

Cholic Acid Feeding Leads to Increased CYP2D6 Expression in CYP2D6-Humanized Mice[§]

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ABSTRACT

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme, but the factors governing transcriptional regulation of its expression remain poorly understood. Based on previous reports of small heterodimer partner (SHP) playing an important role as a transcriptional repressor of CYP2D6 expression, here we investigated how a known upstream regulator of SHP expression, namely cholestasis triggered by cholic acid (CA) feeding in mice, can lead to altered CYP2D6 expression. To this end, CYP2D6-humanized (Tg-CYP2D6) mice were fed with a CA-supplemented or control diet for 14 days, and hepatic expression of multiple genes was examined. Unexpectedly, CA feeding led to insignificant changes in SHP mRNA

but also to significant (2.8-fold) decreases in SHP protein levels. In silico analysis of the SHP gene regulatory region revealed a putative binding site for a microRNA, miR-142-3p. Results from luciferase reporter assays suggest that miR-142-3p targets the SHP gene. Hepatic expression of miR-142-3p was significantly increased in CA-fed mice (~5-fold), suggesting a potential role of miR-142-3p in the regulation of SHP expression in cholestasis. The decreased SHP protein levels were accompanied by increased expression and activity of CYP2D6 in the liver of CA-fed mice. These results suggest potential roles of differential hepatic levels of bile acids in the transcriptional regulation of CYP2D6 expression.

Introduction

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme, responsible for eliminating ~20% of marketed drugs such as opioids (e.g., codeine) and antidepressants (e.g., citalopram). Additionally, CYP2D6 expressed in the brain has been implicated in the pathogenesis of Parkinson disease and the synthesis of neurotransmitters (Yu et al., 2003; Lu et al., 2014). Despite its diverse physiologic functions, the regulation of CYP2D6 expression remains poorly understood. Cairns et al. (1996) reported that the CYP2D6 promoter is transactivated by hepatocyte nuclear factor 4 α (HNF4 α), the most abundant DNA-binding protein in the liver and a master regulator of hepatic functions (Chandra et al., 2013). Recently, we have identified the transcriptional repressor small heterodimer partner (SHP) as a novel regulator of CYP2D6 expression; SHP represses HNF4 α transactivation of the CYP2D6 promoter, and SHP knockdown (using small interfering RNA) led to increased CYP2D6 expression in CYP2D6-humanized transgenic (Tg-CYP2D6) mice (Koh et al., 2014). Furthermore, a chemical inducer of SHP expression (GW4064) repressed CYP2D6 expression in Tg-CYP2D6 mice as well as in human hepatocytes (Pan et al., 2015).

SHP is a representative target gene of bile acid sensor farnesoid X receptor (FXR). Along with FXR, SHP serves as a key player in the maintenance of bile acid homeostasis, especially in patients with

cholestasis. Cholestasis is a chronic liver disease that is characterized by an interruption of bile flow, enlarged bile acid pool size, and altered bile acid composition. When hepatic bile acid levels rise, bile acids bind to FXR, and the ligand-activated FXR transactivates the SHP promoter via a FXR response element in the proximal promoter region (Goodwin et al., 2000; Chanda et al., 2008). SHP in turn represses the transcription of genes involved in bile acid synthesis (e.g., Cyp7a1 and Cyp8b1) by interfering with the actions of transcriptional activators of the genes (Goodwin et al., 2000; Zhang and Chiang, 2001).

The goal of this study is to define the role of SHP and bile acids in the regulation of CYP2D6 expression by examining the effects of cholestasis on hepatic CYP2D6 expression. As a cholestasis model, Tg-CYP2D6 mice were fed a cholic acid (CA)-supplemented diet for over 1 week (Barone et al., 1996; Fickert et al., 2001; Rost et al., 2003; Teng and Piquette-Miller, 2007). The treatment was known to increase bile acid pool size by 2-fold and to replace ~90% of bile acids with CA (Fickert et al., 2001), recapitulating the features of cholestatic conditions in humans (van Berge Henegouwen et al., 1976). Our results revealed unexpected directional changes in SHP expression levels and differential regulation of CYP2D6 and Cyp7a1/Cyp8b1 expression in Tg-CYP2D6 mice upon CA feeding.

