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Changes in Maternal liver Cyp2c and Cyp2d Expression and Activity During Rat Pregnancy

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Abstract

During human pregnancy, CYP2C9, CYP2C19, and CYP2D6 activities are altered. The aim of the current study was to determine if this phenomenon can be replicated in the rat, and to evaluate the mechanisms that contribute to the changes in Cyp2c and Cyp2d activity during pregnancy. The intrinsic clearance of dextromethorphan *O*-demethylation, a measure of Cyp2d2 activity, was decreased 80% at both days 9 and 19 of gestation when compared to nonpregnant controls. The decreased intrinsic clearance was a result of both decreased V_{max} and increased K_m -values at both days of gestation. Quantitative RT-PCR revealed that transcripts of Cyp2d2 and Cyp2d4 were significantly decreased at day 19 of pregnancy ($p<0.05$) when compared to day 9 and nonpregnant controls. The decrease in Cyp2d mRNA levels correlated with a decrease in several nuclear receptor mRNA levels (RARα, RXRα, HNF1 and HNF3β) but not with the mRNA levels of nuclear receptors usually associated with regulation of P450 enzymes (PXR, CAR and HNF4α). In contrast, Cyp2c12 and Cyp2c6 transcription and protein expression were not significantly altered during rat pregnancy although the intrinsic clearance of Cyp2c6-mediated diclofenac 4′-hydroxylation was increased 2-fold on day 19 of gestation when compared to nonpregnant controls. The increase in intrinsic clearance was due to a decrease in the K_m-value for 4′-hydroxydiclofenac formation. These data show that pregnancy significantly alters the expression and activity of drug metabolizing enzymes in an enzyme and gestational stage specific manner. These changes are likely to have toxicological and therapeutic implications.

1. Introduction

There is substantial evidence that pregnancy changes the disposition of many drugs and modifies the activity of drug metabolizing enzymes [1-3]. Increased clearance may lead to a lack of therapeutic effect or prolonged titration to achieve desired effect, while decreased clearance may lead to an increased occurrence of side-effects or toxicity due to elevated drug concentrations. Changes in drug clearance may also change the exposure of the developing fetus to the potentially harmful parent drug or its metabolites.

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CYP2C9, CYP2C19, and CYP2D6 are major drug metabolizing enzymes and together account for approximately 40% of all Phase I drug metabolism [4]. CYP2C9 plays a major role in the metabolism of phenytoin and anti-inflammatory drugs, whereas CYP2D6 is important in the metabolism of almost all antidepressants, including fluoxetine, paroxetine, tricyclic antidepressants and several of the newer antidepressants [5]. Changes in CYP2C9 and CYP2D6 activity are important in the treatment of pregnant women, since many of their substrates are commonly administered to pregnant women. Increases in enzyme activity could result in significantly decreased plasma concentrations of their substrates during pregnancy, leading to therapeutic failure.

The available clinical data, although limited, suggests that CYP2C9 and CYP2D6 mediated metabolism is increased during pregnancy, while metabolism catalyzed by CYP2C19 is decreased. An increased clearance of phenytoin, an anti-epileptic drug predominantly metabolized by CYP2C9, was reported during pregnancy in two studies [6,7] and phenytoin dosage had to be increased in 85% of pregnancies to maintain therapeutic efficacy [8]. In contrast, CYP2C19 mediated metabolism of proguanil to its active metabolite, cycloguanil, appears to decrease during pregnancy [9]. In a study of 44 women, the proguanil to cycloguanil plasma concentration ratio was 63% higher during the second and third trimesters of pregnancy compared to postpartum [9].

Two classical CYP2D6 probe substrates, dextromethorphan and metoprolol, have been used to assess CYP2D6 activity during human pregnancy. The data suggests that CYP2D6 activity increases significantly during pregnancy [3,10,11]. When evaluated at 26-30 weeks of gestation, the oral clearance of metoprolol increased 6-fold and the bioavailability decreased to half when compared to postpartum [10]. A significant decrease in the urinary dextromethorphan to dextrorphan metabolic ratio, indicative of an increase in CYP2D6 activity, was also observed at all trimesters of pregnancy [3]. Finally, in individuals genotyped for CYP2D6, a 53% decrease in dextromethorphan to dextrorphan plasma ratio was observed in extensive metabolizers whereas a 63% increase in the same ratio was observed in poor metabolizers during pregnancy [11].

The mechanisms underlying the indicated changes in CYP2C9, CYP2C19, and CYP2D6-mediated clearance during pregnancy are poorly understood. For this reason, the goal of the current study was to investigate changes in hepatic Cyp2c and Cyp2d expression and activity during pregnancy using the rat as a model and to investigate potential mechanisms behind any observed changes in Cyp2c or Cyp2d expression and activity. Hepatic Cyp2c and Cyp2d transcription and expression were measured by quantifying the mRNA and protein levels, as well as enzyme activity using isoform-specific probe substrates. Messenger RNA levels of nuclear hormone receptors known to regulate P450 expression were also measured and correlated to changes in P450 mRNA levels.

2. Materials and Methods

2.1 Animal studies

Animals were cared for in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication [12]. The experimental studies were approved by the Institutional Animal Care and Use Committee at the University of Washington. Virgin female Sprague-Dawley rats 8 weeks of age were mated and pregnancy was determined by the detection of a vaginal plug. Males were then removed and the females divided in random order into two groups. The rats in group one (n=6) were sacrificed at day 9 of gestation and the rats in group 2 (n=10) on day 19 of gestation. An additional 6 virgin, age-matched females, from the same cohort of animals, were sacrificed as non-pregnant controls at the same days as the pregnant animals were sacrificed. The animals

were sacrificed under deep isoflurane inhalation anesthesia by cardiac puncture and exsanguination between 9 am and 12 noon. Blood was collected and the plasma harvested and stored at -80° C. Upon sacrifice, liver was perfused via the portal vein using ice cold PBS and liver, intestine, kidney, and brain tissues were harvested, weighed, and washed in ice-cold PBS. The tissue samples were snap frozen in liquid nitrogen and stored at -80° C until further processing.

2.2 Preparation of rat liver microsomes

All procedures were performed on ice. Approximately 5 g of rat liver tissue was minced in 10 mL of homogenization buffer (50 mM potassium phosphate, pH 7.4, 0.25 M sucrose) and then homogenized in a glass tissue grinding tube by passing a tight-fitting Teflon pestle through the solution using an electric drill. The samples were then diluted to 100 mL final volume with homogenization buffer and centrifuged at 8,000 × g for 30 min at 4°C. The supernatant was carefully poured off and centrifuged at 110,000 × g for 70 min at 4°C. The supernatant was discarded and the pellet was resuspended in wash buffer (10 mM potassium phosphate, pH 7.4, 0.1 M potassium chloride) by vortexing until the microsomal pellet (and not the clear glycogen pellet) dislodged from the side of the tube. The pellet was then transferred to a glass tissue grinding tube and homogenized by hand using a Teflon pestle and resuspended in a final volume of 100 mL wash buffer. Samples were centrifuged again at 110,000 × g for 70 min at 4°C, the supernatant was discarded and the pellet was resuspended in ~2 mL of storage buffer (50 mM potassium phosphate, pH 7.4, 0.25 M sucrose, 10 mM EDTA) by vortexing and then homogenizing by hand in a small glass tissue grinding tube with a tight-fitting pestle. The resulting rat liver microsome samples were aliquoted and stored at -80° C until further use. Pooled microsomes from nonpregnant rats and from rats of gestational days 9 and 19 were prepared by combining equal amounts of microsomal protein from each liver in the group.

