Impact of polyinosinic:polycytidylic acid on placental and hepatobiliary drug transporters in pregnant rats

Vanja Petrovic and Micheline Piquette-Miller

Department of Pharmaceutical Sciences, University of Toronto,
Toronto, Ontario, Canada
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Corresponding Author:
Micheline Piquette-Miller, Ph.D.
Professor, Leslie Dan Faculty of Pharmacy
University of Toronto
144 College Street
Toronto, ON M5S 3M2, Canada
Tel: (416) 946-3057
Fax: (416) 978-8511
m.piquette.miller@utoronto.ca

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List of non-standard abbreviations:
ABC: ATP-binding cassette
ANOVA: analysis of variance
Bcrp: breast cancer resistance protein
Bsep: bile salt export pump
CYP: cytochrome P-450
ELISA: enzyme-linked immunosorbent assay
IFN: interferon
IL: interleukin
LPS: lipopolysaccharide
Mrp: multidrug resistance-associated protein
Ntcp: sodium-taurocholate-cotransporting polypeptide
Oatp: organic anion-transporting polypeptide
P-gp: P-glycoprotein
Poly I:C: polyinosinic:polycytidylic acid
qRT-PCR: quantitative reverse-transcription polymerase chain reaction
SLC: solute carrier
TNF: tumor necrosis factor
ABSTRACT

While inflammation is known to impose changes in the expression and activity of drug transporters, little is known about the impact of inflammatory stimuli on these transporters during pregnancy. Our objective was to study the effect of viral-induced inflammation on key maternal hepatic and placental drug transporters and their endogenous substrates. Acute inflammation was induced in pregnant Sprague-Dawley rats (G17-18, n=5-6/group) by single i.p. doses of poly I:C (2.5 or 5.0 mg/kg) with saline as control. Tissues were harvested 24 hours later. Expression of transporters was measured via real-time PCR and Western blotting. Maternal plasma levels of cytokines, bile acids and bilirubin, as well as fetal bile acids were examined. Plasma concentrations of IFN-γ, TNF-α and IL-6 were significantly induced in poly I:C-treated rats, as compared to controls (p<0.001). Significant downregulation of placental Abcb1a/b, Abcc1, Abcc3, Abcg2, Slco1a4 and Slco4a1 mRNA, as well as hepatic Abcc2, Abcg2, Slco1a4, Slc10a1 and Cyp3a2 mRNA was observed in poly I:C-treated rats. Hepatic Abcb1b and Abcc3 mRNA levels were significantly induced. Hepatic protein levels of P-gp, Mrp2 and Bcrp were significantly downregulated relative to controls (p<0.05). Total bile acids in maternal plasma were significantly increased at the higher dose of poly I:C. In summary, the poly I:C model of viral infection imposes significant changes in the expression of key drug transporters in placental and hepatic tissues of pregnant rats. Since many clinically important endogenous and exogenous compounds are substrates of these transporters, inflammation-mediated changes in transporter expression could affect their maternal disposition and fetal exposure.
INTRODUCTION

Membrane transport proteins are of critical importance in the cellular uptake and efflux of numerous endogenous and xenobiotic compounds in the body. Pharmacokinetic processes often involve active transport of substrates across epithelial membranes, and this frequently involves members of the ATP-binding cassette (ABC) transporter family. ABC proteins are efflux transporters, several of which are known to move many clinically important drugs, as well as endogenous substrates such as biliary compounds. P-glycoprotein (P-gp, encoded by \textit{ABCB1} in humans and \textit{Abcb1a} and \textit{Abcb1b} in rodents), several of the multidrug resistance-associated proteins (MRPs, encoded by \textit{Abcc} genes) and the breast cancer resistance protein (Bcrp, encoded by \textit{Abcg2}) are of particular importance in this regard. These drug transporters are highly expressed in the epithelium of the liver, gastrointestinal tract, brain and placenta (Schinkel and Jonker, 2003).

