Biphasic changes in fetal heart rate variability in preterm fetal sheep developing hypotension after acute on chronic lipopolysaccharide exposure

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ABSTRACT

Perinatal exposure to infection is highly associated with adverse outcomes. Experimentally, acute, severe exposure to gram negative bacterial lipopolysaccharide (LPS) is associated with increased fetal heart rate variability (FHRV). It is unknown whether FHRV is affected by subclinical infection with or without acute exacerbations. We therefore tested the hypothesis that FHRV would be associated with hypotension after acute on chronic exposure to LPS. Chronically instrumented fetal sheep at 0.7 gestation were exposed to a continuous low-dose LPS infusion (n = 12, 100 ng/kg over 24 hours, followed by 250 ng/kg/24 hours for a further 96 hours) or the same volume of saline (n = 10). Boluses of either 1 μg LPS or saline were given at 48, 72 and 96 hours. Low-dose infusion was not associated with hemodynamic or FHRV changes. The first LPS bolus was associated with tachycardia and suppression of nuchal electromyographic activity in all fetuses. 7/12 fetuses developed hypotension (a fall in mean arterial blood pressure ≥5 mmHg). FHRV was transiently increased only at the onset of hypotension, in association with increased cytokine induction and EEG suppression. FHRV then fell before the nadir of hypotension, with transient suppression of short-term FHRV. After the second LPS bolus, the hypotension group showed a biphasic pattern of a transient increase in FHRV followed by more prolonged suppression. These findings suggest that infection-related hypotension in the preterm fetus mediates the transient increase in FHRV and that repeated exposure to LPS leads to progressive loss of FHRV.
INTRODUCTION

Perinatal infection is associated both with greater risk of preterm labor (24) and with greater risk of adverse outcomes (42, 49). The mechanisms of this increased risk likely include both direct effects of the inflammatory cascade, and cardiovascular impairment (41, 42). Chorioamnionitis has been associated with lower blood pressure and hemodynamic instability in preterm infants (72), while hypotension in itself may also contribute to neurodevelopmental impairment (50, 61). Experimentally, acute high-dose exposure to the lipopolysaccharide (LPS) component of the gram negative bacterial cell wall is associated with cardiovascular impairment as shown by hypotension with peripheral vasodilation, and subsequent neural injury in preterm fetal sheep (16, 43, 54).

Fetal heart rate (FHR) changes and associated parameters including heart rate variability (HRV) are key clinical indices of fetal wellbeing (52). Early-onset sepsis in preterm infants has been associated with reduced HRV and transient decelerations (27, 28), which can support early diagnosis and so help reduce infant mortality (17, 47). In contrast, in preterm fetal sheep, a single LPS bolus (i.e. rapid intravenous injection) was associated with increased fetal HRV (FHRV) between 2 and 4 hours after exposure (6). Although this could simply reflect differences between newborns and fetuses, potentially, it could reflect the speed of onset of sepsis.

Acute injection of LPS is consistent with extremely rapid-onset sepsis. However, both subclinical and subacute infections that progress relatively slowly before acute clinical manifestations are more common and also associated with adverse outcomes (26, 71). For example, in a prospective multicenter study of very-low birth weight infants, inflammatory markers such as plasma interleukin (IL)-6 were elevated several days before the onset of clinical symptoms (38). It is striking that in preclinical studies, exposure to LPS rapidly results in self-tolerance, that is to say, attenuated responses to further doses of LPS (16, 43).
For example, 48 hours of chronic low-dose infusion of LPS is associated with reduced mortality and less severe hypotension after subsequent high-dose boluses in preterm fetal sheep (43).

Given the discordance between the experimental and clinical literature on the effects of infection on HRV we examined the hypothesis that induction of self-tolerance by low-dose LPS infusion may modify the FHRV response to acute LPS exposure in preterm fetal sheep from previously published studies (8, 43), at 0.7 gestation, when neural maturation of the sheep is broadly equivalent to 28-32 weeks of human development (2). We examined whether the FHRV responses were associated with fetal hypotension, impaired peripheral vascular tone as shown by changes in femoral blood flow (FBF) and conductance (FVC, the reciprocal of resistance) and induction of circulating cytokines. Given the evidence that septic shock may be associated with impaired adrenal stress responses (57), fetal plasma cortisol levels were measured. Fetal movements can contribute to FHRV (12, 13, 40), while seizures have also been associated with increased FHRV, both clinically and in fetal sheep (69). Therefore the relationship between FHRV, nuchal electromyographic (EMG) and EEG activity were examined. Finally, FHR and FHRV show distinctive diurnal rhythms (13), that may be in part related to autonomic activity (34). We therefore examined whether LPS exposure altered these rhythms.
MATERIAL AND METHODS

