RESEARCH PAPER

Inherent sex-dependent regulation of human hepatic CYP3A5

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BACKGROUND AND PURPOSE

Expression of hepatic cytochromes P450 (CYP) in all species examined, including humans, is generally sexually dimorphic. We examined the sex-dependent expression of CYP3A5 and the hormone-regulated molecular mechanism(s) responsible for any dimorphism.

EXPERIMENTAL APPROACH

CYP3A5 levels as well as nuclear translocation and promoter binding of transcription factors regulating CYP3A5 expression were measured in primary hepatocyte cultures derived from men and women exposed to physiological-like levels of growth hormone alone, dexamethasone alone and the combined regimen.

KEY RESULTS

We observed a dramatic inherent CYP3A5 sexual dimorphism (women > men) with all treatments as a result of a ~2-fold greater level of hormone-induced activation and nuclear accumulation of hepatocyte nuclear factor-4 α (HNF-4 α), pregnane X receptor (PXR) and retinoic X receptor α (RXR α) in female hepatocytes. Furthermore, PXR : RXR α exhibited significantly higher DNA binding levels to its specific binding motif on the CYP3A5 promoter in female hepatocytes, inferring a possible explanation for the elevated expression of the isoform in women. Results from experiments using HepG2 cells treated with siRNA-induced knockdown of HNF-4 α and/or transfected with luciferase reporter constructs containing the CYP3A5 promoter were in agreement with the basic mechanism observed in primary hepatocytes of both sexes.

CONCLUSIONS AND IMPLICATIONS

Female-predominant expression of human CYP3A5 is due to an inherent, sex-dependent suboptimal activation of the transcription networks responsible for hormone-induced expression of the isoform in men. Accordingly, in conjunction with previous studies of other human CYPs, men and women are intrinsically unlikely to handle many drugs in the same way; thus, sex should be a requisite component factored into the design of personalized drug therapies.

Abbreviations

ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; ER6, everted repeat 6; GH, growth hormone; HNF-4 α , hepatocyte nuclear factor-4 α ; PXR, pregnane X receptor; rhGH, recombinant human GH; RXR α , retinoic X receptor α ; Scr, scrambled; siRNA, small inhibitory RNA

Introduction

The human cytochrome P450 3A (CYP3A) gene family is responsible for phase I metabolism of at least one-half of all consumed drugs and is expressed at the highest concentration (i.e. 30% to 50% of the total pool of hepatic CYPs) (Shimada *et al.*, 1994; Rendic, 2002). The CYP3A subfamily consists of four members, CYP3A4, CYP3A5, CYP3A7 and CYP3A43, which are aligned in tandem on chromosome 7 (Gellner *et al.*, 2001). Although the nucleotide sequences of the CYP3A genes are relatively similar, their expression patterns vary. CYP3A4, the predominant isoform, is basically expressed in adult human liver and intestine (Beaune *et al.*, 1986; Watkins *et al.*, 1987; Shimada *et al.*, 1994), whereas CYP3A7 is generally limited to the fetal human liver (Komori *et al.*, 1990; Rendic, 2002). CYP3A5 is polymorphically expressed in various tissues, especially the adult liver, kidney and lung (Schuetz *et al.*, 1992; Kuehl *et al.*, 2001; Biggs *et al.*, 2007). CYP3A43 has been detected in liver, kidney, prostate and pancreas; but its mRNA levels are so low as to make an insignificant contribution to the systemic clearance of drugs (Gellner *et al.*, 2001; Rendic, 2002).

As regards CYP3A5, only individuals with at least one CYP3A5*1 allele express large amounts of the isoform. A commonly inherited single-nucleotide polymorphism in CYP3A5*3 as well as CYP3A5*5, *6 and *7 can cause alternative splicing and protein truncation resulting in the absence of CYP3A5 protein and/or activity in most people. Nevertheless, CYP3A5 can represent at least 50% of the total hepatic CYP3A activity in people polymorphically expressing a functional allele and thus could be a major contributor to the biotransformation of drugs and their subsequent clearance in a considerable portion of the population (Kuehl *et al.*, 2001; Lamba *et al.*, 2002).

Sex-dependent differences in CYP-dependent drug metabolism are quite common, existing in numerous diverse species from trout to humans (c.f. Shapiro *et al.*, 1995). In the case of humans, expression of many of the major isoforms of CYP is sexually dimorphic, including CYP3A4, the only member of the CYP3A subfamily so examined (Dhir *et al.*, 2006). The endogenous factor known to maintain sexually dimorphic expression of hepatic CYPs is growth hormone (GH) (Legraverend *et al.*, 1992; Shapiro *et al.*, 1995). Moreover, in all species examined, including humans (Hartman *et al.*, 1993; Van den Berg *et al.*, 1996; Engstrom *et al.*, 1998), GH is secreted in a sexually dimorphic pattern; the masculine profile is deemed 'episodic'; and the feminine is referred to as 'continuous' (Jansson *et al.*, 1985; Shapiro *et al.*, 1995), which in turn is responsible for the sexually dimorphic expression of hepatic CYPs (Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000).

