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Acute on chronic exposure to endotoxin in preterm fetal sheep

Sam Mathai, Lindsea C. Booth, Joanne O. Davidson, Paul P. Drury, Mhoyra Fraser, Ellen C. Jensen, Sherly George, Andrew Naylor, Alistair J. Gunn, and Laura Bennet

Department of Physiology, the University of Auckland, New Zealand

Submitted 24 August 2012; accepted in final form 11 December 2012

Mathai S, Booth LC, Davidson JO, Drury PP, Fraser M, Jensen EC, George S, Naylor A, Gunn AJ, Bennet L. Acute on chronic exposure to endotoxin in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 304: R189–R197, 2013. First published December 12, 2012; doi:10.1152/ajpregu.00388.2012.—Acute, high-dose exposure to endotoxin lipopolysaccharide (LPS) in preterm fetal sheep can trigger periventricular white matter lesions (PVL), in association with severe hypotension/hypoxemia and significant mortality. Intriguingly, however, chronic or repeated exposure to LPS can induce tachyphylaxis. We therefore tested the hypothesis that progressive, acute on chronic fetal infection would be associated with white matter injury with little fetal mortality. Chronically instrumented preterm (0.7 gestational age) fetal sheep were exposed to a continuous low-dose LPS infusion (100 ng over 24 h, followed by 250 ng/24 h for 96 h) or saline. Boluses of 1 μg LPS or saline were given at 48, 72, and 96 h; sheep were killed at day 10. Six of 11 fetal sheep exposed to saline infusion + LPS boluses died 4–7 h after the first bolus. In contrast, there was no fetal mortality after saline infusions alone (n = 9), low-dose LPS infusion + saline boluses (n = 5), or low-dose LPS + LPS boluses (n = 9). Low-dose LPS infusion + LPS boluses was associated with greater microglial induction than low-dose LPS + saline boluses but a similar area of periventricular white matter inflammation. One fetus developed severe focal white matter necrosis after LPS infusion + boluses. The acute cardiovascular compromise associated with high-dose, acute exposure to LPS is markedly attenuated by previous low-dose infusions, with limited apparent exacerbation of periventricular white matter injury compared with low-dose infusion alone.

lipopolysaccharide: white matter damage; oligodendrocytes; microglia; inflammation; infection; hypotension

EXPOSURE TO INFECTION/INFLAMMATION by gram negative and other bacteria before birth is highly associated with perinatal mortality and neurodevelopmental handicap in survivors (22). Supporting a causal link, fetal sheep exposed to acute boluses of lipopolysaccharide (LPS), a cell wall component of gram negative bacteria, commonly develop white matter damage (8–10, 12, 14, 17, 18, 24, 31).

Conceptually, acute injection of LPS corresponds broadly with acute, rapidly progressive sepsis. Although this is a well-recognized situation, subclinical infection followed by acute exacerbations is more common and associated with adverse outcomes (20, 39). An important practical limitation of bolus administration of high-dose LPS is that it triggers hypotension and impaired placental flow, a decrease in oxygen delivery to the brain, and an increased risk of fetal death (9, 18, 31). Conversely, there is consistent evidence for tachyphylaxis with LPS, as shown by attenuation of cardiovascular responses with successive boluses of LPS (13), and reduced monocyte reactions after chronic LPS exposure (25, 27). We have recently shown that low-dose systemic endotoxin exposure for 5 days is associated with neural inflammation without hemodynamic disturbances in preterm fetal sheep (26), at 0.7 gestation, when the sheep is broadly equivalent to 28–32 wk of human development (2). Systemic inflammation was transient, suggesting that this paradigm may also induce self-tolerance. In the present study we tested the hypothesis that chronic exposure to a low dose of LPS in preterm fetal sheep would reduce acute fetal mortality from cardiovascular compromise after large boluses of LPS but still trigger neural injury.

MATERIALS AND METHODS

Experimental Preparation

All procedures were approved by the Animal Ethics Committee of the University of Auckland, New Zealand. Singleton 97 ± 1-day-old Romney-Suffolk fetal sheep of either sex (term = 147 days) were operated on as previously described (26). Food but not water was withdrawn 18 h before the surgery. Five milliliters of Streptopen (procaine penicillin 250,000 IU/ml and dihydrostreptomycin 250 mg/ml, Pitman-Moore, Wellington, New Zealand) were given as prophylaxis intramuscularly to the ewes 30 min before surgery. Anesthesia was induced by maternal intravenous injection of 3 mg/kg Alfaxan (Alfaxalone, Jurox, Rutherford, NSW, Australia) and maintained using 2–3% isoflurane in oxygen throughout the surgery and monitored by trained anesthetic staff. The ewes were infused with 1 liter of isotonic saline intravenously to maintain fluid balance during surgery.

