Estrogen-Induced Cholestasis Leads to Repressed CYP2D6 Expression in CYP2D6-Humanized Mice[§]

Xian Pan and Hyunyoung Jeong

Departments of Pharmacy Practice (H.J.) and Biopharmaceutical Sciences (X.P., H.J.), College of Pharmacy, University of Illinois, Chicago, Illinois

Received March 6, 2015; accepted May 5, 2015

ABSTRACT

Cholestasis activates bile acid receptor farnesoid X receptor (FXR) and subsequently enhances hepatic expression of small heterodimer partner (SHP). We previously demonstrated that SHP represses the transactivation of cytochrome P450 2D6 (CYP2D6) promoter by hepatocyte nuclear factor (HNF) 4 α . In this study, we investigated the effects of estrogen-induced cholestasis on CYP2D6 expression. Estrogen-induced cholestasis occurs in subjects receiving estrogen for contraception or hormone replacement, or in susceptible women during pregnancy. In CYP2D6-humanized transgenic (Tg-CYP2D6) mice, cholestasis triggered by administration of 17 α -ethinylestradiol (EE2) at a high dose led to 2- to 3-fold decreases in CYP2D6 expression. This was accompanied by increased hepatic

SHP expression and subsequent decreases in the recruitment of HNF4 α to CYP2D6 promoter. Interestingly, estrogen-induced cholestasis also led to increased recruitment of estrogen receptor (ER) α , but not that of FXR, to Shp promoter, suggesting a predominant role of ER α in transcriptional regulation of SHP in estrogen-induced cholestasis. EE2 at a low dose (that does not cause cholestasis) also increased SHP (by ~50%) and decreased CYP2D6 expression (by 1.5-fold) in Tg-CYP2D6 mice, the magnitude of differences being much smaller than that shown in EE2-induced cholestasis. Taken together, our data indicate that EE2-induced cholestasis increases SHP and represses CYP2D6 expression in Tg-CYP2D6 mice in part through ER α transactivation of Shp promoter.

Introduction

Cholestasis is a disease caused by impaired bile formation in liver cells (i.e., intrahepatic cholestasis) or obstruction of bile flow via bile ducts (i.e., extrahepatic cholestasis) (Trauner et al., 1999; Kullak-Ublick and Meier, 2000; Trauner and Boyer, 2003). Intrahepatic cholestasis can be caused by medications (e.g., cyclosporine A) or pregnancy, and extrahepatic cholestasis by bile duct blockade from gallstones or tumors. Regardless of the underlying causes, cholestasis results in hepatic and systemic accumulation of cytotoxic bile acids. This induces liver damage accompanied by pruritus and indigestion, ultimately leading to biliary fibrosis and cirrhosis (Trauner et al., 1998; Rodriguez-Garay, 2003). Treatment of cholestasis involves surgical removal of underlying causes, if possible, and drug therapy using ursodeoxycholic acid and immunosuppressive drugs (EASL, 2009). The management of symptoms accompanying cholestasis may also require the use of drug therapy (e.g., serotonin reuptake inhibitors) (Kremer et al., 2008).

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme and is responsible for eliminating approximately 20% of

marketed drugs such as opioid analgesics and antidepressants. Recently, we have identified small heterodimer partner (SHP) as a novel transcriptional regulator of CYP2D6 expression (Koh et al., 2014); SHP represses hepatocyte nuclear factor (HNF) 4 α transactivation of CYP2D6 promoter (Cairns et al., 1996; Koh et al., 2014). Also, in CYP2D6-humanized transgenic (Tg-CYP2D6) mice, SHP knockdown led to a significant increase in CYP2D6 expression (Koh et al., 2014). SHP is a representative target gene of the bile acid sensor, farnesoid X receptor (FXR). Upon binding to bile acids, FXR transactivates SHP promoter and upregulates SHP expression (Goodwin et al., 2000). SHP in turn represses the transcription of genes involved in bile acid synthesis such as CYP7A1 and CYP8B1 (Goodwin et al., 2000). Whether enhanced expression of SHP in cholestasis leads to altered CYP2D6 expression remains unknown.