In humans, numerous reports, generally using GH-deficient individuals, have shown that GH replacement can restore drug-metabolizing enzymes to normal levels (Cheung *et al.*, 1996). The inductive effects of restored sex-dependent, GH-like profiles on CYP3A4-dependent activity has been assessed in GH-deficient young boys and girls (Sinués *et al.*, 2004) and GH-deficient men and women (Jaffe *et al.*, 2002). Extending these studies, we examined the effects of physiological-like exposure doses of episodic or continuous human GH on expression levels of several CYPs, including

CYP3A4, in hepatocyte cultures derived from men and women donors (Dhir *et al.*, 2006). Whether in the presence or absence of dexamethasone (a positive regulator for all members of the CYP3A family) (Gonzalez *et al.*, 1986; Dhir *et al.*, 2006), and independent of sex, the masculine-like episodic GH profile suppressed CYP3A4 expression, whereas the feminine-like continuous GH profile was inductive.

In addition to observing the differential effects of the masculine and feminine GH profiles on CYP3A4 expression, we noted an apparent intrinsic sexually dimorphic response of several CYP isoforms, in that the episodic GH profile, when suppressive, was more so in hepatocytes from men than women, while the continuous GH profile, when inductive, was more effective in hepatocytes from women than men (Dhir *et al.*, 2006; Thangavel *et al.*, 2011). In this regard, the same once daily GH replacement regimen was significantly more suppressive of CYP3A4 enzymatic activity in boys than girls (Sinués *et al.*, 2004). Similar inherent sexually dimorphic responses in humans to the same episodic GH regimen have been reported for insulin-like growth factor 1, bone mineralization, lipid metabolism, growth rates and growth hormone-binding protein; men > women (Burman *et al.*, 1997; Kuromaru *et al.*, 1998; Johansson *et al.*, 1999; Span *et al.*, 2001; Soares *et al.*, 2004).

In the present study, we have compared the responsiveness of hepatocyte CYP3A5 from adult men and women exposed to the same regimen of either dexamethasone alone, GH alone or the combined hormones and have observed at every treatment a female predominance. In addition, we have identified a possible molecular mechanism that could explain the irreversible sexual dimorphism.

Methods

Human hepatocyte culture

Male and female hepatocytes were isolated from human liver (Strom *et al.*, 1996) and plated on rat tail collagen-coated flasks (T-25) in DMEM and were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA). All of the samples were obtained with donors' consent and with approval of the appropriate hospital ethics committee. Male and female donors varied in age from 21 to 56 years. About 80% was Caucasian; the remainder was African American and Hispanic. Alcohol consumption, smoking and drug history as well as causes of death varied between donors. Approximately 50% of livers had some degree of steatosis (5–40%). Unlike other human CYP3A isoforms, diverse and highly expressed aberrant allelic variants in the CYP3A5 gene reduces the functional expression of the isoform to about one-third of Caucasians and a higher percentage Asian and African Americans (Kuehl *et al.*, 2001; Lamba *et al.*, 2002). Accordingly, only those hepatocytes expressing CYP3A5 protein have been included in the present study. To limit the effects of interperson variability, hepatocytes from each donor were used in all determinations. Approximately 48 h after isolation and plating, the primary hepatocyte cultures arrived at our laboratory. The replacement medium and culture conditions were described previously (Thangavel *et al.*, 2004; Dhir *et al.*, 2006).

Hormonal conditions¹

In order to replicate the more continuous feminine-like GH profile shown to be favoured for human CYP3A4 expression (Dhir *et al.*, 2006), the primary hepatocytes were constantly exposed ($2 \text{ ng}\cdot\text{mL}^{-1}$) to recombinant human growth hormone (rhGH) purchased from the National Hormone and Peptide Program (Torrance, CA). Other cells were exposed to dexamethasone ($4 \text{ ng}\cdot\text{mL}^{-1}$) alone or to both the glucocorticoid and rhGH. Some hepatocytes were exposed to neither hormone. The medium was changed every 12 h. After 5 days in culture, cells were harvested 60 min following the final change of media as previously described (Thangavel *et al.*, 2004; Dhir *et al.*, 2006).

Preparation of whole cell and nuclear extracts

To isolate protein for immunoblots, harvested hepatocytes were centrifuged (800 g for 10 min), and the resulting cell pellets were resuspended in lysis buffer (Garcia *et al.*, 2001). The crude extract was passed through a 22-gauge needle 10 times. The solution was then gently mixed at 4°C for 20 min and centrifuged at $12\,000\times \text{g}$ for 20 min. The supernatant (whole cell extract) was then removed and stored at -80°C until analyses. Briefly, nuclei were isolated as described (Dignam *et al.*, 1983) by a series of centrifugations of resuspended, homogenized, and dialysed crude nuclear extract originating from the low-speed pellet. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Western blot analysis

Using standard protocol (Dhir *et al.*, 2006; Thangavel and Shapiro, 2007), 25 to $50 \mu\text{g}$ of the $12\,000\times \text{g}$ supernatant (i.e. whole cell extract) and $50 \mu\text{g}$ of nuclear extract were resolved in 10% SDS-PAGE and transferred electrophoretically onto PVDF membranes with a Bio-Rad transfer unit. The membranes were then blocked with 5% non-fat dry milk and incubated with primary antibody raised against recombinant human CYP3A5 (kindly provided by Dr F Peter Guengerich) as well as rabbit anti-human CYP3A5 (Bioscience Research Reagents, Temecula, CA), both producing similar results, hepatocyte nuclear factor- 4α (HNF- 4α), pregnane X receptor (PXR) or retinoic X receptor α (RXR α) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The primary antibody was located by using HRP conjugated to anti-rabbit IgG. The blots, incubated with SuperSignal West Femto (Pierce, Rockford, IL), were visualized, captured and quantified by using an Alpha Innotech FluoroChem 8800 Imager (San Leandro, CA) with a movie mode. Signals were normalized to a control sample which was repeatedly run on each blot and exhibited

