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## Characterization of Maternal and Fetal CYP3A-Mediated Progesterone Metabolism

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### Abstract

**Introduction**—Progesterone is critical for maintaining pregnancy and onset of labor. We evaluated CYP450-mediated progesterone metabolism, specifically the contribution of CYP3A isoforms.

**Materials and Methods**—*In vitro* progesterone metabolism was characterized in human liver microsomes (HLMs) with and without selective cytochrome P450 inhibitors and in recombinant CYP3A4, CYP3A5, and CYP3A7. 6 $\beta$ -hydroxyprogesterone (6 $\beta$ -OHP) and 16 $\alpha$ -hydroxyprogesterone (16 $\alpha$ -OHP) metabolites were quantified by HPLC/UV and fit to the Michaelis-Menten equation to determine  $K_m$  and  $V_{max}$ . The effect of CYP3A5 expression on progesterone clearance was determined by *in vitro in vivo* extrapolation.

**Results**—Ketoconazole inhibited formation of both 6 $\beta$ -OHP and 16 $\alpha$ -OHP more than 90%. 6 $\beta$ -OHP and 16 $\alpha$ -OHP were both produced by CYP3A4 (2.3 and 1.3  $\mu$ L/min/pmol, respectively) to a

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**Conflict of Interests**

The Author(s) declare that there is no conflict of interest

greater extent than by CYP3A5 (0.09 and 0.003  $\mu\text{L}/\text{min}/\text{pmol}$ ) and CYP3A7 (0.004 and 0.003  $\mu\text{L}/\text{min}/\text{pmol}$ ).

**Conclusions**—Maternal clearance of progesterone by hepatic CYP450's is driven primarily by CYP3A4, with limited contributions from CYP3A5 and CYP3A7.

### Keywords

Progesterone; Metabolism; Cytochrome P450; Fetal; Pharmacokinetics

### Chemical Compounds

Chemical compounds studied in this article are Progesterone (PubChem CID: 5994); 16 $\alpha$ -hydroxyprogesterone (PubChem CID: 243761); 6 $\beta$ -hydroxyprogesterone (PubChem CID: 200149)

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## INTRODUCTION

Since the 1960s, there has been extensive examination of the role of progestogens in human parturition. A physiologic withdrawal of progesterone activity at the level of the uterus is associated with the subsequent onset of labor.(1–3) However, the molecular mechanisms responsible for progesterone's maintenance of uterine quiescence are not clear. Proposed mechanisms include an increase in the myometrial ratio of progesterone receptor A to progesterone receptor B, a decrease in spontaneous myometrial contractions with an increased threshold for stimulation, and the inhibition of downstream signaling of the oxytocin receptor by progesterone metabolites.(1, 2, 4, 5) Additionally, several derivatives of progesterone have been identified, which modulate human myometrial contractility with variable potency.(6–9) Preliminary work by our group found that in a murine model, oxytocin-induced uterine contractility frequency was increased by 6 $\beta$ -hydroxyprogesterone (6 $\beta$ -OHP) but decreased by 16 $\alpha$ -hydroxyprogesterone (16 $\alpha$ -OHP). The opposing effects of these progesterone metabolites on oxytocin-induced uterine contractility suggests that the relative amounts of the metabolites may influence myometrial contractility.(10)

Progesterone and other steroids are substrates of cytochrome P450 (CYP) enzymes. Adrenal CYP17A1 metabolizes progesterone to 17 $\alpha$ -hydroxyprogesterone and 16 $\alpha$ -hydroxyprogesterone (16 $\alpha$ -OHP). (11) Yamazaki and Shimada demonstrated that CYP3A, the major CYP450 expressed in human liver, metabolizes progesterone to 6 $\beta$ -hydroxyprogesterone (6 $\beta$ -OHP) and 16 $\alpha$ -hydroxyprogesterone (16 $\alpha$ -OHP), (12) a finding that has been confirmed by other groups. (13, 14) However, these studies did not examine the individual isoforms of CYP3A expressed in humans (CYP3A4, CYP3A5, and CYP3A7), which are known to have different substrate selectivity. CYP3A4 and CYP3A5 are the major isoforms in adults and are highly expressed in the liver and gastrointestinal tract. On the other hand, CYP3A7 is primarily expressed in fetal liver, although variants of CYP3A7 have been described that result in expression into adulthood.(15) Shortly after birth, hepatic CYP3A undergoes a transition from CYP3A7 expression in the fetus, to CYP3A4 and CYP3A5 in the first year of life.(16) CYP3A mRNA has also been detected in first and third trimester human placenta and in endometrium during pregnancy and the secretory phase of

the menstrual cycle. (17–19) However, the CYP3A activity in these tissues is negligible; reported testosterone 6 $\beta$ -hydroxylation in the placenta is less than 2% of that fetal liver. (20, 21) There is large inter- and intra-individual variability in CYP3A activity due to both genetic and non-genetic factors.(22) CYP3A5 is highly polymorphic, and more than 80% of Caucasians, but only 33% of African Americans, express a variant allele, most commonly CYP3A5\*3 (rs776746) which produces a non-functional protein.(23) Activity of CYP3A4 is also known to increase during pregnancy, leading to increased hepatic clearance of CYP3A substrates.(24–27) Alterations in drug metabolism in pregnancy may also be due to by placental aromatase (CYP19A1), which has been shown to metabolize several drugs including methadone and buprenorphine. (28, 29)

While it was known that 6 $\beta$ -OHP and 16 $\alpha$ -OHP are produced by CYP3A4, we recently showed that 6 $\beta$ -OHP and 16 $\alpha$ -OHP are also produced by CYP3A5 and CYP3A7.(10) To our knowledge, the contribution of CYP3A5, CYP3A7, or placental CYP19A1 to the production of 6 $\beta$ -OHP and 16 $\alpha$ -OHP has not been evaluated in detail. Therefore, the objectives of this *in vitro* drug metabolism study were to evaluate the ability of hepatic CYP450's and CYP19A1 to form 6 $\beta$ -OHP and 16 $\alpha$ -OHP from progesterone and to determine the Michaelis-Menten kinetic parameters for these metabolites for individual CYP3A isoforms. Using *in vitro in vivo* extrapolation, we also evaluated the effect of CYP3A5 polymorphism on the maternal and fetal hepatic production of 6 $\beta$ -OHP and 16 $\alpha$ -OHP.