After a maternal midline abdominal incision was made and the uterus was exteriorized, fetal catheters were placed in the left femoral artery and vein, right brachial artery and vein, and the amniotic sac for recording of blood pressure, blood sampling, and drug infusions. An ultrasound blood flow probe (3S, Transonic Systems, Ithaca, NY) was placed around the left carotid artery to measure carotid blood flow, as an index of cerebral blood flow (19). A pair of multistranded Teflon-coated stainless steel electrodes was placed on both sides of the fetal chest to measure the fetal heart rate. Two small Burr holes were drilled at 5 and 10 mm anterior to bregma and 5 mm lateral on both sides of the skull without rupturing the dura, and EEG electrodes (AS 633-5SSF, Cooner Wire; Chatsworth, CA) were fixed on top of the dura. The fetus was then returned to the uterus. Gentamicin (80 mg; Pharmacia and Upjohn, Australia) was given into the amniotic sac. All fetal leads were exteriorized through the maternal flank. Bupivacaine hydrochloride 0.5% (10 ml) (Astra Zenca, North Ryde, NSW, Aus-
tria) was injected around the maternal incision before being closed.

A vascular catheter was inserted into the maternal tarsal vein for postoperative maternal care and euthanasia.

After surgery the ewes were housed together in a temperature-controlled vivarium (16 ± 1°C and relative humidity of 50 ± 10%) in separate metabolic cages with a 12 h light/12 h dark regimen. All the experimental animals were given postsurgical care in accordance with the NZ Animal Welfare Act (1999). Antibiotics [Crystapen, 600 mg (Biochemie, Vienna, Austria) daily for 4 days and gentamycin, 80 mg (Pharmacia), daily for 2 days] were given intravenously to the ewe. Fetal catheters were kept patent by infusing heparinized saline (20 IU/ml at 0.2 ml/h), and the maternal catheter was maintained by daily flushing with heparinized saline.

Fetal arterial blood was collected every morning starting from 24 h before the experiment until the day of post mortem for pH, blood gases, electrolytes, hematocrit, hemoglobin (845 Blood Gas Analyzer and Co-oximeter, Ciba-Corning Diagnostics, MA), and glucose and lactate content (model 2300, YSI, Yellow Springs, OH). Additional blood samples were collected just before the infusion/bolus and 2 and 6 h after injections of LPS or saline, and plasma was analyzed for cortisol, ACTH, interleukin (IL)-6, and IL-10 levels.

Recordings

All signals were acquired at 512 Hz, processed online, and stored to disk as 1-min averaged intervals by custom software (Labview for Windows, National Instruments, Austin, TX). Fetal mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure (Novatrans II MX860, Medex, Hilliard, OH), fetal heart (FHR), and carotid artery blood (CaBF) were recorded using an 125I RIA kit (24130, DiaSorin, Stillwater, MN) validated for ovine maternal and fetal plasma. The intra-assay coefficient of variation was 9.7% and the inter-assay coefficient was 12.8%. The mean ACTH assay sensitivity was 9.7 pg/ml, and samples showing less than this value were assigned this value for analysis.

IL-6 and IL-10

Plasma cytokine concentrations were measured using in house-developed enzyme-linked immunosorbent assays. IL-6 was detected using antibodies specific to ovine species (Epitope Technologies, Melbourne, Australia). Ovine recombinant IL-6 was used as standards (Protein Express, Cincinnati, OH). The standard series ranged from 0 to 5 ng/ml. Each sample was run in duplicate, and the averages were obtained. Internal quality controls were included in each assay. The mean assay sensitivity was 0.097 ng/ml. Cytokine concentrations were within the detection limit in all samples. IL-10 was detected using antibodies specific to the bovine species (AbD Serotec, Oxford, UK). Standards were recombinant bovine IL-10 (kindly provided by Prof. G. Ehrlich, Moredun Research Institute, Scotland) and ranged from 0 to 11 BU/ml with a detection sensitivity of 0.086 BU/ml.