Materials and Methods

Animals. Tg-CYP2D6 mice were previously described (Corchero et al., 2001). Adult male mice (8 weeks of age and weighing 20–25 g) were used for the experiments. Mice were fed with normal chow or 1% (w/w) CA-supplemented diet (Teklad Laboratory Animal Diets; Envigo, Indianapolis, IN). After feeding for 14 days, mice were sacrificed, and blood and liver tissue samples were

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ABBREVIATIONS: ALP, alkaline phosphatase; ALT, alanine aminotransferase; CA, cholic acid; ChIP, chromatin immunoprecipitation; CPR, cytochrome P450 reductase; Cyb5, cytochrome-*b*₅; CYP2D6, cytochrome P450 2D6; EE2, ethinylestradiol; FXR, farnesoid X receptor; HNF4 α , hepatocyte nuclear factor 4 α ; miRNA, microRNA; Pol II, RNA polymerase II; qRT-PCR, quantitative real-time polymerase chain reaction; SHP, small heterodimer partner; UTR, untranslated region.

collected. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

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

Immunoprecipitation and Western Blot. SHP protein was detected by using immunoprecipitation followed by Western blotting. Liver tissues were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP40, 1 mM EDTA, complete protease inhibitor cocktail; Roche, Mannheim, Germany). For immunoprecipitation, equal amounts of lysates were incubated with the indicated antibody overnight at 4°C and then incubated with Dynabeads (Invitrogen, Carlsbad, CA) for 2 hours. After washing with phosphate-buffered saline, precipitated proteins were extracted from dynabeads by boiling in 1 \times sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue). Western blotting was performed as described previously (Koh et al., 2014). CYP2D6 (Corning, Corning, NY) and SHP (Santa Cruz Biotechnology, Dallas, TX) protein expression levels were determined by using the respective antibodies. The results of validating SHP detection by using two SHP antibodies (i.e., H160 and Q14) are shown in Supplemental Fig. 1.

Cell Culture. HEK293T cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), 10 mM HEPES, 100 μ M nonessential amino acids, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies, Carlsbad, CA).

Plasmids. To construct a reporter vector for microRNA (miRNA) binding to the 3'-untranslated region (UTR) of SHP, the putative miRNA-142-3p (miR-142-3p) binding region of mouse SHP (110–129) was cloned into the pMIR-REPORT luciferase vector (Life Technologies, Carlsbad, CA) by using 5'-phosphorylated primers listed in Supplemental Table 1. Additionally, the pMIR-REPORT vector harboring the complementary sequence of miR-142-3p was constructed by using a primer set (Supplemental Table 1). miR-142-3p mimic and the control (i.e., negative control 1) were purchased from ThermoFisher Scientific (Waltham, MA).

Luciferase Reporter Assay. HEK293T cells were seeded in 24-well plates at a density of 1×10^5 cells/ml. On the next day, the cells were transfected with 250 ng of luciferase construct, 30 nM miR-142-3p mimic or null control mimic, and 10 ng of *Renilla* vector (Promega, Madison, WI) using Fugene HD transfection reagent (Promega) according to the manufacturer protocol. The transfected cells were grown for 48 hours and were harvested for the determination of luciferase activity using a Dual-Luciferase Reporter Assay Kit (Promega). At least two independent experiments were performed in triplicate.

Measurement of CYP2D6 Activity. Hepatic S9 fractions were prepared as described previously (Felmlee et al., 2008; Koh et al., 2014). S9 fractions were incubated with debrisoquine (a CYP2D6 probe substrate; 200 μ M) based on the report that endogenous mouse CYP2D6s play minor roles in debrisoquine hydroxylation (Koh et al., 2014). The concentration of 4-hydroxydebrisoquine was determined by liquid chromatography-tandem mass spectrometry (1200 HPLC; Agilent; interfaced with Qtrap 3200; Applied Biosystems) using an electrospray ion source. Multiple reaction monitoring data acquisition was employed: m/z 192.3/132.2 for 4-hydroxydebrisoquine and 181.1/124.1 for the internal standard paraxanthine (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) and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Using the cDNA as template, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus Real-Time PCR System (ThermoFisher Scientific) and primers listed in Supplemental Table 1. The results were expressed as fold changes under treatment using the gene expression levels normalized to those of GAPDH ($2^{-\Delta\Delta Ct}$ method). For analysis of mature miR-142-3p, total RNA was converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The specific primers and probes used for miR-142-3p and snoRNA202 were listed in Supplemental Table 1. The results are expressed as fold changes under treatment using the gene expression levels normalized to those of snoRNA202 ($2^{-\Delta\Delta Ct}$ method).

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation (ChIP) assays were performed as previously described, with minor modifications (Koh et al., 2014). Briefly, livers were finely minced and incubated in PBS

containing 1% formaldehyde at room temperature for 15 minutes, and glycine was added to stop the crosslinking 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, 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 base pairs (bp). After centrifugation, the chromatin sample was immunoprecipitated with 2 μ g of antibody [HNF4 α , sc-6556x; RNA polymerase II (Pol II), sc-899x; SHP, sc-30169; Santa Cruz Biotechnology] or IgG (normal goat IgG, sc-2028; normal rabbit IgG, sc-2027; Santa Cruz Biotechnology) at 4°C 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) and was used as a template for PCR. Primer sequences are listed in Supplemental Table 1.