Animals and experimental procedures

All procedures were approved by the Animal Ethics Committee of the University of Auckland. Singleton Romney/Suffolk fetal sheep were surgically instrumented at 98-100 days of gestation (term = 147 days) as previously reported (8, 43). Ewes were given 5 mL of Streptocin (procaine penicillin, 250,000 IU/mL, and dihydrostreptomycin, 250 mg/mL, Stockguard Labs, Hamilton, New Zealand) intramuscularly 30 minutes before surgery for prophylaxis. Anesthesia was induced by intravenous injection of Alfaxan (Alphaxalone, 3 mg/kg, Jurox, Rutherford, New South Wales, Australia), and general anesthesia was maintained by 2-3% isoflurane in oxygen. A midline incision was made to expose the uterus, and the fetus was partially exteriorized for instrumentation. Polyvinyl catheters were placed in the amniotic sac, left femoral artery and vein and right brachial artery to measure blood pressure and for pre-ductal blood sampling. A 2 R-type ultrasonic blood flow probe (Transonic Systems, Ithaca, New York, United States) was placed around the femoral artery to measure FBF. Two pairs of electrodes (Cooner Wire, Chatsworth, California, United States) were placed over the parietal cortex bilaterally, 10 mm lateral to bregma and 5 mm and 10 mm anterior to measure EEG activity. A reference electrode was sewn over the occiput. The nuchal muscle was then exposed on the right side of the neck, and two electrodes sewn into the muscle to record nuchal EMG activity. A pair of electrodes was placed across the fetal chest to measure the fetal electrocardiogram (ECG). All fetal leads were exteriorized through the maternal flank, and a maternal saphenous vein was catheterized for post-operative care and euthanasia.

Antibiotics were administered into the amniotic sac (80 mg Gentamicin, Pharmacia and Upjohn, Rydalmere, New South Wales, Australia) before the uterus was closed. The maternal midline skin incision was infiltrated with local analgesic (10 mL 0.5% bupivacaine plus
adrenaline, AstraZeneca Ltd., Auckland, New Zealand). After surgery, ewes were housed together in separate metabolic cages with *ad libitum* access to food and water. Rooms were temperature and humidity controlled (16 ± 1°C, humidity 50 ± 10%) with a 12 hour light/dark cycle (light 0600 to 1800 hours). Ewes were given daily intravenous antibiotics (600 mg Crystapen, Biochemie, Vienna, Austria and 80 mg Gentamicin, Pharmacia and Upjohn) for four days after surgery. Fetal catheters were maintained patent with continuous infusion of heparinized saline (20 U/mL at 0.2 mL/h).

Fetal arterial blood was collected every morning starting from 24 hours before the experiment until the day of post mortem for pH, blood gases (845 Blood Gas Analyzer and Co-oximeter, Ciba-Corning Diagnostics, Massachusetts, United States), and glucose and lactate content (model 2300, YSI, Yellow Springs, Ohio, United States). Further samples were taken at 2 and 6 hours after the start of low-dose LPS infusion and the first LPS bolus and subsequently at 6 hours after the remaining two LPS boluses. At each of these time points additional blood samples were taken for tumor necrosis factor α (TNFα), IL-6, IL-10 and cortisol analysis, as described below.

**Recordings**

Fetal mean arterial pressure (MAP), ECG, EEG, EMG and FBF were recorded continuously for offline analysis using custom data acquisition programs (LabView for Windows, National Instruments, Austin, Texas, United States). Fetal MAP was recorded using Novatrans II, MX860 pressure transducers (Medex Inc., Hilliard, Ohio, United States) and corrected for maternal movement by subtraction of amniotic fluid pressure. The blood pressure signal was collected at 64 Hz and low-pass filtered at 30 Hz. The analogue fetal EEG signal was low-pass filtered with the cut-off frequency set with the -3 dB point at 30 Hz, and digitized at a sampling rate of 512 Hz. The intensity (power) was derived from the intensity spectrum...
signal between 0.5 and 20 Hz while spectral edge was calculated as the frequency below which 90% of the intensity was present. For data presentation, the total EEG power was normalized by log transformation (dB, $20 \times \log$ intensity). The nuchal EMG signal was band-pass filtered between 100 Hz and 1 kHz, the signal was then integrated using a time constant of 1 second and digitalized at 512 Hz. The fetal ECG was analog filtered using a first-order, high-pass filter at 0.05 Hz and a low-pass, eight-order Bessel filter at 100 Hz and digitalized at 512 Hz, and used to derive FHR and FHRV, as described below.

**Experimental Protocol**

Experiments started 5 days post-surgery. Fetuses were randomized into 2 experimental groups: (1) chronic saline infusion and saline boluses (Saline controls, $n = 10$) and (2) chronic LPS infusion and LPS boluses ($n = 12$). LPS was dissolved in saline and infused at 100 ng/kg (50 ng/mL at 83 µL/hour) for the first 24 hours followed by 250 ng/kg/24 hours (50 ng/mL at 207.5 µL/hour) for the next 96 hours. Boluses were administered as 1 µg LPS dissolved in 1 mL of saline, or the same volume of saline, at 48, 72 and 96 hours from the start of infusion. The chronic infusions were chosen to cause minimal cardiovascular alterations, consistent with a subclinical infection (35). The 1 µg bolus is known to be associated with neuroinflammation in previous studies (15, 16, 18), but little mortality when given after the chronic infusion (43). At 100 hours after the start of infusion 4 fetuses from the Saline control group and 7 fetuses from the LPS group then received complete umbilical cord occlusion for inclusion in a separate study (8). Instrumentation for these fetuses was identical except an inflatable silicone occluder was also placed around their umbilical cord. For this reason data were only analyzed until 99 hours after the start of infusions.

