Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain

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Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. Am J Physiol Regul Integr Comp Physiol 286: R1024–R1029, 2004. First published February 26, 2004; 10.1152/ajpregu.00664.2003.—Perinatal infections are a risk factor for fetal neurological pathologies, including cerebral palsy and schizophrenia. Cytokines that are produced as part of the inflammatory response are proposed to partially mediate the neurological injury. This study investigated the effects of intraperitoneal injections of lipopolysaccharide (LPS) to pregnant rats on the production of cytokines and stress markers in the fetal environment. Gestation day 18 pregnant rats were treated with LPS (100 μg/kg body wt) ip, and maternal serum, amniotic fluid, placenta, chorioamnion, and fetal brain were harvested at 1, 6, 12, and 24 h posttreatment to assay for LPS-induced changes in cytokine protein (ELISA) and mRNA (real-time RT-PCR) levels. We observed induction of proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) as well as the anti-inflammatory cytokine IL-10 in the maternal serum within 6 h of LPS exposure. Similarly, proinflammatory cytokines were induced in the amniotic fluid in response to LPS; however, no significant induction of IL-10 was observed in the amniotic fluid. LPS-induced mRNA changes included upregulation of the stress-related peptide corticotropin-releasing factor in the fetal whole brain, TNF-α, IL-6, and IL-10 in the chorioamnion, and TNF-α, IL-1β, and IL-6 in the placenta. These findings suggest that maternal infections may lead to an unbalanced inflammatory reaction in the fetal environment that activates the fetal stress axis.

endotoxin; tumor necrosis factor; interleukin; brain; cerebral palsy; chorioamnionitis

MATERNAL INFECTIONS DURING pregnancy, including urinary tract and dental infections, have long been associated with the risk of preterm labor (9, 13, 22, 25, 42) and most recently with an increased risk of fetal neurological injury (8, 11, 35, 44, 46, 49). Although likely acting via ascending rather than systemic routes, symptomatic vaginitis also is associated with an increased risk of preterm labor (41). In addition, intra-amniotic infection or chorioamnionitis may represent the etiology of spontaneous preterm labor in up to 37.5% of patients with intact membranes and 30% of patients with preterm rupture of membranes (15, 30, 33). Furthermore, chorioamnionitis may result from conservative therapy of preterm premature rupture of membranes, exposing infants to risks associated with an infected amniotic environment.

Recent evidence suggests an association of fetal infection with newborn neurological injury. Although infants born of mothers with chorioamnionitis have an increased risk of cerebral palsy (47, 48), it is unknown if this risk is the result of maternal or fetal primary infection. Furthermore, questions remain as to whether the putative insult results from localized infectious agents within the fetal central nervous system, fetal sepsis (with or without hypoxia), or localized and/or systemic effects of fetal cytokines. Cytokines are intracellular mediators that are intrinsic to the inflammatory/immune response necessary for the organism’s ability to counteract infections. Cytokines can be broadly categorized as proinflammatory cytokines, which act to mobilize immune system cells to proliferate and produce more cytokines creating an inflammatory cascade, and anti-inflammatory cytokines, which function to dampen or control the inflammatory response. During pregnancy, women maintain both pro- and anti-inflammatory cytokine responses, although there is a shift toward increased anti-inflammatory and reduced proinflammatory cytokine production (26, 38, 40). Similarly, both term and preterm fetuses have intact cytokine responses, with the preterm exhibiting a more robust proinflammatory response (50). Furthermore, the trophoblast, decidua, and chorioamnion have the ability to produce cytokines (3, 20, 27, 31), as do numerous other cell types (24). Because cytokines represent molecules of <50 kDa, there is potential transfer of cytokines between mother, fetus, and amniotic fluid, although these routes are poorly understood.