¹We realized that because of radical difference in metabolism, it was not possible to translate normal circulating hormone levels into equivalent in vitro doses, but we did base the selected hormone concentrations in the hepatocyte cultures on physiologic levels. Dexamethasone is a highly potent, synthetic glucocorticoid. However, when comparing its biologic potency (e.g. gluconeogenic and glycogenolytic) with cortisol, the present levels (10 nM) would be comparable with resting plasma concentrations of the natural steroid in men and women (Haynes and Murad, 1985). In addition, our rhGH dose of $2 \text{ ng}\cdot\text{mL}^{-1}$ is also physiological (Murad and Haynes, 1985).

a concentration variant between blots of 3.2% to 6.7% for the different proteins. Lastly, blots were stripped and reprobed with loading controls actin or p97 antibody and found to be comparable with those obtained with internal controls of the assayed samples.

Chromatin immunoprecipitation assay (ChIP)

Following the hormonal regimen described above, ChIP assays (Aparicio *et al.*, 2005) were performed on primary human hepatocytes as well as HepG2 cells (American Type Culture Collections, HB-8065, Manassas, VA) according to our previously described procedure (Thangavel and Shapiro, 2007; 2008). Lysed, purified nuclei were sonicated to generate DNA fragments with an average length of 200 to 500 bp. Equal concentrations of chromatin from all treatment groups were pre-cleared with protein A agarose beads in the presence of $1 \text{ mg}\cdot\text{mL}^{-1}$ BSA and $2 \mu\text{g}$ of sonicated salmon sperm DNA to reduce the non-specific background. After removal of beads by centrifugation, $2 \mu\text{g}$ of PXR or RXR α specific antibody (Santa Cruz Biotechnology) were added and kept at 4°C for overnight on a rotary platform. The immunoprecipitates were washed sequentially, eluted and prepared as previously described (Thangavel and Shapiro, 2007). Immunoprecipitated DNA was purified using a PCR purification kit (Qiagen, Valencia, CA) and resuspended in $50 \mu\text{L}$ of sterile water. The purified DNA from immunoprecipitation was subjected to semi-quantitative PCR using a binding site for PXR : RXR α on the CYP3A5 flanking region (Iwano *et al.*, 2001) with a forward primer of 5'-CTA GAA TGA AGG CAG CCA TGG A-3' (-268/-247) and a reverse primer 5'-TTA GCT GAG TGC TGC TGT TTG CTG-3' (+49/+25). PCR products resolved on agarose gels were quantified using a FluoroChem IS-8800 Imager.

Confirmation of PXR and RXR α binding to the CYP3A5 promoter by southern blotting

The PCR products from the ChIP assays were denatured and transferred onto Nytran N filters from Schleicher and Schuell (Keene, NH) and subjected to Southern blotting as described (Thangavel and Shapiro, 2007; 2008) to confirm the PXR : RXR α binding motif in the PCR products by using a γ - ^{32}P -labelled nucleotide sequence 5'-aTG AAC TCA AAA GAG GTC Agc-3' (-121/-101) of the everted repeat with a six-nucleotide spacer region (ER6) of the human CYP3A5 promoter (Iwano *et al.*, 2001; Biggs *et al.*, 2007). The signals were scanned and quantitated by using a FluoroChem TM IS-8800 Imager. The signals were normalized with a positive control, which was repeatedly run on each blot.

HNF- 4α and PXR knockdown in HepG2 cells

HepG2 cells were cultured in DMEM/F-12 containing 10% FBS, penicillin ($100 \text{ U}\cdot\text{mL}^{-1}$) and streptomycin ($100 \mu\text{g}\cdot\text{mL}^{-1}$) under 5% CO_2 . Upon 50% to 70% confluency, small inhibitory (si)RNA-mediated knockdown of HNF- 4α or PXR was carried out as per manufacturer's instructions (Santa Cruz Inc.) as previously reported (Zhou *et al.*, 2007; Iwazaki *et al.*, 2008; Selva and Hammond, 2009). Twenty-four hours after transfection with the siRNA or scrambled (Scr) siRNA (controls), the HepG2 cells were exposed for 2 days to the hormonal treatments described above. Cells were harvested on

the third day and assayed for whole cell CYP3A5 protein, nuclear HNF-4 α or PXR protein as well as PXR binding to its putative CYP3A5 promoter (ChIP) and for confirmation of the occupied binding motif (Southern blotting) by procedures described above for primary hepatocytes.

CYP3A5 luciferase assay in HNF-4 α and PXR knockdown HepG2 cells

Dual luciferase reporter assays were conducted to determine the emulative transcriptional activity of the CYP3A5 promoter containing the PXR:RXR α binding site (i.e. ER6) in HNF-4 α or PXR knockdown HepG2 cells. HepG2 cells were cultured to 50% to 70% confluency and transfected with either HNF-4 α knockdown siRNA or PXR siRNA or the control Scr siRNA as we have described above. Twenty-four hours later, the cells were transiently transfected with a CYP3A5 promoter containing luciferase (a generous gift from Dr Garold S Yost) using Lipofectamine LTX transfection reagent (Invitrogen, Carlsbad, CA) as previously reported (Biggs *et al.*, 2007). The *Renilla reniformis* luciferase plasmid was co-transfected as an internal control. After 24 h, the cells were exposed for 2 days to the same hormonal treatments as described for the primary human hepatocytes. On the third day, cells were lysed and the respective luciferase activities were determined (Boopathi *et al.*, 2004) using the dual-luciferase assay system (Promega, Madison, WI). Firefly luciferase activities for the experimental constructs were normalized for transfection efficiency and cell loading using *R. reniformis* luciferase activity and total protein concentration respectively.

