Isoform-specific regulation of cytochrome P450 expression and activity by estradiol in female rats

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A B S T R A C T

Estradiol (E2) is the major endogenous estrogen, and its plasma concentration increases up to 100-fold during pregnancy in humans. Accumulating evidence suggests that an elevated level of E2 may influence hepatic drug metabolism, potentially being responsible for altered drug metabolism during pregnancy. We characterized effects of E2 on expression and activities of cytochrome P450 enzymes (CYPs) in an in vivo system using rats. To this end, female rats were treated with estradiol benzoate (EB) or known CYP inducers. Liver tissues were obtained after 5 days of treatment, and mRNA and protein expression levels as well as activities of major hepatic CYPs were determined by qRT-PCR, immunoblot, and microsomal assay. E2 increased CYP1A2 expression and activity to a smaller extent than β-naphthoflavone did. E2 also enhanced CYP2C expression (CYP2C6, CYP2C7, and CYP2C12) to levels comparable to those observed by phenobarbital. E2 upregulated CYP3A9 expression, while expression of CYP3A1 was downregulated. Expression of hepatic nuclear receptors (PXR and CAR) and the obligate redox partner of CYPs (POR) was downregulated in EB-treated rats, suggesting their potential involvement in regulation of CYP expression and activity by E2. In summary, in female rats E2 regulates expression of hepatic CYPs in a CYP isoform-specific manner although the directional changes are different from those clinically observed during human pregnancy. Further study is warranted to determine whether the changes in drug metabolism during human pregnancy are attributable to involvement of hormones other than E2.

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1. Introduction

Medication use during pregnancy is prevalent; over 50% of pregnant women take at least one medication [1]. Pharmacokinetic profiles of drugs are different in these women as compared to those in nonpregnant women that typical drug doses established in males or nonpregnant women often result in treatment failure or toxicity [2]. Results from clinical studies suggest that altered hepatic metabolism is mainly responsible for the changes in drug disposition during pregnancy [3–5]. Pregnancy influences drug metabolism in a CYP isoform-dependent manner; the activities of CYP2A6, CYP3A4, CYP2C9, and CYP2D6 are increased by 50%, 100%, 20%, and 50%, respectively, whereas the activities of CYP2C19 and CYP1A2 are decreased by 50% and 40% in the 3rd trimester as compared to nonpregnant controls [3–5]. Activities of CYP1A2 and CYP2D6 have shown to change gradually during pregnancy, in a gestational period dependent manner [4,5]. Causative factors responsible for the changes in CYP activities during pregnancy remain unknown.

17β-Estradiol (E2) is the major endogenous estrogen. Its plasma concentration increases up to 100-fold during pregnancy in humans [6]. Accumulating evidence suggests that E2 may influence the rate and extent of drug metabolism, potentially being responsible for the CYP isoform-dependent changes in drug metabolism during pregnancy. For example, changes in hepatic drug elimination are similar for CYP1A2, CYP2A6, and CYP2C19 substrates in pregnant women and users of estrogen-based oral contraceptives [5]. Also, elimination of CYP2A6, CYP2B6, and CYP3A4 substrates is faster in females than in males whereas CYP1A2-mediated elimination is slower in females [7]. These directional changes in CYP activity in females are similar to those in pregnant women (as compared to the nonpregnant). Despite the evidence, the effects of E2 on major hepatic CYP expression and activities remain largely unknown.

Rats have been extensively used as an *in vivo* animal model for drug metabolism studies. The interspecies similarities and differences in CYP activities and the transcriptional regulators of CYP expression are well established. Between rats and humans, catalytic activities of CYP1A2 and CYP2E1 are well conserved while isoforms in the rest of major CYP families (i.e., CYP2B, CYP2C, CYP2D and CYP3A) show considerable differences in substrate specificity due to the distinct structures of the catalytic sites [8]. Interestingly however, mechanisms underlying transcriptional regulation of CYP expression appear well conserved between rats and humans regardless of CYP isoform. For example, in both humans and rats, expression of CYP1A, CYP2B/2C, and CYP3A is strongly enhanced upon activation of transcription regulators, aromatic hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), respectively, while the CYP expression is downregulated by inflammatory mediators. This suggests that rats may serve as an animal model for studying regulation of CYP expression.

