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RESEARCH PAPER

Pregnancy Represses Induction of Efflux Transporters in Livers of Type I Diabetic Mice

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ABSTRACT

Purpose To determine whether down-regulation of transcription factor signaling during pregnancy disrupts the induction of efflux transporters in type I diabetic mice.

Methods Type I diabetes was induced in female C57BL/6 mice with multiple low dose intraperitoneal injections of streptozotocin (STZ) at least 2 weeks prior to mating with normoglycemic male mice. On gestation day 14, livers were collected from vehicle- and STZ-treated non-pregnant and pregnant mice for quantification of efflux transporter and transcription factor signaling.

Results STZ treatment up-regulated expression of Mrp1-5, Mdr1, Abcg5, Abcg8, Bcrp, and Bsep mRNA and/or protein in the livers of non-pregnant mice. Interestingly, little to no change in transporter expression was observed in STZ-treated pregnant mice compared to vehicle- and STZ-treated non-pregnant mice.

Conclusions This study demonstrates the opposing regulation of hepatobiliary efflux transporters in response to diabetes and pregnancy and points to PPARγ, Nrf2, and FXR as candidate pathways underlying the differential expression of transporters.

KEY WORDS ABC transporter · diabetes · efflux transporters · liver · pregnancy

ABBREVIATIONS

Abc	ATP-binding cassette
Bsep	bile salt export pump
Bcrp	breast cancer resistance protein
CAR	constitutive androstane receptor
Cyp	cytochrome P450
FXR	farnesoid x receptor
Mrp	multidrug resistance-associated proteins
Mdr	multidrug resistance proteins
Nqo1	NAD(P)H quinone oxidoreductase 1
Nrf2	nuclear factor E2-related factor 2
PPARγ	peroxisome proliferator activated receptor gamma
PGC-1α	PPARγ coactivator-1alpha
PXR	pregnane X receptor
Shp	short heterodimer protein
STZ	streptozotocin

INTRODUCTION

Hepatobiliary transporters participate in nutrient and xenobiotic trafficking within the liver (reviewed in (1)). ATP-binding cassette (Abc) transporters remove chemicals and their metabolites from hepatocytes into bile or

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back to the blood. These transporters consist of the Multidrug resistance proteins 1a and 1b (Mdr1a/1b, Abcb1a/1b), Multidrug resistance-associated proteins 1–6 (Mrp1-6, Abcc1-6), and the Breast cancer resistance protein (Bcrp, Abcg2) and mediate the efflux of drugs as well as endogenous chemicals from hepatocytes. The Bile salt export pump (Bsep, Abcc11), Abcg5/8, and Abca1 are additional efflux transporters that participate in the biliary excretion of bile acids, sterols, and cholesterol, respectively (reviewed in (1)). Expression and function of hepatobiliary transporters are regulated by multiple mechanisms including, but not limited to, pathological conditions, chemical exposure, and developmental age. Work by our groups and others demonstrate dysregulation of liver transporter expression in rodent models of hyperglycemia, fatty liver, and obesity (2–4). In general, these conditions tend to up-regulate expression of Mrp efflux transporters. Functionally, this can lead to altered biliary disposition of drugs, such as pravastatin (5), as well as anionic dyes (6) and endogenous chemicals (bile acids, phospholipids) (7).

A number of signaling pathways, including the nuclear factor E2-related factor 2 (Nrf2, Nfe2l2) and the peroxisome proliferator activated receptor gamma (PPARγ, Nr1c3), are activated in mouse liver in response to hyperglycemia (8,9). Nrf2 up-regulates expression of drug metabolizing enzymes such as NAD(P)H quinone oxidoreductase 1 (Nqo1) and efflux transporters during periods of oxidative stress (10). PPARγ regulates the expression of cytochrome P450 (Cyp) 4a genes and works in concert with the transcriptional activator PPARγ coactivator-1alpha (PGC-1α) to control glucose- and fatty acid-related gene networks (11,12). More recent evidence has demonstrated that Nrf2 can also regulate the mRNA expression of PPARγ in livers of mice (13,14).

Liver transporter expression is also regulated by physiological conditions, such as pregnancy. Prior research in pregnant rats demonstrates global down-regulation of uptake and efflux transporters in late gestation (15–17). Coupled with changes in synthetic and conjugating enzymes, altered regulation of transporters likely contributes to elevated hepatic and serum bile acids during pregnancy (reviewed in (18)). Recent work in rodents and humans has suggested that altered farnesoid x receptor (FXR, Nr1h4) transcription factor signaling contributes to pregnancy-induced cholestasis (19). FXR has been suggested to be functionally decreased during pregnancy leading to reduced mRNA expression of hepatic uptake and efflux transporters in mice (20). Increased mRNA levels of the short heterodimer protein (Shp, Nr0b2) are routinely used to monitor activation of hepatic FXR signaling (21), however, some recent data suggest that Nrf2 may also regulate Shp in mouse livers (13).

Because of the opposing effects of pregnancy and diabetes on the expression of hepatic transporters, mainly efflux transporters, it is necessary to investigate how the combination of hyperglycemia and pregnancy influences drug and toxicant disposition. Although there are multiple types of diabetes (type I, II, gestational) of clinical relevance during pregnancy, we selected the streptozotocin (STZ) model of type I diabetes for this initial work to minimize variables associated with the other models such as chemical administration during pregnancy or confounding pathological changes such as obesity and steatohepatitis. Therefore, the purpose of the current study was to 1) assess the regulation of hepatic efflux transporters during diabetes, pregnancy, and diabetic pregnancy in female mice, 2) identify potential physiological regulators (such as hormones, ketones, lipids, and bile acids) that may contribute to transporter regulation, and 3) evaluate transcriptional pathways known to regulate mRNA levels of hepatic transporters. PPARγ, Nrf2, and FXR were assessed as candidate regulators of hepatic transporters during diabetic pregnancy.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