2.3 Spectral characterization of rat liver microsomes

Spectral studies were performed using a Varian Cary 3E UV-Visible Spectrophotometer. Spectral P450 contents were measured according to published methods [13] and P450 reductase levels were measured by reduction of cytochrome c according to the method of Phillips and Langdon [14] using the $\Delta A_{550\ nm}$/min and an ε= 21 $mM^{-1}cm^{-1}$ to calculate the nmol of cytochrome c reduced/min ([$\Delta A_{550\ nm}$/min]/0.021 = nmol cytochrome c reduced/min). Total protein was measured using the Pierce BCA Protein assay (Rockford, IL) according to manufacturer's protocol with bovine serum albumin as the standard.

2.4 RNA isolation and cDNA synthesis

Total liver RNA was isolated using TRI reagent® (Ambion, Austin, TX). Briefly, 25-50 mg of tissue was homogenized in 1 mL of TRI reagent® and further processed according to manufacturer's protocol. Purified RNA was resuspended in 100 μL of TE-buffer (10mM Tris, 1mM EDTA pH 7.5) and stored at -80° C until further use. Total RNA was quantified using the fluorescence based RiboGreen RNA Quantitation Assay (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The assay was prepared in a black 96-well plate (Molecular Devices, Sunnyvale, CA) using the high-range standard curve (0-1 μg/mL RNA) and 5 μL of a 1:100 dilution of purified liver RNA in a final volume of 200 μL. Fluorescence was measured using a Spectra Max Gemini EM (Molecular Devices) at excitation and emission wavelengths of 480 and 520 nm, respectively. RNA degradation was assessed by running 1 μg of RNA on a 1% agarose gel stained with SYBR Safe dye (Invitrogen, Carlsbad, CA). cDNA was prepared using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Total RNA (5 μg) and random hexamers were used in a final volume of 50 μL. After completion of cDNA synthesis, the reactions were

diluted to 250 μL final volume with TE and 5 μL of this was used per reaction for real-time PCR.

2.5 Real-Time PCR

Real-time reactions were set up in a MicroAmp optical 96-well reaction plate (Applied Biosystems, Foster City, CA). Cyp2c12, Cyp2d1-4, HNF1, HNF3β, HNF4α, HNF6, PXR, CAR, RARα, RXRα, RXRβ and GhR TaqMan assays were commercially available and purchased from Applied Biosystems. Both the target and reference (β2-microglobulin) reactions were run in duplicate per plate. Each TaqMan reaction contained 12.5 μL 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 μL appropriate 20× primer and FAM-labeled probe mix, 5 μL cDNA, and 6.25 μL water. Cycling conditions were an initial 95° C soak for 10 min followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min. A Cyp2c6 TaqMan assay was not commercially available and Cyp2c6 mRNA quantification was performed using a SYBR Green assay. Oligos were made to the exon 7/exon 9 junction [15] such that the Cyp2c7 pseudogene sequence would not be amplified. Cyp2c6 oligo sequences were: forward 5′-TCA GCA GGA AAA CGG ATG TG-3′, reverse 5′-AAT CGT GGT CAG GAA TAA AAA TAA CTC-3′ and β2-microglobulin sequences were: forward 5′-CGT GCT TGC CAT TCA GAA AA-3′ and reverse” 5′-GAA GTT GGG CTT CCC ATT CTC-3′. Each SYBR Green reaction contained 12.5 μL 2× SYBR Green PCR Master Mix (Applied Biosystems), 10 pmol of both the forward and reverse oligo, and 5 μl cDNA in a final volume of 25 μL. Cycling conditions were as above. Dissociation curves indicated only one amplicon at ~100bp for both the Cyp2c6 and β2-micorglobulin reactions. Data was analyzed using the comparative C_T ($\Delta\Delta C_T$) method (Applied Biosystems (1997) Relative Quantification of Gene Expression User Bulletin #2: ABI PRISM 7700 Sequence Detection System. http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf) with β2-microglobulin as the reference and the average of the non-pregnant ΔC_T values as the calibrator.

2.6 Western blot analysis for Cyp2c12 and Cyp2d1 content

Each of the 22 rat liver microsomal preparations was diluted in sample buffer to yield a final concentration of 4 μg per 15 μL. The diluted microsomal preparations were boiled (3 minutes), loaded onto 0.25% SDS-10% polyacrylamide gels (8 × 15 cm), and the proteins were separated by electrophoresis. The proteins were transferred for 1 hour at 100V and 1.5 A to nitrocellulose membranes (Immobilon-NC, Millipore, Billeria, MA) after which the membranes were placed overnight in blocking buffer [50% Odyssey block (LI-COR Biosciences, Lincoln, NE) 50% PBS] at room temperature. TWEEN 20 (final concentration 0.1%) was added together with the primary antibodies. The membranes were incubated with either rabbit anti-rat-CYP2C12 (1:4000) (Research Diagnostics Inc, Flanders, NJ) or rabbit anti-rat-CYP2D1 (1:6000) (Research Diagnostics Inc, Flanders, NJ) antibody and mouse anti-β-actin antibody (1:5000) (SIGMA, St Louis) for 1 hour after which the membrane was rinsed 4 times with PBS-Tween and incubated for 1 hour with the secondary antibody mixture (IRDye 800, Rockland Gilbertsville, PA anti-mouse 1:5000 and Alexa Fluor 680, Molecular Probes, Eugene, OR anti-rabbit; 1:3000) in 1:1 mixture of Odyssey blocking buffer and PBS-0.1% Tween. The membrane was rinsed again with PBS-0.1% Tween and stored in PBS at 4° C until imaged. The proteins of interest were visualized by fluorescence using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Integrated fluorescence intensities were obtained using the Odyssey software (version 2.1) and the ratio of fluorescence intensities between the enzyme of interest and β-actin was calculated. The relative expression of Cyp2c12 and Cyp2d1 on different days of pregnancy was calculated by dividing the fluorescence intensity ratio of CYP to β-actin of individual samples on day 9 and day 19 by the average intensity ratio in non-pregnant controls. All westerns were run in triplicate. The antibody selectivity was tested using a panel of recombinant rat P450 enzymes (Supersomes®, BD Gentest), including Cyp1a2,

Cyp2b1, Cyp3a2, Cyp2d1, Cyp2d2, Cyp2c6, Cyp2c11 and Cyp2c12. The Cyp2c12 antibody was found to cross react mildly with Cyp2c6, whereas the Cyp2d1 antibody was selective towards this isozyme.

2.7 Diclofenac incubations and metabolite analysis

Incubations contained 50 μg total protein (0.2 mg/mL), 0-200 μM diclofenac, and 100 mM potassium phosphate buffer in a final volume of 250 μL. Reactions were incubated for 3 min at 37° C and initiated by adding 1 mM final concentration of NADPH. Standard curves using 3′-, 4′- and 5-hydroxydiclofenac metabolites were set up in a similar fashion minus the addition of NADPH. All incubations were conducted in triplicate. After 15 min, the reactions were quenched by adding 100 μL of 75% acetonitrile/1% formic acid. The reactions were centrifuged at 16,000 × g and 100 μL was used for HPLC analysis. Formation of 3′-, 4′-, and 5-hydroxydiclofenac was determined using a Hewlett Packard series 1100 HPLC system equipped with a series 1100 UV-detector and HP Chemstation software. The mobile phase consisted of (A) acetonitrile/0.1% formic acid and (B) water/0.1% formic acid. The initial mobile phase conditions were 35% (A):65% (B) with a gradient to 80% (A):20% (B) starting at 18 min and ending at 25 min and a gradient back to initial conditions between 25 and 30 min with a 5 min post-run. The column used was a Synergi Max-RP, 150 × 4.6 mm, the flow rate was 1 mL/min, and the UV_{max} was 270 nm. Under these conditions all three metabolites were separated to baseline resolution.