Estrogen is the major component in oral contraceptives and hormone replacement therapy. Estrogen regulates growth and differentiation as well as multiple physiologic functions by activating its cognate receptor, estrogen receptor (ER) α and ER β . In the liver, expression of ER β is localized to the cholangiocytes (Alvaro et al., 2002), and ER α is the major isoform expressed in the parenchymal cells (Kuiper et al., 1997). Of note, estrogens can cause intrahepatic cholestasis in premenopausal women receiving oral contraceptives, in postmenopausal women on hormone replacement therapy (Schreiber and Simon, 1983), or in men who receive estrogen

This work was supported by National Institutes of Health National Institute of Child Health and Human Development [Grant R01-HD065532].
dx.doi.org/10.1124/mol.115.098822.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ChIP, chromatin immunoprecipitation; EE2, 17 α -ethinylestradiol; ER, estrogen receptor; FXR, farnesoid X receptor; GW4064, 3-[2-[2-chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid; HNF, hepatocyte nuclear factor; Pol II, polymerase II; qRT-PCR, quantitative real-time polymerase chain reaction; SHP, small heterodimer partner.

therapy for the treatment of prostate cancer (Kontturi and Sotaniemi, 1969). Estrogen-induced cholestasis can also occur during pregnancy; intrahepatic cholestasis of pregnancy is the most common liver disease in pregnant women (Reyes, 1997; Riely and Bacq, 2004). An experimental intrahepatic cholestasis model established by 17 α -ethinylestradiol (EE2) administration in rodents has been commonly used to study the pathogenesis of estrogen-induced intrahepatic cholestasis (Bossard et al., 1993; Trauner et al., 1999; Rodriguez-Garay, 2003; Yamamoto et al., 2006).

In this study, we examined the effects of estrogen-induced intrahepatic cholestasis on CYP2D6-mediated drug metabolism. Our results indicate that EE2-induced cholestasis increases SHP and represses CYP2D6 expression in Tg-CYP2D6 mice. Studies of the underlying mechanisms revealed a role of ER α in CYP2D6 regulation in estrogen-induced cholestasis.

Materials and Methods

Animals. CYP2D6-humanized transgenic (Tg-CYP2D6) mice were previously described (Corchero et al., 2001). Tg-CYP2D6 mouse harbors the wild-type CYP2D6 gene with its 2.5-kb upstream regulatory region in the mouse genome (Corchero et al., 2001). Adult male mice (8 weeks of age and weighing 20–25 g) were used for the experiments. For estrogen-induced intrahepatic cholestasis, mice received subcutaneous injections of EE2 (10 mg/kg) or vehicle (olive oil) daily for 5 days (Yamamoto et al., 2006). For the activation of ER α alone (without cholestasis), mice received intraperitoneal injections of EE2 (5 μ g/kg) or vehicle daily for 5 days (Yoshikawa et al., 2012). Mice were sacrificed on the sixth day (i.e., 24 hours after the last dose), and blood and liver tissues were collected. All procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and Use Committee at the University of Illinois (Chicago, IL).

Chemicals and Reagents. Debrisoquine, ($\pm$)-4-hydroxydebrisoquin, and paraxanthine were purchased from Biomol (Plymouth Meeting, PA). EE2 was purchased from Sigma-Aldrich (St. Louis, MO).

Western Blot. Western blot was performed, as described previously (Koh et al., 2014), using SHP antibody from Santa Cruz Biotechnology (Dallas, TX).

Determination of CYP2D6 Activity. Liver S9 fractions were prepared, as described previously (Koh et al., 2014). S9 fractions were incubated with debrisoquine (a CYP2D6 probe substrate; 200 μ M) based on the finding that mouse endogenous CYP2Ds have minor roles in debrisoquine hydroxylation (Koh et al., 2014). The concentration of 4-hydroxydebrisoquine was determined by liquid chromatography–tandem mass spectrometry using paraxanthine as the internal standard (Koh et al., 2014).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated from mouse liver tissues using TRIzol (Life Technologies, Carlsbad, CA) and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Using the cDNA as template, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using StepOnePlus Real-Time PCR System and primers listed in Supplemental Table 1. The results are expressed as fold changes under treatment using the gene expression levels normalized to those of glyceraldehyde-3-phosphate dehydrogenase (2^{– $\Delta\Delta C_t$} method).

