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Hepatic Cyp2d and Cyp26a1 mRNAs and Activities Are Increased During Mouse Pregnancy^S

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ABSTRACT

There is considerable evidence that drug disposition is altered during human pregnancy and based on probe drug studies, CYP2D6 activity increases during human pregnancy. The aim of this study was to determine whether the changes of CYP2D6 activity observed during human pregnancy could be replicated in the mouse, and explore possible mechanisms of increased CYP2D6 activity during pregnancy. Cyp2d11, Cyp2d22, Cyp2d26 and Cyp2d40 mRNA was increased (P < 0.05) on gestational days (GD) 15 and 19 compared with the non-pregnant controls. There was no change (P > 0.05) in Cyp2d9 and Cyp2d10 mRNA. In agreement with the increased Cyp2d mRNA, Cyp2d-mediated dextrorphan formation from dextromethorphan was increased 2.7-fold (P < 0.05) on GD19 (56.8±39.4 pmol/min/mg protein) when compared with the non-pregnant controls (20.8±11.2 pmol/min/mg protein). An

increase in Cyp26a1 mRNA (10-fold) and retinoic acid receptor (Rar) β mRNA (2.8-fold) was also observed during pregnancy. The increase in Cyp26a1 and Rar β mRNA during pregnancy indicates increased retinoic acid signaling in the liver during pregnancy. A putative retinoic acid response element was identified within the Cyp2d40 promoter and the mRNA of Cyp2d40 correlated (P < 0.05) with Cyp26a1 and Rar β . These results show that Cyp2d mRNA is increased during mouse pregnancy and the mouse may provide a suitable model to investigate the mechanisms underlying the increased clearance of CYP2D6 probes observed during human pregnancy. Our findings also suggest that retinoic acid signaling in the liver is increased during pregnancy, which may have broader implications to energy homeostasis in the liver during pregnancy.

Introduction

Altered drug disposition during human pregnancy could compromise the therapy to the mother or affect the risk of medications to the fetus. Chronic or pregnancy-related conditions such as epilepsy, depression, asthma, preeclampsia or gestational diabetes often require medical treatment during pregnancy, and as a consequence the use of prescription and over-the-counter medications during pregnancy is common (Andrade et al., 2004). For example, 95.8% of pregnant women in rural West Virginia were administered prescription medications, 92.6% took over-the-counter medications, and 45.2% used herbal medicines (Glover et al., 2003). However, little is known about whether the same dosing regimens of drugs used in non-pregnant women and in men can

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be used during pregnancy. In fact, there is accumulating evidence that pregnancy alters the disposition of drugs and hence drug doses need to be adjusted during pregnancy (Anderson, 2005).

Pharmacokinetic studies using probe drugs have shown that the activity of liver cytochrome P450 enzymes (P450s) changes during pregnancy in an enzyme specific fashion (Anderson, 2005). The activity of CYP3A4 is increased approximately 2-fold during human pregnancy based on midazolam clearance (Hebert et al., 2008). The 6-fold increase in metoprolol oral clearance (Hogstedt et al., 1983) and decrease in dextromethorphan urinary ratio (Tracy et al., 2005) during pregnancy indicate up to 6-fold increase in CYP2D6 activity during pregnancy. In contrast, CYP1A2-mediated metabolism of caffeine and theophylline is decreased approximately 50% during human pregnancy, and CYP2C19 activity appears to be decreased based on proguanil metabolism (Anderson, 2005; Tracy et al., 2005). Together these studies show a complicated network of pregnancy mediated pharmacokinetic changes. However, the mechanisms causing these changes during pregnancy are still largely unknown. Due to the lack of mechanistic understanding, extrapolation of the findings from one gestational age to another cannot be done for specific P450s.

Animal models have been used to identify specific mechanisms by which pregnancy alters drug disposition. For example, in the mouse an

ABBREVIATIONS: atRA, all-trans-retinoic acid; GD, gestational day; Kpi, potassium phosphate; MLH, mouse liver homogenates; PMSF, phenylmethylsulfonyl fluoride; P450, cytochrome P450; RA, retinoic acid; RAR or Rar, retinoic acid receptor; RARE, retinoic acid response element.

increase was detected in liver Cyp3a16, Cyp3a41, and Cyp3a44 mRNA, in liver Cyp3a activity and in the activity of the human CYP3A4 promoter during pregnancy (Mathias et al., 2006; Zhang et al., 2008). This increase in CYP3A mRNA, activity and transcription is in agreement with the observed increase in CYP3A4 activity during human pregnancy. In addition, similar to what is observed during human pregnancy, the clearance of nelfinavir increased during mouse pregnancy (Mathias et al., 2006). Together these data suggest that the mouse is a good model for investigating the mechanisms responsible for changes in CYP3A4 activity during human pregnancy. Similarly, the observed 50% decrease in caffeine clearance during human pregnancy was replicated in the pregnant rat with CYP1A2 activity, expression and mRNA decreasing during rat pregnancy (Walker et al., 2011). However, Cyp2d mRNA and activity were also decreased during rat pregnancy, contradictory to the apparent increase in CYP2D6 activity in pregnant humans (Dickmann et al., 2008), suggesting that the rat does not replicate some aspects of human pregnancy. Nevertheless, the study demonstrated that Cyp2d mRNA and activity were altered during pregnancy. This is important since there are no known xenobiotic inducers of CYP2D6 expression and classic inducers of P450 enzymes do not affect CYP2D6 activity. Interestingly, in the rat, a correlation between the Cyp2d isoform mRNA and retinoic acid receptor $(RAR)\alpha$ and hepatocyte nuclear factor (HNF) 3β mRNA was found, and retinoic acid (RA) signaling was suggested as potential mechanism of Cyp2d regulation (Dickmann et al., 2008).

