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Acute Effects of Viral Exposure on P-Glycoprotein Function in the Mouse Fetal Blood-Brain Barrier

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Key Words
P-glycoprotein (P-gp) • Polyinosinic:polycytidylic acid (PolyI:C) • Placenta • Blood-brain barrier (BBB) • Toll-like receptor 3 (TLR-3)

Abstract

Background/Aims: Viral infection during pregnancy is known to affect the fetal brain. The toll-like receptor (TLR)-3 is a pattern recognition receptor activated by viruses known to elicit adverse fetal neurological outcomes. The P-glycoprotein (P-gp) efflux transporter protects the developing fetus by limiting the transfer of substrates across both the placenta and the fetal blood-brain barrier (BBB). As such, inhibition of P-gp at these blood-barrier sites may result in increased exposure of the developing fetus to environmental toxins and xenobiotics present in the maternal circulation. We hypothesized that viral exposure during pregnancy would impair P-gp function in the placenta and in the developing BBB. Here we investigated whether the TLR-3 ligand, polyinosinic:polycytidylic acid (PolyI:C), increased accumulation of one P-gp substrate in the fetus and in the developing fetal brain. Methods: Pregnant C57BL/6 mice (GD15.5) were injected (i.p.) with PolyI:C (5 mg/kg or 10 mg/kg) or vehicle (saline). [\textsuperscript{3}H]digoxin (P-gp substrate) was injected (i.v.) 3 or 23h post-treatment and animals were euthanized 1h later. Maternal plasma, ‘fetal-units’ (fetal membranes, amniotic fluid and whole fetus), and fetal brains were collected. Results: PolyI:C exposure (4h) significantly elevated maternal plasma IL-6 (P<0.001) and increased \textsuperscript{3}H]digoxin accumulation in the fetal brain (P<0.05). In contrast, 24h after PolyI:C exposure, no effect on IL-6 or fetal brain accumulation of P-gp substrate was observed. Conclusion: Viral infection modeled by PolyI:C causes acute increases in fetal brain accumulation of P-gp substrates and by doing so, may increase fetal brain exposure to xenobiotics and environmental toxins present in the maternal circulation.

E. Bloise and S. Petropoulos share joint first-authorship.
Introduction

Infective viral agents pose serious threats to pregnancy outcome. Viral disease predisposes pregnant women to severe obstetric outcomes including spontaneous abortion or fetal congenital viral syndromes, which may disrupt optimal fetal or offspring development [1, 2]. Viral infections can also elicit preterm labor and injury to the developing brain [3]. As such, understanding potential mechanisms of how viral infections elicit such adverse outcomes in pregnancy is of great importance.

Antiviral innate immune responses are mediated by a subset of toll-like receptors (TLRs) namely TLR-3, TLR-7/8, and TLR-9. These pattern recognition receptors trigger the production of antiviral cytokines responsible for mounting an intricate immunological response [4]. Of special importance is TLR-3, which mediates antiviral responses to the zika virus (ZIKV) [5], and cytomegalovirus (CMV) [6, 7], both of which are related to adverse fetal neurological outcomes [8].

It is currently unknown whether fetal exposure to xenobiotics and environmental toxins is linked to the severe fetal phenotype commonly associated with antenatal ZIKV and CMV infections. In this context, the efflux transporter P-glycoprotein (P-gp; encoded by the ABCB1 gene) plays a key role in fetal protection against xenobiotics and environmental toxins [9]. P-gp is localized in blood-barrier sites and actively effluxes numerous xenobiotics, steroids, toxins and cytokines out of cells. In pregnancy, it functions as a "gatekeeper" preventing fetal accumulation of detrimental substances circulating in the maternal blood stream [9].

P-gp is localized to the apical surface of the microvillus membrane of the syncytiotrophoblast [10, 11], facing the placental intervillous space in both human and the mouse. Thereby preventing the entry of its substrates into the fetal compartment. In the brain, P-gp is localized to the luminal surface of the blood-brain barrier (BBB) and effluxes its substrates out of the brain, against concentration gradients, preventing accumulation of many different substances [12, 13]. Inhibition of P-gp function therefore reduces the ability of these barriers to protect the developing fetus.

