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

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Lear CA, Davidson JO, Booth LC, Wassink G, Galinsky R, Drury PP, Fraser M, Bennet L, Gunn AJ. Biphasic changes in fetal heart rate variability in preterm fetal sheep developing hypotension after acute on chronic lipopolysaccharide exposure. Am J Physiol Regul Integr Comp Physiol 307: R387–R395, 2014. First published June 18, 2014; doi:10.1152/ajpregu.00110.2014.—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 h, followed by 250 ng·kg−1·24 h−1 for a further 96 h) or the same volume of saline (n = 10). Boluses of either 1 μg LPS or saline were given at 48, 72, and 96 h. 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. Seven of twelve fetuses developed hypotension (α 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 electroencephalogram 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.

fetal sheep; lipopolysaccharide; fetal heart rate variability; tolerance

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), whereas 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 well being (52). Early-onset sepsis in preterm infants has been associated with reduced HRV and transient decelerations (27, 28), which can support early diagnosis and also 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 h 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 h 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 wk 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 femoral vascular 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), whereas seizures have also been associated with increased FHRV, both

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clinically and in fetal sheep (69). Therefore, the relationship among FHRV, nuchal electromyographic (EMG), and electroencephalogram (EEG) activity were examined. Finally, FHR and FHRV show distinctive diurnal rhythms (13), which may be in part related to autonomic activity (34). We therefore examined whether LPS exposure altered these rhythms.

**MATERIALS 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 (250,000 IU/ml procaine penicillin and 250 mg/ml dihydrostreptomycin; Stockgaard Labs, Hamilton, New Zealand) intramuscularly 30 min 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 preductal blood sampling. A 2-R-type ultrasonic blood flow probe (Transonic Systems, Ithaca, NY) was placed around the femoral artery to measure FBF. Two pairs of electrodes (Cooner Wire, Chatsworth, CA) were placed over the parietal cortex bilaterally, 10 mm lateral to bregma 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 were 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 postoperative 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 epinephrine, AstraZeneca, 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-h light-dark cycle (light 0600 to 1800 h). Ewes were given daily intravenous antibiotics [600 mg Crystapen (Biochemie, Vienna, Austria) and 80 mg gentamicin (Pharmacia and Upjohn)] for 4 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 h before the experiment until the day of post mortem for pH, blood gases (845 blood gas analyzer and co-oximeter, Ciba-Corning Diagnostics, Medfield, MA), and glucose and lactate content (model 2300, DrellMed, Darnstadt, Germany). After removal of the organic supernatant, samples were ovine recombinant IL-6 (Protein Express, Cincinnati, OH). The standard series ranged from 0 to 5 ng/ml and the assay sensitivity was 0.354 ng/ml. IL-10 was detected using antibodies specific to the bovine species (AbD Serotec, MorphoSys, Kidlington, UK) (43). Standards used were recombinant bovine IL-10 (kindly supplied by Professor G. Entrican, Moredun Research Institute) and ranged from 0 to 11 BU (biological units/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). One hundred microliters of internal standard (20 ng/ml cortisol-d4 in water) were added to 200 μl plasma. Steroids were extracted using 1 ml of ethyl acetate (Merck, Darmstadt, Germany). After removal of the organic supernatant, samples were dried by vacuum concentration (Savant SC250EXP, Thermo Scientific, Asheville, NC), resuspended in 60 μl of mobile phase 72% methanol (Merck) and 28% water, and transferred to HPLC injector vials. Twelve microliters were 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, San Jose, CA). The mobile phase was isocratic, flowing at 250 μl/min through a Luna HST 2.6-μm C18 (2) 100 × 3.0 mm column at 40°C (Phenomenex, Auckland, New Zealand). Retention time was 3.1 min for both cortisol and cortisol-d4. Ionization was in positive mode and Q2 had 1.2 mTorr of argon. The mass transitions followed were cortisol-d4 367.2 → 121.2 at 28 V and cortisol 363.2 → 122.2 at 28 V. Mean inter-and intra-assay coefficient of variation values for cortisol were 5.8% and 6.0%, respectively.

**Fetal cytokine measurements.** Cytokine levels in the plasma were measured using an in-house enzyme-linked immunosorbent assay (43). TNF-α was detected using antibodies specific to the ovine species (Epitope Technologies, Melbourne, Australia). Standards were ovine recombinant TNF-α and ranged from 0 to 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, OH). The standard series ranged from 0 to 5 ng/ml and the assay sensitivity was 0.354 ng/ml. IL-10 was detected using antibodies specific to the bovine species (AbD Serotec, MorphoSys, Kidlington, UK) (43).