The first aim of this study was to determine whether Cyp2d mRNA and activity increases during mouse pregnancy. The second aim was to explore whether RA signaling is also increased during mouse pregnancy and correlates with Cyp2d mRNA similar to what was observed in the rat. The overall hypothesis of the study was that according to the changes observed during human pregnancy, the mRNA and activity of Cyp2d enzymes is increased during pregnancy and as suggested by the rat data this increase will correlate with RA signaling in the mouse liver.

Materials and Methods

Chemicals and Reagents. All-trans-retinoic acid (atRA), dextromethorphan, and dextrorphan were purchased from Sigma-Aldrich (St. Louis, MO). 4-OH-RA and 4-oxo-RA were synthesized as previously described (Samokyszyn et al., 2000; Topletz et al., 2012). Optima-grade water, optima-grade acetonitrile, and ethyl acetate used for chemical analyses were purchased from Thermo Fisher Scientific. Water used for mRNA analysis was purchased from Qiagen (Valencia, CA). Potassium phosphate (KPi) buffers used were prepared in house and included a tissue homogenization buffer (50 mM KPi, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF) and a 100 mM KPi buffer (pH 7.4) for incubations.

Breeding and Tissue Preparation. Wild-type FVB mice (Taconic) were cared for and housed in the specific pathogen free facility at the University of Washington. The studies were approved by the Institutional Animal Care and Use committee of the University of Washington. Wild-type female mice 8–10 weeks of age were mated and pregnancy was determined by the detection of a vaginal plug. The mice were then divided in random order into two groups. The mice in group one (n = 9) were sacrificed at gestational day (GD) 15 and the mice in group 2 (n = 9) were sacrificed at GD 19. The mice were sacrificed using CO₂, and the livers were collected. In addition, virgin, age matched females (n = 9), from the same cohort of animals, were sacrificed as non-pregnant controls. The tissues were flash-frozen in liquid N₂ and stored at -80° C until use.

All tissue processing and preparation was performed on ice. For activity assays, 0.1–0.2 g of liver tissue of non-pregnant (n = 5), GD 15 (n = 3), and GD 19 (n = 3) mice were homogenized in 2 ml of Omni Hard Tissue Homogenizing tubes containing 1.4 mm ceramic beads, 0.3 ml of homogenization buffer (50 mM KPi, 0.25 M Sucrose, 1 mM EDTA, 1 mM PMSF), and the tissue using an

Omni Bead Ruptor 24 containing dry ice in acetone (Omni International, Kennesaw, GA). The mixture was homogenized for $2\times 20\,\mathrm{s}$. The homogenates were aliquoted and stored at $-80^{\circ}\mathrm{C}$ until further use. Protein content was determined using a Pierce BCA Protein Assay (Thermo Fisher Scientific) according to manufacturer's instructions with albumin as the standard.

mRNA Extraction and Real-Time PCR Assav. Total RNA was extracted from livers of non-pregnant (n = 4), GD 15 (n = 6), and GD 19 (n = 6) mice. To 0.1-0.2 g tissue, 1 ml of TRIzol reagent (Invitrogen, Grand Island, NY) was added, and mRNA extracted according to manufacturer's recommendations. Total RNA content was quantified using the Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and RNA quality was confirmed by gel electrophoresis. cDNA was generated from 1 µg of mRNA using the TaqMan Gene expression reagents (Applied Biosystems, Carlsbad, CA) as previously described (Tay et al., 2010). TaqMan Real-Time Universal polymerase chain reaction (PCR) master mix and PCR fluorescent primers and probes were purchased from Applied Biosystems (Foster City, CA). Probes used were as follows: Cyp2d9 (Mm00651731), Cyp2d10 (Mm00731648), Cyp2d11 (Mm04205381), Cyp2d22 (Mm01306302), Cyp2d26 (Mm00472520), Cyp2d40 (Mm01303815_m1), Cyp26a1 (Mm00514486), Rarα (Mm01296312_m1), Rar β (Mm01319677), and β -actin (Mm00607939_s1). β-Actin was used as the endogenous control (housekeeping gene). RT-PCR reactions were conducted using a StepOnePlus Real-Time PCR instrument (Applied Biosystems) according to manufacturer's recommendations. The foldchanges in Cyp2d9, Cyp2d10, Cyp2d11, Cyp2d22, Cyp2d26, and Cyp2d40 mRNA as well as Cyp26a1 and Rarβ mRNA during pregnancy in comparison with non-pregnant mice were quantified using RT-PCR.

Dextromethorphan Metabolism in Mouse Liver Homogenates. To quantify Cyp2d activity, the formation of dextrorphan from dextromethorphan by mouse liver homogenates (MLH) was determined. A total of eleven MLHs were used from non-pregnant (n = 5), GD 15 (n = 3) and GD 19 (n = 3) mice. All incubations were conducted at the protein and time linear range of dextromethorphan metabolism. MLHs (0.4 mg/ml) were incubated individually with two concentrations (1 and 50 µM) of dextromethorphan. These concentrations were chosen based on prior data of dextromethorphan metabolism in rat liver microsomes (Dickmann et al., 2008) and the known K_m for CYP2D6-mediated formation of dextrorphan (Lutz and Isoherranen, 2012). The 1 μ M concentration is below the $K_{\rm m}$ of dextromethorphan in rat liver microsomes and for CYP2D6 allowing evaluation of intrinsic clearance changes as reported previously (Dickmann et al., 2008), whereas 50 μM is much greater than the $K_{\rm m}$ and allows determination of $V_{\rm max}$ in rat liver microsomes and for human CYP2D6. Michaelis-Menten kinetic constants for dextrorphan formation were determined using MLH from 7 mice (3 nonpregnant, 2 GD 15 and 2 GD 19). The MLHs (0.4 mg/ml) were incubated with six concentrations of dextromethorphan (0.5, 1, 5, 10, 25, 50 µM). All incubations were performed using 96-well plates in 100 mM KPi buffer (pH 7.4) in a total volume of 0.2 ml per well. Samples were preincubated for 5 min before initiation with NADPH (1 mM final concentration). After an incubation period of 10 min, 0.125 ml of each sample was removed and quenched into cold acetonitrile (0.125 ml) in a fresh 96-well plate. Plates were then centrifuged for 20 min at 612g, 4°C, and 0.125-ml supernatant was removed and transferred into a fresh 96-well plate for UFLC-MS/MS (ultra fast liquid chromatography-tandem mass spectrometry) analysis.

