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Restoration of Enterohepatic Bile Acid Pathways in Pregnant Mice Following Short Term Activation of Fxr by GW4064

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Abstract

The farnesoid X receptor (Fxr) controls bile acid homeostasis by coordinately regulating the expression of synthesizing enzymes (Cyp7a1, Cyp8b1), conjugating enzymes (Bal, Baat) and transporters in the ileum (Asbt, Ostα/β) and liver (Ntcp, Bsep, Ostβ). Transcriptional regulation by Fxr can be direct, or through the ileal Fgf15/FGF19 and hepatic Shp pathways. Circulating bile acids are increased during pregnancy due to hormone-mediated disruption of Fxr signaling. While this adaptation enhances lipid absorption, elevated bile acids may predispose women to develop maternal cholestasis. The objective of this study was to determine whether short-term treatment of pregnant mice with GW4064 (a potent FXR agonist) restores Fxr signaling to the level observed in virgin mice. Plasma, liver and ilea were collected from virgin and pregnant mice administered vehicle or GW4064 by oral gavage. Treatment of pregnant mice with GW4064 induced ileal Fgf15, Shp and Ostα/β mRNAs, and restored hepatic Shp, Bal, Ntcp, and Bsep back to vehicle-treated virgin levels. Pregnant mice exhibited 2.5-fold increase in Cyp7a1 mRNA compared to virgin controls, which was reduced by GW4064. Similarly treatment of mouse primary hepatocytes with plasma isolated from pregnant mice induced Cyp7a1 mRNA by nearly 3-fold as compared to virgin plasma, which could be attenuated by co-treatment with either GW4064 or recombinant FGF19 protein. Collectively, these data reveal that repressed activity of intestinal and hepatic Fxr in pregnancy, as previously demonstrated, may be restored by pharmacological activation. This study provides the basis for a novel approach to restore bile acid homeostasis in patients with maternal cholestasis.

Graphical Abstract

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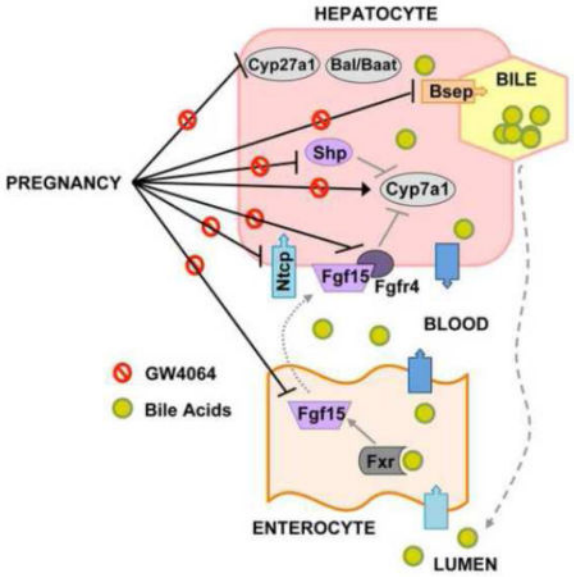

Keywords

Fxr; GW4064; pregnancy; Cyp7a1; bile acids

Introduction

Bile acids are required for the absorption of lipids and lipid-soluble vitamins from the intestine. During periods of high nutritional demand such as pregnancy, the body adapts to increase the size of the bile acid pool. While this is necessary for enhanced absorption of nutrients to support placental and fetal growth, hypercholanemia can lead to maternal cholestasis or a condition called intrahepatic cholestasis of pregnancy (ICP). ICP is observed in 0.5–2% of pregnancies in the United States, and carries with it an increased risk of fetal distress, spontaneous preterm delivery and stillbirth (Glantz *et al.*, 2004; Geenes *et al.*, 2014; Henderson *et al.*, 2014; Williamson and Geenes, 2014). Likewise, women with ICP are more likely to have concomitant gestational diabetes or pre-eclampsia, and may be predisposed to develop subsequent liver disease, such as hepatitis C, fibrosis and gallstones (Marschall *et al.*, 2013; Wikstrom Shemer *et al.*, 2013).

The synthesis, metabolism, and excretion of bile acids is tightly regulated by transcription factors, enzymes, transporters, and signaling mediators in the liver and intestine. Only 5% of bile acids are excreted into the feces each day. The remaining 95% are conserved through enterohepatic recirculation (Danielsson and Sjovall, 1975). Bile acids are synthesized in the liver via the classic (key enzymes cholesterol-7α-hydroxylase, Cyp7a1, and sterol 12α-hydroxylase, Cyp8b1) or alternative (key enzyme steroid 27-hydroxylase, Cyp27a1) pathways and released into the intestinal lumen to promote the absorbance of lipids and lipid-soluble vitamins. Bile acids are reabsorbed into enterocytes by the apical sodium-dependent bile acid transporter (Asbt). Bile acids are thought to be bound by intestinal binding proteins such as ileal bile acid binding protein (I-babp), and secreted into the portal

circulation by the basolateral organic solute transporter alpha/beta (Ostα/β) heterodimer (Dawson *et al.*, 2005). Portal bile acids are then taken up into hepatocytes predominantly by the sinusoidal transporter Na^+-taurocholate cotransporting polypeptide (Ntcp). Removal of bile acids from the liver is mainly accomplished by the canalicular efflux transporters bile salt export pump (Bsep) and multidrug resistance-associated protein (Mrp) 2 (Keppler *et al.*, 1997; Stieger *et al.*, 2007). However, the sinusoidal efflux transporters Ostα/β and Mrp3 can also pump bile acids into the circulation. Sinusoidal efflux of bile acids into the blood can be enhanced and is critical for removing bile acids from the liver during cholestasis (Belinsky *et al.*, 2005; Landrier *et al.*, 2006; Teng and Piquette-Miller, 2007).