In view of the potential contribution of maternal and fetal cytokine responses, production, and transfer to fetal neurological injury, we sought to determine maternal and fetal inflammatory and stress responses to maternal intraperitoneal injections of lipopolysaccharide (LPS). Intraperitoneal administration of LPS is used to model common maternal infections (such as pylonephritis) that lead to a local inflammatory response. The inflammatory response was determined by LPS-induced changes in proinflammatory [interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α] and anti-inflammatory (IL-10) cytokines, whereas the stress response was characterized by corticotropin-releasing factor (CRF) and proopiomelanocortin (POMC) changes.

METHODS

Animals and Treatments

Sprague-Dawley female pregnant rats (Harlan Sprague Dawley) were obtained at gestational day 15 (term = 21). Animals were allowed to acclimate for 72 h before beginning experiments. Animals were maintained in temperature (37°C) and light (0600 lights on; 1800 lights off)-controlled facilities with access to food (LabDiet...
5001 Rodent Diet; PMI Nutrition International) and water ad libitum throughout the study. At gestational day 18, animals were administered LPS (100 µg/kg body wt ip, Escherichia coli, serotype 0111:B4, reconstituted in physiological saline; Calbiochem). Control animals received intraperitoneal injections of physiological saline. The protocols and procedures for this study were approved by the Institutional Animal Care and Utilization Committee at Harbor-University of California Los Angeles Research and Education Institute.

**Sample Collection**

At 1, 6, 12, and 24 h after LPS or saline injections, pregnant rats were anesthetized with pentobarbital sodium (intramuscular), and the hearts and peritoneal cavities were exposed via midline incision. Maternal blood was collected via cardiac puncture and centrifuged at 4 °C to isolate serum. The uterus was removed and placed in a chilled petri dish. Each chorioamnion was punctured, and amniotic fluid was collected individually in microcentrifuge tubes and quickly frozen in liquid nitrogen. Individual chorioamnions, placentas, and fetal brains were also collected and frozen in liquid nitrogen. All samples were subsequently stored at −80 °C for further processing and analyses. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining.

**Real-Time RT-PCR**

Real-time RT-PCR were conducted using Applied Biosystems (ABS) Sequence Detection System 7000. Primer Express software (ABS) was used to design specific rat primers (Table 1) for IL-1β, IL-6, IL-10, TNF-α, CRF, POMC, and β-actin.

**Reactions.** All real-time RT-PCR reactions were performed as One-Step RT-PCR using SYBR Green PCR Master Mix (ABS PN: 4309155). RT-PCR reactions were performed in a total volume of 25 µl containing 12.5 µl of 2× SYBR Green master mix (containing SYBR Green I Dye, AmpliTag Gold DNA polymerase, dNTPs with dUTP, passive reference, and optimized buffer components), 0.25 U/µl MultiScribe RT, 0.4 U/µl RNase inhibitor, 200 nM each of forward and reverse primer for the gene of interest, and 1 µl sample RNA. All reactions were reverse transcribed at 48 °C for 30 min followed by activation of DNA polymerase at 95 °C for 10 min followed by 40 PCR cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Melting curve analysis was used to verify single product amplification. Levels of β-actin were used to control for sample amount.

**Quantitation.** Detection of PCR product is accomplished by real-time detection of the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. After each RT-PCR experiment, data were analyzed to select a threshold level of fluorescence that was in the linear phase of the PCR product accumulation. The cycle at which each reaction reached threshold fluorescence was defined as the threshold cycle (C_T) for that reaction. Hence, samples that had higher C_T values for a particular signal had correspondingly lower C_T values. For each sample, we obtained a C_T for TNF-α, IL-1β, IL-6, IL-10, POMC, CRF, and β-actin. The C_T for β-actin was subtracted from the C_T of TNF-α, IL-1β, IL-6, IL-10, POMC, and CRF to obtain a delta C_T (ΔC_T) value for each signal. ΔC_T values were used as normalized C_T values to semiquantitatively compare relative amounts of a signal among different samples.