Dextrorphan was separated using a Thermo Scientific Hypersil Gold column (1.9 $\mu m, 2.1 \times 100$ mm; Thermo Fisher Scientific) with a Shimadzu UFLC XR DGU-20A5 (Shimadzu Scientific Instruments, Columbia, MD) coupled to an AB Sciex 3200 Mass Spectrometer (AB Sciex, Framingham, MA). The analytes were separated using a linear seven minute gradient from an initial mobile phase of 10% acetonitrile 90% aqueous with 0.1% formic acid to a final condition of 90% acetonitrile 10% aqueous with 0.1% formic acid. Dextrorphan was detected using positive ion electrospray mass spectrometry, with a declustering potential of 61, a collision energy of 49, and a collision exit potential of 4.5 (Lutz and Isoherranen, 2012). The parent-fragment MS/MS transition of m/z 258.2 > 157.2 Da was monitored. The injection volume was 10 μ l. A six point standard curve for dextrorphan was constructed using concentrations between 5 and 500 nM and dextrorphan concentrations were quantified based on dextrorphan peak height.

All-trans-Retinoic Acid Metabolism in Mouse Liver Homogenates. Changes in hepatic all-trans-retinoic acid (atRA) metabolism were

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determined by quantifying the formation of 4-OH-RA, 4-oxo-RA and 16-OH-RA from atRA by MLHs. All atRA incubations were conducted under red light. MLHs (0.4 mg/ml) were incubated with atRA (1 μ M) in borosilicate glass tubes (8 × 13 cm). A total incubation volume of 0.5 ml in 100 mM KPi buffer (pH 7.4) was used. Samples were preincubated for 5 minutes before reactions were initiated with NADPH. After a 25-min incubation, reactions were quenched by adding 3 ml of ethyl acetate to each incubation together with an internal standard (20 µl of 2.5 µM 4-oxo-RA-d₃). Samples were then centrifuged, and the organic layer was extracted and evaporated under a stream of N₂ and then reconstituted in 100 µl of optima grade acetonitrile for MS/MS analysis. Analytes were separated and analyzed using an Agilent Zorbax C18 column (3.5 μ m, 2.1 \times 100 mm) with an AB Sciex API 5500 Q/LIT mass spectrometer (AB Sciex) equipped with an Agilent 1290 Infinity UHPLC. The analytes were separated over a linear 35-min gradient using an initial mobile phase of 5% acetonitrile 95% aqueous with 0.1% formic acid to a final condition of 95% acetonitrile 5% aqueous with 0.1% formic acid. Analytes were detected using negative ion electrospray mass spectrometry, with declustering potentials of -80 (4-OH-RA), -90 (16-OH-RA and 4-oxo-RA- d_3), and -95 (4-oxo-RA); collision energies of -28 (4-OH-RA), -25(16-OH-RA and 4-oxo-RA-d₃), and -22 (4-oxo-RA); and collision exit potentials of -7 (4-OH-RA), -16 (16-OH-RA), -5 (4-oxo-RA), and -10(4-oxo-RA-d₃). The parent-fragment MS/MS transitions of m/z 315 > 253 Da (4-OH-RA), 315 > 241 Da (16-OH-RA), 313 > 269 Da (4-oxo-RA), and 316 > 272 Da (4-oxo-RA-d₃) were monitored. Standard curves were constructed at concentrations between 2.5 and 100 nM for 4-OH-RA and 4-oxo-RA. Due to the lack of a synthetic standard for the 16-OH-RA, the relative changes in 16-OH-RA formation were measured from analyte to internal standard peak height ratio. As 4-oxo-RA is formed from 4-OH-RA sequentially, the sum of 4-OH-RA and 4-oxo-RA formation rates was used to determine total 4-OH-RA formed by MLHs.

RARE Identification within Promoter Regions of Cyp2d, Cyp26a1 and **Rar\beta.** The DNA sequences of the mouse Cyp2d genes, Cyp26a1 and Rar β were scanned to determine if any retinoic acid response elements (RAREs) existed within the respective promoter regions. In-house Perl scripts were written to collect and parse stretches of genomic information for potential RARE promoter sequences in a two-step process. The first step uses the BioMart API (http://www.biomart.org) to access Ensembl (http://www. ensemble.org) and record the user-defined gene information to a text file in FASTA format. Genes of interest are listed in FASTA format and are accessed by the Perl script. For each gene in the input file, 10,000 DNA base pairs are captured directly upstream of the gene and amended to an output file. The second step uses an independent Perl script to parse the aforementioned collection of gene information for RARE sequences. Perl string matching is employed to match sequences with the following requirement: $(A|G)G(G|T)T(C|G)A(A|G|T|C)^n(A|G)G(G|T)T(C|G)A$, where n is one, two or five bases as defined by DR1, DR2 and DR5 RARE sequences, respectively. As defined by Perl, bases listed within parentheses and separated by the character "I" allow the string matching algorithm to match only one of the listed bases. If a series of base pairs in a gene match the string representing a potential RARE sequence based on the above rule, the gene name and the determined RARE sequence found are listed in a FASTA format output file.