Female and male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Female mice (6 to 8 weeks old) were injected intraperitoneally with citrate buffer vehicle (0.01 M, pH4.5) or streptozotocin (STZ, 50 mg/kg/d, 5 ml/kg) (Sigma, St. Louis, MO) for 4 consecutive days. STZ-treated mice were considered diabetic when blood glucose levels peaked over 250 mg/dl on two consecutive occasions. Blood glucose levels were assessed from tail vein blood using a TRUEtrack glucometer in fed mice (Home Diagnostics, Fort Lauderdale, FL). Up to two additional doses of STZ were administered to female mice to achieve sustained non-fasted blood glucose levels over 250 mg/dl. After at least 2 weeks of hyperglycemia, STZ-treated mice, as well as, vehicle-treated female mice were mated overnight with normoglycemic male C57BL/6 mice until a vaginal plug was observed (denoting gestation day 0). At gestation day 14, non-fasted blood glucose levels, weights (body, liver, fetal, placental), and the number of fetuses were recorded during necropsy. There was no difference in litter number between vehicle- and STZ-treated mice. Portions of liver were either snap frozen or fixed in formalin for mRNA, protein, histologic and immunofluorescence analysis. Serum

and frozen samples were stored at −80°C until used for analysis. The Rutgers University Institutional Animal Care and Use Committee approved these studies which followed the "Principles of Laboratory Animal Care".

Histology

Portions of the left lobe of the liver were fixed in 10% neutral-buffered formalin and embedded in paraffin. Livers were sectioned into 5 μm thick sections and stained with hematoxylin and eosin. Sections were examined by light microscopy for histopathological changes.

Serum Analyses

An ELISA assay kit was used to quantify serum insulin (Millipore, Billerica, MA, EZRMI-13K). Colorimetric assay kits were used to quantify serum alanine aminotransferase (ALT, Fisher Scientific, St. Louis, MO, 23-666-087), triglycerides (Fisher Scientific, 23-666-410), total cholesterol (Fisher Scientific, 23-666-200), bile acids (Bioquant San Diego, CA BQ-042A-EALD), and beta-hydroxybutyrate (Stanbio, Boerne, TX, 2440). All assays were performed according to the manufacturers' protocol.

RNA Isolation and Real-Time Quantitative PCR (qPCR)

Total RNA was isolated from frozen livers using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm, and purity confirmed by 260/280 nm ratio. One microgram of total RNA was converted to single-stranded cDNA using oligo$(dT)_{18}$ primers. Messenger RNA levels were then quantified by real-time qPCR using SYBR Green PCR Master Mix (Roche Applied Science, Indianapolis, IN) and analyzed on a Roche Lightcycler detection system (Roche Applied Science, Indianapolis, IN). Target gene expression was normalized to the level of the reference gene, ribosomal protein l13a (Rpl13a) (9).

Western Blot Analysis

Livers were homogenized in sucrose (250 mM)-Tris (10 mM) buffer containing protease inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Proteins (50 μg/lane) were electrophoretically resolved using polyacrylamide 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Gels were transblotted overnight at 4°C onto polyvinylidene fluoride membrane. Western blot staining of Mrp2-6, Bsep, Bcrp, and Mdr1a/b was performed as described previously (22). Antibodies included: Mrp2 (M_2III-5, Enzo Life Sciences, Farmingdale, NY), Mrp3 (M_3II-2 G. Scheffer, VU Medical Center, Amsterdam, The Netherlands), Mrp4 (M_4I-10, Enzo Life Sciences), Mrp5 (M_5I-10, G. Scheffer), Mrp6 (M_6II-68, G. Scheffer), Bcrp (BXP-53, Enzo Life Sciences), Bsep (K44, B. Steiger, University Hospital, Zurich, Switzerland) and Mdr1a/b (C219, Abcam, Cambridge, MA). β-actin protein was used as a reference protein for normalization. Anti-β-actin antibody was purchased from Abcam (ab8227). After incubation with secondary antibodies, antibody-protein complexes were detected and quantified using a Fluorchem imager (Alpha Innotech, San Leandro, CA).

Indirect Immunofluorescence

Livers were embedded in Optimal Cutting Temperature compound and brought to −20°C. Cryosections (5 μm) were thaw-mounted onto Superfrost glass slides (Fisher Scientific) and stored at −80°C with a desiccant until use. Tissue sections were fixed with 4% paraformaldehyde for 5 min. Immunofluorescence analysis was limited to Mrp2-4, 6, and Bsep. Liver sections were blocked with 5% goat serum/phosphate-buffered saline with 0.1% Triton X (PBS-Tx) for 1 h and then incubated with primary antibodies against Mrp2 (the antibody for immunofluorescence was obtained from B. Steiger, University Hospital, Zurich, Switzerland), Mrp3 (M_3II-2), Mrp4 (M_4I-10), Mrp6 (M_6II-68), or Bsep (K44) diluted 1:100 in 5% goat serum/PBS-Tx for 2 h at room temperature. After incubation with primary antibody, the sections were washed and incubated for 1 h with goat anti-rat Alexa 488 IgG (Invitrogen Corporation, Carlsbad, California) diluted 1:200 in 5% goat serum/PBS-Tx. Sections were air dried and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (Invitrogen Corp.). Images were acquired on a Zeiss Observer D1 microscope with an x-cite series 120Q fluorescent illuminator (Zeiss Inc., Thornwood, NY) and a Jenoptik camera with ProgRes CapturePro 2.8 software (Jenoptik, Easthampton, MA). All sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were included to ensure minimal nonspecific staining (data not shown).

Transcription Factor Binding Assays

Nuclear extracts were prepared with the NE-PER nuclear extraction kit (Pierce Biotechnology, Rockford, IL) and used to quantify DNA binding of Nrf2 and PPARγ using Transcription Factor Assay Kits (Kit 50296 Active Motif, Carlsbad, CA and Kit 10006855, Cayman Chemicals, Ann Arbor, MI, respectively).

Statistical Analysis

The software program GraphPad Prism© version 5 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Differences among groups were determined by a one-way analysis of variance followed by a Newman-Keul's posthoc test. Differences were considered statistically significance at $p<0.05$.

RESULTS

Hepatic mRNA Expression of Efflux Transporters During Diabetic Pregnancy

Non-pregnant mice treated with STZ exhibited up to a 2-fold increase in the mRNA expression of canalicular efflux transporters Mrp2, Bcrp, Abcg5, and Abcg8 relative to vehicle-treated mice (Fig. 1a). A similar trend was observed for Mdr1a mRNA that was not statistically significant. Interestingly, pregnant mice treated with STZ demonstrated little (Bcrp, Abcg5/8) to no (Mrp2, Mdr1a) induction of apical transporters compared to vehicle, non-pregnant mice. Bsep mRNA was unchanged by STZ treatment and pregnancy.