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation (ChIP) assays were performed in mouse liver tissues, as described previously (Koh et al., 2014). Briefly, livers were finely minced and incubated in phosphate-buffered saline containing 1% formaldehyde at room temperature for 15 minutes, and glycine was added to stop the cross-linking reaction. Cell pellets were resuspended

in hypotonic buffer (15 mM HEPES, pH 7.9, 60 mM KCl, 2 mM EDTA, 0.5% bovine serum albumin, 0.15 mM spermine, 0.5 mM spermidine, and 0.32 M sucrose) and lysed by homogenization. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% SDS). The samples were sonicated to shear DNA to the length ranging from 100 to 500 bp by using Misonix S-4000 sonicator (Farmingdale, NY) with 80% amplitude for 12 minutes. After centrifugation, the chromatin sample was immunoprecipitated with 2 μ g antibody (ER α ; FXR; HNF4 α ; RNA polymerase II; SHP; Santa Cruz) or IgG (normal goat IgG; normal rabbit IgG; Santa Cruz) at 4°C for overnight. The immune complex was collected, the magnetic beads were extensively washed, and the bound chromatin was eluted. Genomic DNA was purified by PCR Clean-up kit (Promega, Madison, WI) and used as a template for quantitative PCR. Primer sequences are listed in Supplemental Table 2.

Alkaline Phosphatase and Alanine Aminotransferase Measurement. Plasma alkaline phosphatase (ALP) and alanine aminotransferase (ALT) levels were measured by chemistry analyzer (Olympus AU 680; Center Valley, PA) following the manufacturer's protocol.

Statistical Analysis. Values were reported as mean $\pm$ S.E.M. Statistical differences were determined by using Student's *t* test.

Results

EE2-Induced Cholestasis Leads to Decreased HNF4 α Transactivation of CYP2D6 Promoter. Previous studies have shown that administration of high-dose estrogen triggers intrahepatic cholestasis (Bossard et al., 1993; Trauner et al., 1999; Rodriguez-Garay, 2003; Yamamoto et al., 2006). To investigate the effects of EE2-induced cholestasis on CYP2D6 expression and activity in vivo, Tg-CYP2D6 mice were treated with EE2 or vehicle control. As expected from EE2-induced cholestasis, the plasma levels of ALP [a marker for cholestasis (Krones et al., 2014)] and ALT (a marker for liver injury) were significantly increased in EE2-treated mice (Fig. 1).

To examine potential effects of EE2-induced cholestasis on hepatic CYP2D6 expression, CYP2D6 mRNA and protein levels were determined in the mice by using qRT-PCR and Western blot. mRNA expression levels of Cyp7a1 and Cyp8b1 are known to be repressed in cholestasis (Jahan and Chiang, 2005; Yamamoto et al., 2006) and were examined as controls. The results showed that EE2-induced cholestasis led to significant decreases in Cyp7a1 and Cyp8b1 expression, as expected (Fig. 2A). EE2 also decreased mRNA level of CYP2D6 2-fold (Fig. 2A). Results from S9 phenotyping (by using debrisoquine as a probe drug for CYP2D6) revealed a ~3-fold decrease in CYP2D6 activity (Fig. 2B). The decreased

Fig. 1. EE2 induces cholestasis in Tg-CYP2D6 mice. Tg-CYP2D6 mice were injected subcutaneously with EE2 (10 mg/kg) or vehicle (olive oil) daily for 5 days ($n = 5$ mice/group). ALP and ALT activities were measured in mouse plasma. ** $P < 0.01$. conc., concentration.

CYP2D6 expression was accompanied by increased expression of SHP at mRNA and protein levels (Fig. 2, C and D). EE2 did not alter mRNA expression levels of *Hnf4α* (data not shown).

