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Research Article

Mechanisms of CYP3A Induction During Pregnancy: Studies in HepaRG Cells

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Abstract. Activity of CYP3A, an enzyme responsible for metabolism of many marketed drugs, is induced by ~2-fold in pregnant women. Through studies in sandwich-cultured human hepatocytes (SCHH) and HepaRG cells, our laboratory has shown that this induction is likely mediated by the increase in cortisol plasma concentrations during pregnancy. Cortisol, at plasma concentrations observed during the third trimester (~800 nM), either alone or in combination with other pregnancy-related hormones, induces CYP3A activity in SCHH and HepaRG cells when cultured in dexamethasone-free media. To determine the mechanism(s) by which cortisol induces CYP3A activity, HepaRG cells were pre-incubated in dexamethasone-free medium and then incubated for 72 h with cortisol (798 nM). Glucocorticoid receptor (GR), pregnane X receptor (PXR), and CYP3A4 or CYP3A5 were knocked down using siRNA, and mRNA expression of these genes was measured. CYP3A4, and not CYP3A5, was found to be the dominant contributor to total CYP3A activity in control- and cortisol-treated HepaRG cells. Constitutive mRNA expression of CYP3A4 in HepaRG cells was regulated by both PXR and GR whereas constitutive expression of CYP3A5 in HepaRG cells was regulated by GR alone. Cortisol-mediated CYP3A4 induction in HepaRG cells was primarily mediated by GR-dependent PXR induction pathway and to a smaller extent via a PXR-independent pathway. Cortisol-mediated CYP3A5 induction was regulated by GR-dependent PXR-independent pathway. These data indicate that PXR plays a central role in cortisol-mediated induction of CYP3A activity during pregnancy and suggests that other enzymes and transporters, such as CYP2B6 and P-glycoprotein, may also be induced during pregnancy via the same mechanism(s).

KEY WORDS: cortisol; CYP3A regulation; HepaRG cells; pregnancy.

INTRODUCTION

Pregnancy is a dynamic process resulting in a variety of physiological changes. One of these is induction of cytochrome P450 (CYP) 3A activity (1,2). During the third trimester of pregnancy, hepatic CYP3A activity, as measured by 1′-OH-midazolam formation clearance, is induced by ~2-fold (2). Additionally, through physiologically based pharmacokinetic modeling and simulation, we have shown that this induction is likely hepatic and not intestinal (3). Such induction is clinically relevant as it can result in sub-therapeutic plasma concentrations of CYP3A substrate drugs such as indinavir (4).

As pregnancy proceeds, plasma concentration of pregnancy-related hormones increases (5). We have shown that, of pregnancy-related hormones, cortisol alone or in combination with other pregnancy-related hormones was the only inducer of CYP3A activity in HepaRG cells and sandwich-cultured human hepatocytes (SCHH) (5,6). Based on the above data, we concluded that CYP3A activity is induced during pregnancy by an increase in cortisol plasma concentration observed during pregnancy.

The plasma concentration of cortisol, an endogenous corticosteroid, increases significantly during pregnancy from 418 nM in the first trimester (T1) to 798 nM in the third trimester (T3) (5). Previously, we have shown that HepaRG cells faithfully replicate induction of CYP3A activity in SCHH by cortisol at plasma concentrations observed during T1, T2, and T3 (5). HepaRG cells express all the nuclear receptors and enzymes present in human hepatocytes and their use circumvents inter-individual variability in induction, a major concern when using human hepatocytes.

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Abbreviations: *SCHH*, Sandwich-cultured human hepatocytes; *GR*, Glucocorticoid receptor; *PXR*, Pregnane X receptor; *UGT1A1*, UDP-glucuronosyltransferase 1A1; *TAT*, Tyrosine aminotransferase; *NP*, Non-pregnant; *T1*, First trimester; *T2*, Second trimester; *T3*, Third trimester; *CYP*, Cytochrome P450; *DPBS*, Dulbecco's phosphate-buffered saline

Cortisol regulates biological functions predominantly by glucocorticoid receptor (GR), a member of the steroid hormone receptor subfamily (7,8). Although GR response elements are not present on CYP3A4 regulatory region, GR binding sites have been discovered on proximal promoter of pregnane X receptor (PXR), a xenobiotic sensing nuclear receptor (9). PXR is well documented to regulate CYP3A4 induction in the liver by binding to several sites upstream of the gene (10–13). Our mechanistic studies focused on the role of GR and PXR because these receptors have previously been implicated in the regulation of CYP3A enzymes by other corticosteroids (e.g., dexamethasone) (14). Based on these data, we hypothesized that the induction of CYP3A activity by cortisol was due to transcriptional regulation of the CYP3A4/5 genes via GR and/or PXR. There is considerable evidence that the expression of CYP3A4/5 mRNA is highly correlated with the expression of the respective protein and/or activity (15–17). Therefore, the focus of our study was measurement of GR, PXR, and CYP3A4/5 mRNA rather than CYP3A4/5 protein or activity. In addition, we determined the contribution of CYP3A4 and CYP3A5 enzymes to this induction by quantifying mRNA expression and CYP3A activity after Cyp3a4 or Cyp3a5 knockdown.

