoform expression. Relative expression of \textit{Cyp2j} isoforms in mice treated with influenza A were 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 to saline-treated controls, influenza A-treated lungs showed a significant decrease in expression of \textit{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 \textit{Cyp2j5} (p=0.05), \textit{Cyp2j6} (p=0.06), \textit{Cyp2j8} (p=0.07), and \textit{Cyp2j11} (p=0.07) isoforms with the TaqMan® primer/probes. In contrast, with the SYBR® Green primers, only \textit{Cyp2j6} tended to be reduced in influenza a-treated females relative to saline-treated controls (p=0.05).

**Expression of mouse \textit{Cyp2j} isoforms in lungs of LPS-treated mice.** \textit{Cyp2j} isoform expression was measured in mouse lungs four hours after LPS or saline treatment. Compared to saline-treated controls, LPS treated mice showed significantly reduced lung expression of \textit{Cyp2j6}, \textit{Cyp2j9}, and \textit{Cyp2j11} (Fig. 10). There were no differences in expression of the other \textit{Cyp2j} isoforms. These findings were consistent in both TaqMan® and SYBR® Green assays.

**Expression of mouse \textit{Cyp2j} isoforms in lungs of HDM-treated mice.** To assess \textit{Cyp2j} isoform expression in a model of allergic asthma, mice were sensitized and exposed to HDM in an 18-day protocol as described in Methods. Both the TaqMan® primer/probes and SYBR® Green primer sets detected significantly reduced expression of \textit{Cyp2j6} and \textit{Cyp2j9} in 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 of consecutive daily doses of corn pollen. After acute exposure to corn pollen, Cyp2j6 and Cyp2j9 isoforms were significantly reduced compared to saline-treated controls (Figs. 12A & B). Chronic corn pollen exposure also strongly reduced Cyp2j6 and Cyp2j9 transcript levels (Figs. 12C & D). There was a smaller, but significant, reduction in Cyp2j8 expression detected with the TaqMan® primer/probe set (Fig. 12C). Since 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 Figure 6.

**DISCUSSION**

Cytochromes P450 are a large gene superfamily of ubiquitous hemoproteins that have physiologically relevant hepatic and extrahepatic functions (Campbell & Harder, 1999; Node et al, 2001; Wu et al, 1996; Zou et al, 1996). Members of the CYP2J subfamily metabolize AA and linoleic acid into bioactive lipids (Graves et al, 2013; Ma et al, 2002; Ma et al, 1999; Qu et al, 2001). Identifying tissue expression patterns of the mouse Cyp2j isoforms and quantifying their expression levels after various treatments is
important for determining the physiological role of these enzymes. The tissue distribution of the 7 members of the mouse Cyp2j subfamily had previously been determined by RT-PCR and western blot analysis (Graves et al, 2013; Ma et al, 2002; Ma et al, 1999; Qu et al, 2001). In a recent study, reverse transcriptase, multiplex, ligation-dependent probe amplification (RT-MLPA) was used to measure mouse Cyp2j gene expression (Zhou et al, 2013); however, qPCR is a more widely available quantitative assay which 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 which 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 7 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, non-quantitative RT-PCR results (Graves et al, 2013; Ma et al, 2002; Ma et al, 1999; Qu et al, 2001) with a few notable exceptions. For example, non-quantitative RT-PCR had detected Cyp2j8 in mouse kidney and heart, whereas both qPCR methods confirmed abundant expression in kidney, but not in heart. In addition, we previously
reported that \textit{Cyp2j8} was abundantly expressed in brain (Graves et al, 2013); however, qPCR revealed highest expression in skin, a tissue which was not included in the previous analysis.

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

Cytochromes P450s located in the gastrointestinal tract may play a role in the biotransformation of dietary nutrients as well as xenochemicals (Kaminsky & Fasco, 1992). In the current study, \textit{Cyp2j6} was shown by both TaqMan\textsuperscript{®} and SYBR\textsuperscript{®} Green qPCR methods to have the highest expression in small intestine, duodenum, stomach, and large intestine. This is in agreement with the published data on the expression of \textit{Cyp2j6} in small intestine by non-quantitative RT-PCR (Ma et al, 2002; Scarborough et al, 1999). It is noteworthy that in previous studies, stomach and large intestine were not included in the tissue panel used for non-quantitative 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 (Holla et al, 1999; Ma et al, 1999; Oyekan et al,
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 hormones which 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 to female kidney, and Cyp2j13 is almost exclusively expressed in male kidney. 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 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 upregulation 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 (Graves et al, 2013; Ma et al, 1999). Further studies will be needed to determine if 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, while Cyp2j5 disruption induced Cyp2j6 expression in the kidney,
it suppressed \textit{Cyp2j6} expression in the liver. Thus, compensatory changes in \textit{Cyp2j6} expression in \textit{Cyp2j5} knockout mice appear to occur in a tissue-specific fashion.