The farnesoid X receptor (Fxr) is a bile acid-activated transcription factor that belongs to the nuclear receptor superfamily. Fxr cooperatively regulates the expression of bile acid transporters including intestinal Ostα/β and hepatic Ntcp, Bsep and Ostβ (Lu *et al.*, 2000; Denson *et al.*, 2001; Thomas *et al.*, 2010), as well as the inhibitory hepatic transcription factor small heterodimer partner (Shp) and the intestinal endocrine factor fibroblast growth factor (Fgf) 15 (FGF19 in humans). Fxr-mediated induction of Fgf15 in the ileum of the small intestine has emerged as a major pathway to suppress bile acid synthesis in the liver (Kong *et al.*, 2012). In detail, Fxr activation induces Fgf15/19 in enterocytes, which is secreted into the portal vein and then binds to its cell surface receptor Fgfr4 on hepatocytes where it represses the expression of bile acid synthesizing genes (Cyp7a1 and Cyp8b1) (Inagaki *et al.*, 2005; Yu *et al.*, 2005). In response to bile acid binding in the liver, Fxr up-regulates the transcription of Shp, which works with Fgf15 to suppress the expression of Cyp7a1. Furthermore, Fxr transactivates the *Abcb11*/Bsep and *Slc51*/Ostβ genes (Zhu *et al.*, 2011; Kong *et al.*, 2012). During pregnancy, elevated bile acid levels have been attributed to suppressed hepatic Fxr function by increased circulating levels of the steroid hormones estradiol and progesterone, and their metabolites, which results in enhanced synthesis and reduced hepatic uptake and secretion of bile acids (Abu-Hayyeh *et al.*, 2010; Milona *et al.*, 2010; Aleksunes *et al.*, 2012; Abu-Hayyeh *et al.*, 2013).

Despite recent advancements in the ICP and bile acid fields, there are a number of knowledge gaps that remain. It is not known whether intestinal Fxr function is modified during pregnancy. Moreover, the potential to intervene and/or reverse the adaptive modifications to bile acid homeostasis has not been tested in pregnant animals. Therefore, the purposes of this study were to 1) characterize ileal Fxr functions during pregnancy in mice and 2) test the ability to overcome repressed Fxr functions using a selective Fxr agonist. Therefore, pharmacologic approaches to modulate Fxr activity *in vivo* and *ex vivo* were used to delineate the molecular mechanisms by which ileal and hepatic Fxr regulate bile acid homeostasis during pregnancy.

Materials and methods

Chemicals

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO). GW4064 was synthesized at the Chemical Discovery Laboratory at the University of Kansas (Lawrence, KS). Recombinant FGF19 protein was synthesized by the laboratory of Dr. Guo at Rutgers University according to a previous report (Kong and Guo, 2014).

Animal treatment

Adult male and female wild-type C57BL/6 mice 8–12 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Female mice were mated overnight with male mice and checked for the presence of a vaginal sperm plug the next morning (designated gestation day 0). Additional female mice were used as virgin controls. Mice had access to standard chow and water *ad libitum*. On gestation days 13 and 14, pregnant females (n=5–6) and time-matched virgin females received two doses of vehicle (PBS with 1% Tween-80 and 1% methylcellulose, 10 mL/kg) or the Fxr synthetic agonist GW4064 (100 mg/kg, 10 ml/kg) 12 hours apart by oral gavage. Plasma, whole liver and intestines were collected from vehicle- and GW4064-treated virgin and pregnant mice 3 hours after the second dose on gestation day 14. Small intestines were divided into three equal segments, representing the duodenum, jejunum and ileum. Ileal fragments were utilized for all studies.

Liver and total body weights were recorded for female mice at the time of sacrifice. Tissues were collected within a 60 minute period in the morning (Zhang *et al.*, 2011), snap frozen in liquid nitrogen, and stored at −80°C. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal care facility in temperature-, light- and humidity-controlled rooms. All animal studies were approved by the Rutgers University Institutional Animal Care and Use Committee, and were in accordance with national guidelines.

Sandwich-cultured primary hepatocytes

Freshly isolated female C57BL/6 mouse hepatocytes (n=3 donors/independent experiments in triplicate) were obtained from Triangle Research Labs (Research Triangle Park, NC). Hepatocytes were sandwich-cultured in 12-well or 6-well plates with a Matrigel overlay, and shipped overnight in cold preservation media. Upon receipt, the preservation media was replaced with serum-free and phenol red-free Williams' E media. Cells were allowed to recover from shipping for 24 hours at 37°C in an atmosphere containing 5% CO_2. After recovery, the hepatocytes were treated with phenol red-free William's E media containing 20% pooled virgin or pregnant (gestation day 17 or greater) mouse plasma, in the presence of GW4064 (5 μM), recombinant FGF19 protein (20 μg/mL), or vehicle (DMSO), for 1 or 24 hours. Total RNA and protein were isolated.