Histology

The fetal brains were further fixed for a period of 7 days in 10% neutral buffered formalin, then sliced into 4, 4- to 5-mm thick, coronal blocks from rostral to caudal using a sheep brain matrix. Paraffin-embedded coronal sections were cut at 10-μm thickness and used for histology and immunohistochemistry.

Basic histological evaluation was conducted on thionin and acid-fuchsin-stained slides under a Nikon Eclipse 80i microscope. An experimenter blind to different treatments assessed these slides for neuronal death, structural damage, gross tissue infarction, and necrosis in the cortex, hippocampus, striatum, and thalamus. Neuronal death was identified by the presence of pink nuclei and contracted nuclei (21). White matter lesions and infarctions were assessed in the periventricular white matter (PVWM), intra-gyral white matter (IGWM), and other deep subcortical white matter areas. Measurements of microglia-infiltrated areas and cell counting were undertaken with Stereo Investigator software (Micro Bright Field, Williston, VT). The region of interest was initially traced at 2× magnification, then a grid was randomly translated onto the sections, and the fractionator
probe consisting of a counting frame for object inclusion/exclusion was performed at 40× magnification. Cell counts for the region of interest were converted to density (cells/mm²) by applying the equation [(total markers counted/number of sampling sites × counting frame area)] × 10⁶ (11).

Immunohistochemistry

Reactive microglia were labeled with biotin-conjugated lectin from Lycopersicon esculentum (Isolectin B4, Sigma-Aldrich, Sydney, Australia). Deparaffinized, rehydrated, antigen unmasked (0.1 M citrate buffer, pH 6.0, 20 min, pressure cooking method), and endogenous peroxidase-quenched (1% H₂O₂ in methanol) sections from the level of the striatum were incubated with biotinylated lectin (diluted 1:100 with Tris-buffered saline) at 4°C overnight. Sections were then incubated with ExtrAvidin peroxidase in PBS (1:200, Sigma) for 3 h at room temperature before developing color with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma).

The following primary antibodies were used for immunohistochemical analysis: monoclonal mouse anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) for labeling oligodendrocytes (Abcam, www.abcam.com, diluted 1:200), monoclonal mouse anti-tumor necrosis factor (TNF)-α (1:200, AbD Serotec) for labeling TNF-α-positive cells and rabbit cleaved anti-caspase 3 ASP 175 for labeling cells undergoing apoptosis (diluted 1:200; Cell Signaling Technology, Beverly, MA). The deparaffinized, rehydrated, antigen-retrieved and peroxidase-quenched sections were incubated with respective 5% serums for 1 h at room temperature followed by primary antibody prepared in 0.1 M PBS + 2.5% serum for two nights at 4°C. Biotin-conjugated secondary antibody (1:200 in PBS + 2.5% serum, overnight at 4°C), ExtrAvidin peroxidase (Sigma, 1:200 for 3 h at RT), and DAB were used to visualize positive signal. Negative (no antibody) controls were run in parallel.

Gyri assessment. To assess whether LPS infusion influenced gyral development, thionin-acid fuchsin-stained sections at +17 and 26 mm from stereotaxic zero were analyzed. A contour was traced horizontal to the midline of the hemispheres touching the upper limit of the lateral ventricle using StereoInvestigator. A comparable area in the whole hemisphere dorsal to this line (A) and the total length of the boundary (L) was measured for calculating the gyral surface folding index (GSFI). The mathematical formula for calculating GSFI is L²/A (33). GSFI is not affected by shrinkage of tissue, and given that the SFI of a circle is 12.56, higher values suggest greater expansion of the surface relative to total volume (7).

Data Analysis

Body and organ weights were analyzed by one-way analysis of variance (ANOVA, SPSS v15, SPSS, Chicago, IL). Physiological data were processed in minute averages for the 24-h, baseline, and 10-day experimental period and subsequently averaged into time periods as outlined in RESULTS. The effect of the infusions on physiological variables were evaluated by ANOVA with time as a repeated measure followed by Fisher’s protected least-significant difference (LSD) post hoc test when a significant overall effect was found. Blood gas analysis and hormone and cytokine results were assessed by one-way ANOVA at each time point with subsequent LSD post hoc tests. Where baseline differences were found an ANCOVA was used for the remainder of those data. Nonparametric data were tested with Mann-Whitney U tests. Statistical significance was accepted when P < 0.05. Data were means ± SE.