Alkaline Phosphatase and Alanine Aminotransferase Activities. Plasma alkaline phosphatase (ALP) and alanine aminotransferase (ALT) levels were measured by chemistry analyzer (AU 680; Olympus, Center Valley, PA).

Cytochrome P450 Reductase Activity. Cytochrome P450 reductase (CPR) activity in hepatic S9 fractions was measured by using the Cytochrome c Reductase (NADPH) Assay Kit (catalog #CY0100; Sigma-Aldrich) following the manufacturer protocol.

Cytochrome-*b*₅ Concentration. The contents of cytochrome-*b*₅ (Cyb5) in hepatic S9 fractions were determined by measuring the differential absorbance between NADH-reduced and oxidized S9 fractions as previously described (Venkatakrisnan et al., 2000; Gan et al., 2009).

Statistical Analysis. Values were reported as the mean \pm S.D. Comparison between the control and CA group was made by using Student's *t* test.

Results

CA Feeding Decreases SHP Expression Via miR-142-3p in Mice.

To determine the effects of cholestasis on hepatic gene regulation, CA or normal chow (control) was fed to mice for 14 days to establish cholestatic conditions. Plasma levels of ALP (a marker for cholestasis (Krones et al., 2015) and ALT (a marker for liver injury) were significantly increased in CA-fed mice (Fig. 1), as previously reported (Barone et al., 1996; Fickert et al., 2001; Rost et al., 2003; Teng and Piquette-Miller, 2007). CA feeding had insignificant effects on hepatic expression of *Hnf4 α* (data not shown). Interestingly, the expression levels of hepatic *Shp* mRNA did not differ between the CA-fed and control groups (Fig. 2A), contrary to the expectation that CA feeding and subsequent cholestasis would lead to increased *Shp* expression. On the other hand, the Western blot results showed that SHP protein was markedly decreased in CA-fed mice compared with the control mice (Fig. 2B).

Post-transcriptional regulation of gene expression via miRNA is known to cause a disconnection between mRNA and protein levels (van Rooij, 2011), and previous studies have shown that cholestasis leads to

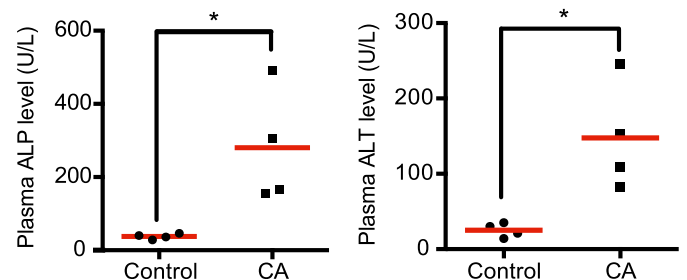


Fig. 1. CA feeding induces cholestasis in Tg-CYP2D6 mice. Tg-CYP2D6 mice were fed with normal chow (control) or 1% (w/w) CA-supplemented diet (CA) for 14 days ($n = 4$ mice/group). ALP and ALT activities were measured in mouse Plasma. * $p < 0.05$.