Plasma analyses

Plasma estradiol and progesterone were quantified using ELISA kits from Calbiotech (Spring Valley, CA) and Genway (San Diego, CA), respectively. Triglycerides and cholesterol were quantified using an enzymatic colorimetric assay from Pointe Scientific (Canton, MI).

RNA isolation and quantitative PCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). RNA purity and concentration were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL). mRNA expression was quantified by real time-qPCR using SYBR Green-based method (Applied

Biosystems) for detection of amplified products. qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Ct values were converted to delta delta Ct values by adjusting to a reference gene (ribosomal protein l13a, Rpl13a) (Livak and Schmittgen, 2001). Primer sequences are listed in Supplemental Table 1.

Western blot analysis

For animal tissues, whole livers and ilea were homogenized in sucrose-Tris buffer (pH=7.4–7.5) using the TissueLyser LT Adapter (Qiagen), per the manufacturer's protocol. Sandwich-cultured primary mouse hepatocytes were collected in PBS and cell pellets were resuspended in lysis buffer with 1% protease inhibitor. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL). Fifty micrograms of whole liver or ilea homogenate or 30 micrograms of cell lysate protein were loaded onto a SDS-PAGE gel (4–12%, Life Technologies). Semi-quantification of expression was determined using primary antibodies raised against Fgf15 (Sc-27177, 1:1000, Santa Cruz), Ntcp (K4, 1:5000, Dr. Bruno Stieger), Bsep (K44, 1:5000, Dr. Bruno Stieger), phosphor-Erk1/2 (9101, 1:1000, Cell Signaling Technology, Danvers, MA) and Erk1/2 (9102, 1:1000, Cell Signaling Technology) followed by incubation with appropriate secondary antibody. The intensity of band luminescence was acquired using a FluorChem E System Imager (ProteinSimple, Santa Clara, CA). β-Actin (ab8227, Abcam, Cambridge, MA) or Gapdh (ab2302, Abcam) were used as loading controls.

Indirect immunofluorescence

Liver cryosections (6 μm) were fixed in 4% paraformaldehyde for 5 minutes. Sections were blocked with 5% goat serum/PBS with 0.1% Triton X-100 (PBS-Tx) for 1 hour and then incubated with a primary antibody against Bsep diluted 1:100 in 5% goat serum, PBS-Tx for 2 hours at room temperature. Sections were then washed and incubated with an anti-rabbit secondary antibody linked to AlexaFluor488 (Life Technologies). Images were acquired on a Zeiss Observer D1 microscope at 20x using a Jenoptik camera. All sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included (data not shown).

Plasma bile acid profiling

Plasma bile acid extracts were analyzed by a Thermo Accela Ultra Performance Liquid Chromatography system (Thermo Fisher Scientific) coupled with a Thermo LTQ XL Ion Trap Mass Spectrometer (ITMS, Thermo Fisher Scientific). Ionization was accomplished using Electrospray and the ITMS was operated in MS/MS mode with Selective Ion Monitoring. Simultaneous determination of 23 bile acids using commercial standards (Sigma-Aldrich and Steraloids, Newport, RI), was as previously reported (Zhan *et al.*, 2016). The limit of quantification for all bile acids was 3.11 ng/μL plasma.

Statistical analysis

Data are presented as mean ± SE (animal studies) or mean ± SD (hepatocyte studies). Statistical analysis was performed using GraphPad Prism v6 (La Jolla, CA). mRNA and

protein expression were analyzed by a 2-way ANOVA or a 1-way ANOVA followed by a Newman-Keul's multiple comparison post-hoc test when appropriate, to compare overall mean differences between groups. Significance was set at $p \leq 0.05$.

Results

Pregnancy outcomes following GW4064 treatment

To determine the effects of Fxr activation on pregnancy adaptations, plasma sex hormone concentrations and other indicators of physiological/pathological changes were quantified in pregnant mice treated with GW4064 (Table 1). As expected, plasma estradiol and progesterone levels increased by 1.5- and 6.5-fold with pregnancy, respectively. Progesterone levels were reduced by 20% with GW4064 treatment (20.3 ng/mL) compared to vehicle-treated pregnant mice (28.0 ng/mL), though still within the normal range for gestation day 14. Pregnancy increased plasma triglycerides by nearly 2-fold, which was not altered by treatment with GW4064. No significant differences were observed in plasma cholesterol levels among treatment groups on gestation day 14. The number of resorption sites at the time of sacrifice was similar between vehicle- and GW4064-treated mice. All fetuses appeared grossly normal. Additionally, both vehicle- and GW4064-treated pregnant mice had increased liver-to-body weight ratios as compared to virgin control mice.

Expression of hepatic bile acid-related genes in virgin and pregnant mice treated with GW4064

Similar to previously published reports, pregnancy had no effect on Fxr mRNA levels (Fig. 1) (Milona *et al.*, 2010; Aleksunes *et al.*, 2012). Vehicle-treated pregnant mice exhibited a 55% reduction in Shp mRNA, as well as 35 to 40% decreased expression of Fgfr4, Cyp27a1, Bal and Baat mRNAs compared to vehicle-treated virgin mice. A 2-fold increase in Cyp7a1 expression, and a 55% elevation in Cyp8b1, was also observed in pregnant mice. Treatment of pregnant mice with GW4064 restored mRNAs of Shp, Cyp7a1, Cyp27a1 and Bal to virgin control levels.