Messenger RNAs of basolateral efflux transporters (Mrp1, Mrp3-5) were up-regulated by STZ treatment in non-pregnant mice, in particular a 6-fold induction of Mrp4 mRNA (Fig. 1b). Similar to canalicular transporters, pregnant mice treated with STZ demonstrated little (Mrp4) to no (Mrp1, Mrp5) induction of basolateral transporters. There was an exception to this trend. Mrp3 mRNA was induced to the same extent in non-pregnant and pregnant mice treated with STZ. In addition, Abca1 mRNA was unchanged in all groups of mice whereas Mrp6 mRNA was reduced 45% in pregnant mice, regardless of STZ treatment.

Hepatic Protein Expression and Immunofluorescent Staining of Efflux Transporters During Diabetic Pregnancy

Protein expression of canalicular (Mrp2, Mdr1, Bcrp, Bsep, Fig. 2a) and basolateral (Mrp3-6, Fig. 2b) transporters was limited to proteins for which there were available antibodies that detect mouse isoforms. Non-pregnant mice treated with

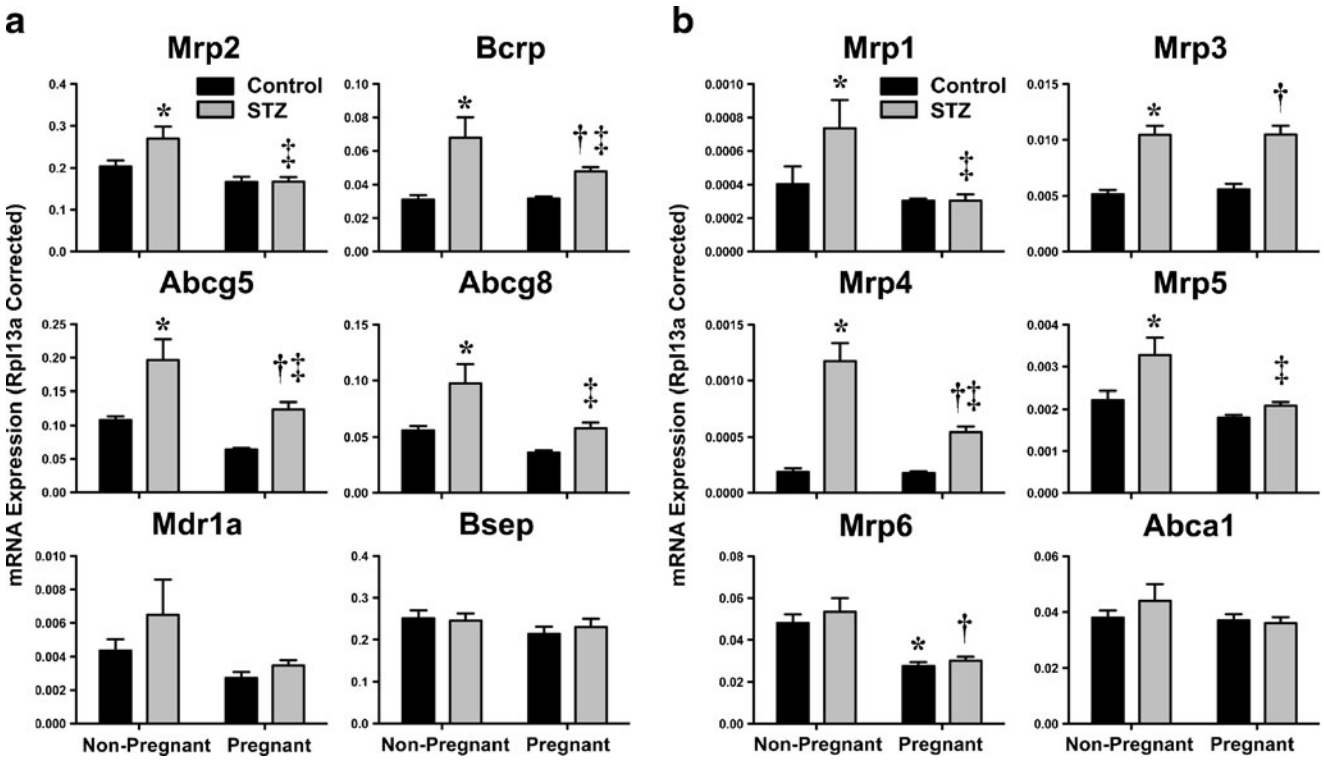

Fig. 1 Hepatic mRNA expression of efflux transporters in diabetic pregnancy. Messenger RNA expression of canalicular (**a**) and basolateral (**b**) efflux transporters was quantified using total hepatic RNA from vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. *Black bars* represent vehicle-treated mice and *light gray bars* represent STZ-treated mice. Data were normalized to mRNA levels of ribosomal protein 113A (Rpl13A) and presented as mean ± SE ($n=4$–11). Asterisks (*) represent statistically significant differences ($p<0.05$) compared to vehicle-treated, non-pregnant mice. Daggers (†) represent statistically significant differences ($p<0.05$) compared to vehicle-treated, pregnant mice. Double daggers (‡) represent statistically significant differences ($p<0.05$) compared to STZ-treated, non-pregnant mice.

