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Short Communication

Pregnancy Decreases Rat CYP1A2 Activity and Expression

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ABSTRACT:

Pregnancy results in increased CYP3A- and CYP2D6-mediated clearance but decreases the clearance of CYP1A2 probe drugs. The aim of this study was to determine whether the decreased CYP1A2 activity during human pregnancy could be explained by decreased expression of CYP1A2 protein and mRNA using the rat as a model. Potential mechanisms leading to decreased CYP1A2 activity and expression were also investigated. Hepatic CYP1A2 activity, protein, and mRNA were measured during mid- and late gestation and compared to nonpregnant control levels. In addition, the effect of 17-β-estradiol and progesterone on CYP1A2 mRNA levels was assessed using rat hepatocytes, and the effect of estrogens or progesterone on CYP1A2 activity in vitro was tested. CYP1A2-mediated probe clearance decreased between 48 and 62% ($p < 0.05$) during pregnancy, with no difference in CYP1A2 activity between mid- and late pregnancy. This decrease in probe clearance was accompanied by a 33 ± 8% (midpregnancy) and 29 ± 27% (late pregnancy) decrease in CYP1A2 protein expression ($p < 0.05$) and a 53% decline in methoxyresorufin *O*-demethylation V_{max} ($p < 0.05$). CYP1A2 mRNA was not significantly different from controls at midpregnancy and decreased by 27 ± 20% ($p < 0.05$) of control during late pregnancy. Estradiol and progesterone had no effect on CYP1A2 mRNA in rat hepatocytes and did not inhibit CYP1A2 activity. These data demonstrate that pregnancy decreases CYP1A2 activity and expression with a modest effect on CYP1A2 mRNA and suggest that the rat can be used as a model to study mechanisms by which pregnancy decreases CYP1A2 activity in humans.

Introduction

Pregnancy alters the disposition of drugs, and efforts have been made to better understand the enzyme-specific changes as well as the mechanisms that cause these changes. Based on probe substrate studies, the activity of CYP3A4, CYP2C9, and CYP2D6 is increased up to 5-fold during pregnancy but CYP1A2 activity is decreased (Tracy et al., 2005; Anderson, 2005). During late pregnancy, the oral clearance of theophylline decreased by 37% (Carter et al., 1986) and concentrations of melatonin increased by 80% (Kivela, 1991). In a similar manner, caffeine salivary clearance decreased by 33, 42, and 65% during the first, second, and third trimesters of pregnancy, respectively (Tracy et al., 2005). These studies suggest that the mechanisms by which pregnancy alters CYP1A2 activity differ from those leading to increased CYP3A4, CYP2C9, and CYP2D6 activity.

Some of the changes in cytochrome P450 (P450) activity observed in humans have been replicated in animal models, whereas others have shown discrepant results. In the mouse, Cyp3a activity and protein expression increased during pregnancy (Mathias et al., 2006; Zhang et al., 2008), whereas in pregnant rats, CYP2D2 activity and mRNA decreased and CYP2C was unchanged (Dickmann et al., 2008). After oral administration to pregnant rats, the area under the plasma concentration time curve of caffeine was not different from that for nonpregnant rats, but the area under the plasma concentration time curve of theobromine and theophylline (formed by rat CYP1A2) decreased, suggesting reduced CYP1A2 activity (Abdi et al., 1993). Decreased methoxyresorufin *O*-demethylation (MROD) was also reported during rat pregnancy with an opposing increase in CYP1A2 protein expression (Czekaj et al., 2005). Due to these conflicting results, the aim of this study was to determine whether the decrease in CYP1A2 activity during pregnancy is due to decreased expression of CYP1A2 protein, decreased CYP1A2 mRNA, alteration of intrinsic CYP1A2 activity, or competitive inhibition of CYP1A2 using the rat as a model.