**Data Analyses**

All results are expressed as means ± SE. All ELISA values were transformed to log values for normalization before being statistically

### Table 1. RT-PCR primers

<table>
<thead>
<tr>
<th>Signal</th>
<th>Primers</th>
<th>Segment (Accession no.)</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>Fwd: 5’-GACCTCTGCAAGCAGAGCA CAGA-3’</td>
<td>793–873 (NM_031512)</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-ACGCTTCCATGTTGGAAGTC-3’</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Fwd: 5’-ATATGTCTCTAGGAGATGTGGA-3’</td>
<td>232–311 (NM_012589)</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-GGCGATCATGTGTTACTAGA-3’</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Fwd: 5’-GCCAAGGCTTCTGCAAATAA-3’</td>
<td>283–357 (NM_012854)</td>
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<tr>
<td></td>
<td>Rev: 5’-TTTCGATGCGATGTCCTTCT-3’</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Fwd: 5’-GTGATGCTGTTCCCAAAAGA-3’</td>
<td>163–233 (X66539)</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-AGGCTTGGGCGCATGGA-3’</td>
<td>613–713 (NM_030199)</td>
</tr>
<tr>
<td>CRF</td>
<td>Fwd: 5’-GGAGGGGCGATGCTGCT-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-GCTCTGCTGCTGCTC-3’</td>
<td>109–216 (NM_139326)</td>
</tr>
<tr>
<td>POMC</td>
<td>Fwd: 5’-CTCAGACAGACAAAAGCAGCT-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-TTTAGCTGAGCCGGTCTT-3’</td>
<td>338–438 (NM_031144)</td>
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</table>

IL, interleukin; TNF, tumor necrosis factor; CRF, corticotropin-releasing factor; POMC, proopiomelanocortin; Fwd, forward; Rev, reverse.
analyzed. Data were analyzed using one-way ANOVA followed by post hoc tests for pairwise comparisons (Tukey’s test). Differences were considered to be significant only for $P < 0.05$. All experiments were repeated at least four times. Hence, there were four pregnant rats ($n = 4$) in each treatment group. For fetal measurements, eight fetuses were harvested from each pregnant rat (32 fetuses were analyzed in each treatment group).

RESULTS

Maternal Response

Maternal serum cytokine responses to systemic LPS are depicted in Fig. 1. A–D. Both pro- and anti-inflammatory cytokines were induced by LPS. However, each cytokine had a unique temporal response profile.

Serum levels of TNF-α (Fig. 1A), which was undetectable in saline-treated group, were maximally induced to $1,382 \pm 162$ pg/ml ($P < 0.001$) within 1 h after LPS treatment and remained significantly elevated at 6 h ($478 \pm 162$ pg/ml, $P < 0.05$) with no other significant induction observed at 12 and 24 h post-LPS treatment. In response to LPS, serum levels of IL-1β (Fig. 1B) were increased from a basal level of 10 ± 7 to $245 \pm 192$ pg/ml (1 h after LPS, $P < 0.05$), 2,228 ± 829 pg/ml (6 h, $P < 0.001$), and 231 ± 52 pg/ml (12 h, $P < 0.01$), returning to near basal levels at 24 h. Serum concentrations of IL-6 (Fig. 1C) were significantly elevated (basal concentration = $31 \pm 17$ pg/ml) at 1 h ($1,080 \pm 222$ pg/ml; $P < 0.01$) and 6 h ($4,586 \pm 1,547$ pg/ml; $P < 0.001$), although not at 12 or 24 h. In response to LPS, maternal serum concentrations of IL-10 (Fig. 1D) were significantly elevated above baseline (9 ± 8 pg/ml) at 1 h ($683 \pm 191$ pg/ml; $P < 0.05$) and 6 h ($1,176 \pm 728$ pg/ml; $P < 0.05$) posttreatment.

