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Induction of hepatic CYP3A enzymes by pregnancy-related hormones: studies in human hepatocytes and hepatic cell lines

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Nonstandard abbreviations:
CYP, cytochrome P450; GH, pituitary growth hormone; PGH, placental growth hormone; E2, 17β-estradiol; E3, estriol; C, cortisol; P, progesterone; T, testosterone; DEX, dexamethasone; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; qPCR, quantitative PCR; MDZ, midazolam; 1'-OH-MDZ, 1'-hydroxymidazolam; SCHH, sandwich-cultured human hepatocytes; PXR, pregnane X receptor; CAR, constitutive androstane receptor; HNF6, hepatocyte nuclear factor 6.
ABSTRACT

CYP3A activity is induced by ~2-fold during the 3<sup>rd</sup> trimester of human pregnancy. Placental growth hormone (PGH), estrogens (primarily 17β-estradiol), cortisol, and progesterone have the potential to modulate CYP3A activity. Therefore, we determined whether the elevated plasma concentrations of these hormones during pregnancy induce hepatic CYP3A expression. We incubated sandwich-cultured human hepatocytes (SCHH) from pre-menopausal female donors (n=2) with the physiologic (unbound, 1X-total) and the 10X-total 3<sup>rd</sup> trimester hormone plasma concentrations (individually and in combination) and determined their effect on CYP3A activity and the transcripts of CYP3A4, CYP3A5 and the respective hormone receptors (growth hormone receptor, GHR; glucocorticoid receptor, GR; estrogen receptor alpha, ER<sub>α</sub>). Of all the hormones, cortisol was the most potent inducer of CYP3A activity and CYP3A4, CYP3A5 mRNA expression. The combination of PGH/GH and cortisol induced CYP3A activity and expression significantly more than cortisol alone. When incubated with the unbound or total plasma concentration of all the hormones, CYP3A activity in SCHH was induced to an extent comparable to that observed in vivo during the 3<sup>rd</sup> trimester. These hormones had only a modest effect on the mRNA expression of the hormone receptors. The pattern of induction observed in SCHH was reproduced in HepaRG cells but not in HuH7/HepG2 cells. SCHH or HepaRG cells could be used to determine the mechanistic basis of CYP3A induction during pregnancy and to predict the magnitude of induction likely to be observed during the 1<sup>st</sup> and 2<sup>nd</sup> trimester, when phenotyping studies to measure in vivo CYP3A activity are logistically difficult to perform.
INTRODUCTION

Third trimester pregnancy significantly induces in vivo CYP3A activity as measured by midazolam oral clearance (~2-fold; (Hebert et al., 2008). This induction can result in subtherapeutic plasma concentrations of drugs. For example, the oral clearance of the HIV protease inhibitor, indinavir, a CYP3A substrate, is much greater (~3-fold) during the 3rd trimester than post-partum (or in non-pregnant women or men) (Unadkat et al., 2007). Similarly, pregnancy induces hepatic Cyp3a activity in mice (~3-fold). This induction is due to increased transcription of Cyp3a isoforms Cyp3a41 (the dominant Cyp3a isoform in female mice), Cyp3a16 and Cyp3a44, resulting in increased Cyp3a protein expression (Zhang et al., 2008). Moreover, pregnancy transcriptionally activates mouse Cyp3a isoforms and the human CYP3A4 promoter in the liver (but not in the intestine) of pregnant CYP3A4-promoter-luciferase transgenic (CYP3A4-tg) mice (Zhang et al., 2008). Although the above studies demonstrate that pregnancy induces CYP3A/3a activity by transcriptional activation of CYP3A4/Cyp3a enzymes, the hormones and mechanisms responsible for this induction are not known. In this manuscript, we report for the first time studies in sandwich-cultured human hepatocytes (SCHH) and hepatic cell lines that identify the responsible hormones.

While the plasma concentration of several hormones is increased during pregnancy, evidence in the literature (as detailed below) suggests that only four of these: pituitary growth hormone (GH), cortisol (C), estradiol (E2), and progesterone (P) have the potential to modulate hepatic CYP3A activity. There are considerable data to show that GH regulates CYP3A activity in humans. When hepatocytes (from male and female donors) are exposed to the feminine-like continuous GH profile, CYP3A activity and expression (protein and mRNA) are induced (Dhir et al., 2006). Similarly, GH replacement in GH-deficient individuals, restores CYP3A4 activity (as measured by erythromycin breath test) to levels observed in healthy individuals (Jaffe et al., 2002). In humans, as pregnancy proceeds, the continuous secretion of placental growth hormone (PGH) by the placenta gradually replaces
the pituitary isoform (Table 1) which remains at a constant low concentration from mid-gestation onwards (Igout et al., 1995). PGH differs from GH by only 13 amino acid residues (Frankenne et al., 1988), but they both bind with similar affinity to the growth hormone receptor (GHR) (Baumann et al., 1991) and activate the same signaling pathways (Silva et al., 2002). However, the effect of PGH on hepatic CYP3A activity has never been investigated. Here we report for the first time the effect of PGH on CYP3A activity and expression in SCHH and hepatic cell lines. The major circulating corticosteroid in humans is cortisol while in mice it is corticosterone (Barlow et al., 1973). In humans (Table 1) and mice, the serum concentrations of these corticosteroids increase during pregnancy. Corticosteroids are well known inducers of hepatic CYP3A/3a expression (Pascussi et al., 2001). Hepatic expression of the female-predominant Cyp3a41 isoform depends on estradiol plasma concentrations in female non-pregnant mice (Yamada et al., 2002). Administration of medroxyprogesterone acetate to post-menopausal women increases CYP3A activity as measured by the erythromycin breath test (Tsunoda et al., 1998).

