Effect of endotoxin on the expression of placental drug transporters and glyburide disposition in pregnant rats

Vanja Petrovic, Jing-Hung Wang and Micheline Piquette-Miller

Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ontario, Canada
Running title: Effect of endotoxin on placental drug transporters

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

Number of text pages: 16
Number of tables: 2
Number of figures: 6
Number of references: 31
Number of words in the Abstract: 241
Number of words in the Introduction: 452
Number of words in the Discussion: 1494

List of non-standard abbreviations:
ABC transporters: ATP-binding cassette transporters
ANOVA: analysis of variance
Bcrp: breast cancer resistance protein
CYP: cytochrome P-450
ELISA: enzyme-linked immunosorbent assay
IL-6: interleukin-6
LPS: lipopolysaccharide
Mdr: multidrug resistance
Mrp: multidrug resistance-associated protein
Oatp: organic anion transporting polypeptide
PCR: polymerase chain reaction
Pgp: P-glycoprotein
RT-PCR: reverse transcription polymerase chain reaction
SLC: solute carrier
TNF-α: tumor necrosis factor-α
ABSTRACT

On average, 80% of pregnant women consume over the counter and/or prescription medications. The placenta is a crucial organ that can restrict fetal drug exposure. ABC drug transporters play an important role in placenta, as they limit the transplacental transfer of xenobiotics. However, the impact of infection or inflammation on placental drug transporters is not well established. We thus examined the impact of endotoxin-induced inflammation on the placental expression of several key drug transporters in rats and its impact on fetal exposure to a drug substrate. Real-time PCR results demonstrated a significant time- and dose-dependent downregulation of Bcrp/Abcg2 mRNA in the placentas of endotoxin-treated rats with a corresponding decrease in protein levels. Likewise, the mRNA levels of several other ABC transporters (Mdr1a/Abcb1a, Mdr1b/Abcb1b, Mrp1-3/Abcc1-3) as well as members of the organic anion transporting polypeptides (Oatp1a4/Slco1a4, Oatp2b1/Slco2b1, Oatp4a1/Slco4a1) were downregulated. A biodistribution study was carried out with glyburide, a hypoglycemic sulfonylurea substrate of both ABC efflux and Oatp uptake transporters. While administration of endotoxin resulted in comparable plasma concentrations of glyburide, a pronounced increase in the accumulation of glyburide was seen in the fetuses of endotoxin-treated rats (162% of controls, p < 0.01). Glyburide plasma protein binding was not affected by endotoxin treatment. Overall, our results demonstrated a significant reduction in the placental expression of several important drug transporters during endotoxin-induced inflammation. Alterations in glyburide distribution highlight the potential importance of both influx and efflux placental transporters in impacting fetal drug exposure.
INTRODUCTION

Inflammation is a component of many common conditions encountered in pregnancy, including infection, preeclampsia and gestational diabetes (Slaviero et al., 2003). The inflammatory response is associated with the induction of endogenous pro-inflammatory cytokines which impact gene regulation. Inflammation mediated either by vaccines, chemicals, cytokines or endotoxins has been shown to down-regulate several of the cytochrome P450 (CYP) isoforms (Morgan, 1997; Renton, 2001; Slaviero et al., 2003). In addition to drug metabolism, inflammatory responses also affect drug distribution and clearance through changes in drug transporters. Indeed, a downregulation in the expression and activity of several ATP-Binding Cassette (ABC) drug transporters, such as P-glycoprotein (Pgp or Mdr1) and the multidrug resistance-associated proteins (Mrp) have been reported in endotoxin, cytokine and turpentine models of inflammation (Piquette-Miller et al., 1998; Hartmann et al., 2001; Kalitsky-Szirtes et al., 2003). It has only recently been recognized that the breast cancer resistance protein (Bcrp/Abcg2), a member of the ABC superfamily of transporters, also plays an important role in drug distribution and clearance. However, very little is known regarding the impact of inflammation on the in vivo expression of Bcrp.

Bcrp, originally discovered in breast cancer cells with a multidrug resistance phenotype, is thought to play an important protective role in normal tissues (Doyle and Ross, 2003). Similar to other ABC transporters, Bcrp is a high capacity efflux transporter with a large variety of substrates, including anticancer and anti-retroviral drugs, toxins, endogenous nutrients, hormones and food additives (Staud and Pavek, 2005). As Bcrp is highly expressed in placenta and is possibly involved in the placental transport of
numerous compounds, the regulation and activity of Bcrp could be of critical importance for fetal development and safety. It has been reported that glyburide, a sulfonylurea drug for treating hyperglycemia, is a substrate of Bcrp (Gedeon et al., 2006; Zhou et al., 2008). To date, more than 800 pregnant women have participated in clinical trials with glyburide and its usage as first-line therapy for gestational diabetes has been growing (Feig et al., 2007). Fetal distribution of glyburide was recently shown to be significantly limited by Bcrp1 in mice (Zhou et al., 2008). Other transporters such as the organic anion transporting polypeptides (Oatps), which belong to the solute carrier family, as well as Mrp3 have also been reported to interact with glyburide (Shitara et al., 2002; Gedeon et al., 2006).

