Special Section on Pregnancy

Induction of Hepatic CYP3A Enzymes by Pregnancy-Related Hormones: Studies in Human Hepatocytes and Hepatic Cell Lines

Ioannis Papageorgiou, Susan Grepper, and Jashvant D. Unadkat

Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, Washington (I.P., J.D.U.), and LifeTechnologies, Carlsbad, California (S.G.)

Received September 10, 2012; accepted December 6, 2012

ABSTRACT

CYP3A activity is induced by approximately 2-fold during the third 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 premenopausal female donors (n = 2) with the physiologic (unbound, 1× total) and the 10× total third 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, glucocorticoid receptor, and estrogen receptor alpha). Of all the hormones, cortisol was the most potent inducer of CYP3A activity and CYP3A4, CYP3A5 mRNA expression. The combination of PGH/growth hormone and cortisol induced CYP3A activity and expression significantly more than did 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 third 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 first and second trimesters, 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 MDZ (midazolam) oral clearance (approximately 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 (approximately 3-fold) during the third trimester than in postpartum (or in nonpregnant women or men) (Unadkat et al., 2007). Similarly, pregnancy induces hepatic Cyp3a activity in mice (approximately 3-fold). This induction is attributable to increased transcription of Cyp3a isoforms Cyp3a4l (the dominant Cyp3a isoform in female mice), Cyp3a10, 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 aforementioned studies showed that pregnancy induces CYP3A3a activity by transcriptional activation of CYP3A4/Cyp3a enzymes, the hormones and mechanisms responsible for this induction are not known. In this article, we report, for the first time to our knowledge, studies in sandwich-cultured human hepatocytes (SCHH) and hepatic cell lines that identify the responsible hormones.

Although 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, estradiol, and progesterone, 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.

ABBREVIATIONS: DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ERa, estrogen receptor alpha; GH, pituitary growth hormone; GHR, growth hormone receptor; GR, glucocorticoid receptor; HNF6, hepatocyte nuclear factor 6; MDZ, midazolam; PGH, placental growth hormone; PXR, pregnane X receptor; qPCR, quantitative polymerase chain reaction; SCHH, sandwich-cultured human hepatocytes.
concentrations (total or unbound) of these hormones and also pregnancy-related hormones in SCHH at the circulating plasma concentrations during pregnancy. Therefore, we alone or in combination, induce hepatic CYP3A enzymes at their activity, as measured by the erythromycin breath test (Tsunoda et al., 2001). Hepatic expression of the female-predominant well-known inducers of hepatic CYP3A/3a expression (Pascussi et al., 1973). In humans (Table 1) and mice, the serum concentrations of humans is cortisol, and in mice, it is corticosterone (Barlow et al., 1989). PGH differs from GH by only 13 amino acid residues (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 onward (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 GH 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, and 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 nonpregnant mice (Yamada et al., 2002). Administration of medroxyprogesterone acetate to postmenopausal women increases CYP3A activity, as measured by the erythromycin breath test (Tsunoda et al., 1998).

Because the concentrations of the aforementioned hormones are 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 1 and unbound) of these hormones and also 10-fold their total plasma concentrations (10×). 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, estril, cortisol, progesterone, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO); placental PGH 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, and testosterone were prepared in methanol; GH and PGH were diluted in dH2O (pH, 9). In all subsequent experiments, the cells were treated at 37°Cin 5% CO2 for 72 hours 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 10× total third trimester plasma concentrations (Table 1). In all experiments, incubations with 10 μM rifampin were always included as an internal biologic control. Medium containing the hormones or rifampin was changed every 24 hours. 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). The only difference from the manufacturer’s protocol was that we excluded the marginal amount of dexamethasone (DEX) normally included in the incubation medium, which is not critical for maintaining cell health. After 72 hours, the SCHH were incubated without or with the pregnancy hormone(s) at the unbound, total, and 10× total third trimester plasma concentrations. During culture, morphologic changes, if any, of the SCHH were examined by a phase contrast microscope. Because DEX is widely used in many in vitro studies using primary human hepatocytes, the morphology of SCHH cultivated in the presence of this agent was used as a normal morphologic control. There were no substantial effects observed on the morphology of SCHH cultivated in the absence of DEX.