The placenta plays an integral role in the protection of fetus from potentially harmful xenobiotics and endogenous substrates that may be found in the maternal circulation. Moreover, since the fetal hepatobiliary system is not fully developed during gestation, the placenta also plays a crucial role in detoxification of cholephilic compounds during intrauterine life (Macias et al., 2009). Key placental ABC drug transporters are believed to aid in the efflux or removal of such endogenous substrates as well as other exogenous substrates, thus serving a protective role in this tissue. Dramatically increased accumulation of P-gp substrates (digoxin, saquinavir, and paclitaxel) in the fetus of P-gp deficient mice has been demonstrated (Smit et al., 1999). Likewise, studies in Bcrp1\textsuperscript{−/−} mice demonstrated a significant increase in fetal accumulation of Bcrp substrates.
(nitrofurantoin, glyburide) as compared to wildtype mice (Zhang et al., 2007; Zhou et al., 2008).

It is well-established that a number of inflammation-inducing agents, such as vaccines, chemicals, cytokines or endotoxins can lead to changes in drug metabolism and disposition by downregulating cytochrome P450 (CYP) metabolizing enzymes (Morgan, 1997; Renton, 2001). An ever increasing amount of data now also points to the involvement of drug transporting proteins in the phenomenon of inflammation-mediated changes in drug disposition, a process thought to be regulated by pro-inflammatory mediators and nuclear hormone receptors (Petrovic et al., 2007; Morgan et al., 2008; Teng and Piquette-Miller, 2008). Previous studies have demonstrated that an acute inflammatory response induced by bacterial endotoxin (lipopolysaccharide, LPS) downregulates the expression and activity of key ABC transporters in rodent liver, brain, renal, intestinal and placental tissues with a corresponding change in drug disposition (reviewed in Morgan et al., 2008; Petrovic et al., 2007). Additionally, other disease models associated with inflammation, such as induced chronic renal failure, diabetes, cholestasis or cancer have been shown to modulate the hepatic expression of several transporters (Nolin et al., 2008; Anger et al., 2009; Teng and Piquette-Miller, 2007; Yang et al., 2009; Slaviero et al., 2003).

Viral infections are a significant concern in pregnancy. Many women may be afflicted by some form of a viral infection throughout the course of their gestation. While some are fairly mild and easily treatable, infections can range in severity to include influenza, herpes viruses and hepatitis B virus, as well as cytomegalovirus and HIV infections which are still ravaging in many parts of the world. Viral infections are
commonly modeled *in vivo* by administration of a synthetic viral-like double stranded (ds) RNA – polyinosinic:polycytidylic acid (poly I:C). Best known as an inducer of interferon (IFN), poly I:C is also associated with the induction of other pro-inflammatory cytokines such as interleukin (IL)-6, IL-10, IL-12 and tumor necrosis factor (TNF-α), as well as with the induction of an acute phase response (APR) (Fortier et al., 2004). Poly I:C acts via Toll-like receptor (TLR) 3 and TLR2 pathways and elicits an inflammatory response through a partially different cytokine cascade than bacterial infections, which are commonly modeled by the administration of LPS, which acts via TLR4 (Barbalat et al., 2009; Vercammen et al., 2008). Since very little information exists as to the effects of viral infection on the expression of drug transporters, our objective was to examine the expression of several key drug transporters in livers and placentae of poly I:C-treated pregnant rats. Furthermore, as inflammation can mediate the disturbance of maternal hepatobiliary homeostasis, which can affect fetal outcomes, we also examined several important bile acid uptake transporters, such as the organic anion-transporting polypeptides (Oatp/Slco) and the sodium-taurocholate-cotransporting polypeptide (Ntcp/Slc10a1), and examined levels of biliary substrates.
MATERIALS AND METHODS

Animals and experimental design

Pregnant near-term Sprague-Dawley rats (gestational day (GD) 17-18; Charles River Laboratories, Senneville, QC) were injected i.p. with single 2.5 or 5.0 mg/kg doses of poly I:C (Amersham Biosciences, Piscataway, NJ) dissolved in phosphate buffered saline, or with a single 0.5 mg/kg dose of bacterial LPS (Escherichia coli serotype O55:B5; Sigma-Aldrich, St Louis, MO) dissolved in saline. Control pregnant rats (GD 17-18) were injected with sterile saline. Animals were sacrificed at 24 h post-injection (n = 4-7/group). Maternal livers, placentae, and fetuses were immediately harvested and preserved in liquid nitrogen for mRNA and protein analyses. Maternal blood was collected and plasma obtained via centrifugation (3000 g, 4 °C, 15 min) and preserved at -80 °C for further analysis. All animal studies were approved by the Office of Research Ethics at the University of Toronto and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Cytokine measurements