Data and Statistical Analysis. Changes in mRNA were quantified as fold-difference from control using the comparative C_T method ($\Delta\Delta C_T$) with β -actin as the housekeeping gene. All data were fitted via linear and nonlinear regression using Prism v.5 (GraphPad Software, Inc., La Jolla, CA) and all statistical analyses were performed using Prism v.5 (GraphPad). One-way analyses of variance were performed to determine statistical significance of differences in mRNA expression and Cyp2d and Cyp26a1 activity between non-pregnant mice and pregnant mice at GD 15 and GD 19. A P value < 0.05 was considered significant. Significant differences between non-pregnant GD 15 and GD 19 mice were then determined using Bonferroni's Multiple Comparison Test. All correlations between mRNA of Cyp2d isoforms, Cyp26a1 and Rar β across the gestational ages were tested using linear regression. Regressions with P values < 0.05 were considered significant.

All enzyme kinetic data were fitted using nonlinear regression. All parameter estimates are given as means \pm S.D. Michaelis-Menten parameters for dextrorphan formation were estimated using Eq. 1, where ν is the formation

velocity of the metabolite and [S] is the initial substrate concentration (dextromethorphan).

$$v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \tag{1}$$

Intrinsic clearance (CLint) of dextrorphan formation was calculated using Eq. 2.

$$CL_{\rm int} = \frac{V_{\rm max}}{K_m} \tag{2}$$

Formation velocity of 4-OH-RA + 4-oxo-RA from *at*RA was used as a measure of *at*RA metabolism due to the sequential oxidation of 4-OH-RA to 4-oxo-RA (Lutz et al., 2009).

The changes in the in vivo intrinsic clearance of dextromethorphan and atRA were predicted using in vitro-to-in vivo extrapolation. The intrinsic clearances were scaled up to in vivo $CL_{\rm int}$ at different gestational stages and in non-pregnant controls according to Eq. 3 using total milligrams of protein per gram of liver measured in each animal (Supplemental Table 1) and the average maternal mouse liver weight for each gestational age. Values for the average liver weight at each gestational age were used as previously reported: 1.35 \pm 0.25 g (non-pregnant), 2.48 \pm 0.39 g (GD 15), and 2.62 \pm 0.49 g (GD 19) (Dai et al., 2011). The values of mg protein per gram of liver were calculated for each animal and used to scale up to total $Cl_{\rm int,\ liver}$ before each gestational age was averaged.

$$CL_{\text{int},liver} = CL_{\text{int }per \ mg \ protein} \times (mg \ protein \ per \ g \ of \ liver) \times (total \ g \ of \ liver)$$
(3)

Results

Changes in Cyp2d mRNA during Mouse Pregnancy. Cyp2d10, Cyp2d11, Cyp2d22, Cyp2d26, and Cyp2d40 are all found in both murine genders (Renaud et al., 2011). The mRNA of Cyp2d11, Cyp2d22, Cyp2d26, and Cyp2d40 was increased significantly (P <0.01) on GD 15 and GD 19 when compared with the non-pregnant control mice (Fig. 1A), with Cyp2d40 showing the greatest increase in mRNA (6-fold) during gestation. The mRNA of Cyp2d10 at GD 15 and GD 19 was not different from the non-pregnant controls (P >0.05). The mRNA of the male-specific enzyme Cyp2d9 was also unchanged during pregnancy (P > 0.05). No differences (P > 0.05)were found between GD 15 and GD 19 for any of the Cyp2d enzymes. Baseline (non-pregnant) C_T values indicated that average mRNA levels were highest for Cyp2d26 ($C_{T,Ave\ NP} = 23.3$), Cyp2d22 ($C_{T,Ave\ NP}$ $_{NP}$ = 25.8), and Cyp2d10 (C_{T,Ave NP} = 24.7), with lower mRNA baseline quantification for Cyp2d40 (C_{T,Ave NP} = 32.6), Cyp2d9 $(C_{T,Ave\ NP} = 32.9)$ and Cyp2d11 $(C_T = 36.8)$.

Changes in Dextromethorphan Metabolism during Mouse Pregnancy. To determine whether increased Cyp2d mRNA levels translated to increased Cyp2d activity, dextrorphan formation from dextromethorphan was evaluated in MLHs (non-pregnant, n=5; GD 15, n=3; and GD 19, n=3) at two concentrations: 1 μ M (below apparent $K_{\rm m}$ in rat liver microsomes) and 50 μ M (~10* $K_{\rm m}$ in rat liver microsomes providing estimate of $V_{\rm max}$). A 2.7-fold increase in dextrorphan formation was observed between non-pregnant (20.8 \pm 11.2 pmol/min/mg protein) and GD 19 (56.8 \pm 39.4 pmol/min/mg protein) MLHs at 50 μ M dextromethorphan (P < 0.05), but the 1.7-fold increase in dextrorphan formation between non-pregnant and GD 15 (36.3 \pm 8.4 pmol/min/mg protein) MLHs was not significant (P > 0.05) (Fig. 1B). Formation of dextrorphan at 1 μ M was comparable (P > 0.05) between all three gestational ages (Fig. 1C).