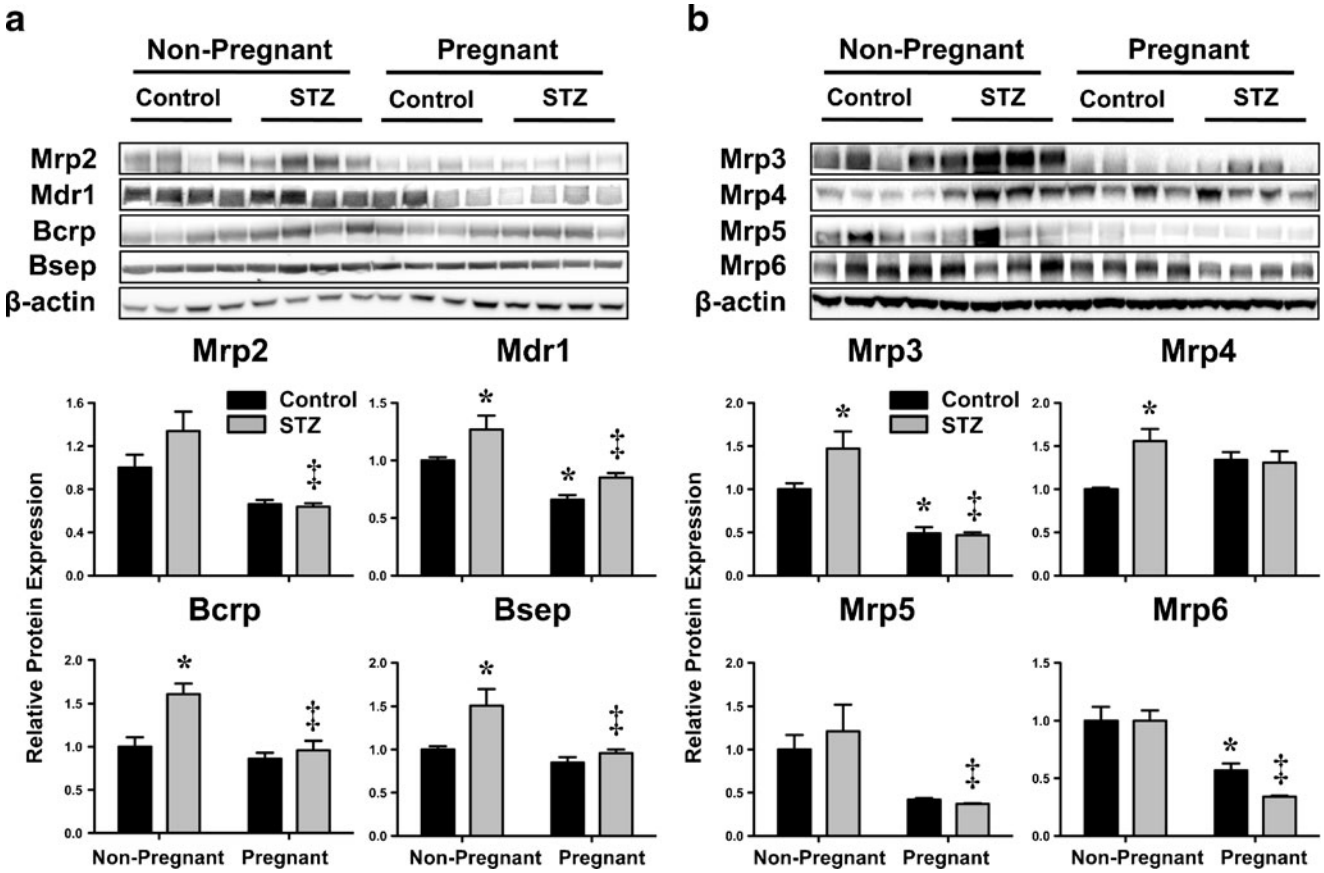

Fig. 2 Hepatic protein expression of efflux transporters in diabetic pregnancy. Liver expression of canalicular (**a**) and basolateral (**b**) efflux proteins was quantified by western blot (50 μg protein homogenates/lane) from vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. β-actin was used as a loading control. The western blot data are presented as individual blots and mean relative protein expression. *Black bars* represent vehicle-treated mice and *light gray bars* represent STZ-treated mice. Data are normalized to vehicle-treated, non-pregnant controls and presented as mean ± SE ($n = 4$). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, non-pregnant mice. Double daggers (‡) represent statistically significant differences ($p < 0.05$) compared to STZ-treated, non-pregnant mice.

STZ had 27–56% increases in expression of Mrp3-4, Mdr1, Bcrp, and Bsep compared to vehicle-treated mice. A trend for increased Mrp2 after STZ was also observed. Similar to mRNA expression, STZ-mediated induction of the Mrp3-4, Mdr1, Bcrp, and Bsep transporters was not observed in pregnant mice. Mrp5 and 6 proteins were not altered by STZ treatment, but were down-regulated by pregnancy. Mrp1 protein could not be detected (data not shown). There are no available antibodies that reliably detect mouse Abca1, Abcg5, and Abcg8 proteins.

As expected, immunofluorescent staining of liver sections demonstrated canalicular staining for Mrp2 and Bsep proteins and basolateral staining for Mrp3, 4, and 6 proteins (Fig. 3). Liver sections from non-pregnant mice treated with STZ had enhanced staining of Mrp2 and Bsep proteins throughout the lobule that was not observed in any other treatment group. In non-pregnant mice that were treated with vehicle, Mrp3 staining was primarily observed in centrilobular hepatocytes. In response to STZ treatment, Mrp3 staining was enhanced and extended throughout the liver lobule. Liver sections from pregnant mice treated with either vehicle or STZ demonstrated reduced Mrp3 protein compared to their non-pregnant counterparts. Mrp4 staining was very low in non-pregnant mice treated with vehicle, which was enhanced in hepatocytes 1 to 2 rows from the central vein in livers from STZ-treated mice. A modest increase in Mrp4 staining was also detected in pregnant mice treated with STZ. Similar to its mRNA and protein expression, Mrp6 staining was unaffected by STZ but was slightly reduced in liver sections from pregnant mice.

Clinical and Anatomic Pathology During Diabetic Pregnancy

Indicators of physiological and pathological changes were investigated because they can alter transporter expression, and thus are potentially regulators of transporters. STZ treatment increased circulating glucose levels over 2-fold in