Therefore the primary objective of this study was to investigate the effect of endotoxin-induced inflammation on the placental expression of several key ABC drug efflux (Bcrp, Mdr1a, Mdr1b, Mrp1, Mrp2, and Mrp3) and solute carrier drug uptake (Oatp1a4, Oatp2b1, Oatp4a1) transporters in pregnant rats. A biodistribution study of glyburide was conducted in order to further investigate the impact of inflammation on placental drug transfer.
MATERIALS AND METHODS

Animals and Experimental Design

All animal studies were approved by the University of Toronto Animal Care Committee (protocol number 2000-6622) and conducted in accordance with the guidelines of the Canadian Council on Animal Care. Pregnant Sprague-Dawley rats (gestational day - GD 17, Charles River Laboratories, Saint-Constant, QC) were injected i.p. with single 0.1, 0.5 or 1.0 mg/kg doses of bacterial endotoxin lipopolysaccharide (LPS; from Escherichia coli serotype O55:B5; Sigma-Aldrich, St Louis, MO) dissolved in saline. Control pregnant rats (GD 17) were injected with sterile saline. Animals were sacrificed at 6, 12, 18 or 24 h post-injection (n = 3-7/group). Placentas were immediately harvested and preserved in liquid nitrogen for mRNA and protein analyses. Another set of placentas was fixed in 4% paraformaldehyde (PFA) for immunohistochemistry analysis. Maternal blood was collected and plasma obtained via centrifugation (3000 × g at 4°C) and preserved at -80°C for measurement of cytokines.

Determination of placental transporter mRNA expression

The mRNA levels of each transporter were examined by quantitative real time PCR. Methods for RNA isolation, cDNA synthesis and real time PCR have been previously described (Goralski et al., 2003; Teng and Piquette-Miller, 2005; Wang et al., 2005). Briefly, RNA was extracted from tissues by use of the QuickPrep total RNA extraction kit (Amersham, Oakville, ON), and single-stranded complementary DNA (cDNA) was synthesized from 2.5 μg of RNA by use of the First Strand cDNA synthesis kit (MBI Fermentas, Burlington, ON) according to the manufacturer’s protocol. Amplification
using real-time PCR was performed with the Roche Light Cycler™ (Roche, Laval, QC) using 25 ng of cDNA product and specific primers for each transporter (Table 1). The reaction was carried out by incubating with the LC FastStart DNA Master SYBR Green I®. The mRNA levels for each gene were normalized to Gapdh and mRNA ratios are presented as the percentage of control values.

**Western blot analysis**

Total membrane protein was isolated as previously reported (Hartmann et al., 2001; 2002; Wang et al., 2005). Protein samples (20 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to a Hybond nitrocellulose membrane (Amersham, Oakville, ON) and blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% nonfat milk at 4°C overnight. Membranes containing transporter protein were then cut in half and the upper portion (MW > 64 kD, based on the Kaleidoscope Molecular weight Standards, Bio-Rad Laboratories, Mississauga, ON) was incubated with M-70 antibody which recognizes rat Bcrp [(1:500); Santa Cruz Biotechnology, Santa Cruz, CA] followed by horseradish peroxidase conjugated anti-rabbit secondary antibody [(1:2000); Santa Cruz Biotechnology, Santa Cruz, CA]. To control for variability in protein loading, the lower part of the membrane (MW < 64 kD) was incubated with anti-β-actin antibody [AC-15 (1:2000); Sigma-Aldrich, St Louis, MO] followed by horseradish peroxidase conjugated anti-mouse secondary antibody [(1:2000); Amersham, Oakville, ON]. 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). Protein band
intensity was quantified by Alpha Ease FC™ imaging software (Alpha Innotech Corporation, San Leandro, CA).

