

## Special Section on Pregnancy

# Isoform-Specific Regulation of Cytochromes P450 Expression by Estradiol and Progesterone

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### ABSTRACT

Results from clinical studies suggest that pregnancy alters hepatic drug metabolism in a cytochrome P450 (P450) isoform-specific manner, and rising concentrations of female hormones are potentially responsible for the changes. The objective of this study was to comprehensively characterize the effects of estrogen and progesterone on the expression and activity of major drug-metabolizing P450s. To this end, primary human hepatocytes were treated with estradiol and progesterone, and mRNA expression and activity levels of 10 different P450 isoforms were determined. The results showed that estradiol enhances CYP2A6, CYP2B6, and CYP3A4 expression, whereas progesterone induces CYP2A6,

CYP2B6, CYP2C8, CYP3A4, and CYP3A5 expression. The induction was mainly observed when the average hormone concentrations were at the levels reached during pregnancy, suggesting that these effects are likely pregnancy-specific. Estradiol also increased enzyme activities of CYP2C9 and CYP2E1 without affecting the mRNA expression levels by unknown mechanisms. Taken together, our results show differential effects of estrogen and progesterone on P450 expression, suggesting involvement of different regulatory mechanisms in female hormone-mediated P450 regulation. Our findings potentially provide a basis in mechanistic understanding for altered drug metabolism during pregnancy.

### Introduction

Medication use during pregnancy is prevalent; over 50% of pregnant women take at least one prescription drug (Glover et al., 2003; Andrade et al., 2004). Pregnancy is known to influence the pharmacokinetics of drugs administered to pregnant women. For example, the rate of hepatic drug metabolism changes during pregnancy in a cytochrome P450 (P450) isoform-specific manner; metabolism of CYP2A6, CYP3A4, CYP2C9, and CYP2D6 substrates is increased by ~50, 100, 20, and 50%, respectively, in the third trimester as compared with nonpregnant controls, whereas that of CYP2C19 and CYP1A2 substrates is decreased by 40–50% (Glover et al., 2003; Anderson, 2005a; Hodge and Tracy, 2007; Anderson and Carr, 2009). The factors responsible for the changes in P450-mediated drug metabolism during pregnancy remain unknown.

Estradiol and progesterone are major female hormones, and their plasma concentrations gradually increase during pregnancy, reaching up to 100 nM and 1  $\mu$ M for estradiol and progesterone, respectively, at term. These concentrations are significantly higher than the concentrations of estradiol (0.2–1 nM) and progesterone (5–50 nM) during

menstrual cycle (Tulchinsky et al., 1972; Mathur et al., 1980; Stricker et al., 2006). Accumulating evidence suggests that female hormones may influence the rate and extent of drug metabolism. For example, changes in hepatic drug elimination are similar for CYP1A2, CYP2A6, and CYP2C19 substrates in pregnant women and users of oral contraceptives (Hodge and Tracy, 2007). Also, the elimination of CYP2A6, CYP2B6, and CYP3A4 substrates is faster in females than in males, whereas CYP1A2-mediated elimination is slower in females (Anderson, 2005b). These directional changes in P450 activity are similar to those occurring during pregnancy. Despite the evidence, whether and how the female hormones modulate expression and/or activities of major hepatic P450 enzymes remains unclear.

The effects of estrogens and progesterone on hepatic P450 expression have been examined in animals (Dean and Stock, 1975; Waxman et al., 1985; Dannan et al., 1986; Ochs et al., 1986; Waxman and Holloway, 2009). In rats, estradiol is known to enhance expression of female-predominant CYP2C7 and female-specific CYP3A9 (Bandiera and Dworschak, 1992; Wang and Strobel, 1997). In contrast, progesterone alters the rate of drug metabolism in a substrate-specific manner, suggesting P450 isoform-specific effects of progesterone on drug metabolism (Dean and Stock, 1975; Ochs et al., 1986). Extrapolation of these results to humans, however, is difficult in part due to significant divergence in hepatic drug-metabolizing enzyme genes and apparent differences in regulation of drug-metabolizing enzymes expression between humans and rodents. For example,

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**ABBREVIATIONS:** b5, NADH-cytochrome b5 reductase; CAR, constitutive androstane receptor; CITCO, 6-(4-Chlorophenyl) imidazo[2,1-b] [1,3] thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime; ER, estrogen receptor; HH, human hepatocyte; LC/MS/MS, tandem mass spectroscopy; P450, cytochrome P450; POR, P450 oxidoreductase; PXR, pregnane X receptor; qRT-PCR, quantitative real-time polymerase chain reaction.

sexual dimorphism of P450 expression in humans is not as prominent as in rodents.

In MCF-7 (human breast cancer) and HepG2 (human hepatoma) cells, estradiol is shown to enhance expression of CYP2A6 and CYP2B6 while decreasing CYP2C19 expression by activating estrogen receptor  $\alpha$  (ER $\alpha$ ) (Higashi et al., 2007; Chen et al., 2009; Lo et al., 2010; Mwinyi et al., 2010; Koh et al., 2012); however, the effects of estradiol on expression of other P450s remain unknown. In contrast, progesterone has been shown to enhance activities of pregnane X receptor (PXR)-driven promoters in various immortalized cell lines (Lehmann et al., 1998; Masuyama et al., 2003; Jeong et al., 2008). PXR, along with constitutive androstane receptor (CAR), is a ligand-activated nuclear receptor known to mediate upregulation of drug-metabolizing enzymes including CYP2A6, CYP2B6, and CYP3A4 (Burk et al., 2004; Dickins, 2004; Ferguson et al., 2005). Whether progesterone influences expression of other P450 enzymes or whether regulatory mechanisms other than PXR signaling are involved in progesterone actions on P450 expression remains unknown.