A number of researchers have examined the effects of E2 on hepatic CYP expression in rats [9–11]. However, these studies mainly focused on mechanisms underlying sex differences in CYP expression such that they involved surgical manipulation of animals (e.g., ovariectomy or hypophysectomy) and concentrated mostly on the CYP isoforms that show prominent sexual dimorphism such as CYP2C11 or CYP3A2. To date, there have not been comprehensive studies that address how E2 influences expression of major CYPs in intact female rats, reflecting the net effects of E2 on CYP expression.

The objective of this study was to determine whether E2 is potentially responsible for the altered CYP-mediated drug metabolism during pregnancy. To this end, we characterized the effects of E2 on the expression and activities of major hepatic CYPs in intact rats. In addition, the effects of E2 on the mRNA levels of major CYP transcriptional regulators and P450 oxidoreductase (POR) were examined to explore potential mechanisms underlying the regulation of CYP expression and activity by E2.

2. Materials and methods

2.1. Chemicals and reagents

Resorufin, 7-ethoxyresorufin, diclofenac, *p*-nitrophenol, *p*-nitrocatechol, 1′-hydroxymidazolam, phenobarbital (PB), dexamethasone (DX), β-naphthoflavone (BNF), estradiol benzoate (EB), potassium fluoride, sodium arsenate, NADP$^+$, isocitric acid, magnesium chloride and isocitric acid dehydrogenase were obtained from Sigma (St. Louis, MO). Midazolam was purchased from Cerilliant (Round Rock, Texas). 4′-Hydroxydiclofenac was purchased from Axxora (San Diego, CA). Bufuralol and 1′-hydroxybufuralol were purchased from BD Biosciences (Franklin Lakes, NJ). Formic acid (ACS grade) and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Drug treatment

Adult female Sprague–Dawley rats weighing 180–200 g (8-week old) were purchased from Harlan (Indianapolis, IN). After 1 week of acclimation, rats were administered with EB (1 mg/kg/day subcutaneous injection), BNF (40 mg/kg/day intraperitoneal injection), PB (60 mg/kg/day intraperitoneal injection), DX (60 mg/kg/day intraperitoneal injection), or vehicle (corn oil subcutaneous injection) [*n* = 4 each group except for control and DX groups (*n* = 3)]. On day 5, the livers were removed and weighed. Microsomes and total RNA were prepared from the liver tissues.

In parallel, two separate groups of rats (*n* = 3 per group) were treated with EB (1 mg/kg subcutaneous injection) for either 1 day or 5 days. After EB administration, 200 μl of blood was collected at various time points (pretreatment, 0.5, 1, 2, 4, 7, 11, and 24 h post-injection). Plasma samples were obtained by immediate centrifugation of the blood and added with 50 μl of 2 M sodium arsenate and 10 μl of 50% potassium fluoride/ml plasma (plasma esterase inhibitors).

2.3. Pharmacokinetic analysis

Concentration of E2 in plasma was determined by an enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). Maximum plasma concentration (C_{max}) and time to reach C_{max} after injection (T_{max}) were determined by visual examination of the concentration *vs.* time profile. Area under the curve over dosing interval ($AUC_{0-24\ h}$) was estimated by using the linear trapezoidal rule. Average plasma concentration (C_{ave}) over dosing interval (τ, 24 h) was estimated by using the equation: $C_{ave} = AUC_{0-24\ h}/\tau$.

2.4. Hepatic microsomal assays

Hepatic microsomes were prepared by differential ultracentrifugation of hepatic tissues as previously described [12]. Protein concentration of the prepared microsomes was determined by using BCA Protein Assay kits (Pierce, Rockford, IL), and CYP amount was measured by the method of Omura and Sato [13]. For microsomal reactions, an NADPH-generating system (1 mM NADP$^+$, 5 mM isocitric acid, 0.2 U/ml isocitric acid dehydrogenase, and 5.0 mM MgCl$_2$) was used. The reactions were initiated by adding NADP$^+$ to drug-containing reaction media and terminated by adding three volumes of ice-cold acetonitrile. A control reaction was performed in the absence of NADP$^+$. Preliminary experiments were conducted for each substrate compound to determine the microsomal protein concentration and the incubation time that lead to proportional increases in metabolite production. For each substrate compound, V_{max} and K_m were determined using Prism 5 software (GraphPad, La Jolla, CA). Statistical analysis was performed using Student's *t*-test.