Maternal plasma cytokine concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined via commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The samples were examined in duplicates and results within the standard curve range reported. The minimum detectable levels were typically less than 10 pg/ml, 5 pg/ml, and 21 pg/ml, for IFN-γ, TNF-α and IL-6, respectively.
qRT-PCR and transporter mRNA expression

Methods for RNA isolation, cDNA synthesis and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) have been previously described (Petrovic et al., 2008; Wang et al., 2005). Briefly, RNA was extracted from tissues (50-100 mg) using the QuickPrep total RNA extraction kit (Amersham Biosciences, Piscataway, NJ) or TRIzol reagent (Invitrogen, Burlington, ON). RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and then reverse-transcribed to cDNA by use of the First Strand cDNA synthesis kit (Fermentas, Burlington, ON) according to the manufacturer’s protocol. Placental and hepatic mRNA expression levels of efflux and uptake transporters were measured by qRT-PCR using the Roche LightCycler™ with the LC FastStart DNA Master SYBR Green I® kit (Roche, Laval, QC). Expression levels of Cyp3a2 were also examined, as the hepatic expression of this gene has been reported to be downregulated in numerous models of acute and chronic inflammation. Oligonucleotides for previously reported primer sequences were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON) (Petrovic et al., 2007; Naud et al., 2007; Macias et al., 2005). All mRNA levels were normalized to 18s rRNA and the ratios are presented as the percentage of control values.

Western blotting and transporter protein expression

Methods for protein isolation and Western blotting have been previously described (Wang et al., 2005). Briefly, crude membrane fractions were isolated from tissue (0.1-0.3 g) homogenized in lysis buffer (0.1 M Tris-HCl, pH 7.5, containing Protease Inhibitor Cocktail 1-3 µL/ml and Phenylmethylsulfonyl fluoride (PMSF) 50 µg/ml; Sigma-Aldrich, Oakville, ON). Homogenates were centrifuged at 2000 g for 20 min, the
supernatant centrifuged at 100,000 g for 60 min, and the protein pellet washed and resuspended in a small volume of lysis buffer. Protein concentrations were measured via Bradford assay. Samples (60 μg or less) were separated via SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Mississauga, ON). Membranes were blocked with Tris-buffered saline (TBST) containing 5% skim milk powder and blotted with the following primary antibodies: C-219 (P-gp), M2 III-6 (Mrp2) or BXP-21 (Bcrp) (1:500; Abcam, Cambridge, MA). Subsequently, membranes were washed with TBST and blotted with an anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; Jackson ImmunoResearch Laboratories, West Grove, PA). Bound antibody was detected using an Enhanced Chemiluminescence (ECL) detection kit (Amersham, Oakville, ON) and visualized after exposure on Kodak BioMax® MS films (Eastman Kodak, Rochester, NY) or by using the Alpha Innotech FluorChem® Xplor imager. Protein band intensity was quantified by Alpha Ease FC™ imaging software (Alpha Innotech Corporation, San Leandro, CA). Molecular weight markers were obtained from Bio-Rad. To control for variability in protein loading, all results were normalized to AC-15 (β-actin) (1:10000; Sigma-Aldrich, Oakville, ON).