Materials and Methods

Chemicals. Potassium phosphate, glucose 6-phosphate, glucose 6-phosphate-dehydrogenase, NADPH, $NADP^+$, magnesium chloride, caffeine, paraxanthine, theobromine, and theophylline were obtained from Sigma-Aldrich (St. Louis, MO). Caffeine was purified from contaminating metabolites using an Agilent 1100 high-pressure liquid chromatography with UV detection system (Agilent Technologies, Santa Clara, CA) equipped with an Econosphere C18 column (250 × 10 mm, 10 μm; Alltech Associates, Deerfield, IL) using an isocratic elution with 12% ammonium acetate (5 mM) and 88% methanol at 3 ml/min. Deuterium-labeled paraxanthine was prepared by reacting D_3-methyl iodide with 7-methylxanthine under basic conditions and purified by high-pressure liquid chromatography as described above. D_3-theophylline and D_3-theobromine were gifts from Tom Kalhorn and Dr. Kent Kunze (University of Washington, Seattle, WA).

Animal Studies and Preparation of Tissue Samples. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Washington, and the animals were housed with free access to

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ABBREVIATIONS: P450, cytochrome P450; MROD, methoxyresorufin *O*-demethylation; RLMs, rat liver microsomes; PCR, polymerase chain reaction.

food and water under 12-h light/dark cycles. Female Sprague-Dawley rats were mated at 8 weeks of age, and pregnancy was determined by detection of a vaginal plug. Pregnant rats were sacrificed at midpregnancy at day 9 ($n = 6$) and late pregnancy at day 19 ($n = 10$). Unmated, age-matched, female rats ($n = 6$) were used as controls. Livers were harvested, weighed, rinsed in phosphate-buffered saline and stored at −80°C. Microsomes were prepared from individual rat livers according to previously published methods (Rettie et al., 1989). Total microsomal protein concentrations were measured using a Pierce BCA protein assay (Pierce, Rockford, IL), according to the manufacturer's instructions, using bovine serum albumin as a standard.

Analysis of CYP1A2 Activity using Caffeine as a Probe. Caffeine *N*-demethylation to paraxanthine, theobromine, and theophylline was determined using rat Supersomes (CYP1A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2D1, CYP2D2, and CYP3A2; BD Gentest, Woburn, MA). Caffeine (500 μM) was incubated with each isoform (20 pmol) for 30 min with 1 mM NADPH in 100 mM potassium phosphate (pH 7.4). For CYP1A2, the Michaelis-Menten parameters (K_m and V_{max}) were determined after incubating 50 to 3000 μM caffeine with CYP1A2 (5 pmol) for 15 min. Caffeine *N*-demethylation was measured as CYP1A2 probe in individual rat livers at a sub-K_m concentration of caffeine (100 μM) with 0.2 mg/ml microsomal protein for 30 min. All incubations were conducted at the linear range for microsomal protein and incubation time.

Paraxanthine, theophylline, and theobromine were extracted using methylene chloride, separated using a SphereClone phenyl column (150 × 4.6 mm; 3 μm; Phenomenex, Torrance, CA) on a Waters 2690 Separations Module (Waters, Milford, MA) with a mobile phase of 18% 10 mM ammonium acetate (pH 7) and 82% methanol at 0.25 ml/min and detected using a Micromass Platform LCZ mass spectrometer (Waters) with positive-mode, selected-ion monitoring of *m/z* 180.9 for paraxanthine, theophylline, and theobromine and *m/z* 183.9 for D_3-paraxanthine, D_3-theophylline, and D_3-theobromine. Peak area ratios were compared to a standard curve (0.2–50 ng/ml) of known concentrations. Intra- and interday variability were <16% for theobromine, paraxanthine, and theophylline, and the limit of quantification was 0.2 ng/ml.

Analysis of CYP1A2 Activity using Methoxyresorufin as a Probe. MROD was measured in rat Supersome panel (5 pmol/ml for CYP1A2 and 100 pmol/ml for all other isoforms) and in individual rat livers (0.04 mg/ml microsomal protein) using an NADPH-regenerating system (100 mM potassium phosphate, 4 units/ml glucose 6-phosphate-dehydrogenase, 0.13 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM magnesium chloride, 200-μl total reaction volume). For the Supersome panel, methoxyresorufin was incubated at 300 nM, and for rat liver microsomes (RLMs) methoxyresorufin was incubated at 25 nM in potassium phosphate buffer. Resorufin formation was measured by fluorescence (excitation, 530 nm; emission, 590 nM) in a multiwell plate reader (Molecular Devices, Sunnyvale, CA) and quantified from fluorescence intensity according to a resorufin standard curve (1–50 nM). Fluorescence was recorded every 30 s for 20 min. Resorufin formation was linear in the rat liver microsomes over a 20-min time frame and between protein concentrations of 0.02 and 0.08 mg/ml, but values after 10 min were not used for quantification due to significant substrate depletion. For all incubations, the percent depletion of substrate was calculated and the substrate concentration was corrected for depletion if necessary.