2.5. Determination of metabolite concentrations

The microsomal samples were analyzed 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 methanol. Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6 mm × 50 mm, 3.5 μm) (Agilent Technologies, Santa Clara, CA) at a flow rate of 0.4 ml/min. MS detection of metabolites and internal standards was followed in a positive ion mode by examining multiple MRM pairs as described previously [14]. Concentration of resorufin, the metabolite of 7-ethoxyresorufin, was measured by a fluorescence plate reader (Synergy 4) (BioTek, Winooski, VT) at excitation and emission wavelengths of 530 and 582 nm, respectively.

2.6. RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNAs were isolated from liver tissues using Trizol® (Invitrogen, Carlsbad, CA). cDNA was prepared using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. qRT-PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems). TaqMan® Gene expression Primers (Applied Biosystems) were used for CYP1A2 (Rn00561082_m1), CYP2B1 (Rn01457880_m1), CYP2D2 (Rn00562419_m1), CYP2E1 (Rn00580624_m1), CYP3A1 (Rn01412959_g1), CYP3A9 (Rn00595977_m1), and β-actin (Rn0066789_m1). Expression levels of CYP2C6, CYP2C7, CYP2C12,

Table 1
Liver weight, microsomal protein and total cytochrome P450 contents after drug treatment (mean ± S.D.).

	Control	EB	BNF	PB	DX
n[a]	3	4	4	4	3
Liver (g)	10.4 ± 1.8	9.2 ± 0.7	11.3 ± 0.5	10.3 ± 0.6	11.9 ± 1.9
Microsomal protein (mg/ml)	6.7 ± 0.8	9.6 ± 1.4*	8.3 ± 2.4*	9.3 ± 1.6*	8.1 ± 1.5*
CYP (pmol/mg protein)	0.32 ± 0.10	0.44 ± 0.04	0.78 ± 0.15*	0.45 ± 0.13	0.63 ± 0.24*

[a] n is the number of animals used in each group.
* $p < 0.05$ vs. control.

pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), and P450 oxidoreductase (POR) were determined by using SYBR® green expression master mix (Applied Biosystems). The following primers were designed by using Primer 3 software [15]: CYP2C6 (F: 5′-ATGGCAGCCCTGCCTCCTCT-3′, R: 5′-GGCATGCGGCTCCTGTCCTG-3′), CYP2C7 (F: 5′-TGCCTTTCTCAGCAGGAAAACGAGC-3′, R: 5′-ACAACTGCATGCGGGC-CAGG-3′), CYP2C12 (F: 5′-TGATTGGGAGACACCGCAGCC-3′, R: 5′-AGGGCATGTGGATCCTGTCCAAC-3′), PXR (F: 5′-GGAGGGCAGGGGC-TGACAGA-3′, R: 5′-GAAACACCGCAGGTAGCCGGA-3′), CAR (F: 5′-GGCGCCCACACTCGTCATGT-3′, R: 5′-GCCGGAGGCCTGAACTGCAC-3′), AhR (F: 5′-ATGAGCAGCGGCGCCAACAT-3′, R: 5′-ACTGTTTTCTG-CACCGGCTTGC-3′), POR (F: 5′-AACCCGCCACGCACCAATGT-3′, R: 5′-ACAGCTCCTTGCCCTCGCCT-3′), and β-actin (F: 5′-AAGTCCCT-CACCCTCCCAAAAG-3′, R: 5′-AAGCAATGCTGTCACCTTCCC-3′). The fold change in mRNA levels of CYP upon drug treatment was determined by normalizing the gene expression levels by those of β-actin ($2^{-\Delta\Delta Ct}$ method, [16]).