The objective of this study was to comprehensively characterize the effects of estrogen and progesterone on the expression of major P450s using human hepatocytes. Our results show differential effects of estradiol and progesterone on P450 expression in an isoform-dependent manner, which suggests the potential involvement of multiple regulatory mechanisms in P450 regulation by the female hormones.

## Materials and Methods

**Chemicals and Reagents.** Acetaminophen, chlorzoxazone, coumarin, dexamethasone, diclofenac, 17 $\beta$ -estradiol, ethinylestradiol, indomethacin, (Insulin-Transferrin-Selenium) ITS, S-mephenytoin, 7-hydroxycoumarin, 4-hydroxymephenytoin, 4-methylumbelliferone, phenobarbital, *s*-nirvanol, phenytoin, *p*-nitrophenol, *p*-nitrocatechol, phenacetin, phenytoin, progesterone, rifampin, and William's E medium were obtained from Sigma-Aldrich (St. Louis, MO). Midazolam, 1-hydroxymidazolam, d4-acetaminophen, and prazepam were purchased from Cerilliant (Round Rock, TX). 4'-Hydroxydiclofenac was purchased from Axxora (San Diego, CA). CITCO, i.e., 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-3,4-dichlorobenzyl oxime, was purchased from Biomol (Plymouth Meeting, PA). Bufuralol and 1-hydroxybufuralol were purchased from BD Biosciences (Franklin Lakes, NJ). 6-Hydroxychlorzoxazone was purchased from Cayman Chemical (Ann Arbor, MI). Formic acid (American Chemical Society [ACS] grade), acetonitrile (Optima grade), and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA).

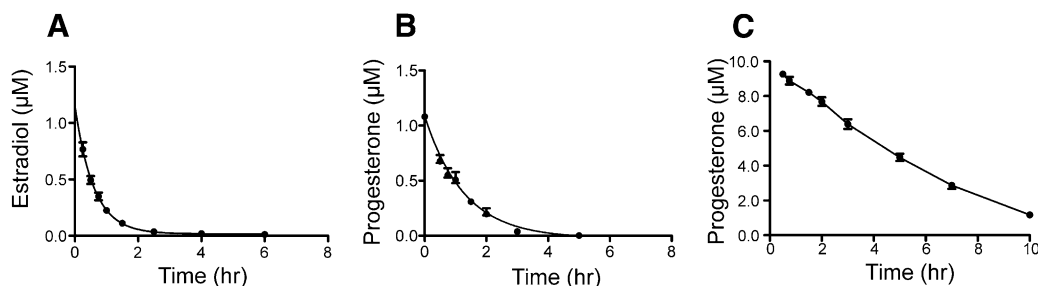
**Primary Human Hepatocytes Culture.** Freshly isolated primary human hepatocytes from different donors (age ranging from 29 to 70, no known history of liver diseases, alcohol abuse, or hepatitis B or C infection) were obtained from CellZDirect (Pittsboro, NC) or the Liver Tissue Cell Distribution System (Pittsburgh, PA). Hepatocytes ( $1 \times 10^6$  cells/well in 12-well plates) were shipped overnight in cold preservation media. Upon receipt, media were replaced with serum-free Williams' E media (without phenol red)

containing 0.1  $\mu$ M dexamethasone, 10  $\mu$ g/ml gentamicin, 15 mM HEPES (pH 7.4), 2 mM L-glutamine, and ITS. Cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After overnight incubation, cells were treated with vehicle control (0.1% ethanol), female hormones [estradiol (1  $\mu$ M) or progesterone (10  $\mu$ M)] or known inducers [rifampin (10  $\mu$ M), CITCO (100 nM), ethanol (100 mM), or phenobarbital (0.5 mM)] for 72 hours. The media were replaced every 6, 12, and 24 hours for estradiol, progesterone, and P450 inducer treatment, respectively.