To evaluate the reasons for the increased dextrorphan formation at 50 μ M dextromethorphan, Michaelis-Menten constants were determined in MLHs from seven mice; three non-pregnant, two GD 15,

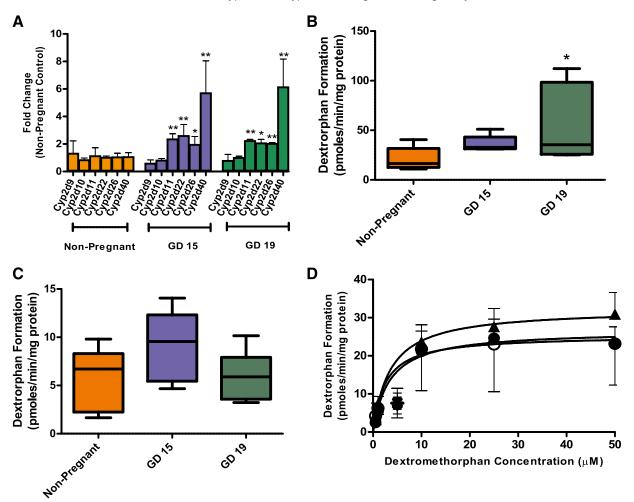


Fig. 1. (A) The fold change in Cyp2d mRNA on GD 15 (n = 6, blue bars), and GD 19 (n = 6, green bars) in comparison with non-pregnant mice (n = 4, yellow bars). The measured dextrorphan formation velocity at 50 μ M (B) and 1 μ M (C) concentration of dextromethorphan is shown at two gestational ages and in non-pregnant controls using MLH from a separate group of animals. The panels show box and whiskers plots of dextrorphan formation velocity in non-pregnant (n = 5), GD 15 (n = 3) and GD 19 (n = 3) mice. The line shows the median, the box the 75th and 25th percentile and the error bar the range of the measurements in each group. The Michaelis-Menten parameters of dextrorphan formation from dextromethorphan were measured in MLHs from individual animals (D) using three control (open circles), two GD15 (closed circles) and two GD19 (closed triangles) MLHs. The lines indicate the fit of Michaelis-Menten equation to the average data from all animals on that gestational age and the error bars show the range of formation velocities observed at a given concentration of dextromethorphan in the different animals. The Eadie-Hofstee plots for each GD are shown in Supplemental Fig. 1. The K_m and V_{max} values are listed in Table 1. Significant changes in comparison with nonpregnant controls are indicated as **P < 0.01 and *P < 0.05.

and two GD 19. A 1.3-fold increase (P < 0.05) in the $V_{\rm max}$ of dextrorphan formation was observed at GD 19 when compared with non-pregnant controls and GD 15 mice (Fig. 1D; Table 1), suggesting increased Cyp2d protein expression. The average $V_{\rm max}$ on GD 15 was not different from non-pregnant mice (Table 1). The increase in the apparent $K_{\rm m}$ for dextrorphan formation during gestation was also not significant (Table 1). The Eadie-Hofstee plots of dextrorphan

formation in the three gestational ages are shown in supplemental Fig. 1. The average intrinsic clearance of dextrorphan formation from dextromethorphan in MLHs did not change on GD 15 and GD 19 when compared with that of non-pregnant mice (Table 1). However, the in vivo intrinsic clearance of dextromethorphan was predicted to increase 1.5- to 1.8-fold during mouse pregnancy (GD 15 and GD 19) in comparison with the non-pregnant controls. The predicted in vivo

TABLE 1

Characterization of changes in dextromethorphan metabolism in mouse liver during gestation
The incubations were conducted as described in *Materials and Methods*.

Michaelis-Menten Parameters for Dextrorphan Formation in MLH from Pregnant and Nonpregnant mice					
Gestational Age	$V_{ m max}$	$K_{ m m}$	$CL_{ m int}$		
	pmol/min/mg protein	μM	μl/min/mg protein		
Nonpregnant $(n = 3)$	25.4 ± 1.0	2.5 ± 0.5	10.1 ± 2.1		
15 days $(n = 2)$	26.6 ± 1.6	3.4 ± 1.0	7.9 ± 2.3		
19 days $(n = 2)$	$32.3 \pm 0.7*$	3.5 ± 0.4	9.2 ± 1.0		

^{*} Indicates statistical significance at P < 0.05 compared with nonpregnant and GD15 mice.

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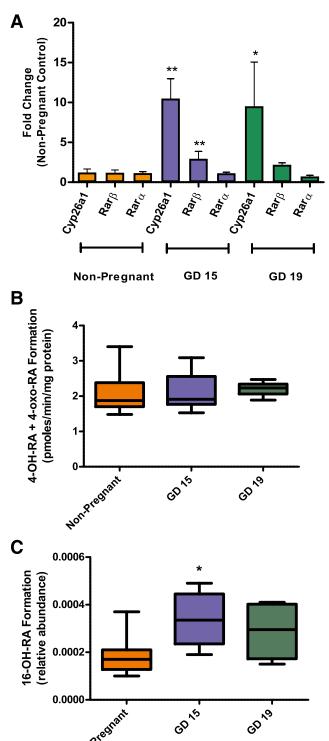


Fig. 2. (A) The fold change in Cyp26a1, Rarα and Rarβ mRNA in GD 15 (n=6, blue bars) and GD 19 (n=6, green bars) mice in comparison to non-pregnant (n=4, yellow bars) mice. The fold differences were calculated relative to non-pregnant values. (B) A box and whiskers plot of 4-OH-RA + 4-oxo-RA formation from atRA (1 μM) in MLHs in non-pregnant (n=5), GD 15 (n=3) and GD 19 (n=3) mice. (C) The relative quantification of 16-OH-RA formation from atRA in MLHs from non-pregnant (n=5), GD 15 (n=3) and GD 19 (n=3) mice. Significant changes in comparison with non-pregnant controls are indicated as **t=10.01 and *t=11.