2.8 Dextromethorphan incubations and metabolite analysis

Incubations with dextromethorphan contained 2 μg of rat liver microsomal protein (0.01 mg/mL) and 0 – 1000 μM dextromethorphan in 100 mM potassium phosphate (pH = 7.4) buffer. The reaction was initiated with NADPH (1mM final concentration) to make final incubation volume of 200 μL and samples were incubated for 10 minutes at 37° C. The reaction was terminated with 200 μL 100% acetonitrile, samples were centrifuged at 10,000 g for 10 minutes, and 200 μL of supernatant was transferred to autosampler vials. 20 μL of each sample was injected onto a 2.1 × 150 mm Zorbax Extend-C18 column. The samples were analyzed using a Micromass Platform LCZ mass spectrometer equipped with a Waters 2690 separations module and autosampler and Masslynx V3.4 data analysis software. Mobile phase A was 0.1% formic acid in 99.9% water and B was 0.1% formic acid in acetonitrile. The mobile phase gradient was as follows: 0 – 1.5 min: 90% A, 10% B; 1.5 min: 90% A, 10% B; 4 min: 70% A, 30% B; 6 min: 70% A,30% B; 9 min: 25% A, 75% B; 13 min: 25% A, 75% B; 13.5 min: 90% A, 10% B. Total run time was 20 min. Electrospray mass spectrometry, operating in the positive ion mode, was used to monitor m/z 258 and m/z 272 for dextrorphan and dextromethorphan, respectively. Cone voltages were 45 and 60 volts for dextrorphan and dextromethorphan, respectively.

2.9 Cell culture

HepG2 cells were maintained in Dulbecco's MEM (GIBCO) media supplemented with 10% (v/v) fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, and 50 mg/ml penicillin-streptomycin under 5% carbon dioxide atmosphere at 37°C. Cells were plated at 1×10^6 cells per well in 6 well plastic tissue culture plates and were given 24 hours to adhere before treatment began. The HepG2 cells were treated with either 10 nM or 500 nM *all-trans* retinoic acid for 24 hours and then harvested with 1 mL TRI reagent® and stored at -80° C. RNA was extracted and CYP2D6, HNF1 and HNF3α and β transcripts were quantified using real time PCR and GAPDH as the housekeeping gene as described for the rat liver tissue. TaqMan assays for these genes were commercially available and purchased from Applied Biosystems.

2.10 Data Analysis

Kinetic parameters were determined using WinNonlin (Pharsight, Mountain View, CA) and linear regression analysis was performed using Stata (College Station, TX). Differences in mRNA transcripts, protein expression, and activity were tested by ANOVA with unpaired two-sided Student's t-test as the post hoc test. A p-value <0.05 was considered significant. Differences between the K_m and V_{max} values were evaluated by use of the one-tailed Z-test. The upper and lower limit of the 95% confidence interval provided by the nonlinear fits were used to calculate the standard error and the values were then compared to determine statistical significance. A Bonferroni adjustment for multiple comparisons was applied so that a p-value <0.01 was considered significant. The first 3000bp of the rat cyp2d2 promoter were analyzed using AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) to determine potential nuclear hormone receptor binding sites and identify possible HNF1, HNF3, HNF4α and RAR binding sites.

3. Results

3.1 Characterization of pregnant rat liver microsomes

Liver weights, microsomal protein, cytochrome P450 content and reductase activity for non-pregnant (control), day 9 and day 19 pregnant animals are shown in Table 1. The liver weight increased significantly from 9.2 g in non-pregnant animals to 11.2 and 13.6 g at day 9 and 19 of gestation, respectively. Liver microsomal protein content (in mg protein/g liver) and microsomal P450 content were significantly lower at day 19 of pregnancy than in nonpregnant animals (Table 1). However, when scaled to the whole liver, there was no overall change in total liver microsomal protein with values of 82 ± 8, 84 ± 13, and 87 ± 21 mg microsomes/liver in control, day 9, and day 19 samples, respectively. In contrast, total P450 content was decreased from 76 ± 12 nmol P450 in controls to 60 ± 10 nmol P450 for day 19 samples ($p<0.01$). Hepatic cytochrome P450 reductase activity remained unchanged at the gestational ages tested.

3.2 Cyp2d and Cyp2c transcription and protein expression in the pregnant rat

To determine whether pregnancy alters Cyp2c and Cyp2d transcription and expression in the rat, the mRNA levels of Cyp2c6, Cyp2c12, Cyp2d1, Cyp2d2, Cyp2d3 and Cyp2d4 and the protein levels of Cyp2c12 and Cyp2d1 were measured at different gestational ages in the rat using quantitative real-time PCR and Western blotting. The average mRNA levels of Cyp2c6 and Cyp2c12 and the relative protein levels of Cyp2c12 were unaltered ($p>0.05$) by pregnancy (Figure 1). In contrast, at day 19 of gestation, transcript levels were significantly decreased for Cyp2d1, Cyp2d2, and Cyp2d4 (54%, 65%, and 77%, respectively, $p<0.05$) when compared to non-pregnant values (Figure 2A). Day 19 values were also significantly decreased compared to day 9 values for Cyp2d2 and Cyp2d4 (Figure 2A). The decrease in Cyp2d1 and Cyp2d2 transcripts at day 9 of gestation when compared to non-pregnant rats was not significant (Cyp2d1, $p=0.4$; Cyp2d2, $p=0.06$) and the transcription of Cyp2d3 was not changed on the different days. Despite the decreased transcription, there was no significant decrease in the relative protein expression at day 9 and 19 for Cyp2d1 (Figure 2B).

3.3 Cyp2d2 and Cyp2c activity in the pregnant rat liver

The rate of dextromethorphan *O*-demethylation, a measure of Cyp2d2 activity, was significantly decreased ($p<0.05$) during pregnancy in individual rat liver microsomes when compared to non-pregnant controls when dextromethorphan concentration of 1μM was used (Figure 3A). On average, a 50% decrease in dextromethorphan *O*-demethylation was observed in both days 9 and 19 of gestation when compared to non-pregnant rats. Kinetic parameters for dextromethorphan *O*-demethylation were measured using pooled hepatic microsomes from

non-pregnant, day 9 and day 19 pregnant rats (Figure 3C). The V_{max} for dextromethorphan *O*-demethylation was decreased from the control value of 0.72 pmol/min/pmol P450 to 0.60 and 0.40 pmol/min/pmol P450 at day 9 and 19 of gestation, respectively, whereas the K_m was increased 3-4 fold during pregnancy when compared to controls (Table 2). The decrease in V_{max} coupled with the increase in K_m led to an 80% decrease in the Cl_{int} at both days 9 and 19 of gestation (Table 2).