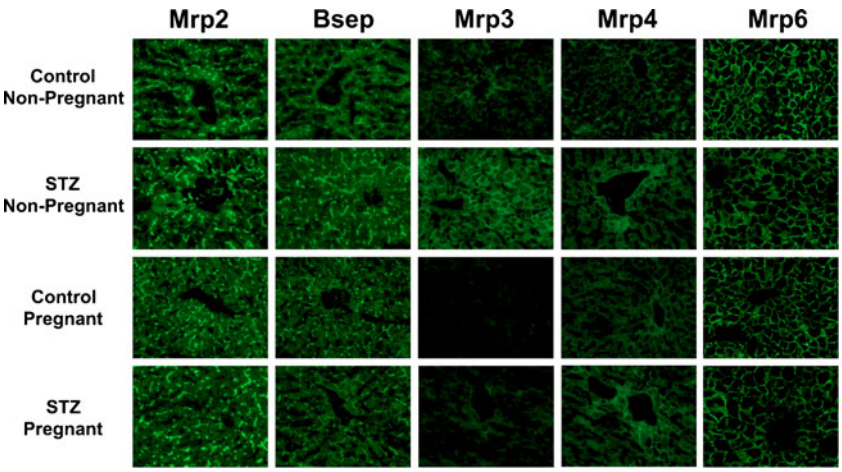

Fig. 3 Immunofluorescent staining of efflux transporters in diabetic pregnancy. Indirect immunofluorescence against canalicular (Mrp2 and Bsep) and sinusoidal (Mrp3, 4, 6) transporters (*green*) was conducted on liver cryosections (5 μm) obtained on gestation day 14 from vehicle- and STZ-treated non-pregnant and pregnant mice. Representative regions are shown. Magnification, ×320.

fed non-pregnant and pregnant mice (Table I). Corresponding decreases in serum insulin levels were observed in non-pregnant and pregnant mice treated with STZ. Compared to vehicle-treated mice, there was a trend towards slight increases (2-fold) in serum ALT activity, an indicator of hepatocyte damage, in non-pregnant and pregnant mice treated with STZ. Serum triglyceride levels were normal in non-pregnant mice, but were elevated approximately 2- to 3-fold in pregnant mice as demonstrated previously (23). Circulating total cholesterol levels were largely unchanged (data not shown). There were trends for slight increases in serum bile acids in pregnant mice treated with vehicle that were even higher in pregnant mice treated with STZ. Livers from non-pregnant mice regardless of treatment exhibited normal histology (Fig. 4). Mild microvesicular vacuolation was observed in livers from pregnant mice treated with STZ, which was not evident in livers from pregnant mice treated with vehicle.

Transcriptional Signaling Pathways During Diabetic Pregnancy

Multiple transcriptional pathways have been shown to regulate efflux transporter expression in rodent liver (24–26). PPAR signaling, notably through PPARα, has been shown to induce Mrp2-4 and Bcrp mRNA expression (27,28). Because PPARα and PPARγ share common response elements and PPARγ is important in the progression of diabetes, both isoforms were quantified in the present study. Liver mRNA levels of PPARγ were induced 3-fold by STZ treatment in non-pregnant mice, which was not observed in pregnant mice (Fig. 5a). Similarly, increased nuclear PPARγ binding was observed only in livers from non-pregnant mice treated with STZ (Fig. 5b). Conversely, PPARα mRNA was unchanged by STZ but was reduced by pregnancy (Fig. 5a). The common PPAR-target gene cytochrome P450 4a14 (Cyp4a14) and its co-regulator PGC-1α were up-regulated up to 2-fold only in livers of non-pregnant mice treated with STZ (Fig. 5c).

Expression of Nrf2 mRNA and nuclear Nrf2 binding were increased 80 and 90%, respectively, in livers of non-pregnant mice administered STZ (Fig. 6a and b). Pregnancy reduced Nrf2 mRNA and binding to baseline levels in STZ-treated mice. Nqo1 is a target gene of Nrf2 signaling and is up-regulated during periods of oxidative stress. Nqo1 mRNA was induced 2-fold in non-pregnant and pregnant mice treated with STZ (Fig. 6a). FXR mRNA was unchanged by STZ treatment but was decreased 30% in pregnant mice (Fig. 6c). Shp is under the direct transcriptional activation of FXR and acts mostly as a transcriptional repressor of

Table I Clinical Pathology of Non-Pregnant and Pregnant Mice Treated with Vehicle or STZ

Treatment groups	Glucose (mg/dl)	Insulin (ng/ml)	ALT (U/l)	Triglycerides (mg/dl)	Total bile acids (μmol/l)
Non-pregnant control	152.3 ± 7.9	0.69 ± 0.07	8.7 ± 1.0	29.3 ± 1.8	2.8 ± 0.2
Non-pregnant STZ	474.3 ± 29.1*	0.43 ± 0.01*	19.0 ± 2.5	21.6 ± 2.3	3.8 ± 0.5
Pregnant control	123.3 ± 1.9	0.83 ± 0.14	7.4 ± 0.9	104.9 ± 14.8*	5.9 ± 2.0
Pregnant STZ	488.8 ± 25.2†	0.47 ± 0.03†	19.4 ± 4.3	69.8 ± 9.1†‡	10.7 ± 3.5

Blood glucose as well as serum insulin, ALT activity, triglycerides and total bile acids were quantified in vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. Data are presented as mean ± SE ($n = 4$–11). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, non-pregnant mice. Daggers (†) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, pregnant mice. Double daggers (‡) represent statistically significant differences ($p < 0.05$) compared to STZ-treated, non-pregnant mice

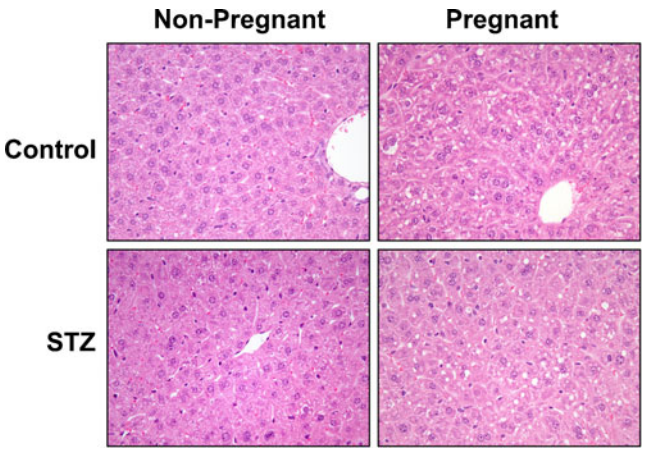

Fig. 4 Liver histology in diabetic pregnancy. Livers were collected on gestation day 14 from vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. Samples were fixed in zinc formalin prior to routine processing and paraffin embedding. Sections (5 μm) of livers were stained with hematoxylin and eosin and examined by light microscopy for histopathological changes. Paraffin-embedded liver sections from non-pregnant mice, regardless of treatment, and vehicle-treated pregnant mice had normal histology. Minimal to mild microvesicular vacuolation was observed in STZ-treated, pregnant mice. Magnification, ×400.

numerous genes involved in bile acid synthesis and transport. Shp mRNA was increased 1.7-fold by STZ in non-pregnant mice with no change in pregnant mice.