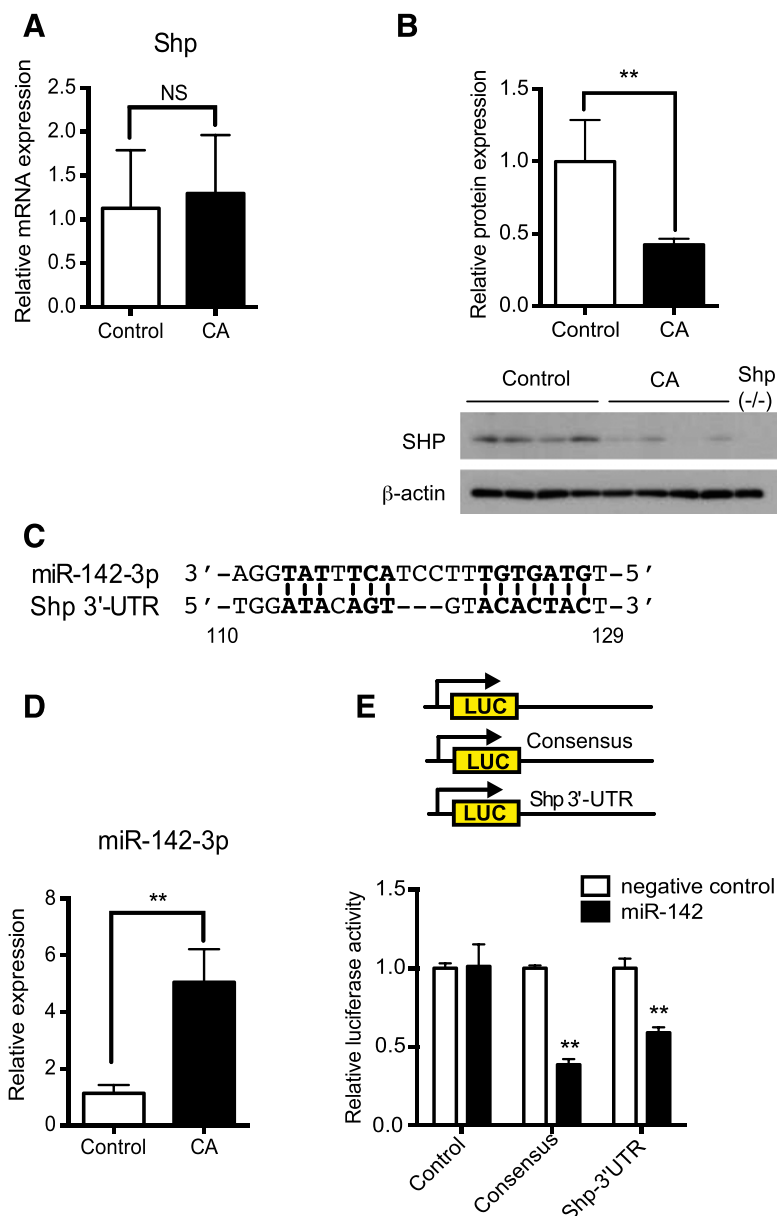


Fig. 2. CA feeding leads to decreased SHP protein level in Tg-*CYP2D6* mice. Tg-*CYP2D6* mice were fed with normal chow (control) or 1% (w/w) CA-supplemented diet for 14 days ($n = 4$ mice/group). (A) Hepatic Shp mRNA expression level was determined by qRT-PCR. NS, not statistically significant. (B) SHP protein expression level was determined by immunoprecipitation (using H160 antibody against SHP) followed by Western blotting (using Q14 antibody against SHP). Liver tissue lysate from *Shp*-null mice was used as a negative control. $**p < 0.01$. (C) Schematic representation of miR-142-3p putative binding sites in Shp 3'-UTR. (D) Hepatic miR-142-3p expression level was determined by qRT-PCR. $**p < 0.01$. (E) HEK293T cells were cotransfected with luciferase (LUC) reporter vector, *Renilla* expression vector, and miR-142-3p expression vector (or negative control vector), and dual luciferase assays were performed. The experiment was performed in triplicate. $**p < 0.01$, versus negative control-transfected cells.

differential expression of multiple hepatic miRNAs (Hirota et al., 2008; Rieger et al., 2013). In silico analysis of the 3'-UTR of *Shp* (by using a combination of DIANA-T, miRanda, miRDB, miRWalk, and Targets-can) revealed putative binding sites for miRNAs (i.e., miR-141-3p and miR142-3p) (miR142-3p shown in Fig. 2C). To determine whether CA feeding alters the expression levels of these miRNAs, their expression levels were measured by using qRT-PCR. The level of miR-142-3p was increased ~5-fold in the livers of CA-fed mice compared with the control (Fig. 2D), whereas miR-141-3p expression did not differ between the groups (data not shown). To validate whether miR-142-3p regulates *Shp* post-transcriptionally, we constructed a luciferase reporter vector that contains the putative miR-142-3p binding sequence of the *Shp* 3'-UTR. HEK293T cells were cotransfected with miR-142-3p mimic, luciferase vector, and *Renilla* expression vector, followed by measurement of luciferase activity. Overexpression of miR-142-3p led to a significant decrease in the activity of the *Shp* 3'-UTR-luciferase reporter (Fig. 2E). To examine whether miR-142-3p also modulates basal *Shp* expression, Raw264.7 cells (a mouse macrophage cell line

where miR-142-3p expression was ~9-fold higher than that in HepG2 cells; data not shown) were cotransfected with the antagonist of miR-142-3p (or negative control), luciferase vector, and *Renilla* expression vector, followed by measurement of luciferase activity. The results, however, showed that the miR-142-3p antagonist did not enhance the expression of the luciferase reporter (Supplemental Fig. 2), potentially due to differences in gene dose (e.g., much larger copy number of luciferase gene compared with miR-142-3p expression level). Together, our data suggest that CA feeding increases the hepatic miR-142-3p level, and this in turn leads to decreased SHP expression in mice.