Since the concentrations of the above hormones is increased during pregnancy, it is critical to identify which hormones, alone or in combination, induce hepatic CYP3A enzymes at their circulating plasma concentrations during pregnancy. Therefore, we studied the induction of CYP3A enzymes (activity and expression) by pregnancy-related hormones in SCHH at the circulating plasma concentrations (total or 1X and unbound) of these hormones and also ten-fold their total plasma concentrations (10X). Furthermore, we determined whether the pregnancy hormone mediated induction of CYP3A observed in SCHH could be replicated in the commercially available human hepatic cell lines, HepG2, Huh7, and HepaRG. If it is, these cells could be used in the future to elucidate the mechanistic basis of this induction.

MATERIALS AND METHODS
Chemicals and reagents

Growth hormone, 17β-estradiol, estriol, cortisol, progesterone, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO); placental growth hormone was purchased from GenWay Biotech. Inc (San Diego, CA). Midazolam was purchased from Cerilliant Corporation (Round Rock, TX). Acetic acid (ACS grade) and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA).

Primary human hepatocytes and Hormone Treatment

Stock solutions of rifampin (10 μM), estradiol, cortisol, progesterone, estriol, and testosterone were prepared in methanol; growth hormone and placental growth hormone were diluted in dH2O, pH=9. In all subsequent experiments the cells were treated at 37°C, 5% CO2 for 72h with vehicle (0.1% dH2O, pH=9 and 0.9% methanol) or the pregnancy-specific hormones (individually or in combinations) at the unbound, total, and 10X-total 3rd trimester plasma concentrations (Table 1). In all experiments, incubations with 10 μM rifampin were always included as an internal biological control. Medium containing the hormones or rifampin was changed every 24h. All incubations were performed in triplicate.

Primary human hepatocytes. Cryopreserved or freshly isolated SCHH from healthy female donors (Table 2) were obtained commercially (Life Technologies, Carlsbad, CA). Hepatocytes were plated in collagen-coated 96-well plates according to the manufacturer's instructions. The cells were maintained (37°C, 5% CO2) in incubation medium which consists of Williams E medium supplemented with serum-free Hepatocyte Maintenance Supplements (Life Technologies, Carlsbad, CA). The only difference from the manufacturer's protocol was that we excluded the marginal amount of dexamethasone normally included in the incubation medium, which is not critical for maintaining cell health. After 72h, the SCHH were incubated without or with the pregnancy hormone(s) at the unbound, total, and 10X-total 3rd trimester plasma concentrations. During culture, morphological changes, if any, of the SCHH were examined by a phase contrast...
microscope. Because dexamethasone is widely used in many in vitro studies utilizing primary human hepatocytes, the morphology of SCHH cultivated in the presence of this agent was used as a normal morphological control. There were no substantial effects observed on the morphology of SCHH cultivated in the absence of dexamethasone.

**Huh7 cells.** Huh7 cells were cultured in complete DMEM which consists of 10% fetal bovine serum, 100 units ml\(^{-1}\) penicillin, and 100 \(\mu\)g ml\(^{-1}\) streptomycin. For all DMSO experiments, Huh7 cells were seeded in 24-well plates at a cell density of \(2 \times 10^4\) cells per well. At 90 to 100% confluence, culture medium was replaced with 1 ml complete DMEM containing 1% DMSO (Sigma-Aldrich). Cultures were incubated for 20 days, as previously described (Choi et al., 2009), during which time complete DMEM containing 1% DMSO was replenished every 3 days. After this period, medium was aspirated, and the cells were treated (in triplicate) with serum-free culture medium containing vehicle or the pregnancy-specific hormones at the 10X-total plasma concentrations (individually or in various combinations) observed at the 3\(^{rd}\) trimester of human pregnancy.

**HepaRG cells.** Cryopreserved differentiated HepaRG cells were kindly provided by Biopredic International (Overland Park, KS). The cells were plated in collagen-coated 96-well plates according to the manufacturer’s instructions. Briefly, after plating the differentiated HepaRG cells were maintained for 72h (37\(^{\circ}\)C, 5% CO\(_2\)) in general purpose medium composed of Williams E Medium with GlutaMAX-I, supplemented with HepaRG\(^\circledR\) Thaw, Seed, and General Purpose supplement 670 (ADD670). At the end of this period medium was aspirated and replaced with induction medium composed of Williams E Medium with GlutaMAX-I, without or with the pregnancy-specific hormone(s), at the 1X or 10X-total 3\(^{rd}\) trimester plasma concentrations. The only difference from the manufacturer’s protocol was that we excluded hydrocortisone hemisuccinate from the incubation medium. During the culture, the morphological changes of HepaRG cells were examined by a phase contrast microscope. Because hydrocortisone hemisuccinate is normally included in the serum-free induction supplements (ADD650) provided by the manufacturer (Biopredic International), the
morphology of HepaRG cells cultivated in the presence of this agent was used as a normal morphological control. There was no substantial effect observed on the morphology of HepaRG cells cultivated in the absence of hydrocortisone hemisuccinate. All incubations were performed in triplicate.