**Biodistribution of glyburide**

Pregnant rats that had received 0.5 mg/kg endotoxin or saline (controls) 24 h earlier were used for the glyburide biodistribution study (n = 3-5 per group). Glyburide (24 \( \mu \)g/kg dissolved in ethanol and diluted in saline containing 25% DMSO; Sigma-Aldrich, St Louis, MO) was administered intravenously through the tail vein. Animals were sacrificed 1 h after glyburide administration. The 1 hour time period was chosen based on previous reports of glyburide biodistribution in pregnant rats and mice (Sivan et al., 1995; Zhou et al., 2008). Individual placentas and fetuses, as well as other maternal organs (liver, intestine, kidney, and brain) were separated, snap-frozen in liquid nitrogen, and preserved at -80°C until use. Glyburide concentration in maternal plasma, as well as in fetal and maternal tissues, was determined by a validated HPLC-UV assay. Plasma protein binding of glyburide was measured in individual plasma samples obtained from endotoxin and control rats using Millipore Centrifree® devices (Millipore, Woodbridge, ON).

**HPLC-UV Assay**

Plasma and tissue glyburide concentrations were determined by a validated HPLC-UV assay (Gedeon et al, 2008). The assay was adapted for fetal and maternal tissue homogenates. Chromatography analysis was carried out using the Agilent 1100 HPLC system (Agilent Technologies, Mississauga, ON) and a Waters 2487 Dual \( \lambda \) Absorbance
Detector (Waters, Milford, MA). The mobile phase consisted of acetonitrile: 50 mM ammonium acetate buffer (pH = 5.35) (55:45, v/v). Separation was performed at a flow rate of 1.0 mL/min on a Waters Xterra MS C18 column (4.6 × 250 mm, 5 µm) and the detection wavelength was set at 254 nm.

Tissue samples (approximately 0.3 g) were homogenized in double distilled water (4.0 mL of water/g of tissue) using a sonic dismembrator (Model 100, Fisher Scientific, Ottawa, ON). Plasma samples (600 µL) and tissue homogenates (600 µL) were vortexed with 8.0 mL of tert-methyl butyl ether, shaken for 10 min and centrifuged at 3750 rpm for 20 min. Organic phases were transferred into clean test tubes, evaporated dry under nitrogen, and reconstituted with 100 µL of the mobile phase.

Standard calibration curves were constructed by adding glyburide to drug free plasma or tissue homogenates (600 µL) to give final concentrations ranging from 0.025 to 0.500 µg/mL. The detection sensitivity of this HPLC-UV assay was 4.2 ng/mL and 5.6 ng/g for plasma and tissue samples, respectively.

**Immunohistochemistry**

Placental tissues fixed in 4% PFA were paraffin-embedded and cut into 5 µm sections. Tissue sections were deparaffinized in xylene, and rehydrated. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in methanol for 20 min. Before staining, paraffin sections were preheated in a microwave. A rabbit anti-BCRP antibody M-70 (1:50; Santa Cruz, CA) was incubated with the sections. The slides were then incubated for 40 min with a Nova Red conjugated goat anti-rabbit antibody (1:400; University Health
Network, Toronto, ON). After staining, the slides were visualized under a microscope and areas of interest recorded by a digital camera.

Measurement of Plasma Cytokine Levels

Maternal plasma cytokine levels of TNF-α and IL-6 were determined by using an enzyme-linked immunosorbent assay (ELISA) at 6 h and 24 h post-endotoxin administration. ELISA protocols for TNF-α (ABCam Laboratories, Cambridge, MA) and IL-6 (R&D Systems, Minneapolis, MN) were followed according to manufacturer’s instructions. The samples were examined in duplicates and results within the standard curve range are reported.

Data and Statistical Analysis

A two-tailed Student’s $t$ test was employed for statistical comparison of biodistribution and ELISA results between the endotoxin and control groups. For analysis of dose effects and comparison to controls at each time point, a one-way analysis of variance (ANOVA) was applied followed by the Newman-Keuls multiple comparison test for post hoc analysis. Multiple comparisons of data for all dose and time points were subsequently analyzed using two-way ANOVA (GraphPad Prism® Software, San Diego, CA). A difference in means with a $p$ value of less than 0.05 was considered statistically significant.
RESULTS

Effect of endotoxin on expression of ABC drug efflux transporters

We observed a significant dose-dependent downregulation in the mRNA levels of Bcrp at 18 to 24 h in placentas obtained from endotoxin-treated rats \((p < 0.05)\) (Figure 1A). A corresponding decrease in immunodetectable levels of Bcrp protein was seen in placentas isolated at 24 h (Figure 1B). Immunohistochemical analysis detected high levels of Bcrp expression on trophoblast cell membranes. Expression in syncytiolized (multi-nucleus) trophoblast cells was relatively moderate but detectable as compared to un-syncytiolized (single-nucleus) trophoblast cells. Strong immunodetectable levels of Bcrp were also seen in the endothelium of some fetal capillaries. Expression of Bcrp, both on the trophoblasts and fetal capillaries, was noticeably diminished in endotoxin-treated animals (Figure 2D-2F) as compared to saline-treated animals (Figure 2A-2C).