2.7. Western immunoblot analysis

Liver microsomes were resolved by SDS gel electrophoresis on an 8% polyacrylamide gel (8 μg microsomal protein/lane). Proteins were transferred to a nitrocellulose membrane (1.5 h, 300 mA); loading of equal sample amounts was ensured by comparing signals after Ponceau S staining. The membrane was then blocked at room temperature for 1 h in 5% (w/v) milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST). Membranes were incubated for overnight at 4 °C in anti-CYP1A2 (1:1000 in 2% milk powder in TBST) (Chemicon International, Billerica, MA), anti-CYP3A1 (1:1500 in 2% milk in TBST) (Chemicon International), or anti-CYP2C (1:1000 in 2% milk in TBST) (Abcam, Cambridge, MA). Then, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody at room temperature for 1 h (1:10,000 in 5% milk in TBST) (Abcam). Protein expression was detected by Supersignal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL) on Kodak films, and the signals were quantitated by using Adobe Photoshop.

3. Results

3.1. Effects of E2 on liver size, microsomal protein and CYP contents

To investigate the effects of E2 on CYP expression and activity, female rats were administered with vehicle, EB, or known inducers of CYP enzymes for 5 days. BNF, PB, and DX were used as prototypical inducers for CYP1A, CYP2C, and CYP3A, respectively [17]. EB treatment did not affect liver size or microsomal CYP contents (Table 1) as compared to the vehicle treatment. However, EB treatment increased concentration of total protein in the microsome by unknown mechanisms. BNF, DX, and PB increased the microsomal proteins and CYP contents as expected.

3.2. Plasma concentration of E2 after EB injection

Plasma E2 concentration vs. time profiles obtained after a single or multiple doses (over 5 consecutive days) of EB are shown in Fig. 1. A single subcutaneous injection of EB led to C_{max} of 1.68 ± 0.45 ng/ml (mean ± SEM) and T_{max} of 2 h. E2 concentration at 24 h (0.34 ± 0.09 ng/ml) was higher than the pretreatment basal level (0.023 ± 0.001 ng/ml; Fig. 1A). After multiple dosing, C_{max} was 1.84 ± 0.46 ng/ml at T_{max} of 2 h. E2 concentration before the 5th dose was 0.83 ± 0.12 ng/ml, showing accumulation of E2 in the bodies. The C_{ave} of E2, estimated from $AUC_{0-24 h}$, was also elevated from 0.56 ± 0.15 ng/ml on day 1 to 0.99 ± 0.04 ng/ml on day 5. These concentrations are comparable to the plasma E2 concentrations attainable during human pregnancy.

3.3. Effects of E2 on mRNA expression of major CYPs

qRT-PCR was performed to investigate the effects of E2 on the mRNA expression of major hepatic CYPs in rat livers: CYP1A2, CYP2B1, CYP2C6, CYP2C7, CYP2C12, CYP2D2, CYP3A1, CYP3A9 and CYP2E1. The results showed that E2 differentially regulated the expression of individual CYP isoforms (Fig. 2). EB treatment increased expression of CYP1A2 by 2.0-fold ($p = 0.049$) as compared to vehicle treatment, whereas BNF increased CYP1A2 expression by over 20-fold. EB treatment led to upregulation of
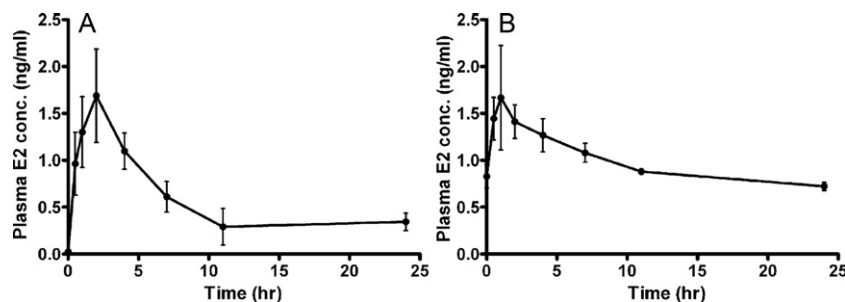