Altered SHP Expression Exhibits Differential Effects on SHP Target Gene Expression in CA-Fed Mice. To determine whether decreased SHP protein level leads to altered *CYP2D6* expression, mRNA levels of *CYP2D6* were measured in the livers of CA-fed mice. CA feeding led to a slight increase in mRNA expression of *CYP2D6* (Fig. 3A). Conversely, contrary to the well-accepted notion that SHP is a transcriptional repressor of genes involved in bile acid synthesis, *Cyp7a1* and *Cyp8b1* expression was dramatically decreased in CA-fed

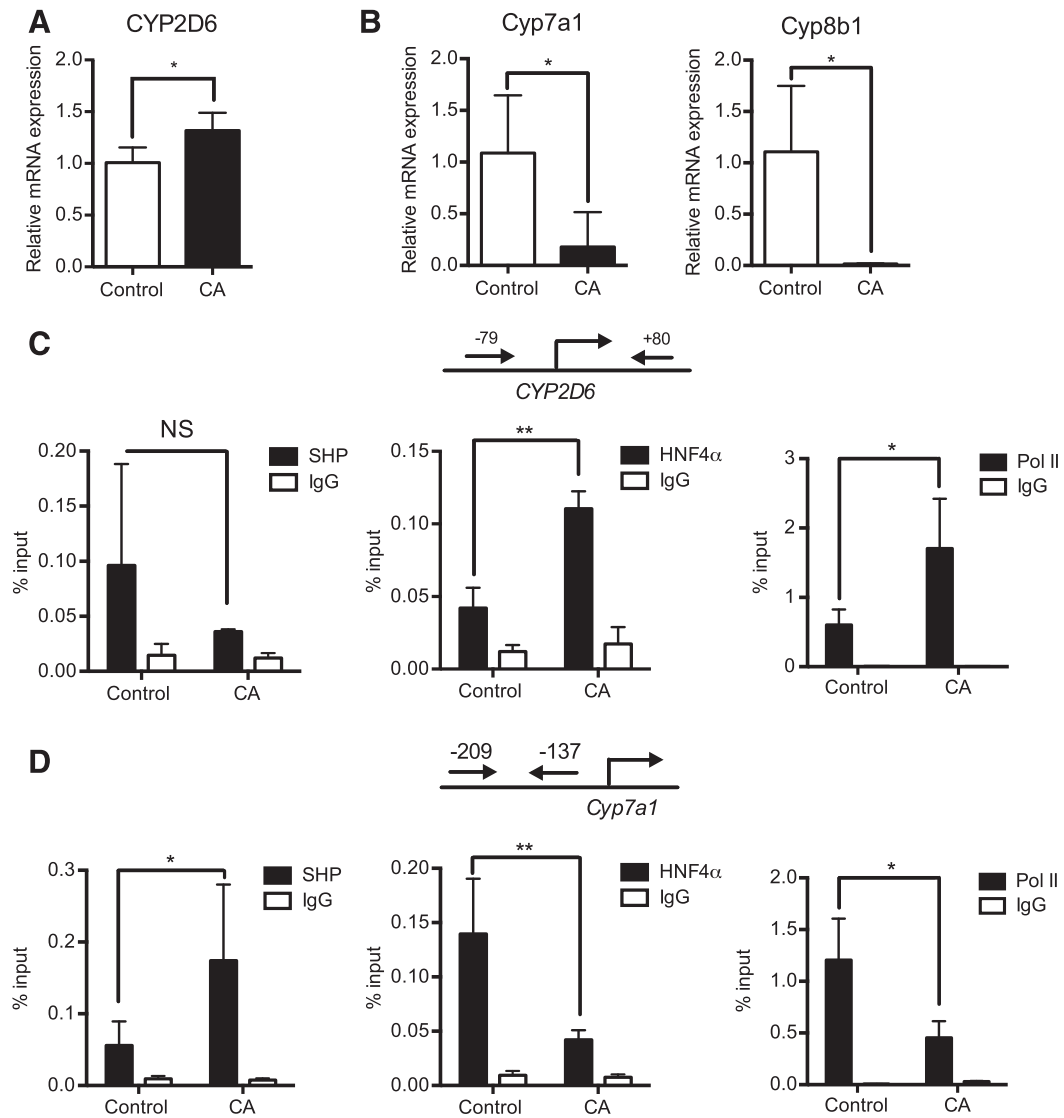


Fig. 3. Expression of CYP2D6 and Cyp7a1/Cyp8b1 is differentially regulated in CA-fed Tg-CYP2D6 mice. Tg-CYP2D6 mice were fed with normal chow (control) or 1% (w/w) CA-supplemented diet (CA) for 14 days ($n = 4$ mice/group). Hepatic mRNA expression levels of CYP2D6 (A) and Cyp7a1/Cyp8b1 (B) were determined by qRT-PCR. Recruitment of SHP, HNF4 α , and Pol II to promoters of CYP2D6 (C) and Cyp7a1 (D) was analyzed by ChIP assay using mouse liver tissues. The locations of primers used in qRT-PCR for ChIP are shown. Values are presented as the mean \pm S.D. ($n = 4$ mice/group). * $p < 0.05$; ** $p < 0.01$. NS, not statistically significant.