RESULTS

Mortality and Postmortem Findings

Six of 11 fetal sheep exposed to saline infusion + LPS boluses died after the first bolus (Sal+LPS); 1 died after 4 h, 4 after 5 h, and 1 after 7 h (Fig. 1). In contrast, no LPS+LPS (n = 9) or LPS+Sal (n = 5) fetuses died during the experiment. This indicates that LPS preinfusion was associated with reduced mortality after subsequent acute LPS boluses (P = 0.014, Fisher’s exact test), with an absolute risk reduction of −0.55 (95% CI, −0.04 to −0.55).

There were no significant differences in the total body weight (Sal+Sal 2.1 ± 0.1 vs. LPS+Sal 2.2 ± 0.1 vs. LPS+LPS 2.3 ± 0.1 vs. Sal+LPS 1.92 ± 0.07 kg) or organ weights (brain weight: 29.7 ± 2.3 vs. 33.3 ± 4.3 vs. 27.2 ± 1.1 vs. 26.3 ± 5.1 g) between groups at postmortem.

Fetal Arterial pH, Blood Gases, Glucose, and Lactate Levels

There was no significant effect of LPS+Sal compared with Sal+Sal (Table 1). In the LPS+LPS and Sal+LPS (lived) groups, the first bolus on day 3 was associated with a significant increase in PaCO₂, HCO₃⁻, base excess, and lactate, and a fall in oxygen content and glucose by 6 h compared with Sal+Sal (P < 0.05). Sal+LPS (died) fetuses showed a fall in pH, base excess, and a trend for a greater rise in lactate levels at 5 to 6 h after the bolus. In surviving fetuses, all measurements subsequently returned to control values, and there was no significant effect of the subsequent boluses, except for a small increase in PaCO₂ in the Sal+LPS (lived) group after the
second bolus of LPS. There were no significant changes in pH, PaO$_2$, hematocrit, or hemoglobin concentration.

**Cardiovascular Changes**

There was a consistent increase in MAP over the 10-day period of observation in all surviving fetuses ($P < 0.001$, Fig. 2). Low-dose LPS infusion with saline boluses was not associated with any significant effect either during the period of infusion or afterwards compared with saline controls. In contrast, in the Sal+LPS (lived) and LPS-LPS groups, LPS boluses were associated with a consistent pattern of increased MAP in the first hour followed by significant hypotension from 4 to 10 h, reaching similar nadirs but more rapidly in the Sal+LPS (lived group), at 5 ± 0.8 h and 8.3 ± 0.3 h, respectively ($P = 0.01$). Blood pressure changes with the second and third boluses were less than those after the first bolus ($P < 0.05$) but in a similar pattern in both groups. Fetuses in the Sal+LPS (died) group showed a fall in MAP within the first hour after LPS ($P < 0.05$ vs. fetuses that lived), were not significantly different at 4 h after LPS boluses than saline boluses from 7 to 8 h after the first bolus.

**Table 1. Fetal arterial pH, blood gases, glucose, and lactate levels**

<table>
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<tr>
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<th>Baseline</th>
<th>Bolus 1</th>
<th>Bolus 1 + 2 h</th>
<th>Bolus 1 + 6 h</th>
<th>Bolus 2 + 6 h</th>
<th>Bolus 3 + 6 h</th>
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<tr>
<td>pH</td>
<td>Sal+Sal</td>
<td>7.38 ± 0.01</td>
<td>7.36 ± 0.01</td>
<td>7.36 ± 0.01</td>
<td>7.37 ± 0.01</td>
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<td>LPS+Sal</td>
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<td>7.38 ± 0.00</td>
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<tr>
<td></td>
<td>Sal+LPS (died)</td>
<td>7.38 ± 0.00</td>
<td>7.36 ± 0.01</td>
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<td>7.22 ± 0.06*</td>
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</tr>
<tr>
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Data are means ± SE. 1LPS infusion was started on day 0, boluses of saline or lipopolysaccharide (LPS) were given at the start of days 3, 4, and 5, and the LPS infusion was stopped on day 6. PaCO$_2$, fetal arterial pressure of carbon dioxide; PaO$_2$, fetal arterial pressure of oxygen; O2ct, fetal arterial oxygen content; BE, base excess; SaO$_2$, arterial oxygen saturation. *$P < 0.05$ vs. Sal+Sal, †$P < 0.05$ vs. LPS+Sal (ANOVA).
bolus. There was no effect on FHR after the end of the LPS infusion. Within the Sal/Sal group, surviving fetuses reached maximal FHR after the first LPS bolus more rapidly than the LPS/LPS group, after 4.4 ± 0.5 h versus 7.8 ± 1.0 h, respectively, to similar maximum values. There were no significant subsequent differences. In contrast, Sal/LPS (died) fetuses showed an immediate increase in FHR compared with Sal/Sal (lived) fetuses (P < 0.01). FHR then returned to near control values at 2 h, then rose in parallel with fetuses that survived LPS, followed by severe bradycardia coincident with the onset of terminal hypotension (Fig. 1).