Down-regulation of Ntcp (53%) transporter transcripts was also observed on gestation day 14 compared to virgin controls (Fig. 2A). A 40% decrease in Bsep mRNA of vehicle-treated pregnant mice was noted but not statistically significant when compared to virgin controls. Treatment of pregnant mice with GW4064 up-regulated (25 to 150%) the expression of all hepatic transporters tested. Whereas GW4064 enhanced Ntcp, Bsep and Ostβ expression in pregnant mice, little to no change in transporter expression was observed in virgin mice treated with GW4064.

At the protein level, down-regulation of Ntcp and Bsep by 50 and 38%, respectively, was confirmed in pregnant vehicle-treated mice (Fig. 2B). GW4064 treatment of pregnant mice moderately increased the expression of both Ntcp (15%) and Bsep (17%) proteins. Additionally, indirect immunofluorescent staining confirmed localization of Bsep to the canaliculi (Fig. 2C). Compared to vehicle-treated virgin mice, pregnancy reduced the intensity of Bsep staining which was restored by GW4064 treatment.

Vehicle-treated pregnant mice had reduced mRNA expression of bile acid transporters not known to be regulated by Fxr, including organic anion-transporting polypeptide (Oatp) 1a4 and 1b2, and Mrp3 (Fig. 3). Alternatively, Mrp4 mRNA expression was enhanced in pregnant mice, regardless of treatment group. Interestingly, GW4064 induced the mRNA expression of Oatp1b2, Mrp3 and Mdr2 in pregnant mice.

Expression of intestinal bile acid-related genes in virgin and pregnant mice treated with GW4064

Decreased mRNAs of Fgf15, Shp and I-babp by 65%, 95% and 50%, respectively, were noted on gestation day 14 in vehicle-treated pregnant mice (Fig. 4A). While treatment of virgin mice with GW4064 nearly doubled Fgf15 expression, even greater induction was observed in pregnant mice (9-fold compared to vehicle-treated pregnant mice). GW4064 treatment also increased Ostα and Ostβ mRNA by 67% and 85% compared to virgin controls, respectively. Although I-babp mRNA levels returned to those seen in virgin controls following GW4064 treatment of pregnant mice, these data were not statistically significant. Neither pregnancy nor GW4064 treatment altered expression of Fxr or Asbt transporter mRNAs. Interestingly, Shp mRNA was nearly undetectable in vehicle-treated pregnant mice, but enhanced by 3.3-fold in GW4064-treated pregnant mice as compared to vehicle-treated virgin mice. Similarly, Fgf15 protein was only detectable in pregnant mice following GW4064 treatment (Fig. 4B).

Bile acid pool analysis of plasma from virgin and pregnant mice treated with GW4064

Overall, a trend for increased total bile acids was observed for vehicle-treated pregnant mice as compared to vehicle-treated virgin mice (Fig. 5A). This corresponded with a relative increase (100%) in the ratio of taurine:glycine conjugated bile acids. We further analyzed the bile acid pool composition by comparing relative percentages of individual bile acids (Fig. 5B). Of the bile acids that are most highly concentrated in the plasma of mice, a trend for reduced taurocholic acid (TCA) was observed in pregnant mice with GW4064 treatment compared to vehicle treatment (Fig. 5C). Short-term treatment of pregnant mice with GW4064 increased the relative percentage of the secondary bile acid ω-murcholic acid (ωMCA) compared to vehicle-treated virgin mice (18%) and compared to vehicle-treated pregnant mice (13%). Though deoxycholic acid (DCA) concentrations in the plasma were low in comparison to other bile acids, levels were significantly increased in GW4064-treated pregnant mice as compared to vehicle-treated virgin and pregnant mice (nearly doubled).

Modulation of Cyp7a1 expression by pregnant plasma in sandwich-cultured primary mouse hepatocytes

To investigate the hypothesis that circulating factors present in blood lead to pregnancy-related bile acid changes, female naïve sandwich-cultured primary mouse hepatocytes were treated with 20% plasma isolated from virgin or pregnant (gestation day 17 or greater) mice. Treatment with plasma had no effect on hepatocyte cell viability as evidenced by negligible lactate dehydrogenase leakage (data not shown).

Subsequent studies tested whether the Fxr agonist GW4064 or recombinant FGF19 protein (the human ortholog of mouse Fgf15) could rescue pregnancy-related changes in bile acid

synthesis pathways. For this effort, sandwich-cultured primary mouse hepatocytes were concomitantly treated with 20% plasma from virgin and pregnant mice and GW4064 or exogenous recombinant FGF19 protein. Activity of recombinant FGF19 protein was confirmed by increased phosphorylation of Erk1/2 protein after 1 hour of treatment (Fig. 6A) (Kong *et al.*, 2012; Kong and Guo, 2014). Cyp7a1 mRNA expression was induced by 2.7-fold in the presence of pregnant plasma as compared to hepatocytes exposed to virgin plasma for 24 hours (Fig. 6B). Co-treatment of hepatocytes with pregnant plasma and either GW4064 or FGF19 reduced Cyp7a1 mRNA to levels at or below controls. For example, co-treatment with pregnant serum and recombinant FGF19 protein was able to reduce Cyp7a1 mRNA by 70 to 75%.