Infection and inflammation lead to dysregulation of P-gp activity/expression in both placenta and BBB. We have recently demonstrated that pregnant mice acutely exposed to lipopolysaccharide (LPS, modeling gram –ve bacterial infection) displayed decreased placental P-gp activity, which resulted in increased fetal accumulation of the P-gp substrate [3H]digoxin [14]. Further, we have shown that human placental explants exposed to LPS and polyinosinic:polycytidylic acid (PolyI:C), a synthetic analog of double stranded RNA (dsRNA) that stimulates TLR-3-viral immunological responses [15], exhibit reduced expression of P-gp [16]. Additionally, brain endothelial cell (BEC) cultures exposed to the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 or tumor necrosis factor (TNF)-α displayed impaired P-gp function [17]. Together, these results suggest that both bacterial and viral infections have the potential to decrease fetal protection and increase exposure to xenobiotics and environmental toxins that may have teratogenic consequences.

Our current hypothesis is that viral exposure during pregnancy impairs P-gp function in the placenta and in the developing BBB, exposing the fetus and the developing brain to xenobiotics environmental toxins. Therefore, we sought to investigate whether the TLR-3 ligand PolyI:C, can increase accumulation of P-gp substrates in the fetus and in the developing fetal brain using an in vivo mouse model.

Materials and Methods

Experimental Design

Virgin female C57BL/6 mice (6-8 weeks of age; Charles River, Germantown, NY) were bred in our colony with male C57BL/6 mice. Pregnancy was defined after presence of a vaginal plug and designated as gestational day (GD) 0.5 (average gestation period ~ 19.5 days). Pregnant mice were arbitrarily assigned to treatment (PolyI:C) or vehicle (saline) groups. These studies were performed using protocols approved
Assessing the Functional Effects of PolyI:C on P-gp

The influence of PolyI:C on P-gp activity was investigated in two cohorts. In the first, pregnant dams (GD15.5) were injected (i.p.) with 5 mg/kg (n = 5) or vehicle control (n = 6) 4 h prior to euthanasia. In the second cohort, pregnant dams (GD14.5) were injected (i.p.) with 5 mg/kg (n = 7) or 10 mg/kg (n = 4) or vehicle control (n = 7) 24 h prior to euthanasia [18]. We have previously determined that in the mouse, placental expression of P-gp declines with gestation while P-gp in the fetal BBB increases [12, 19]; as such we chose mid-gestation (GD15.5) as the time-point to investigate the influence of PolyI:C on P-gp function in these blood-barrier tissues. In both cohorts, [3H]digoxin (50 μg/kg and 1 μCi/animal), a specific P-gp substrate, was injected directly into the tail vein, and dams (GD15.5) were euthanized 1 h later by isoflurane overdose [12, 14, 19-21]. Maternal blood was collected via cardiac puncture and plasma was separated for analysis. ‘Fetal-units’ (~2-5/litter arbitrarily selected) comprising the fetus, amniotic fluid and intact fetal membranes, but not the placenta, were weighed at the time of dissection [12, 14, 19-21]. Considering that the amniotic fluid and fetal membranes contain substrate that has traversed the placenta from maternal circulation, substrate accumulation in the ‘fetal-unit’ provides an index of net transplacental transfer. From the remainder of the litter, fetal brains and fetal bodies were collected, weighed at the time of dissection and stored at ~80°C. Maternal brains were also collected, weighed and immediately frozen. Levels of maternal [3H]digoxin accumulation were calculated as DPM counts and standardized as a drug equivalent per weight or volume. For ‘fetal-unit’ and brain:body drug ratios, drug distribution was expressed as the ratio of tissue concentration (DPM/gram tissue weight) to maternal plasma concentration (DPM/ml plasma); designated ‘drug ratio’ [12, 19]. For each dam, ‘drug ratio’ values were derived for individual ‘fetal-units’ (~2-5/dam) or fetal brain:body (~2-5/dam) and were averaged to provide a litter mean, which was used for subsequent statistical analysis. One entire litter, consisting of 7 fetuses displayed fetal death in the 10 mg/kg cohort, this animal was excluded.

ELISA

The pro-inflammatory cytokine IL-6 was measured in the maternal plasma to examine maternal inflammatory response after PolyI:C treatment. Analysis was performed by ELISA using a commercially available kit (R&D systems, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions (minimum detectable concentration = 1.6 pg/mL; intra-assay variation = 4.7%).

Statistical Analysis

Group data are presented as means ± standard error of the mean (SEM) and analyzed using Prism (GraphPad Software Inc., San Diego, California, USA). [3H]Digoxin accumulation was averaged for fetuses within a litter and Student’s t-test was performed for the 4 h time point and one-way ANOVA for the 24 h time point, comparing treatment to corresponding control. Partial data from the control animals (fetal unit accumulation of [3H]digoxin and maternal plasma IL-6 levels) has been reported previously [14]. Significance was set at p < 0.05.