To determine whether the altered SHP expression upon EE2 administration leads to changes in *HNF4α* transactivation of CYP2D6 promoter, ChIP assays were performed using mouse liver tissues. Livers from vehicle- or EE2-treated mice were collected and subjected to ChIP assays using antibodies against HNF4α, SHP, or RNA polymerase II (Pol II, a marker of transcription initiation). Based on previous reports that HNF4α binds to -53/-41 of CYP2D6 (Cairns et al., 1996) and SHP suppresses HNF4α transactivation of CYP2D6 promoter (Koh et al., 2014), recruitment of the transcription factors to the proximal promoter region of CYP2D6 was examined. The results showed a significant increase in the recruitment of SHP to CYP2D6 promoter, accompanied by decreased recruitment of both HNF4α and Pol II to CYP2D6 promoter (Fig. 3A). A similar pattern of changes in SHP, HNF4α, and Pol II recruitment was observed for *Cyp7a1* and *Cyp8b1*, the known target genes of SHP (Inoue et al., 2006) (Fig. 3B; Supplemental Fig. 1 for *Cyp7a1*). Such changes in the transcriptional factor recruitment were absent when a PCR primer set detecting a downstream region (+3913/+4112) of CYP2D6 gene was used as a negative control (Supplemental Fig. 2). Also, the decrease in HNF4α recruitment to the promoter region was not observed for other target genes of HNF4α (i.e., *ApoC2* and *Hes6* (Nikolaïdou-Neokosmidou et al., 2006; Martinez-Jimenez et al., 2010) (Supplemental Fig. 3), suggesting that the repressive action of SHP on HNF4α transactivation may be target gene-specific. Together, these results suggest that EE2-induced cholestasis leads to decreased HNF4α transactivation of CYP2D6 promoter, potentially through enhanced SHP expression.

ERα Plays a Predominant Role in SHP Upregulation in EE2-Induced Cholestasis. FXR transactivates SHP promoter by binding to -294/-281 of *SHP* in humans (Goodwin et al., 2000). Interestingly, results from a previous study

indicate that ERα also transactivates SHP promoter by binding to an overlapping promoter region (Lai et al., 2003). As the SHP promoter sequence is highly conserved between humans and mice (Lai et al., 2003), we examined the comparative roles of ERα and FXR in the regulation of SHP expression in mice with EE2-induced cholestasis. To this end, recruitment of ERα and FXR to *Shp* promoter was compared between the livers of EE2-versus vehicle-treated mice by using ChIP assay. Previously known target genes of ERα and FXR, *Stat5a* and *Ostβ*, respectively (Zollner et al., 2006; Hewitt et al., 2010), were used as positive controls. The results showed that ERα recruitment to *Shp* promoter was increased significantly upon EE2 treatment (Fig. 4A). Such increase in ERα recruitment was not observed when a PCR primer set detecting a downstream region (+4018/+4209) of *Shp* was used as a negative control (Supplemental Fig. 4). EE2 treatment also led to a significant increase in ERα recruitment to *Stat5a* promoter (Fig. 4B) and increased mRNA expression of *Stat5a* (Fig. 4C). In contrast to the increased ERα recruitment to *Shp* promoter, the recruitment of FXR to the promoter region did not differ between the EE2-treated and the control groups (Fig. 4D). In contrast, FXR recruitment to *Ostβ* promoter and mRNA expression of *Ostβ* were significantly increased (Fig. 4, E and F), indicating a robust FXR activation by EE2-induced cholestasis. Together, these results suggest that when both ERα and FXR are activated in EE2-induced cholestasis, ERα may play a predominant role in the regulation of SHP expression.