Discussion

The current study assessed the ability of activated Fxr to modulate hepatic and intestinal regulation of bile acid synthesis and transport pathways during pregnancy. As previously reported, pro-cholestatic adaptive changes in hepatic gene expression have been observed in pregnant mice in the absence of changes in the mRNA expression of Fxr itself (Fig. 1) (Milona *et al.*, 2010; Aleksunes *et al.*, 2012; Song *et al.*, 2014). This included the repression of gene expression of Shp, Fgfr4, Cyp27a1, Bal, Baat, Ntcp, and Mrp3, and induction of the gene expression of Cyp7a1 on gestation day 14. This was further reflected in decreased protein levels of major basolateral and canalicular bile acid transporters, Ntcp and Bsep (Aleksunes *et al.*, 2012). These data support the postulated functional impairment of Fxr activity during pregnancy more than a change in its expression. The coordinated down-regulation of sinusoidal uptake and efflux, as well as canalicular efflux transporters suggests that there is reduced bile acid enterohepatic recirculation, which may contribute to increased plasma bile acid levels late in pregnancy. A trend towards increased total bile acids in plasma was already apparent on gestation day 14 (Fig. 5). Interestingly, treatment of pregnant mice with a specific Fxr agonist GW4064 restored the expression of bile acid synthesis enzymes and transporters towards levels typically observed in virgin mice without negatively impacting plasma hormone and other biochemistries, resorptions or viable fetuses.

It has been demonstrated in the rat that Fxr indirectly suppresses Ntcp expression through the inhibitory transcription factor Shp (Denson *et al.*, 2001). Namely, activation of Fxr in the rat liver induces expression of Shp, which in turn inhibits the gene expression of Ntcp, a bile acid uptake transporter in hepatic sinusoidal membranes (Denson *et al.*, 2001). However, analysis of the same Shp response element in human and mouse suggests this may not be the dominant or singular pathway for bile acid regulation of NTCP/Ntcp in other species (Jung *et al.*, 2004). Previously published literature confirms that during pregnancy, both Shp and Ntcp gene expression are concomitantly reduced (Milona *et al.*, 2010; Aleksunes *et al.*, 2012). In this study, activation of Fxr in pregnant mice with the agonist GW4064 similarly induced both Shp and Ntcp (Fig. 1 and 2). This co-regulation phenomenon for Shp and Ntcp has also been noted in newborn mice with the bile acid surge that initiates Fxr signaling. At birth, a positive correlation between Fxr and Ntcp mRNA is observed (Cui *et al.*, 2012). Though not well-explored, Fxr response elements in the 5′ promoter region of the Ntcp/*Slc10a1* gene have been identified in adult mice (Thomas *et al.*, 2010). Thus, a secondary

mechanism for Fxr regulation of the bile acid uptake transporter Ntcp may occur in certain physiological states such as pregnancy.

GW4064 was identified and characterized as a potent, nonsteroidal and selective Fxr agonist in 2000 (Maloney *et al.*, 2000). GW4064 is commonly utilized as a pharmacological activator of Fxr in mice. It has been shown to decrease bile acid synthesizing enzymes Cyp7a1 and Cyp8b1 mRNA levels in an Fxr dependent manner, and not in Fxr-null mice (Kong *et al.*, 2012). The ability of GW4064 to reduce expression of Cyp7a1 and Cyp8b1 and induce Shp and Fgf15 mRNA is mitigated by deletion of Fxr in all tissues of mice (Moschetta *et al.*, 2004; Kong *et al.*, 2012). Interestingly, more pronounced increases in intestinal and hepatic mRNA expression of Fxr targets were consistently observed following GW4064 treatment in pregnant mice as compared to virgin mice. This may result from reduced gastrointestinal motility in mice during pregnancy (Datta *et al.*, 1974), similar to humans, which could lead to greater residence time in the intestinal lumen and subsequent absorption into the body. It should be recognized that GW4064 is a chemical probe used for research purposes and possesses little pharmaceutical utility. As a result, pharmacokinetic analysis of GW4064 in pregnant mice is unwarranted. Future studies utilizing long-term treatment of mice with an FDA-approved Fxr agonist, such as obeticholic acid, could help elucidate differences in pharmacokinetics and/or pharmacodynamics as potential mechanisms of action during pregnancy, in addition to providing a clearer understanding of the ability of increased Fxr activation to alter bile acid pool composition.

The current study highlights the relationship between the restoration of intestinal and hepatic Fxr target genes by GW4064. Intestinal Fgf15 was inducible by 9-fold with GW4064 treatment during pregnancy (Fig. 4). This newly identified responsiveness in pregnant mice likely led to the attenuation of Cyp7a1 mRNA induction in the liver (Fig. 1). Further, in an isolated system where only primary mouse hepatocytes were exposed to pregnant plasma in the media, Cyp7a1 mRNAs were induced (Fig. 6). Similar to *in vivo* results it was observed that this enhanced expression could be abolished by not only a pharmacological Fxr agonist, but also the addition of FGF19, a positively regulated signaling molecule produced by activation of ileal Fxr. The lack of a sensitive method has precluded our ability to quantify Fgf15 protein levels in serum. Nonetheless, this study supports the novel hypothesis that intestinal bile acid pathways can be altered in pregnancy in an Fxr-dependent manner. Further, it is likely that Fxr in the ileum contributes to changes in hepatic gene expression due to the Fgf15/FGF19-mediated communication between the two organs as part of the coordinated regulation of bile acid homeostasis.