Statistics

All data were subjected to analysis of variance. Significant differences were determined with *t* statistics and the Bonferroni procedure for multiple comparisons.

Results

Sexually dimorphic response to hormonal regulation of CYP3A5 protein levels in primary hepatocytes derived from men and women

Hepatic CYP3A5 induction was greatest when the cells were exposed to the combined treatment of continuous dexamethasone and GH (Figure 1). Alone, GH was moderately inductive and dexamethasone considerably more so. Sex differences in the induction of CYP3A5 were indicated by the significantly greater expression levels of the isoform in female hepatocytes following all hormonal treatments.

Sex-dependent hormonal regulation of nuclear HNF-4 α , PXR and RXR α concentrations in human hepatocytes

Regardless of sex, the apparent nuclear translocation of HNF-4 α , PXR and RXR α exhibited different responses to dexamethasone and GH when each hormone was administered separately. That is, nuclear concentrations of HNF-4 α were about three to four times greater following exposure to GH

Figure 1

Sex-dependent hormonally regulated expression levels of CYP3A5 protein in hepatocytes derived from adult men and women. The cells were exposed to either continuous dexamethasone (DEX) alone, continuous GH alone, both hormones (DEX + GH) or vehicle alone (-DEX-GH) for 5 days in culture after which the cells were harvested and analysed. Each data point is a mean $\pm$ SD for cells from five or more individuals. **P* < 0.01 compared with the DEX alone treatment of the same sex. †*P* < 0.01 compared women with men exposed to the same hormone treatment. A representative immunoblot of CYP3A5 and its respective loading control (actin) is presented in the figure.

than dexamethasone (Figure 2A). In contrast, nuclear levels of PXR and RXR α were three to four times greater in hepatocytes exposed to dexamethasone than GH (Figure 2B and C). Simultaneous exposure to both hormones increased nuclear concentrations of HNF-4 α , PXR and RXR α in both sexes to levels that exceeded the additive effects of individual hormone treatments. Lastly, nuclear accumulation of all three transcription factors was dramatically greater, at all hormone treatments, in hepatocytes from women. Control hepatocytes (no GH and no dexamethasone treatment) from both sexes exhibited no detectable nuclear concentrations of HNF-4 α , PXR and RXR α levels and data not presented.

Sex-dependent hormonally regulated PXR binding to the CYP3A5 promoter

Consistent with Figure 2B, dexamethasone alone induced greater levels of PXR binding to the CYP3A5 promoter than GH alone (Figure 3A). The combined treatment of continuous dexamethasone and continuous GH was clearly the most effective, stimulating PXR binding to the CYP3A5 promoter to levels exceeding the additive effects of the two hormones administered separately. Again, an inherent sexual dimorphism was observed in that PXR binding to the CYP3A5 promoter was significantly greater with all hormone treatments in primary hepatocytes derived from women. In confirmation, using Southern blotting, we observed very much similar levels of the PXR-binding motif of the CYP3A5 promoter bound to the activated transcription factor (Figure 3B), reflecting the results of the ChIP assay (Figure 3A). Control hepatocytes (no GH and no dexamethasone treatment) from

Figure 2

Sex-dependent, hormonal regulation of nuclear HNF-4α, nuclear PXR and nuclear RXRα protein levels in hepatocytes derived from adult men and women. The cells were exposed to either continuous DEX alone, continuous GH alone or both hormones (DEX + GH) for 5 days in culture after which the hepatocytes were harvested and analysed. Each data point is a mean $\pm$ SD for cells from five or more individuals. * $P < 0.01$ compared with the DEX alone treatment of the same sex. † $P < 0.01$ compared with the DEX alone treatment of the same sex. † $P < 0.01$ compared with men exposed to the same hormone treatment. Representative immunoblots of nuclear HNF-4α (A), PXR (B) and RXRα (C) and their respective loading controls (p97) are presented in the figure. Positive controls (HNF-4α, PXR and RXRα) were repeatedly run on all blots for procedural integrity (not shown).

promoter than GH alone (Figure 4A). The combined treatment of continuous dexamethasone and continuous GH was clearly the most effective, stimulating RXRα binding to the CYP3A5 promoter to levels exceeding the additive effects of the two hormones administered separately. Again, an inherent sexual dimorphism was observed in that RXRα binding to the CYP3A5 promoter was significantly greater with all hormone treatments in primary hepatocytes derived from women. In confirmation, using Southern blotting, we observed very much similar levels of the RXRα-binding motif of the CYP3A5 promoter bound to the activated transcription factor (Figure 4B), reflecting the results of the ChIP assay (Figure 4A). Control hepatocytes (no GH and no dexamethasone treatment) from both sexes exhibited no detectable CYP3A5 promoter bound RXRα and the data not presented.