**Huh7 Cells.** Huh7 cells were cultured in complete DMEM (Dulbecco’s modified Eagle’s medium), which consists of 10% fetal bovine serum, 100 units ml–1 penicillin, and 100 mg ml–1 streptomycin. For all DMSO (dimethylsulfoxide) experiments, Huh7 cells were seeded in 24-well plates at a cell density of 2×104 cells per well. At 90–100% 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 10× total plasma concentrations (individually or in various combinations) observed during the third trimester of human pregnancy.

### Table 1

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Unbound</th>
<th>Total</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>

### Table 2

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Age, years</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>Not available</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>
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. In brief, after plating, the differentiated HepaRG cells were maintained for 72 hours (37°C, 5% CO2) in general purpose medium composed of Williams E medium with GlutaMAX-I supplemented with HepaRG 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 1× or 10× total third 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 morphologic 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 morphologic 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 MDZ as a probe drug. In brief, after the third day, culture medium was aspirated; the cells were rinsed twice with prewarmed phosphate-buffered saline and were incubated (37°C, 5% CO2) with serum-free incubation medium containing MDZ (2 μM) for 1 hour. The supernatant was collected for 1′-hydroxymidazolam 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 and reconstituted in acetonitrile/water (9/1), and the concentration of 1′-hydroxymidazolam was determined by liquid chromatography–mass spectrometry [Waters Alliance 2695 HPLC (Waters, Milford, MA) 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, Santa Clara, CA) at a flow rate of 0.25 ml min−1.

Isolation of RNA and Measurement of CYP3A4/5 mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction (qPCR)

At the end of the hormone treatments, total RNA was extracted (according to the manufacturer’s instructions) from SCHH and HepaRG cells with use of the Ambion RNeasy-96 Kit (Life Technologies; Austin, TX) or from Hu7 cells using the TRIzol reagent (Life Technologies). This was followed by reverse transcription to cDNA with the TaqMan Reverse Transcription Reagents (Invitrogen, Carlsbad, CA). Universal Polymerase Chain Reaction Master Mix and TaqMan primer-probe mixes were purchased from Applied Biosystems, Inc. (Carlsbad, CA) for the detection of human mRNA sequences for CYP3A4 (Hs00430021_m1), CYP3A5 (Hs00244147_m1), estrogen receptor alpha (ERα; Hs00174860_m1), glucocorticoid receptor (GR; Hs00353740_m1), 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 (unpublished data). 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 (Prism v3.02; GraphPad Software, Inc., La Jolla, CA). Correlation was determined by the Spearman rank analysis of data across all concentrations of hormones (GraphPad Prism, version 3.02) and
expressed as the corresponding correlation coefficient $r$. 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 Postmenopausal and Premenopausal Women. To establish that pregnancy hormones can indeed induce CYP3A4 and to determine the effect of DEX on this induction, we conducted the following preliminary studies. Cryopreserved SCHH from three postmenopausal donors (Hu0737, Hu1037, and Hu4152) (Table 2) were incubated with PGH, GH, cortisol, and progesterone (individually or in combinations) at the 10× total third trimester plasma concentrations (Table 1). We included the 10× total concentration in our experiments to provide a measure of CYP3A induction produced at this supraphysiological concentration, against which induction at lower but physiologic concentrations can be compared. For two of these donors (Hu0737 and Hu1037), the incubations were performed in the absence and presence of DEX (100 nM), an agent that is traditionally included in hepatocyte incubations. CYP3A4 induction was measured by the most sensitive index available (i.e., CYP3A4 mRNA). In the presence of DEX, as expected, rifampin induced the expression of CYP3A4 transcripts by ~14-fold but not by any of the pregnancy hormones or their combinations (unpublished data). In contrast, in the absence of DEX, 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 GHs (GHs; i.e., PGH and GH) plus cortisol (but not GHs and progesterone) 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 GHs (GHs; i.e., PGH and GH) plus cortisol (but not GHs and progesterone) consistently and significantly induced CYP3A4 mRNA expression greater than did cortisol alone (Fig. 1A). In addition, the combination of cortisol and progesterone consistently and significantly induced CYP3A4 greater than did progesterone or cortisol alone (Fig. 1A). Rifampin induced CYP3A4 mRNA expression by ~90–340-fold. Because in the absence of DEX, the pregnancy hormones dramatically and significantly induced CYP3A4 transcripts, all subsequent experiments detailed below were conducted without DEX.