MATERIALS AND METHODS

Chemicals and Reagents

Cortisol was purchased from Sigma-Aldrich (St. Louis, MO). Midazolam and 1-OH-midazolam-d_5 were purchased from Cerilliant Corporation (Round Rock, TX). Acetic acid and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). William's Medium E, Dulbecco's phosphate-buffered saline (DPBS), GltaMAX-I, insulin-transferrin-selenium, penicillin-streptomycin (10,000 U/ml), Trizol reagent, TaqMan® reverse transcription reagents, universal Polymerase Chain Reaction Master Mix, CYP3A4, CYP3A5, GR, PXR, Tyrosine aminotransferase (TAT), UDP-glucuronosyltransferase (UGT) 1A1, and human beta glucuronidase (GUSB) Taqman primer-probe sets and CYP3A4, CYP3A5, GR, and PXR siRNAs were purchased from Life Technologies (Carlsbad, CA).

Treatment of HepaRG Cells

Proliferative state HepaRG cells were expanded and differentiated according to the provider's protocol (Biopredic International). Differentiated PXR knock out (*NR1I2*−/−) and *NR1I2*+/+ HepaRG cells were purchased from Sigma-Aldrich (St. Louis, MO). Differentiated HepaRG cells were seeded in 24-well plates at a density of ~ 0.8×10^6 cells/ml. Cells were initially maintained for 48 h in differentiation medium before switching to dexamethasone-free maintenance medium (prepared using William's Medium E supplemented with GltaMAX-I, insulin-transferrin-selenium, and penicillin-streptomycin) for 72 h. Afterwards, maintenance medium was removed and the cells were treated for 72 h with the total plasma concentration of cortisol observed during T3 (798 nM). As a control, the cells were treated with the unbound cortisol plasma concentration observed in non-pregnant (NP) women (16 nM) to represent the constitutive expression of the *CYP3A4/5* genes. Previously, we have shown that CYP3A activity is not induced in HepaRG cells irrespective of whether the unbound or total NP cortisol plasma concentration is used (5). For knockdown studies, freshly trypsinized cells (2.5×10^6 cells) were resuspended in 0.5 ml of electroporation buffer (HEPES 21 nM, NaCl 137 nM, KCl 5 nM, Na_2HPO_4-$7H_2O$ 0.7 nM, dextrose 6 mM; pH 7.15) and electroporated (300 V, 500 μF one pulse in 0.4 cm cuvette) with siRNA cocktail (equal amounts [120 ng each] of 3 siRNAs targeting different areas of same mRNA transcript) using an electroporation system (Gene Pulser II; Bio-Rad, CA). The electroporated cells were then transferred to 2 ml of dexamethasone-free maintenance medium and centrifuged. Pelleted cells were resuspended in fresh medium and plated in 24-well plate for 24 h. Next day, medium was removed and the cells were treated for 72 h with cortisol (798 or 16 nM).

Quantification of CYP3A Activity

To quantify CYP3A activity, the cortisol containing medium was aspirated and cells were rinsed twice with pre-warmed DPBS. Then, cells were incubated for 1 h with 2 μM midazolam dissolved in serum-free William's Medium E. At the end, supernatant was collected and equal volume of ice-cold acetonitrile containing 100 nM 1′-OH-midazolam-d_5 was immediately added to the supernatant and vials were sealed with airtight covers. 1′-OH-midazolam formation was quantified by using liquid chromatography–tandem mass spectrometry method as described previously (18).

Quantification of mRNA

For mRNA quantification, cells were washed and lysed using Trizol reagent and total RNA was extracted, reverse transcribed, quantified, and analyzed as described previously (19).

Data Analysis

All experiments were conducted in triplicate. Unless otherwise stated, each experiment was independently repeated 3 or 4 times, and the mean of each experiment was expressed relative to the corresponding control to arrive at the overall mean ± SD. For comparisons of treatments, *t* test or one-way analysis of variance (ANOVA) followed by *post hoc* Tukey multiple comparison was performed using Prism (Graphpad Prism 6.0, La Jolla, CA).

RESULTS

Induction of CYP3A4 and CYP3A5 in HepaRG Cells by T3 Cortisol Treatment

HepaRG cells were pre-incubated in dexamethasone-free medium for 72 h and then incubated for 72 h with T3 cortisol (798 nM) or control (16 nM). T3 cortisol (798 nM) robustly induced CYP3A4 mRNA (~76-fold vs. control) ($p < 0.05$). However, CYP3A5 mRNA induction was modest