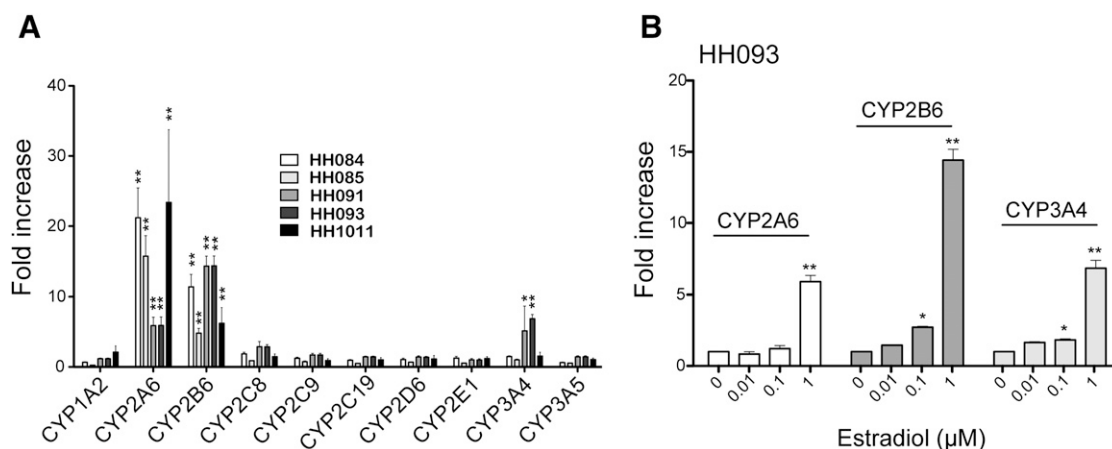
**Determination of Estradiol and Progesterone Concentration in Media.** Hepatocytes were treated with estradiol (1  $\mu$ M) or progesterone (1 or 10  $\mu$ M), and media were sampled at various time points up to 12 hours. Estradiol and progesterone concentrations in culture media were measured by tandem mass spectroscopy (LC/MS/MS) (Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200 using an electrospray ion source). Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6  $\times$  50 mm, 3.5  $\mu$ m; Agilent Technologies) at a flow rate of 0.4 ml/min. For measurement of estradiol concentrations, linear gradient of the mobile phase, consisted of water [0.02% ammonium hydroxide (v/v)] and acetonitrile, was used for separation: 15% acetonitrile at time 0 increased to 90% at 7 minutes. Multiple Reaction Monitoring (MRM) pairs used for estradiol and ethinylestradiol (internal standard) were 271.2/145.1 and 295.2/145.1, respectively. For measurement of progesterone concentrations, linear gradient of mobile phase of water [0.1% HCOOH (v/v)] and acetonitrile was used for separation: 20% acetonitrile at time 0 increased to 80% at 6 minutes. MRM pairs used for progesterone and prazepam (internal standard) were 315.2/109.2 and 325.0/271.1, respectively. Average concentrations of estradiol or progesterone in media were calculated as follows:  $C_{ave} = AUC_{0-\tau}/\tau$ , where  $AUC_{0-\tau}$  is the area under the curve (hormone concentration versus time from 0 to  $\tau$  h), and  $\tau$  is the media change interval.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction.** Total RNAs were isolated from human hepatocytes using TRIzol (Invitrogen, Carlsbad, CA). RNA purity was confirmed by 260:280 nm absorbance ratio (greater than 1.7), and the integrity of ribosomal RNAs was visually confirmed by gel electrophoresis. cDNA was synthesized using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) amplification of cDNA corresponding to 40 ng of total RNA was performed in a reaction mixture (10  $\mu$ l) containing 1 $\times$  concentration of TaqMan Universal Master Mix (Applied Biosystems) and the following TaqMan Gene Expression Assay (individual assay ID is listed below): CYP1A2 (Hs01070369\_m1), CYP2A6 (Hs00868409\_s1), CYP2B6 (Hs03044634\_m1), CYP2C8 (Hs00258314\_m1), CYP2C9 (Hs00426397\_m1), CYP2C19 (Hs00426380\_m1), CYP2D6 (Hs02576167\_m1), CYP2E1 (Hs00559367\_m1), CYP3A4 (Hs00430021\_m1), CYP3A5 (Hs00241417\_m1), CAR (Hs00901571\_m1), PXR (Hs00243666\_m1), POR (Hs00287016\_m1), b5 (Hs00157217\_m1), and GAPDH (Hs99999905\_m1). Cycling conditions were initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative mRNA expression P450 isoforms upon hormone or inducer treatment was determined by normalizing the gene expression levels to GAPDH ( $2^{-\Delta\Delta C_t}$  method).

**Determination of P450 Activity.** Hepatocytes were treated with vehicle control (ethanol), a female hormone, or one of the known inducers for 72 hours, and P450 activities were determined as previously described elsewhere (Choi et al., 2009; Choi et al., 2011). Briefly, the media were replaced with fresh media containing one of P450 isoform-selective probe substrates for CYP1A2 (50  $\mu$ M phenacetin), CYP2A6 (100  $\mu$ M coumarin), CYP2B6



**Fig. 1.** Elimination of estradiol and progesterone in a representative batch of human hepatocytes. Primary human hepatocytes were treated with estradiol (1  $\mu$ M, A), or progesterone (1  $\mu$ M, B; 10  $\mu$ M, C), and the media were sampled at various time points. The hormone concentrations were determined by LC/MS/MS.