## MATERIALS AND METHODS

### Chemicals

Progesterone, 6 $\beta$ -OHP, and 16 $\alpha$ -OHP were obtained from Steraloids, Inc. (Newport, RI). 6 $\beta$ -hydroxytestosterone,  $\beta$ -nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH), and ketoconazole were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Letrozole,  $\alpha$ -naphthoflavone, omeprazole, pilocarpine, quercetin, quinidine, and sulfaphenazole were obtained from Sigma-Aldrich, Santa Cruz Biotechnology (Dallas, TX), or Cayman Chemical (Ann Arbor, MI). Pooled human liver microsomes (HLMs) from 150 individual donors and baculovirus–expressed CYP3A4+b5, CYP3A5+b5 and CYP3A7+b5, CYP2A6, CYP19A1 were purchased from Corning Gentest (Woburn, MA). Acetonitrile (ACN) and methyltert-butyl ether were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were of high performance liquid chromatography (HPLC) grade and purchased from reputable manufacturers.

### Analytical Methods

Quantification of the progesterone metabolites 6 $\beta$ -OHP and 16 $\alpha$ -OHP were performed by high performance liquid chromatography (HPLC, Agilent 1100, Santa Clara, CA) with UV detection (254 nm). Separation was achieved on a Luna 5 $\mu$  C18 100A 250  $\times$  4.6 mm column (Phenomenex, Torrance, CA) with a gradient elution. Mobile phase A consisted of 0.25% acetic acid, 10% ACN, 90% H<sub>2</sub>O. Mobile phase B consisted of 0.25% acetic acid, 90% ACN, and 10% H<sub>2</sub>O. Mobile phase composition was as follows: 40% B at 0 minutes,

increasing linearly from 40 to 95% B from 1 to 28 minutes before resetting to the initial condition of to 40% B over 0.1 minute. Flow was set at 1.0 ml/min.

Stock solutions of 16 $\alpha$ -OHP, 6 $\beta$ -OHP, and 6 $\beta$ -hydroxytestosterone (internal standard) dissolved in methanol (1 mg/mL) were prepared separately in polypropylene tubes and, and stored at  $-20^{\circ}\text{C}$ . Standards were serially diluted into sodium phosphate buffer (100 mM, pH 7.4) to attain desired concentrations. Standard curves were constructed by comparing the area under the curve (AUC) of metabolite (16 $\alpha$ -OHP or 6 $\beta$ -OHP) to the AUC of 6 $\beta$ -hydroxytestosterone. Limit of quantification (LOQ) was 0.05  $\mu\text{g/mL}$  for 16 $\alpha$ -OHP and 0.25  $\mu\text{g/mL}$  for 6 $\beta$ -OHP. Quality controls were constructed in triplicate. Inter- and intra-day coefficient of variation was less than 15%.

### Progesterone metabolism in HLMs

Kinetic studies for 6 $\beta$ -OHP and 16 $\alpha$ -OHP formation from progesterone were conducted in pooled HLMs. Progesterone in methanol (20–250  $\mu\text{M}$ , final concentration) was added to glass tubes and evaporated to dryness prior to addition of HLMs (0.5 mg/mL) and microsomal buffer (100 mM sodium phosphate containing 5 mM  $\text{MgSO}_4$ , pH 7.4) to a final reaction volume of volume 250  $\mu\text{L}$ . The mixtures were equilibrated at  $37^{\circ}\text{C}$  for 5 minutes, and reactions initiated by addition of 5  $\mu\text{L}$  of NADPH (10 mM). After 30-minutes, reactions were terminated by 1:1 addition of ice-cold acetonitrile. Ten  $\mu\text{L}$  of 10 mg/mL internal standard (6 $\beta$  - hydroxytestosterone), 250  $\mu\text{L}$  citric acid (0.1 M, pH 3.2), and 3 ml methyltert-butyl ether were added. Samples were vortex-mixed for 30 seconds prior to centrifugation for 5 minutes at  $1200\times g$ . The supernatant was transferred to clean glass tubes, and evaporated to dryness. The residues were reconstituted by adding 100  $\mu\text{L}$  of mobile phase A. 6 $\beta$ -OHP and 16 $\alpha$ -OHP were quantified as described above. Assays were conducted in duplicate.

### Inhibition Analyses

Rates 6 $\beta$ -OHP and 16 $\alpha$ -OHP formation in HLMs was evaluated in the absence (control) and presences of known isoform-selective CYP inhibitors: ketoconazole (1  $\mu\text{M}$ , CYP3A4),  $\alpha$ -naphthoflavone (1  $\mu\text{M}$ , CYP1A2), letrozole (50  $\mu\text{M}$ , CYP2A6), omeprazole (10  $\mu\text{M}$ , CYP2C19), pilocarpine (50  $\mu\text{M}$ , CYP2A6), quercetin (50  $\mu\text{M}$ , CYP2C8), quinidine (1  $\mu\text{M}$ , CYP2D6), or sulfaphenazole (10  $\mu\text{M}$ , CYP2C9).(30) Progesterone (30  $\mu\text{M}$ ) and inhibitors were added to glass culture tubes and evaporated to dryness before the addition of HLMs (0.5 mg/mL) and microsomal buffer sufficient to bring the final reaction volume to 250  $\mu\text{L}$ . Reactions were conducted in duplicate as described above.