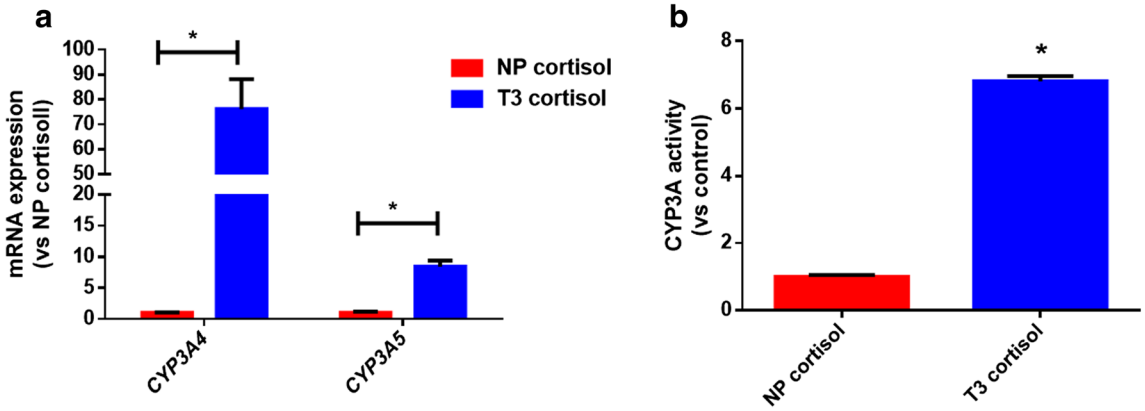

Fig. 1. Induction of *CYP3A4/5* mRNA and CYP3A4 activity in HepaRG cells by T3 cortisol. *CYP3A4* and *CYP3A5* mRNA (**a**) as well as total CYP3A activity (**b**) was robustly induced when T3 cortisol concentration (798 nM) was incubated for 72 h with terminally differentiated HepaRG cells that were pre-incubated for 72 h in dexamethasone-free media. Data shown are expressed relative to control (cortisol 16 nM) and are mean ± SD of triplicates. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. *$P < 0.05$ compared with the respective control

(~ 8-fold) (Fig. 1a). As a result, total CYP3A activity was induced ~ 7-fold (Fig. 1b).

Contribution of CYP3A4 and CYP3A5 Towards Total CYP3A Activity in HepaRG Cells

HepaRG cells were pre-incubated in dexamethasone-free medium for 72 h and CYP3A4 and/or CYP3A5 were knocked down prior to cortisol treatment using siRNA. Knockdown of CYP3A4 or CYP3A5 resulted in approximately equal and significant decrease in their mRNA expression after T3 cortisol and control (data not shown). Since mRNA expression of *CYP3A4* in control-treated HepaRG cells was ~ 3-fold greater than *CYP3A5* (data not shown), CYP3A4 knockdown resulted in 72% decrease ($p < 0.05$) in total CYP3A activity while CYP3A5 knockdown decreased total CYP3A activity by 27% ($p < 0.05$) (Fig. 2). Simultaneous knockdown of CYP3A5 and CYP3A4 resulted in a greater decrease (79%; $p < 0.05$) in total CYP3A activity. T3 cortisol (798 nM) resulted in ~ 6.7-fold induction in total CYP3A activity in HepaRG cells (labeled scRNA). CYP3A4 and CYP3A5 knockdown in T3 cortisol-treated HepaRG cells significantly decreased induction of CYP3A activity (86% and 20%, respectively) while simultaneous knockdown of both CYP3A4 and CYP3A5 resulted in a greater decrease (93.5%; $p < 0.05$) in induction of CYP3A activity (Fig. 2).

Role of GR and PXR on Constitutive mRNA Expression of *CYP3A4* and *CYP3A5* in HepaRG Cells

HepaRG cells were pre-incubated in dexamethasone-free medium for 72 h and PXR and/or GR were knocked down prior to cortisol (16 nM) treatment. GR knockdown significantly decreased constitutive *GR* mRNA expression (~ 60%; $p < 0.05$) without any effect on constitutive *PXR* mRNA expression (Fig. 3). Consequently, mRNA expression of *CYP3A4* and *CYP3A5* was significantly decreased (~ 56% and ~ 30%, respectively; $p < 0.05$) (Fig. 3). PXR knockdown decreased constitutive expression of *PXR* (64%; $p < 0.05$) but did not influence constitutive expression of *GR* mRNA. As a result, *CYP3A4* mRNA was decreased by ~ 55% ($p < 0.05$) but did not affect *CYP3A5* constitutive expression (Fig. 3). Repression of *UGT1A1* and *TAT* mRNA (positive controls