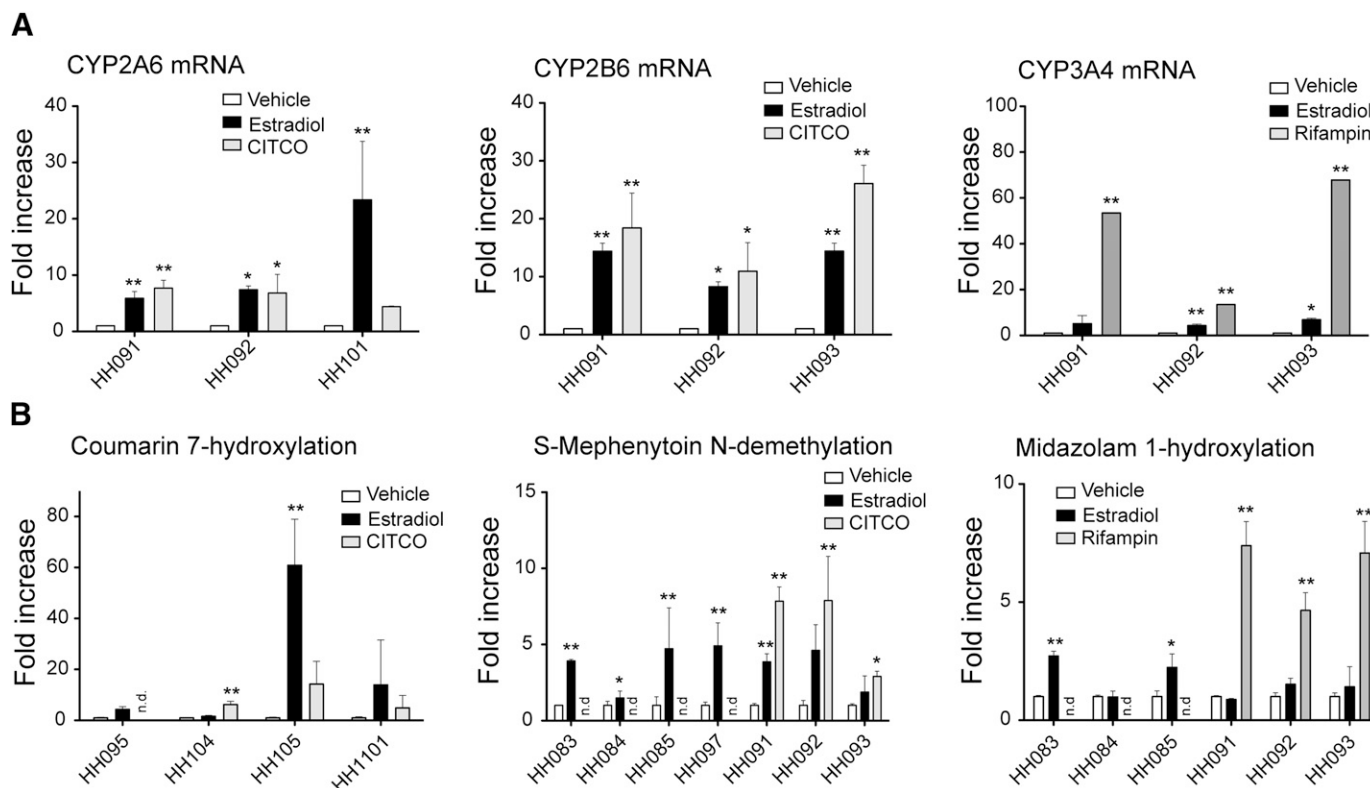


**Fig. 2.** Effects of estradiol on mRNA expression levels of major P450s. (A) Primary human hepatocytes from five different donors ( $n = 3/\text{batch}$ ) were treated with estradiol ( $1 \mu\text{M}$ ) or vehicle (ethanol) for 72 hours (with regular media change; see text for details). mRNA levels of P450 isoforms were measured by qRT-PCR. Results are expressed as relative expression in mRNA levels by estradiol relative to vehicle treatment. (B) A batch of human hepatocytes (HH093) was treated with estradiol ( $0.01\text{--}1 \mu\text{M}$ ) or vehicle (ethanol) for 72 hours (with regular media change; see text for details) ( $n = 3$ ). mRNA levels of P450 isoforms were measured by qRT-PCR.  $*P < 0.05$ ;  $**P < 0.01$  in comparison with vehicle-treated control.

( $100 \mu\text{M}$  S-mephenytoin), CYP2C9 ( $100 \mu\text{M}$  diclofenac), CYP2C19 ( $100 \mu\text{M}$  S-mephenytoin), CYP2D6 ( $60 \mu\text{M}$  bufuralolol), CYP2E1 ( $100 \mu\text{M}$  *p*-nitrophenol), and CYP3A4 ( $15 \mu\text{M}$  midazolam) and sampled at various time points (30 minutes to 4 hours). The concentration of a metabolite of each probe substrate was measured by LC/MS/MS (Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200) using an electrospray ion source. The mobile phase consisted of water ( $0.1\%$  formic acid) and acetonitrile. Separation was performed with a Zorbax Eclipse XDB-C8 column ( $4.6 \times 50 \text{ mm}$ ,  $3.5 \mu\text{m}$ ) (Agilent Technologies, Santa Clara, CA) except for acetaminophen and 7-hydroxycoumarin (YMC C8  $4.6 \times$

$150 \text{ mm}$ ,  $3.5 \mu\text{m}$ , Waters, Milford, MA). The quantitative analytical conditions for the metabolites (e.g., internal standards, MRM pairs, and mobile phase gradients) have been previously described (Choi et al., 2009; Choi et al., 2011).

**Statistical Analysis.** Each experiment (from an individual donor) was performed in triplicates and data are expressed as means  $\pm$  S.D. The statistical difference between hormone-treated versus vehicle-treated control groups was determined by Student's *t* test. For statistical testing among different treatment groups, one-way analysis of variance test was performed for multiple comparisons followed by posthoc Dunnett's test.



**Fig. 3.** Comparison of the effects of estradiol and prototypical inducers on expression and activities of CYP2A6, CYP2B6, and CYP3A4. Human hepatocytes from different donors ( $n = 3/\text{batch}$ ) were treated with vehicle (ethanol), estradiol ( $1 \mu\text{M}$ ), CITCO ( $100 \text{ nM}$ ), or rifampin ( $10 \mu\text{M}$ ) for 72 hours (A) mRNA expression levels of CYP2A6, CYP2B6, and CYP3A4 were determined by qRT-PCR. (B) P450-isoform selective probe substrates [coumarin ( $100 \mu\text{M}$ ) for CYP2A6, S-mephenytoin ( $100 \mu\text{M}$ ) for CYP2B6, or midazolam ( $15 \mu\text{M}$ ) for CYP3A4] were added to media, and metabolite concentrations were measured by using LC/MS/MS.  $*P < 0.05$ ;  $**P < 0.01$  in comparison with vehicle-treated control. n.d., not determined.

## Results

**Estradiol and Progesterone Are Rapidly Metabolized by Human Hepatocytes.** Estradiol and progesterone are known to be rapidly eliminated from the body via hepatic metabolism (Goldzieher and Brody, 1990; Kuhl, 1990). In studying the effects of estradiol and progesterone on the expression of major P450s using human hepatocytes, we first determined elimination rates of estradiol and progesterone in the hepatocyte cultures. Human hepatocytes were treated with estradiol (1  $\mu$ M) or progesterone (1  $\mu$ M), and hormone concentrations at various time points were measured. The result (Fig. 1A) showed that estradiol was metabolized at a first-order elimination rate by the human hepatocytes (half-life:  $0.57 \pm 0.12$  hours). The average concentration of estradiol over 6 hours was  $157 \pm 39$  nM, which corresponds to the average concentration during the third trimester of human pregnancy (Tulchinsky et al., 1972). Similar results were obtained in different batches of human hepatocytes (unpublished data). On the basis of the results, throughout the study, culture media containing 1  $\mu$ M estradiol were replenished every 6 hours during the daytime and 12 hours for overnight incubation to maintain the average estradiol concentration at the level of the third trimester of pregnancy. Progesterone was also metabolized rapidly by human hepatocytes at a first-order elimination rate (Fig. 1B, half-life:  $0.73 \pm 0.16$  hours). This led to the average concentration of  $92 \pm 16$  nM over 12 hours, which corresponds to the progesterone concentration at the early phase of pregnancy, lower than the concentration at full-term ( $\sim 1$   $\mu$ M) (Tulchinsky et al., 1972). Accordingly, a 10-fold higher concentration of progesterone (i.e., 10  $\mu$ M) was used throughout the study to treat hepatocytes. After treatment with 10  $\mu$ M progesterone (Fig. 1C), the estimated average concentration of progesterone over 12 hours in culture media was  $3.28 \pm 0.47$   $\mu$ M.