Pregnancy did not alter Cyp2c activity in the rat liver based on diclofenac 3′- and 4′-hydroxylation rate in individual liver microsomes at 5 μM concentration of diclofenac (Figure 3B). However, the V_{max} value for Cyp2c12 and Cyp2c6 mediated formation of 3′-hydroxydiclofenac was significantly decreased ($p<0.005$) from 0.53 pmol/min/pmol P450 (non-pregnant value) to 0.42 and 0.33 pmol/min/pmol P450 at days 9 and 19 respectively, while the V_{max} values for 4′-hydroxydiclofenac formation (Cyp2c6 mediated) were unchanged ($p>0.025$) at day 9 and 19 of pregnancy (Table 3). At day 19, the K_m values for 3′- and 4′-hydroxylation were decreased approximately 50% compared to both nonpregnant and day 9 values (Table 3). Eadie-Hofstee plots for all gestational ages were linear (data not shown). Due to the decrease in K_m at day 19, the Cl_{int} for 4′-hydroxydiclofenac doubled at this time point compared to the non-pregnant and day 9 pregnant Cl_{int} values.

When the intrinsic clearance was scaled to whole liver, the intrinsic clearance for dextromethorphan *O*-demethylation decreased almost 90% by day 19 of pregnancy despite the increase in liver weight. The whole liver Cl_{int} values were 22.1mL/min in controls and 4.0 mL/min and 3.0 mL/min at day 9 and day 19 of gestation. For diclofenac, the 3′-hydroxylation whole liver intrinsic clearance did not change during pregnancy (0.76, 0.47 and 0.60 mL/min at control, day 9 and day 19 of pregnancy, respectively) whereas a 50% increase in the whole liver intrinsic clearance was observed for diclofenac 4′-hydroxylation by day 19 of gestation (1.22, 0.94 and 1.92 mL/min for controls, day 9 and day 19 of pregnancy, respectively).

3.4 Changes in the transcription of nuclear hormone receptors during pregnancy

To investigate possible mechanisms behind the changes in Cyp2d mRNA levels, the transcript levels of nuclear receptors, which are known to be involved in the regulation of cytochrome P450 expression, were examined. The relative fold changes in nuclear receptor mRNA levels at day 9 and 19 of gestation are summarized in Table 4. The mRNA levels of HNF3β and RARα were decreased by approximately 50% ($p<0.05$) at day 19, compared to non-pregnant values, while growth hormone receptor (GhR) transcripts were increased at day 19 by approximately 50% ($p<0.05$). Correlation analysis was performed between the fold change in Cyp2d mRNA levels and the fold change in nuclear hormone receptor mRNA levels. Associations between changes in Cyp2d levels and changes in several nuclear receptor levels were observed (Table 5). Significant correlations between all four Cyp2ds and HNF3β and RARα were observed (Table 5 and Figure 4), as well as between Cyp2d1, 2d2, and 2d3 and HNF1; Cyp2d1, 2d3, and 2d4 and RXRα; and between Cyp2d1 and HNF6 (Table 5 and Figure 4).

Multiple regression analysis was conducted between Cyp2d transcripts and HNF1, HNF3β and RAR as well as between the three nuclear hormone receptors. For all four Cyp2d enzymes, neither HNF1 nor HNF3β improved significantly the correlation observed with the Cyp2ds and RAR. HNF3β did not improve the correlation between HNF1 and Cyp2d enzymes either. HNF1, HNF3β and RAR correlated with one another as well but multiple regression analysis showed that the correlation between HNF1 and RAR was not improved by HNF3β.

Analysis of the rat Cyp2d2 promoter yielded four potential RARα binding sites at locations −558, -1521, -1563 and −1731 bp from the transcription initiation site. Two putative HNF1

binding sites at −48 and −1052 bp and one putative HNF4α binding site at −404 bp were also identified. No putative HNF3 binding sites were discovered.

3.5 Cell culture experiments

To investigate the potential role of retinoic acid and RAR in CYP2D regulation, we tested the effect of increasing retinoic acid concentration in CYP2D6 transcription using HepG2 cells. Following 500 nM retinoic acid treatment, CYP2D6 transcripts were undetectable in all samples despite a robust transcription in control cells, demonstrating a significant downregulation of CYP2D6 by retinoic acid. After 10 nM treatments, two out of the three treated samples had undetectable CYP2D6 transcript levels and one sample was unchanged. No fold change in CYP2D6 transcription could be calculated because of the total disappearance of the transcripts, but based on the control values the decrease was >100-fold at 500 nM retinoic acid. In contrast to CYP2D6, HNF3α, HNF3β and HNF1 transcript levels were unchanged following retinoic acid treatments.

4. Discussion

The disposition of many drugs studied in pregnant women is altered during gestation and pharmacokinetic studies using probe drugs suggest that P450 and UGT activity is also altered during pregnancy [3,16]. Increased P450 activity observed during pregnancy could be due to increased transcription (mRNA) of the P450 genes, increased protein translation, improved stability of mRNA or protein or allosteric modulation of enzyme activity. The goal of this study was to establish, using the rat as a model, whether hepatic CYP transcription, expression and activity are altered during pregnancy focusing on Cyp2c and Cyp2d isoforms. Of these, Cyp2d1, Cyp2d2 and Cyp2c6 were of special interest as they have been suggested to be the rat orthologs of human CYP2D6 and CYP2C9 respectively.

Significant changes were observed in the liver physiology during pregnancy. As observed by others [17-19], rat liver weight was increased by 67% by day 19 of gestation. Our data showed, however, that despite the increased liver weight, total liver microsomal protein was unaltered during pregnancy. In agreement with other studies, the total hepatic P450 content in the rat was decreased by day 19 of gestation [17,18,20,21].

In parallel with the decrease in P450 content, a significant decrease in Cyp2d2 activity was observed. The intrinsic clearance and V_{max} for dextromethorphan *O*-demethylation decreased approximately 50% by day 19 of gestation when compared to non-pregnant controls. Interestingly, in addition to the change in V_{max}, the K_m value for dextromethorphan *O*-demethylation was increased approximately 4-fold at day 9 and 3-fold at day 19 when compared to nonpregnant values. The increase in K_m as well as the decrease in V_{max} could be due to pregnancy related increased concentrations of an endogenous inhibitor that behaves as a mixed type inhibitor or other allosteric changes in Cyp2d2 protein. Alternatively these changes could be due to changes in other enzymes that contribute to dextromethorphan *O*-demethylation. Cyp2c6 could potentially be one such enzyme, as data from our lab using a panel of rat CYP supersomes® (Gentest) indicates that this reaction can also be catalyzed by Cyp2c6 at high (100-1000 μM) substrate concentrations (data not shown). The Eadie-Hofstee plots, however, indicated a single enzyme model (data not shown) and dextromethorphan *O*-demethylation has been previously shown to be catalyzed specifically by Cyp2d2 [22].

The decrease in the Cl_{int} and V_{max} for dextromethorphan *O*-demethylation paralleled the changes in Cyp2d2 mRNA levels. The excellent correlation between pregnancy mediated decrease in V_{max} and mRNA supports the hypothesis that changes in Cyp2d activity during pregnancy are at least in part due to transcriptional regulation. Unfortunately, no antibody for Cyp2d2 was commercially available and it was not possible to confirm decreased Cyp2d2

protein levels. Cyp2d1 and Cyp2d4 mRNA levels were also decreased gradually in gestational stage-specific manner, although this was not reflected in Cyp2d1 protein levels. The lack of significant decrease in Cyp2d1 protein content could be due to the semiquantitative nature of western blot assays, or too small sample size with high basal variability in expression, to obtain sufficient power to detect 50% decrease in protein levels.