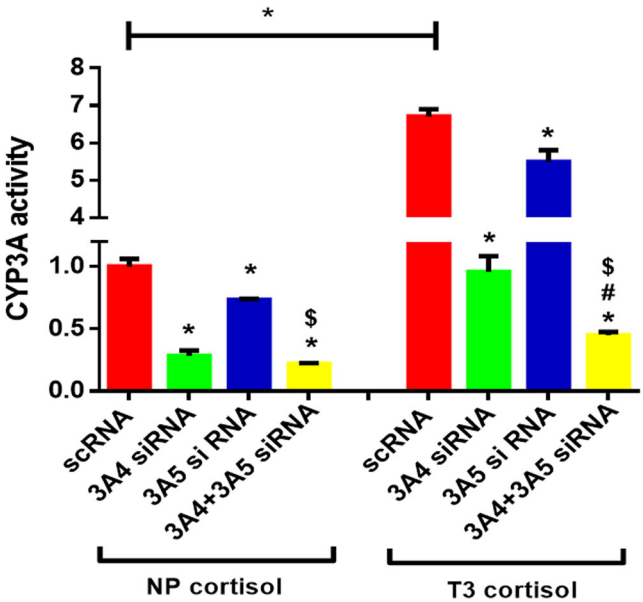

Fig. 2. Induction of CYP3A activity in HepaRG cells by T3 cortisol treatment in the presence and absence of knockdown of CYP3A4, CYP3A5, or both. CYP3A activity was reduced to a greater extent by siRNA-mediated knockdown of CYP3A4 vs. CYP3A5 in both control- and cortisol-induced conditions. That is, induction of CYP3A activity in HepaRG cells by T3 cortisol (798 nM) treatment was mediated primarily by induction of CYP3A4 enzyme. Terminally differentiated HepaRG cells, pre-incubated for 72 h in dexamethasone-free media, and *CYP3A4* and/or *CYP3A5* siRNA cocktail were electroporated. Afterwards, HepaRG cells were treated with T3 cortisol (798 nM) or control cortisol concentration (16 nM) for 72 h. Data shown are expressed relative to scRNA-treated control (cortisol 16 nM) cells and are mean ± SD of triplicates. Data were analyzed using one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. *$P < 0.05$ compared with respective control (cortisol 16 nM or scRNA). \$$P < 0.05$ compared with the respective 3A5 siRNA. #$P < 0.05$ compared with the respective 3A4 siRNA

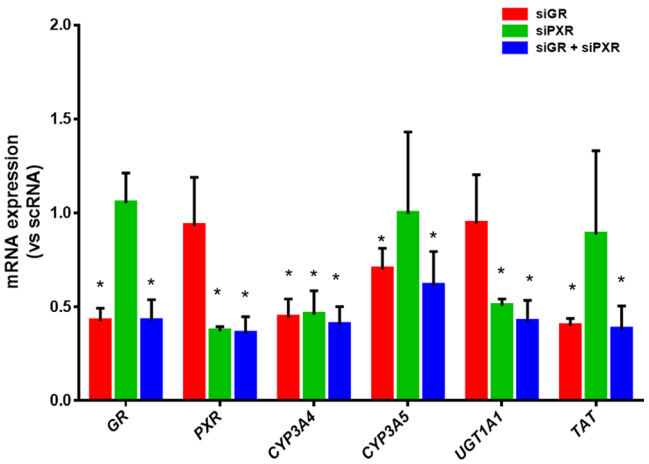

Fig. 3. The effect of PXR and/or GR knockdown on constitutive *CYP3A4*, *CYP3A5*, *UGT1A1*, or *TAT* mRNA expression in control-treated HepaRG cells. GR or PXR knockdown caused a significant decrease in the constitutive expression of *CYP3A4* mRNA whereas only GR knockdown decreased *CYP3A5* mRNA constitutive expression. Repression of *UGT1A1* and *TAT* mRNA (positive controls for PXR and GR regulation, respectively) closely followed the repression of PXR and GR mRNA, respectively. Terminally differentiated HepaRG cells, pre-incubated for 72 h in dexamethasone-free media, electroporated with *GR*, *PXR*, or *GR* plus *PXR* siRNAs (or scRNA). Afterwards, cells were treated with control cortisol concentration (16 nM) for 72 h. Data shown are expressed relative to scRNA-treated cells and are mean ± SD of four independent experiments, each conducted in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. $^*P < 0.05$ compared with the respective control (scRNA). *UGT1A1* and *TAT* are positive controls for PXR and GR-mediated induction

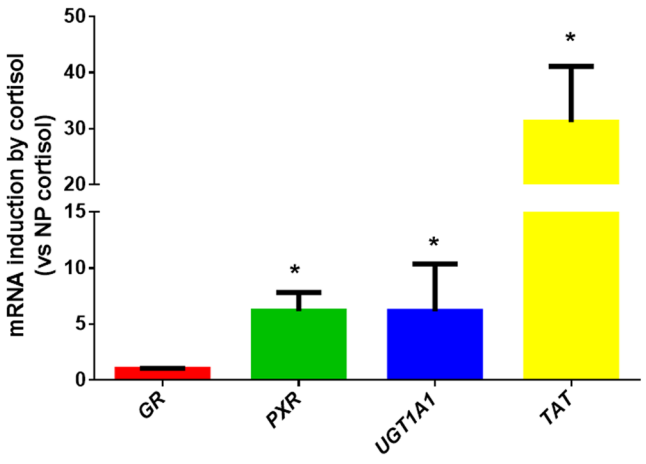

Fig. 4. Effect of T3 cortisol treatment on *GR* and *PXR* mRNA expression in HepaRG cells. Cortisol treatment induced *PXR*, *UGT1A1*, *TAT* but not *GR* mRNA. Terminally differentiated HepaRG cells, pre-incubated for 72 h in dexamethasone-free media, were treated with T3 cortisol concentration (798 nM) for 72 h before quantifying the target mRNA. Data shown are expressed relative to control-treated (cortisol 16 nM) cells and are mean ± SD of four independent experiments, each conducted in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. $^*P < 0.05$ compared with the respective control-treated (cortisol 16 nM) cells. *UGT1A1* and *TAT* are positive controls for PXR and GR-mediated induction

for PXR and GR regulation, respectively) closely followed the repression of *GR* and *PXR* mRNA, respectively. Concomitant knockdown of both PXR and GR did not result in further decrease in *GR*, *PXR*, *CYP3A4*, or CY*P3A5* mRNA.