**Fetal cytokine measurements**

Cytokine levels in the plasma were measured using an in-house enzyme-linked
immunosorbent assay (43). TNFα was detected using antibodies specific to the ovine species (Epitope Technologies, Melbourne, Australia). Standards were ovine recombinant TNFα and ranged from 0-10 ng/mL with a detection sensitivity of 0.354 ng/mL. IL-6 was detected using antibodies specific to ovine IL-6 (Epitope Technologies). Standards were ovine recombinant IL-6 (Protein Express, Cincinnati, Ohio, United States). The standard series ranged from 0-5 ng/mL and the assay sensitivity was 0.097 ng/mL. IL-10 was detected using antibodies specific to the bovine species (AbD Serotec, MorphoSys, Kidlington United Kingdom) (43). Standards used were recombinant bovine IL-10 (kindly supplied by Professor G. Entrican, Moredun Research Institute, Scotland) and ranged from 0-11 BU/mL with a detection sensitivity of 0.086 BU/mL. Internal quality controls were included in each assay and cytokine concentrations were within the detection limit in all samples.

Cortisol analysis

Fetal plasma cortisol levels were measured using triple quadrupole mass spectrometry (43). 100 µL of internal standard (20 ng/mL cortisol-d4 in water) was added to 200 µL plasma. Steroids were extracted using 1 mL of ethyl acetate (Merck KGaA, Darmstadt, Germany). After removal of the organic supernatant, samples were dried by vacuum concentration (Savant SC250EXP, Thermo Scientific, Asheville, North Carolina, United States), re-suspended in 60 µL of mobile phase 72% methanol (Merck KGaA) and 28% water, and transferred to HPLC injector vials. 12 µL was injected onto an HPLC mass spectrometer system consisting of an Accela MS pump and autosampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcaliber software (Thermo Electron Corporation, San Jose, California, United States). The mobile phase was isocratic, flowing at 250 µL/min through a Luna HST 2.6 µm C18 (2) 100 x 3.0 mm column at 40°C (Phenomenex, Auckland, New Zealand). Retention time was 3.1 minutes for both cortisol and cortisol-d4. Ionisation was in
positive mode and Q2 had 1.2 mTorr of argon. The mass transitions followed were: cortisol-d4 367.2 \( \Pi \) 121.2 at 28 V and cortisol 363.2 \( \Pi \) 122.2 at 28 V. Mean inter- and intra-assay CV values for cortisol were 5.8% and 6.0% respectively.

Data analysis

In order to assess cardiovascular impairment following LPS administration, we compared fetuses that developed a fall in MAP of \( \geq 5 \) mmHg (equivalent to a fall of \( \geq 2 \) standard deviations of baseline MAP) after the first LPS bolus (LPS-Hypotension, \( n = 7 \)) with normotensive fetuses (LPS-Normotension, \( n = 5 \)). Long-term FHRV was calculated as described by Dawes and colleagues (14) to obtain the mean minute range (MMR, the difference between the maximum and minimum R-R intervals every minute). FHRV was not measured during accelerations or decelerations of \( \geq 10 \) beats/minute for more than 1 minute or \( \geq 20 \) beats/minute for more than 30 seconds (14). For comparison, we also calculated short term variability (STV) (14) and RMSSD (the root mean square of successive differences in R-R intervals)(63). Changes in STV showed an essentially identical pattern to MMR (within-subjects \( p < 0.001 \), \( R^2 = 0.93 \), \( n = 13 \)), and therefore are not shown. The diurnal rhythm of FHR was determined as the time to the daily peak in FHR relative to 9 am and shown for the first five days of recording. FVC was calculated as FBF / MAP (56).