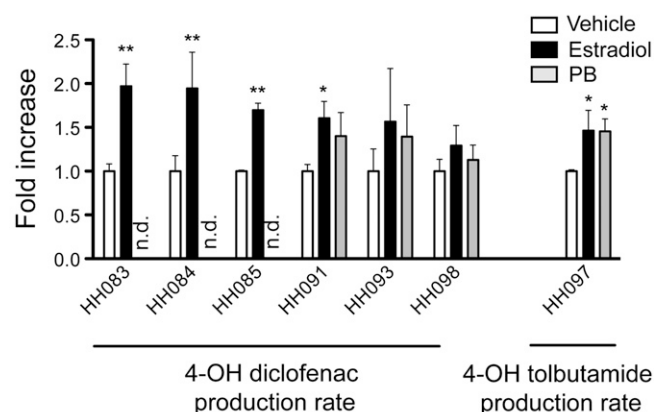
**Estradiol Induces CYP2A6, CYP2B6, and CYP3A4 Expression.** To determine the effects of estradiol on P450 expression, human hepatocytes (from five different donors) were treated with estradiol, and the mRNA expression of major drug-metabolizing CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) was determined by qRT-PCR. Estradiol increased the expression levels of CYP2A6 (5.9- to 23.4-fold), CYP2B6 (4.8- to 14.4-fold) and CYP3A4 (1.5- to 6.8-fold) but not those of the remaining P450 enzymes we examined (Fig. 2A). Of note, induction of CYP3A4 with estradiol was overall in a smaller magnitude than that of CYP2A6 and CYP2B6, and the induction was observed only in two of five different batches of hepatocytes. The effects of estradiol on CYP2A6, CYP2B6, and CYP3A4 showed a concentration dependency (Fig. 2B).

The extent of induction of CYP2A6 and CYP2B6 mRNA levels by estradiol was comparable to that by a prototypical inducer of P450 expression, CITCO (a CAR activator) (Fig. 3A); induction of CYP2A6 mRNA levels by estradiol was 76–530% of the induction by CITCO, whereas the induction of CYP2B6 was 55–78%. In contrast, the extent of CYP3A4 induction by estradiol was much smaller as compared with that by rifampin (a PXR activator) (Fig. 3A), indicating minor effects of estradiol on CYP3A4 expression. The effects of estradiol on activities of CYP2A6, CYP2B6, and CYP3A4 in comparison with those of known P450 inducers showed results similar to the estradiol effects on mRNA expression of those genes (Fig. 3B); estradiol increased the activities of CYP2A6 (1.5- to 60.8-fold), CYP2B6 (1.5- to 4.9-fold), and CYP3A4 (0.9- to 2.7-fold), whereas CITCO and rifampin increased the activities of CYP2A6, CYP2B6 and CYP3A4 by 6.2- to 14.2-fold, 2.9- to 7.9-fold, 4.6- to 7.4-fold, respectively. The activities of CYP1A2, CYP2C19, and CYP2D6 were not affected by estradiol treatment (unpublished data).

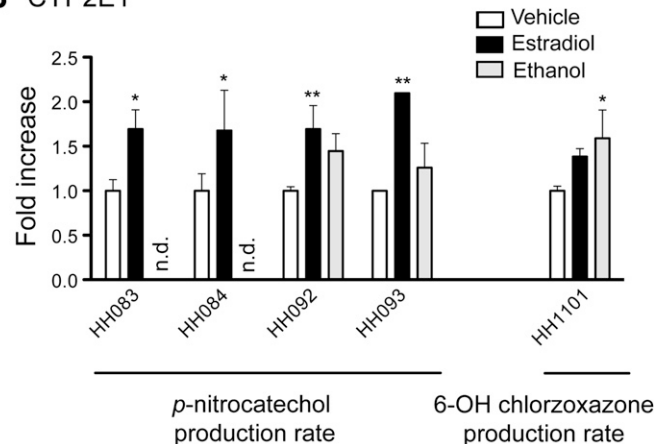
Taken together, estradiol enhanced expression of CYP2A6 and CYP2B6 to the level comparable to that by CITCO. Estradiol also enhanced CYP3A4 expression, although the effect was variable, and the magnitude of induction was small.

**Estradiol Enhances CYP2C9 and CYP2E1 Activities but not Their mRNA Expression.** Estradiol did not affect mRNA levels of CYP2C9 or CYP2E1 in human hepatocytes (Fig. 2). Also, determination of protein levels of CYP2C9 and CYP2E1, by using Western blotting, showed a lack of estradiol effects on protein levels of CYP2C9 and CYP2E1 (unpublished data). Interestingly, however, estradiol enhanced activities of CYP2C9 (diclofenac 4'-hydroxylation) and CYP2E1 (*p*-nitrophenol hydroxylation) by 1.3- to 2.0- and 1.7- to 2.1-fold, respectively, as compared with vehicle treatment in human hepatocytes (Fig. 4). Although the magnitude was small, the induction was comparable to that by known inducers of CYP2C9 (phenobarbital) and CYP2E1 (ethanol) (Roberts et al., 1995; Gerbal-Chaloin et al., 2002). The enhanced activities of CYP2C9 and CYP2E1 upon estradiol treatment were further verified using different probe drugs for the enzymes, tolbutamide and chlorzoxazone. Estradiol increased tolbutamide hydroxylation (CYP2C9) by 1.8-fold, as compared with vehicle treatment, similar to the results obtained using diclofenac as a probe drug (Fig. 4). In contrast, although the effect of estradiol on