DISCUSSION

This study investigated the opposing regulation of hepatobiliary transporters in response to diabetes and pregnancy. Type I diabetes was induced by multiple low doses of STZ and was confirmed by increased serum glucose and decreased serum insulin after 4 weeks. Because STZ is cleared from the plasma and liver within 2 and 24 h, respectively (29), changes in hepatic expression of genes is likely due to hypoinsulinemia and/or hyperglycemia rather than STZ itself. As expected, mice treated with STZ had induced mRNA and/or protein expression of liver efflux transporters, Mrp1-5, Mdr1, Abcg5/8, Bcrp and Bsep. Interestingly, up-regulation of these transporters was largely attenuated by pregnancy. This response did not appear to be related to levels of circulating glucose, insulin, cholesterol, bile acids, or triglycerides since their relative abundance differed from the patterns of transporter expression. Furthermore, there was little evidence of hepatic injury with only 2-fold increases in serum ALT activity and mild microvesicular vacuolation of unknown etiology in the livers of STZ-treated pregnant mice. Rather, the pattern of efflux transporter expression resembled key transcriptional pathways previously shown to regulate transporters. STZ treatment enhanced PPARγ, Nrf2, and FXR signaling, as quantified by nuclear binding and target gene regulation (Cyp4a14, Nqo1, and Shp, respectively). Activation of these transcriptional

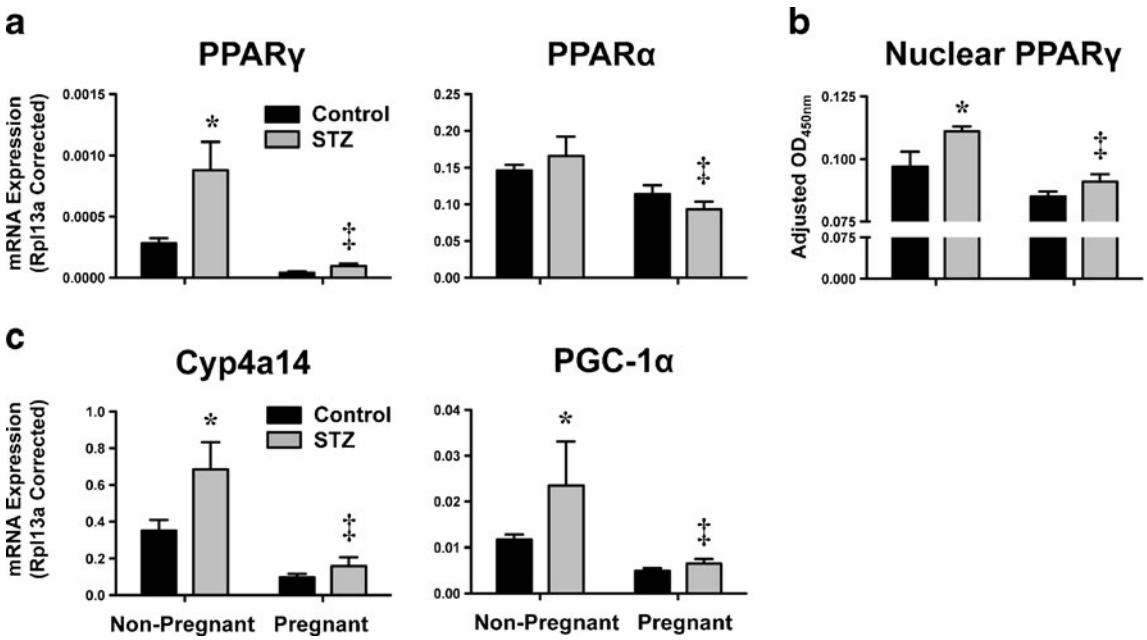

Fig. 5 Hepatic PPAR transcription factor signaling in diabetic pregnancy. (**a**) mRNA expression of transcription factors, (**b**) nuclear binding, and (**c**) mRNA of target genes were quantified in livers from vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. *Black bars* represent vehicle-treated mice and light gray bars represent STZ-treated mice. Data were normalized to mRNA levels of ribosomal protein l13A (Rpl13A) and presented as mean ± SE ($n = 4$–11). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, non-pregnant mice. Double daggers (‡) represent statistically significant differences ($p < 0.05$) compared to STZ-treated, non-pregnant mice.