ERα Activation Leads to CYP2D6 Repression. To determine whether ERα activation alone (without accompanying cholestasis) can alter CYP2D6 expression via upregulating SHP, mice were administered with EE2 at a low dose (5 μg/kg) for 5 days, and CYP2D6 expression was examined. Plasma ALP levels were slightly higher in mice treated with low-dose EE2 (Fig. 5A), but within the normal range (i.e., 113 ± 49 U/l) for 8-week-old mice (Krones et al., 2014). ALT levels did not increase in EE2-treated mice (Fig. 5A). Also, the dramatic decreases in *Cyp7a1* and *Cyp8b1* expression or increased *Ostβ* expression (i.e., hallmarks of cholestasis and

Fig. 2. EE2-induced cholestasis represses CYP2D6 expression in Tg-CYP2D6 mice. Tg-CYP2D6 mice were injected subcutaneously with EE2 (10 mg/kg) or vehicle (olive oil) daily for 5 days ($n = 5$ mice/group). (A) Hepatic CYP2D6, *Cyp7a1*, *Cyp8b1*, and (C) *Shp* mRNA expression were determined by using qRT-PCR. (B) S9 fractions were prepared from the liver tissues, and CYP2D6 activity in S9 fractions was measured using debrisoquine (200 μM) as a probe drug. Data shown are metabolite production rates in pmol/min per milligram protein. (D) SHP protein expression level was determined by Western blot. The image of Western blot (right) and the quantified band intensities (left; SHP level normalized by β-actin) are shown. Values are presented as mean ± S.E.M. ** $P < 0.01$, versus vehicle treatment.

Fig. 3. EE2-induced cholestasis represses HNF4 α transactivation of CYP2D6 promoter. Tg-CYP2D6 mice were injected subcutaneously with EE2 (10 mg/kg) or vehicle (olive oil) daily for 5 days ($n = 5$ mice/group). Recruitment of HNF4 α , SHP, and RNA Pol II to (A) CYP2D6 promoter and (B) Cyp8b1 promoter was analyzed by ChIP assay using the mouse liver tissues. Values are presented as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$, versus vehicle treatment.

FXR activation) were not observed in these mice (Fig. 5B). Together, the data indicate a lack of cholestasis in mice treated with low-dose EE2.

The treatment with EE2 at the low dose led to increased Shp and decreased CYP2D6 mRNA levels (Fig. 6A). The mRNA expression of Stat5a (an ER α target gene) was also increased in EE2-treated mice, as expected (Fig. 6A). In accordance with increased Shp mRNA levels, SHP protein level was increased upon EE2 treatment, but to an extent smaller than that by EE2 at the high dose (1.5-fold versus 2.5-fold for low- and high-dose EE2-treated groups, respectively) (Figs. 2D and 6B). CYP2D6 activity was decreased in the EE2-treated group only by ~20% as compared with the vehicle control group (Fig. 6C). Consistent with the changes in SHP and CYP2D6 expression, ChIP results showed increased recruitment of SHP to CYP2D6 promoter, and decreased recruitment of HNF4 α and Pol II to CYP2D6 promoter (Supplemental Fig. 5A). Such changes in transcription factor recruitment were not observed for Cyp8b1 (Supplemental Fig. 5B). ER α recruitment to Shp promoter was increased upon low-dose EE2 treatment, indicating enhanced ER α transactivation of Shp promoter (Fig. 6D). A similar pattern was observed for ER α recruitment to the promoter of Stat5a (Fig. 6D). Together, these results suggest that ER α activation leads to CYP2D6 repression through enhanced SHP expression, although the magnitude of CYP2D6 repression was smaller than that in EE2-induced cholestasis.

Discussion

In previous studies, we have shown that SHP is a transcriptional repressor of CYP2D6 expression (Koh et al., 2014) and that FXR activation (by using an FXR agonist GW4064

[3-[2-[2-chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid] leads to decreased CYP2D6 expression via enhancing SHP expression (Pan et al., 2015). In this study, we examined whether cholestasis (a condition known to enhance FXR activity) also alters CYP2D6 expression by upregulating SHP expression. To this end, we employed the estrogen-induced cholestasis model that is widely used to examine the mechanisms involved in intrahepatic cholestasis (Bossard et al., 1993; Trauner et al., 1999; Rodriguez-Garay, 2003; Yamamoto et al., 2006).