Knockdown and transfection studies

Having identified sex differences in the signalling pathways mediating GH/dexamethasone regulation of CYP3A5 expression, we proceeded to examine the role of the individual signalling molecules in knockdown and transfection studies. Having obtained poor efficiency with primary hepatocytes from men and women, we performed our experiments on HepG2 cells. Although the use of a transformed cell line does not allow us to draw conclusions regarding the sexual dimorphisms identified above, the fact that these differences are quantitative and not qualitative suggests that findings obtained with HepG2 cells may reflect similar mechanisms in both sexes.

siRNA interference with hormonal regulation of nuclear HNF-4α and PXR accumulation as well as CYP3A5 synthesis in HepG2 cells

In order to identify the role of HNF-4α in the hormonal regulation of CYP3A5 expression for subsequent experiments, we examined the effectiveness of a siRNA designed to knockdown HNF-4α in HepG2 cells (Figure 5). The control HepG2 cells transfected with the nonspecific (scrambled) siRNA exhibited the same HNF-4α responsiveness to hormone exposure as the primary human hepatocytes (Figure 2A). That is, vehicle treatment resulted in no measurable levels of nuclear HNF-4α (not presented), dexamethasone alone induced the smallest increase in nuclear HNF-4α concentrations whereas GH alone was several fold more effective and the combined hormonal treatment was clearly the most inductive regimen

both sexes exhibited no detectable CYP3A5 promoter bound RXR and the data not presented.

Sex-dependent hormonally regulated RXRα binding to the CYP3A5 promoter

Consistent with Figure 2C and similar to levels of PXR binding to the CYP3A5 promoter (Figure 3A), dexamethasone alone induced greater levels of RXRα binding to the CYP3A5

Figure 3

Sex-dependent hormonal regulation of PXR binding to the CYP3A5 promoter (ChIP assay, A) and confirmation of the occupied PXR-binding motif in the CYP3A5 promoter (Southern blot, B) in hepatocytes derived from adult men and women. The cells were exposed to either continuous DEX alone, continuous GH alone or both hormones (DEX + GH) for 5 days after which the hepatocytes were harvested and analysed. Each data point is a mean $\pm$ SD for cells from five or more individuals. * $P < 0.01$ compared with the DEX alone treatment of the same sex. † $P < 0.01$ compared women with men exposed to the same hormone treatment. A representative ChIP assay-agarose gel picture (A) with its negative control demonstrating the specificity of the PXR antibody by immunoprecipitating with IgG, input control (albumin) demonstrating the presence of the albumin promoter only in those samples not 'pulled down' by PXR antibodies and a Southern blot (B) are presented in the figure.

(Figure 5A). Exposure of the HepG2 cells to siRNA reduced the effectiveness of each hormone treatment to stimulate HNF-4 α nuclear accumulation by about 60%. While this level of knockdown is somewhat modest when compared with the effects of other species of siRNA, it is in agreement with reports specifically using siRNAs against HNF-4 α (Zhou *et al.*, 2007; Selva and Hammond, 2009).

Figure 4

Sex-dependent hormonal regulation of RXR α binding to the CYP3A5 promoter (ChIP assay, A) and confirmation of the occupied RXR α -binding motif in the CYP3A5 promoter (Southern blot, B) in hepatocytes derived from adult men and women. The cells were exposed to either continuous DEX alone, continuous GH alone or both hormones (DEX + GH) for 5 days in culture after which the hepatocytes were harvested and analysed. Each data point is a mean $\pm$ SD for cells from five or more individuals. * $P < 0.01$ compared with the DEX alone treatment of the same sex. † $P < 0.01$ compared women with men exposed to the same hormone treatment. A representative ChIP assay-agarose gel picture (A) with its negative control demonstrating the specificity of the RXR α antibody by immunoprecipitating with IgG, input control (albumin) demonstrating the presence of the albumin promoter only in those samples not 'pulled down' by the RXR α antibodies and a Southern blot (B) are presented in the figure.

As regards nuclear levels of PXR, the control HepG2 cells transfected with Scr siRNA exhibited the same PXR responsiveness to the hormone treatments as the primary hepatocytes (Figure 2B). There were no detectable levels of nuclear PXR following vehicle treatment (not presented), GH alone induced the smallest increase in nuclear PXR concentrations,

whereas dexamethasone alone was several fold more effective and the combined hormonal treatment was the most inductive regimen (Figure 5B). In general, exposure of the HepG2 cells to the HNF-4 α knockdown siRNA reduced the effectiveness of each hormone treatment to stimulate PXR nuclear translocation by about 55%.

In contrast to primary hepatocytes that contained nominal concentrations of baseline CYP3A5 protein following exposure to just vehicle (Figure 1), HepG2 cells similarly treated (–DEX–GH) contained no statistically measurable levels of the protein, irrespective of HNF-4 α knockdown. Otherwise, similar to primary hepatocytes, GH alone induced

Figure 5

Hormonal regulation of nuclear HNF-4 α (A) and PXR (B) as well as whole cell CYP3A5 protein (C) levels in HNF-4 α proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either the control non-specific scrambled (Scr) siRNA (proficient) or HNF-4 α knockdown siRNA (deficient) and 24 h later exposed to either continuous DEX alone, continuous GH alone or both hormones (DEX + GH) for 2 days after which the cells were harvested and analysed. Each data point is a mean $\pm$ SD with an $n = 6$ or more. * $P < 0.01$ compared the effects of the knockdown siRNA with the control cells exposed to the same hormone treatment. † $P < 0.01$ compared with DEX alone in cells transfected with the same siRNA. A representative immunoblot of HNF-4 α , PXR and CYP3A5 and their respective loading controls are presented in the figure. A positive control (HNF-4 α and PXR) was repeatedly run on all blots for procedural integrity (not shown).

the smallest increase in CYP3A5 in the HepG2 cells, whereas dexamethasone alone was 2.5-fold more effective and the combined hormonal treatment was clearly more inductive than the sum effects of the individual hormones (Figure 5C). In general, exposure of the HepG2 cells to the HNF-4 α knockdown siRNA reduced the effectiveness of each hormone treatment to induce CYP3A5 protein by about 55%.