### Enzymatic Characterization of Metabolite Formation

To further characterize the role of individual CYP isoforms, rates of formation of 6 $\beta$ -OHP and 16 $\alpha$ -OHP were determined with recombinant human CYP3A4, 3A5, and 3A7. Progesterone in methanol (10–250  $\mu\text{M}$  for CYP3A4 and 20–250  $\mu\text{M}$  for CYP3A5 and CYP3A7; 100  $\mu\text{M}$  for CYP19A1) was added to glass tubes and evaporated to dryness prior to addition of CYP3A4 (25 pmol), CYP3A5 (20 pmol), CYP3A7 (20 pmol), or CYP19A1 (25 pmol). Microsomal buffer (100 mM sodium phosphate containing 5 mM  $\text{MgSO}_4$ , pH 7.4) was added to bring the total volume to 250  $\mu\text{L}$ . After a 5 minute equilibration, reactions

were initiated by addition of 5  $\mu\text{L}$  of NADPH (10 mM). After 30-minutes, reactions were terminated by 1:1 addition of ice-cold acetonitrile. Protein was then precipitated by centrifugation and transferred to new glass tubes as described above. Control incubations with no cofactor were performed concurrently to validate CYP-dependent metabolism.

## Statistics

Metabolite formation in the presence of inhibitors was compared to metabolite formation in the absence of inhibitors using one-way analysis of variance with post-hoc analysis by t-test with Bonferroni correction for multiple comparisons. All analyses were carried out in GraphPad Prism. Enzyme kinetic values,  $V_{\text{max}}$  (maximum rate of metabolism) and  $K_m$  (substrate concentration at which metabolic velocity is half of  $V_{\text{max}}$ ) were determined by fitting the data to the Michaelis-Menten equation using GraphPad Prism v6 (GraphPad Software, Inc. La Jolla, CA), and are presented as mean  $\pm$  standard error. Intrinsic Clearance ( $CL_{\text{int}}$ ) was calculated as  $V_{\text{max}}/K_m$ .

## In vitro in vivo extrapolation

The *in vivo* formation clearances of 16 $\alpha$ -OHP and 6 $\beta$ -OHP by maternal and fetal livers were estimated by *in vitro in vivo* extrapolation. Hepatic intrinsic clearance ( $CL_{\text{int,H}}$ ) was estimated as previously described: (31)

$$CL_{\text{int,H}} = \sum CL_{\text{int,enz}(j)} \times ISEF_{\text{enz}(j)} \times Abund_{\text{enz}(j)} \times MPPGL \times Wt_H$$

where  $CL_{\text{int,enz}(j)}$  represents the intrinsic clearance for each CYP isoform (i.e. CYP3A4, CYP3A5, CYP3A7); ISEF is an intersystem extrapolation factor to account for intrinsic differences between recombinantly expressed enzymes and HLMS,  $Abund_{\text{enz}(j)}$  represents the abundance of each individual CYP isoform, MPPGL is the microsomal protein content per gram of liver, and  $Wt_H$  is liver weight.

For scaling to maternal liver, 16 $\alpha$ -OHP and 6 $\beta$ -OHP were assumed to be formed by CYP3A4 and CYP3A5. For fetal liver, only CYP3A5 and CYP3A7 activity were considered, as CYP3A4 activity is negligible prior to birth.(21, 32, 33) Maternal parameters were obtained from Simcyp v.14 (Pharsight): hepatic abundance of CYP3A4 and CYP3A5 were set to 137 and 103 pmol/mg microsomal protein; liver was estimated as 1728 g and MPPGL as 39.8 mg/g. ISEF for CYP3A4 and CYP3A5 supersomes was set at 0.24 based on the SimCyp default values. Clearance of CYP3A substrates increases approximately 2-fold during pregnancy, apparently due to increased hepatic clearance.(24–27) To account for this, the estimate  $CL_{\text{int,H}}$  was multiplied by a factor of two to determine final maternal hepatic intrinsic clearance.

Fetal abundance of CYP3A7 was estimated to be 235 pmol/mg microsomal proteins.(21) Recently, Vyhldal *et al.* reported that an average of 42.1 pmol/mg CYP3A5 protein is expressed in fetal livers that carry the CYP3A5\*1\*1 genotype.(33) Estimated fetal liver weight at 38 weeks gestation is 130g,(34) and MPPGL in fetal liver is estimated to be 26 mg/g.(35) In the absence of data, ISEF for CYP3A7 was conservatively set at 1.

## RESULTS

### Progesterone metabolism by HLMs

A representative HPLC chromatogram of metabolite formation after progesterone (100  $\mu\text{M}$ ) incubation with HLMs (0.5  $\mu\text{g/mL}$ ) is shown in Figure 1. Major peaks eluted at times consistent with known standards: 16 $\alpha$ -OHP (8.1 min), 6 $\beta$ -OHP (10.9 min), and progesterone (20.1 min). We were unable to chromatographically separate the 2 $\alpha$ - and 17 $\alpha$ -hydroxyprogesterone metabolites, which both eluted at 14.2 minutes. However, the peak area was lower than that of the other metabolites, indicating that these metabolites were poorly formed by human liver microsomes. Therefore, we did not further investigate the formation kinetics of 2 $\alpha$ - and 17 $\alpha$ -hydroxyprogesterone. Further, incubation of 16 $\alpha$ -OHP and 6 $\beta$ -OHP with HLMs did not yield additional metabolism products (data not shown).