The K_m and V_{max} of MROD in pooled rat liver microsomes was determined using 0.04 mg/ml RLMs from each gestational stage and 20 to 2500 nM methoxyresorufin. The contribution of CYP1A2 to MROD was confirmed in inhibition experiments with α-naphthoflavone (200 nM) and methoxyresorufin (25 nM) with pooled rat liver microsomes (0.04 mg/ml). The inhibition of CYP1A2 by sex steroids was measured with 25 nM methoxyresorufin and 1 pmol/ml rat CYP1A2 Supersomes. The potential inhibitors (17-β-estradiol, estrone, estriol, and progesterone) were added at concentrations of 5 to 20,000 nM, and resorufin production was determined using fluorescence as described above.

Analysis of CYP1A2 Expression and mRNA. Protein levels of CYP1A2 were determined by Western blotting using 10 μg of liver microsomal protein from each animal as described previously (Dickmann et al., 2008). Membranes were probed with sheep anti-rat CYP1A2 (1:6000 dilution; Research Diagnostics, Flanders, NJ) and mouse anti-β-actin (1:4000 dilution; Sigma-Aldrich) primary antibodies in Odyssey blocking solution (LI-COR Biosciences, Lincoln, NE) with 0.1% Tween 20, rinsed, and incubated with IRDye 700DX donkey anti-sheep (1:3000) and IRDye 800CW donkey anti-mouse (1:5000) secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA). The fluorescence intensities were quantified on an Odyssey infrared imaging system (LI-COR Biosciences). All samples were blotted in triplicate, and the relative abundance of CYP1A2 protein was expressed as the CYP1A2/β-actin ratio. The antibody was confirmed to be specific toward CYP1A2 by the supplier.

Total liver RNA was isolated, quantified, and reverse transcribed to cDNA as described previously (Dickmann et al., 2008). CYP1A2, β2M, 18-*S*-Vic, and GusB transcripts were measured using commercially available TaqMan real-time polymerase chain reaction (PCR) primers and probes with PCR Master Mix on a StepOnePlus Real-Time PCR instrument (Applied Biosystems, Foster City, CA). CYP1A2 mRNA expression in pregnant rat liver was normalized by two housekeeping genes (β2M and 18-*S*-Vic) using the geometric mean described previously by Vandesompele et al. (2002).

Regulation of CYP1A2 in Primary Rat Hepatocytes. Primary hepatocytes from a 12-week-old female rat were purchased from CellzDirect (Durham, NC). Cells were plated in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 1× penicillin-streptomycin-L-glutamine (Invitrogen, Carlsbad, CA), dexamethasone (Sigma-Aldrich), insulin (Sigma-Aldrich), and 10% fetal bovine serum (Invitrogen) on collagen-coated plates (BD BioCoat; BD Biosciences, San Jose, CA) with a Matrigel overlay (Sigma-Aldrich). Treatments were started after a 24-h adaptation period in Williams' E medium. Rat hepatocytes were treated with 17-β-estradiol (10, 100, and 1000 pM), progesterone (10, 100, and 1000 nM), or β-naphthoflavone (1 μM, positive control) for 24 and 48 h, and the medium was changed every 24 h. At the end of the treatment, mRNA was harvested using Tri Reagent (Ambion; Applied Biosystems, Austin, TX), and CYP1A2 mRNA was measured using real-time PCR and normalized to the housekeeping gene β2M. CYP1A2 activity was measured by phenacetin *O*-dealkylation.