As depicted in Figure 3A, we observed a pronounced decrease in the placental mRNA expression of Mdr1a \((Abcb1a)\) in the endotoxin-treated rats. A significant dose- and time-dependent downregulation was seen from 18 to 24 h post-endotoxin injection \((p < 0.05)\). Endotoxin administration also imposed a significant decrease in mRNA levels of Mdr1b \((Abcb1b)\) at 18 and 24 h (Figure 3B).

Significant decreases in the mRNA levels of Mrp1 \((Abcc1)\), Mrp2 \((Abcc2)\) and Mrp3 \((Abcc3)\) were seen in the endotoxin-treated rats (Figure 4). Downregulation of Mrp1 mRNA was observed as early as 6 h post-endotoxin injection with a profound dose- and time-dependent effect seen at 24 h (Figure 4A). Decreases in mRNA levels of Mrp2 were seen between 12 to 24 h in the endotoxin-treated rats but this was only significant at
12 and 18 h (Figure 4B). Significant decreases in mRNA levels of Mrp3 were seen between 18 to 24 h in the endotoxin-treated rats (Figure 4C).

**Effect of endotoxin on expression of Oatp drug transporters**

As rat Oatp1a4 and human OATP2B1 have been reported to interact with glyburide, we also examined the impact of endotoxin on the expression of several Oatp isoforms. The mRNA levels of the predominant isoforms of placental influx transporter Oatp, which include Oatp1a4 (Slco1a4; also known as Oatp2), Oatp2b1 (Slco2b1; also known as Oatp-B) and Oatp4a1 (Slco4a1; also known as Oatp-E) (Mikkaichi et al., 2004) were analyzed. In general, endotoxin-mediated reduction in mRNA levels of the Oatp isoforms occurred at earlier time points and recovered by 24 h post-endotoxin administration (Figure 5). As compared to controls, Oatp1a4 mRNA was significantly reduced at 6 to 12 h in the endotoxin-treated rats (Figure 5A), whereas significant decreases in Oatp2b1 and Oatp4a1 mRNA were seen between 12 to 18 h (Figures 5B, 5C) ($p < 0.05$).

**Effect of endotoxin on the biodistribution of glyburide**

The biodistribution of glyburide was examined in order to assess the potential impact of inflammation-mediated changes in the expression of transporters on fetal drug exposure. While glyburide levels tended to be higher in control as compared to endotoxin-treated rats at 1 h post-administration, these differences did not reach statistical significance (control: $166 \pm 48$ ng/mL; endotoxin: $77 \pm 13$ ng/mL). However, a significant increase of glyburide accumulation was seen in fetuses of endotoxin-treated
rats (162% of saline, \( p < 0.01 \)). Normalizing fetal tissue glyburide levels to maternal plasma levels also revealed a 3.5 fold increase in the glyburide concentration ratio for fetuses obtained from endotoxin-treated rats as opposed to those from saline controls (\( p < 0.001 \)) (Figure 6). While absolute amounts of glyburide accumulation (ng/g tissue) were significantly different only in the fetal tissue, normalized tissue:plasma levels of glyburide were significantly higher in maternal tissues obtained from endotoxin-treated rats. In the placenta, glyburide concentration ratio was 2.5 fold higher in endotoxin-treated rats (\( p < 0.01 \)), whereas the difference was 1.7 to 2.9 fold higher in maternal liver, intestine, kidney, and brain (\( p < 0.05 \)) (Figure 6). Plasma protein binding of glyburide was not significantly different between treatment groups (control: 80 ± 1.6%; endotoxin: 82 ± 1.6%).

**Maternal plasma cytokine levels after endotoxin administration**

*In vivo* administration of endotoxin is known to induce plasma levels of the pro-inflammatory cytokines. TNF-\( \alpha \) and IL-6 are two of the major cytokines induced upon endotoxin administration in the circulation system of rodents. Our ELISA results demonstrated a dose-dependent 2-26 fold induction in maternal plasma TNF-\( \alpha \) levels at 6 h post-endotoxin administration, with levels subsiding by 24 h (Table 2A). At 6 hr, there was a pronounced 3-4 fold elevation in the plasma IL-6 levels of rats that received 0.5 – 1.0 mg/kg of endotoxin (Table 2B). By 24 h, plasma IL-6 levels decreased in rats treated with the lower endotoxin doses, but were dramatically elevated in the 1.0 mg/kg endotoxin treatment group (Table 2B).
DISCUSSION