EETs have been shown to possess significant anti-inflammatory properties (Campbell et al., 1996; Larsen et al., 2006; Node et al., 1999). Inflammation is known to affect the expression and metabolic capacity of hepatic and extrahepatic P450s (Morgan, 2001; Theken et al., 2011). P450 expression is usually suppressed in inflammatory states, but may also be induced or unaffected (Morgan, 1997). In the current study, we examined expression of the \textit{Cyp2j} isoforms in four lung inflammatory models. Influenza A infection induces inflammation in the lungs of mice (Dawson et al., 2000; Virelizier et al., 1979). LPS is part of the outer membrane of gram-negative bacteria and induces airway inflammation and hyperresponsiveness (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 \textit{Cyp2j} expression in the lung during inflammation in these 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 modest reduction of most \textit{Cyp2j} isoforms; only the reduction in \textit{Cyp2j9} was statistically significant. Sex differences in immune responses are well established (Carey et al., 2007). The reduction of \textit{Cyp2j9} expression was similar in males and females; however, we observed a trend for reduction of several other \textit{Cyp2js} in female mice (\textit{p}=0.05-0.1). Interestingly, no significant change in any of the mouse \textit{Cyp2j}
isoforms was observed using a human respiratory syncytial virus (RSV) model (Supplemental Figure 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 which found no change in Cyp2j5 but reduced Cyp2j9 expression following LPS exposure (Theken et al, 2011). We found that Cyp2j5, Cyp2j11, Cyp2j12, and Cyp2j13 are expressed at very low levels in the lung; therefore, 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 P450s 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 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 Cyp2j isoforms are expressed at 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 Cyp2j isoforms 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 we developed in our laboratory. Overall, similar expression profiles were observed for all seven isoforms with
the two qPCR methods. We found compensatory upregulation of several mouse Cyp2j isoforms in the kidneys of Cyp2j5 KO mice. In addition, 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 pathological conditions.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Graves, Gruzdev, Edin, and Zeldin

Conducted experiments: Graves

Contributed new reagents or analytical tools: Bradbury, DeGraff, Li

Performed data analysis: Graves, Gruzdev, and Zeldin

Wrote or contributed to the writing of the manuscript: Graves, Gruzdev, Hoopes, House, Edin, and Zeldin
REFERENCES


FOOTNOTES

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Figure Legends

Figure. 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 highest expression of Cyp2j5 in male kidney with lower expression in female kidney and liver. Data shown are mean ± SE, n=6 per group except for sex-specific tissues where n=3 per group; *p < 0.05 (male versus female kidney).

Figure. 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 small intestine, duodenum, stomach and large intestine. Data shown are mean ± SE, n=6 per group.

Figure. 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 skin using both the TaqMan® primer/probe (C) and the SYBR® Green primer set (D). Data shown are mean ± SE, n=6 per group.

Figure. 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. Highest expression was in brain, liver and lung using both the TaqMan® primer/probe (C) and
the SYBR® Green primer set (D). Abundant expression was also observed in ovary, skin, small intestine, and visceral fat for the Cyp2j9 SYBR® Green primer set. Data shown are mean ± SE, n=6 per group.

**Figure. 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 ± SE, n=6 per group.

**Figure. 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 ± SE, n=6 per group.

**Figure. 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 ± SE, n=6 per group except for sex-specific tissues where n=3 per group. *p < 0.05 (male versus female kidney).

**Figure. 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 ± SE, n=4 per group; *p<0.05 Cyp2j5 KO versus WT.

**Figure. 9.** Detection of the mouse *Cyp2j* isoforms in lungs of mice treated with influenza A virus. Similar expression profiles were observed in female mice using 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 ± SE, n=4 per group; *p < 0.05* (compared to saline control).

**Figure. 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 ± SE, n=6 per group; *p < 0.05* (compared to saline control).

**Figure. 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 ± SE, n=6 per group for HDM-treated lungs and n=5 per group for saline-treated lungs; *p<0.05* (compared to saline controls).

**Figure. 12.** Detection of the mouse *Cyp2j* isoforms in lungs from corn pollen-treated mice. Using both qPCR methods, in acute (A & B) and chronic (C & 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 ± SE, n=4 per group; *p<0.05 (compared to saline controls).
TABLE 1

TaqMan® primer/probes for the mouse Cyp2j subfamily

<table>
<thead>
<tr>
<th>Subfamily Member</th>
<th>TaqMan® Primer/Probe</th>
</tr>
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<tbody>
<tr>
<td>Cyp2j5</td>
<td>Mm00487292_m1</td>
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<td>Cyp2j6</td>
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<td>Cyp2j8</td>
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<tr>
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<td>Cyp2j13</td>
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<tr>
<td>Gapdh</td>
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<td>18s</td>
<td>Mm03928990_g1</td>
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</tbody>
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**TABLE 2**

SYBR® Green primer set sequences for the mouse *Cyp2j* subfamily

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<th>Transcript</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
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<td>GCAAGTCTTGCTGCCCTTTCT</td>
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<td><em>Cyp2j</em>8</td>
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<td><em>Cyp2j</em>9</td>
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<tr>
<td><em>Cyp2j</em>11</td>
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<td>GTGATGGCCATTACTTGAGG</td>
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<tr>
<td><em>Cyp2j</em>12</td>
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<td>GACTGTCCTCATACTCAAAAGGC</td>
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<tr>
<td><em>Cyp2j</em>13</td>
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<td>GTCTCATCTGCGGCAACGC</td>
<td>Graves et al, 2013</td>
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<td>Gapdh</td>
<td>TGTATGGGCAAATATCCTCACA</td>
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<tr>
<td>18s</td>
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<td>TCTGCTATGGGGCGGTGAT</td>
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</tr>
</tbody>
</table>
Figure 1
Figure 2

A and B: Relative expression of CYP266 and CYP256 in cDNA.

C and D: Relative expression to Gapdh across different tissues.

Tissue categories include: Aorta, Brain, Duodenum, Esophagus, Eye, Heart, Kidney, Large Intestine, Liver, Lung, Lymph Node, Ovary, Skeletal Muscle, Small Intestine, Skin, Stomach, Spleen, Testis, Tongue, Uterus, Visceral Fat.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Figure 12