Pregnancy Decreases Rat CYP1A2 Activity and Expression

Alysa A Walker, Leslie Dickmann¹, Nina Isoherranen

Department of Pharmaceutics, University of Washington, Seattle, Washington
Running Title:  CYP1A2 expression is decreased by pregnancy

Corresponding Author:

Nina Isoherranen, PhD
Department of Pharmaceutics, University of Washington
Box 357610
Seattle, WA 98195-7610
Phone: (206) 543-2517
Fax: (206) 543-3204
E-mail: ni2@u.washington.edu

Number of text pages: 11
Number of tables: 0
Number of figures: 1
Number of references: 15
Number of words in the abstract: 250
Number of words in the introduction: 295
Number of words in results and discussion: 1374

Nonstandard abbreviations:  AUC, Area under the plasma concentration time curve;
CYP, cytochrome P450; KPi, Potassium phosphate; MROD, methoxyresorufin O-
demethylation; RLM, rat liver microsomes
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 controls. Additionally, 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% (mid-pregnancy) 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 mid-pregnancy 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 whereas 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). Similarly, caffeine salivary clearance decreased by 33%, 42% and 65% during the first, second and third trimester 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 CYP 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). The AUC of caffeine after oral administration to pregnant rats, was not different from non-pregnant rats but the AUC of theobromine and theophylline, formed by rat CYP1A2, decreased suggesting reduced CYP1A2 activity (Abdi et al., 1993). Decreased methoxyresorufin O-demethylation 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 HPLC-UV system (Santa Clara, CA) equipped with an Econosphere C18 column (250 mm x 10 mm, 10 µm, Alltech, Deerfield, IL), using an isocratic elution with 12% 5 mM ammonium acetate and 88% methanol at 3 mL per minute. Deuterium-labeled paraxanthine was prepared by reacting D3-methyl iodide with 7-methylxanthine under basic conditions and purified by HPLC as described above. D3-theophylline and D3-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 food and water under 12 hour 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 mid-pregnancy 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 PBS 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 (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, CYP3A2, BD Biosciences, Woburn, MA). Caffeine (500 μM) was incubated with each isoform (20 pmol) for 30 minutes with 1 mM NADPH in 100 mM KPi (pH 7.4). For CYP1A2, the Michaelis-Menten parameters (Km and Vmax) were determined after incubating 50 – 3000 μM caffeine with CYP1A2 (5 pmol) for 15 minutes. Caffeine N-demethylation was measured as CYP1A2 probe in individual rat livers at sub-Km concentration of caffeine (100 μM) with 0.2 mg/mL microsomal protein for 30 minutes. 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 150x4.6 mm 3 μm phenyl column (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, Milford, MA) with positive-mode selected ion monitoring of m/z 180.9 for paraxanthine, theophylline and theobromine and m/z 183.9 for D3-paraxanthine, D3-theophylline and D3-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: Methoxyresorufin O-demethylation (MROD) to resorufin, was measured in rat supersome panel (5 pmol/mL CYP1A2, 100 pmol/mL 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 RLM’s at 25 nM in KPi buffer. Resorufin formation was measured by fluorescence (Ex 530 nm/ Em 590 nM) in a multi-well 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 seconds until 20 minutes. Resorufin formation was linear in the rat liver microsomes over a 20 min time and between protein concentrations of 0.02 and 0.08 mg/mL, but values after 10 minutes were not used for quantification due to significant substrate depletion. For all incubations the % depletion of substrate was calculated and the substrate concentration was corrected for depletion if necessary.

The Km and Vmax of MROD in pooled rat liver microsomes was determined using 0.04 mg/mL RLMs from each gestational stage and 20 – 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 concentration 5 nM - 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 blot as described before (Dickmann et al., 2008) using 10 μg of
liver microsomal protein from each animal. Membranes were probed with sheep anti-rat CYP1A2 (1:6000 dilution, RDI, Flanders, NJ) and mouse anti-β-actin (1:4000 dilution, Sigma, St. Louis) primary antibodies in Odyssey blocking solution with 0.1% Tween 20, rinsed and incubated with IRDye700DX donkey anti-sheep (1:3000) and IRDye800CW donkey anti-mouse (1:5000) secondary antibodies (Rockland, Gilbertsville, PA). The fluorescence intensities were quantified on an Odyssey infrared imaging system (LICOR 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 towards CYP1A2 by the supplier.

Total liver RNA was isolated, quantified and reverse transcribed to cDNA as described before (Dickmann et al., 2008). CYP1A2, β2M, 18-S-Vic and GusB transcripts were measured using commercially available TaqMan real-time 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 as described by Vandesompele, et al. (Vandesompele et al., 2002).