### A CYP2C9



### B CYP2E1



**Fig. 4.** Increased CYP2C9 and CYP2E1 activities by estradiol. Human hepatocytes from different donors ( $n = 3$ /batch) were treated with vehicle (ethanol; 0.1%), estradiol (1  $\mu$ M), phenobarbital (PB; 0.5 mM), or ethanol (100 mM) for 72 hours. P450-isoform selective probe substrates [(A) diclofenac (100  $\mu$ M)] or tolbutamide (100  $\mu$ M); (B) *p*-nitrophenol (100  $\mu$ M) or chlorzoxazone (100  $\mu$ M)] were added to media. The metabolite concentrations in the media were measured by using LC/MS/MS. \* $P < 0.05$ ; \*\* $P < 0.01$  in comparison with vehicle-treated control. n.d., not determined.

chlorzoxazone 6-hydroxylation (CYP2E1) did not reach statistically significance, estradiol increased the activity by 1.4-fold (Fig. 4). Considering that the activities of CYP2C9 and CYP2E1 are sensitive to expression levels of redox partners such as P450 oxidoreductase (POR) or NADH-cytochrome b5 reductase (b5) (Kaminsky and Guengerich, 1985; Wortham et al., 2007), mRNA levels of POR and b5 in estradiol- or vehicle-treated hepatocytes were determined by qRT-PCR. The expression of POR and b5 was not altered by estradiol (data not shown). Taken together, our results indicate that estradiol moderately enhances activities of CYP2C9 and CYP2E1 while not affecting their expression levels.

**Progesterone Induces Expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5.** To determine the effects of progesterone on expression of P450 enzymes, we treated human hepatocytes with vehicle or progesterone for 72 hours and examined mRNA expression of major CYPs by using qRT-PCR. The results (Figure 5A) showed that progesterone increased the expression of CYP2A6 (1.7- to 3.4-fold), CYP2B6 (1.8- to 7.6-fold), CYP2C8 (1.4- to 9.2-fold), CYP3A4 (1.4- to 12.0-fold), and CYP3A5 (1.4- to 6.7-fold). The effects of progesterone on CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5 showed concentration dependency (Fig. 5B). Progesterone did not show significant effects on the mRNA expression or enzyme activities of the remaining CYPs (data not shown).

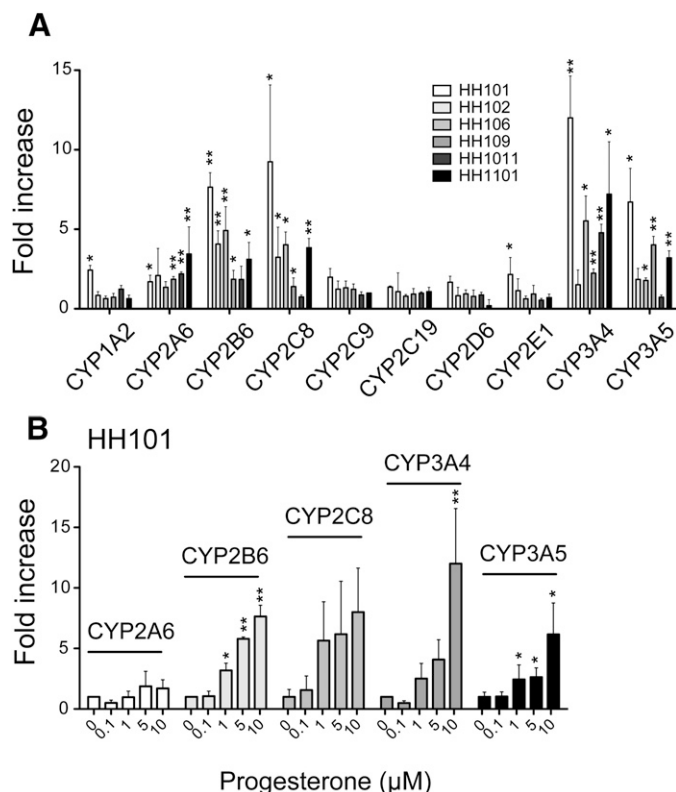
When compared with the action of prototypical inducers of P450 expression (i.e., CITCO and rifampin), the magnitude of induction in CYP2A6, CYP2B6, and CYP3A4 mRNA levels by progesterone was smaller and exhibited large interindividual-variability (Fig. 6A); induction of CYP2A6 and CYP2B6 by progesterone was 33–74% and 17–54%, respectively, of the induction by CITCO whereas induction of CYP3A4 was 14–45% of the induction by rifampin. Induction of CYP2C8 and CYP3A5 was comparable to the induction by rifampin (46–108% and 83–107%). The progesterone-mediated changes in activities of CYP2A6, CYP2B6, and CYP3A4 appeared to show patterns similar to those in mRNA expression of the genes (Fig. 6B). Taken together, our data indicate that progesterone enhances expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5, although the effects appear minor when compared with those by typical P450 inducers.

## Discussion

Results from clinical studies suggest that increased plasma levels of female hormones are potentially responsible for altered drug metabolism during pregnancy. However, there have been no comprehensive studies conducted using human liver tissues for the effects of female hormones on expression and activity of major P450s. In this study, we characterized the effects of major female hormones, estradiol and progesterone, on the expression of 10 different P450 isoforms in human hepatocytes.

