Endotoxin has acute and chronic effects on the cerebral circulation of fetal sheep

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Feng SY, Phillips DJ, Stockx EM, Yu VY, Walker AM. Endotoxin has acute and chronic effects on the cerebral circulation of fetal sheep. Am J Physiol Regul Integr Comp Physiol 296: R640–R650, 2009. First published December 24, 2008; doi:10.1152/ajpregu.00087.2008.—We studied the impact of endotoxia on cerebral blood flow (CBF), cerebral vascular resistance (CVR), and cerebral oxygen transport (O2 transport) in fetal sheep. We hypothesized that endotoxia impairs CBF regulation and O2 transport, exposing the brain to hypoxic-ischemic injury. Responses to lipopolysaccharide (LPS; 1 µg/kg iv on 3 consecutive days, n = 9) or normal saline (n = 5) were studied. Of LPS-treated fetuses, five survived and four died; in surviving fetuses, transient cerebral vasoconstriction at 0.5 h (ΔCVR approximately +50%) was followed by vasodilatation maximal at 5–6 h (ΔCVR approximately −50%) when CBF had increased (approximately +60%) despite reduced ABP (approximately −20%). Decreased CVR and increased CBF persisted 24 h post-LPS and the two subsequent LPS infusions. Cerebral O2 transport was sustained, although arterial O2 saturation was reduced (P < 0.05). Histological evidence of neuronal injury was found in all surviving LPS-treated fetuses; one experienced grade IV intracranial hemorrhage. Bradykinin-induced cerebral vasodilatation (ΔCVR approximately −20%, P < 0.05) was abolished after LPS. Fetuses that died post-LPS (n = 4) differed from survivors in three respects: CVR did not fall, CBF did not rise, and O2 transport fell progressively. In conclusion, endotoxin disrupts the cerebral circulation in two phases: 1) acute vasoconstriction (1 h) and 2) prolonged vasodilatation despite impaired endothelial dilatation (24 h). In surviving fetuses, LPS causes brain injury despite cerebral O2 transport being maintained by elevated cerebral perfusion; thus sustained O2 transport does not prevent brain injury in endotoxia. In contrast, cerebral hypoperfusion and reduced O2 transport occur in fetuses destined to die, emphasizing the importance of sustaining O2 transport for survival.

Neither chooroamnionitis nor elevated cytokine concentrations were associated with impaired cerebral Doppler blood flow velocities in preterm neonates (74). Similarly, in a recent study in premature neonates (63), infection-related white matter injury (WMI) was not related to hypotension or to loss of autoregulation, and presumably not to hypoperfusion. In endotoxin-induced brain injury in immature animals, cerebral perfusion was maintained (7, 8, 13, 15) or even increased (56). Because WMI was found to be induced despite increased cerebral perfusion, it has been suggested that cerebral ischemia is not a main etiological factor in endotoxin-induced injury (56).

Arterial O2 saturation (SaO2) is normally low in fetal life and falls further during endotoxia (7, 8, 10, 15, 56). As a consequence, oxygen (O2) transport to the brain may be compromised and neuronal injury may result even if cerebral blood flow (CBF) were to be maintained. Several studies have described falls in cerebral O2 transport during endotoxia (7, 8), an effect ascribed to the inability of CBF to increase in the face of falling arterial blood pressure and hypoxemia. In another study (56), no decrements in carotid blood flow were evident in endotoxin-induced injury. As yet, it remains uncertain whether CBF increases to sustain O2 transport in the fetus exposed to endotoxin.

In this study we aimed to clarify the uncertainty surrounding the changes of CBF and O2 transport in endotoxia because of their importance in understanding the pathogenesis of brain injury in intrauterine infection. We hypothesized that endotoxin would impair cerebral perfusion and oxygenation, exposing the brain to hypoxic-ischemic injury. Because variability between studies might arise from intermittent measurements, we continuously monitored changes of CBF and O2 transport in fetal sheep exposed to LPS. Also, we repeated the study on successive days, because circulatory tolerance to endotoxin may develop with repeated LPS exposure (10, 70). In addition, because nitric oxide (NO) synthesis might be critical for sustaining CBF in endotoxia (56), we included functional tests of the cerebral endothelium, a primary source of NO (18), in the study. LPS-induced responses of the vasoconstrictor and proinflammatory cytokine tumor necrosis factor-α (TNF-α) were also measured to assess its role in cerebral regulation in conditions of endotoxia.