Cyp2d transcription may be altered by changes in nuclear hormone receptor expression, changes in availability of ligands of nuclear hormone receptors or by epigenetic mechanisms. We investigated the possibility that during pregnancy, the transcription of nuclear hormone receptors is changed, leading to increased or decreased transcription of P450 genes. Of the nuclear hormone receptors studied, only HNF3β, GhR and RARα changed significantly in their transcription during pregnancy. The mRNA levels for HNF1, HNF3β, RARα, and RXRα correlated significantly with the mRNA levels for several Cyp2d genes suggesting that these nuclear hormone receptors may contribute to the decreases in Cyp2d mRNA levels. As two putative HNF1 and several potential RAR binding sites were identified in the Cyp2d2 promoter, the contribution of these transcription factors requires further investigation.

Based on multiple regression analysis, variation in RAR transcripts when compared to HNF1 or HNF3β explains the majority of changes in Cyp2d1, Cyp2d2 and Cyp2d4 transcription. Therefore we used cell culture experiments to investigate whether RAR and retinoic acid play a role in CYP2D6 regulation. The cell culture experiments demonstrated that altered retinoic acid concentrations lead to changes in CYP2D6 transcription in retinoic acid concentration dependent manner, supporting the hypothesis that RAR is involved in regulating CYP2D transcription during pregnancy. Despite the fact that HNF3α has been shown to be a direct target of retinoic acid [25] no effect of HNF3 transcription was observed after retinoic acid treatments.

Based on the Cyp2d2 promoter analysis and the cell culture experiments it is likely that HNF1 and RAR regulate Cyp2d2 transcription independent of each other. The role of retinoic acid in CYP2D6 regulation has not been previously reported whereas HNF family has been shown to affect Cyp2d transcription. In HNF1α null mice, Cyp2d10 expression was decreased whereas Cyp2d9 expression was significantly elevated [24]. A role of HNF4α in CYP2D transcriptional regulation has been suggested by anti-sense targeting of HNF4α in human hepatocytes [26]. As no putative response element for HNF3 was found in Cyp2d2, HNF3β may be involved in CYP2d regulation via altering HNF transcription. HNF3β has been shown to be involved in regulation of HNF1 transcription [23].

In contrast to Cyp2d enzymes, there was no significant change in the transcription of Cyp2c12 and Cyp2c6 or in the expression of Cyp2c12, demonstrating that transcriptional changes caused by pregnancy are P450 isoform-specific. In the female rat, Cyp2c6 and Cyp2c12 are considered the predominant Cyp2c isoforms, while Cyp2c11 is male-specific and Cyp2c7 encodes for a non-sense splice variant of Cyp2c6 [15,26]. Despite unchanged Cyp2c protein and mRNA levels, the K_m was halved for diclofenac 3′ and 4′-hydroxylation at day 19 of gestation and V_{max} decreased significantly on day 9 and 19 for 3′-hydroxylation suggesting that physiological changes that occur in the liver during pregnancy alter intrinsic P450 function. Alternatively, these changes may be a result of induction of an unidentified low K_m enzyme. We have observed that diclofenac 3′-hydroxylation can be mediated by both Cyp2c6 and Cyp2c12 equally well at 5 and 25 μM substrate concentrations, while 4′-hydroxylation is mediated by Cyp2c6 and to a lesser extent by Cyp3a1 and 3a2 (approximately 50% at 5 μM and 20% at 25 μM compared to Cyp2c6; data not shown). Since Cyp3a1 and 3a2 are male-predominant isoforms [27,28], it is assumed that Cyp3a will not contribute significantly to diclofenac 4′-hydroxylation in the female rat. As other P450 enzymes tested did not catalyze diclofenac hydroxylation and Eadie-Hofstee plots were linear, diclofenac 3′-hydroxylation was

accepted as a measure of combined Cyp2c6 and Cyp2c12 activity and diclofenac 4′-hydroxylation as a measure of Cyp2c6 activity. Total diclofenac hydroxylation has also been previously reported to be specifically catalyzed by Cyp2c6 among the same panel of rat isoforms [22].

Changes in lipid content, microsomal membrane composition, and allosteric protein modification would all be plausible explanations for the simultaneous decrease in K_m and V_{max} of diclofenac hydroxylation. Alteration of the enzyme membrane environment as well as an endogenous allosteric modulation could change both substrate binding and catalysis of the enzyme substrate complex simultaneously leading to a mixed type inhibition. Lipid content has been shown to influence P450 structure and substrate binding [29]. For CYP1A, changes in phospholipid content coincided with spectral changes associated with substrate binding [30,31] whereas the K_m of cholesterol binding to CYP11A1 is modified by lipid fatty acyl content. Pregnancy in the rat reduces the total phospholipid concentrations of microsomal membranes, and decreases the ratio of microsomal phosphatidylcholine to phosphatidylethanolamine leading to a reduction in the high spin form of ferricytochrome P450 [32,33]. Other protein-protein interactions, as shown between albumin and several CYP2C9 substrates *in vitro* [34,35] could also alter substrate turnover. Direct modulation of the enzyme itself could make it difficult to extrapolate from one substrate to another for the same isoform. Consequently, pharmacokinetic changes for different substrates of the same CYP isoform during pregnancy warrant further investigation both in animal models and in humans.

Finally, characterization of P450 activity in the pregnant rat is an important step towards better understanding of the appropriateness of the rat as a model for drug disposition and fetal safety of xenobiotics during human pregnancy. Several studies using coumarin, progesterone, dehydroepiandrosterone, aminopyrine, styrene, toluene, and trichloroethylene have indicated a general decrease in drug metabolism during pregnancy in the rat although no studies using probe substrates have been published [20,36-38]. The results of this study clearly demonstrate that the effects of pregnancy on cytochrome P450 expression and activity are enzyme-specific and that there is no general “pregnancy effect”. The rat is used regularly in pre-clinical pharmacokinetic and toxicological studies and the resulting data is routinely extrapolated to humans. It should be noted that based on clinical observations of increased CYP2D6 activity during human pregnancy and Cyp2d2 being the proposed ortholog of CYP2D6 in the rat, there is poor concordance between the human and the rat pregnancy.

In conclusion, we have shown that pregnancy has enzyme and gestational age-specific effects on CYP transcription, expression and activity in the rat liver emphasizing the importance of studying pharmacokinetic changes in pregnant women for different substrates and at multiple gestational stages. We have also identified several mechanisms that could contribute to the changes in CYP activity during pregnancy. These include changes in nuclear hormone receptor transcription, which may lead to altered CYP transcription, and altered K_m values. Additional studies will need to be conducted to further decipher these mechanisms.