Hormonally regulated PXR binding to the CYP3A5 promoter in HNF-4 α knockdown HepG2 cells

Having established the effectiveness of the siRNA to inhibit the nuclear accumulation of PXR (Figure 5B), we proceeded to examine the role of the transcription factors in mediating hormonal induction of CYP3A5 expression. Consistent with our findings using primary human hepatocytes (Figure 3A), in control (Scr siRNA) HepG2 cells, dexamethasone alone induced greater levels of PXR binding to the CYP3A5 promoter than GH (Figure 6A). The combined treatment of continuous GH and dexamethasone was clearly the most effective stimulating PXR binding to the CYP3A5 promoter to levels greatly exceeding the sum of the individual effects of the two hormones. In contrast, HepG2 cells exposed to vehicle alone exhibited no measurable PXR binding to the CYP3A5 promoter (not presented). An ~50% reduction in PXR nuclear accumulation in HNF-4 α -deficient HepG2 cells (Figure 5B) resulted in a similar percent reduction in the binding of PXR to the CYP3A5 promoter at every hormone treatment, demonstrating the importance of HNF-4 α in PXR recruitment by all the hormone regimens in their induction of CYP3A5 expression. In confirmation, using Southern blotting, we observed very much similar levels of the occupied PXR-binding motif of the CYP3A5 promoter (Figure 6B) as seen in the ChIP assay (Figure 6A).

Hormonal regulation of CYP3A5 promoter activity in HNF-4 α or PXR proficient and deficient HepG2 cells transfected with the PXR : RXR α -containing ER6 binding motif

The aim of this experiment was to correlate the requirement(s) for hormone-induced nuclear accumulation and/or promoter binding of HNF-4 α and PXR with transactivation of

Figure 6

Hormonal regulation of PXR binding to the CYP3A5 promoter (ChIP assay, A) and confirmation of the occupied PXR binding motif in the CYP3A5 promoter (Southern blot, B) in HNF-4 α proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either the control non-specific scrambled (Scr) siRNA (proficient) or HNF-4 α knockdown siRNA (deficient) and 24 h later exposed to either continuous DEX alone, continuous GH alone or both hormones (DEX + GH) for 2 days after which the cells were harvested and analysed. Each data point is a mean $\pm$ SD with an $n = 6$ or more. * $P < 0.01$ compared the effects of the knockdown siRNA with the control cells exposed to the same hormone treatment. † $P < 0.01$ compared with DEX alone in cells transfected with the same siRNA. A representative ChIP assay-agarose gel picture (A) with its input control (albumin) and a Southern blot (B) are presented in the figure.

the CYP3A5 promoter as measured by luciferase activity. Whereas hormone treatment (DEX + GH > GH > DEX) stimulates nuclear accumulation of HNF-4 α in primary hepatocytes (women > men) and HepG2 cells (Figures 2A and 5A), we observed, in agreement with earlier conclusions (Burk and Wojnowski, 2004; Biggs *et al.*, 2007), no significant HNF-4 α binding to the CYP3A5 promoter by ChIP assay and confirmatory Southern blotting (not presented). Nevertheless,

interference with HNF-4 α expression by siRNA knockdown depressed hormone-induced CYP3A5 promoter activity by ~60% in HepG2 cells at all hormone regimens (Figure 7A).

Hormone-induced expression levels of CYP3A5 as determined by luciferase activity was basically the same when comparing proficient PXR to proficient HNF-4 α (Scr siRNA transfected) HepG2 cells (Figure 7A and B). That is, GH alone induced the smallest increase in CYP3A5 promoter activity, whereas dexamethasone was more than twice as effective, and the combined hormone treatment was clearly the most effective. PXR knockdown reduced CYP3A5 promoter activity <50% with all hormone regimens (Figure 7B), agreeing with the reported requirement for PXR:RXR α transactivation of the ER6 motif in the xenobiotic induction of the CYP3A5 isoform (Burk and Wojnowski, 2004; Biggs *et al.*, 2007).