Michaelis-Menten parameters for metabolite formation by HLMs were determined (Figure 2).  $V_{\text{max}}$  and  $K_{\text{m}}$  were  $1640 \pm 111$  (95% CI, 1398–1881) pmol/min/mg protein and  $27.7 \pm 8.1$  (95% CI, 10.1–45.3)  $\mu\text{M}$  for formation of 6 $\beta$ -OHP and  $192.1 \pm 8.9$  (95% CI, 172.7–211.5) pmol/min/mg protein and  $29.8 \pm 5.7$  (95% CI, 17.2–42.3)  $\mu\text{M}$  for formation of 16 $\alpha$ -OHP, respectively. Overall, the formation of 6 $\beta$ -OHP was 9 times faster than that of 16 $\alpha$ -OHP, with intrinsic clearances ( $\text{CL}_{\text{int}}$ ) of 59.2 and 6.45  $\mu\text{L}/\text{min}/\text{mg}$  protein, respectively.

### Inhibition Assays

Inhibition of 6 $\beta$ -OHP and 16 $\alpha$ -OHP formation by isoform-selective inhibitors was evaluated in HLMs (Figure 3). Ketoconazole inhibited formation of both 6 $\beta$ -OHP and 16 $\alpha$ -OHP by over 95% ( $p < 0.01$ ), indicating that CYP3A plays a major role in production of these metabolites. 6 $\beta$ -OHP formation was significantly inhibited by co-incubation with the CYP2C8 inhibitor quercetin (89%,  $p < 0.05$ ); CYP2A6 inhibitor letrozole (73%,  $p < 0.05$ ); CYP2A6 inhibitor pilocarpine (33%,  $p < 0.05$ ); and CYP2C9 inhibitor sulfapahenazole (29%,  $p < 0.05$ ). 16 $\alpha$ -OHP formation was significantly inhibited by quercetin (62%,  $p < 0.05$ ) and letrozole (75%,  $p < 0.05$ ), indicating potential roles for CYP2C8 and CYP2A6 metabolism.

### Characterization of progesterone metabolite formation by recombinant enzymes

Progesterone metabolism was further studied in recombinant CYP3A4, CYP3A5, CYP3A7, and CYP19A1 (aromatase) microsomes. Incubation of progesterone (100  $\mu\text{M}$ ) with recombinant CYP19A1 microsomes resulted in minimal formation of 6 $\beta$ -OHP and 16 $\alpha$ -OHP metabolites (data not shown), so further characterization was not performed.

The Michaelis-Menten kinetic parameters for progesterone metabolite (6 $\beta$ -OHP and 16 $\alpha$ -OHP) formation catalyzed by recombinant CYP3A isoforms are illustrated in Figure 4 and summarized in Table 1. As observed in the HLM experiments, 6 $\beta$ -OHP formation was greater than that of 16 $\alpha$ -OHP formation, with CYP3A4 and CYP3A5 preferentially forming 6 $\beta$ -OHP. Intrinsic clearance of both products was highest in CYP3A4-expressing microsomes, with 25-fold greater enzymatic activity for 6 $\beta$ -OHP ( $\text{CL}_{\text{int}}$  2.3  $\mu\text{L}/\text{min}/\text{pmol}$  protein vs. 0.09  $\mu\text{L}/\text{min}/\text{pmol}$  protein) and 419-fold greater enzymatic activity than rCYP3A5 for 16 $\alpha$ -OHP ( $\text{CL}_{\text{int}}$  1.3  $\mu\text{L}/\text{min}/\text{pmol}$  protein vs. 0.003  $\mu\text{L}/\text{min}/\text{pmol}$  protein). CYP3A5 had similar activity to CYP3A7 for 16 $\alpha$ -OHP ( $\text{CL}_{\text{int}}$  0.003  $\mu\text{L}/\text{min}/\text{pmol}$  protein,



0.003  $\mu\text{L}/\text{min}/\text{pmol}$  protein), but 23-fold greater activity for 6 $\beta$ -OHP ( $\text{CL}_{\text{int}}$  0.09 pmol protein, 0.004 pmol protein).

### In vitro in vivo extrapolation

Based on formation of 6 $\beta$ -OHP and 16 $\alpha$ -OHP, maternal hepatic clearance of progesterone in CYP3A5 expressers is estimated as 1026 L/h. CYP3A5 nonexpressers are expected to exhibit a 5% reduction in clearance of progesterone (Table 2). 6 $\beta$ -OHP formation is 2-fold greater than 16 $\alpha$ -OHP (679 L/h and 347 L/h, respectively). Overall, clearance from fetal liver is less than 0.1% that of the maternal liver. However, fetal CYP3A5 genotype appears to be an important determinant of fetal clearance. Fetuses that are CYP3A5 expressers (CYP3A5\*1\*1 genotype) are predicted to have 2.7-fold greater clearance of progesterone than nonexpressers, primarily due to increased formation of 6 $\beta$ -OHP by CYP3A5 in expressers.

## DISCUSSION

Preterm birth continues to be a major cause of infant morbidity and mortality.(36) Progesterone is known to influence parturition, likely through multifactorial mechanisms. (37, 38) We have recently reported that the primary metabolites of progesterone, 6 $\beta$ -OHP and 16 $\alpha$ -OHP, have contradictory effects on oxytocin-induced contractile frequency.(10) While previous studies have shown that progesterone is metabolized in the liver by CYP3A enzymes, the relative importance of the three human isozymes CYP3A4, CYP3A5, and CYP3A7 had not been described.(12, 13, 39) Therefore, we undertook the current study to examine the CYP3A isozyme specific metabolic formation of 6 $\beta$ -OHP and 16 $\alpha$ -OHP *in vitro*.