mice (Fig. 3B). To identify potential mechanisms underlying this finding, the extent of SHP recruitment to the promoter regions of CYP2D6, Cyp7a1, and Cyp8b1 was analyzed via ChIP assays by using antibodies against SHP, HNF4 α , and Pol II (a marker of transcription). The results showed that CA feeding increased the recruitment of HNF4 α and Pol II to CYP2D6 promoter (Fig. 3C), consistent with elevated CYP2D6 expression upon CA feeding. This was accompanied by decreased recruitment of SHP to the CYP2D6 promoter, although the difference did not reach statistical significance due to large variability (Fig. 3C). Interestingly, despite the decreased SHP protein levels in CA-fed mice (Fig. 2B), CA feeding led to increased SHP (and decreased HNF4 α and Pol II) recruitment to the promoters of Cyp7a1 (Fig. 3D) and Cyp8b1 (Supplemental Fig. 3), consistent with the decrease in Cyp7a1 and Cyp8b1 mRNA expression observed with CA feeding.

CA Feeding Leads to Increased CYP2D6 Activity in Mice. To determine whether increased CYP2D6 mRNA expression leads to increased protein and enzyme activity levels, S9 fractions were isolated from the livers of CA-fed or control mice. Western blot results showed that CYP2D6 protein levels in the S9 fraction were increased in CA-fed

mice (Fig. 4A) to a greater extent than the increase in mRNA levels. The production rate of 4-hydroxylated debrisoquine in the S9 fractions (determined as a marker for CYP2D6 activity) was also significantly higher in CA-fed mice (Fig. 4B) but exhibited large variability. Considering that CYP2D6 activity can be regulated by the rate of electron transfer from CPR and Cyb5 (Henderson et al., 2015), we measured the activity of CPR and the content of Cyb5 in S9 fractions. Neither CPR activity nor Cyb5 content in hepatic S9 fractions differed between the control and CA-fed groups (Fig. 4C). Together, these results indicate that CA feeding increases CYP2D6 activity in Tg-CYP2D6 mice.

Discussion

We have previously shown that SHP is a transcriptional repressor of CYP2D6 expression (Koh et al., 2014), and activation of the FXR and SHP pathways by using a synthetic FXR agonist leads to decreased CYP2D6 expression and activity (Pan et al., 2015). Bile acids are endogenous activators of FXR that are capable of upregulating SHP