Data were processed in minute averages for the 24 hour baseline and the first 99 hours of the experimental period and subsequently averaged into 1 hour averages. Statistical analysis was performed using SPSS (v22, SPSS Inc., Chicago, Illinois, United States). For physiological, cytokine and blood gas data, differences between groups were evaluated using analysis of variance (ANOVA) followed by a Fisher’s protected least-significant difference (LSD) post hoc test when a significant overall effect was found. FHR was divided into periods of 4 hour averages prior to being assessed as above. Changes in FBF were further assessed after the first LPS bolus using one way ANOVA by comparing hourly averages with the hour
immediately before bolus administration. The within-subjects relationship between MAP and FVC was examined for the first 10 hours after the first LPS bolus using the method of Bland and Altman (7). Data are presented as means ± SEM. Statistical significance was accepted when p < 0.05.
RESULTS

Fetal arterial pH, blood gases, lactate and glucose levels

There were no significant differences in pH, pO₂, pCO₂, lactate and glucose measurements during the baseline period (Table 1). There were also no significant differences during the initial 48 hours of low-dose infusion, and so these time points are not shown. After the first LPS bolus, pH was significantly lower at 2 hours while lactate was significantly higher at 6 hours in the LPS-Hypotension group compared to both the Saline control and LPS-Normotension groups (p < 0.05). There were no significant differences in pO₂ and pCO₂ except for a trend for an increase in pCO₂ at 2 hours after the first LPS bolus in the LPS-Hypotension group (p = 0.05). Glucose was significantly lower in the LPS-Hypotension group at 6 hours after the first LPS bolus compared to both the Saline control and LPS-Normotension groups (p < 0.05).

Mean arterial pressure

There were no significant differences in MAP between groups in the baseline period and initial 48 hours of low-dose infusion. With the first LPS bolus, MAP fell in the LPS-Hypotension group between 55-57 hours compared to Saline controls (Figure 1, p < 0.05). During this time, the LPS-Normotension group did not significantly differ from Saline controls. Thereafter, there were no significant differences between groups.

Femoral blood flow and vascular conductance

There was a temporal increase in FBF in all groups over the course of this study (Figure 1, p < 0.01). Two fetuses from both LPS groups did not have continuous FBF recordings. There was a significant interaction over time with group in FBF after the first LPS bolus, from 48-71 hours (p < 0.005). After the first LPS bolus, FBF was significantly lower in the LPS-Hypotension group at 51-52 hours compared to Saline controls (p < 0.05), and then
significantly higher at 54-68 hours compared to the hour before the first LPS bolus (p < 0.05). There was a significant within-subjects correlation between the magnitude of the increase in FVC and the fall in MAP during the first 10 hours after the first LPS bolus across both the LPS groups (Figure 2, p < 0.05, R² = 0.70, n = 8).

Fetal heart rate

There were no significant differences in FHR between groups during the baseline period and initial 48 hours of low-dose infusion (Figure 1). After the first LPS bolus, FHR was significantly higher in the LPS-Hypotension group from 52-59 hours and in the LPS-Normotension group from 52-53 hours compared to Saline controls (p < 0.05). After the second LPS bolus FHR was significantly higher in the LPS-Hypotension group from 76-79 hours and in the LPS-Normotension group from 76-87 hours compared to Saline controls (p < 0.05). Fetal heart rate was significantly lower in the LPS-Hypotension group between 84-91 hours compared to the LPS-Normotension group (p < 0.05).

Fetal heart rate variability

There were no significant differences in MMR or RMSSD during the baseline period and initial 48 hours of low-dose infusion (Figure 1). After the first LPS bolus MMR was significantly higher in the LPS-Hypotension group between 50-51 hours compared to Saline controls (p < 0.05). After the second LPS bolus there was initially an apparent trend towards a higher MMR between 75-76 hours (p = 0.05), followed by a fall in MMR in the LPS-Hypotension group between 83-91 hours to lower values than both the Saline control and LPS-Normotension groups (p < 0.05). Thereafter, there were no significant differences between groups.

After the first LPS bolus, RMSSD was significantly higher in the LPS-Hypotension group between 50-52 hours compared to both the Saline control and LPS-Normotension groups
(Figure 1, p < 0.05), subsequently RMSSD was significantly lower in the LPS-Hypotension group at 58-59 hours compared to Saline controls (p < 0.05). After the second LPS bolus, RMSSD was significantly decreased in the LPS-Hypotension group at 80-91 hours compared to the Saline control and LPS-Normotension groups (p < 0.05). Thereafter, there were no significant differences between groups.

**Fetal heart rate diurnal rhythm**

There were no significant differences in time to peak FHR between groups before the start of low-dose LPS (Figure 1 and 3). The diurnal rhythm of FHR in Saline controls did not differ with time over the experimental period. After the start of the LPS infusion, both the LPS-Hypotension and LPS-Normotension groups showed a significantly earlier FHR peak compared to Saline controls (p < 0.05). There was no significant difference between groups on the second day of low-dose infusion. After the first and second LPS boluses, both LPS-Hypotension and LPS-Normotension groups again showed a significantly earlier FHR peak compared to Saline controls (p < 0.05).

**EEG activity**

There were no significant differences in EEG spectral edge frequency between the groups (Figure 4). There were no significant differences in EEG power between the groups during the baseline period and initial 48 hours of low-dose infusion. After the first LPS bolus, EEG power fell significantly between 49-53 hours in the LPS-Hypotension group (p < 0.05) but not in the LPS-Normotension group compared to Saline controls. Thereafter there were no significant differences between groups.