 $CL_{\rm int}$ values were 2,041 \pm 590 μ l/min (non-pregnant), 2969 \pm 1267 μ l/min (GD15) and 3773 \pm 772 μ l/min (GD 19).

Changes in Cyp26a1, RAR α , and RAR β mRNA during Mouse Pregnancy. The mRNA of Cyp26a1 was increased (P < 0.05) on both GD 15 (10-fold) and GD 19 (9.7-fold) compared with non-pregnant controls (Fig. 2A). Rar β mRNA was also increased on GD 15 (2.8-fold, P < 0.01) but the increase on GD 19 (2.0-fold) was not significant (P > 0.05). There was no significant change in Rar α mRNA at GD 15 and GD 19 when compared to non-pregnant controls. No differences in mRNA expression were observed between GD 15 and GD 19 for Cyp26a1, Rar α , or Rar β (P > 0.05).

Changes in 4-OH-RA, 4-oxo-RA, and 16-OH-RA Formation during Mouse Pregnancy. Since Cyp26a1 mRNA was increased during pregnancy, atRA metabolism by MLHs was quantified using the additive formation rates of 4-OH-RA and 4-oxo-RA. While 4-OH-RA is formed by multiple P450 enzymes, 16-OH-RA appears to be a specific metabolite for CYP26A1 (Thatcher et al., 2011). Hence, the relative formation of 16-OH-RA was measured as a specific Cyp26a1 probe. Formation of 4-OH-RA, 4-oxo-RA and 16-OH-RA from atRA was detected in all livers, regardless of gestational age. The combined formation rate of 4-oxo-RA and 4-OH-RA (Fig. 2B) was not different between the different gestational days: 2.05 ± 0.57 pmol/min/mg protein (non-pregnant), 2.11 ± 0.55 pmol/min/mg protein (GD 15), and 2.21 ± 0.20 pmol/min/mg protein (GD 19). The relative formation of 16-OH-RA (Fig. 2C), increased 1.8-fold (P < 0.05) on GD 15 compared with the non-pregnant animals. The in vivo CL_{int} for 4-OH-RA and 4-oxo-RA formation was predicted to increase 1.7- and 2.2-fold on gestational days 15 and 19, respectively, when compared with the non-pregnant controls. The predicted CL_{int} , values were 391.0 \pm 105.6 μ L/min (non-pregnant), 673.4 \pm 314.8 μ L/min (GD15) and 873.4 \pm 358.0 μ L/min (GD19) suggesting that atRA clearance is increased during mouse pregnancy.

Correlations between Cyp2d, Cyp26a1 and Rar\(\beta \) mRNA. To explore the role of atRA in regulating Cyp2d expression, correlation between Cyp2d mRNA and the mRNA of marker genes of Rar activation was evaluated. The mRNA of Cyp2d11, Cyp2d22, Cyp2d26, and Cyp2d40 positively correlated (P < 0.05) with Cyp26a1 mRNA (Table 2; Supplemental Fig. 2). There was no correlation (P > 0.05) between Cyp26a1 mRNA and the mRNA of Cyp2d9 and Cyp2d10 (Table 2; Supplemental Fig. 2). Similar to the correlation between Cyp2d enzymes and Cyp26a1, a correlation (P < 0.05) was observed between Rarβ mRNA and Cyp2d22, Cyp2d26, and Cyp2d40 mRNA. There was no correlation between Rarβ mRNA and Cyp2d11, Cyp2d9 and Cyp2d10 mRNA (P > 0.05) (Table 2; Supplemental Fig. 3). In addition, of the Cyp2d enzymes, Cyp2d22 and Cyp2d40 mRNA correlated (P < 0.05) with Cyp2d11, the mRNA of Cyp2d26 and Cyp2d40 correlated with Cyp2d22 mRNA, and the mRNA of Cyp2d40 correlated with Cyp2d26 mRNA (Supplemental Table 2). A positive correlation was also detected between Cyp26a1 and Rar\(\beta\) mRNA (Table 2; Supplemental Fig. 4).

Identification of an RARE within Mouse Cyp2d40, Cyp26a1 and Rarβ Promoter Regions. The location of the *Cyp2d11*, *Cyp2d22*, *Cyp2d10*, *Cyp2d40* and *Cyp2d26* genes in the *Cyp2d* gene locus is shown in Fig. 3A. An RARE-DR2 promoter sequence was found within *Cyp2d40* promoter (Fig. 3, B and E) but due to the location of the Cyp2d genes it is possible that this RARE also contributes to the regulation of the other Cyp2d genes in this locus. No additional RARE sequences were found in the *Cyp2d* gene locus. *Cyp26a1* and *Rarβ* have previously been shown to contain identical RARE-DR5 promoter regions (Loudig et al., 2000; Loudig et al., 2005), and these RAREs were identified in the current study as well (Fig. 3, C, D, and E).