Consistent with other studies, we found that progesterone metabolism in HLMs could largely be inhibited with ketoconazole, indicating that CYP3A is the primary metabolic route.(12–14) The formation of both 6 $\beta$ -OHP and 16 $\alpha$ -OHP were also inhibited >50% by letrozole and quercetin. Letrozole is a selective inhibitor of CYP2A6 (40). However, additional studies with recombinant CYP2A6 did not produce detectable metabolites (data not shown), consistent with reports from Niwa *et al.* indicating minimal formation of 6 $\beta$ -OHP in of CYP2A6-expressing microsomes.(13) Quercetin is a non-selective inhibitor of CYP2C8 that has also been shown to inhibit CYP3A, which may explain the moderate inhibition of progesterone metabolism observed in this study. Niwa *et al.* did not find evidence of CYP2C8 metabolism of progesterone. (13)

Previous studies of progesterone metabolism by CYP450 enzymes have examined its metabolism in CYP3A4, but not in CYP3A5 or CYP3A7. Therefore, we further explored the selectivity of CYP3A4, CYP3A5, and CYP3A7 isozymes towards progesterone metabolism using recombinantly expressed enzymes. The CYP3A4 isozyme most efficiently formed both 6 $\beta$ -OHP and 16 $\alpha$ -OHP, with both metabolites formed to a similar extent. However, CYP3A5 was found to preferentially form 6 $\beta$ -OHP over 16 $\alpha$ -OHP, although both were formed at a much lower rate than by CYP3A4. Progesterone is a weak substrate for the fetal CYP3A7 enzyme, indicating minimal metabolism of progesterone in fetal liver. Our study is

consistent with previous studies identifying 6 $\beta$ -OHP and 16 $\alpha$ -OHP as the primary metabolites of progesterone in human liver microsomes.(41)

Utilizing an *in vitro in vivo* extrapolation approach, we probed the effect of CYP3A5 genotype on the production of 6 $\beta$ -OHP and 16 $\alpha$ -OHP in maternal and fetal livers. As shown in Table 2, it is expected that CYP3A5 genotype has a minimal effect on the overall metabolism of progesterone from the maternal system. While fetal clearance of progesterone is minute with respect to overall clearance, fetuses who express an active CYP3A5 were estimated to form ~3-times more 6 $\beta$ -OHP than nonexpressers. Based on our prior *in vitro* work indicating pro-contraction effects of 6 $\beta$ -OHP and anti-contraction effects of 16 $\alpha$ -OHP (10), we hypothesize that the higher 6 $\beta$ -OHP/16 $\alpha$ -OHP ratio in CYP3A5 expressers may contribute to increased preterm labor risk in CYP3A5 expressers. Additional studies are needed to evaluate this hypothesis.

Our study does not address the metabolism of progesterone by extra-hepatic CYPs, other than CYP19A1. Swart et al. studied progesterone metabolism in microsomes derived from CYP17A1-transfected COS1 cells. 17 $\alpha$ -OHP and 16 $\alpha$ -OHP were the primary metabolites identified, with a  $K_m$  of 0.75  $\mu$ M and  $V_{max}$  of 0.46 nmol/h/mg protein for 17 $\alpha$ -OHP and 0.70  $\mu$ M and 0.12 nmol/h/mg protein for 16  $\alpha$ -OHP. (11) CYP17A1 is expressed in adult and fetal adrenal glands, thecal cells of the ovary, kidney, thymus, spleen, heart, and adipose tissue. (42) Therefore, extrahepatic metabolism likely plays a primary role in the formation of 16 $\alpha$ -OHP and contributes greatly to the overall clearance of progesterone. However, because there is limited data on the quantitative expression level of CYP17A1 in these tissues and in the *in vitro* expression systems studied, it is not possible to estimate the contribution of CYP17A1 to progesterone metabolism using *in vitro in vivo* extrapolation techniques. However, we do note that inclusion of CYP17A1 pathway in our prediction may further minimize the role of CYP3A5 polymorphisms.

Our study is also limited by the inadequacies inherent to *in vitro* studies. Specifically, the use of recombinant systems may be confounded by the absence of endogenous cofactors (including progesterone itself) that are known to impact enzyme activity. The HLM's used in these experiments were from men and women, and may not represent the CYP450 enzyme activity of pregnant women. The formation of progesterone metabolites by CYP3A5 and CYP3A7 were near the limit of detection for the assay, contributing to increased variability in formation rates at lower concentrations of substrate. This variability contributes to the wide confidence interval of  $K_m$  for these enzymes. We also lacked access to fetal liver microsomes for confirmation of role of CYP3A7 in fetal liver metabolism. However, despite this limitation, the use of recombinantly expressed CYPs, including rCYP3A7, allowed investigation of the contribution of individual enzymatic isoforms to the biotransformation pathway. Based on literature reports of CYP3A5 and CYP3A7 expression in fetal liver and *in vitro in vivo* extrapolation techniques, we are able to estimate the contribution of CYP3A5 and CYP3A7 to fetal formation of 6 $\beta$ -OHP and 16 $\alpha$ -OHP.(33) However, it should be noted that extrapolation of the *in vitro* data to *in vivo* clearance is limited by the availability of data regarding physiologic changes and enzyme expression during pregnancy.