**Nuchal EMG activity**

There were no significant differences between groups in nuchal EMG activity during the baseline period and the initial 48 hours of low-dose infusion (Figure 4). After the first LPS
bolus, nuchal EMG was significantly lower in the LPS-Normotension group between 49-51
hours and in the LPS-Hypotension group between 50-51 hours compared to Saline controls (p
< 0.05). Thereafter, there was no significant difference between groups.

Cytokine analysis

There were no significant differences in plasma cytokines before the first LPS bolus (Figure
5). After the first LPS bolus, TNFα levels were significantly elevated on Day 3 in the LPS-
Hypotension group compared to both the Saline control and LPS-Normotension groups (p <
0.05). IL-6 was significantly elevated on Day 3 in the LPS-Hypotension group compared to
both the Saline control and LPS-Normotension groups and remained significantly elevated
until prior to the second LPS bolus (p < 0.05). After the start of low-dose infusion, IL-10 was
significantly elevated on Day 1 in the LPS-Hypotension group compared to both the Saline
control and LPS-Normotension groups (p < 0.05). After the first LPS bolus, IL-10 values
were elevated on Day 3 in the LPS-Hypotension group (p < 0.05) and then elevated again on
Day 4 after the second LPS bolus compared to both the Saline control and LPS-
Normotension groups (p < 0.05).

Cortisol

There were no significant differences in plasma cortisol levels before the first LPS bolus
(Figure 5). After the first LPS bolus there was a sustained increase in cortisol levels in the
LPS-Hypotension group on Days 3 and 4 compared to Saline controls (p < 0.05). Cortisol
levels were also significantly elevated in the LPS-Normotension group after the first LPS
bolus on Day 3 and remained significantly elevated compared to Saline controls until before
the second LPS bolus (p < 0.05). There were no significant differences between the LPS-
Hypotension and LPS-Normotension groups at any time point.
This study shows that induction of cardiovascular self-tolerance by low-dose LPS to subsequent high-dose LPS is highly variable and in turn, this markedly affected the pattern of changes in FHRV. We have previously demonstrated that low-dose LPS infusion reduces mortality and the severity of hypotension during subsequent acute, high-dose exposure to LPS (43). We now show that nearly half of the fetuses were able to sustain normal blood pressure even during acute LPS exposure. In fetuses that developed hypotension after high-dose LPS, FHRV as measured by MMR was markedly but transiently increased at the onset of hypotension, with resolution to saline control values before the nadir of hypotension. Intriguingly, after the second LPS bolus, these fetuses developed a biphasic pattern of an initial, statistically borderline, increase in MMR followed by more prolonged suppression. Moreover, although the pattern of changes in MMR and RMSSD were very similar, RMSSD, a measure of higher frequency variability, showed a biphasic pattern after the first LPS bolus, with prolonged suppression after the second LPS bolus. In contrast, no changes in FHRV were seen during low-dose infusion or after LPS boluses in fetuses that did not develop hypotension, despite a similar pattern of tachycardia and suppression of nuchal EMG activity.

Exposure to LPS is well known to induce tolerance to further LPS exposure in a variety of settings, as previously reviewed (5). This is partly mediated through the reprogramming of the innate immune system. In human monocytes this leads to decreased production of TNFα and increased release of IL-10 following subsequent *in vitro* exposure to LPS (45). Down regulation of LPS receptor CD14 has also been reported in tolerant monocytes from both adult humans (45) and preterm fetal sheep (37). Consistent with these findings, low-dose or repeated LPS boluses in fetal and newborn sheep are associated with attenuation of cardiovascular impairment as well as the release of pro-inflammatory cytokines (16, 18, 19, 43). The finding that a subset of fetuses in the present study were actually able to maintain
normal blood pressure after a dose of LPS that typically kills approximately 40% of LPS-naïve sheep illustrates the remarkable potential of this adaptation (16, 43).

Both the LPS-Hypotension and LPS-Normotension groups showed similar FHR responses to the acute LPS boluses. Given that stroke volume is largely constrained in the fetus (25), combined ventricular output is primarily determined by FHR. Conversely, the severity of hypotension was strongly correlated with increased FVC. A limitation of this study is that not all fetuses had continuous FBF recordings. Nevertheless, although FBF was significantly increased in the LPS-Hypotension group after the first high-dose bolus compared to values immediately before the bolus, this increase was modest. Considering that hypotension developed in the face of tachycardia and only moderate vasodilation, this suggests that myocardial contractility was impaired in the LPS-Hypotension group. Thus, maintenance of arterial pressure in the LPS-Normotension group was likely mediated by a combination of reduced peripheral vasodilation and preservation of myocardial contractility during high-dose LPS.