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Nonstandard abbreviations used

RT-PCR
: real time PCR

RAR
: retinoic acid receptor

RXR
: retinoid X receptor

HNF
: hepatic nuclear factor

PXR
: pregnane X receptor

CAR
: constitutive androstane receptor

PBS
: phosphate buffered saline

TE

tris-EDTA

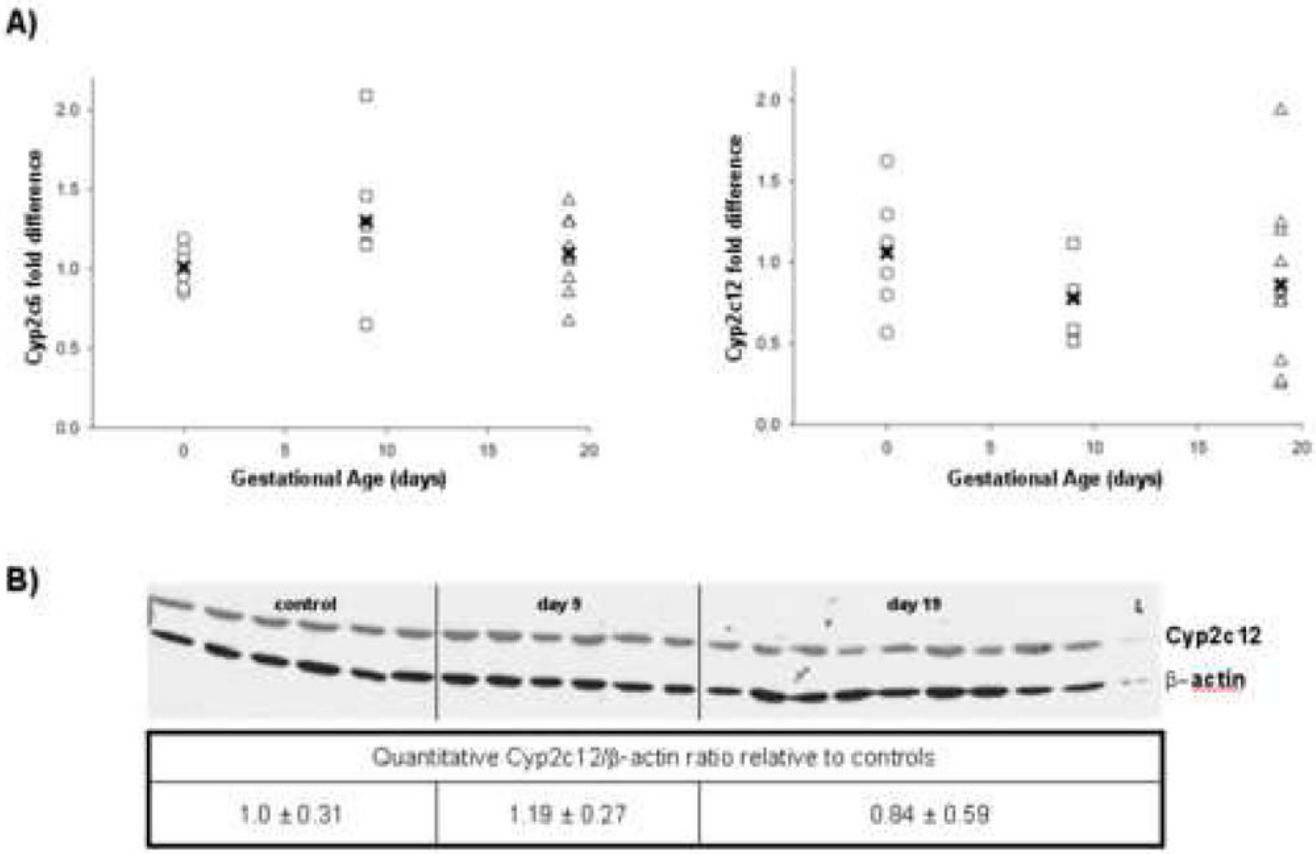

Figure 1.

Effects of pregnancy on rat hepatic Cyp2c6 and Cyp2c12 mRNA (A) and Cyp2c12 protein (B) levels, measured in non-pregnant (n=6), day 9 (n=6) and day 19 (n=10) pregnant rats. A) The fold difference in mRNA at days 9 and 19 were calculated relative to non-pregnant values. The bold **x** indicates mean values for that time point. Each symbol depicts an individual animal. Mean values for Cyp2c6 fold change in mRNA were 1.01 ± 0.14, non-pregnant; 1.30 ± 0.47, day 9; and 1.10 ± 0.24, day 19. Mean values for Cyp2c12 mRNA were 1.06 ± 0.38, non-pregnant; 0.78 ± 0.21, day 9; and 0.86 ± 0.52, day 19. No significant ($p>0.05$) differences were observed for both mRNA and protein at any of the gestational ages.

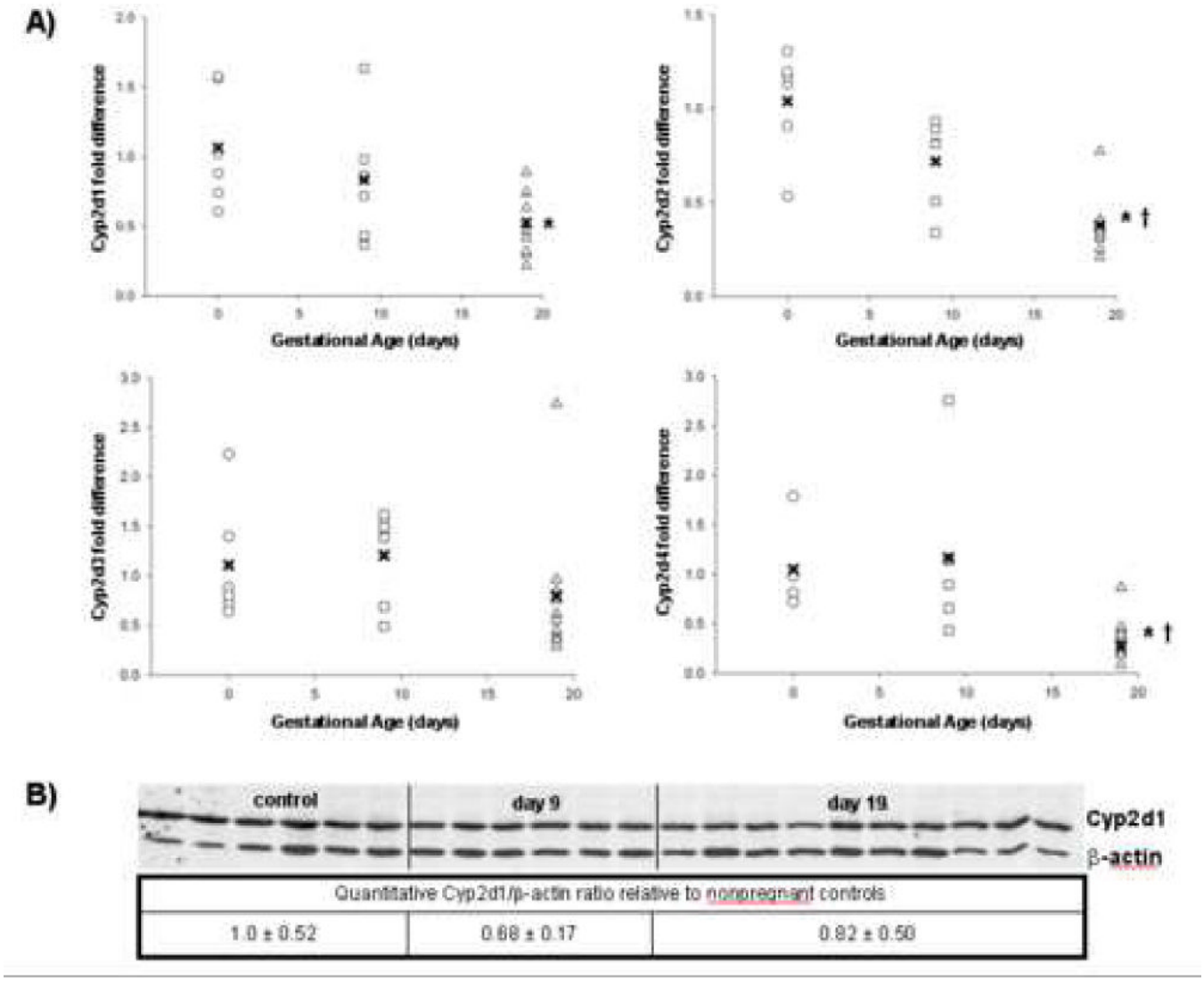

Figure 2.