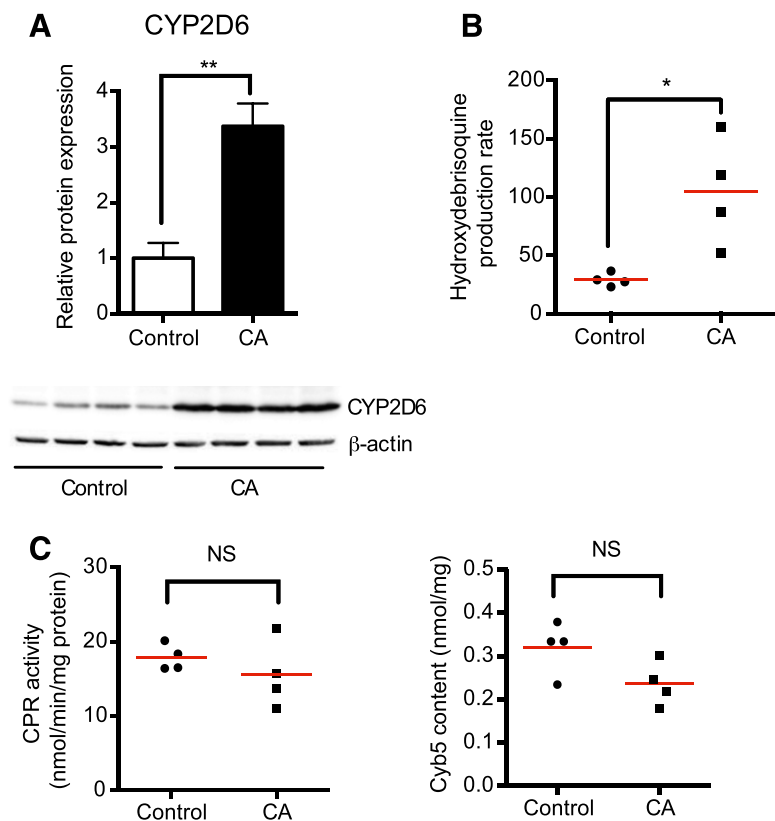


Fig. 4. CA feeding increases CYP2D6-mediated drug metabolism in Tg-CYP2D6 mice. Tg-CYP2D6 mice were fed with normal chow (control) or 1% (w/w) CA-supplemented diet (CA) for 14 days ($n = 4$ mice/group). (A) S9 fractions were prepared from the liver tissues, and protein levels of CYP2D6 in the S9 fractions were determined by Western blot. A gel image (bottom) and the quantified band intensities (top) are shown. $**p < 0.01$. (B) CYP2D6 activity was measured in hepatic S9 fractions using debrisoquine (200 μ M) as a CYP2D6 probe drug. Data are shown as the metabolite production rate in pmoles per minute per milligram protein. $*p < 0.05$. (C) CPR activity and Cyb5 contents were measured in hepatic S9 fractions. NS, not statistically significant.

within hours (Fang et al., 2007; Miao et al., 2009); however, it remains unclear how chronically elevated concentrations of bile acids (e.g., in cholestatic conditions) affect SHP expression/activity and thus its regulation of CYP2D6 expression. In this study, we employed CA feeding in mice to mimic cholestatic conditions and unexpectedly found that CA feeding decreased SHP protein levels, thus increasing CYP2D6 expression and activity.

In this study, mRNA expression levels of SHP were similar between the control and CA-fed mice, but SHP protein expression was decreased upon CA feeding. The lack of SHP induction by bile acids was also observed in a previous study where CA feeding for 12 weeks had insignificant effects on mRNA levels of SHP in mice (Wang et al., 2003), suggesting that cholestasis by CA feeding may have differential effects on SHP expression depending on the duration of CA feeding. Indeed, SHP is known to repress its own expression by inhibiting the action of transcriptional activators on the SHP promoter (Goodwin et al., 2000). Also, bile acids were shown to phosphorylate CCAAT-enhancer-binding protein α (via activation of phosphatidylinositol 3 kinase signaling), which subsequently binds to the SHP promoter and represses the promoter activity (Benet et al., 2015). It appears likely that the 2-week time point used in this study (to examine long-term cholestasis effects on CYP2D6 expression) allowed negative transcriptional regulators of SHP to be activated. Interestingly, despite the lack of difference in SHP mRNA levels between the CA-fed and control groups, SHP protein levels were significantly decreased in CA-fed mice. This is potentially attributable to the action of miR-142-3p. CA feeding significantly increased hepatic levels of miR-142-3p, which is consistent with a previous report (Rieger et al., 2013). A sequence complementary to miR-142-3p was identified within the 3'-UTR region of Shp mRNA, and the overexpression of miR-142-3p significantly repressed the expression of the luciferase gene that is flanked by the Shp 3'-UTR. Considering the pleiotropic roles of SHP in repressing fibrosis and

tumorigenesis in the liver (Zhang et al., 2011), upregulation of miR-142-3p and subsequent downregulation of SHP in cholestatic livers may provide insights for a better understanding of the liver damage that accompanies cholestasis.

Our study showed that CA feeding led to increased CYP2D6 expression and activity in mice. Interestingly, however, the magnitude of the increase in CYP2D6 protein and activity in the S9 fraction was greater than the changes in CYP2D6 mRNA levels (i.e., ~ 1.5 -fold versus ~ 3.4 -fold increases in mRNA and protein/activity levels, respectively). CYP2D6 mRNA has a very short 3'-UTR (89 bp). This is much shorter than the average length of the 3'-UTRs of most (if not all) miRNA target genes (i.e., 1730 bp) (Hu, 2009), suggesting that the involvement of miRNAs in CYP2D6 regulation is unlikely. Furthermore, *in silico* prediction by using several programs (miRanda, picTar, and TargetScan) did not reveal any putative miRNA binding sites in 3'-UTR of CYP2D6 mRNA (data not shown). The underlying mechanisms for the disconnect between CYP2D6 mRNA and protein/activity levels remain to be elucidated.

Previously, we demonstrated that CYP2D6 expression and activity were *repressed* in an ethinylestradiol (EE2)-induced cholestasis mouse model (Pan and Jeong, 2015). On the other hand, we observed *enhanced* CYP2D6 expression and activity in this study when CA feeding (to mice) was used as a model for cholestatic conditions. Furthermore, the SHP protein level was higher in the EE2-induced cholestasis model (Pan and Jeong, 2015), whereas it decreased in CA-fed mice, suggesting that the differential regulation of SHP expression may underlie the apparent discrepancy in the directional changes in CYP2D6 expression/activity. While both cholestatic mouse models lead to significant increases in intrahepatic bile acid levels, they are distinct with respect to certain pathologies associated with cholestasis, such as altered bile flow. Bile acids are surfactants by nature (Begley et al., 2005) and also modulate the expression of intestinal genes involved in local innate immune