Effect of T3 Cortisol Treatment on *GR* and *PXR* mRNA Expression in HepaRG Cells

HepaRG cells were pre-incubated in dexamethasone-free media for 72 h and then incubated for 72 h with T3 cortisol (798 nM) or control (16 nM). T3 cortisol treatment did not affect GR expression but *PXR* mRNA was induced by ~ 6-fold ($p < 0.05$) (Fig. 4). Also, T3 cortisol resulted in mRNA induction of PXR target gene, *UGT1A1*, and GR target gene, *TAT*, by ~ 6.1-fold and ~ 31-fold, respectively.

Role of GR and PXR in T3 Cortisol-Mediated Induction of CYP3A4 and CYP3A5 in HepaRG Cells

HepaRG cells were pre-incubated in dexamethasone-free medium for 72 h and PXR and/or GR were knocked down prior toT3 cortisol (798 nM) treatment. GR knockdown decreased GR expression by ~ 55% ($p < 0.05$). This resulted in decreased mRNA induction of *PXR*, *CYP3A4*, and *CYP3A5* by ~ 45%, ~ 78%, and ~ 6%, respectively ($p < 0.05$) (Fig. 5). In contrast, PXR knockdown resulted in ~ 60% decrease in *PXR* induction without any effect on *GR* mRNA expression (Fig. 5). PXR knockdown also resulted in significant decrease in *CYP3A4* mRNA induction (~ 82%; $p < 0.05$) and concomitant knockdown of both GR and PXR resulted in further decrease ($p < 0.05$) in *CYP3A4* mRNA induction (~ 90%). However, PXR knockdown did not affect *CYP3A5* mRNA induction. Similar to GR knockdown, concomitant knockdown of both GR and PXR resulted in 59% ($p < 0.05$) decrease in *CYP3A5* mRNA induction. Decrease in *UGT1A1* and *TAT* induction followed *GR* and *PXR* expression.

Effect of T3 and Control Cortisol Treatment on GR, CYP3A4, and CP3A5 mRNA Expression in *NR1I2−/−* HepaRG Cells

NR1I2−/− HepaRG cells were pre-incubated in dexamethasone-free media for 72 h and then incubated for 72 h with T3 cortisol (798 nM) or control (16 nM). In control-treated *NR1I2−/−* HepaRG cells, constitutive *CYP3A4* mRNA expression was 90% lower than *NR1I2+/+* HepaRG cells whereas *CYP3A5* mRNA expression was not affected (Fig. 6). T3 cortisol resulted in ~ 47-fold ($p < 0.05$) induction in *CYP3A4* in *NR1I2+/+* cells whereas only a modest ~ 7.7-fold ($p < 0.05$) induction was observed in *NR1I2−/−* cells. *CYP3A5* mRNA was induced by ~ 4.5-fold ($p < 0.05$) in both *NR1I2+/+* and *NR1I2−/−* cells. PXR deletion marginally (but not significantly) decreased constitutive expression of *UGT1A1* and prevented T3 cortisol-mediated UGT1*A1* induction. In *NR1I2−/−* HepaRG cells, constitutive expression and T3 cortisol-induced mRNA expression of GR were not affected. Consequently, *TAT* constitutive mRNA expression as well as T3 cortisol-mediated *TAT* induction in *NR1I2+/+* and *NR1I2−/−* cells was not significantly different.

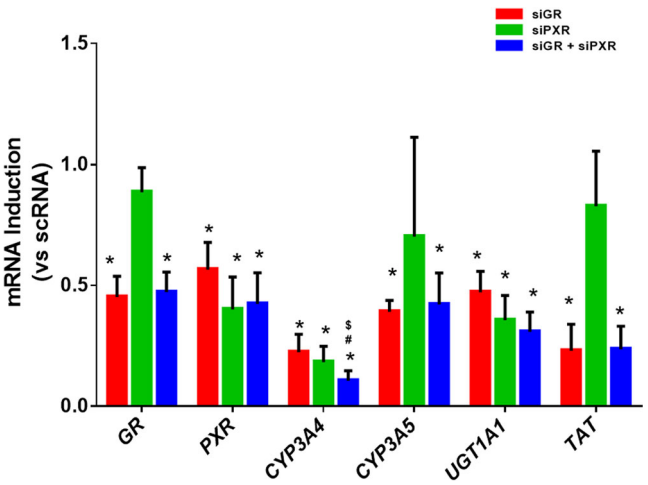

Fig. 5. Role of GR and PXR in cortisol-mediated induction of CYP3A4 and CYP3A5 in HepaRG cells treated with T3 cortisol concentration. GR knockdown decreased induction of *PXR*, *CYP3A4*, and *CYP3A5* mRNA by T3 cortisol treatment. PXR knockdown decreased T3 cortisol-mediated mRNA induction of *CYP3A4* but not of *CYP3A5* or *GR* mRNA expression. Knockdown of both GR and PXR resulted in a further decrease in *CYP3A4* but not *CYP3A5* mRNA. Terminally differentiated HepaRG cells, pre-incubated for 72 h in dexamethasone-free media, and electroporated with *GR*, *PXR*, or *GR* plus *PXR* siRNAs (or scRNA). Afterwards, cells were treated with T3 cortisol concentration (798 nM) for 72 h before quantifying the target mRNA. Data shown are expressed relative to scRNA-treated T3 cortisol-treated cells and are mean ± SD of four independent experiments, each conducted in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. $^*P < 0.05$; compared with the respective scRNA control, $\$P < 0.05$ compared with *GR* siRNA and $\#P < 0.05$ compared with *PXR* siRNA. *UGT1A1* and *TAT* are positive controls for PXR and GR-mediated induction

DISCUSSION

The dual expression of functional CYP3A4 and CYP3A5 isozymes in HepaRG cells allowed us to determine the magnitude and mechanism of induction of these enzymes in response to cortisol treatment. CYP3A5 is a polymorphic enzyme; therefore, CYP3A activity depends on differential expression of CYP3A5 functional protein. Our data indicate a relatively small but definitive role of CYP3A5 in mediating total CYP3A activity toward midazolam in control HepaRG cells (Fig. 2).