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

Fig. 2. CYP3A activity in SCHH from two premenopausal donors (Hu4197 and Hu4232). SCHH when exposed to unbound (A), total (B), or 10× total (C) third trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± S.D. of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way analysis of variance 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 < 0.001, compared with SCHH exposed to estradiol alone. †††P < 0.001, compared with SCHH exposed to progesterone alone. *P < 0.05; †††P < 0.001, compared with SCHH exposed to GHs and cortisol (C). E2, estradiol; E3, estriol; T, testosterone.
Effect of Pregnancy-Related Hormones on CYP3A Activity and CYP3A4/5 Expression in Premenopausal 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. First, estradiol, estriol, and testosterone were included in the panel of hormones studied. Second, three different concentrations of the hormones were studied, namely the unbound, total, and 10× total third trimester plasma concentrations. Only PGH was studied at the unbound third trimester plasma concentrations, because the unbound plasma concentrations of the GH in the period were negligible. Third, CYP3A activity and CYP3A4 and CYP3A5 transcripts were quantified. Fourth, the transcripts of the receptors of these hormones were quantified, namely ERα, GR, and 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 approximately 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 10× total third trimester plasma concentrations (Figs. 2, 3, and 4). The combination of the GHs (or PGH at the unbound plasma concentration observed during the third 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 cortisol plus progesterone versus cortisol or estradiol plus cortisol versus cortisol was compared, no consistent pattern emerged. However, as was observed for the postmenopausal SCHH, at 10× total third trimester plasma concentration, cortisol plus progesterone induced CYP3A activity more than did cortisol alone. With the exception of 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, and progesterone) at the unbound, total, and 10× total third 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 estradiol and testosterone were included (data not shown). The combination of all the hormones (GHs, estradiol, cortisol, and progesterone) at the unbound or total third trimester plasma concentrations of the hormones induced CYP3A activity (but not CYP3A4 expression) to a lesser extent than did GHs and cortisol, suggesting that, at these concentrations, estradiol and/or progesterone suppress the induction caused by GHs and cortisol (Figs. 2 and 3). Of interest, the fold-induction in CYP3A activity produced by the combined unbound or total third trimester hormone plasma concentrations was ~2–4-fold (Fig. 2, A and 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

![Fig. 3. CYP3A4 expression in SCHH from two premenopausal donors (Hu4197 and Hu4232) when exposed to unbound (A), total (B), or 10× total (C) third trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± S.D. of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way analysis of variance was followed by Tukey’s multiple comparisons test. *P < 0.05; **P < 0.01; ***P < 0.001, compared with control.}{attachment}
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 ERα, GHR, and 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 (ERα and ERβ), we chose to determine the expression of ERα, because it is the only one that is expressed in adult human hepatocytes (Taylor and Al-Azzawi, 2000). Of all the hormones incubated individually, only estradiol at the total and 10× total third trimester plasma concentrations consistently induced ERα mRNA expression (Fig. 5). In addition, the combination of the GHs and estradiol at the total and 10× total plasma third trimester concentrations consistently and significantly induced ERα mRNA expression, exceeding those induced by estradiol alone (Fig. 5, B and C). Of interest, under certain hormone treatments (Fig. 5), ERα expression decreased, compared with control. For example, at the 1× and 10× total third trimester plasma concentrations of the hormones, GHs, cortisol, and estradiol or GHs, cortisol, +estradiol, and progesterone induced ERα expression to a lesser degree than did GHs and estradiol. In addition, all the hormone treatments had a modest or no effect on the expression of GHR and GR (Supplemental Figs. 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 10× total third 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, because it has been shown that DMSO induces basal expression of CYP3A4 to a measureable level (Choi et al., 2009). Likewise, we decided to use the 10× total third 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 attributable to the lower expression in these cells of various transcription factors that have the potential to regulate CYP3A4 expression (e.g., constitutive androstane receptor, hepatocyte nuclear factor 6) (Sivertsson et al., 2010). In addition, we decided to determine only the fold-induction in CYP3A4 transcripts, because 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 10× total third 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 compared with estradiol plus cortisol or GHs plus cortisol (Fig. 6A).