**Experimental protocol.** Experiments started 5 days postsurgery. Fetuses were randomized into two 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/l at 83 μl/h) for the first 24 h followed by 250 ng/kg·1·24 h·1 (50 ng/ml at 207.5 μl/h) for the next 96 h. Boluses were administered as 1 μg LPS dissolved in 1 ml of saline or the same volume of saline at 48, 72, and 96 h 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 h after the start of infusion four fetuses from the saline control group and seven 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 h after the start of infusions.

**Material and methods.** The intensity spectrum signal between 0.5 and 20 Hz, while spectral edge intensity was calculated as the frequency below which 90% of the intensity was present. For data presentation, the total ECG power was normalized by log transformation (dB, 20 × log intensity). The nuchal EMG signal was band-pass filtered between 100 Hz and 1 kHz, and the signal was then integrated using a time constant of 1 s 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.
Data analysis. To assess cardiovascular impairment after 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/min for more than 1 min or $\geq 20$ beats/min for more than 30 s (14). For comparison, we also calculated short-term variability (STV) (14) and the root-mean-square of successive differences in R-R interval (RMSSD) (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 5 days of recording. FVC was calculated as FBF/MAP (56).

Data were processed in minute averages for the 24-h baseline and the first 99 h of the experimental period and subsequently averaged into 1-h averages. Statistical analysis was performed using SPSS (v22, SPSS, Chicago, IL). 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 differences (LSD) post hoc test when a significant overall effect was found. Differences between groups were evaluated using analysis of variance (ANOVA) followed by a Fisher’s protected least-significant differences (LSD) post hoc test when a significant overall effect was found. 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 5 days of recording. FVC was calculated as FBF/MAP (56).

RESULTS

Fetal arterial pH, blood gases, lactate, and glucose levels. There were no significant differences in the pH, PO2, PCO2, lactate, and glucose measurements during the baseline period (Table 1). There were also no significant differences during the initial 48 h of low-dose infusion, and so these time points are not shown. After the first LPS bolus, pH was significantly lower at 2 h, whereas lactate was significantly higher at 6 h in the LPS-hypotension group compared with 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 h after the first LPS bolus in the LPS-hypotension group ($P = 0.05$). Glucose was significantly lower in the LPS-hypotension group at 6 h after the first LPS bolus compared with 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 h of low-dose infusion. With the first LPS bolus, MAP fell in the LPS-hypotension group between 55 and 57 h compared with saline controls (Fig. 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.

FBF and vascular conductance. There was a temporal increase in FBF in all groups during this study (Fig. 1, $P < 0.01$). Two fetuses from both LPS groups did not have continuous FBF recordings. There was a significant interaction between time and group for FBF after the first LPS bolus from 48 to 71 h ($P < 0.005$). After the first LPS bolus, FBF was significantly lower in the LPS-hypotension group at 51–52 h compared with saline controls ($P < 0.05$) and then significantly higher at 54–68 h compared with 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 h after the first LPS bolus across both the LPS groups (Fig. 2, $P < 0.05$, $R^2 = 0.70$, $n = 8$).

Fetal heart rate. There were no significant differences in FHR between groups during the baseline period and initial 48 h of low-dose infusion (Fig. 1). After the first LPS bolus, FHR was significantly higher in the LPS-hypotension group from 52 to 59 h and in the LPS-normotension group from 52 to 53 h compared with saline controls ($P < 0.05$). After the second LPS bolus FHR

<table>
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<th>Group</th>
<th>Baseline</th>
<th>Before Bolus 1</th>
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<th>Bolus 1 + 6 h</th>
<th>Bolus 2 + 6 h</th>
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<td>46.9 ± 1.9</td>
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<td>PO2, mmHg</td>
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Data are means ± SE. LPS, lipopolysaccharide; PCO2, arterial pressure of carbon dioxide; PO2, arterial pressure of oxygen. *$P < 0.05$, LPS-hypotension vs. saline controls; †$P < 0.05$, LPS-normotension vs. saline controls.
FHR variability. There were no significant differences in MMR or RMSSD during the baseline period and initial 48 h of low-dose infusion (Fig. 1). After the first LPS bolus MMR was significantly higher in the LPS-hypotension group between 50 and 51 h compared with saline controls (P < 0.05). After the second LPS bolus there was initially an apparent trend toward a higher MMR between 75 and 76 h (P = 0.05), followed by a fall in MMR in the LPS-hypotension group between 83 and 91 h 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 and 52 h compared with both the saline control and LPS-normotension groups (Fig. 1, P < 0.05), subsequently RMSSD was significantly lower in the LPS-hypotension group at 58–59 h compared with saline controls (P < 0.05). After the second LPS bolus, RMSSD was significantly decreased in the LPS-hypotension group at 80–91 h compared with the saline control and LPS-normotension groups (P < 0.05). Thereafter, there were no significant differences between groups.