Fetal Responses

Cytokines in amniotic fluid. The induction of cytokines in the amniotic fluid in response to maternal LPS is shown in Fig. 2, A–D. The temporal induction of cytokines in the amniotic fluid was similar to that observed in the maternal serum. However, the magnitude of induction was decreased markedly. Amniotic fluid levels of TNF-α (Fig. 2A) were elevated significantly ($P < 0.05$) from baseline (undetectable) at 1 h ($140 \pm 22$) and 6 h ($107 \pm 33$) post-LPS treatment, although not at 12 or 24 h. Levels of IL-1β in the amniotic fluid (Fig. 2B) were elevated significantly ($P < 0.05$; basal concentration = $14 \pm 3$ pg/ml) at 1 h ($96 \pm 14$ pg/ml) followed by a maximum induction at 6 h ($943 \pm 172$ pg/ml). Levels declined at 12 h ($252 \pm 38$) but remained significantly elevated. There was no significant induction at 24 h after LPS treatment. The baseline level of IL-6 (62 ± 10 pg/ml) increased ($P < 0.05$) at 6 and 12 h after LPS treatment ($781 \pm 157$ and $332 \pm 63$ pg/ml, respectively).

Although maternal serum IL-10 levels showed significant induction in response to LPS, there was no significant LPS-induced increase in IL-10 in the amniotic fluid (Fig. 2D).

Cytokines in chorioamnion, placenta, and fetal brain. Changes in the cytokine mRNA expression in the chorioamnion, placenta, and fetal whole brain are shown in Fig. 3, A–C. TNF-α, IL-1β, IL-6, and IL-10 mRNAs were all present in these tissues under basal conditions. In response to maternal LPS, cytokine mRNA levels were upregulated in the chorioamnion and placenta, but no changes were observed in the fetal whole brain.

The chorioamnion (Fig. 3A) had significantly increased levels of TNF-α mRNA at 1 and 6 h after LPS treatment. Levels of IL-6 were increased at 6 and 12 h; meanwhile, IL-10 mRNA was upregulated at 1, 6, and 12 h. No significant
induction of IL-1β was observed in the chorioamnion in response to LPS.

LPS significantly increased placental (Fig. 3B) mRNA levels of TNF-α at 1, 6, and 12 h posttreatment. LPS-induced increases were also observed for IL-1β at 12 h and for IL-6 at 6 and 12 h post-LPS. No significant induction of placental IL-10 mRNA was observed.

**Stress factors in fetal brain.** Assessment of the effects of maternal LPS on mRNA induction of putative stress factors in the fetal whole brain is presented in Fig. 4. We observed significant LPS-induced upregulation of fetal brain CRF mRNA levels at 6 and 12 h post-LPS treatment. No significant changes in POMC mRNA were observed, although there was a decreasing trend at 6 and 12 h posttreatment.

**DISCUSSION**

Our findings show cytokine protein induction in the maternal serum and amniotic fluid in response to maternal intraperitoneal LPS. In response to LPS, the proinflammatory cytokine TNF-α was the first to peak (usually within 1 h), in both serum and amniotic fluid, followed by a quick return to baseline within 12 h. Levels of the other two proinflammatory cytokines, IL-1β and IL-6, had a more delayed response. In the serum and amniotic fluid, both cytokines exhibited peak responses at 6 h followed by a gradual decline to baseline by 24 h post-LPS treatment. Interestingly, levels of the anti-inflammatory cytokine IL-10 were induced significantly in the maternal serum at 1 and 6 h posttreatment but did not change in the amniotic fluid.

Assessment of changes in the mRNA levels of these four cytokines revealed LPS-induced increases of TNF-α, IL-6, and IL-10 in the chorioamnion and of TNF-α, IL-1β, and IL-6 in the placenta. No LPS-induced changes in cytokine mRNA in the fetal whole brain were observed. However, the mRNA level of fetal whole brain CRF was elevated in response to maternal LPS at 6 and 12 h.