Fig. 1. Plasma concentration of E2 vs. time profile after a single injection (A) and 5-day injections (B) of EB. Rats were administered with EB (1 mg/kg subcutaneous injection), and blood samples were collected at various time points after EB administration. Concentration of E2 in plasma was determined by ELISA. The values are mean ± SEM (ng/ml).
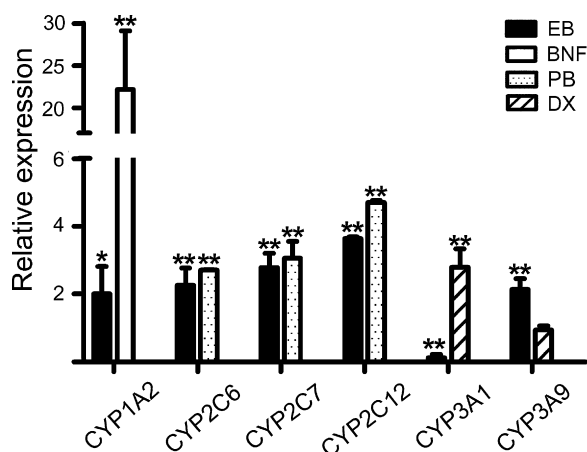
Fig. 2. Effects of E2 on mRNA levels of rat CYPs. Rats were administered with EB (1 mg/kg/day), BNF (40 mg/kg/day), PB (60 mg/kg/day), DX (60 mg/kg/day), or vehicle (corn oil) for 5 days ($n = 3$–4/group). mRNA levels were determined by qRT-PCR. Data shown are relative CYP expression as compared to the control group (corn oil). $^*p < 0.05$; $^{**}p < 0.01$ *vs.* control.

Table 2
Kinetic parameters for metabolite formation from each probe substrate. Results are expressed as mean $\pm$ S.D. ($n = 3$–4/group).

	K_m (μM)	V_{max} (pmol/min/mg protein)	CL_{int} (ml/min/mg protein)
Ethoxyresorufin *O*-dealkylation (CYP1A2)			
Control	0.48 ± 0.06	53 ± 2	109 ± 13
EB	0.45 ± 0.05	$80 \pm 2^*$	$183 \pm 37^*$
BNF	$1.35 \pm 0.33^*$	$631 \pm 4^{**}$	$463 \pm 54^{**}$
Diclofenac 4′-hydroxylation (CYP2C6/7)			
Control	18.6 ± 5.5	432 ± 40	23.9 ± 5.2
EB	20.0 ± 4.0	434 ± 27	24.1 ± 8.6
PB	28.0 ± 0.6	$902 \pm 197^*$	$32.3 \pm 7.7^*$
Midazolam 1′-hydroxylation (CYP3A)			
Control	8.0 ± 6.8	178 ± 42	16.1 ± 9.2
EB	10.7 ± 3.0	71 ± 10	7.1 ± 2.4
DX	8.6 ± 4.3	$643 \pm 72^{**}$	$85.2 \pm 20.9^*$
p-Nitrophenol hydroxylation (CYP2E1)			
Control	4.8 ± 1.5	1308 ± 94	296 ± 139
EB	7.8 ± 1.9	$649 \pm 38^*$	$89 \pm 24^*$
Bufuralol 1′-hydroxylation (CYP2D2)			
Control	5.6 ± 1.3	383 ± 30	97 ± 7
EB	4.0 ± 0.8	492 ± 23	120 ± 16

$^*p < 0.05$, $^{**}p < 0.01$ *vs.* control.

CYP2C isoforms by 2.2-, 2.7- and 4.0-fold for CYP2C6, CYP2C7, and CYP2C12, respectively. The induction in CYP2C expression by EB treatment was to a similar extent as the induction by PB (2.7-, 3.0- and 4.6-fold for CYP2C6, CYP2C7, and CYP2C12, respectively). EB treatment also increased CYP3A9 expression by 2.3-fold ($p = 0.007$) while downregulating CYP3A1 expression by 8.0-fold ($p < 0.001$). DX increased CYP3A1 expression (by 2.8-fold) but had no effect on CYP3A9 expression. EB treatment had insignificant effects on mRNA expression of CYP2B1, CYP2E1, and CYP2D2 (data not shown).