A wide array of pathological, physiological and hormonal changes occur in pregnancy, which can significantly impact the pharmacokinetics of drugs (Anger and Piquette-Miller, 2008). Overall, our findings demonstrated that endotoxin-induced inflammation imposes a pronounced decrease in the placental expression of several key drug transporters in pregnant rats. While the temporal pattern and magnitude of endotoxin-mediated changes differed between transporters, a downregulation was seen in mRNA levels of both efflux and influx transporters including: Bcrp, Mdr1a, Mdr1b, Mrp1, Mrp2, Mrp3, Oatp1a4, Oatp2b1 and Oatp4a1. It is important to note that these particular transporters influence the transplacental passage of numerous clinically important drugs and toxins, therefore alterations in their expression and activity have the potential to greatly impact fetal exposure to xenobiotics. As there are many maternal conditions that are associated with an inflammatory response, this could represent an important source of variability in fetal drug exposure and teratogenicity.

Placental efflux transporters such as Bcrp and Pgp are believed to protect placental and fetal tissues by removing potentially toxic xenobiotics and endogenous metabolites. It has been previously demonstrated that endotoxin and other inflammatory stimuli impose a downregulation of Pgp in epithelial tissues of the liver, intestine, brain and placenta in rodents (Piquette-Miller et al., 1998; Hartmann et al., 2001; Goralski et al., 2003; Kalitsky-Szirtes et al., 2004; Wang et al., 2005). Recently, it has been reported that intestinal expression of BCRP is decreased in patients with ulcerative colitis (Englund et al., 2007), however the effect of inflammation on the placental Bcrp expression is unknown. Bcrp is highly expressed in the apical syncytiotrophoblast
membrane as well as fetal capillary endothelium of human term placenta (Maliepaard et al., 2001; Litman et al., 2002; Ceckova-Novotna et al., 2006). Our results demonstrated a significant dose-dependent reduction in Bcrp mRNA levels 18 to 24 h after endotoxin administration with a corresponding decrease in the immunodetectable levels of protein. Interestingly, a downregulation in Bcrp expression was detected in both the placental trophoblast cells and fetal capillary endothelium of endotoxin-treated animals. A decrease in the in vitro expression of Bcrp in primary term trophoblasts was recently reported after exposure to tumor necrosis factor-alpha (TNF-α) or interleukin (IL)-1β (Evseenko et al., 2007). Consistent with these in vitro findings, our results demonstrate a significant induction of TNF-α and IL-6 in the maternal plasma of endotoxin-treated rats. This suggests that these cytokines could be involved as mediators of downregulation of placental Bcrp in endotoxin-treated rats.

Bcrp has been shown to restrict the passage of topotecan and mitoxantrone to the fetus in pregnant mice, thus it is believed that Bcrp plays an important role in the protection of fetus from exposure to toxic chemicals (Jonker et al., 2000). In order to investigate the impact of endotoxin-mediated downregulation of transporter expression on fetal drug exposure we conducted a biodistribution study of glyburide, a sulphonylurea hypoglycemic agent that can be used to manage gestational diabetes. Glyburide has been shown to be actively transported by the human isoforms of BCRP and MRP3 but not PGP, MRP1 or MRP2 (Gedeon et al., 2006). Furthermore, it has recently been shown that fetal distribution of glyburide is increased in Bcrp1−/− pregnant mice as compared to wild-type controls (Zhou et al, 2008). As Bcrp is involved in the active efflux of glyburide, we anticipated that downregulation of Bcrp in endotoxin-treated rats would
result in increased fetal exposure. Indeed, we observed increased absolute and plasma normalized concentrations of glyburide in the fetuses of endotoxin-treated rats. Hence, inflammation-mediated changes in the expression of Bcrp appear to impact the overall fetal exposure of glyburide. Although we cannot rule out the possibility that glyburide distribution at other time points could reveal additional changes, previous studies have reported a time delay in glyburide distribution to the fetus with concentrations reaching a maximum at 1 hour (Zhou et al., 2008; Sivan et al., 1995). Moreover, as concentrations of glyburide in fetal tissues decrease in parallel to maternal plasma concentrations after initial distribution, fetal:maternal plasma concentration ratios have been found to remain relatively constant between 1 to 4 hr after glyburide administration. Therefore it is probable that endotoxin-mediated changes in the fetal accumulation of glyburide are likely to persist at later time points. Glyburide concentrations were comparable to controls in maternal tissues, but when normalized to plasma levels, a significant increase in the ratio of tissue:plasma concentration was found in placental, liver, intestinal, kidney, and brain tissues of endotoxin-treated rats. Some of these changes could result, in part, due to Bcrp downregulation in maternal tissues, however changes in Bcrp alone cannot fully explain these findings. While alterations in plasma protein binding could result in an increased tissue distribution, we did not detect significant differences in glyburide protein binding in plasma obtained from endotoxin and control rats.