**CYP3A Activity Measurement**

At the end of the incubations, CYP3A activity in SCHH and HepaRG cells was measured using midazolam as a probe drug. Briefly, after the third day, culture medium was aspirated; the cells were rinsed twice with pre-warmed PBS and were incubated (37°C, 5% CO₂) with serum-free incubation medium containing midazolam (MDZ, 2 μM) for 1 h. The supernatant was collected for 1'-hydroxymidazolam (1'-OH MDZ) formation analysis. In brief, the reaction was stopped by adding an equal volume of ice-cold methanol containing the internal standard diazepam (100 ng/mL). The samples were dried down, reconstituted in acetonitrile/water (9/1) and the concentration of 1'-OH MDZ was determined by LC/MS (Waters Alliance 2695 HPLC interfaced with Waters Micromass Quattro Micro) using an electrospray ion source. The mobile phase consisted of 0.1% (v/v) acetic acid in water and 0.1% (v/v) acetic acid in acetonitrile as described previously (Kirby et al., 2006). The separation was performed with a Zorbax Eclipse SB-C18 column (2.1 × 150 mm, 5 μm; Agilent Technologies) at a flow rate of 0.25 ml min⁻¹.

**Isolation of RNA and measurement of CYP3A4/5 mRNA expression by real-time PCR (qPCR)**

At the end of the hormone treatments, total RNA was extracted (as per the manufacturer's instructions) from SCHH and HepaRG cells using the Ambion RNAqueous®-96 Kit (Life Technologies, Austin, TX) or from Huh7 cells using the TRIzol reagent (Life Technologies, Carlsbad, CA). This was followed by reverse transcription to cDNA with the TaqMan® Reverse Transcription Reagents (Invitrogen, Carlsbad, CA). Universal PCR
Master Mix and TaqMan primer-probe mixes were purchased from Applied Biosystems Inc. for the detection of human mRNA sequences for CYP3A4 (Hs00430021_m1), CYP3A5 (Hs00241417_m1), estrogen receptor alpha (ERα; Hs00174860_m1), glucocorticoid receptor (GR; Hs00353740_m1), growth hormone receptor (GHR; Hs00174872_m1), and β-glucuronidase (GUS; Hs00939627_m1). Because the concentration of methanol used in the hormone treatments (0.9%) might promote changes in gene expression, the mRNA levels of the studied genes in the presence and absence of methanol (in the absence of the hormones) was compared and found not to be different (data not shown). In all cases, qPCR reactions and analysis of the results were performed as described previously (Dixit et al., 2007).

**Statistical and Data Analysis**

CYP3A activity and mRNA levels in the treated groups were expressed relative to that observed in the vehicle control (negative control). One-way analysis of variance was used when multiple comparisons were made, followed by Tukey's Multiple Comparison Test (GraphPad Prism, v.3.02). Correlation was determined by the Spearman rank analysis of data across all concentrations of hormones (GraphPad Prism, v.3.02), and expressed as the corresponding correlation coefficient $r_s$. Correlations with $p$ values $< 0.001$ were designated as strong or excellent.

**RESULTS**

*The effect of PGH, GH, cortisol, and progesterone on CYP3A4 mRNA expression and activity in SCHH from post-menopausal and pre-menopausal women*

To establish that pregnancy hormones can indeed induce CYP3A4 and to determine the effect of dexamethasone on this induction, we conducted the following preliminary studies.
Cryopreserved SCHH from three post-menopausal donors (Hu0737, Hu1037, and Hu4152 – Table 2) were incubated with PGH, GH, cortisol, and progesterone (individually or in combinations) at the 10X-total 3rd trimester plasma concentrations (Table 1). We included the 10X-total concentration in our experiments to provide a measure of CYP3A induction produced at this supraphysiological concentration against which induction at lower but physiological concentrations can be compared. For two of these donors (Hu0737, Hu1037), the incubations were performed in the absence and presence of dexamethasone (100 nM), an agent which is traditionally included in hepatocyte incubations. CYP3A4 induction was measured by the most sensitive index available i.e. CYP3A4 mRNA. In the presence of dexamethasone, as expected, rifampin induced the expression of CYP3A4 transcripts by ~14-fold but not at all by any of the pregnancy hormones or their combinations (data not shown). In contrast, in the absence of dexamethasone, of all the hormones incubated individually, only cortisol consistently and significantly induced CYP3A4 mRNA expression (Fig. 1A). Progesterone was able to induce CYP3A4 expression in only one donor (Fig. 1A). The combination of the growth hormones (GHs, i.e. PGH and GH) plus cortisol (but not GHs+progesterone) consistently and significantly induced CYP3A4 mRNA expression greater than cortisol alone (Fig. 1A). In addition, the combination of cortisol and progesterone consistently and significantly induced CYP3A4 greater than progesterone or cortisol alone (Fig. 1A). Rifampin induced CYP3A4 mRNA expression by ~90-340 fold.