3.4. Immunoblot analysis

To determine whether E2-mediated mRNA changes in CYPs led to corresponding changes in the protein levels, immunoblot analysis was performed for CYP1A2, CYP3A1, and CYP2C. The results showed that EB treatment increased protein levels of CYP1A2 by 1.4-fold ($p = 0.022$), but to a much smaller extent than the increase observed with BNF (4.0-fold, $p < 0.001$) (Fig. 3A). EB treatment also increased CYP2C protein expression by 3.1-fold ($p < 0.001$), an increase comparable to that by PB (3.5-fold, $p < 0.001$) (Fig. 3B). On the other hand, EB treatment markedly decreased CYP3A1 expression (by 4.8-fold, $p < 0.001$) (Fig. 3C), consistent with the qRT-PCR results. The increased mRNA level of CYP3A9 by E2 (Fig. 2) was not confirmed by immunoblot analysis

due to a lack of commercially available antibodies that specifically detect rat CYP3A9.

3.5. Effects of E2 on CYP activities

To examine the effects of E2 on CYP activities, microsomal assays were performed using CYP-isoform specific probe substrates: 7-ethoxyresorufin (CYP1A2), diclofenac (CYP2C6/7), bufuralol (CYP2D2), p-nitrophenol (CYP2E1) and midazolam (CYP3A) [18]. EB treatment increased V_{max} and intrinsic clearance (CL_{int}) of 7-ethoxyresorufin *O*-dealkylation activity as compared to the vehicle treatment (by 1.5-fold and 1.7-fold, respectively) (Table 2); however, it did not affect K_m, suggesting that intrinsic function of CYP1A2 was likely not influenced by EB treatment. The CL_{int} and V_{max} of p-nitrophenol hydroxylation were decreased by EB treatment by 70% and 50%, respectively. EB treatment had insignificant effects on diclofenac 4′-hydroxylation, bufuralol 1′-hydroxylation, and midazolam 1′-hydroxylation activities.

3.6. Effects of E2 on modulators of drug metabolism

PXR, CAR, and AhR are transcriptional regulators that play key roles in modulating CYP expression [19]. On the other hand, POR is an obligate redox partner of CYP enzymes [20]. To explore
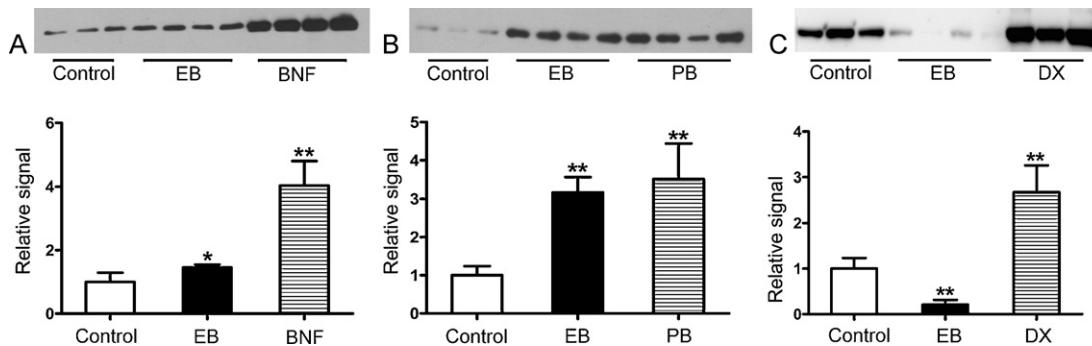

Fig. 3. Effects of E2 on protein levels of CYP1A2 (A), CYP2C (B), and CYP3A1 (C). Western blot analysis was performed using hepatic microsomes prepared from rats administered with EB (1 mg/kg/day), BNF (40 mg/kg/day), PB (60 mg/kg/day), DX (60 mg/kg/day), or vehicle (corn oil) for 5 days. Eight micrograms of microsome from each treatment group were resolved on SDS-PAGE gel (8%). Results from quantitative analysis of the blots were shown at the bottom panel, expressed as relative signals in comparison with the control group (corn oil). $^*p < 0.05$; $^{**}p < 0.01$ *vs.* control.
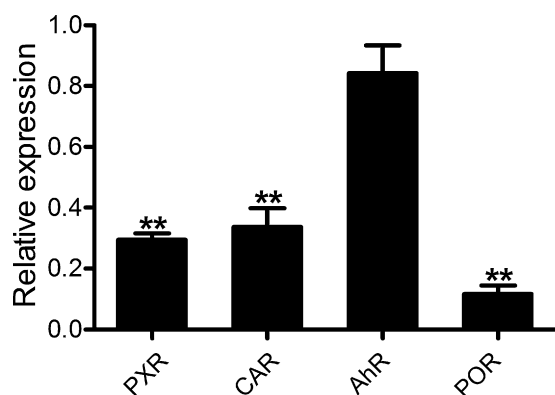
Fig. 4. Effects of E2 on mRNA levels of transcription factors and POR. Female rats were administered with EB (1 mg/kg/day) or vehicle (corn oil) for 5 days (n = 3–4/group). mRNA expression levels of PXR, CAR, AhR, and POR in the livers were determined by qRT-PCR. Data shown are relative CYP expression as compared to the control group (corn oil). $^{**}p < 0.01$ vs. control.