**Regulation of CYP1A2 in Primary Rat Hepatocytes:** Primary hepatocytes from a 12-week-old female rat were purchased from CellzDirect. Cells were plated in Dulbecco’s Modified Eagle Medium (Sigma, St. Louis) supplemented with 1X Penicillin-Streptomycin-L-Glutamine (Invitrogen, San Diego, CA), Dexamethasone (Sigma, St. Louis), Insulin (Sigma, St. Louis) and 10% FBS (Invitrogen, San Diego, CA) on collagen coated plates (BD Biocoat) with a matrigel overlay. Treatments were started after a 24-hour adaptation period in Williams E media. Rat hepatocytes were treated with 17-β-
estradiol (10, 100 and 1000 pM), progesterone (10 nM, 100 nM, 1000 nM) or β-naphthoflavone, (1 μM, positive control) for 24 and 48 hours and media was changed every 24 hours. At the end of the treatment, mRNA was harvested using Tri-reagent 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 (La Jolla, 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 was compared between pregnant and unmated controls by one-way ANOVA followed by Tukey’s test. Cells treated with 17-β-estradiol were compared to untreated controls using Student’s $t$-test. A p-value of 0.05 was considered statistically significant. All values are reported as means ± standard deviation.
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 as CYP2C6 and CYP2C11, a male specific isoform, also metabolized caffeine to theophylline. CYP2C6 and CYP2C11 also had a minor activity towards the other N-demethylation reactions. The \( V_{\text{max}} \) 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), respectively. 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, \( \alpha \)-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 nL/min/mg (control) to 4.4 ± 1.3 nL/min/mg (p < 0.01) during mid-pregnancy and to 4.8 ± 2.0 nL/min/mg (p < 0.01) during late-pregnancy. Paraxanthine formation clearance decreased from 5.4 ± 1.4 nL/min/mg to 2.7 ± 0.9 nL/min/mg (p < 0.01) at mid-pregnancy and to 2.9 ± 1.2 nL/min/mg (p < 0.01) during late-pregnancy. Theophylline formation decreased from 7.4
± 1.2 nL/min/mg to 3.3 ± 1.4 nL/min/mg (p < 0.001) and 4.6 ± 1.8 nL/min/mg (p < 0.01) during mid- and late-pregnancy, respectively. In agreement with the results obtained with caffeine as a probe, methoxyresorufin clearance decreased from 220 ± 77 μL/min/mg (control) to 119 ± 52 (p < 0.05) and 133 ± 53 (p < 0.05) μL/min/mg during mid- and late-pregnancy, respectively.

The V_{max} of MROD in pooled RLMs decreased significantly (p<0.0001) from 46 ± 9.1 pmol/min/mg (control) to 21 ± 3.8 pmol/min/mg (mid-pregnancy) and 22 ± 3.8 pmol/min/mg (late-pregnancy) suggesting decreased expression of CYP1A2 or presence of a non-competitive 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 (ANOVA p < 0.05) during mid- and late-pregnancy to 67 ± 7.8 % (p < 0.05) and 71 ± 27 % (p < 0.05) of control rats, respectively (figure 1c). Despite the significant decrease in CYP1A2 protein expression, no change in CYP1A2 mRNA was observed in mid-pregnany and CYP1A2 mRNA decreased during late pregnancy to 73 ± 20 % (p < 0.05) of unmated controls (figure 1d). Interestingly, 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 is of interest as with most other CYP enzymes the largest magnitude of change is observed in mRNA. It is possible that the decreased CYP1A2 protein expression during pregnancy is due to post-translational 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 is noteworthy as 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 CYP enzyme, and the mechanisms that result in altered CYP 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) decrease 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 1,000 pM) and progesterone (10, 100 and 1,000 nM) had no effect (p > 0.05) on CYP1A2 mRNA either following 24 or 48 hours of treatment except for a <15% decrease (p<0.05) in CYP1A2 mRNA after 1,000 nM progesterone treatment for 24 hours. In addition, treatment of the rat hepatocytes with estrogen or progesterone for up to 48 hours
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 following 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 is in agreement with previously determined Ki of 17-β-estradiol (114 μM) and progesterone (710 μM) towards human CYP1A2 (Eugster et al 1993., Karjalainen et al 2008). Therefore, downregulation 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 well, 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 suggests that mechanisms beyond regulation of CYP1A2 mRNA levels may be responsible for the early downregulation 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 CONTRIBUTION:

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.

REFERENCES


**FOOTNOTES:**

1 Current address for Leslie Dickmann, Amgen, Seattle, Washington.

The project described was supported in part by National Institutes of Health grants [T32GM007750] and [U10HD047892] from the Eunice Kennedy Shriver National Institute Of Child Health & Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute Of Child Health & Human Development or the National Institutes of Health.
LEGENDS FOR FIGURES

Figure 1: Rat CYP1A2 activity, and protein expression are decreased during pregnancy. Panel A depicts caffeine N-demethylation and methoxyresorufin O-demethylation clearances as averages of individual mid- and late- gestation rat livers. The data is reported as a percent of the clearance determined in nonpregnant controls. Panel B shows the 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 ($p < 0.05$). Panel C shows a 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. Panel D shows the relative CYP1A2 mRNA during pregnancy as measured by real-time PCR. An asterisk (*) indicates that values were significantly different from unmated controls ($p < 0.05$).
Figure 1

(A) Activity in Individual Rat Livers (Relative to Control)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>MID</th>
<th>LATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theobromine</td>
<td>100%</td>
<td>85%</td>
<td>70%</td>
</tr>
<tr>
<td>Paraxanthine</td>
<td>100%</td>
<td>90%</td>
<td>80%</td>
</tr>
<tr>
<td>Theophylline</td>
<td>100%</td>
<td>95%</td>
<td>85%</td>
</tr>
<tr>
<td>MROD</td>
<td>100%</td>
<td>95%</td>
<td>85%</td>
</tr>
</tbody>
</table>

* Indicates significant difference from control.

(B) Resorufin in Pooled Rat Liver Microsomes (pmoles/min/mg)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyresorufin (nM)</td>
<td>0</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Resorufin (pmoles/min/mg)</td>
<td>0</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>

(C) Standard

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>1.0 ± 0.24</td>
<td>0.67 ± 0.08 *</td>
<td>0.71 ± 0.27 *</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(D) CYP1A2 mRNA (fold-change)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational Stage</td>
<td>1.0</td>
<td>0.5</td>
<td>*</td>
</tr>
</tbody>
</table>

* Indicates significant difference from control.