In Tg-CYP2D6 mice, EE2 at a dose of 10 mg/kg per day led to increased plasma concentrations of liver enzymes and decreased expression of Cyp7a1 and Cyp8b1, a hallmark of cholestasis. In the EE2-treated mice, CYP2D6 expression and activity were significantly repressed, suggesting that CYP2D6-mediated drug metabolism may be decreased in cholestasis. Cholestasis is caused by factors that impair bile formation or bile flow. For example, in 0.4–1% of pregnant women in North America and up to 15–20% of pregnant women in some areas of Europe, intrahepatic cholestasis occurs most likely due to high plasma concentrations of estrogen during pregnancy (Reyes et al., 1978; Reyes, 1997; Riely and Bacq, 2004; Puls and Beuers, 2007). Based on our results, it appears possible that CYP2D6-mediated drug metabolism is lower in pregnant women with increased hepatic bile acid levels (than in pregnant women with normal bile acid levels), and this may lead to greater interindividual variability in CYP2D6 activity in pregnant women as compared with the nonpregnant subjects. Indeed, it was previously shown that CYP2D6 activity (i.e., clearance of metoprolol) exhibited greater variability during pregnancy than after delivery (Hogstedt et al., 1985). Whether altered hepatic bile acid levels are indeed responsible for the

Fig. 4. ER α plays a major role in SHP upregulation in EE2-induced cholestasis. Tg-CYP2D6 mice were injected subcutaneously with EE2 (10 mg/kg) or vehicle (olive oil) daily for 5 days ($n = 5$ mice/group). Recruitment of ER α to (A) Shp promoter and (B) Stat5a promoter; recruitment of FXR to (D) Shp promoter and (E) Ost β promoter was analyzed by ChIP assay using the mouse liver tissues. (C) Stat5a and (F) Ost β mRNA expression levels in the liver tissues were determined by using qRT-PCR. Values are presented as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; n.s., not statistically significant, versus vehicle treatment.

increased interindividual variability in CYP2D6 activity during pregnancy by controlling hepatic CYP2D6 expression remains to be determined.

CYP2D6 repression in EE2-induced cholestasis was accompanied by increased SHP expression and subsequent changes in transcription factor recruitment to CYP2D6 promoter, that is, increased SHP and decreased HNF4 α recruitment to the promoter. These results suggest increased SHP expression may be responsible for CYP2D6 repression in cholestasis. SHP promoter is transactivated by FXR and ER α (Goodwin et al., 2000; Lai et al., 2003), both of which are most likely activated in EE2-induced cholestasis. Of note, FXR and ER α bind to overlapping promoter regions (Lai et al., 2003), suggesting that the roles of ER α and FXR in SHP upregulation could be mutually exclusive in EE2-induced cholestasis.

To determine which one of two transcription factors (ER α or FXR) plays a major role in SHP induction in EE2-induced cholestasis, the extent of ER α and FXR recruitment to Shp promoter was examined. Our results showed that ER α recruitment (but not that of FXR) to Shp promoter was increased in mice administered with EE2 at a high dose. The results suggest that ER α transactivation of Shp promoter is potentially responsible for CYP2D6 repression in EE2-induced cholestasis.

Based on the results indicating an important role of ER α in the regulation of SHP expression, we further examined the effects of ER α activation alone (without cholestasis and subsequent FXR activation) on CYP2D6 expression. To this end, Tg-CYP2D6 mice were administered with EE2 at a low dose that does not cause cholestasis or liver damage. At the

Fig. 5. Low-dose EE2 does not induce cholestasis in Tg-CYP2D6 mice. Tg-CYP2D6 mice were injected intraperitoneally with EE2 (5 μ g/kg) or vehicle control (olive oil) daily for 5 days ($n = 4$ mice/group). (A) ALP and ALT activities in mouse plasma were measured. (B) Hepatic mRNA levels of Cyp7a1, Cyp8b1, and Ost β were determined by using qRT-PCR. Values are presented as mean $\pm$ S.E.M. ** $P < 0.01$; n.s., not statistically significant. conc., concentration.