The early and transient duration of the TNF-α induction in response to LPS is supported by previous findings that report rapid induction of plasma TNF-α relative to IL-6 or IL-1β in response to both intravenous (within 30 min) and intraperitoneal (within 1.5 h) LPS (18). Others have reported a short-term duration (3 h) of the TNF-α response to systemic LPS (1). In LPS-stimulated cultures, TNF-α protein was induced within 1 h followed by IL-1β and IL-6 at 3 h or greater (29). TNF-α was also more robustly induced by LPS in culture compared with IL-1β induction (14). These and our current findings suggest that TNF-α functions as a first responder to combat infections (particularly bacterial infections) while initiating the inflammatory cascade that leads to the induction of other cytokines. However, it is not clear that TNF-α is the sole initiator of the inflammatory cascade, since others have shown an attenuated (but significant) LPS-induced upregulation of IL-1β in the presence of TNF-α antibodies (20).

Induction of the proinflammatory cytokines in the amniotic fluid followed the same time course as the induction in the maternal serum. However, for each cytokine, the magnitude of induction in the amniotic fluid was lower than that observed in maternal serum. This may suggest that cytokines in the amniotic fluid are from the maternal serum. However, clinical studies have shown elevated amniotic fluid cytokines (IL-1β, IL-6, and TNF-α) in the absence of maternal serum cytokines (36), which suggests that LPS may simultaneously trigger both maternal and fetal tissues to produce cytokines. This is supported by a recent report demonstrating the induction of IL-6 in the amniotic fluid after intra-amniotic injections of LPS (34). Additionally, in vitro stimulation of amniochorionic membranes by LPS leads to the release of cytokines in a ratio that mirrors that observed in amniotic fluid in vivo (12).

**Fig. 3. Real-time RT-PCR measurements of cytokine mRNA signals in the chorioamnion (A), placenta (B), and fetal whole brain (C) collected from pregnancies exposed to LPS (100 μg/kg body wt ip) for 1, 6, 12, and 24 h. Threshold cycle (C_T) values were normalized to β-actin. *P < 0.05 compared with C (saline-treated).**

**Fig. 4. Real-time RT-PCR measurements of corticotropin-releasing hormone (CRH) and pro-opiomelanocortin (POMC) mRNA signals in fetal whole brain collected from pregnancies exposed to LPS (100 μg/kg body wt ip) for 1, 6, 12, and 24 h. C_T values were normalized to β-actin. *P < 0.05 compared with C (saline-treated).**
Interestingly, despite a significant induction in the maternal serum, the anti-inflammatory cytokine IL-10 was not significantly induced in the amniotic fluid; proinflammatory cytokines showed significant induction in both maternal serum and amniotic fluid. These findings are indicative of an unbalanced induction of pro- and anti-inflammatory cytokines in fetal compared with adult tissues. This is supported by previous reports that show no significant elevations of IL-10 in amniotic fluid collected from women with preterm labor associated with chorioamnionitis (11). Others have reported negligible increases in IL-10 mRNA levels in amnion and choriodecidual membranes from pregnancies complicated by chorioamnionitis, despite >30-fold increases in the mRNA levels of IL-1β, IL-6, and TNF-α (20). Mice studies show an enhanced IL-10 response to LPS during pregnancy (44), and rabbits inoculated with *E. coli* exhibit increased levels of TNF-α in amniotic fluid but no change in the anti-inflammatory cytokine IL-1 receptor antagonist (23). It is also feasible that, because the magnitude of cytokine induction in the amniotic fluid was lower than in the maternal serum, a higher dose of LPS may trigger a significant induction of IL-10 in the amniotic fluid. Presumably, levels of proinflammatory cytokines would also increase, thus maintaining a deleterious imbalance between pro- and anti-inflammatory cytokines. We observed basal mRNA expression of IL-1β, IL-6, TNF-α, and IL-10 in the chorioamnion, placenta, and fetal whole brain. Basal expression of cytokines in the chorioamnion and placenta is not surprising, since cytokines have been reported previously to be produced by placental and extra-placental tissue and to have multiple functions during pregnancy, including initiation of uterine contractions and dilation of the cervix (see Refs. 3 and 20 for review). Maternal LPS increased the mRNA expression of IL-6, IL-10, and TNF-α in the chorioamnion and of IL-1β, IL-6, and TNF-α in the placenta. These findings are supported by a recent report of TNF-α, IL-6, and IL-1β induction in the placenta and fetal membranes after intra-amniotic LPS (34). These increases in cytokine mRNA likely contributed to the elevated amniotic fluid cytokine proteins observed in our current study.