TABLE 2
Statistical analyses of mRNA correlations between Cyp2d isoforms and the retinoic acid responsive genes Cyp26a1 and $Rar\beta$

	Correlation with Cyp26a1			
	R^2	P Value	Significant	
Cyp2d9	0.0002	0.95	No	
Cyp2d10	0.09	0.27	No	
Cyp2d11	0.62	< 0.001	Yes	
Cyp2d22	0.65	0.008	Yes	
Cyp2d26	0.38	0.01	Yes	
Cyp2d40	0.62	0.005	Yes	

	Correlation with $Rar\beta$			
	R^2	P Value	Significant	
Cyp2d9	0.14	0.15	No	
Cyp2d10	0.001	0.92	No	
Cyp2d11	0.19	0.09	No	
Cyp2d22	0.72	<.001	Yes	
Cyp2d40	0.31	0.03	Yes	
Cyp2d26	0.49	0.03	Yes	
Cyp26a1	0.34	0.02	Yes	

Discussion

Based on probe studies, one of the largest effects of pregnancy on specific P450 activity is observed with CYP2D6. The increased activity of CYP2D6 is clinically important as approximately 30% of drugs on the market are metabolized by CYP2D6 (Shimada et al., 1994), including many drugs administered to pregnant women such as antidepressants, antipsychotics, and β -blockers (Wadelius et al., 1997). The oral clearance of metoprolol, a CYP2D6 probe, increased 6-fold and the bioavailability decreased to half at 26-30 weeks of gestation compared with postpartum (Hogstedt et al., 1985). A decrease in the urinary dextromethorphan-dextrorphan metabolic ratio was also observed at all trimesters (Tracy et al., 2005). In CYP2D6 extensive metabolizers, at 36 weeks of gestation a 50% decrease in the plasma dextromethorphan-dextrorphan metabolic ratio was detected when compared with post-partum, suggesting CYP2D6 induction (Wadelius et al., 1997). In individuals lacking functional CYP2D6, a 50% increase in parent/metabolite ratio was detected (Wadelius et al., 1997). Together these data provide evidence that CYP2D6 activity is increased during human pregnancy.

The apparent induction of CYP2D6 during pregnancy is puzzling since CYP2D6 is not considered to be inducible by xenobiotics. It has been shown that classic xenobiotic inducers that activate PXR, AhR and CAR, do not induce CYP2D6 (Niemi et al., 2003; Dixit et al., 2007; Hewitt et al., 2007; Westerink and Schoonen, 2007). There does, however, appear to be transcriptional regulation of CYP2D6. The variation of CYP2D6 activity correlates well with CYP2D6 mRNA in humans (Carcillo et al., 2003) and previous studies have shown that the orphan nuclear receptor HNF 4α regulates CYP2D6 transcription via binding to a direct repeat site (DR1) on the CYP2D6 promoter (Cairns et al., 1996). In addition, in human hepatocytes, analysis of P450 gene expression after adenoviral HNF4 α antisense RNA transfection resulted in a 45% decrease in CYP2D6 gene expression (Jover et al., 2001). In CYP2D6 humanized mice lacking HNF4 α in the liver, a 50% decrease in CYP2D6 mRNA and activity was observed (Corchero et al., 2001). However, HNF4 α is a common regulator of many liver P450 enzymes, which all change in different manner during pregnancy, and as such it is unlikely that changes in CYP2D6 activity during human pregnancy can be completely explained by changes in HNF4 α -mediated transcriptional activity.

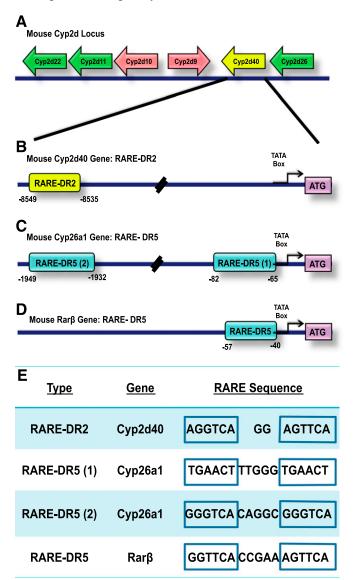


Fig. 3. (A) The orientation of the Cyp2d genes in the Cyp2d gene locus. The promoter region of Cyp2d40 gene is enlarged in (B) and the identified RARE is shown. The promoters of Cyp26a1 and $Rar\beta$ with the RAREs identified are shown in (C and D). Panel (E) shows sequences of the identified RAREs in the respective genes.

Studies in pregnant rats have shown that both Cyp2d mRNA and activity change during pregnancy, but a decrease instead of an increase in Cyp2d mRNA and activity was observed during rat pregnancy (Dickmann et al., 2008). In contrast to the findings in the rat, this study shows that during mouse pregnancy the mRNA of Cyp2d40 was increased up to 6-fold and Cyp2d11, Cyp2d22 and Cyp2d26 were increased approximately 2-fold. This increase in Cyp2d mRNA is in agreement with the observed increase in CYP2D6 activity in the human, and suggests that the mouse can replicate changes in Cyp2d activity and may be an appropriate model to study the mechanisms underlying CYP2D regulation during pregnancy. Indeed, when the in vitro intrinsic clearance of dextrorphan during mouse pregnancy was extrapolated to in vivo, a 2.2- to 3.7-fold increase in dextromethorphan intrinsic clearance was predicted on GD15 and GD19, respectively. This predicted increase is in agreement with the 2-fold increase in dextromethorphan clearance observed in humans (Wadelius et al., 1997). The increase in predicted Cyp2d activity and dextromethorphan

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intrinsic clearance is most likely due to increased apparent $V_{\rm max}$ for dextrorphan formation. The apparent $V_{\rm max}$ value was increased by 30% at GD 19, and the dextrorphan formation velocity in a different set of mice was increased by 2.8-fold at GD 19 at dextromethorphan concentration of 50 μ M.