CaBF increased gradually in all groups over time, similarly to MAP. The low-dose infusion of LPS with saline boluses had no effect compared with saline controls. In the LPS/LPS group, each bolus of LPS was associated with a marked, transient increase (P < 0.001), reaching a maximum 7–9 h after the bolus, with no significant change in this pattern with repeated boluses. Oxygen delivery (oxygen content × CaBF) was not significantly affected by LPS boluses (6 h after the first bolus, Sal+Sal: 0.137 ± 0.006 vs. LPS+LPS: 0.134 ± 0.006 mmol/min).

Endocrine and IL-6 and IL-10 Responses

ACTH levels were not significantly affected by LPS infusion or boluses (Fig. 3). In contrast, although fetal cortisol levels were not significantly affected by the low-dose LPS infusion, there was a sustained increase after the first 1-μg LPS bolus on day 3 and day 4 (P < 0.01), which resolved by day 5, with no further increase after the third bolus.

IL-6 levels did not increase significantly during saline or low-dose LPS infusions. There was a transient increase in IL-6 levels after bolus 1 (P < 0.001), that resolved by the next day, with no further response to subsequent doses. In contrast, IL-10 levels increased 2 h after the start of the low-dose LPS infusion (P < 0.001), resolved to control levels by 6 h, with no significant change after the increased

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Fig. 2. Time sequence of changes in fetal MAP, FHR, and fetal carotid artery blood flow (CaBF). Dashed vertical lines show the timing of bolus infusions. Data are 1 h means ± SE. Short horizontal bars P < 0.05, LPS+LPS vs. Sal+Sal.

Fig. 3. Time sequence of changes in IL-6, IL-10, cortisol, and ACTH levels over the 10-day experimental period in the Sal+Sal, LPS+Sal, and LPS+LPS groups. Data are means ± SE. Each time point was tested by one-way ANOVA. Light shaded area: low-dose infusion (100 ng over 24 h); dark shaded area: increased infusion (250 ng over 24 h). B1, bolus 1; B2, bolus 2; B3, bolus 3. ***P < 0.01, **P < 0.001.
infusion rate on day 2. IL-10 levels increased 2 h after the first bolus and then resolved rapidly, with no significant response to subsequent boluses.

**Histology**

There was no evidence of structural injury or neuronal death in the hippocampus, striatum, or thalamus in all but one LPS-LPS fetus. This fetus developed a large area of PVWM necrosis with associated hemorrhage (Fig. 4B). The lesion extended into the dorsal subcortical white matter and to the anterior end of the ventral external capsule. Because of the extensive nature of this lesion, this brain was excluded from remaining analyses. There was infiltration of thionin blue-positive small cells in the lesion area, which were probably leucocytes and other macrophages. Intraventricular hemorrhage was not observed in any other animals.

**Immunohistochemistry**

In saline-treated control brains, resting microglia (38) were sparsely distributed in the PVWM, IGWM, and cortical gray matter in the parietal and temporal lobes, external capsule, and subventricular zone (Fig. 4A). Both LPS groups showed patchy infiltration of reactive microglia in the PVWM (Sal+Sal: 1.3 ± 1.3%; LPS+Sal: 14.3 ± 4.5%; LPS+LPS: 15.5 ± 10.3% of PVWM area, P < 0.001 vs. Sal+Sal; not significant vs. LPS+Sal). A similar but less extensive pattern of patchy infiltration was seen in the IGWM, subventricular zone, and external capsule in both LPS groups (data not shown). The increase in activated microglia in the PVWM was significantly greater in the LPS+LPS group than the LPS+Sal group (P < 0.001, Fig. 5).