Data Analysis. Michaelis-Menten curve fitting was done in GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The *F* test was used to determine whether K_m or V_{max} values were statistically different during pregnancy. CYP1A2 activity during pregnancy was measured as v/[S] for caffeine and MROD after correction for substrate depletion for methoxyresorufin if necessary. CYP1A2 activity, expression, and mRNA were compared between pregnant and unmated controls by one-way analysis of variance followed by Tukey's test. Cells treated with 17-β-estradiol were compared to untreated controls using the Student's *t* test. A *p* value of 0.05 was considered statistically significant. All values are reported as means ± S.D.

Results and Discussion

Probe Validation. Caffeine metabolism to theobromine and paraxanthine and MROD were confirmed to be selective markers of CYP1A2 activity in rat liver after incubating caffeine and methoxyresorufin with a panel of rat Supersomes (data not shown). Of caffeine metabolites, theobromine instead of paraxanthine was the major metabolite observed in CYP1A2 incubations. Formation of theophylline was less selective for CYP1A2 because CYP2C6 and CYP2C11, a male-specific isoform, also metabolized caffeine to theophylline. CYP2C6 and CYP2C11 also had a minor activity toward the other *N*-demethylation reactions. The V_{max} and K_m estimates for caffeine metabolism by CYP1A2 were 1.7 ± 0.1 pmol/(min · pmol) and 295 ± 42 μM (theobromine), 1.0 ± 0.1 pmol/(min · pmol) and 230 ± 27 μM (paraxanthine), and 0.6 ± 0.1 pmol/(min · pmol) and 323 ± 49 μM (theophylline). MROD appeared more specific than caffeine for CYP1A2 in the Supersome panel (data not shown). The specificity of MROD as a CYP1A2 probe was confirmed in pooled RLMs. At 200 nM, α-naphthoflavone inhibited 85% of MROD, demonstrating that MROD is selective for CYP1A2.

Pregnancy Mediated Changes in CYP1A2 Activity, Expression, and mRNA during Rat Pregnancy. CYP1A2-mediated metabolism was decreased by approximately 50% during rat pregnancy regardless of the stage of pregnancy (Fig. 1A). Theobromine formation clearance decreased from 9.2 ± 2.9 (control) to 4.4 ± 1.3 nl/(min · mg) ($p < 0.01$) during midpregnancy and to 4.8 ± 2.0 nl/(min · mg) ($p < 0.01$) during late pregnancy. Paraxanthine formation clearance decreased from 5.4 ± 1.4 to 2.7 ± 0.9 nl/(min · mg) ($p < 0.01$) during midpregnancy and to