Effects of pregnancy on rat hepatic Cyp2d1, 2d2, 2d3, and 2d4 mRNA (A) and Cyp2d1 protein (B) levels measured in non-pregnant (n=6), day 9 (n=6), and day 19 (n=10) pregnant rats. A) The fold difference in mRNA at days 9 and 19 were calculated relative to non-pregnant values. The bold **x** indicates mean values for that time point. Mean values for fold change in Cyp2d1 mRNA are 1.07 ± 0.42, non-pregnant; 0.83 ± 0.46, day 9; and 0.52 ± 0.22, day 19. Mean values for Cyp2d2 mRNA are 1.04 ± 0.28, non-pregnant; 0.72 ± 0.24, day 9; and 0.38 ± 0.15, day 19. Mean values for Cyp2d3 mRNA are 1.11 ± 0.61, non-pregnant; 1.21 ± 0.49, day 9; 0.80 ± 0.72, day 19. Mean values for Cyp2d4 mRNA are 1.05 ± 0.38, non-pregnant; 1.17 ± 0.82, day 9; and 0.28 ± 0.14, day 19. Statistical significance ($p<0.05$) when compared to non-pregnant values is indicated by an asterisk and to day 9 values by a dagger.

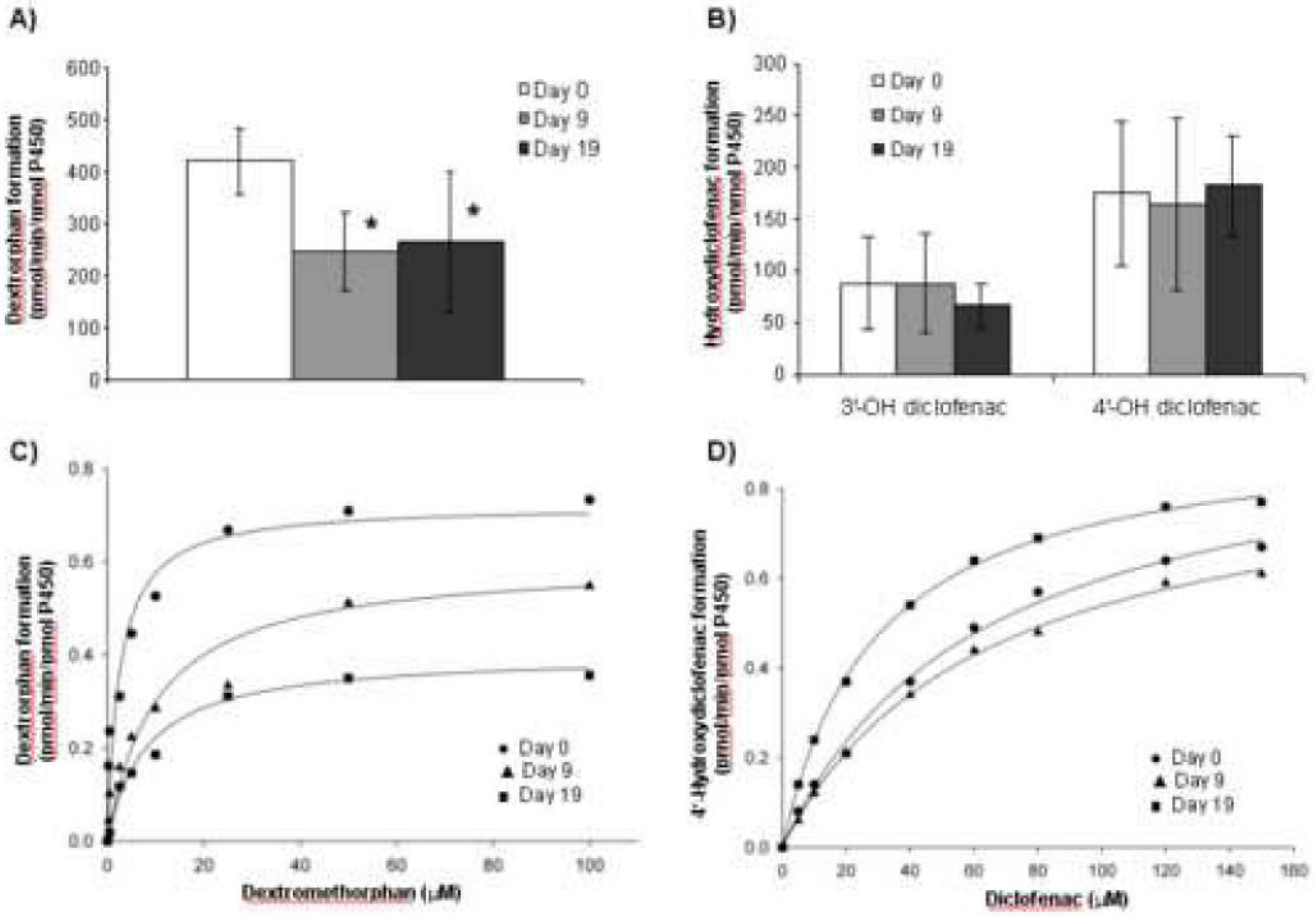

Figure 3.

Diclofenac hydroxylase and dextromethorphan *N*-demethylase activity in microsomes from non-pregnant, day 9, and day 19 pregnant rats. Individual microsomes from non-pregnant (n=6), day 9 (n=6), and day 19 (n=10) were incubated with either A) 1 μM dextromethorphan or B) 5 μM diclofenac as described in the Materials and Methods section. Data were compared using the student t-test and statistical significance ($p<0.05$) to non-pregnant values is indicated by an asterisk. Pooled microsomes from each time point were used to obtain velocity versus substrate concentration curves for C) dextrorphan and D) 4′-hydroxydiclofenac formation. Each data point represents the average of two separate values.