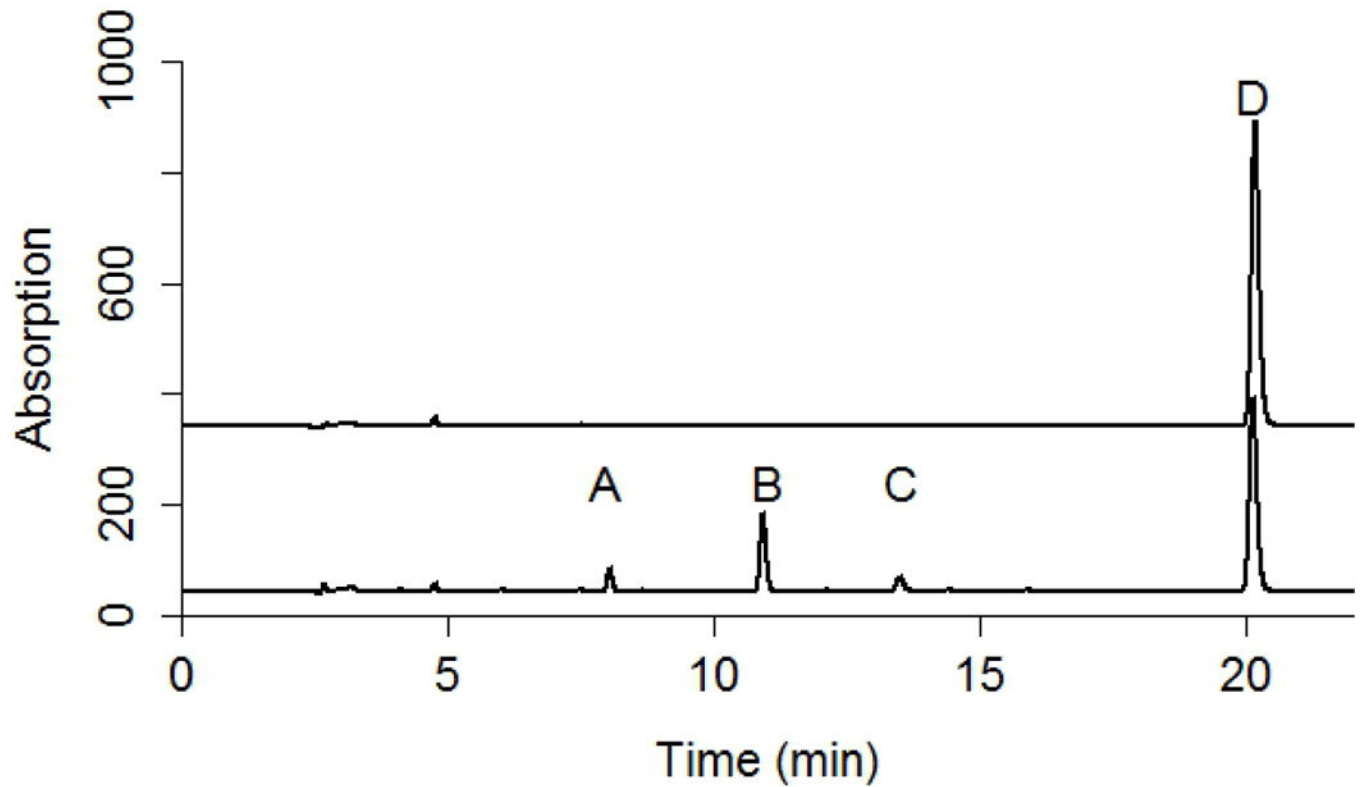
Our understanding of the role of progesterone in the onset of parturition continues to evolve. Progesterone and its metabolites appear to elicit characteristic effects on oxytocin-induced uterine contractility, suggesting that relative amounts of each compound may alter myometrial contractility.<sup>(10)</sup> Our study identifies the primary sources of progesterone metabolite formation and describes the influence of CYP3A isoforms on the relative amounts of 6 $\beta$ -OHP and 16 $\alpha$ -OHP produced from progesterone. While our in vitro in vivo extrapolation does not indicate that maternal CYP3A5 genotype will influence formation of progesterone metabolism, it is unknown whether increased 6 $\beta$ -OHP by fetal liver will influence localized 6 $\beta$ -OHP/16 $\alpha$ -OHP ratio and whether this will effect uterine contractility. Future work may consider whether variation in CYP3A enzyme function alters the relative amounts of progesterone metabolites produced in vivo and the effect of these hormones on uterine contractility.