Sandwich-cultured fresh primary hepatocytes were utilized in this study because they behave similarly to hepatocytes *in vivo* and form bile canaliculi in culture to better mimic the liver and bile acid pathway. However, this is still not an ideal system to assess transporter expression and activity, which decrease over time in culture. This was evidenced by higher Ct values of hepatic transporters in sandwich-cultured fresh primary hepatocytes as compared to Ct values in mice (data not shown). Therefore, mRNA expression of uptake and efflux transporters measured in the primary mouse hepatocytes showed limited correlation to data from intact animals.

Data presented in this paper suggest that activation of Fxr can restore bile acid-related gene expression both in the intestine and the liver. Potential mechanisms for the restoration of liver bile acid pathways may occur through 1) activation of hepatic Fxr or 2) suppression of Cyp7a1 by up-regulation of Fgf15 levels. These may be important findings for the treatment of pregnancy-specific cholestatic liver disease. Pharmacological activation of Fxr in rodents prevents or resolves both intra- and extra-hepatic cholestasis (Liu *et al.*, 2003; Moschetta *et al.*, 2004). Other studies have shown that constitutively active Fxr in the intestine can reduce total bile acids and decrease bile acid pool hydrophobicity, and presumably, toxicity (Modica *et al.*, 2012). Intestinal activation of Fxr specifically attenuated liver injury in models of extrahepatic cholestasis (bile duct ligation), as well as intrahepatic cholestasis (treatment with α-naphthylisothiocyanate and genetically induced). In this study, FGF19 treatment abrogated the pregnancy-related increase in Cyp7a1 expression *ex vivo*. Additional reports in the literature have confirmed that administration of FGF19 not only reduced CYP7A1 activity in healthy human patients but also protected mice from hepatotoxicity due to bile duct ligation and α-naphthylisothiocyanate treatment (Luo *et al.*, 2014). The present study implicates not only hepatic, but for the first time, intestinal Fxr activation as a potential mechanism to regulate bile acid signaling during pregnancy and may represent a new therapeutic target for treating ICP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Asbt	apical sodium-dependent bile acid transporter
Baat	bile acid CoA:amino acid N-acetyltransferase
Bal	bile acid CoA ligase
Bsep	bile salt export pump
Cyp	cytochrome P450
Fgf	fibroblast growth factor
Fxr	farnesoid X receptor
I-babp	ileal bile acid binding protein
ICP	intrahepatic cholestasis of pregnancy

Mdr	multidrug resistance protein
Mrp	multidrug resistance-associated protein
Ntcp	Na^+-taurocholate cotransporting polypeptide
Oatp	organic anion-transporting polypeptide
Ost	organic solute transporter
Shp	small heterodimer partner

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Highlights

- Ileal bile acid pathways are altered in pregnancy in an Fxr-dependent manner.
- Ileal Fxr/Fgf contributes to changes in hepatic bile acid synthesis and transport.
- Treatment of pregnant mice with an Fxr agonist restores bile acid homeostasis.

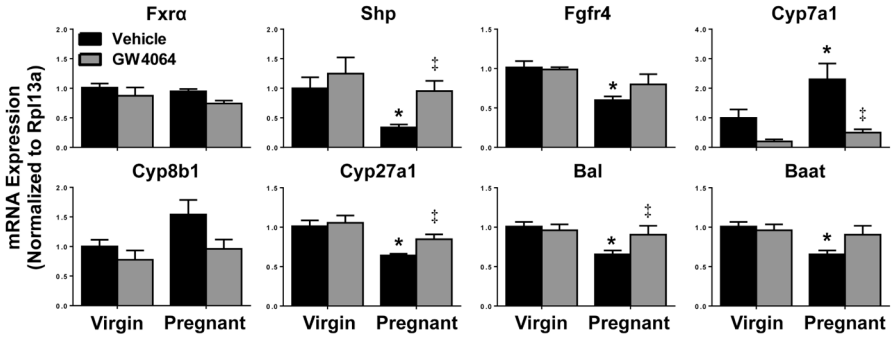

Fig. 1.

Hepatic mRNA expression in vehicle- or GW4064-treated pregnant mice. Hepatic mRNA expression of Fxr signaling and bile acid synthesis and metabolic enzyme genes was quantified in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5–6). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

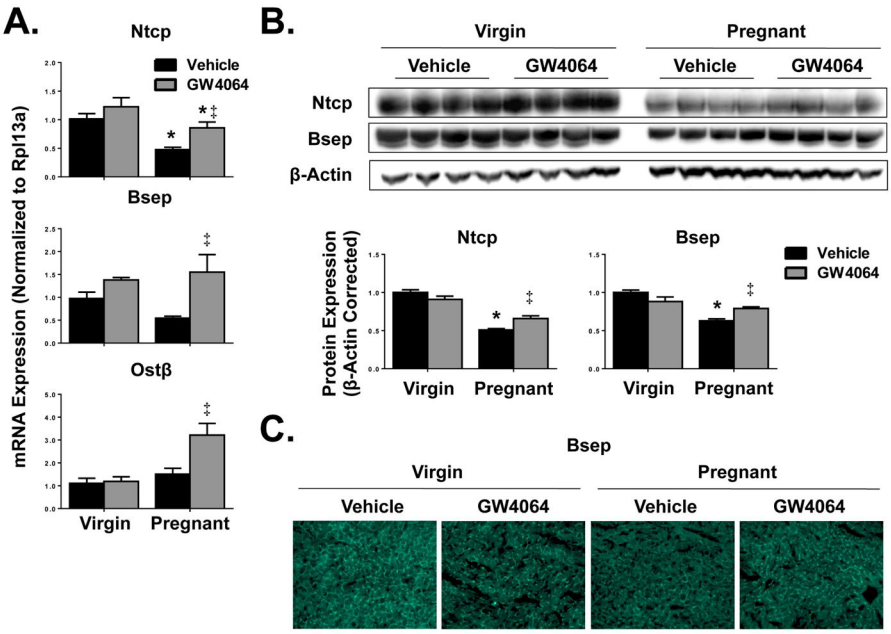

Fig. 2.