Given that in the absence of dexamethasone the pregnancy hormones dramatically and significantly induced CYP3A4 transcripts, all subsequent experiments detailed below were conducted without dexamethasone.

We confirmed that the above observations, with respect to CYP3A activity and mRNA expression, were mostly replicated in SCHH from a pre-menopausal female (Hu8116 – Table 2) incubated with 1X-total and 10X-total plasma concentrations of the hormones. Similar to that observed with post-menopausal donors, cortisol induced CYP3A activity and CYP3A4 mRNA expression in these SCHH at both the concentrations used (Figs 1B, 1C).
Progesterone was able to induce CYP3A activity and CYP3A4 mRNA expression only at the 10X-total plasma concentration (Figs 1B, 1C). The combination of the growth hormones (GHs, i.e. PGH and GH) plus cortisol (but not GHs+progesterone) consistently and significantly induced CYP3A activity and CYP3A4 mRNA expression greater than cortisol alone (Figs. 1B, 1C). Moreover, the combination of cortisol and progesterone consistently and significantly induced CYP3A activity and CYP3A4 mRNA greater than progesterone or cortisol alone (Figs. 1B, 1C). Rifampin induced CYP3A activity (~15-fold) and CYP3A4 mRNA (~120-fold).

**Effect of pregnancy-related hormones on CYP3A activity and CYP3A4/5 expression in pre-menopausal SCHH**

To conduct a detailed study of the role of the pregnancy-related hormones in the induction of CYP3A enzymes, we expanded the above study as follows: 1) estradiol, estriol and testosterone were included in the panel of hormones studied; 2) three different concentrations of the hormones were studied, namely the unbound, total, and 10X-total 3rd trimester plasma concentrations. Only PGH was studied at the unbound 3rd trimester plasma concentrations as the unbound plasma concentrations of the GH in the period is negligible; 3) CYP3A activity as well as CYP3A4 and CYP3A5 transcripts were quantified; 4) the transcripts of the receptors of these hormones were quantified, namely estrogen receptor alpha (ER$\alpha$), glucocorticoid receptor (GR), and growth hormone receptor (GHR). These studies were conducted in three different donors of SCHH. Results from only two donors (Hu4197, Hu4232 – Table 2) are presented below because, for the third donor (Hu8130 – Table 2), none of the hormones investigated induced CYP3A activity or CYP3A4/5 mRNA expression even though, in this donor, 10 µM rifampin induced CYP3A activity by ~5-fold.

Of all the hormones incubated individually (PGH, GH, estradiol, estriol, cortisol, progesterone, and testosterone) only cortisol consistently and significantly induced CYP3A
activity and CYP3A4/5 mRNA levels at the unbound, total, and 10X-total 3rd trimester plasma concentrations (Figs. 2, 3, and 4). The combination of the GHs (or PGH at the unbound plasma concentration observed during the 3rd trimester) plus cortisol induced CYP3A activity and CYP3A4 mRNA to levels surpassing those induced by cortisol alone (Figs. 2 and 3). However, the same combination did not consistently induce CYP3A5 mRNA more than cortisol alone (Fig. 4). When the induction by C+P vs. C or E2+C vs. C was compared, no consistent pattern emerged. However, as was observed for the post-menopausal SCHH, at 10x-total 3rd trimester plasma concentration, C+P induced CYP3A activity more than C alone. Except for those discussed below, all other combination of hormones did not consistently or significantly induce CYP3A activity or CYP3A4/5 mRNA expression.

The combined treatment with the five hormones (PGH, GH, estradiol, cortisol, progesterone) at the unbound, total, and 10X-total 3rd trimester plasma concentrations induced CYP3A activity and CYP3A4/5 expression (Figs. 2, 3, and 4). Moreover, this induction was not significantly different from that observed when estriol and testosterone were included (data not shown). The combination of all the hormones (GHs+E2+C+P), at the unbound or total 3rd trimester plasma concentrations of the hormones, induced CYP3A activity (but not CYP3A4 expression) to a lesser extent than GHs+C, suggesting that at these concentrations, E2 or P or both suppress the induction caused by GHs+C (Figs. 2 and 3). Interestingly, the fold-induction in CYP3A activity produced by the combined unbound or total 3rd trimester hormone plasma concentrations was ~2-4 fold (Fig. 2A, B). Rifampin induced CYP3A activity by ~14-25 fold, CYP3A4 expression by ~85-250 fold, and CYP3A5 expression by ~3-4 fold. The ability of the hormones to induce CYP3A activity was strongly and significantly correlated with the induction of CYP3A4 transcripts ($r_s = 0.825$, $p < 0.001$). In addition the induction of CYP3A5 transcripts under all the hormonal treatments was strongly and significantly correlated with that of CYP3A4 ($r_s = 0.801$, $p < 0.001$).
Effect of pregnancy-related hormones on estrogen receptor alpha (ERα), growth hormone receptor (GHR), and glucocorticoid receptor (GR) mRNA expression in SCHH