We treated primary human hepatocytes with estradiol and progesterone with frequent media change, and measured the expression and activity levels of different P450s. Estradiol and progesterone are noncharged lipophilic compounds that can freely cross cell membranes via passive diffusion (Stumpf, 1969; Revelli et al., 1998). Accordingly, we expect that after 72 hours of drug treatment, the concentration of estradiol and progesterone between media and intracellular compartment would reach equilibrium and that the media concentration likely represents the intracellular concentrations of estradiol and progesterone.

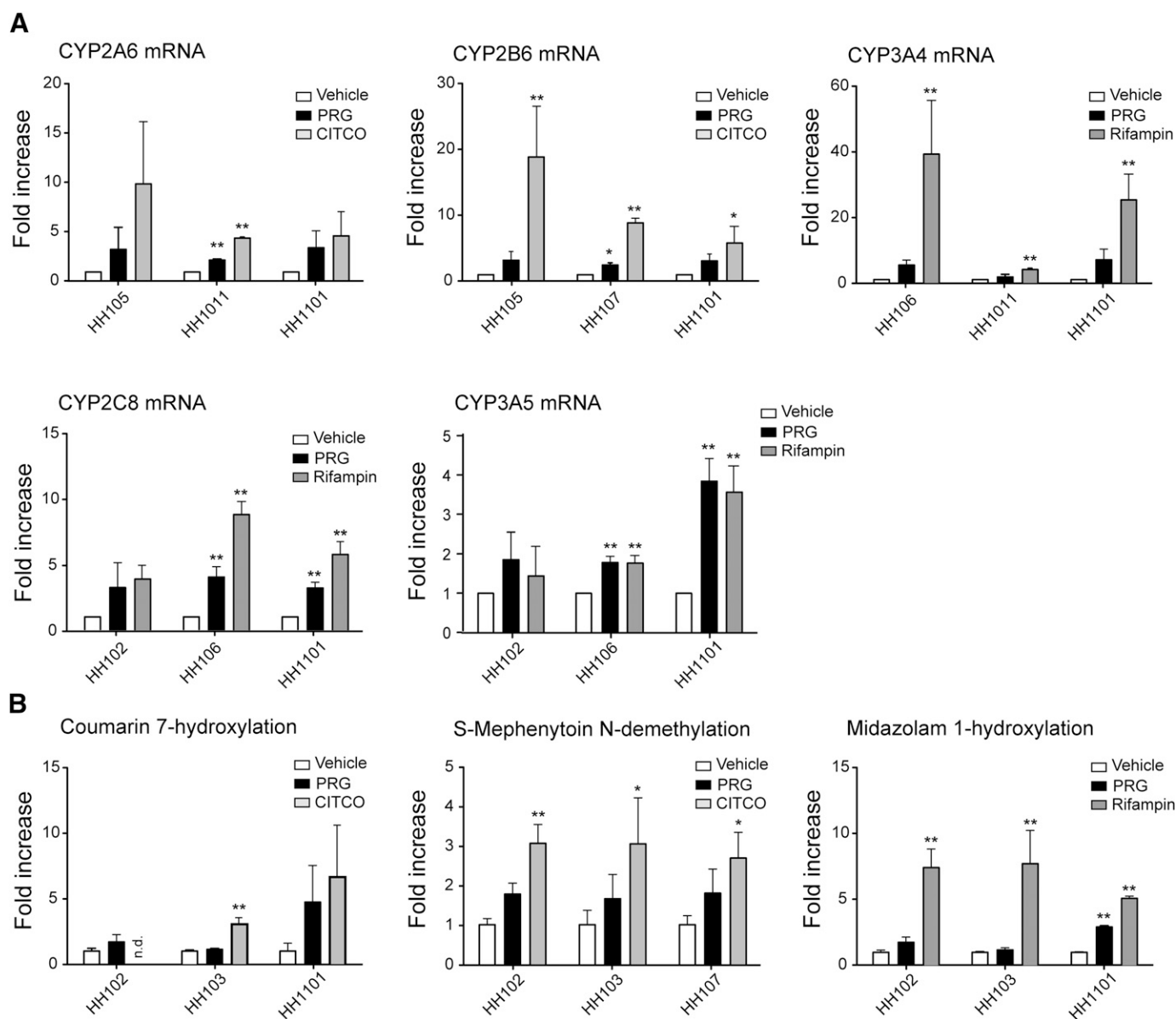
Our results showed that estradiol induces the expression of CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes. The induction was mainly observed when the average estradiol concentration



**Fig. 5.** Effects of progesterone on mRNA expression levels of major CYPs. (A) primary human hepatocytes from six different donors ( $n = 3$ /batch) were treated with progesterone (10  $\mu$ M) or vehicle (ethanol) for 72 h (with regular media change; see text for details). mRNA levels of CYP enzymes were measured by qRT-PCR. (B) a batch of human hepatocytes (HH101) were treated with progesterone (0.1–10  $\mu$ M) or vehicle (ethanol) for 72 h with regular media change ( $n = 3$ ). mRNA levels of CYP isoforms were measured by qRT-PCR. \* $P < 0.05$ ; \*\* $P < 0.01$  in comparison with vehicle-treated control.

was at the level reached during pregnancy, suggesting that the hormonal effects on P450 expression are likely pregnancy specific. The induction of CYP2A6 by estradiol is in agreement with a previous study where estradiol increased transcriptional activity of CYP2A6 via activation of ER $\alpha$  (Higashi et al., 2007). The magnitude of induction in CYP2A6 mRNA levels by estradiol shown in our study was to a similar extent to that by CITCO, a known inducer of CYP2A6 (Maglich et al., 2003). This suggests that induction of CYP2A6 by estradiol potentially leads to clinically significant outcomes and may be in part responsible for the reported increases in metabolism of CYP2A6 substrates (e.g., nicotine) during pregnancy (Dempsey et al., 2002). CYP2B6 induction by estradiol was also comparable to that by CITCO, suggesting potential increases in elimination of CYP2B6 substrates (e.g., bupropion) during pregnancy. Whether pregnancy indeed influences CYP2B6-mediated drug metabolism in humans remains to be determined. The induction of CYP3A4 expression by estradiol was relatively weak when compared with that by rifampin, rendering its clinical significance questionable.