There was loss of CNPase +ve oligodendrocytes in PVWM in LPS+LPS compared with Sal+Sal fetuses (Fig. 5). Al-

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Fig. 4. Top: photomicrographs showing activated microglia [isolectin B4 (IB4), A–C] and oligodendrocytes [2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), D–F] in periventricular white matter (PVWM) in the Sal+Sal (A, D), LPS-Sal (B, E), and LPS-LPS (C, F) groups. Note the areas of patchy microglial infiltration (arrows) with reduced CNPase labeling. Scale bar: 40 μm. Bottom: white matter necrosis in the PVWM of a fetus in LPS+LPS group (A). Note IB4-positive cells around the area of hemorrhage (arrows show microglia) (B). CNPase staining (C) is shown. Note that there are few CNPase-positive cells in the same area. Scale bar C: 350 μm; B: 40 μm.
though some individual fetuses in the LPS + Sal group showed severe apparent loss (Fig. 4A), overall survival of CNPase positive cells was intermediate and not significantly different from the other two groups. This was associated with an increase in total number of caspase-3 positive cells in the PVWM in both LPS groups compared with the Sal + Sal group ($P < 0.01$). Finally, there was an increase in TNF-α-positive cells in the PVWM in both LPS groups compared with the Sal + Sal group ($P < 0.0001$).

Cortex and Periventricular White Matter Measurements

The LPS + Sal and LPS + LPS groups were not associated with any change in GSFI (Sal + Sal: 49.6 ± 3.9; LPS + Sal: 47.7 ± 3.0; LPS + LPS: 55.8 ± 3.8).

DISCUSSION

The present study confirms that high-dose, acute exposure to LPS is associated with acute cardiovascular compromise and demonstrates that this can be markedly attenuated by 48-h exposure to a low-dose infusion of LPS in chronically instrumented preterm fetal sheep. Mild fetal hypotension, tachycardia, and increased carotid blood flow with mixed respiratory and metabolic acidosis were seen after the first bolus of LPS. However, in contrast with the group that received saline preinfusion with the same LPS boluses, and previous studies using similar doses of LPS (9, 15), there were no fetal deaths. Intriguingly, there was further attenuation of hypotension with successive LPS boluses, showing that the infusion did not induce complete or maximal insensitivity to LPS. The historical outcomes also support a relative reduction in sensitivity to brain injury after low-dose LPS infusion. Although LPS + LPS was associated with greater induction of activated microglia in the PVWM, excluding one fetus that developed a unilateral white matter infarct, the area of microglial infiltration, loss of CNPase-positive oligodendrocytes, numbers of activated caspase-3 and TNF-α-positive cells, and gyral complexity were strikingly similar after infusion + boluses compared with infusion alone.

Acute exposure to LPS, consistent with acute ascending *E. coli* infection, is associated with fetal hypotension, hypoxia, and metabolic acidosis in the fetal sheep (9, 13, 15), consistent with the present study. Although no mortality was reported by Duncan and colleagues in a slightly less mature cohort (0.65 of gestation) (13), others report acute loss of a third to over 40% of fetuses associated with terminal hypotension (9, 15, 31), similar to the deaths of 6/11 fetuses in our preliminary studies. Although it is unknown what determines whether or not fetuses are able to tolerate acute exposure to LPS, it is intriguing to note that the fetuses that died after the first bolus of LPS showed an immediate fall in blood pressure from the first hour after the LPS bolus, in contrast with initial hypertension for the first 2 h in fetuses that survived, consistent with a marked preexisting difference in tolerance to LPS. The initial hypotension in those that died was associated with rapid, transient tachycardia consistent with a baroreflex response, with no apparent impairment of cardiac chronotropic responses (4). Consistent with this, surviving fetuses also developed tachycardia, in parallel with delayed hypotension (4). Thus the mechanism of profound hypotension is likely a combination of impaired cardiac contractility with systemic vasodilation and reduced placental perfusion (9, 17). The findings in the present study, and previously, of increased carotid blood flow despite hypotension are consistent with marked fetal vasodilation (15, 31).

These data of course relate to LPS naive animals. Self-induced tolerance to LPS is well recognized in many settings. For example, repeated boluses of LPS 24 h apart are associated with markedly reduced hypotension after successive boluses (13, 15), similar to the pattern seen in surviving fetuses in the Sal + LPS group and the LPS + LPS group in the present study. Our study demonstrates that preinfusion with LPS for 48 h before the first bolus of LPS significantly reduced mortality in preterm fetal sheep. Furthermore, the nadir of hypotension and the corresponding maximum increase in fetal heart rate were significantly delayed by the LPS preinfusion compared with fetuses that survived after receiving an LPS bolus with saline preinfusion. Isolated monocytes also show tolerance after chronic or repeated exposure to endotoxin in vitro (25, 27). This has been related at least in part to downregulation of receptors, including CD14 (28).