The increased $V_{\rm max}$ value for dextrorphan formation is in agreement with the increase in Cyp2d40, Cyp2d11, Cyp2d22 and Cyp2d26 mRNA. Nevertheless, it is not possible to differentiate the relative contributions of individual Cyp2d enzymes to dextrorphan formation. Using Cyp2d knock-out mice, it has been shown that Cyp2d enzymes mediate the overall elimination of dextromethorphan in mice (Scheer et al., 2012), but dextromethorphan metabolism has been kinetically characterized only for Cyp2d22 ($K_{\rm m}$ = 250 μ M) (Yu and Haining, 2006). The observed $K_{\rm m}$ values for dextrorphan formation in the mouse liver homogenates (2.5–3.5 μ M), were ~40-fold lower than that shown for Cyp2d22, suggesting that multiple enzymes contribute to dextrorphan formation in mouse liver. However, the Eadie-Hofstee plots were linear preventing identification of multiple contributing enzymes. The observed $K_{\rm m}$ values in MLH were similar to those previously determined in rat liver microsomes (2.5-10 µM) from pregnant and nonpregnant animals (Dickmann et al., 2008) and in agreement with the $K_{\rm m}$ values of dextrorphan formation determined for all four recombinant rat Cyp2d enzymes ($K_{\rm m}$ values 0.3–49 μ M) (Narimatsu et al., 2009). The trend toward higher $K_{\rm m}$ during pregnancy is similar to that observed in rats, and could be entirely a result of increased contribution of a low affinity Cyp2d enzyme to dextrorphan formation in relation to a high affinity Cyp2d enzyme. Assuming similar PCR amplification efficiencies and the obtained absolute C_T values for the Cyp2d enzymes, Cyp2d26, Cyp2d22 and Cyp2d10 are the predominant Cyp2d enzymes in female mouse liver. Therefore, it is likely that the obtained $K_{\rm m}$ is a combination of the activities of these three enzymes together with increased contribution of Cyp2d40 to dextromethorphan metabolism during pregnancy. It is also likely that at low dextromethorphan concentrations ($< 5 \mu M$) dextrorphan formation is mainly catalyzed by a high affinity Cyp2d enzyme which does not change during pregnancy. This high affinity Cyp2d enzyme could be Cyp2d10. The metabolism at 50 μ M is likely to be catalyzed by low affinity enzymes, possibly Cyp2d22 and Cyp2d40, that are significantly increased during pregnancy. It is also possible that the recombinant enzyme system used for Cyp2d22 characterization is different in the lipid content and protein-protein interactions than MLH resulting in possible differences in measured enzyme kinetic values. Together these data suggest that the increased dextrorphan formation on GD19 is due to increased expression of Cyp2d proteins. However, due to the likely contribution of multiple mouse P450 enzymes to dextromethorphan metabolism a humanized CYP2D6 mouse model may be a better option for studying changes in CYP2D6 activity during pregnancy.

It has been proposed that RA signaling contributes to CYP2D regulation during pregnancy and RA was shown to alter CYP2D6 mRNA in HepG2 cells (Dickmann et al., 2008). To further evaluate this relationship the changes in the mRNA of two classic model atRA responsive genes, Cyp26a1 and $Rar\beta$, were measured during mouse pregnancy. The mRNA of both of these genes was increased during pregnancy in agreement with activation of Rars and atRA signaling. The promoter regions of Cyp26a1 and $Rar\beta$ have characteristic RAREs with direct repeats separated by 5 bases (DR5) (Fig. 3) that are typically associated with increased transcription (Chambon, 1996). The increase in Cyp26a1 mRNA was greater than Rar β during pregnancy, similarly to what has been previously observed following RA treatment in HepG2 cells (Tay et al., 2010). The increase in Cyp26a1 mRNA was coupled with increase in 16-OH-RA formation,

a reaction indicative of increased Cyp26a1 activity. The increased Cyp26a1 and Rar β mRNA during pregnancy suggest that atRA signaling is also increased in the maternal liver. atRA is a critical signaling molecule during mammalian development (Clagett-Dame and DeLuca, 2002; Maden, 2007; Duester, 2008) and plays a role in fetal organogenesis.

Positive correlations between activation of Rars (using Cyp26a1 and Rar\(\beta \) mRNA as markers), and Cyp2d mRNA were observed in this study. On the basis of these correlations and previous data, it was speculated that RA signaling may contribute to Cyp2d regulation. A DR2 RARE was identified in the Cyp2d40 promoter (Fig. 3). The DR2 RARE has been shown to be associated with an RA mediated increase in transcription of the CRABPII gene (Lefebvre et al., 1995; Chambon, 1996). The DR2 element may be responsible for the correlation between Cyp2d40 mRNA and Cyp26a1 mRNA, the increased mRNA of Cyp2d22, Cyp2d11, Cyp2d40 and Cyp2d26 and the correlation of the mRNA of these four genes due to the orientation of these genes and a co-regulation of the linked genes. However, further detailed biochemical studies are needed to demonstrate that the identified RARE is a functional promoter element and to determine whether RA signaling contributes to the increased Cyp2d mRNA during pregnancy.

In conclusion, this study shows that Cyp2d mRNA and activity together with Cyp26a1 and Rar β are increased during mouse pregnancy. As hypothesized, the increased Cyp2d mRNA correlated with increased RA signaling in the mouse liver during pregnancy. This is the first study to demonstrate that Cyp2d mRNA is increased during pregnancy and hence the data presented provides important evidence toward potential transcriptional regulation of CYP2D6 and Cyp2d enzymes during human and mouse pregnancy. Further mechanistic characterization of the possible involvement of RA signaling in CYP2D6 regulation is needed.

Authorship Contributions

Participated in research design: Topletz, Isoherrranen

Conducted experiments: Topletz, Le, Lee

Contributed new reagents or analytic tools: Kelly, Wang, Lee, Chapman

Performed data analysis: Topletz, Le, Isoherranen, Chapman

Wrote or contributed to writing of the manuscript: Topletz, Le, Chapman, Isoherranen

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