FHR diurnal rhythm. There were no significant differences in time to peak FHR between groups before the start of low-dose LPS (Figs. 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 with 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 with saline controls (P < 0.05).

EEG activity. There were no significant differences in EEG spectral edge frequency between the groups (Fig. 4). There were no significant differences in EEG power between the groups during the baseline period and initial 48 h of low-dose infusion. After the first LPS bolus, EEG power fell significantly between 49 and 53 h in the LPS-hypotension group (P < 0.05).

was significantly higher in the LPS-hypotension group from 76 to 79 h and in the LPS-normotension group from 76 to 87 h compared with saline controls (P < 0.05). FHR was significantly lower in the LPS-hypotension group between 84 and 91 h compared with the LPS-normotension group (P < 0.05).
DISCUSSION

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,

0.05) but not in the LPS-normotension group compared with 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 h of low-dose infusion (Fig. 4). After the first LPS bolus, nuchal EMG was significantly lower in the LPS-normotension group between 49 and 51 h and in the LPS-hypotension group between 50 and 51 h compared with 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 (Fig. 5). After the first LPS bolus, TNF-α levels were significantly elevated on day 3 in the LPS-hypotension group compared with 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 with both the saline control and LPS-normotension groups and remained significantly elevated until before 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 with 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 with 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 (Fig. 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 with 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 with 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.

**Fig. 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 ± SE. aP < 0.05, LPS-hypotension vs. saline controls; bP < 0.05, LPS-hypotension vs. saline controls.

**Fig. 4.** Time sequence of changes in spectral edge (Hz, change from baseline), electroencephalogram (EEG) power (dB, change from baseline) and nuchal electromyographic activity (EMG, % baseline) from 24 h before until 99 h 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, whereas solid vertical lines show the timing of bolus administration. Data are 1 h means ± SE. aP < 0.05, LPS-hypotension vs. saline controls; bP < 0.05, LPS-normotension vs. saline controls; cP < 0.05, LPS-hypotension vs. LPS-normotension.
hypotension vs. saline controls; b following subsequent in vitro exposure to LPS (45). Down-decreased production of TNF-α/H9251 innate immune system. In human monocytes this leads to (5). This is partly mediated through the reprogramming of the LPS exposure in a variety of settings, as previously reviewed and suppression of nuchal EMG activity.

Develop hypotension, despite a similar pattern of tachycardia bolus. In contrast, no changes in FHRV were seen during LPS bolus, with prolonged suppression after the second LPS frequency variability, showed a biphasic pattern after the first RMSSD were very similar, RMSSD, a measure of higher 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 boluses and those that did not, suggesting that the development of hypotension was not due to impaired hypothalamic-pituitary-adrenal axis responses. Nevertheless, we cannot ex-

Fig. 5. Time course of changes in tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-10, and cortisol over the 5-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, whereas 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 ± SE. aP < 0.05, LPS-hypotension vs. saline controls; bP < 0.05, LPS-normotension vs. saline controls; cP < 0.05, LPS-hypotension vs. LPS-normotension.

Consistent with a key role for cytokine release, in the present study the LPS-normotension group showed markedly lower production of IL-6 and TNF-α throughout the period of LPS exposure relative to 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 likely mediated by a combination of reduced peripheral vasodilation and preservation of myocardial contractility during 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 ~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 with values immediately before the bolus, this increase was modest. Considering that hypotension developed in the fetus with recovery of glycogen in the mesentery, 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 TNF-α 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).

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clude 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 with 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 sinoatrial 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 intraplacental 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-neuronal 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 depolarization (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 depolarization. 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. Furthermore, 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 the transient increase was followed by prolonged suppression of HRV.

Further studies are now essential to fully understand how sepsis affects HRV before and after birth.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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