**Measurement of bile acids and bilirubin in maternal plasma and fetal pools**

Bilirubin (total and direct) levels were measured in maternal plasma and bilirubin standards (Verichem Laboratories, Providence, RI) using a commercially available reagent kit (Thermo Fisher Scientific, Mississauga, ON) according to manufacturer’s instructions. Total bile acids (BA) in plasma and fetal tissue were measured using a kit from Trinity Biotech (Jamestown, NY). BA in plasma were analyzed directly, whereas fetal BA were first extracted with ethanol using a modified procedure from Lee et al.
(Lee et al., 2008). Three to five fetuses from the same mother were pooled together for analysis. Briefly, thawed fetal torsos including the entire gastrointestinal tract were placed in 50 ml of ethanol, minced well and boiled down to approximately 20 ml. Samples were filtered through no. 2 Whatman paper, and the volume brought to 25 ml. Samples were then evaporated dry with nitrogen, redissolved in 2 ml of ethanol and centrifuge filtered using a PVDF membrane (Millipore Corp, Bedford, MA). In order to avoid interference of ethanol with assay reagents, samples (100 μl) were evaporated dry again, and reconstituted in 1 ml of assay reagent before analysis.

**Statistical analysis**

Data were analyzed using Prism 5 for Windows (GraphPad Software, San Diego, CA). All results are expressed as means ± standard error (SE). For comparison of the effects of each dose to control, either a two-tailed independent-samples t-test (†) or a one-way ANOVA with Dunnett’s multiple comparison test (*) was used. Significance was assigned at α = 0.05, and is indicated as follows: * or †: p<0.05, ** or ††: p<0.01, and *** or †††: p<0.001.
RESULTS

Cytokine response to poly I:C

Pro-inflammatory cytokines were examined in maternal plasma samples from poly I:C-treated and vehicle control rats. Administration of stress stimuli such as poly I:C is known to trigger an acute phase reaction and a release of cytokines. Indeed, our ELISA results demonstrated a significant induction of IFN-γ 24 hours after administration of 2.5 or 5.0 mg/kg poly I:C, as compared to control (Figure 1a). Measurements of IFN-γ in control samples were below detection limit and were thus considered to be <10 pg/ml. Furthermore, IL-6 and TNF-α levels were also increased as compared to control, with significant differences at the 5.0 mg/kg poly I:C dose (Figures 1b and 1c).

Effect of poly I:C and LPS on placental and hepatic mRNA

The impact of poly I:C on the expression of transporters in placental and hepatic tissues was examined by qRT-PCR. In placenta, we observed significant changes in the mRNA expression of several transporters (Figure 2a and b). Placental *Abcb1a* and *Abcb1b* were significantly downregulated at one or both doses of poly I:C, to approximately half of the value of control mRNA levels (*p*<0.01). Expression of *Abcg2* was also significantly decreased to 51 ± 9% of control, at the higher poly I:C dose (*p*<0.05). While *Abcc1* and *Abcc3* were significantly downregulated at both doses (*p*<0.05), we did not observe significant changes in *Abcc2* mRNA levels at either dose. As compared to controls, placental mRNA levels of *Slco1a4* and *Slco4a1* were significantly reduced in the poly I:C-treated rats, with values ranging from 53-77% of controls (*p*<0.05). No changes were observed in the placental mRNA expression of *Slco2b1*. 
Administration of poly I:C also imposed significant changes in the expression of several transporters in the liver (Figure 3). After administration of 5.0 mg/kg poly I:C, Abcg2 and Abcc2 were significantly reduced to 20 ± 5% and 31 ± 6% of control values, respectively, while Abcc3 was increased to 305 ± 67% of controls (p<0.05). While we did not observe any changes in Abcb1a mRNA levels, we found a pronounced upregulation of Abcb1b (p<0.01). Significant downregulation at both doses of poly I:C was seen for Slc10a1 (Ntcp) and Slco1a4 (Oatp1a4, also known as Oatp2) genes, with values ranging from 8-38% of controls (p<0.05). Expression of hepatic Abcb11 (Bsep) was reduced to 54-70% of control levels. Additionally, the hepatic metabolizing enzyme Cyp3a2 was significantly decreased to 24 ± 2% of control mRNA value (p<0.05).