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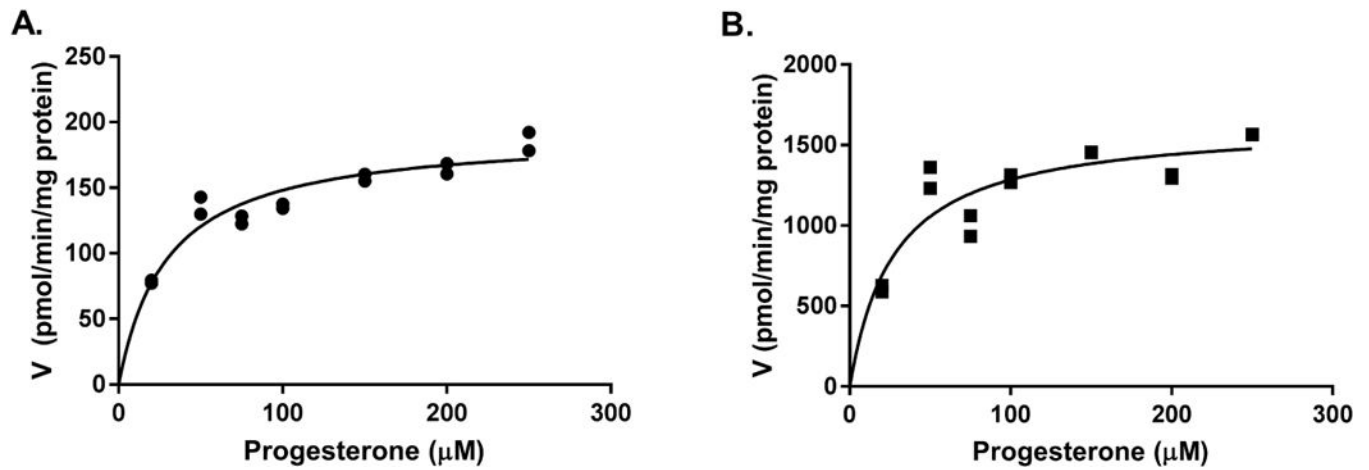
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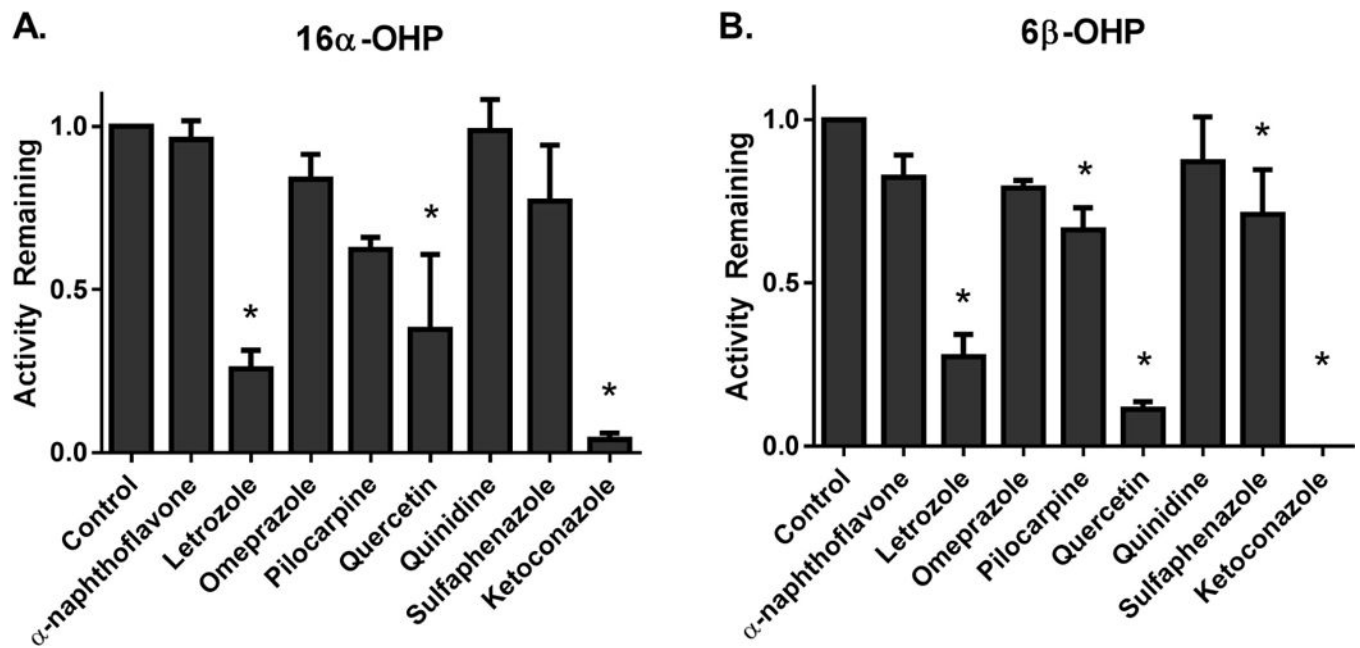
**Figure 1.**

HPLC chromatograph of progesterone with human liver microsomes in the presence (lower line) and absence (upper line) of NADPH. A) 16 $\alpha$ -OHP, 8.1 minutes; B) 6 $\beta$ -OHP, 10.9 minutes; C) 2 $\alpha$ - and 17 $\alpha$ -hydroxyprogesterone co-eluted at 13.5 minutes; and D) progesterone, 20.1 minutes. The internal standard (6 $\beta$ -OHT), which eluted at 4.7 minutes is not shown.



**Figure 2.**

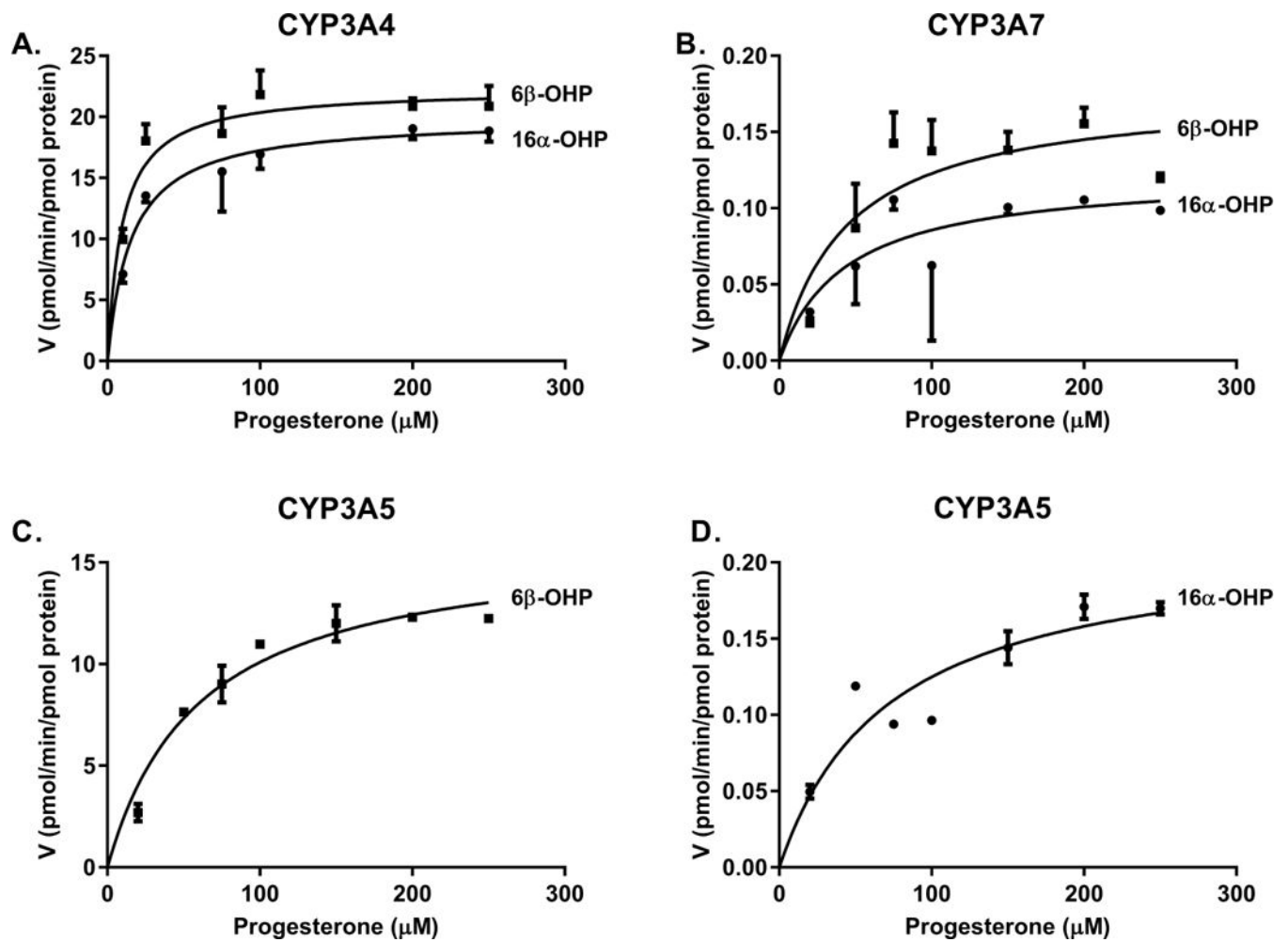
Kinetics for (A) 6β-OHP ( $V_{\max} = 1640 \pm 111$  pmol/min/mg protein;  $K_m = 27.7 \pm 8.1$  μM) and (B) 16α-OHP ( $V_{\max} = 192.1 \pm 8.9$  pmol/min/mg protein;  $K_m = 29.8 \pm 5.7$  μM) formation in human liver microsomes. Progesterone 20μM, 50μM, 75μM, 100μM, 150μM, 200μM and 250μM were incubated with 0.5mg/ml of HLM for 30min. Formation rates of 6β-OHP and 16α-OHP were fit to the Michaelis-Menten equation using GraphPad Prism.