Discussion

Similar to CYP3A4, which exhibits a female predominance when determined *in vivo* in liver extracts (Gleiter and Gundert-Remy, 1996; Jaffe *et al.*, 2002; Wolbold *et al.*, 2003) and in cultured hepatocytes (Dhir *et al.*, 2006), we now report a female predominance in CYP3A5 in those livers of individuals capable of expressing the protein. Moreover, like CYP3A4 (Dhir *et al.*, 2006; Thangavel *et al.*, 2011), the sexually dimorphic expression of human CYP3A5 appears to be intrinsic as the normally inductive effects of continuous GH, alone or when administered with dexamethasone (both at physiological-like levels) or even the glucocorticoid alone are significantly more effective in hepatocytes from women than men. In agreement with the present CYP3A5 results, we have found that other female-predominant human (Dhir *et al.*, 2006) as well as rat (Pampori and Shapiro, 1999; Thangavel *et al.*, 2004; Thangavel and Shapiro, 2008) CYPs are more responsive to the inductive effects of the feminine continuous secretory GH profile when administered, *in vivo* and/or *in vitro*, to females than to males. Similarly, male-predominant human (Sinués *et al.*, 2004; Dhir *et al.*, 2006) as well as rat (Shapiro *et al.*, 1993; Waxman *et al.*, 1995; Thangavel and Shapiro, 2007) CYPs, *in vivo* and/or *in vitro*, are more responsive in males than in females when exposed to the renaturalized masculine episodic GH profile. Curiously though, the existence of intrinsic sexually dimorphic responses of CYPs to the sex-dependent GH profiles seems an unwarranted redundancy since it is highly unlikely that men would be exposed to the feminine GH circulating profile nor would women be exposed to the masculine profile. Nevertheless, several inherent GH-dependent sexually dimorphic responses to non-CYP functions in humans have also been reported (Burman *et al.*, 1997; Kuromaru *et al.*, 1998; Johansson *et al.*, 1999; Span *et al.*, 2001; Soares *et al.*, 2004). Whereas any possible justification(s) for these intrinsic sexual dimorphisms is speculative, the present study has identified possible mechanisms responsible for their expression.

Although the human CYP3A subfamily is composed of at least four members, understandably, the vast majority of investigations have been directed to CYP3A4; likely the most important human CYP isoform (Shimada *et al.*, 1994; Rendic, 2002). Accordingly, those few reports examining the regulation of CYP3A5 expression tend to compare their results with

Figure 7

Hormonal regulation of CYP3A5 promoter activity in HepG2 cells transiently transfected with a CYP3A5 promoter construct as well as either a control non-specific scrambled (Scr) siRNA, HNF-4 α knockdown siRNA (A) or PXR knockdown siRNA (B). HepG2 cells were cultured to 50% to 70% confluency and transfected with either HNF-4 α knockdown siRNA, or PXR siRNA or the control Scr siRNA. Twenty-four hours later the cells were transiently transfected with a CYP3A5 promoter containing luciferase. After another 24 h, the cells were exposed to either continuous DEX alone, continuous GH alone, both hormones (DEX + GH) or vehicle alone (-DEX-GH) for 2 days after which the cells were harvested and promoter activity measured using a dual luciferase reporter system. Luciferase activity was normalized with co-transfected Renilla luciferase activity. * $P < 0.01$ compared the effects of knockdown siRNA with the control (Scr siRNA) cells exposed to the same hormone treatment. † $P < 0.01$ compared with DEX alone in cells transfected with the same siRNA. Schematic explanations of the findings are presented in the Figure 7A and B.

the more completely defined mechanisms describing CYP3A4 regulation. While the 5'-regulatory region of CYP3A5 shows only a very limited sequence identity to the corresponding region of CYP3A4, the more proximal motif on the CYP3A4 and CYP3A5 promoter regions (e.g. ER6) share more than 90% sequence homology (Goodwin *et al.*, 2002; Burk *et al.*, 2004; Biggs *et al.*, 2007). Based on these findings, it has been concluded that CYP3A5 induction could be exclusively regulated by the ER6 motif. In this regard, PXR, functioning as its

heterodimer PXR : RXR α , appears to be the major, if not sole transcription factor that binds ER6 and thus is capable of inducing CYP3A5 expression (Burk *et al.*, 2004; Burk and Wojnowski, 2004; Biggs *et al.*, 2007). In agreement, we have found that hormone treatment (GH < dexamethasone < GH + dexamethasone) increases nuclear uptake of PXR and RXR α as well as increasing PXR and RXR α binding to the CYP3A5 promoter (i.e. ER6) and subsequently leading to an elevated expression of the isoform. Moreover, we report that PXR

siRNA knockdown suppresses transactivation of CYP3A5 in HepG2 cells exposed to the hormone regimens. In agreement, introduction of a PXR siRNA adenoviral vector into human hepatocytes has been shown to reduce the concentration of PXR mRNA and significantly decrease the basal level of CYP3A5 mRNA in a dose-dependent manner (Kojima *et al.*, 2007). Thus, it would appear that PXR, or more correctly the PXR : RXR α heterodimer, is solely capable of mediating GH and/or dexamethasone induction of CYP3A5.