Results

TLR-3 activation may induce fetal death

Number of live fetuses were recorded on GD15.5 at 4 or 24 h following exposure to PolyI:C (5 mg/kg or 10 mg/kg; Table 1). No fetal death was observed 4 or 24 h after PolyI:C exposure with the lower dose (5 mg/kg). However, 24 h after 10 mg/kg of PolyI:C exposure resulted in 60% fetal death. Importantly, as described in the materials and methods, one entire litter, consisting of 7 fetuses displayed fetal death in the 10 mg/kg cohort.

Robust acute maternal inflammatory response after TLR-3 activation by PolyI:C

IL-6 levels were markedly increased 4 h after PolyI:C treatment (p < 0.0001) but had returned to baseline 24 h after exposure (Fig. 1). These results demonstrate that acute PolyI:C
Table 1. Pregnant dams (GD14.5 or 15.5) were injected (i.p.) with: PolyI:C or vehicle (control) for 4 h or 24 h or PolyI:C for 24 h. Number of live or dead fetuses was assessed.

<table>
<thead>
<tr>
<th>PolyI:C Injection Day</th>
<th>Groups (PolyI:C mg/kg)</th>
<th>Exposure (h)</th>
<th>Euthanasia Day</th>
<th>N (dams)</th>
<th>% Litter Death % Fetal Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD15.5</td>
<td>Vehicle</td>
<td>4</td>
<td>E15.5</td>
<td>6</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>5mg/kg</td>
<td>4</td>
<td>E15.5</td>
<td>5</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>24</td>
<td>E15.5</td>
<td>7</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>5mg/kg</td>
<td>24</td>
<td>E15.5</td>
<td>7</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>10mg/kg</td>
<td>24</td>
<td>E15.5</td>
<td>4</td>
<td>60% (3/5)</td>
</tr>
</tbody>
</table>

Fig. 1. Maternal plasma IL-6 levels in pregnant mice exposed to acute PolyI:C treatment. Vehicle and acute PolyI:C (5 mg/kg) treatments were performed followed by maternal plasma collection and IL-6 measurements: Veh for 4 h (n = 6), PolyI:C for 4 h (n = 5) or 24 h (n = 7/gp). Values are means ± SEM. One-way ANOVA followed by the Tukey’s post-hoc test, ***P < 0.0001.

Fig. 2. Accumulation of P-gp substrate in the fetal body and brain 4 h after PolyI:C exposure. Accumulation of [3H]Digoxin in (A) the fetal brain (fetal brain:body ratio), as an index of P-gp function in the fetal BBB, (B) the fetal unit, as a index of P-gp function in the placenta, (C) the maternal brain, as an index of P-gp function in the maternal BBB and (D) the maternal blood. Values are mean ± SEM. Student’s t-test, *P < 0.05 compared to control group; PolyI:C 5 mg/kg for 4 h (n = 5) or Veh for 4 h (n = 6).

PolyI:C treatment increases P-gp substrate accumulation in the fetal brain

[3H]Digoxin accumulation was significantly (p < 0.05) increased in the fetal brain 4 h after PolyI:C exposure (5 mg/kg). However, no changes in fetal brain [3H]Digoxin accumulation were observed 24 h after PolyI:C exposure to two different doses (5 and 10 mg/kg) (Fig. 2A & 3A). No significant changes in overall fetal accumulation of [3H]digoxin were observed after any of the treatments (Fig. 2B & 3B). Furthermore, accumulation of [3H]digoxin in the maternal brain was not significantly modified 4 h (5 mg/kg) or 24 h (5 and 10 mg/kg)
after polyI:C treatments (Fig. 2C & 3C). Importantly, maternal plasma levels of [3H]digoxin remained unaltered in all treatments (Fig. 2D and 3D).

**Discussion**

We have demonstrated that acute PolyI:C treatment (4h) on GD15.5 elicited a marked maternal inflammatory response and increased P-gp substrate accumulation in the fetal brain. However, no changes were observed 24 h after PolyI:C insult, demonstrating a time-dependent effect of PolyI:C in disrupting fetal BBB permeability to P-gp substrates. Higher doses of PolyI:C (10 mg/kg) increased rates of fetal death whereas lower doses (5 mg/kg) did not lead to fetal demise after 24 h.