**Figure 3.**

Effect of CYP450 Inhibitors on 16 $\alpha$ -OHP (A) and 6 $\beta$ -OHP (B) formation following incubation of progesterone (30  $\mu$ M) with human liver microsomes (0.5 mg/mL) for 30 minutes in the absence (control) and presences of known isoform-selective CYP inhibitors: ketoconazole (CYP3A4),  $\alpha$ -naphthoflavone (CYP1A2), letrozole (CYP2A6), omeprazole (CYP2C19), pilocarpine (CYP2A6), quercetin (CYP2C8), quinidine (CYP2D6), or sulfaphenazole (CYP2C9). Data is presented as mean  $\pm$  SD of duplicate experiments. \* indicates  $p < 0.05$  compared to control.





**Figure 4.** Michaelis-Menten plots of 6β-OHP and 16α-OHP formation by recombinant CYP3A isoforms. A.) Formation of 6β-OHP (squares) and 16α-OHP (circles) from progesterone following 10 minute incubation with 25 pmol CYP3A4. B.) Formation of 6β-OHP (squares) and 16α-OHP (circles) following 30 minute incubation with 25 pmol CYP3A7. C) Formation of 6β-OHP following 10 minute incubation of progesterone with 25 pmol rCYP3A5. D) Formation of 16α-OHP from progesterone following 30 minute incubation with CYP3A5. Error bars indicate standard error.

Enzyme kinetic parameters for cytochrome P450 CYP3A catalyzed progesterone metabolism

**Table 1**

Enzyme	16 $\alpha$ -OHP				6 $\beta$ -OHP			
	V <sub>max</sub> (pmol/min/pmol protein)	K <sub>m</sub> ( $\mu$ M)	CL <sub>int</sub> ( $\mu$ L/min/pmol protein)	V <sub>max</sub> (pmol/min/pmol protein)	K <sub>m</sub> ( $\mu$ M)	CL <sub>int</sub> ( $\mu$ L/min/pmol protein)	V <sub>max</sub> (pmol/min/pmol protein)	CL <sub>int</sub> ( $\mu$ L/min/pmol protein)
<b>CYP3A4</b>	19.2 $\pm$ 1.1 (17.5–22.3)	15.6 $\pm$ 4.3 (6.4–24.7)	1.3	22.3 $\pm$ 1.1 (20.1–24.6)	9.7 $\pm$ 2.7 (3.9–15)	2.3		
<b>CYP3A5</b>	0.22 $\pm$ 0.027 (0.16–0.27)	72.5 $\pm$ 24.3 (19.5–125)	0.003	16.2 $\pm$ 1.1 (14.2–18.7)	60.2 $\pm$ 11.9 (40.2–89.7)	0.09		
<b>CYP3A7</b>	0.1 $\pm$ 0.03 (0.08–0.2)	43.8 $\pm$ 31.0 (6.4–199)	0.003	0.2 $\pm$ 0.03 (0.13–0.25)	44.4 $\pm$ 23.5 (15.1–116)	0.004		

K<sub>m</sub>, CL<sub>int</sub>, intrinsic clearance -  $\mu$ L/min/pmol rCYP. Data presented as mean  $\pm$  standard error (95% CI).

**Table 2**Estimated maternal and fetal formation clearances of 16 $\alpha$ -OHP and 6 $\beta$ -OHP

	CYP3A5 Genotype	16 $\alpha$ -OHP CL <sub>f</sub> (L/h)	6 $\beta$ -OHP CL <sub>f</sub> (L/h)	CL <sub>int, H</sub> (L/h)
<b>Maternal</b>	CYP3A5*1*1	347	679	1026
	CYP3A5*0*0	346	624	970
<b>Fetal</b>	CYP3A5*1*1	0.14	0.74	0.88
	CYP3A5*0*0	0.13	0.19	0.32

CL<sub>f</sub>: formation clearance estimated from recombinant enzyme CL<sub>int</sub> and scaled to in vivo values as described in Materials and Methods.

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