As LPS-mediated changes in hepatic expression have not been previously described in pregnant rats, we also examined the impact of LPS on the hepatic mRNA expression of several transporters (Figure 4). Changes seen in the LPS model were consistent with the poly I:C model, with the exception of Abcb1a which was significantly downregulated in LPS- but not poly I:C-treated pregnant rats. Moreover, at 24 hours, levels of Abcg2 and Cyp3a2 were decreased to 65% and 55% of controls, respectively, but did not reach statistical significance. However, at 6 hours post-LPS administration, Abcg2 was significantly downregulated to 47 ± 16% of controls (LPS 0.5 mg/kg, n=3, p<0.05), and Cyp3a2 was significantly reduced to 40 ± 15% (LPS 1.0 mg/kg, n=3, p<0.05).

**Effect of poly I:C on placental and hepatic ABC transport proteins**

Relative to controls, immunodetectable levels of P-gp, Mrp2 and Bcrp were significantly downregulated in the livers of poly I:C-treated rats at both doses (p<0.05) (Figure 5a). P-gp was significantly reduced to 17-49% of control levels, Bcrp was
reduced to 29-58% of control levels, and Mrp2 was reduced to 45-59% of control values. We did not find differences in placental P-gp or Bcrp expression between the control group and the poly I:C-treated groups (Figure 5b); however, the immunodetectable levels in our placental crude membrane preps were very low, with several samples below detection limits.

**Effect of poly I:C on maternal and fetal concentrations of biliary compounds**

As poly I:C-treated rats demonstrated changes in the expression of many of the major hepatic bile acid transporters (Abcb11, Abcc2, Abcc3, Slc10a1, Slco1a4), we examined maternal and fetal levels of bile acids (BA). After 24 hours of administering 5 mg/kg poly I:C, total BA levels in maternal plasma were dramatically increased (8-fold, $p<0.001$) as compared to plasma levels of vehicle control rats (Figure 6a). After normalizing to tissue weight of pooled fetuses, we also observed a trend towards increased magnitudes of total BA in fetuses from poly I:C-treated mothers, as compared to controls, but this did not reach statistical significance, possibly due to high inter-group variation (Figure 6b). On the contrary, maternal plasma levels of direct and total bilirubin did not significantly differ between control and poly I:C groups (Figure 6c and 6d).
DISCUSSION

Pregnancy is associated with a number of physiological and hormonal changes that can alter drug pharmacokinetics by modulating the mechanisms involved in drug uptake, metabolism, and excretion (Anger and Piquette-Miller, 2008). A further complication may be introduced with the onset of an acute or chronic inflammatory condition in the mother. Inflammation is not uncommon in pregnancy and the induction of pro-inflammatory cytokines is seen with many prevalent obstetric complications including infections, chorioamnionitis, cholestasis of pregnancy, gestational diabetes and preeclampsia (Romero et al., 2007). Inflammation-mediated changes in drug metabolizing enzymes and drug transporting proteins have been previously reported (reviewed in Petrovic et al., 2007; Morgan et al., 2008), however very little is known about the effects of inflammatory response in pregnancy. We investigated the effect of viral and bacterial models of inflammation on the placental and hepatic expression of several ABC drug efflux and solute carrier (SLC) uptake transporters, which are known to transport numerous clinically relevant endogenous substrates and xenobiotics. Overall, our findings demonstrated that the induction of a viral-like response by poly I:C imposed a downregulation in the expression of a number of key transporters involved in drug and bile acid transport in the liver and placenta of pregnant rats. Furthermore, significantly increased levels of total bile acids in maternal plasma of poly I:C-treated rats suggest functional changes in the activity of these transporters.

One of the most frequently used and best characterized pathogen-associated molecular patterns for simulating the effects of infection and systemic inflammation is bacterial endotoxin, LPS. LPS-induced inflammation has been shown to impact the expression of
placental transporters and fetal drug exposure (Petrovic et al., 2008). However, the endotoxin model may not be reflective of changes which occur during viral infections. Viruses typically produce double-stranded (ds) RNA during their replication and are thus modeled by synthetic viral-like forms of dsRNA, such as poly I:C. Bacteria and viruses bind to different TLRs, which can activate differential signaling pathways and cytokine cascades. LPS binds to TLR4, whereas poly I:C binds primarily to TLR3 (Vercammen et al., 2008). Differences in the profile and rate of cytokine induction between the two pathogens have been reported (Lee et al., 2007; Reimer et al., 2008). Furthermore, whereas LPS mediates its effects through IRF3 and NF-κB signaling, poly I:C can trigger a robust type-I IFN response without activating those signaling pathways (Reimer et al., 2008). Because of such evidence of differential response pathways in bacterial and viral infections, we felt it was imperative to examine the effect of viral-like infection on the expression of drug transporters that are known to be involved in the disposition of clinically relevant substrates.