Of interest, estradiol increased CYP2C9 and CYP2E1 activities while not affecting their expression. In estradiol-treated hepatocytes, similar increases in the P450 enzyme activity were observed for multiple probe substrates of CYP2C9 (diclofenac and tolbutamide) and CYP2E1 (*p*-nitrophenol and chlorzoxazone). Furthermore, co-treatment of hepatocytes with a phase II enzyme inhibitor (i.e., 2 mM salicylamide) to block the known glucuronidation of *p*-nitrophenol (Zerilli et al., 1997; Hanioka et al., 2001) did not abolish the increased *p*-nitrophenol hydroxylation upon estradiol treatment (data not shown).



**Fig. 6.** Comparison of effects of progesterone and prototypical inducers on expression and activities of CYP2A6, CYP2B6, and CYP3A4. Human hepatocytes from three different donors ( $n = 3$ /batch) were treated with vehicle (ethanol), progesterone (PRG, 10  $\mu$ M), CITCO (100 nM), or rifampin (10  $\mu$ M) for 72 hours (A) mRNA expression levels of CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5 were determined by qRT-PCR. (B) CYP-isoform selective probe substrates [coumarin (100  $\mu$ M) for CYP2A6, S-mephenytoin (100  $\mu$ M) for CYP2B6, or midazolam (15  $\mu$ M) for CYP3A4] were added to media, and metabolite concentrations were measured by using LC/MS/MS. \* $P < 0.05$ ; \*\* $P < 0.01$  in comparison with vehicle-treated control. n.d., not determined.

These results suggest that potential nonspecificity of the substrates unlikely contributes to the enhanced CYP2C9 and CYP2E1 activity by estradiol. Notably, CYP2C9 and CYP2E1 activities are known to be sensitive to the expression levels of redox partners such as POR or b5 (Kaminsky and Guengerich, 1985; Wortham et al., 2007). Our data, however, showed that estradiol does not alter the mRNA expression levels of POR and b5, suggesting that POR and b5 are unlikely responsible for the enhanced CYP2C9 and CYP2E1 activities by estradiol. Previous studies have reported that posttranslational modification of P450 enzymes may influence P450 activities without affecting expression levels (reviewed in Aguiar et al., 2005). Whether estradiol influences posttranslational modification of CYP2C9 and CYP2E1 leading to altered enzyme activity remains unknown.

Progesterone enhanced mRNA expression of CYP2A6 (minor), CYP2B6, CYP2C8, CYP3A4, and CYP3A5. Although this list of

P450 isoforms partly overlaps with that upregulated by estradiol, the pattern of P450 induction by progesterone was found to be different; for example, progesterone showed the largest effect on CYP3A4 expression whereas estradiol enhanced CYP2B6 expression greatest. This appears to suggest that differential regulatory mechanisms may play a role in the hormonal action on P450 expression. Indeed, we and other groups have reported that estradiol is an activator of human CAR (but not of PXR), whereas progesterone is an activator of PXR (but not of CAR) at concentrations reached during pregnancy (Lehmann et al., 1998; Jeong et al., 2008; Koh et al., 2012). Expectedly, estradiol-activated ER $\alpha$  would also play a role in the differential actions of estradiol and progesterone on P450 expression; CYP2A6 and CYP2B6 are known targets of ER $\alpha$  (Higashi et al., 2007; Lo et al., 2010; Koh et al., 2012).

The induction of CYP2C8 and CYP3A5 expression by progesterone is noteworthy. CYP2C8 and CYP3A5 are targets of PXR

action, but PXR-mediated induction of the genes is known to be minor (Gerbal-Chaloin et al., 2001; Burk et al., 2004); the magnitude of CYP2C8 and CYP3A5 induction by a PXR activator (such as rifampin) is typically less than 10% of CYP3A4 induction (Gerbal-Chaloin et al., 2001; Rae et al., 2001). Interestingly, however, in our study, progesterone enhanced CYP2C8 and CYP3A5 expression by the magnitude comparable to CYP3A4, suggesting that regulatory mechanism(s) independent of PXR activation may be involved in CYP2C8 and CYP3A5 induction by progesterone. CYP2C8 is responsible for oxidative metabolism of up to 5% of drugs cleared by phase I processes, and its substrates include repaglinide and arachidonic acids (Totah and Rettie, 2005). In contrast, CYP3A5 has substrate selectivity overlapping with CYP3A4 (e.g., midazolam) (Williams et al., 2002), suggesting that clinically observed increases in midazolam metabolism during pregnancy may be due in part to CYP3A5 induction by progesterone. Whether pregnancy influences pharmacokinetics of CYP2C8 substrates remains to be determined.

In summary, we comprehensively characterized the effects of estradiol and progesterone on expression of major hepatic P450s using human hepatocytes. Estradiol enhances CYP2A6, CYP2B6, and CYP3A4 expression, whereas progesterone induces CYP2A6 (minor), CYP2B6, CYP2C8, CYP3A4, and CYP3A5 expression. Although molecular mechanisms underlying these hormonal effects on P450 expression are yet to be determined, our findings potentially provide a basis for optimizing drug therapy in pregnant women by better understanding for altered drug metabolism during pregnancy.

#### Authorship Contributions

Participated in Research Design: Choi, Koh, Jeong  
 Conducted Experiments: Choi, Koh  
 Performed Data analysis: Choi, Jeong  
 Wrote or contributed to the writing of the manuscript: Choi, Koh, Jeong

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