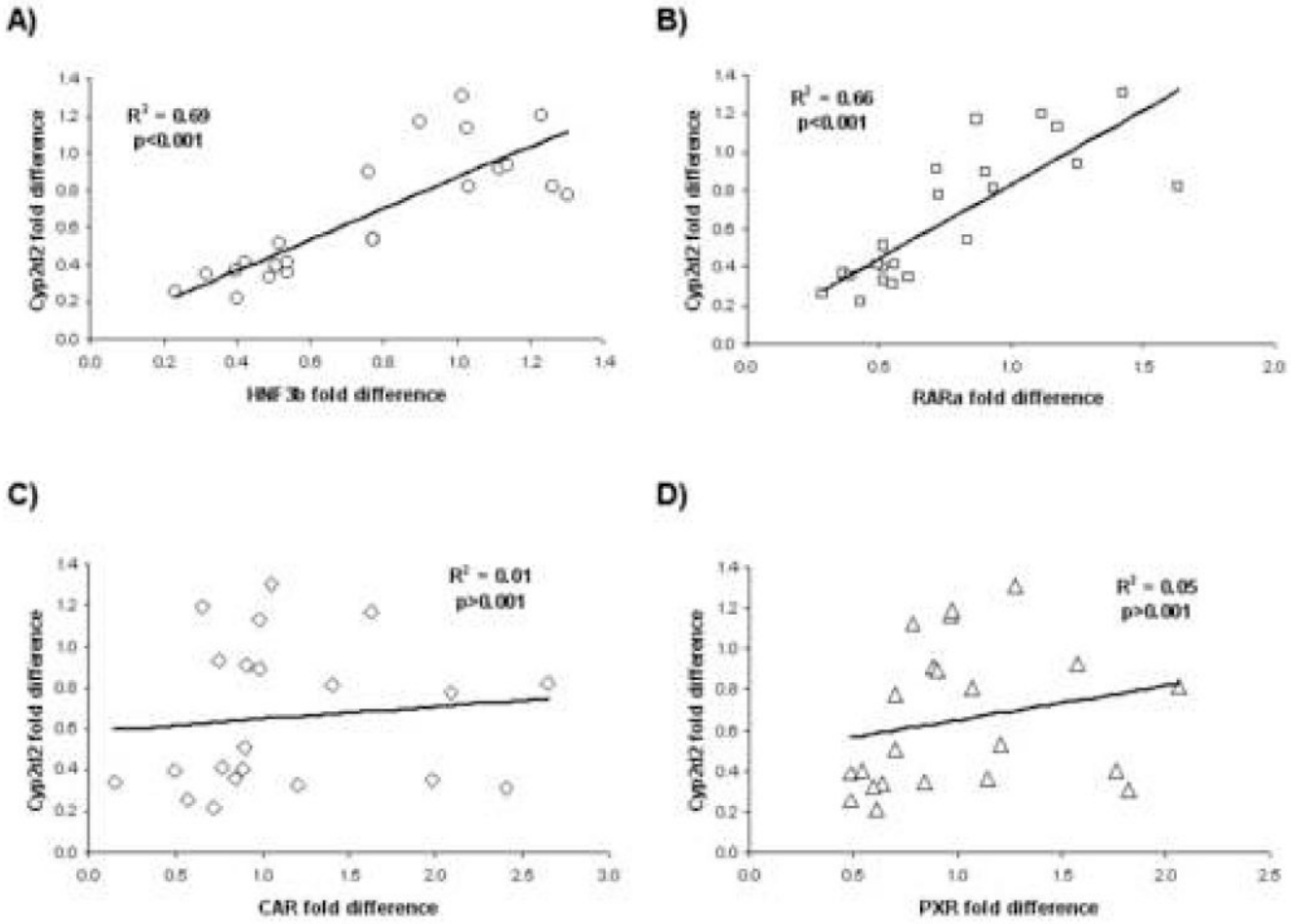

Figure 4.

Correlation between the fold difference in Cyp2d2 and nuclear receptor mRNA levels for non-pregnant, day 9, and day 19 pregnant rats. The fold difference in Cyp2d2 mRNA levels was plotted versus the fold difference in A) HNF3β, B) RARα, C) CAR, and D) PXR. R-squared and *p*-values are indicated on the figure. *P*-values were calculated using linear regression.

Table 1

Liver weight, microsomal protein and total cytochrome P450 contents, and reductase activity of non-pregnant (n=6) and pregnant rats at days 9 (n=6) and 19 (n=6) of gestation

	Non-pregnant	Day 9	Day 19
Liver Weight (g)	9.2 ± 0.9	11.2 ± 1.3[a]	13.6 ± 1.6[a,b]
Microsomal Protein (mg protein/g liver)	8.9 ± 0.6	7.4 ± 1.0	6.4 ± 1.4[a]
Total liver microsomal protein (mg microsomes/liver)	82 ± 8	84 ± 13	87 ± 21
P450 content (nmol P450/mg protein)	0.93 ± 0.10	0.81 ± 0.11	0.69 ± 0.07[a,b]
Total liver P450 content (nmol P450/liver)	76 ± 12	68 ± 10	60 ± 10 [a]
Reductase activity (nmol cyt. c reduced/min/mg)	282 ± 28	248 ± 34	281 ± 39

[a] Significantly different from non-pregnant controls, $p<0.01$

[b] Significantly different from Day 9

Table 2

Kinetic parameters for microsomal dextromethorphan *O*-demethylation at different gestational ages using pooled liver microsomes (n=6-10)

	Non-pregnant	Day 9	Day 19
K_m (μM)	2.5 ± 0.6	10.1 ± 2.2[a]	8.0 ± 1.7[a]
V_{max} (pmol/min/pmol P450)	0.72 ± 0.04	0.60 ± 0.04	0.40 ± 0.02[b]
Cl_{int} (mL/nmol P450/min)	0.29	0.06	0.05

[a] Significantly different from non-pregnant values, p<0.0025

[b] Significantly different from non-pregnant and day 9 pregnant values, p<0.0005

Table 3

Kinetic parameters for microsomal diclofenac hydroxylation at different gestational ages using pooled liver microsomes

(n=6-10). 3′-OH and 4′-OH refer to 3′-hydroxydiclofenac and 4′-hydroxydiclofenac respectively.

		K_m (μM)	
	Non-pregnant	Day 9	Day 19
3′-OH	55 ± 5	62 ± 4	34 ± 1[a]
4′-OH	62 ± 6	63 ± 4	29 ± 1[a]
		V_{max} (pmol/min/pmol P450)	
	Non-pregnant	Day 9	Day 19
3′-OH	0.53 ± 0.02	0.42 ± 0.01[b]	0.33 ± 0.01 [b,c]
4′-OH	0.97 ± 0.04	0.88 ± 0.02	0.94 ± 0.03
		Cl_{int} (ml/nmol P450/min)	
	Non-pregnant	Day 9	Day 19
3′-OH	0.010	0.007	0.010
4′-OH	0.016	0.014	0.032

[a] Significantly different from non-pregnant and day 9 pregnant values, p<0.0005

[b] Significantly different from non-pregnant values, p<0.0005

[c] Significantly different from day 9 pregnant values, p<0.0005

Table 4

Maternal hepatic nuclear receptor mRNA levels at days 9 (n=6) and 19 (n=10) of pregnancy

Values represent the fold difference compared to non-pregnant animals (n=6).

	Day 9	Day 19
HNF1	0.91 ± 0.36	0.76 ± 0.29
HNF3β	0.84 ± 0.47	0.63 ± 0.40[a]
HNF4α	0.76 ± 0.30	0.82 ± 0.21
HNF6	0.94 ± 0.38	0.80 ± 0.24
PXR	1.16 ± 0.55	0.90 ± 0.50
CAR	1.14 ± 0.84	1.20 ± 0.70
RARα	0.98 ± 0.42	0.49 ± 0.12[a]
RXRα	0.91 ± 0.59	0.74 ± 0.28
RXRβ	1.34 ± 0.75	1.21 ± 0.49
GhR	0.73 ± 0.25	1.51 ± 0.44[a]

[a] Significantly different from non-pregnant values, $p<0.01$.

Table 5

Correlations between the fold change in maternal hepatic Cyp2d and receptor mRNA levels (n=22)

Numbers represent the R-squared values obtained for correlation analysis between the fold change in Cyp2d mRNA levels versus the fold change in receptor mRNA levels.

	Cyp2d1	Cyp2d2	Cyp2d3	Cyp2d4
HNF1	0.42[a]	0.49[a]	0.61[a]	0.33
HNF3β	0.57[a]	0.69[a]	0.53[a]	0.48[a]
HNF4α	0.30	0.32	0.13	0.11
HNF6	0.43[a]	0.35	0.38	0.35
CAR	0.09	0.01	0.10	0.13
PXR	0.10	0.05	0.07	0.27
RARα	0.73[a]	0.66[a]	0.68[a]	0.84[a]
RXRα	0.66[a]	0.38	0.45[a]	0.68[a]
RXRβ	0.15	0.03	0.11	0.13
GhR	0.04	0.10	0.01	0.23

[a] $p<0.001$ using linear regression analysis.