Since CYP3A activity is induced during pregnancy (2), we determined if cortisol induces activity of CYP3A4, CYP3A5, or both in HepaRG cells. Although cortisol induced both *CYP3A4* and *CYP3A5* mRNA, the induction of *CYP3A5* mRNA at T3 cortisol was much less than that of *CYP3A4* mRNA. This was confirmed in T3 cortisol-treated HepaRG cells where CYP3A4 knockdown caused 72% decrease in total CYP3A activity while CYP3A5 knockdown resulted in only 27% decrease in activity even though both CYP3A4 and CYP3A5 mRNA were equally knocked down (Fig. 2). Thus, we conclude that, during pregnancy, induction of CYP3A activity by cortisol is primarily due to induction of CYP3A4 enzyme. This conclusion is supported by our previous study in T3 pregnant women where CYP3A activity was induced irrespective of the CYP3A5 genotype of the subjects (2). Moreover, through *in silico* methods, we concluded that it is hepatic (and not intestinal) CYP3A activity that is induced during pregnancy (3).

Based on the above data, we hypothesized that the induction of CYP3A activity by cortisol was due to transcriptional regulation of the CYP3A4/5 genes via GR and/or PXR. Our study focused on measurement of GR, PXR, and CYP3A4/5 mRNA rather than CYP3A4/5 protein or activity

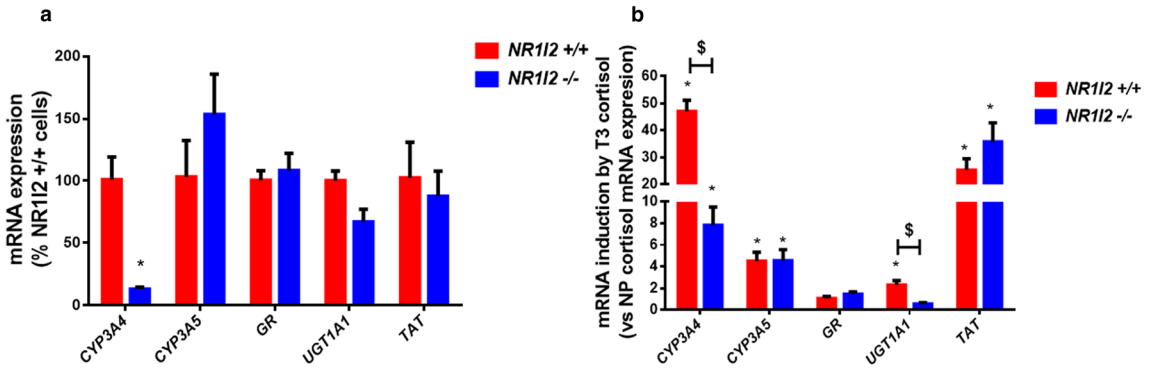

Fig. 6. Effect of *NR1I2* deletion on constitutive and T3 cortisol-mediated induction of *GR*, *CYP3A4*, or *CP3A5* mRNA expression in HepaRG cells. Constitutive (16 nM cortisol) mRNA expression (**a**) and cortisol (798 nM)-mediated mRNA induction (**b**) in *NR1I2*−/− and *NR1I2*+/+ HepaRG cells. *NR1I2* deletion decreased constitutive *CYP3A4* mRNA expression without affecting *CYP3A5* mRNA expression. T3 cortisol treatment (798 nM) robustly induced *CYP3A4* mRNA expression in *NR1I2*+/+ HepaRG cells but this induction was significantly reduced in *NR1I2*−/− cells. *CYP3A5* mRNA induction in *NR1I2*−/− cells was no different from that in *NR1I2*+/+ HepaRG cells. Terminally differentiated *NR1I2*+/+ and NR1I2−/− HepaRG cells were pre-incubated for 72 h in dexamethasone-free media, were treated with T3 or control cortisol concentration (798 nM or 16 nM) for 72 h before quantifying the target mRNA. Data shown are expressed as percent of mRNA expression in *NR1I2*+/+ HepaRG cells (**a**) and fold induction in mRNA expression of *NR1I2*+/+ or *NR1I2*−/− HeparRG cells, respectively (i.e., relative to cells treated with 16 nM of cortisol) (**b**). Data shown are mean ± SD of triplicate. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparison *post hoc* test. $^*P < 0.05$ compared with the respective constitutive expression after control treatment (cortisol 16 nM) in *NRI12*+/+ cells or *NRI12*−/− cells, $\$P < 0.05$ compared with the cortisol-treated *NR1I2*+/+ cells. UGT1A1 and TAT are positive controls for PXR and GR-mediated induction