potential mechanisms underlying E2-mediated regulation of CYP expression and activity, we examined whether EB treatment affects expression of PXR, CAR, AhR and POR. The results from qRT-PCR (Fig. 4) showed that EB treatment downregulated mRNA levels of PXR (3.4-fold, $p < 0.001$), CAR (2.9-fold, $p < 0.001$), and POR (8.6-fold, $p < 0.001$) as compared to the vehicle treatment, whereas EB treatment had an insignificant effect on AhR expression. These results suggest that in rats, E2 may influence CYP expression and activities by downregulating transcription factors and the redox partner.

4. Discussion

Pregnancy influences hepatic drug metabolism in a CYP isoform-dependent manner in humans. However, the responsible factors or the underlying mechanisms remain largely unknown. The objective of this study is to determine whether increasing plasma concentration of E2 during pregnancy is potentially responsible for the altered drug metabolism using rats as a model.

In the present study, to achieve the plasma E2 concentrations attainable during human pregnancy, we administered 1 mg/kg of EB by subcutaneous injection into female rats. This led to C_{max} and C_{ave} of 1.84 ± 0.46 ng/ml and 0.99 ± 0.04 ng/ml, respectively (Fig. 1). The C_{ave} corresponds to the plasma E2 level in pregnant women during the first trimester [6], which is 40-fold higher than the baseline E2 concentrations in non-pregnant rats and women. Of note, high doses of estrogen are known to negatively influence hepatic functions in rats, e.g., increasing liver sizes and causing cholestasis [21], which may indirectly affect hepatic drug metabolism. At the dosage used in this study, EB treatment did not change liver weight or CYP concentration in hepatic microsomes in the rats (Table 1), suggesting an apparent lack of E2 effects on normal liver physiology.

Our results indicate that E2 modulates CYP expression in an isoform-specific manner: upregulation of CYP1A2, CYP2Cs, and CYP3A9 expression and downregulation of CYP3A1. The increased expression and activity of rat hepatic CYP1A2 by EB treatment are, in part, in agreement with a previous study where activity of rat intestinal CYP1A2 is enhanced by EB treatment [22]. The induction of CYP1A2 expression may be attributed to activation of an AhR-mediated regulatory mechanism which is involved in upregulation of hepatic and intestinal CYP1A expression [23]. Results from previous studies have suggested that E2 may activate AhR by an indirect mechanism, potentiating the induction of CYP1A expression by other AhR ligands (e.g., endogenous AhR ligands) [24,25]. Our results show that EB treatment increased mRNA expression of

another representative target gene of AhR, NADPH dehydrogenase quinone 1 (NQO1) [23] (by 2-fold; data not shown). These findings suggest that E2 upregulates CYP1A2 expression potentially by activating AhR-mediated regulatory pathways. On the other hand, the induction of CYP1A2 expression in rats by EB treatment did not correspond to the clinically reported reduction in metabolism of CYP1A2 substrates during human pregnancy. Considering the highly conserved regulatory mechanisms for CYP1A2 between humans and rats [8], our data suggest that pregnancy-specific factors other than E2 may be responsible for the decreased CYP1A2 activity during human pregnancy.