In the present study, PolyI:C effects on P-gp activity were tissue-dependent since placental P-gp activity (determined by Fetal Unit measurements) remained unaltered regardless of time of exposure and dosage, whereas fetal BBB displayed significant impaired P-gp activity. Previous studies from our group corroborate these findings. Maternal exposure to LPS, sertraline or glucocorticoids altered P-gp activity in a tissue dependent-manner [12, 14, 20]. In the rodent, two different gene isoforms, *Abcb1a* and *Abcb1b*, encode distinct P-gp proteins [22]. In the placenta, *Abcb1b* is the predominant isoform expressed, whereas in the BBB, *Abcb1a* predominates [11]. It is likely that tissue-dependent expression of different P-gp isoforms are responsible for the tissue-specific effects of Poly:IC observed in the current study. To the best of our knowledge, no studies have investigated the effect of PolyI:C on the function of different P-gp isoforms in key developmental blood-barrier sites. This hypothesis clearly requires further investigation.

LPS administered to pregnant mice on GD15.5 markedly increased maternal serum levels of IL-6 and concomitantly impaired placental P-gp activity, resulting in increasing fetal [3H]digoxin accumulation 4h after LPS exposure. IL-6 levels and placental P-gp function were restored to baseline 24 h after LPS treatment, demonstrating a time-dependent effect of LPS on placental P-gp. Importantly, maternal myocardial P-gp activity was unaltered at any time point, suggesting that LPS exposure targets P-gp activity in a tissue-dependent manner [14]. Similarly, here we report time and tissue-dependent actions of PolyI:C on P-gp activity. Furthermore, based on our current and previous findings, we can conclude that impairment of placental P-gp activity induced by infection is highly dependent on the
infective agent. Future studies should be undertaken in order to evaluate if the same holds true in the developing BBB.

Impaired P-gp activity in tissue barriers profoundly impacts fetal phenotype [9]. Exposure of pregnant Abcb1a knock-out mice to the teratogen L-652,280, an ivermectin derivative and a P-gp substrate, resulted in 100% of fetuses bearing cleft palate on GD18.5, compared to no cleft palate in controls [23]. In that study, increased incidence of fetal cleft palate was attributed to decreased placental P-gp activity, although impairment of P-gp activity in the fetal BBB could not be ruled out. In fact, many Collie dogs have a deletion mutation in the Abcb1 gene, which impairs P-gp activity in the BBB making them highly susceptible to the neurotoxic effects of ivermectin [24].

The present study has demonstrated increased permeability of the developing BBB to P-gp substrates after PolyI:C-mediated TLR-3 activation. It is unknown whether PolyI:C crosses the placental barrier to directly affect the developing BBB, and as such, whether the reduction of P-gp function at this site was induced by direct or indirect actions. Indirect actions would likely be mediated by induction of pro-inflammatory cytokines. Indeed, pro-inflammatory cytokines such as IL-6 can reduce P-gp activity in the developing BBB [17]. IL-6 had the most potent effect inhibiting P-gp activity in BECs when compared to other pro-inflammatory cytokines [17]. Importantly, increased maternal levels of IL-6 temporally matched the decline in P-gp function at the developing BBB.

It is also possible that PolyI:C disrupts BBB permeability through unknown mechanisms. BBB paracellular permeability may be modulated by different pathological conditions including subarachnoid hemorrhage, autoimmune encephalomyelitis, bacterial meningitis, HIV infection among others [25-28], as well as by therapeutic treatments such as hyperbaric oxygen intervention [26]; highlighting the need for more studies investigating whether TLR-3 activation disrupts BBB paracellular permeability in the developing and adult brains.

Maternal infection with ZIKV, rubella and cytomegalovirus (CMV) can result in adverse neurological outcomes including intellectual disability, ischemic brain damage, cerebral palsy and microcephaly [8]. Importantly, ZIKV and CMV induce TLR-3 activation [5-7] and therefore, may disrupt P-gp activity in the fetal BBB. Whether these viral diseases alter P-gp function in the developing BBB requires further investigation.

In conclusion, acute PolyI:C exposure increases fetal brain accumulation of P-gp substrates, without causing fetal demise. As such, TLR-3 activation has the potential to increase fetal brain exposure to xenobiotics and environmental toxins that may be present in the maternal circulation. Future studies are warranted to investigate whether impaired P-gp function in the fetal BBB underlies, at least in part, the pathogenesis of neurological disorders associated with gestational ZIKV and CMV infections.

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Disclosure Statement

The authors report no conflicts of interest.

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