We investigated the effect of pregnancy-related hormones on the expression of the respective hormone receptors using qPCR. Of the two estrogen receptor isoforms (alpha and beta; ERα and ERβ), we chose to determine the expression of ERα since it is the only one which is expressed in adult human hepatocytes (Taylor and Al-Azzawi, 2000). Of all the hormones incubated individually, only estradiol at the total and 10X-total 3rd trimester plasma concentrations, consistently induced ERα mRNA expression (Fig. 5). Additionally the combination of the GHs and estradiol at the total and 10X-total plasma 3rd trimester concentrations consistently and significantly induced ERα mRNA expression, exceeding those induced by estradiol alone (Fig. 5B, 5C). Interestingly under certain hormone treatments (Fig. 5), ERα expression decreased compared to control. For example, at the 1X- and 10X-total 3rd trimester plasma concentrations of the hormones, GHs+C+E2 or GHs+C+E2+P induced ERα expression to a lesser degree than GHs+E2. In addition, all the hormone treatments had a modest or no effect on the expression of GHR and GR (Supplemental Figures 1 and 2).

Effect of pregnancy-related hormones on CYP3A4 expression in HepG2 and Huh7 cells

To examine whether the hormone-mediated changes in CYP3A4 expression that were observed in SCHH could be replicated in a commercially available human hepatic cell lines, we incubated HepG2 and DMSO-treated Huh7 cells with the 10X-total 3rd trimester plasma concentrations of PGH, GH, estradiol, cortisol, and progesterone (individually or in various combinations). We chose to use DMSO-treated Huh7 cells for these experiments, since it
has been shown that DMSO induces basal expression of CYP3A4 to a measurable level (Choi et al., 2009). Likewise, we decided to use the 10X-total 3rd trimester plasma concentration of the hormones because, in preliminary experiments, no induction of CYP3A transcripts could be observed at lower hormone concentrations. This may be due to the lower expression in these cells of various transcription factors that have the potential to regulate CYP3A4 expression (e.g. CAR, HNF6) (Sivertsson et al., 2010). Additionally we decided to determine only the fold-induction in CYP3A4 transcripts as it was a more sensitive measure of CYP3A induction in SCHH (Figs. 2 and 3). In HepG2 cells the expression of CYP3A4 transcripts was very low, thus we could not determine any hormone-mediated induction of CYP3A4 transcript expression in these cells. In Huh7 cells, of all the hormones incubated individually (PGH, GH, estradiol, cortisol, and progesterone) only cortisol consistently induced CYP3A4 mRNA levels at the 10X-total 3rd trimester plasma concentrations (Fig. 6A). The combination of GHs plus cortisol induced CYP3A4 mRNA levels even further (~30% increase). Moreover, the combination of cortisol with estradiol induced CYP3A4 transcripts greater than that observed after exposure to estradiol or cortisol alone (Fig. 6A). Finally, the combined treatment with the five pregnancy-specific hormones significantly induced CYP3A4 mRNA levels but to a lesser extent than E2+C or GHs+C (Fig. 6A).

**Effect of pregnancy-related hormones on CYP3A activity and CYP3A4 expression in HepaRG cells**

The incubation of HepaRG cells with the pregnancy-specific hormones was performed in the presence and absence of hydrocortisone hemisuccinate, an agent which is normally included in the manufacturer-provided supplements (Steen D., personal communication). However, in the presence of hydrocortisone hemisuccinate, none of the hormones were able to produce a measurable and significant change in CYP3A activity but rifampin did induce
CYP3A activity by ~12-fold (data not shown). Considering that hydrocortisone (cortisol) was the most potent inducer of CYP3A activity and CYP3A4 mRNA in the experiments we performed SCHH (Figs. 2 and 3), all subsequent experiments were performed in the absence of this agent. Given that the expression levels of various CYP enzymes and transcriptions factors is comparable between HepaRG cells and primary human hepatocytes (Hart et al., 2010), we decided to incubate HepaRG cells with the total and 10X-total 3rd trimester plasma concentrations of the studied hormones.

Of all the hormones incubated individually, only cortisol consistently and significantly induced CYP3A activity and CYP3A4 mRNA expression (Fig. 6B, 6C). The combination of the GHs plus cortisol consistently and significantly induced CYP3A activity and CYP3A4 transcripts to levels surpassing those induced by the glucocorticoid alone (Fig. 6B, 6C). Finally, the combined treatment with the five pregnancy-specific hormones (PGH, GH, estradiol, cortisol, and progesterone) consistently and significantly induced CYP3A activity and CYP3A4 expression to a level not significantly different from GHs+C (Fig. 6B, 6C). Rifampin induced CYP3A activity by ~20 fold and CYP3A4 expression levels by ~120 fold (data not shown).