because there is considerable evidence that CYP3A4/5 activity is highly correlated with their mRNA expression (15–17). As shown by our knockdown studies, both control- and T3 cortisol-induced mRNA expression of *CYP3A4* and *CYP3A5* in HepaRG cells is regulated by GR (Figs. 3 and 5). GR knockdown caused a significant decrease in *CYP3A4* in both control- and T3 cortisol-treated HepaRG cells confirming the role of GR in the regulation of CYP3A4 expression (Figs. 3 and 5). For the following reasons, our data also point to the conclusion that GR, at T3 cortisol, regulates induction of CYP3A4 primarily (but not entirely) via PXR induction. First, T3 cortisol simultaneously induced *PXR* and *UGT1A1* mRNA, suggesting that the induction of *PXR* mRNA was critical in induction of PXR-target genes such as *UGT1A1* and *CYP3A4* (Figs. 1a and 4). Second, in T3 cortisol-treated cells, decrease in *CYP3A4* mRNA induction after GR knockdown closely paralleled decrease in *PXR* mRNA (Fig. 5). Third, in T3 cortisol-treated cells, knockdown of PXR alone decreased the induction of *CYP3A4* and *UGT1A1* mRNA without affecting *GR* mRNA expression further confirming that PXR is downstream to GR in cortisol-mediated CYP3A induction pathway. Fourth, in comparison with GR or PXR knockdown alone, the double knockdown of GR and PXR resulted in further decrease in the T3 cortisol-induced expression of *CYP3A4* mRNA without a further decrease in *GR* or *PXR* mRNA expression indicating a minor GR-dependent PXR-independent pathway (Fig. 5). Fifth, when compared to *NR1I2*+/+ HepaRG cells, *NR1I2*−/− resulted in only ~ 16% induction of *CYP3A4* mRNA by T3 cortisol confirming that cortisol-mediated *CYP3A4* mRNA induction during T3 is primarily via the PXR pathway (as discussed below, these data do suggest a minor PXR-independent pathway in the induction of CYP3A4 mRNA by cortisol).

The above data also beg the question: what is the GR but PXR-independent pathway that causes induction of *CYP3A4* mRNA by cortisol? The *CYP3A4* regulatory region does not contain any GR binding sites preventing direct regulation of *CYP3A4* by GR (20). A study showed mutation in the *CYP3A4* proximal promoter of hepatocyte-nuclear-factor-3/CCAAT-enhancer binding protein-α binding site, disrupts GR-dependent CYP3A4 induction without affecting PXR-dependent induction (21). Though these data raise an interesting possible mechanism for this GR but PXR-independent pathway, further research is needed to completely elucidate this pathway. However, the absence of GR binding sites on *CYP3A4* regulatory region makes it likely that this pathway is indirect.

Consistent with these data, a previous study using HepG2 cells showed that the response of CYP3A4 promoter-dependent gene reporter to dexamethasone (a synthetic corticosteroid) is enhanced when co-transfected with GR (22). Moreover, dexamethasone-mediated *CYP3A4* mRNA induction in human hepatocytes is attenuated in the presence of the GR antagonist, RU486 (23). Dexamethasone is known to induce CYP3A activity *in vivo* and in human hepatocytes (14,24). For several reasons, the mechanisms by which dexamethasone induces CYP3A activity cannot be extrapolated to pregnancy. First, dexamethasone is a synthetic corticosteroid. Second, previous induction studies were performed using hepatocytes treated with dexamethasone (100 nM) as a media supplement (25). Third, dexamethasone has greater binding affinity and binding capacity to GR compared to cortisol (26). Furthermore, there is evidence that supra-therapeutic plasma concentrations of dexamethasone, normally employed in these induction studies, may induce CYP3A enzymes through mechanism not relevant at cortisol plasma concentrations observed during pregnancy. For example, supra-therapeutic concentrations of dexamethasone induce CYP3A activity in human hepatocytes by binding to both the GR and PXR (14). Studies have also suggested that dexamethasone causes CYP3A4 induction by activating GR which in turn up-regulates PXR and constitutive androstane receptor (CAR) (14). Indeed, T3 cortisol induced *PXR* mRNA expression in HepaRG cells (Fig. 4) but *CAR* mRNA expression was below the detection limit in both control- and T3 cortisol-treated group.

In contrast to cortisol-mediated CYP3A4 induction, constitutive *CYP3A4* mRNA expression in HepaRG cells was regulated independently by both GR and PXR (Fig. 3). In control, either GR knockdown or PXR knockdown decreased *CYP3A4* constitutive mRNA expression without affecting *PXR* or *GR* mRNA expression, respectively. Therefore, GR is responsible for cortisol-mediated *PXR* induction but not its constitutive expression. In addition, these data further confirm GR-dependent, but PXR-independent, regulation of *CYP3A4* mRNA expression in both control- and T3 cortisol-treated cells. Moreover, either GR