EB treatment significantly upregulated expression of CYP2C isoforms (CYP2C6, CYP2C7, and CYP2C12) (Figs. 2 and 3B), comparable to the induction observed from PB treatment. This result appears consistent with previous studies where administration of E2 enhanced expression of CYP2C7 and CYP2C12 in male rats [26] and increased mRNA expression and activity of CYP2C6 in rat hepatocytes [27,28]. Global upregulation of CYP2C shown in our study as well as others suggests that E2 may be responsible for the increased metabolism of CYP2C9 substrates in pregnant women [3–5]. Interestingly, despite significant induction in CYP2C expression by E2, diclofenac 4′-hydroxylation, a marker for CYP2C6 and CYP2C7 activities [29], did not increase in the EB-treated rats as compared to the vehicle-treated rats (Table 2). Downregulation of POR expression by E2 (Fig. 4) may provide a potential explanation. In both humans and rats, amount of POR in microsomes is the key determinant of CYP2C-mediated reaction rates such as warfarin 7-hydroxylation and 16α-steroid hydroxylation [30]. Whether pregnancy alters POR expression, and subsequently CYP2C9 activity in humans, remains unknown. Taken together, further study is warranted to determine the role of E2 in increased CYP2C9 activity during human pregnancy.

EB treatment had differential effects on expression of CYP3A1 and CYP3A9 (two major female hepatic CYP3A isoforms): marked downregulation of CYP3A1 and upregulation of CYP3A9 (Fig. 2). The opposing effects of E2 on CYP3A1 and CYP3A9 expression are potentially responsible for the minimal changes in CL_{int} of midazolam 1′-hydroxylation (Table 2) in the EB-treated group as compared to the control because the reaction is mediated by both CYP3A1 and CYP3A9 enzymes [31]. The downregulation of CYP3A1 expression in the EB-treated rats may be attributed to the decreased expression of PXR (Fig. 4), a key transcription factor in modulating CYP3A1 expression [32]. Considering that CYP3A1 is the ortholog of human CYP3A4, our finding suggests that increased metabolism of CYP3A4 substrates in pregnant women [3–5] is mediated by yet to be characterized pregnancy factors other than E2. Such candidates include progesterone. Plasma concentration of progesterone rises about 100-fold during human pregnancy, and progesterone has shown to be a PXR activator in in vitro systems [35,36]. Potential involvement of progesterone in altered drug metabolism during human pregnancy is currently under investigation. On the other hand, CYP3A9 expression is not governed by PXR ([33] and Fig. 2). Interestingly, consistent with our data indicating increased CYP3A9 expression upon EB treatment, CYP3A9 expression has shown to be female-specific and enhanced by ethinylestradiol in rats [31,34]. These directional changes in CYP3A9 expression are similar to those reported for CYP3A4 expression in pregnant women. The mechanism underlying estrogen-responsive expression of CYP3A9, although it currently remains unclear, may help us determine how E2 is potentially involved in the increased CYP3A4 expression during pregnancy.

Our results show that EB treatment has insignificant effects on expression and activity of CYP2D6, suggesting a lack of involvement of E2 in the increased metabolism of CYP2D6 substrates in pregnant women. EB treatment also shows minimal effects on CYP2E1 expression while decreasing V_{max} and CL_{int} of p-

nitrophenol hydroxylation as compared to the vehicle-treated group. Although p-nitrophenol hydroxylation is mainly mediated by CYP2E1 in humans, in rats CYP3A1 also mediates the reaction with an efficiency ~40% that of CYP2E1 [18]. Potentially, the decreased p-nitrophenol hydroxylation may reflect the reduced CYP3A1 expression in the EB-treated rats. Our immunoblot results also show that protein levels of CYP2E1 were not affected by EB treatment (data not shown).

Taken together, we have characterized the in vivo effects of E2 (at high E2 concentrations attainable during human pregnancy) on hepatic CYP expression and activities in rats. Our results show that E2 modulates CYP expression in an isoform-specific manner, leading to downregulation of CYP3A1 expression and upregulation of CYP1A2, CYP2C6, CYP2C7, CYP2C12, and CYP3A9. The directional changes mostly did not reflect those clinically reported during human pregnancy, suggesting that pregnancy-specific changes other than elevated E2 level are potentially responsible for the changes in major CYP activities during human pregnancy. Further studies appear warranted to identify such factors and to better understand the mechanisms underlying the discrepancies between our results and the clinically reported data. Information obtained from the current study should be of great value in better understanding the effects of estrogen on drug metabolism and guiding future approaches to investigating CYP regulation during pregnancy.

Acknowledgement

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