DISCUSSION

We found that of all the hormones incubated individually, only cortisol consistently and significantly induced CYP3A activity and CYP3A4 expression in SCHH. These results are in agreement with in vivo studies in mice, which have shown that adrenalectomy decreases hepatic expression of the female-predominant Cyp3a41 (Sakuma et al., 2004). This phenotype could be reversed only after dexamethasone administration. Even though corticosteroids induce CYP3A4, literature data are contradictory on the involvement of GR or PXR in this induction. Using GR(-/-) mice, Schuetz et al., (Schuetz et al., 2000) have shown that induction of CYP3a by dexamethasone is independent of GR. This induction, at
physiological concentrations of cortisol, is also independent of PXR (pregnane X receptor). Cortisol activates human PXR at very high μM concentrations (Blumberg et al., 1998; Mnif et al., 2007), concentration which are considerably higher than the plasma concentrations of cortisol during human pregnancy (Table 1). When rat hepatocytes, transfected with reporter constructs containing the human CYP3A4 promoter and the human PXR, are exposed to 10 μM cortisol, a concentration which far exceeds that observed in pregnant women (Table 1), the expression of the reporter CYP3A construct is not induced (Xie et al., 2000). Moreover, Cyp3a11 induction by oral administration of dexamethasone was not eliminated in PXR knock-out mice (Zimmermann et al., 2009). Therefore, cortisol-mediated induction of CYP3A4 seems to occur by an unknown PXR-independent mechanism(s).

The combination of the two growth hormone isoforms plus cortisol induced CYP3A activity and CYP3A4 mRNA expression greater than that observed after exposure to cortisol alone. This pattern of induction was identical between pre-menopausal and post-menopausal donors (Figs. 1A, 1C) indicating that SCHH from both sources of donors could be used for gain insight into the mechanisms of CYP3A induction by these hormones. This difference in induction by a glucocorticoid in the absence and presence of GH is in agreement with previous studies in human hepatocytes (Dhir et al., 2006; Thangavel et al., 2011). In this regard, our results indicate that PGH behaves very much like GH. Given, that PGH gradually replaces the pituitary GH isoform throughout gestation, and it is continuously secreted, our findings indicate the potential role of PGH in combination with glucocorticoids, in regulating hepatic CYP3A activity in pregnant women. Interestingly, continuous exposure of human hepatocytes to GHs (feminine circulating pattern) and corticosteroids induces CYP3A4 protein to a greater extent in hepatocytes derived from female compared with male subjects (Thangavel et al., 2011). This is due to greater activation and nuclear translocation of the transcriptional factors HNF-4α and PXR and enhanced binding of these factors to the CYP3A4 regulatory motifs (Thangavel et al., 2011). In addition, the combination of cortisol
and progesterone consistently and significantly induced CYP3A activity and CYP3A4 mRNA greater than progesterone or cortisol alone in both post- and pre-menopausal donors (Figs. 1, 3C), thus supporting the notion that the mechanisms of CYP3A induction is common between the two types of donors.

When compared with cortisol, the effect of PGH, GH, estradiol, progesterone, estriol, and testosterone (when incubated individually) in inducing hepatic CYP3A activity and expression was modest. Estradiol was not able to induce hepatic CYP3A activity or CYP3A4/5 transcripts at any of the concentrations used. Progesterone was a less potent inducer compared to cortisol since it was able to generate a statistically significant effect on CYP3A activity and CYP3A4 transcripts in only a few cases. This lack of effect may be due to rapid depletion of these steroids from the incubation medium as previously reported (Choi et al., 2012). In their study, even when the medium was changed frequently to compensate for this depletion, estradiol or progesterone, at the 10X-total 3rd trimester plasma concentrations, resulted in a maximum 3-fold induction of CYP3A activity in some of their SCHH (Choi et al., 2012; Koh et al., 2012). Moreover administration of medroxyprogesterone acetate to post-menopausal women results in a modest increase (23-25%) in in vivo CYP3A activity as determined by prednisolone clearance (Tsunoda et al., 1998). Collectively, these data suggest modest individual contribution of estradiol and progesterone on in vivo induction of CYP3A activity in SCHH.

Despite the modest individual contribution of estradiol and progesterone in inducing hepatic CYP3A activity and expression, their combination with the GHs (or PGH at the unbound plasma concentration observed during the 3rd trimester) and cortisol consistently and significantly induced CYP3A activity and CYP3A4 transcripts. Interestingly this combination at the unbound and 1X 3rd trimester plasma concentrations induced CYP3A activity by ~2-4 fold, a value which is comparable to the in vivo induction of CYP3A activity
(as measured by midazolam oral clearance) during the 3rd trimester (~2-fold) (Hebert et al., 2008). To our knowledge, this in vitro to in vivo correspondence implies that SCHH, when incubated with PGH, GH, estradiol, cortisol, and progesterone (at the unbound and total 3rd trimester plasma concentrations), can mimic the changes observed in hepatic CYP3A activity during the 3rd trimester. Additional in vitro studies are required to determine the potency of these hormones at the lower concentrations observed in early gestation to induce hepatic CYP3A activity. Moreover the combination of all the hormones (GHs+E2+C+P), at the unbound or total 3rd trimester plasma concentrations of the hormones, induced CYP3A activity to a lesser extent than GHs+C, suggesting that at these concentrations E2 or P or both suppress the induction caused by GHs+C. Further studies are needed to confirm and elucidate the mechanistic basis of this suppression. Furthermore, CYP3A activity and the expression of CYP3A4 transcripts in the SCHH exhibited a strong and significant correlation ($r_s = 0.825, p < 0.001$). Thus, either CYP3A activity or CYP3A4 mRNA could be used as markers of hepatic CYP3A induction by pregnancy-related hormones in SCHH.