As evidence exists suggesting that glyburide uptake may occur through several of the Oatp transporters, it is plausible that inflammation-mediated changes in these transporters could contribute to alterations in glyburide uptake and distribution. Glyburide has been shown to be transported by human OATP2B1 (Satoh et al., 2005) and can inhibit
transport activity of rat Oatp1a4 at high concentrations (Shitara et al., 2002). There are several Oatp isoforms expressed in rat placenta, particularly Oatp2b1, Oatp4a1, and to a lesser extent Oatp1a4 (St-Pierre et al., 2004). We detected significantly lower mRNA levels of all three isoforms in placental tissues obtained from endotoxin-treated rats. Likewise, previous studies have reported an endotoxin-mediated downregulation of Oatp1a4 mRNA in the brain and liver of rodents (Hartmann et al., 2002; Goralski et al., 2003). As a downregulation of the Oatp transporters would serve to decrease rather than increase the placental uptake and fetal accumulation of glyburide, it does not appear that in vivo changes in the placental expression of the Oatp transporters play a major role in the fetal accumulation of glyburide. However, it is still conceivable that alterations in Oatps could have attenuated glyburide accumulation in fetal tissues. Thus, changes in fetal glyburide accumulation could result from a combination of the observed endotoxin-mediated suppression of both Bcrp and Oatp transporters. While downregulation of Bcrp appeared to play a larger role on fetal exposure of glyburide in endotoxin-treated rats, the final impact on fetal drug accumulation could differ with other xenobiotics depending on their relative affinity to each transporter. In general, the overall impact of inflammation on transporter expression and transplacental drug transport requires further investigation.

It could also be hypothesized that increased fetal accumulation of glyburide stems from alterations in expression of Mrp3. While mRNA levels of Mrp3 are detectable in placental trophoblasts, it has been reported that this transporter is predominantly confined to the endothelium of fetal capillaries and that directionality in placental transfer of its substrates is unclear (St-Pierre et al., 2000; 2004). Hence it is possible that an increased fetal uptake of glyburide could result from changes in Mrp3. However, we were unable
to detect measurable quantities of Mrp3 protein via western blot analysis of placental samples obtained from either control or endotoxin treated rats. Likewise, other laboratories have reported undetectable protein levels of MRP3 in placental tissues or in human placental BeWo cells (Pascolo et al., 2003). The likelihood that changes in Mrp3 expression play a significant role in altered fetal drug accumulation is less probable when one considers its limited expression.

Many transporters of endogenous and exogenous compounds are highly expressed in placenta. Due to the wide impact of systemic inflammation, compromised activity of placental transporters may vary the amount of nutrients, medications and toxins accumulated in the fetal circulation system, and therefore could rearrange fetal exposure to these compounds. The consequences, such as dystrophy or fetal drug exposure, are serious issues and could be detrimental to fetal health. We detected a substantial downregulation of Bcrp and attempted to examine its impact on fetal accumulation of a Bcrp substrate. Although it has a distinct function, Bcrp shares much similarity in substrate affinity and tissue distribution with other transporters. Therefore, examining the in vivo impact of changes in Bcrp expression without interference from the activity of other transporters represents a challenge. Bcrp is also expressed in mammary glands during late pregnancy and lactation, impacting the secretion of xenobiotics into breast milk (Jonker et al., 2005). Hence, further investigations into the expression of this transporter in diseases associated with inflammation would be an important step in understanding altered drug disposition and patient outcomes during pregnancy and lactation.
In summary, our results demonstrate that endotoxin-induced inflammation significantly downregulates the expression of several important drug transporters in placenta. This includes transporters that are involved in both placental drug uptake as well as drug efflux. Moreover, we observed a significant increase in the distribution of glyburide into fetal tissues in endotoxin-treated rats. As this drug is a substrate of both influx and efflux transporters, our findings highlight the importance of considering the mutual functioning relationship of these transporters, rather than the changes in only a single group of transporters. Different substrates are likely to have different affinities for the transporters that direct their transplacental transfer, and thus the interaction between inflammatory stimuli and transporter activities should be assessed when considering medication usage during pregnancy.
ACKNOWLEDGEMENTS

We thank Ji Zhang, Gregory Anger and Deborah Scollard for their generous help and technical assistance with the biodistribution study.
REFERENCES


Zhou L, Naraharisetti SB, Wang H, Unadkat JD, Hebert MF, and Mao Q (2008) The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide...
FOOTNOTES