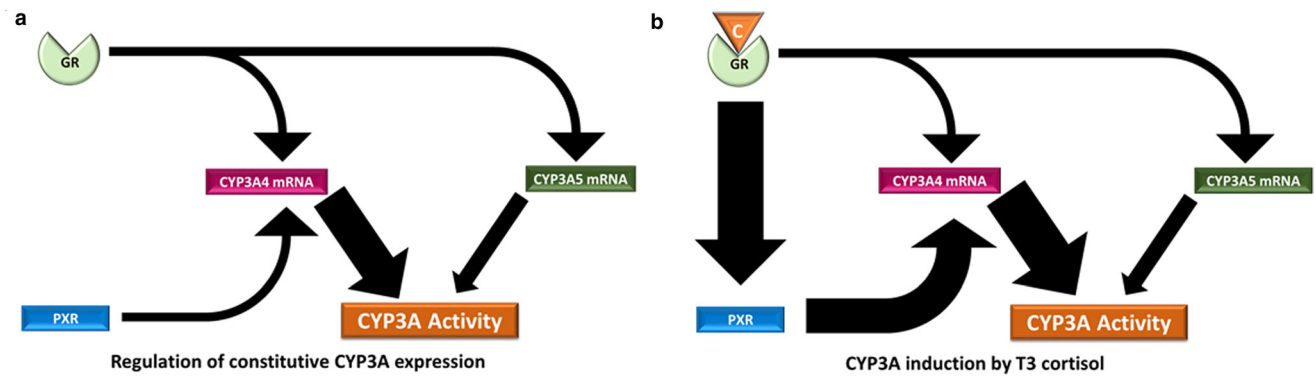

Fig. 7. A schematic illustrating the mechanisms of GR and PXR regulation of constitutive expression of *CYP3A4/5* mRNA (**a**) and T3 cortisol-mediated induction of *CYP3A4/5* mRNA in HepaRG cells (**b**). Broader and narrower arrows, respectively, indicate greater and lesser role of the indicated pathway

or PXR knockdown decreased *CYP3A4* constitutive mRNA expression to a similar extent suggesting that both GR-dependent and PXR-dependent pathways contribute equally to constitutive *CYP3A4* mRNA expression (Fig. 3). Surprisingly, knockdown of both GR and PXR did not result in a further decrease in *CYP3A4* constitutive mRNA expression. We do not have a good explanation of this observation. Perhaps constitutive *CYP3A4* mRNA expression cannot be knocked down further.

In HepaRG cells, the regulation of *CYP3A5* mRNA expression by GR differs from that of *CYP3A4*. GR knockdown decreased *CYP3A5* constitutive as well as T3 cortisol-mediated *CYP3A5* mRNA expression (Figs. 3 and 5). Constitutive expression as well as T3 cortisol-mediated induction of *CYP3A5* was not influenced by PXR knockdown or by *NR1I2* deletion in HepaRG cells (Figs. 3, 5, and 6). In addition, the double knockdown of GR and PXR did not result in a further decrease in *CYP3A5* mRNA expression when compared with GR knockdown alone. These data suggest that GR's regulation of constitutive expression as well as T3 cortisol-mediated induction of *CYP3A5* mRNA is not mediated via PXR. This is consistent with the fact that GR can directly interact with *CYP3A5* gene via the presence of two GR response elements in its promoter region (27). In contrast to our observations, Burk *et al.* (28) have reported presence of a PXR binding site in *CYP3A5* promoter region and a correlation between *PXR* mRNA and *CYP3A5* mRNA expression in a collection of human liver samples which suggests that PXR plays a role in the regulation of *CYP3A5* mRNA. However, correlation studies do not necessarily reflect cause and effect relationship.

In summary, our data indicate that induction of CYP3A4 activity is likely the major contributor to the cortisol-induced CYP3A activity during pregnancy. As illustrated in Fig. 7, *CYP3A4* constitutive mRNA expression in HepaRG cells is regulated by PXR and GR whereas constitutive expression of *CYP3A5* mRNA is regulated by GR with no involvement from PXR. *CYP3A4* mRNA induction in response to cortisol observed during pregnancy is predominately mediated by PXR. Although GR is involved in this induction, it does so primarily via *PXR* induction. In contrast, *CYP3A5* mRNA induction by cortisol is mediated by GR only. Here, GR appears to act independent of PXR. One limitation of knocking down gene products via siRNA is that this approach may non-specifically affect other gene products which, in turn, may affect CYP3A4/5 mRNA expression. Therefore, these findings need to be confirmed *in vivo* such as in *Nr1I2*−/− mice. If confirmed, these data suggest that other enzymes and transporters, such as hepatic CYP2B6 and hepatic/renal P-glycoprotein, may also be induced during pregnancy by cortisol via the same PXR-mediated mechanism. Indeed, we have shown that renal P-glycoprotein is significantly induced during pregnancy as measured by renal secretory clearance of digoxin (2).

CONCLUSION

Our data indicate that the *in vivo* induction of CYP3A activity during pregnancy is primarily caused by induction of CYP3A4 enzyme. This induction appears to be mediated by cortisol mostly via GR-mediated induction of PXR. Since PXR plays a central role in the induction of CYP3A activity during pregnancy, other enzymes and transporters, such as CYP2B6 and P-glycoprotein, that are also regulated by PXR, may also be induced during pregnancy via the same mechanism(s).

AUTHORS' CONTRIBUTIONS

Participated in research design: MS, EJK, and JDU. Conducted experiments: MS. Performed data analysis: MS. Wrote or contributed to the manuscript: MS, EJK, and JDU.FundingThis work was supported by National Institute on Drug Abuse grant P01DA032507.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

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