Cortisol was also the main inducer of CYP3A5 expression levels even though the observed fold-induction of CYP3A5 transcripts was modest. Moreover there was a strong correlation ($r_s = 0.801, p < 0.001$) between the expression of the transcripts of these two CYP3A isoforms. Thus, despite the lower magnitude of induction of CYP3A5 vs. CYP3A4 transcripts, the strong correlation in the expression of these two transcripts suggests a commonality in the regulatory mechanisms of their induction.

The hormones had only a modest effect on the expression of the respective hormone receptors ($GHR$, $GR$, and $ER{\alpha}$). Thus, this does not appear to be a primary mechanism by which the pregnancy-related hormones induce CYP3A activity. However, these results do not necessarily preclude the involvement of these receptors in pregnancy-induced CYP3A
activity because an increase in the plasma concentration of the hormone(s) alone, acting through its respective receptor, could induce CYP3A activity.

The magnitude of the induction of CYP3A4 transcripts in SCHH and HepaRG cells was consistently and considerably larger than that observed in Huh7 cells. In addition, CYP3A activity was measurable in HepaRG but not Huh7 cells. The expression of multiple genes in HepaRG cells is comparable to that observed in primary human hepatocytes and human liver (Hart et al., 2010). Although the fold-induction of CYP3A4 transcripts in HepaRG cells by the pregnancy-related hormones was larger than that in human hepatocytes, the pattern was similar except that, at the 1X-total 3rd trimester plasma concentrations, the combination of five hormones did not suppress CYP3A activity when compared with GHs+C. Thus, our results suggest that HepaRG cells could serve as a readily available, cost-effective, in vitro model, to gain further insight into the mechanistic basis for the hormone-mediated induction of CYP3A4.

In summary, our data show that cortisol alone or in combination with GHs is a potent inducer of CYP3A activity and CYP3A4, CYP3A5 expression in SCHH, including at their physiologically relevant 3rd trimester unbound plasma concentrations. The combination of cortisol and GH induced CYP3A activity and CYP3A4 transcripts greater than cortisol alone. Additional experiments will need to be conducted to confirm whether there is synergy between cortisol and GH in the induction of CYP3A activity and transcripts. Our experiments also showed that PGH and GH are equally effective, when combined with cortisol, in inducing CYP3A activity. In addition, we have shown for the first time that the combination of all the pregnancy-specific hormones at their physiologic (unbound, total) plasma concentrations induce CYP3A activity in SCHH comparable to that observed in vivo. This finding is important for several reasons. First, this suggests that the SCHH could be used to predict the magnitude of in vivo CYP3A induction earlier in gestation when...
phenotyping studies (e.g. midazolam) are difficult to conduct for ethical and logistical reasons. Such in vitro to in vivo extrapolation is reasonable because our previous studies strongly suggest that pregnancy induces hepatic and not intestinal CYP3A activity. In mice expressing the human CYP3A4 promoter-luciferase construct, pregnancy induced only hepatic and not intestinal luciferase activity (Zhang et al., 2003). We confirmed this observation in humans through sensitivity analysis of physiologically based pharmacokinetic modeling of disposition of CYP3A substrate drugs that have low to high intestinal first pass effect (Ban Ke et al., (in press)). Second, the SCHH could be used in the future to elucidate the molecular mechanisms of induction of in vivo CYP3A activity. Last, but not least, we have identified HepaRG cells as an in vitro model that could potentially be used to determine the role of hormone receptors and transcription factors in the hormone-mediated induction of CYP3A4 expression during pregnancy.
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Authorship contribution

Participated in research design: Papageorgiou and Unadkat.

Conducted experiments: Papageorgiou.

Contributed new reagents or analytic tools: Grepper.

Performed data analysis: Papageorgiou and Unadkat.

Wrote or contributed to the writing of the manuscript: Papageorgiou and Unadkat.
REFERENCES


Footnotes

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FIGURE LEGENDS

Figure 1. (A) Induction of CYP3A4 expression in SCHH from three post-menopausal donors (Hu0737, Hu1037, and Hu4152) when exposed to 10X-total 3rd trimester plasma concentrations of the indicated hormones. Induction of (B) CYP3A activity or (C) CYP3A4 expression in SCHH from a pre-menopausal donor (Hu8116) when exposed to 1X- or 10X-total 3rd trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± SD of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey’s multiple comparisons test. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with control. †, p < 0.05; ††, p < 0.01; †††, p < 0.001 compared with SCHH exposed to cortisol alone. §, p < 0.05; §§, p < 0.01; §§§, p < 0.001 compared with SCHH exposed to progesterone alone. PGH: placental growth hormone, GH: pituitary growth hormone, GHs: PGH and GH, C: cortisol, P: progesterone.