Sepsis-related hypotension has been associated with both increased release of vasodilators, and insensitivity to vasoconstrictors. Greater nitric oxide (NO) release is associated with vasodilation in cases of septic shock and after LPS exposure in fetal sheep (18, 65). Conversely, inhibition of NO synthase (NOS) ameliorates the vascular disturbances of LPS (10, 33). LPS exposure is associated with reduced vascular reactivity to catecholamines, angiotensin II and endothelin (59, 65), at least partly related to NO release (62). There is evidence that proinflammatory cytokines including IL-6 and TNFα mediate these changes through upregulation of inducible-NOS (64, 67). Clinically, there is evidence of an association between elevated IL-6 concentrations and hypotension in newborn preterm infants with chorioamnionitis (72).

Consistent with a key role for cytokine release, in the present study the LPS-Normotension
group showed markedly lower production of IL-6 and TNFα throughout the period of LPS infusion and boluses than the LPS-Hypotension group. Speculatively, lower cytokine levels may have limited NO-mediated vasodilation or allowed for improved endothelial sensitivity to vasoconstrictors. However, greater IL-10 production in the LPS-Hypotension group was not associated with improved cardiovascular function.

Further, there is clinical evidence that relative adrenal insufficiency is common in children with septic shock (57), with increasing evidence that physiological doses (rather than high-doses) of hydrocortisone can help reverse septic shock and may improve mortality, as recently reviewed (60). In the present study, however, there was no difference in the cortisol response to LPS between fetuses that developed hypotension and those that did not, suggesting that the development of hypotension was not due to impaired hypothalamic-pituitary-adrenal axis responses. Nevertheless, we cannot exclude the possibility that the LPS-Hypotension group had impaired sensitivity to cortisol.

Both hypotensive and normotensive fetuses developed a tachycardia after acute high-dose LPS. Given that FHRV was not significantly elevated at the time of peak FHR, it is likely that increased FHR was mediated by circulating catecholamines, consistent with the effects of LPS exposure and septic shock (3, 29). LPS interacted with, and hastened, the diurnal rise in FHR in both LPS groups. This was observed after the first two boluses, but intriguingly was also observed to a similar degree on the first day of low-dose infusion at a time when basal FHR was not significantly elevated compared to saline controls. This phase shift did not occur on the second day of low-dose infusion despite the infusion rate being increased, consistent with the progressive development of central tolerance to LPS during this period of low-dose infusion.

FHRV ultimately represents the integration of sympathetic and parasympathetic activity and the intrinsic pacemaker rhythms of the sino-atrial node (51). Fetal body movements,
breathing and activation of baroreflexes and chemoreflexes modulate autonomic activity and FHRV (13, 22, 44, 46, 70, 73). It is highly likely that the transient increase in FHRV after acute LPS in the present study was mediated by sympathetic activity. We observed an essentially identical pattern of changes with three measures of FHRV in the present study, MMR, STV and RMSSD. Although higher frequency activity measured by STV and RMSSD has been suggested to be a measure of parasympathetic activity (20), preterm fetuses show considerably less high frequency activity than adult animals and the frequencies of sympathetic and parasympathetic activity overlap substantially, even in near-term fetal sheep (36). Intravenous LPS potently increases both renal and mesenteric sympathetic activity before the onset of hypotension in adult rats (68). Furthermore, spectral analysis of HRV is consistent with a relative increase in sympathetic activity after LPS injection in adult animals (31, 58).

Conversely, it is improbable that parasympathetic activity contributed to the increase in FHRV, given that FHR was increased at this time. Interestingly, intraperitoneal LPS injection in adult rats impaired the ability of the sino-atrial node to respond to parasympathetic input (23). Similarly, both nuchal EMG and EEG power were transiently suppressed after the first LPS bolus, consistent with the transient suppression of both of these measures in preterm fetal sheep after intrapleural injection of killed gram-positive Streptococcus pyogenes (4, 11), and so it is unlikely that these factors contributed to changes in FHRV.

There is long-standing evidence that there is a major non-neural component to FHRV that is not suppressed by double autonomic blockade (12). This is likely related in part to fetal breathing and body movements; clinically small fetal movements are correlated with FHRV (40). Furthermore, seizures can increase FHRV, both clinically and in brain damaged fetal sheep (69). However, in the present study EEG and nuchal activity were suppressed during the initial increase in FHRV after the first bolus, and conversely were similar to Saline
control values during the phase of suppression of FHRV. We did not measure fetal breathing movements in the present study, however, infection in fetal sheep has previously been associated with acute apnea (53). LPS has limited ability to pass the blood brain barrier (1), and so the central effects of LPS in the present study are likely mediated by cytokines. This may involve both direct transport across the blood-brain barrier, as shown in the mouse (66), and induction of prostaglandins in the paraventricular nucleus (PVN) of the hypothalamus and rostral ventrolateral medulla (74).