Hepatic transporter expression in vehicle- or GW4064-treated pregnant mice. Hepatic (A) mRNA and (B) protein expression of uptake and efflux transporters were quantified in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Western blots were performed using whole liver homogenates, and protein band intensity was semi-quantified. β-Actin was used as a loading control. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5–6). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice. (C) Indirect immunofluorescence against canalicular transporter Bsep (*green*) was conducted on liver cryosections (6 μm). Representative images are shown. Magnification x100.

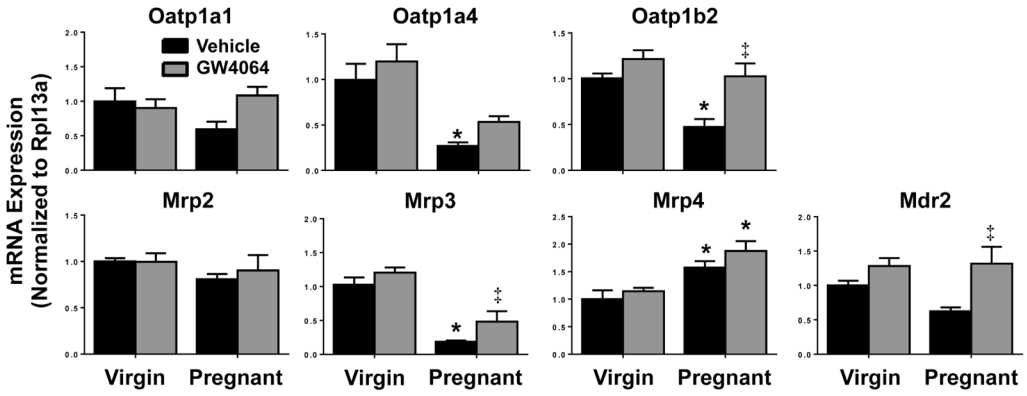

Fig. 3.

Hepatic bile acid transporter mRNA expression in vehicle- or GW4064-treated pregnant mice. Hepatic mRNA expression of bile acid uptake and efflux transoprter genes was quantified in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5–6). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

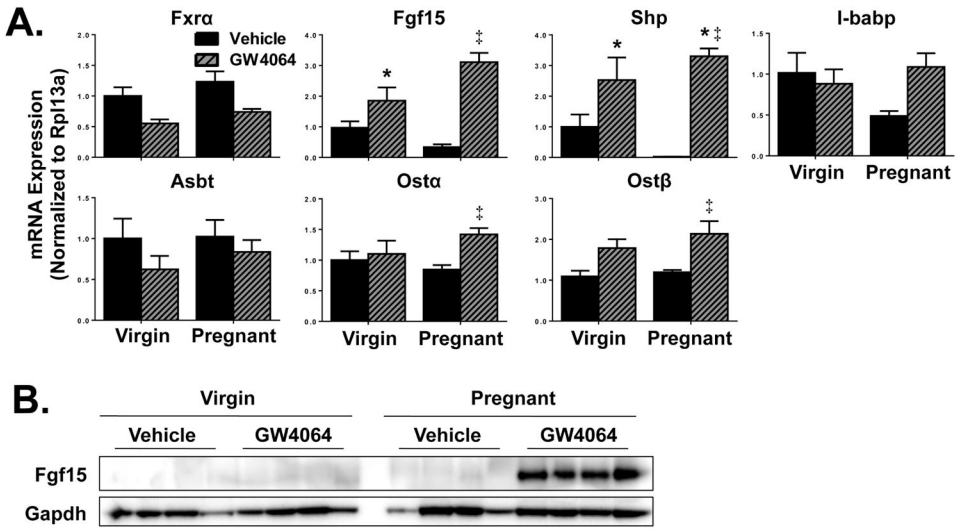

Fig. 4.

Ileal gene and protein expression in vehicle- or GW4064-treated pregnant mice. (A) mRNA and (B) protein expression of Fxr-regulated targets were quantified in ilea from vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Western blot staining of Fgf15 protein was performed using whole ileal homogenates. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5–6). Black bars represent vehicle-treated mice and grey diagonal striped bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