Figure 2. CYP3A activity in SCHH from two pre-menopausal donors (Hu4197, Hu4232). SCHH when exposed to unbound (A), total (B), or 10X-total (C) 3rd trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± SD of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey’s multiple comparisons test. *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with control. †, p < 0.05; ††, p < 0.01; †††, p < 0.001 compared with SCHH exposed to cortisol alone. ¶, p < 0.05; ¶¶, p < 0.01; ¶¶¶, p < 0.001 compared with SCHH exposed to estradiol alone. §, p < 0.05; §§, p < 0.01; §§§, p < 0.001 compared with SCHH exposed to progesterone alone. #, p < 0.05; ##, p < 0.01; ###, p < 0.001 compared with SCHH exposed to GHs+C. E2: estradiol, E3: estriol, T: testosterone.

Figure 3. CYP3A4 expression in SCHH from two pre-menopausal donors (Hu4197, Hu4232) when exposed to unbound (A), total (B), or 10X-total (C) 3rd trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± SD of triplicates) are expressed...
relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with SCHH exposed to cortisol alone. ‡, $p < 0.05$; ‡‡, $p < 0.01$; ‡‡‡, $p < 0.001$ compared with SCHH exposed to estradiol alone. §, $p < 0.05$; §§, $p < 0.01$; §§§, $p < 0.001$ compared with SCHH exposed to progesterone alone.

**Figure 4.** CYP3A5 expression in SCHH from two pre-menopausal donors (Hu4197, Hu4232) when exposed to unbound (A), total (B), or 10X-total (C) 3rd trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± SD of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with SCHH exposed to cortisol alone. ‡, $p < 0.05$; ‡‡, $p < 0.01$; ‡‡‡, $p < 0.001$ compared with SCHH exposed to estradiol alone.

**Figure 5.** ERα expression in SCHH from two pre-menopausal donors (Hu4197, Hu4232) when exposed to unbound (A), total (B), or 10X-total (C) 3rd trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± SD of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with SCHH exposed to estradiol alone. §, $p < 0.05$; §§, $p < 0.01$; §§§, $p < 0.001$ compared with SCHH exposed to GHs+E2.

**Figure 6.** (A) CYP3A4 expression in DMSO-treated Huh7 cells, when exposed to 10X-total 3rd trimester plasma concentrations of the indicated hormones. CYP3A activity (B) and CYP3A4 expression (C) in differentiated HepaRG cells exposed to the total or 10X-total 3rd
trimester plasma concentrations of the indicated hormones. Fold-induction values (n=3, mean ± SD) are expressed relative to the expression levels in the vehicle control (CTRL). One-way ANOVA was followed by Tukey’s multiple comparisons test. **, \( p < 0.01 \); ***, \( p < 0.001 \), compared with control. †, \( p < 0.05 \), ††, \( p < 0.01 \); †††, \( p < 0.001 \) compared with cells exposed to cortisol alone. §, \( p < 0.05 \), §§, \( p < 0.01 \), §§§, \( p < 0.001 \) compared with cells exposed to estradiol alone. RIF: rifampin.
Table 1: Maximum unbound and total plasma concentrations of hormones in humans observed during the 3rd trimester of human pregnancy

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Unbound (nM)</th>
<th>Total (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGH</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>GH</td>
<td>0.015</td>
<td>0.05</td>
</tr>
<tr>
<td>Estradiol</td>
<td>~2</td>
<td>100</td>
</tr>
<tr>
<td>Estriol</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Cortisol</td>
<td>70</td>
<td>800</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7</td>
<td>540</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.2</td>
<td>15</td>
</tr>
</tbody>
</table>

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\(a\): (Wu et al., 2003); \(b\): (Baumann et al., 1988; Caufriez et al., 1993); \(c\): (Lindberg et al., 1974); \(d\): (Boroditsky et al., 1978; Voss, 1999); \(e\): (Lindholm and Schultz-Moller, 1973; Demey-Ponsart et al., 1982); \(f\): (Meulenberg and Hofman, 1989); \(g\): (O’Leary et al., 1991)
<table>
<thead>
<tr>
<th>Lot number</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Medication</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu0737</td>
<td>52</td>
<td>F</td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>Hu1037</td>
<td>57</td>
<td>F</td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>Hu4152</td>
<td>50</td>
<td>F</td>
<td>None</td>
<td>Anoxia</td>
</tr>
<tr>
<td>Hu8116</td>
<td>23</td>
<td>F</td>
<td>NA</td>
<td>Not available</td>
</tr>
<tr>
<td>Hu4197</td>
<td>31</td>
<td>F</td>
<td>None</td>
<td>Blunt trauma</td>
</tr>
<tr>
<td>Hu4232</td>
<td>20</td>
<td>F</td>
<td>Wellbutrin, Lexipro, asthma medications</td>
<td>Anoxia, secondary to drug overdose</td>
</tr>
<tr>
<td>Hu8130</td>
<td>18</td>
<td>F</td>
<td>None</td>
<td>Anoxia, secondary to asphyxiation</td>
</tr>
</tbody>
</table>
Figure 4

A. CYP3A45 mRNA (fold change vs. control)

B. CYP3A45 mRNA (fold change vs. control)

C. CYP3A45 mRNA (fold change vs. control)