Overall we found that poly I:C imposed a significant downregulation of several key ABC efflux and Oatp uptake transporters in the placenta. It is likely that the observed poly I:C-mediated induction of IFN-γ, TNF-α and IL-6 is involved in the downregulation of these placental transporters. Indeed, in vitro studies in human trophoblasts have reported cytokine-mediated changes in the expression of P-gp and Bcrp (Evseenko et al., 2007). In general, the downregulation of ABC efflux and Oatp uptake transporters in placenta is consistent with changes seen in endotoxin-treated rats (Petrovic et al., 2008; Wang et al., 2005). Likewise, a dose-dependent decrease in the placental expression of Abcb1a has been reported in endotoxin-treated mice (Chen et al., 2005). In
contrast, changes at the protein level differed between models of viral and bacterial infection. Although LPS mediates a significant downregulation in the protein expression of Bcrp and P-gp in placenta (Petrovic et al., 2008), significant changes in protein expression were not detected in the poly I:C-treated rats. As the protein expression of P-gp was not significantly changed, the biological significance of the *Abcb1a* and *Abcb1b* downregulation mediated by poly I:C remains to be elucidated. Since the effects of LPS were apparent only at a high dose, it is possible that we would see changes in placental protein expression with higher doses of poly I:C. The maximum reduction of cytochrome P450 enzymes has been reported 24 hours after the administration of 10 mg/kg poly I:C dose (Anari et al., 1995). This dose, however, is not tolerated well by pregnant rats and thus could not be examined in the present study.

We also examined the expression profile of important hepatic drug transporters in pregnant rats. Similar to LPS, poly I:C mediated a significant decrease in the expression of hepatic Bcrp in the pregnant dams, both at the gene and protein level. However, downregulation of *Abcg2* appears to occur more rapidly after LPS administration as we observed a decrease at 6 hours post-LPS treatment, but not at 24 hrs. The temporal pattern of gene expression changes in poly I:C-treated rats may differ from that seen after LPS administration due to quantitative or time-dependent differences in cytokine release which can stimulate alternate cell signaling pathways. While the effect of viral or bacterial models of inflammation on hepatic Bcrp expression has not been previously examined in pregnancy, a decrease in hepatic Bcrp expression has been reported in male rats with extrahepatic cholestasis (Villanueva et al., 2008; Brcakova et al., 2009). On the other hand, in a model of LPS-induced cholestasis, a 5-fold increase in the hepatic
expression of Abcg2 mRNA was recently reported in male rats, accompanied with no changes in Bcrp protein expression (Brackova et al., 2009). It is therefore likely that a complex interplay of cytokines is involved in the regulation of Bcrp. Indeed, in vitro studies in primary human hepatocytes reported that IL-6 mediates a decrease, whereas TNF-α mediates an increase in Bcrp expression (Vee et al., 2009).

Downregulation of Abcc2/Mrp2 and induction of Abcc3 were seen in the livers of both poly I:C- and LPS-treated pregnant rats. As Mrp2 and Mrp3 are believed to have a compensatory relationship in hepatocytes, this is consistent with studies performed in non-pregnant rats (Cherrington et al., 2004; Donner et al., 2004). We also observed endotoxin-mediated downregulation of Abcb1a and a strong induction of Abcb1b in pregnant rats. In line with our observations, previous studies in non-pregnant endotoxemic rats have suggested differential regulation of these two hepatic P-gp gene isoforms (Wang et al., 2005; Cherrington et al., 2004). In contrast, administration of poly I:C did not mediate a strong change in Abcb1a; it led to a drastic induction of Abcb1b, but an overall decrease rather than increase in P-gp protein expression. There is still debate as to the biological significance of the 1b gene isoform of P-gp, but our results indicate that mRNA levels of Abcb1b may not greatly contribute to protein levels of P-gp in rat liver. It is also possible that P-gp expression is regulated by additional post-transcriptional mechanisms activated within this viral model of infection. This could account for the discrepancies between the gene and protein expression of P-gp. Such a phenomenon has been previously observed in case of Mrp2 and Bsep in human liver (Elferink et al., 2004). It is interesting that the expression of Abcb1b was downregulated
in placenta while induced in liver. Organ-specific regulation of \textit{Abcb1b} has been previously reported in LPS-treated mice (Hartmann et al., 2005).