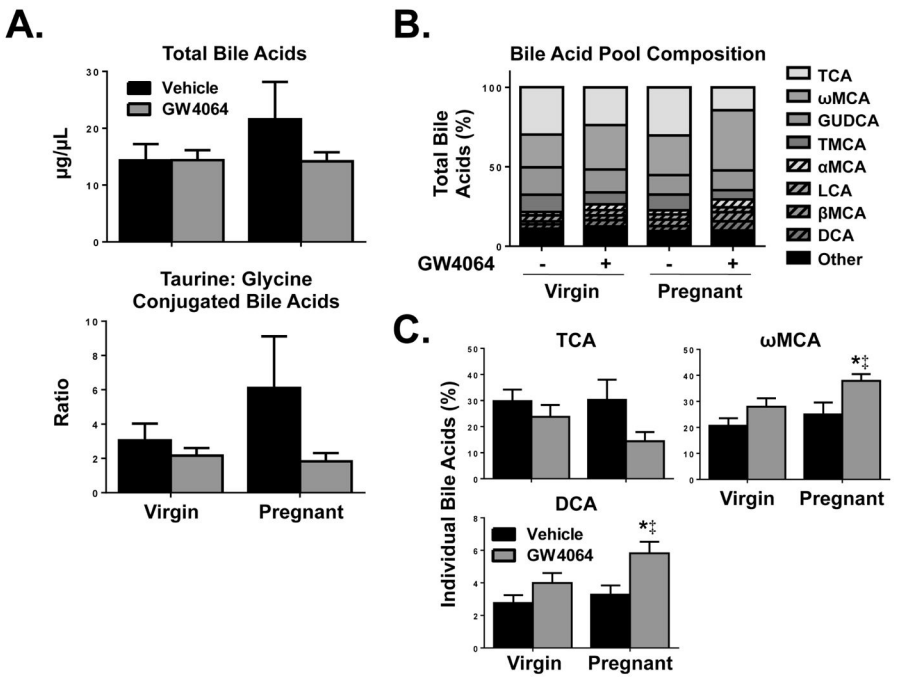

Fig. 5.

Plasma bile acid profiling of virgin and pregnant mice after short-term treatment with GW4064. (A) Total and (B, C) individual bile acids were determined in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Individual bile acids are shown as a percentage of total bile acids per group. Data are presented as mean relative expression ± SE (n=5). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

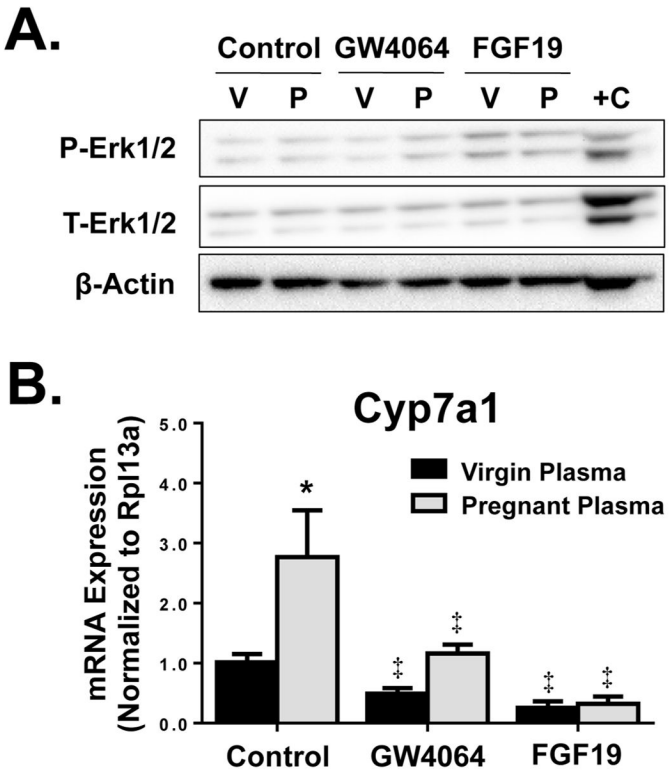

Fig. 6.

Cyp7a1 regulation in sandwich-cultured primary mouse hepatocytes. (A) Erk1/2 activation as indicated by phospho-Erk1/2 (P-Erk) compared to total Erk1/2 (T-Erk) after 1 hour treatment and (B) mRNA expression of Cyp7a1 after 24 hour treatment of sandwich-cultured primary mouse hepatocytes with media containing 20% pooled virgin or pregnant mouse plasma, in the presence of GW4064 (5 μM), recombinant FGF19 protein (20 μg/ml), or vehicle (DMSO). Western blots were performed using whole cell lysates. V, virgin plasma; P, pregnant plasma, mouse liver positive control. Data are presented as mean relative expression ± SD (n=3 donors/independent experiments in triplicate). Black bars represent virgin mouse plasma-treated hepatocytes and grey bars represent pregnant mouse plasma-treated hepatocytes. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with plasma-treated virgin controls. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with plasma-treated pregnant controls.

Table 1

Pregnancy endpoints following GW4064 treatment[1].

	Virgin Vehicle	Virgin GW4064	Pregnant Vehicle	Pregnant GW4064
Liver: Body weight	0.0569±0.0019	0.0554±0.0017	0.0647±0.00070*	0.0681±0.0010*
Resorptions	N/A	N/A	1.00±0.36	0.667±0.33
Estradiol (pg/mL)	7.91±0.4	7.85±1.3	12.8±0.8*	12.1±2.0
Progesterone (ng/mL)	3.70±1.0	2.68±0.6	28.0±1.8*	20.3±3.2*‡
Triglycerides (mg/dL)	36.9±1.1	36.1±2.7	65.0±4.2*	55.7±4.7*
Cholesterol (mg/dL)	91.0±5.7	100.7±3.1	90.9±3.6	92.3±3.1

[1]Body weights, liver weights and number of resorptions were recorded at the time of sacrifice. Total litter sizes ranged from 1 to 9 (mean 5.5). Circulating progesterone, estradiol, total bile acids, triglycerides and cholesterol were quantified for vehicle- and GW4064-treated mice on gestation day 14. Data are presented as mean ± SE (n=5–6).

Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice. N/A: not applicable.