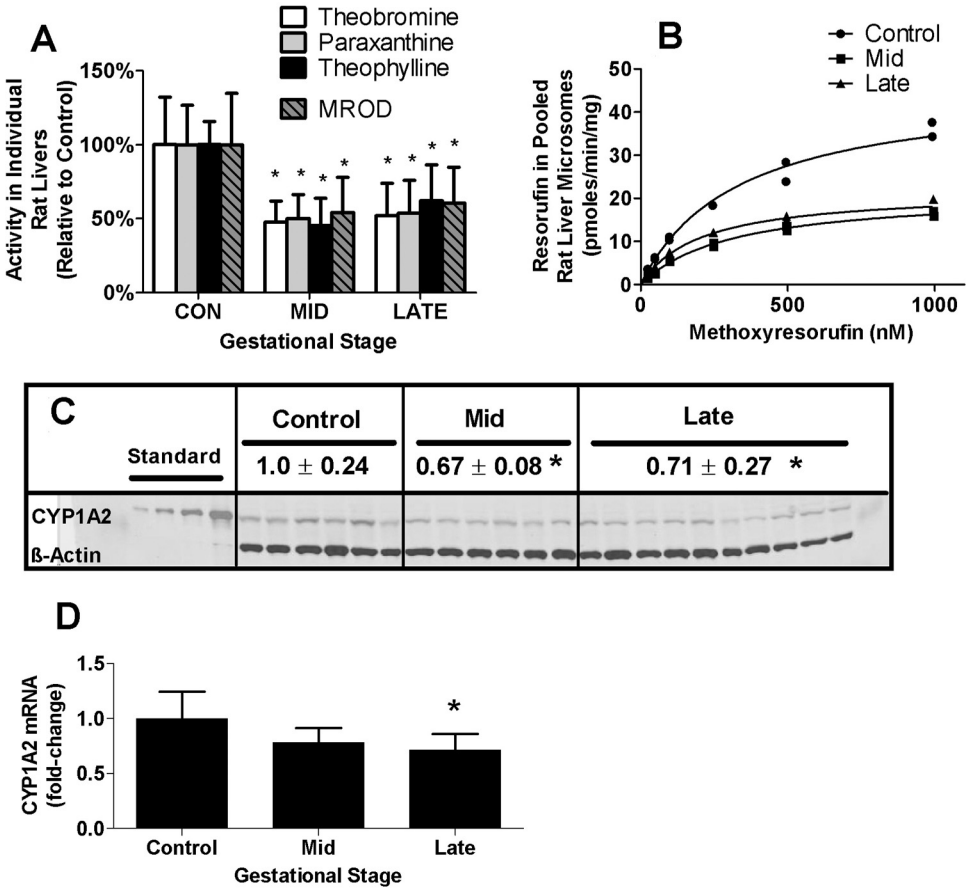

FIG. 1. Rat CYP1A2 activity and protein expression are decreased during pregnancy. A, caffeine *N*-demethylation and MROD clearances as averages of individual mid- and late gestation rat livers. The data are reported as a percentage of the clearance determined in nonpregnant controls (CON). B, Michaelis-Menten curves of methoxyresorufin metabolism in pooled rat liver microsomes from control, mid-, and late gestation rats. The K_m was significantly decreased from 332 nM in control animals to 205 nM in late gestation ($p < 0.05$). C, representative Western blot from individual rat liver microsomes, demonstrating decreased CYP1A2 expression during pregnancy. The values depict the fold change in CYP1A2 expression in each gestation stage. D, relative CYP1A2 mRNA during pregnancy as measured by real-time PCR. An asterisk (*) indicates that values were significantly different from unmated controls ($p < 0.05$).

2.9 ± 1.2 nl/(min · mg) ($p < 0.01$) during late pregnancy. Theophylline formation decreased from 7.4 ± 1.2 to 3.3 ± 1.4 nl/(min · mg) ($p < 0.001$) during midpregnancy and to 4.6 ± 1.8 nl/(min · mg) ($p < 0.01$) during late pregnancy. In agreement with the results obtained with caffeine as a probe, methoxyresorufin clearance decreased from 220 ± 77 (control) to 119 ± 52 μl/(min · mg) ($p < 0.05$) during midpregnancy and to 133 ± 53 μl/(min · mg) ($p < 0.05$) during late pregnancy.

The V_{max} of MROD in pooled RLMs decreased significantly ($p < 0.0001$) from 46 ± 9.1 (control) to 21 ± 3.8 pmol/(min · mg) (midpregnancy) and 22 ± 3.8 pmol/(min · mg) (late pregnancy), suggesting decreased expression of CYP1A2 or presence of a noncompetitive inhibitor in the liver microsomes (Fig. 1B). The K_m for MROD was significantly lower in late pregnancy than controls, suggesting possible modification of CYP1A2 protein itself. In agreement with the decreased V_{max} of MROD, the relative expression of rat CYP1A2 protein significantly decreased (analysis of variance; $p < 0.05$) during mid- and late pregnancy to 67 ± 7.8% ($p < 0.05$) and 71 ± 27% ($p < 0.05$), respectively, of control rats (Fig. 1C). Despite the significant decrease in CYP1A2 protein expression, no change in CYP1A2 mRNA was observed in midpregnancy, and CYP1A2 mRNA decreased during late pregnancy to 73 ± 20% ($p < 0.05$) of unmated controls (Fig. 1D). Note that the greatest effect was observed in CYP1A2 activity, followed by a smaller change in CYP1A2 protein expression, and finally the least effect on CYP1A2 mRNA. This result is of interest because with most other P450 enzymes, the largest magnitude of change is observed in mRNA. It is possible that the decreased CYP1A2 protein expression during pregnancy is due to posttranslational regulation rather than effects on CYP1A2 mRNA. However, during late pregnancy, the magnitude of decrease in CYP1A2 mRNA was similar to the protein, suggesting a contribution of transcriptional regulation as well. Based on these results, it is likely that multiple mechanisms contribute to the decreased CYP1A2 activity during pregnancy. This result is noteworthy because the decreased CYP1A2 activity observed in the rat is in close agreement with the 45 and 65% decrease in caffeine oral clearance during mid- and late pregnancy (Tracy et al., 2005). In contrast to the gestational stage-specific changes in mRNA and protein of CYP2D and CYP2C enzymes during rat pregnancy (Dickmann et al., 2008), no significant differences were observed between gestational stages in CYP1A2 activity or expression. This finding again highlights the fact that the effects of pregnancy are specific for a given P450 enzyme, and the mechanisms that result in altered P450 expression and activity during pregnancy vary between isoforms.