Fig. 4. CYP3A5 expression in SCHH from two premenopausal donors (Hu4197 and Hu4232) when exposed to unbound (A), total (B), or 10× total (C) third trimester plasma concentrations of the indicated hormones. Fold-induction values (mean ± S.D. of triplicates) are expressed relative to the expression levels in the vehicle control (CTRL). One-way analysis of variance was followed by Tukey’s multiple comparisons test. *P < 0.05; **P < 0.01; ***P < 0.001, compared with control. †††P < 0.001, compared with SCHH exposed to cortisol alone. §§§P < 0.001 compared with SCHH exposed to estradiol alone.
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 that is normally included in the manufacturer-provided supplements (D. Steen, 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 that we performed with SCHH (Figs. 2 and 3), all subsequent experiments were performed in the absence of this agent. Because the expression levels of various CYP enzymes and transcriptions factors are comparable between HepaRG cells and primary human hepatocytes (Hart et al., 2010), we decided to incubate HepaRG cells with the total and 10× total third 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. 6, B and C). 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. 6, B and C). 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 that induced by GHs and cortisol (Fig. 6, B and C). Rifampin induced CYP3A activity by ~20-fold and CYP3A4 expression levels by ~120-fold (unpublished data).

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 that have shown that adrenalectomy decreases hepatic expression of the female-predominant Cyp3a41 (Sakuma et al., 2004). This phenotype could be reversed only after DEX administration. Although corticosteroids induce CYP3A4, literature data are contradictory on the involvement of GR or pregnane X receptor (PXR) in this induction. Using GR(−/−) mice, Schuetz et al., (Schuetz et al., 2000) have shown that induction of CYP3a by DEX is independent of GR. This induction, at physiologic concentrations of cortisol, is also independent of PXR. Cortisol activates human PXR at very high micromolar concentrations (Blumberg et al., 1998; Mnif et al., 2007), concentrations that 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 that 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 DEX 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 GH 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 premenopausal and postmenopausal donors (Fig. 1, A and C), indicating that SCHH from both sources of donors
could be used to 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. Because 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. Of interest, 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 attributable 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 did progesterone or cortisol alone in both post- and premenopausal donors (Figs. 1 and 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 than cortisol, because 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 attributable to rapid depletion of these steroids from the incubation medium as previously reported (Choi et al., 2013). In their study, even when the medium was changed frequently to compensate for this depletion, estradiol or progesterone, at the 10× total third trimester plasma concentrations, resulted in a maximum 3-fold induction of CYP3A activity in some SCHHs (Choi et al., 2013; Koh et al., 2012). Moreover administration of medroxyprogesterone acetate to postmenopausal women results in a modest increase (23–25%) 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 third trimester) and cortisol consistently and significantly induced CYP3A activity and CYP3A4 transcripts. Of interest, this combination at the unbound and 1× total third trimester plasma concentrations, resulted in a maximum 3–4-fold induction of CYP3A activity (as measured by MDZ oral clearance) during the third trimester (approximately 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 third trimester plasma concentrations), can mimic the changes observed in hepatic CYP3A activity during the third trimester.
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, estradiol, cortisol, and progesterone) at the unbound or total third trimester plasma concentrations of the hormones induced CYP3A activity to a lesser extent than did GHs and cortisol, suggesting that, at these concentrations, estradiol and/or progesterone suppress the induction caused by GHs and cortisol. Additional 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 versus 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 ERα). 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 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 greater than that in human hepatocytes, the pattern was similar except that, at the 1× total third trimester plasma concentrations, the combination of five hormones did not suppress CYP3A activity when compared with GHs plus cortisol. 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 and CYP3A expression in SCHH, including at their physiologically relevant third trimester unbound plasma concentrations. The combination of cortisol and GH induced CYP3A activity and CYP3A4 transcripts greater than did 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 to our knowledge, 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., MDZ) 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.

Acknowledgments

The authors thank Brian Kirby for valuable discussions, and David Steen for kindly providing the HepaRG cells.

Authorship Contributions

Participated in research design: Papageorgiou, Unadkat.
Conducted experiments: Papageorgiou.
Contributed new reagents or analytic tools: Grepper.
Performed data analysis: Papageorgiou, Unadkat.
Wrote or contributed to the writing of the manuscript: Papageorgiou, Unadkat.

References