Fig. 6. Low-dose EE2 represses CYP2D6 expression in Tg-CYP2D6 mice. Tg-CYP2D6 mice were injected intraperitoneally with EE2 (5 μ g/kg) or vehicle control (olive oil) daily for 5 days ($n = 4$ mice/group). (A) Hepatic mRNA expression levels of CYP2D6, Shp, and Stat5a were measured by using qRT-PCR. (B) SHP protein expression level was determined by Western blot. The image of Western blot (right) and the quantified band intensities (left; SHP level normalized by β -actin) are shown. (C) CYP2D6 activity in hepatic S9 fractions was measured using debrisoquine (200 μ M) as a probe drug. Data shown are metabolite production rates (in pmol/min per milligram protein). (D) Recruitment of ER α to Shp promoter and Stat5a promoter was analyzed by ChIP assay using the mouse liver tissues. Values are presented as mean $\pm$ S.E.M. * $P < 0.05$, versus vehicle treatment.

low dose, EE2 increased SHP and decreased CYP2D6 expression; however, the extent of CYP2D6 repression in these mice was much smaller than that in mice with EE2-induced cholestasis (2.6- versus 1.2-fold decrease in CYP2D6 activity for high- and low-dose EE2 groups, respectively). The greater magnitude of CYP2D6 repression in cholestatic mice could be in part due to bile acid-induced stabilization of SHP protein; bile acids increase stability of hepatic SHP protein by inhibiting its proteasomal degradation in an extracellular signal-regulated kinase-dependent manner (Miao et al., 2009). Indeed, the extent of increases in SHP protein level was greater in mice administered with high-dose EE2 than in those with low-dose EE2. Cholestasis triggers hepatic inflammation that is known to downregulate CYP2D6 expression (Hara and Adachi, 2002), and this may have also contributed to CYP2D6 repression in EE2-induced cholestasis. Our qRT-PCR results showed that expression levels of inflammatory cytokines (i.e., TNF- α , IL-1 β , and IL-6) were significantly higher in the livers of mice treated with high-dose EE2 as compared with the vehicle-treated mice, and such increase was not observed in mice treated with low-dose EE2 (data not shown). Consistent with the relatively small changes in CYP2D6 activity upon administration of EE2 at the low dose, clinical data suggest minor roles of estrogen (if any) in the regulation of CYP2D6 expression/activity in humans. For example, the use of oral contraceptive steroids had no influence on the urinary metabolic ratios of CYP2D6 substrates (sparteine or dextromethorphan) (Bock et al., 1994; Tamminga et al., 1999). Also, sex did not affect the extent of CYP2D6-mediated sparteine oxidation in 194 subjects (Bock et al., 1994) nor the CYP2D6 expression levels in 300 human

liver tissues (Zanger et al., 2005). Overall, these results suggest that CYP2D6 repression in estrogen-induced cholestasis is triggered in part by a combination of 1) ER α activation and

Fig. 7. Working model for CYP2D6 regulation in EE2-induced cholestasis. The working model illustrates the overlapping estrogen response element (ERE) and FXR response element (FXRE) in SHP promoter. In EE2-induced cholestasis, ER α recruitment to SHP promoter increases, leading to higher SHP expression. Also, cholestasis stabilizes SHP protein, further enhancing SHP expression. SHP in turn suppresses HNF4 α transactivation of CYP2D6 promoter, leading to CYP2D6 repression in EE2-induced cholestasis.

2) biologic changes accompanying cholestasis (e.g., SHP protein stabilization and inflammation) (Fig. 7).

We showed that EE2-induced cholestasis increases SHP and represses CYP2D6 expression in Tg-CYP2D6 mice. Importantly, this study presents estrogen and bile acids as potential contributors to the differential regulation of CYP2D6 expression. This potentially provides a mechanistic basis to identify the sources of interindividual variability in CYP2D6-mediated drug metabolism.

Acknowledgments

The authors thank Sung-joon Cho for technical assistance with animal experiments.

Authorship Contributions

Participated in research design: Pan, Jeong.

Conducted experiments: Pan.

Performed data analysis: Pan, Jeong.

Wrote or contributed to the writing of the manuscript: Pan, Jeong.

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Address correspondence to: Dr. Hyunkyung Jeong, Department of Pharmacy Practice (MC 886), College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612. E-mail: yjeong@uic.edu