Basal expression of cytokine mRNA in the fetal brain is a novel finding that suggests noninflammatory roles of cytokines during development (5, 28, 32, 37, 51). We did not observe any effects of maternal LPS on cytokine mRNA expression in the fetal brain. This is likely because of the low dose of LPS, since previous findings report elevated TNF-α and IL-1β mRNA and protein in fetal whole brain in response to higher (1–4 mg/kg) maternal doses of LPS administered intraperitoneally (4, 43). Intracerebral injection of LPS (0.1–3 mg/kg) into embryonic day 15 pregnant rats also led to increased TNF-α immunoreactivity in fetal brains at embryonic day 20 (2).

Despite the absence of cytokine mRNA induction, we observed increased CRF mRNA in fetal whole brain in response to maternal LPS. In nonfetal systems, LPS is a potent activator of the hypothalamic-pituitary-adrenal (HPA) system (see Ref. 2 for review) and acts to increase both the transcription and release of CRH (6, 16, 19, 39). The effect of LPS on the CRH mRNA is proposed to be mediated via cytokine signaling (19). Although LPS can directly induce cytokines, LPS-induced maternal hypoxia may also impact on fetal brain development (7). That is, maternal hypoxia leads to fetal hypoxia, which initiates production of brain inflammatory mediators, including cytokines (21). In addition to activating the HPA axis, elevated cytokines may trigger a series of neurotoxic processes culminating in CNS compromise (17, 24). Further studies are needed to delineate the roles of hypoxia and/or cytokines in LPS-induced changes in the fetal brain.

Not all pregnant rats treated with LPS responded similarly. However, for each pregnant rat that responded, all cytokines were elevated, suggesting that cytokine induction represents a cascade of activity that is triggered by a common stimulus. Failure of some gravid rats to respond likely reflects a differential threshold for the inflammatory response, although factors that determine this threshold are unclear. Perhaps, some animals can clear the LPS before it can trigger an immune response. We also observed differential induction of proinflammatory cytokines among amniotic sacs within a pregnancy, which was not related to sac position in the uterus; this is consistent with previous reports (34). However, as observed in the maternal serum, cytokine induction (except IL-10, which showed no response) in the amniotic fluid was positively correlated. It is unclear what factors determine the differential inflammatory response among the various amniotic sacs.

**Implications**

The differential induction of proinflammatory cytokine over time after an infection may serve as an index of the progress of an infection. Detection of TNF-α may indicate that an infection is in its early stages, whereas detection of IL-1β, IL-6, or IL-10 in the absence of TNF-α suggests latter stages of the infection. Although maternal infections are associated with cytokine changes, which may contribute to or result from preterm labor, these changes also represent a risk for the developing fetus. Our findings suggest that infection within the fetal compartments may have deleterious consequences because of an imbalance of pro- vs. anti-inflammatory cytokines. Presumably, this leads to a prolonged inflammatory response. The fetal brain is also affected by maternal infections, as evidenced by mRNA changes in the fetal stress axis. Although we did not find evidence of cytokine mRNA changes in the fetal brain after a single LPS injection, there were changes in the fetal membranes and placenta. In view of these results, it is feasible that maternal infections during a pregnancy precipitate fetal neuroinflammation, leading to aberrant brain development and offspring neurological disorders.

**GRANTS**

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