PXR has been defined as one of several 'promiscuous' transcription factors because it can be activated by a structurally diverse collection of drugs, environmental chemicals, various endogenous lipophilic compounds (e.g. steroids, bile acids, eicosanoids) as well as collaborative cell signalling pathways (Goodwin *et al.*, 2002; Burk *et al.*, 2004; Burk and Wojnowski, 2004). As regards other signalling/transcription factors, HNF-4 α , a zinc finger protein, is the most abundant transcription factor in the liver having been reported to bind to the promoters of more than 1000 genes involved in most aspects of hepatocyte function (Schrem *et al.*, 2002; Odom *et al.*, 2004). One of these responsive genes is CYP3A4. In addition to activating its own specific binding sites on the CYP3A4 promoter (Burk and Wojnowski, 2004; Biggs *et al.*, 2007), HNF-4 α induces CYP3A4 transcription by increasing PXR expression (Pascucci *et al.*, 2000) and its subsequent transactivation of PXR : RXR α binding elements on the isoform's promoter (Iwahori *et al.*, 2003; Tirona *et al.*, 2003; Kamiyama *et al.*, 2007). Accordingly, we observed that GH alone, dexamethasone less so, and the combined hormone treatment most effectively elevated nuclear levels of HNF-4 α in human hepatocytes as well as in HepG2 cells. Moreover, transfection of HepG2 cells with HNF-4 α siRNA not only decreased hormone-induced nuclear levels of the transcription factor, but it also reduced nuclear concentrations of PXR and PXR binding to the CYP3A5 promoter. But the CYP3A5 promoter appears to contain no HNF-4 α binding elements comparable to that described for the CYP3A4 promoter (Goodwin *et al.*, 2002; Burk *et al.*, 2004; Burk and Wojnowski, 2004). Accordingly, we have proposed (see schematics in Figure 7A and B) in the case of hormone-induced CYP3A5 that HNF-4 α basically acts, along with glucocorticoids, to elevate PXR expression and its subsequent nuclear translocation which in turn transactivates CYP3A5 expression. Suppression of HNF-4 α levels by siRNA knockdown reduces PXR mRNA (Iwano *et al.*, 2001) and nuclear PXR levels depressing CYP3A5 expression (Figure 7A). PXR siRNA knockdown also suppresses CYP3A5 expression, but this is due to directly neutralizing the PXR effect on isoform induction and making moot the actions of HNF-4 α (Figure 7B). The contribution of both HNF-4 α and PXR to CYP3A5 expression is demonstrated by their differential responses to hormone treatments. That is, while GH is the more effective activator of HNF-4 α and dexamethasone is the more effective activator of PXR (as well as RXR α), the combined hormone treatment induces CYP3A5 to levels exceeding the sum of the individual hormones.

It is unlikely that HNF-4 α and PXR : RXR α are the only regulators capable of inducing CYP3A5 expression. Indeed, several additional transcription factors have been implicated in CYP3A5 expression. Whereas these studies are few in number, they have identified nuclear factor-Y (NF-Y) and specificity proteins 1 and 3 (Sp 1 and 3) (Iwano *et al.*, 2001)

as well as constitutively activated receptor (CAR) (Burk *et al.*, 2004) as possible factors involved in CYP3A5 expression. Other studies, however, have questioned the importance of some of these additional factors (Nibourg *et al.*, 2010). A similar controversy has arisen over the involvement of a far larger number of factors required for the expression of CYP3A4 (Burk and Wojnowski, 2004; Tirona *et al.*, 2003; Biggs *et al.*, 2007). While the identification of the requisite factors involved in CYP3A4 and CYP3A5 expression will doubtless require additional studies and some time to sort out, it is possible that all or perhaps most of the identified factors are required for expression of the isoforms, but each under different circumstances. Expression of many constitutive CYP isoforms in all species examined, including some members of the CYP3A subfamily, can be regulated by different mechanisms under different conditions: (i) mechanisms that maintain basal levels, (ii) mechanisms that mediate endogenous (e.g. hormones) inducers, (iii) mechanisms that mediate exogenous (e.g. drugs) inducers and (iv) mechanisms that suppress expression. Thus, depending upon the mechanism examined (e.g. basal expression, glucocorticoid induction, rifampicin induction or immune-based suppression), different signalling/transcription factors may be identified.

Lastly, the present study has also shown that hepatocytes derived from men respond with strikingly lower induction levels of CYP3A5 than hepatocytes from women exposed to the same hormone regimens. This intrinsic sexual dimorphism can be explained, at least in part, by the suboptimal effectiveness of the hormone treatments to recruit PXR and HNF-4 α as well as limiting both their nuclear translocation and/or subsequent transactivation of the CYP3A5 promoter in male hepatocytes. This observation is in agreement with our previous studies examining the molecular mechanisms responsible for the inherent sexually dimorphic responses of human CYP3A4 (Thangavel *et al.*, 2011) as well as several rat CYPs to sex-dependent GH profiles (Dhir *et al.*, 2007; Thangavel and Shapiro, 2007; 2008).

Regarding CYP3A5, there are numerous structural polymorphisms in the gene, particularly single nucleotide polymorphisms that determine the expression/activity of the isoform. However, these polymorphisms are not reported to be sex-linked (Schirmer *et al.*, 2007) and are thus unlikely determinants of the intrinsic sex-differences in CYP3A5 expression. Instead, we have proposed that the intrinsic sexually dimorphic responses of CYP isoforms to sex-dependent GH profiles in humans as well as rats may result, at least in part, from permanent imprinting effects, possibly epigenetic, by perinatal hormones (Thangavel *et al.*, 2011). Whether these intrinsic effects produce a selective advantage or are simply neutral phenotypes that may or may not be linked to beneficial sexual dimorphisms is unknown.

In conclusion, female-predominant expression of human CYP3A5 is due to an inherent, sex-dependent suboptimal activation of transcription networks responsible for hormone-induced expression of the isoform in men. Accordingly, in conjunction with previous studies of other human CYPs, men and women are intrinsically unlikely to handle many drugs in the same way; thus, sex should be a requisite component factored into the design of personalized drug therapies.

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Conflict of interest

None.

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