functions (Vavassori et al., 2009) or hepatic nutrient metabolism (Jiang et al., 2015). The flow of bile from the gallbladder to the gut is critical in preventing intestinal bacteria overgrowth (and bacterial translocation to the liver) and maintaining systemic nutrient homeostasis. In intrahepatic cholestasis (including EE2-induced cholestasis) and biliary obstruction, bile flow was decreased (Yamamoto et al., 2006), whereas the oral administration of bile acids (such as ursodeoxycholic acid and CA) induces bile flow (Ding et al., 1993; Fickert et al., 2001). These differences in bile flow may lead to the establishment of a distinct gut microbiota environment and differential hepatic functions (Slocum et al., 1992; Ding et al., 1993). Indeed, our preliminary results showed significant increases in hepatic expression of inflammatory marker genes (i.e., tumor necrosis factor- α , interleukin-1 β , and interleukin-6) in the mice with EE2-induced cholestasis (which often accompanies intestinal bacteria overgrowth) but not in CA-fed mice (data not shown). Whether and how the altered gut microbiota modulates hepatic regulation of SHP and CYP2D6 expression remains to be further investigated.

SHP is a transcriptional repressor of genes encoding enzymes mediating the synthesis of bile acids, *Cyp7a1*, and *Cyp8b1*. Our results showed that CA feeding for 2 weeks led to decreased SHP protein expression. However, despite decreased SHP protein levels in CA-fed mice, SHP recruitment to the promoter regions of *Cyp7a1* and *Cyp8b1* (but not of *CYP2D6*) increased in these mice. This finding is in part consistent with previous reports (Kemper et al., 2004; Fang et al., 2007) that bile acids induce the closure of the chromatin region harboring the *Cyp7a1* gene by recruiting histone deacetylase and histone methyltransferase G9a. Indeed, we found increased histone methylation of *Cyp7a1* and *Cyp8b1* promoter regions in the CA-fed mouse liver (by using ChIP assays; data not shown). The mechanism through which bile acids trigger the chromatin remodeling of promoter regions in a gene-specific manner remains unclear.

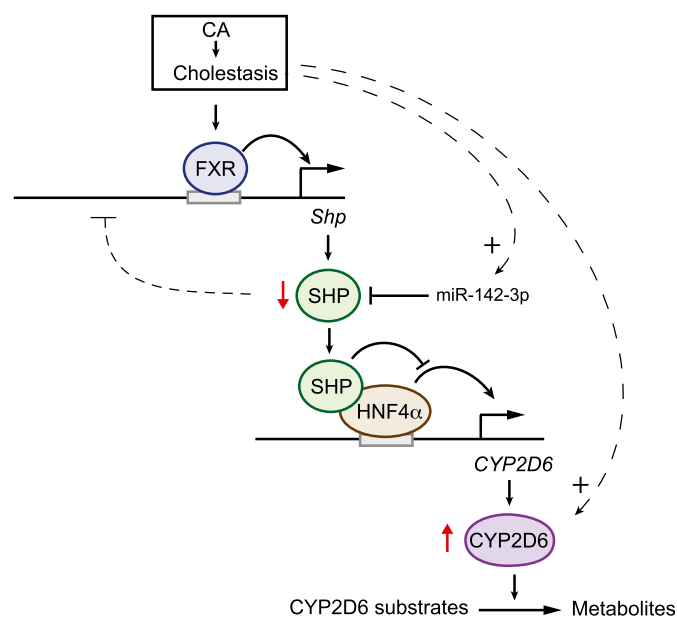


Fig. 5. Working model for CYP2D6 regulation in CA feeding-induced cholestasis. CA feeding (for 2 weeks) leads to increased FXR activity on the *Shp* promoter. SHP is known to negatively regulate its own expression. Together, this causes minor (if any) changes in *Shp* mRNA levels. CA feeding also enhances the expression of miR-142-3p that targets SHP. CA feeding increases CYP2D6 protein amounts in hepatic S9 fractions by unknown mechanisms, subsequently leading to greater CYP2D6-mediated drug metabolism. Red arrows represent directional changes in its hepatic levels in CA-fed Tg-*CYP2D6* mice.

In conclusion, CA feeding in Tg-*CYP2D6* mice led to decreased SHP protein expression, potentially via the upregulation of miR142-3p. This was accompanied by increased CYP2D6 expression and activity (Fig. 5). The results highlight the important role of SHP in the regulation of CYP2D6 expression and provide a better understanding of factors modulating the basal levels of CYP2D6-mediated drug metabolism.

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Authorship Contributions

Participated in research design: Pan, Kent, Won, and Jeong.

Conducted experiments: Pan, Kent, and Won.

Performed data analysis: Pan, Kent, Won, and Jeong.

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

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