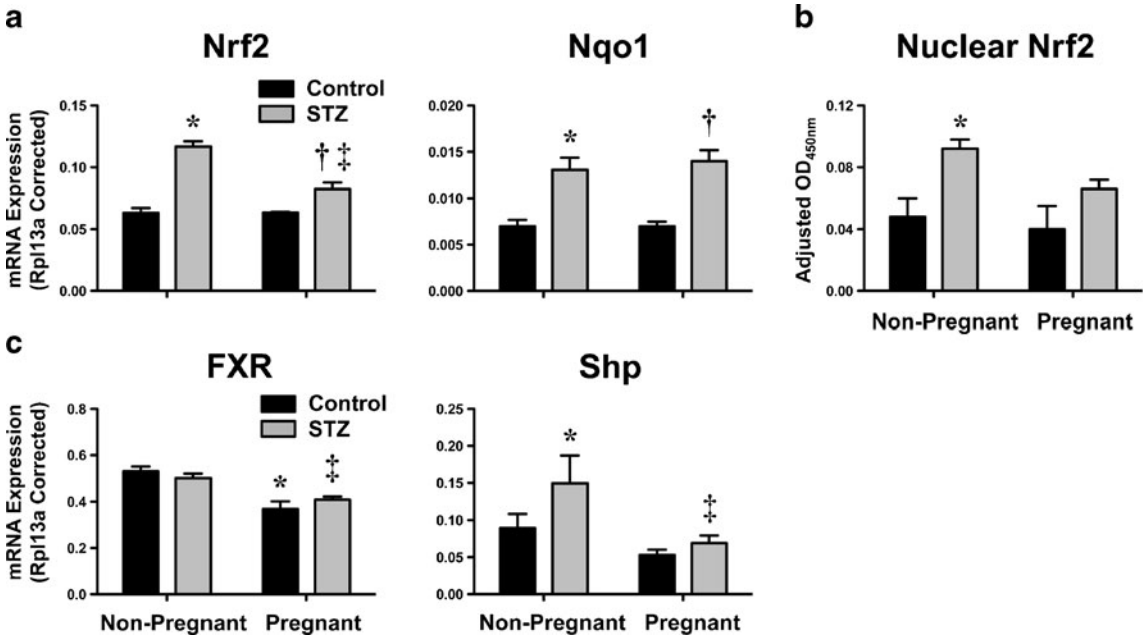

Fig. 6 Hepatic Nrf2 and FXR transcription factor signaling in diabetic pregnancy. (**a**) mRNA expression of Nrf2 and target gene Nqo1, (**b**) Nrf2 nuclear binding, and (**c**) mRNA of FXR and its target gene Shp were quantified in livers from vehicle- and STZ-treated non-pregnant and pregnant mice on gestation day 14. *Black bars* represent vehicle-treated mice and *light gray bars* represent STZ-treated mice. Data were normalized to mRNA levels of ribosomal protein 113A (Rpl13A) and presented as mean ± SE ($n = 4–11$). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, non-pregnant mice. Daggers (†) represent statistically significant differences ($p < 0.05$) compared to vehicle-treated, pregnant mice. Double daggers (‡) represent statistically significant differences ($p < 0.05$) compared to STZ-treated, non-pregnant mice.

pathways was suppressed by pregnancy. Collectively, this study demonstrates that pregnancy represses the induction of hepatobiliary efflux transporters in response to hyperglycemia and hypoinsulinemia caused by STZ and points to PPARγ, Nrf2, and FXR as candidate pathways underlying the differential expression of transporters (Fig. 7).

Hepatobiliary Transport in Rodent Models of Diabetes

Hepatobiliary excretion is altered in rodent models of diabetes. For example, biliary excretion of pravastatin (5) is reduced whereas clearance of rose bengal into bile (6) is enhanced in rats administered STZ. It is important to consider the timing of when pharmacokinetic analysis is performed in diabetic animals since there can be a biphasic response with early reductions in bile flow (< 2 days) followed by subsequent increases (5–10 days) (30,31). Altered hepatic clearance of these chemicals may be due to changes in hepatobiliary transporter expression. For example, diabetic rats have higher secretion of bile acids, cholesterol and phospholipids into bile (7,32,33). Enhanced biliary clearance of these endobiotics may be in part due to up-regulation of efflux transporters such as Bsep, Abca1, and Abcg5/8.

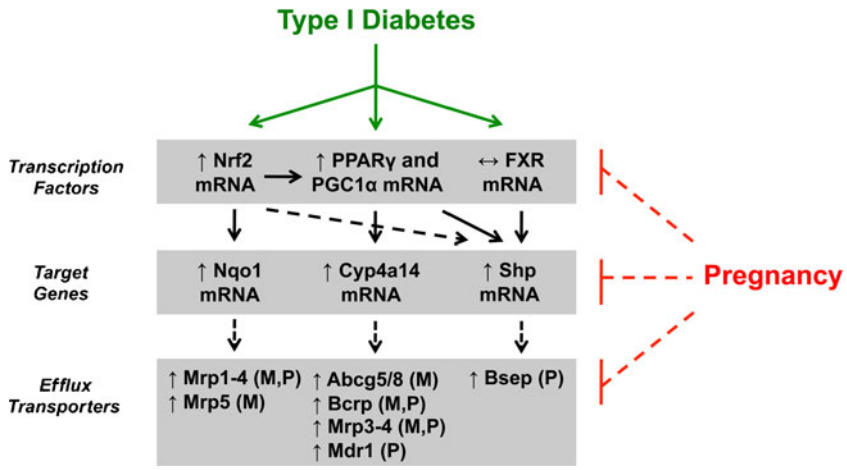

Fig. 7 Proposed hepatic transcription factor and transporter regulation in diabetic pregnancy. STZ treatment induced hyperglycemia and activated PPARγ, Nrf2, and FXR signaling pathways by increasing transcription factor and/or target gene mRNA. Potential downstream efflux transporters were induced at the mRNA (M) and/or protein (P) levels. Pregnancy directly or indirectly repressed expression of transcription factor and transporter pathways in maternal livers.

The STZ model of diabetes has been used in other investigations to assess regulation of hepatic transporters. Administration of a single dose of STZ to rats increases liver Mrp3 and Mrp4 mRNAs and decreases Mdr1a and Bcrp mRNAs (5,34). In two separate studies, Mrp2 mRNA was decreased in rat livers at 9 and 28 days (5,34). However, a recent report demonstrated that Mrp2 protein is elevated in livers of rats 5 weeks after STZ treatment. Induction of Mrp2 protein in STZ-treated rats correlated with a functional increase in the biliary excretion of the Mrp2 substrate sulfobromophthalein (35). This suggests that the mRNA and protein expression of Mrp2 in rat liver in response to STZ may be under different regulatory mechanisms.

Work by Anger *et al.* (2010) went a step further and established gestational diabetes in rats using a single dose of STZ on gestation day 6. Compared to normoglycemic pregnant rats, the expression of Mdr1 protein was increased in diabetic pregnant rats on gestation day 20 (36). This was not observed in the present study, which may reflect differences in experimental design (species, dosing regimen, or gestational day). We initiated hyperglycemia prior to pregnancy to eliminate the direct chemical effects of STZ as a confounder. Therefore, additional work will be necessary to determine whether the current findings are applicable to gestational diabetes.

Pregnancy and Hepatic Transporters

Late in pregnancy rats exhibit down-regulation of several efflux transporters including Mrp2, 3, and 6 (17). To date, most studies investigating transporters in pregnancy have focused on late gestation and the postpartum period (16,17). Recent work from our laboratory has demonstrated that there is a global decline in the mRNA expression of Bsep, Mrp2, 3, 6, Bcrp, and Abcg5/8 at varying time points between gestation days 7 and 17 (37). From this same study, it was also observed that there were declines in various signaling pathways including PPARα and FXR as well as the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) confirming a prior study (23).