Role of Estrogens and Progesterone in Decreased CYP1A2 Activity. CYP1A2 can metabolize endogenous estrogens that have been suggested as potential regulators of CYP1A2. CYP1A2 activity is lower in women than in men (Relling et al., 1992), and hormone replacement therapy (Laine et al., 1999) decreases CYP1A2 activity. Hence, the effect of 17-β-estradiol and progesterone on CYP1A2 mRNA was tested in primary rat hepatocytes, and the effect of common sex steroids as reversible CYP1A2 inhibitors was measured.

In contrast to the clinical findings, estrogens or progesterone had no effect on CYP1A2 mRNA or activity at biologically relevant concentrations. 17-β-estradiol (10, 100, or 1000 pM) and progesterone (10, 100, and 1000 nM) had no effect ($p > 0.05$) on CYP1A2 mRNA after either 24 or 48 h of treatment, except for a <15% decrease ($p < 0.05$) in CYP1A2 mRNA after a 1000 nM progesterone treatment for 24 h. In addition, treatment of the rat hepatocytes with estrogen or progesterone for up to 48 h did not change CYP1A2 activity measured by phenacetin *O*-deethylation (data not shown), suggesting that at physiological concentrations, these hormones do not inactivate CYP1A2, despite the report

of increased CYP1A2 inhibition after preincubation with 300 μM progesterone (Karjalainen et al., 2008). No inhibition of MROD was observed in CYP1A2 Supersomes at physiologically relevant concentrations of 17-β-estradiol, estrone, estriol, and progesterone, and a maximum of 40% inhibition was observed at 20,000 nM concentration of these hormones (data not shown). This result is in agreement with previously determined K_i values of 17-β-estradiol (114 μM) and progesterone (710 μM) toward human CYP1A2 (Eugster et al., 1993; Karjalainen et al., 2008). Therefore, down-regulation or inhibition of CYP1A2 by increasing concentrations of these hormones during pregnancy is an unlikely reason for the observed decrease in CYP1A2-mediated clearance.

In conclusion, these data demonstrate that CYP1A2 protein expression is decreased during pregnancy, resulting in a decreased activity of CYP1A2. This decrease occurs in rat pregnancy and mimics the changes observed in human CYP1A2 activity during pregnancy supporting the use of the rat as a model to study mechanisms by which CYP1A2 changes during pregnancy. The decrease in CYP1A2 mRNA was smaller in magnitude than that observed for CYP1A2 protein and activity and was only observed during late pregnancy. This result suggests that mechanisms beyond regulation of CYP1A2 mRNA levels may be responsible for the early down-regulation of CYP1A2 protein and require further studies. The fact that changes in CYP1A2 activity in the rat during pregnancy closely mimic those observed in humans is important when the rat is used as a model species for studying developmental toxicity of CYP1A2 substrates.

Authorship Contributions

Participated in research design: Walker, Dickmann, and Isoherranen.

Conducted experiments: Walker and Dickmann.

Performed data analysis: Walker, Dickmann, and Isoherranen.

Wrote or contributed to the writing of the manuscript: Walker, Dickmann, and Isoherranen.

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