As many of the affected transporters are also involved in bile acid transport, we examined maternal and fetal concentrations of biliary compounds. Our results demonstrated that the concentration of total bile acids in maternal plasma was significantly increased in poly I:C-treated rats relative to controls, likely a consequence of inflammation-mediated changes in transporter expression. The mechanisms for hepatocyte regulation of bile acids are well known (Wolkoff and Cohen, 2003). The poly I:C-mediated reduction of hepatic Ntcp (\textit{Slc10a1}) and Oatp2 (\textit{Slco1a4}), likely contributed to a decrease in the basolateral uptake of bile acids, whereas decreased levels of the canalicular efflux transporters Mrp2 and P-gp could have resulted in decreased efflux into bile. Additionally, since the basolateral transporter Mrp3 is also capable of effluxing bile acids from hepatocytes, its increased expression may have contributed to greater efflux into blood (Donner et al., 2004). Increased levels of total bile acids have been reported in pregnant rats with obstructive cholestasis (Serrano et al., 2003; Hassan and Subbiah, 1980). Likewise, studies in cholestatic non-pregnant rodents also reported an increase in total bile acids (Teng and Piquette-Miller, 2007; Yang et al., 2009). Repeated poly I:C administration over 28 weeks has been reported to induce the development of primary biliary cirrhosis in mice (Okada et al., 2005); however, it is unknown whether single doses of poly I:C can induce cholestasis.

Notably, we found that although there was a trend towards increased fetal bile acid levels, this did not reach statistical significance. During the intrauterine period, the placenta plays an important role in fetal hepatobiliary-like function (Macias et al., 2009).
As the Oatp uptake transporters were downregulated in placentae of poly I:C-treated dams, and placental Mrp2 efflux transporter was unchanged, it is possible that decreased uptake of bile acids could have played a protective role. Mrp3 levels were also reduced; however the impact of this change is unclear as directionality of Mrp3 transport in the feto-placental unit has not been definitively determined. While we are not aware of any other studies examining the effect of inflammation on fetal bile acid levels, maternal cholestasis studies have yielded conflicting results, as both decreases and increases in rat fetal bile acids have been reported (Serrano et al., 2003; Hassan and Subbiah, 1980). Given that changes in fetal bile acid levels can lead to developmental challenges, a further elucidation of the effects of inflammation on fetal biliary transport is necessary.

Lastly, administration of poly I:C did not elicit significant changes in maternal plasma concentrations of bilirubin, which is transported via similar pathways as bile acids. We detected mildly increased plasma concentrations of both direct and total bilirubin in poly I:C-treated rats, however the levels were comparable to the normal range. Increased renal expression of bilirubin glucuronide transporters and increased renal clearance have previously been shown to play a compensatory role for reducing the cytotoxic effects of accumulating cholephilic compounds (Tanaka et al., 2002). The effect of poly I:C on renal transport in pregnancy awaits elucidation. Furthermore, bilirubin accumulation may be a slower process than changes in accumulation of bile acids. Indeed, a recent study has shown that bile salt levels in the liver were profoundly higher in infected mice much sooner than the onset of direct hyperbilirubinemia (Yang et al., 2009). Studies of duration longer than 24 hours may be helpful in clarifying the regulation of bilirubin during maternal infections.
In summary, our results demonstrate that viral mimic, poly I:C-induced inflammation significantly downregulates the expression of several key drug transporters in placentae and liver of pregnant rats. The observed changes are consistent, but not identical to those seen with bacterial endotoxin-mediated inflammation, likely due to the involvement of different cytokine pathways in bacterial and viral infections. Moreover, we found that poly I:C mediated a significant increase in maternal plasma bile acid levels, but did not affect pooled fetal levels of bile acids. However, different substrates are likely to have different outcomes. As the combined action of the placenta and maternal liver protect the fetus from deleterious effects of endogenous and xenobiotic compounds, it is imperative to further investigate the effect of infections and inflammatory changes on maternal and fetal drug exposure.
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REFERENCES


Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvadi MR, Charles KA, Clarke SJ, Kacevska M, Liddle C, Richardson TA, Sharma R, and


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LEGENDS FOR FIGURES

Figure 1: Pro-inflammatory cytokine concentrations in maternal plasma. Pregnant rats were injected with poly I:C and sacrificed 24 h later. IFN-γ, IL-6 and TNF-α plasma concentrations were determined via ELISA and are presented as pg/ml of plasma. Data represent the mean ± SEM (n=5-6/group). †: p<0.05 (t-test), ***: p<0.001 (one-way ANOVA with Dunnett’s multiple comparison test), as compared to saline control. †≠: levels not detectable.

Figure 2: Effect of poly I:C on the mRNA expression of placental efflux (A) and uptake (B) transporters. Placentae were collected from near term pregnant rats at 24 h-post poly I:C administration. Analysis of mRNA expression was performed via qRT-PCR and gene expression normalized to 18s, as described in methods. Data represent the mean ± SEM as a percentage of control value (n=4-6/group) with statistics calculated by ANOVA (*) or t-test (†). Significance as compared to saline control is indicated as follows: *, †: p<0.05, **, ††: p<0.01, ***: p<0.001.

Figure 3: Effect of poly I:C on hepatic mRNA expression. Hepatic tissue was collected from near term pregnant rats at 24 h-post poly I:C administration. Analysis of mRNA expression was performed via qRT-PCR and gene expression normalized to 18s, as described in methods. Data represent the mean ± SEM as a percentage of control value (n=4-7/group) with statistics calculated by ANOVA (*) or t-test (†). Significance as compared to saline control is indicated as follows: *, †: p<0.05, **: p<0.01, ***: p<0.001.
Figure 4: Effect of bacterial endotoxin on hepatic mRNA expression. Hepatic tissue was collected from near term pregnant rats at 24 h-post LPS administration. Analysis of mRNA expression was performed via qRT-PCR and gene expression normalized to \(18s\), as described in methods. Data represent the mean ± SEM as a percentage of control value (n=6/group). Statistics were calculated by t-test. †: \(p<0.05\), as compared to saline control.

Figure 5: Immunohistochemical detection of ABC drug efflux transporters in livers (A and C) and placentae (B and D) of poly I:C- treated rats. Protein levels were determined by Western blotting and normalized to β-actin, as described in methods. A and B: Data represent the mean ± SEM as a percentage of control value (n=4-6/group) with statistics calculated by ANOVA (*) or t-test (†). *,†: \(p<0.05\), **,††: \(p<0.01\), ***: \(p<0.001\), as compared to saline control. C and D: representative Western blots.

Figure 6: Bile acids and bilirubin in maternal plasma and fetal tissue. Plasma and fetuses (3-5/dam) were harvested from rats 24 h after administration of poly I:C. Total bile acid concentration (µmol/L) was measured in maternal plasma (A) and pooled fetal tissue (B). Direct and total bilirubin concentrations (µmol/L) were measured in maternal plasma (C and D). Data represent the mean ± SEM (n=4-6/group) with statistics calculated by ANOVA. ***: \(p<0.001\), as compared to saline control.
Figure 1

A) IFN-γ

B) IL-6

C) TNF-α
Figure 5

A. Hepatic protein

B. Placental protein

C. Bcrp, Mrp2, P-gp

D. Bcrp, P-gp
Figure 6

A. Maternal bile acids

B. Fetal bile acids

C. Direct bilirubin

D. Total bilirubin