PPAR Signaling

Livers of diabetic db/db mice exhibit elevated PPARγ mRNA with little to no change in PPARα mRNA (38,39). Similarly, we observed up-regulation of hepatic PPARγ mRNA as well as nuclear protein binding following STZ treatment in non-pregnant mice. In addition, PPAR-related genes Cyp4a14 and PGC-1α were also increased by STZ. No change in PPARα mRNA was observed in STZ-treated mice. Treatment with STZ has been previously shown to increase PGC-1α mRNA in livers of mice (40). The ability of PPARs to regulate efflux transporters has largely focused upon PPARα. Activation of PPARα with clofibrate increases mRNA expression of Mrp3, Mrp4, and Bcrp in wild-type, but not PPARα-null mice (27). Furthermore, *in silico* analysis demonstrated potential PPRE sites in the 5′-flanking regions of Mrp3, Mrp4, and Bcrp that may participate in receptor-mediated transactivation (27). Similar up-regulation of liver Mdr1a and 1b mRNAs by the PPARα ligand ciprofibrate has been demonstrated in mice (41). Likewise, induction of Abcg5 and 8 mRNAs in response to fasting is observed in livers of wild-type, but not PPARα-null mice (42). Additional studies have demonstrated that PPARγ agonists up-regulate Bcrp, Abcg5, and Abcg8 mRNA. For example, rosiglitazone increases BCRP mRNA and protein human intestinal Caco-2 (43) and hepatoma Huh-7 cells (44). In addition, pioglitazone enhances Abcg5 and Abcg8 mRNA in rat livers (45). Given the overlap in PPRE sequences for PPARα and γ, it is feasible that up-regulation of PPARγ may be critical for the up-regulation of not only Abcg5/8 and Bcrp, but also Mrp3-4 and Mdr1 mRNA and/or protein.

FXR Signaling

FXR regulates the mRNA expression of mouse and human Bsep (46). Prior studies demonstrate that STZ treatment has little to no effect on Bsep mRNA and protein in rat livers (7,47). However, in the present study, Bsep protein was elevated in STZ-treated non-pregnant mice with no change in mRNA expression. While FXR is the primary regulator of Bsep in rodent liver, it is unclear the exact mechanism for selective induction of protein only. Although FXR mRNA was unchanged by STZ, its target gene Shp was induced suggesting FXR signaling was activated. Because only one time point was analyzed in this study, it is possible that induction of Bsep mRNA may have occurred at an earlier time point after STZ treatment and initiation of hyperglycemia. It should also be noted that PGC-1α can regulate mRNA expression of Shp (48) and act as a transactivator of FXR (49). Thus, activation of FXR signaling may be occurring through an indirect mechanism in response to STZ treatment. Interestingly, up-regulation of Bsep protein by STZ was attenuated in livers of pregnant mice in a fashion similar to Shp and PGC-1α mRNA. A recent study has pointed to the ability of a 3β-sulfated progesterone metabolite (epiallopregnanolone sulfate) to inhibit ligand-dependent FXR activation (50). This metabolite is associated with intrahepatic cholestasis of pregnancy and may be a potential mediator for repression of FXR signaling during diabetic pregnancy.

Nrf2 Signaling

STZ-induced hyperglycemia activates Nrf2 signaling in the livers of mice (9). Unpublished data from our laboratory has demonstrated that the hepatic induction of Mrp2-4 mRNA and protein in response to STZ treatment in male mice is absent in Nrf2-null mice. Nrf2 has been previously demonstrated to regulate hepatic induction of Mrp2-4 mRNA in response to acetaminophen hepatotoxicity (22) and perfluoroctanoic acid treatment (28). Work by other groups also suggests that Nrf2 regulates the expression of Mrp1 (51) and Mrp5 (25). In the present study, expression of Nrf2 mRNA was enhanced in STZ-treated non-pregnant mice but was attenuated in pregnant mice. A similar pattern was observed for Nrf2 binding, although the 30% decline in binding between STZ-treated non-pregnant and pregnant mice was not statistically significant. We have determined that the basal regulation of Nrf2 is unchanged by pregnancy, however, the present study suggests that the activation of Nrf2 is dampened by pregnancy. Combined with the fact that Nrf2 regulates Mrp expression in mouse liver, it is probable that the observed patterns of transporter regulation (induction with STZ and repression in pregnancy) may be regulated, in part, through Nrf2 signaling. Interestingly, the Nrf2 target gene, Nqo1, remained elevated in pregnant mice treated with STZ suggesting that other redox-related transcriptional pathways continue to be activated in pregnant mice. Furthermore, there is increasing evidence that Nrf2 regulates Shp and PPARγ expression (13,52) and this transcription factor may be a master regulator of the transporter gene changes observed in this study.

Other Transcription Factor Signaling

Work by Dong *et al.* have demonstrated induction of drug metabolism genes such as the Cyp2b10 in wild-type mice treated with STZ, but not mice lacking CAR (40). Because of the critical role of CAR in regulating expression of drug efflux transporters (reviewed in (1)), we similarly quantified mRNA levels of CAR and Cyp2b10 and observed no change in expression in non-pregnant mice treated with STZ (data not shown). In addition, the mRNA levels of PXR and its target gene Cyp3a11 were not changed with STZ treatment (data not shown). These data suggested that CAR and PXR played a minimal role in the transporter changes, particularly in response to STZ, observed in the present study. In addition, we observed overall declines in the mRNAs of other transcription factors including the liver x receptor and hepatocyte nuclear factors 1a, 1b, 3 and 4 in pregnant mice (data not shown). However, there was little enhancement of these pathways in response to STZ. Given the fact that these pathways are suppressed in response to pregnancy, these transcription factors could play a role in the general repression of transporters in pregnant mice rather than their induction in non-pregnant mice treated with STZ.

CONCLUSION

The present study demonstrates that pregnancy attenuates the hepatic induction of efflux transporters in response to uncontrolled hyperglycemia in female mice. Additional studies are needed to definitively test the roles of Nrf2, PPARγ, and FXR signaling in regulating transporter expression during diabetes and pregnancy. Moreover, it is critically important to identify the roles for sex hormones in the down-regulation of transcription factor and transporter mRNA during pregnancy. Not only will these findings be critical for understanding the regulation of transporters by opposing pathways, but also the potential changes in maternal-fetal drug and toxicant disposition during normal and diabetic pregnancies.

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