This study was supported by an Operating Grant from the Canadian Institutes of Health Research (CIHR).
LEGENDS FOR FIGURES

FIG. 1. *Effect of endotoxin on placental Bcrp (A) mRNA and (B) protein expression in rats.* As described in methods, mRNA levels were determined by real-time PCR and normalized to *Gapdh*. Protein levels were determined by Western blotting. Data represent the mean ± SEM as a percentage of control value (n = 3-7/group). Statistics were calculated by ANOVA as described in methods. At each time point, Asterisk (*): control vs. each endotoxin dose; Dagger (†): 0.1 mg/kg vs. 0.5 mg/kg or 1.0 mg/kg endotoxin; Hash (#): 0.5 mg/kg vs. 1.0 mg/kg endotoxin. * † # p < 0.05; ** †† # # p < 0.01; *** ††† # # # p < 0.001.

FIG. 2. *Immunohistochemical detection of Bcrp in placentas of 24 h saline (A-C) and endotoxin (0.5 mg/kg) (D-F) treated rats.* Representative areas of interest (Bcrp was detected using M-70 and visualized by Novo Red stain) are indicated by arrows. Bcrp expression was detected on the trophoblast cells outside the chorionic villi (as indicated by green arrows) (A, D) or the endothelium of fetal capillary (as indicated by red arrows) (B, C, E, F). Fetal capillary stains depicted in B and E represent vertical sections, while C and F represent horizontal sections.

FIG. 3. *Effect of endotoxin on placental (A) Mdr1a and (B) Mdr1b mRNA levels in rats.* As described in methods, mRNA levels were determined by real-time PCR and normalized to *Gapdh*. Data represent the mean ± SEM as a percentage of control value (n=3-7/group). Statistics were calculated by ANOVA as described in methods. At each time point, Asterisk (*): control vs. each endotoxin dose; Dagger (†): 0.1 mg/kg vs. 0.5
mg/kg or 1.0 mg/kg endotoxin; Hash (#): 0.5 mg/kg vs. 1.0 mg/kg endotoxin. * † # p < 0.05; ** †† ## p < 0.01; *** ††† ### p < 0.001.

FIG. 4. Effect of endotoxin on placental (A) Mrp1, (B) Mrp2 and (C) Mrp3 mRNA levels in rats. Levels of mRNA were determined by real-time PCR and normalized to Gapdh, as described in methods. Data represent the mean ± SEM as a percentage of control value (n=3-7/group). Statistics were calculated by ANOVA as described in methods. At each time point, Asterisk (*): control vs. each endotoxin dose; Dagger (†): 0.1 mg/kg vs. 0.5 mg/kg or 1.0 mg/kg endotoxin; Hash (#): 0.5 mg/kg vs. 1.0 mg/kg endotoxin. * † # p < 0.05; ** †† ## p < 0.01; *** ††† ### p < 0.001.

FIG. 5. Effect of endotoxin on placental (A) Oatp1a4, (B) Oatp2b1 and (C) Oatp4a1 mRNA levels in rats. Levels of mRNA were determined by real-time PCR and normalized to Gapdh, as described in methods. Data represent the mean ± SEM as a percentage of control value (n = 3-7/group). Statistics were calculated by ANOVA as described in methods. At each time point, Asterisk (*): control vs. each endotoxin dose; Dagger (†): 0.1 mg/kg vs. 0.5 mg/kg or 1.0 mg/kg endotoxin; Hash (#): 0.5 mg/kg vs. 1.0 mg/kg endotoxin. * † # p < 0.05; ** †† ## p < 0.01; *** ††† ### p < 0.001.