Alternatively, counter-regulatory responses may be important. In the present study, the potent immunoregulatory cyto-
kine IL-10 was induced 2 h after the start of the continuous LPS infusion and after the first bolus of LPS, before levels of the pro-inflammatory cytokine IL-6 increased. In mouse astrocyte culture, IL-10 provides rapid onset suppression of IL-6 induction after IL-1β stimulation (32). In rats, maternal treatment with IL-10 prevented E. coli-related brain injury in the pups (34). Of particular interest for the present study, treatment with IL-10 within 24 h after LPS injection to pregnant rats prevented fetal growth retardation or death (37), whereas, in newborn mice, IL-10 attenuated both excitotoxic and IL-1β-related neural injury (30).

Finally, cortisol has direct cardiac effects, mediated by augmented coupling of the β-adrenoreceptors to cellular post-receptor signal transduction (3) and likely by augmenting sympathetic responses (6, 35) and activation of the fetal renin-angiotensin system (16, 40). Therefore, it is plausible that the sustained, largely ACTH independent, increase in fetal cortisol levels in the first and second days after the LPS boluses in the present study may have helped to limit the fall in fetal blood pressure after the first two boluses.

A limitation of the present study is that there were insufficient brains available from the Sal+LPS group to assess whether the low-dose infusion also protected the brain from LPS boluses. Nevertheless, the limited additional histopathological impact of the LPS boluses compared with the continuous LPS infusion with saline boluses suggests that the infusion also induced relative neural tolerance to LPS. The effects are likely to be indirect because, although LPS has a high affinity for microglia and astrocytes (36), it shows limited penetration of the blood barrier in mice, even after repeated injections (1). It is unlikely that LPS was associated with a material degree of cerebral ischemia. Although the LPS+LPS group developed hypotension after LPS boluses, with a significant fall in oxygen content after the first bolus, this was associated with increased carotid blood flow, mediated by marked vasodilation, such that oxygen delivery remained normal, at least up to 6 h after the LPS bolus.

Consistent with other, indirect mechanisms of toxicity, LPS can stimulate microglial activation in fetal white matter after maternal infusions (24). In the present study for example, there was greater expression of TNF-α-positive cells in the PVWM that potentially may represent an indirect mechanism of LPS-induced neural inflammation. In vitro, high concentrations of TNF-α induce oligodendrocyte death and vacuolation of myelin (29). Furthermore, in the present study, the major feature of LPS exposure was patchy microgliosis covering a mean of 15% of the area of the PVWM in both LPS chronic infusion groups. There was no apparent reduction in brain weight or loss of gyral white matter complexity in either group. Although microgliosis were markedly more induced after LPS boluses, there was relatively modest loss of the immature/mature myelinating oligodendrocytes labeled by CNPase, in the LPS+LPS group compared with saline controls. Furthermore, there was a similar increase in white matter apoptosis between groups, suggesting that the potential toxic effects of microgliosis were comparatively limited in this setting. However, we cannot exclude the possibility that greater toxic effects might have manifested after a longer period of recovery.

**Perspectives and Significance**

Clinically, severe infection is associated with a high risk of cerebral palsy in survivors of preterm birth. In animals, acute boluses of LPS, representing rapid onset, severe *E. coli* infection, can trigger periventricular necrosis suggesting that infection is indeed one potential mechanism of periventricular leukomalacia after preterm birth. It is striking that such overt lesions are gradually becoming infrequent for unclear reasons (5, 23). The present study demonstrates that a relatively short and low-dose infusion, which did not trigger any hemodynamic disturbance, was associated with marked reduced cardiovascular compromise after subsequent large boluses of LPS and a low risk of severe white matter injury. Given that milder or subclinical infection is much more common and also associated with adverse outcomes (20, 39), it is possible that rapid self-induction of tolerance to LPS may be one factor helping to reduce the risk of severe white matter damage after perinatal infection.

**GRANTS**

This study was funded by grants from the Health Research Council of New Zealand, the Auckland Medical Research Foundation, and the Lotteries Board of New Zealand.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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*AJP-Regul Integr Comp Physiol* • doi:10.1152/ajpregu.00388.2012 • www.ajpregu.org

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