After the second bolus of LPS, the LPS-Hypotension group showed a biphasic increase followed by suppression of FHRV despite normal EEG and EMG activity. The mechanism of this response is unknown. The phase of suppressed variation corresponded with both resolution of the fetal tachycardia as well as a relative increase in MAP to above Saline control values. Thus, reduced FHRV may reflect, in part, relative suppression of sympathetic neural activity. Although RMSSD was suppressed after the first two LPS boluses, the duration of suppression was markedly longer after the second LPS bolus. This again supports that the progressive induction of self-tolerance to LPS contributes to suppression of FHRV. A limitation of the present study is that it was not possible to analyze the changes in FHRV after the third LPS bolus because many of the fetuses were enrolled in a separate study of umbilical cord occlusion. Further studies would be valuable to clarify the longer-term impact on FHRV.

For the same reason, this study cannot resolve whether induction of cardiovascular tolerance was paralleled by a greater tolerance to neural injury. However, we may reasonably observe that the LPS-Hypotension group showed EEG suppression after the first high-dose LPS bolus. This marked suppression of EEG power may potentially reflect either a direct effect of cytokines to inhibit synaptic activity (9, 39, 55), or may be secondary to arterial hypotension. Hypotension in turn initially triggers active suppression of EEG activity which is mediated by
the release of inhibitory neuromodulators such as adenosine (30, 32). As hypotension becomes more severe, it can lead to overt anoxic depolarisation (48). Further studies of cerebral metabolism and oxygenation are needed to clarify whether decreased EEG power in the present study reflected active suppression or a passive effect of anoxic depolarisation. The observation of an increase in plasma lactate values may suggest an impairment of oxidative phosphorylation and greater anaerobic metabolism, similar to previous studies (18, 19, 43). It is interesting to note that consistent with our finding that chronic LPS infusion reduced mortality after subsequent exposure to high-dose LPS (43), clinically, chorioamnionitis is associated with a lower rate of neonatal mortality in extremely immature newborns, but does not necessarily reduce the risk of neurological impairment (21). Further studies would be valuable to help understand this dissociation.

Perspectives and Significance

Although severe infection is associated with a high risk of cerebral palsy in survivors of preterm birth, milder or subclinical infection is more common and also associated with adverse outcomes (26, 71). There is increasing clinical evidence that reduced HRV can help identify developing sepsis and sepsis-like states in preterm newborn infants (17). In the present study we found that low-dose, stable exposure to LPS did not affect FHRV. However, we found that acute on chronic LPS leading to hypotension was associated with a transient increase in FHRV, presumptively reflecting transient sympathetic activation. After repeated boluses, a biphasic pattern developed, such that the transient increase was followed by prolonged suppression. The present study was intended to better understand how subacute, progressive exposure to infection/inflammation affects fetal responses, using very structured doses and timing of exposure to LPS. Further, suppression of FHRV was only found in association with hypotension after high-dose boluses of LPS, whereas, in preterm infants suppressed HRV may precede clinical presentation of late onset sepsis (27, 28). Thus, the
present findings should not be taken to directly reflect the heterogeneous nature of clinical infection. Nevertheless, many cases of infection in preterm infants are associated with increased inflammation several days before clinical diagnosis (38). This in turn is consistent with evidence in preterm infants that reduced HRV and transient decelerations can be used to detect infection earlier (27, 28). The present findings support the hypothesis that repeated exposure to LPS and partial self-tolerance contribute to prolonged suppression of HRV. Further studies are now essential to fully understand how sepsis affects HRV before and after birth.

Acknowledgments

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Table 1: Arterial pH, blood gases, glucose and lactate values

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Before Bolus 1</th>
<th>Bolus 1 +2 h</th>
<th>Bolus 1 +6 h</th>
<th>Bolus 2 +6 h</th>
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<tbody>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
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<tr>
<td>Saline controls</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>
<td>7.35 ± 0.01</td>
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<tr>
<td>LPS-Hypotension</td>
<td>7.37 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.32 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.38 ± 0.01</td>
</tr>
<tr>
<td>LPS-Normotension</td>
<td>7.38 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.36 ± 0.01</td>
<td>7.37 ± 0.01</td>
</tr>
<tr>
<td><strong>pCO₂ (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline controls</td>
<td>49.5 ± 0.8</td>
<td>50.1 ± 1.5</td>
<td>50.4 ± 0.9</td>
<td>48.3 ± 1.0</td>
<td>49.2 ± 1.2</td>
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<tr>
<td>LPS-Hypotension</td>
<td>47.4 ± 1.7</td>
<td>48.9 ± 1.4</td>
<td>53.9 ± 1.9</td>
<td>52.2 ± 1.8</td>
<td>48.0 ± 1.2</td>
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<tr>
<td>LPS-Normotension</td>
<td>47.2 ± 1.0</td>
<td>47.4 ± 1.8</td>
<td>46.2 ± 3.2</td>
<td>49.2 ± 3.1</td>
<td>46.9 ± 1.9</td>
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<tr>
<td><strong>pO₂ (mmHg)</strong></td>
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<td></td>
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<tr>
<td>Saline controls</td>
<td>25.2 ± 1.3</td>
<td>26.3 ± 1.9</td>
<td>24.6 ± 2.0</td>
<td>23.2 ± 2.1</td>
<td>25.1 ± 2.4</td>
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<td>LPS-Hypotension</td>
<td>26.4 ± 0.8</td>
<td>26.4 ± 1.3</td>
<td>24.9 ± 1.7</td>
<td>21.8 ± 0.8</td>
<td>23.6 ± 2.1</td>
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<td>LPS-Normotension</td>
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<td>22.6 ± 2.3</td>
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<td><strong>Lactate (mmol/L)</strong></td>
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<tr>
<td>Saline controls</td>
<td>0.8 ± 0.1</td>
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<td>0.8 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.0</td>
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<td>LPS-Hypotension</td>
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<td>0.7 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.9 ± 0.1</td>
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<td>Glucose</td>
<td>LPS-Normotension</td>
<td>Saline controls</td>
<td>LPS-Hypotension</td>
<td>LPS-Normotension</td>
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<tr>
<td>(mmol/L)</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.2</td>
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<tr>
<td></td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
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<tr>
<td></td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
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<tr>
<td></td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>0.7 ± 0.1 &lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.0 ± 0.1</td>
<td></td>
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<tr>
<td></td>
<td>1.6 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
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</table>