FIG. 6. Glyburide biodistribution in pregnant rats. Pregnant rats injected with endotoxin (0.5 mg/kg) or saline control 24 hours earlier were administered glyburide (24 µg/kg, iv) and sacrificed after 1 h. Glyburide concentrations were measured by HPLC-UV, as
described in methods. Data represent the mean ± SEM of the ratio of tissue to maternal plasma concentrations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to control group.
TABLE 1. Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>gapdh</td>
<td>5’-CCATCACCATCTTCCAGGAG-3’</td>
<td>5’-CCTGCTTCACCACCTTCTTG-3’</td>
</tr>
<tr>
<td>bcrp</td>
<td>5’-AGTCCGGAAAACAGCTGAGA-3’</td>
<td>5’-CCCATCACAACGTCATCTTG-3’</td>
</tr>
<tr>
<td>mdr1a</td>
<td>5’-GACGGAATTGATAATGTGGACA-3’</td>
<td>5’-AAGGATCAGGAACAATAAAA-3’</td>
</tr>
<tr>
<td>mdr1b</td>
<td>5’-GAAATAATGCTTATGAATCCCCAAA-3’</td>
<td>5’-GGTTTCATGGTGTCGTCTCTTGA-3’</td>
</tr>
<tr>
<td>mrp1</td>
<td>5’-TTCTAGTGTGGACGAGGCT-3’</td>
<td>5’-TGGCCATGCTATAGAAGACG-3’</td>
</tr>
<tr>
<td>mrp2</td>
<td>5’-GTCACGGGCTTCTTTTCTG-3’</td>
<td>5’-AACCCCCAACACCTGCTAA-3’</td>
</tr>
<tr>
<td>mrp3</td>
<td>5’-GTGCTGAAGAATTTGACTCTG-3’</td>
<td>5’-GACCAGGACCCGGTTGTAAGTC-3’</td>
</tr>
<tr>
<td>oatp1a4</td>
<td>5’-TTGGTGTTGGATGTGCAGTT-3’</td>
<td>5’-GCCAATGGTCATTCTTGTGT-3’</td>
</tr>
<tr>
<td>oatp2b1</td>
<td>5’-GACTATGGCTCCAGCCTCTG-3’</td>
<td>5’-GTGCTATGCTGGTGGAATGTC-3’</td>
</tr>
<tr>
<td>oatp4a1</td>
<td>5’-CTGGGACGTGCTGACTAAT-3’</td>
<td>5’-GGCGAATAGCTCGACTTGAC-3’</td>
</tr>
</tbody>
</table>
TABLE 2. *Maternal plasma cytokine levels after endotoxin administration.* Pregnant rats were administered various doses of endotoxin (LPS), and sacrificed 6 or 24 h later. Plasma was immediately isolated from the collected blood and the levels of TNF-α (A) and IL-6 (B) were determined via ELISA. Cytokine levels are calculated in the unit of pg/mL of plasma. Data represent the mean ± SEM. *p < 0.05, as compared to saline control.

A

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>0.1 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.0 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>17.65 ± 0.12</td>
<td>37.43 ± 12.56</td>
<td>306.78 ± 67.18 *</td>
<td>459.00 ± 172.21</td>
</tr>
<tr>
<td>24 h</td>
<td>16.93 ± 1.59</td>
<td>21.73 ± 5.41</td>
<td>18.97 ± 2.38</td>
<td>37.95 ± 0.12</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>0.1 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.0 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>83.52 ± 48.22</td>
<td>245.27 ± 131.53</td>
<td>274.79 ± 40.41 *</td>
<td>369.02 ± 98.42</td>
</tr>
<tr>
<td>24 h</td>
<td>103.80 ± 15.67</td>
<td>121.72 ± 83.52</td>
<td>134.22 ± 10.50</td>
<td>3033.06 ± 639.85 *</td>
</tr>
</tbody>
</table>
Figure 1

A

![Bar chart showing the expression of bcrp/gapdh as a percentage of control over time after endotoxin administration.](chart1)

B

![Immunoblot showing Bcrp and β-actin expression under different conditions.](blot1)

- **A**
  - Bars represent bcrp/gapdh expression as a percentage of control over time (6 h, 12 h, 18 h, 24 h).
  - Saline, Endotoxin (0.5 mg/kg), and Endotoxin (1.0 mg/kg) groups.
  - Significant differences indicated by ***, **, *, #,

- **B**
  - Immunoblot of Bcrp and β-actin under Saline, Endotoxin (0.5 mg/kg), and Endotoxin (1.0 mg/kg) conditions.

---

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

A

B
**Figure 4**

**A**

![Bar graph showing mrp1/gapdh as % of control with time after endotoxin administration.](image)

**B**

![Bar graph showing mrp2/gapdh as % of control with time after endotoxin administration.](image)

**C**

![Bar graph showing mrp3/gapdh as % of control with time after endotoxin administration.](image)
Figure 5

A

![Graph showing oatp1a4/gapdh as % of control over time after endotoxin administration for different treatments (Saline, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg).](image)

B

![Graph showing oatp2b1/gapdh as % of control over time after endotoxin administration for different treatments (Saline, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg).](image)

C

![Graph showing oatp4a1/gapdh as % of control over time after endotoxin administration for different treatments (Saline, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg).](image)
Figure 6

Tissue:plasma glyburide concentration ratios (ng/g tissue) / (ng/mL plasma)

- **Placenta**
- **Fetus**
- **Brain**
- **Kidney**
- **Intestine**
- **Liver**

- **Saline**
- **Endotoxin**

Significant differences indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.