Table 1. Data are means ± SEM. pCO<sub>2</sub>, arterial pressure of carbon dioxide; pO<sub>2</sub>, arterial pressure of oxygen. <sup>a</sup>p < 0.05, LPS-Hypotension vs. Saline controls; <sup>b</sup>p < 0.05, LPS-Normotension vs. Saline controls.
Figure Legends

Figure 1. Time sequence of changes in mean arterial blood pressure (MAP, mmHg), femoral blood flow (FBF, mL/min), fetal heart rate (FHR, bpm) and fetal heart rate variability as measured by MMR (ms) and the root mean square of successive differences in R-R intervals (RMSSD, ms) from 24 hours before until 99 hours after the start of infusions in the Saline control (n = 12), LPS-Hypotension (n = 7) and LPS-Normotension (n = 5) groups. Dashed vertical lines show the timing of stepwise low-dose infusions while solid lines show the timing of bolus administration. Data are 1 hour means ± SEM. \(^{a}p < 0.05\), LPS-Hypotension vs. Saline controls; \(^{b}p < 0.05\), LPS-Normotension vs. Saline controls; \(^{c}p < 0.05\), LPS-Hypotension vs. LPS-Normotension.

Figure 2. The relationship between changes in femoral vascular conductance (FVC, mL/min/mmHg) and mean arterial pressure (MAP, mmHg) during the first 10 hours after the first LPS bolus in the LPS-Hypotension group (black symbols, n = 5) and the LPS-Normotension group (grey symbols, n = 3). Different symbols represent individual animals, while the dotted lines indicate their regression analysis. There is a consistent negative within-subjects relationship between femoral vascular conductance and mean arterial pressure (p < 0.05, \(R^2 = 0.70\), n = 8).

Figure 3. Timing of the diurnal peak in fetal heart rate from the day before the onset of low-dose infusion until the day of the second LPS bolus in the Saline control (n = 12), LPS-Hypotension (n = 7) and LPS-Normotension (n = 5) groups. Times are shown relative to 9 am every day. Data are means ± SEM. \(^{a}p < 0.05\), LPS-Hypotension vs. Saline controls; \(^{b}p < 0.05\), LPS-Normotension vs. Saline controls.

Figure 4. Time sequence of changes in spectral edge (Hz, change from baseline), EEG power (dB, change from baseline) and nuchal electromyographic activity (EMG, % baseline) from
24 hours before until 99 hours after the start of infusions in the Saline control (n = 12), LPS-Hypotension (n = 7) and LPS-Normotension (n = 5) groups. Dashed vertical lines show the timing of stepwise low-dose infusions while solid vertical lines show the timing of bolus administration. Data are 1 hour means ± SEM. ^p < 0.05, LPS-Hypotension vs. Saline controls; _p < 0.05, LPS-Normotension vs. Saline controls; ¨p < 0.05, LPS-Hypotension vs. LPS-Normotension.

Figure 5. Time course of changes in tumor necrosis factor α (TNFα), interleukin (IL)-6, IL-10 and cortisol, over the five day experimental period in the Saline control (open circles, n = 12), LPS-Hypotension (closed squares, n = 7) and LPS-Normotension (open squares, n = 5) groups. The light shaded area represents the period of low-dose infusion while the dark shaded region represents the period of increased infusion. Each of the three boluses are indicated as B1, B2 and B3. Data are means ± SEM. ^p < 0.05, LPS-Hypotension vs. Saline controls; _p < 0.05, LPS-Normotension vs. Saline controls; ¨p < 0.05, LPS-Hypotension vs. LPS-Normotension.


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of early and late periventricular/intraventricular hemorrhage in premature infants. 


Baseline  
Day 1  
Day 2  
Day 3  
Day 4  

Time (hours)

Saline controls  
LPS-Normotension  
LPS-Hypotension

a,b