METHODS

Fourteen ewes (Merino/Border-Leicester cross) were brought into the animal house 2 wk before surgery. Ewes were fed ad libitum and, once feeding well, were surgically prepared for chronic study. All surgical and experimental procedures were performed with the use of sterile surgical...
techniques and in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes established by the National Health and Medical Research Council of Australia and were approved by the Monash University-Monash Medical Centre Committee on Ethics in Animal Experimentation.

Surgical and Experimental Procedures

Ewes were anesthetized at 123 ± 1 days of gestation (halothane 1–2%, nitrous oxide 60%, and oxygen 38–39%), and both ewe and fetus were then instrumented for study. A nonocclusive double-lumen catheter (1.7-mm OD; Argyle, Tyco Healthcare Group, Mansfield, MA) was inserted into the fetal carotid artery for mean arterial blood pressure (ABP) monitoring, bradykinin (BK) injection (61), and blood sampling, and a nonocclusive single-lumen catheter (0.86-mm ID, 1.52-mm OD) was inserted into the amniotic cavity for measuring amniotic fluid pressure (PAF). A catheter (1.57-mm ID, 2.41-mm OD) was also positioned under the dura to record fetal intracranial pressure (ICP). Nonocclusive jugular venous catheters (1.57-mm ID, 2.41-mm OD) were inserted in both the fetus and ewe for drug administration and blood sampling. To record fetal CBF, we positioned a transit-time ultrasonic flow probe (2-mm diameter; Transonic Systems, Ithaca, NY) around the fetal superior sagittal sinus as previously described (20). In brief, a 2 × 2-cm section of the skull overlying the intersection of the lambdoid and sagittal sutures was removed to access the superior sagittal sinus. The flow probe was carefully positioned around the superior sagittal sinus, with care taken not to damage neural or vascular tissue. A rigid cap of dental acrylic was formed over the probe to stabilize it and to replace the section of skull that had been removed. This technique provides a simple, quantitative, and beat-by-beat measurement of CBF that has been validated for use on the sagittal sinus of the lamb (20). In combination with nonocclusive catheters in the carotid artery and jugular vein, values of CBF estimated with the sagittal sinus method in lambs are quantitatively similar to those measured with radioactive microspheres (20). At the completion of surgical procedures, both the fetus and ewe were treated with antibiotics (Fortum 50 mg/kg, Glaxo SmithKline Australia; and gentamicin 2.5 mg/kg, Pharmacia Australia) and analgesic (Finadyne 1 mg/kg; Schering-Plough Australia).

Study Conditions

Fetuses were studied over a 5-day period beginning at a gestational age of 125 ± 1 days (mean ± SE, 0.85 gestation, term at 147 days) after 48-h postoperative recovery. During the study, the ewe’s cage was partitioned to prevent the sheep from turning while still allowing freedom to move forward and backward and to stand up and lie down. Room temperature was maintained between 22 and 25°C. The flow probe was connected to the flowmeter (model T101 Ultrasonic blood flowmeter; Transonic Systems). Vascular and intracranial catheters were connected to calibrated strain-gauge manometers (Cobe CDX III; Cobe Laboratories, Lakewood, CO). Pressure and flow signals were low-pass filtered at 100 Hz and recorded to hard disk (Powerlab, Chart v5.4.1; ADInstruments, Sydney, Australia).

Experimental Protocol

Responses to LPS infusion. LPS (1 μg/kg; Escherichia coli O127-B8, L3129; Sigma) or normal saline (NS) was infused intravenously to fetuses by syringe pump (sp 100 μl infusion pump; WP) over 30 min on 3 consecutive days (LPS, n = 9; NS, n = 5). Fetoplacental parameters including ABP, ICP, PAF, and CBF were recorded continuously before and up to 12 h after each LPS infusion; measurements were repeated 24 h post-LPS. Blood samples were collected from fetuses for arterial blood gas, lactate, and pH analysis and the measurement of TNF-α and total nitrate/nitrite at 0, 1, 2, 4, 6, 8, 10, 12, and 24 h post-LPS. TNF-α concentrations in plasma were determined using a previously validated immunoassay developed to measure ovine TNF-α, as described previously (35). The TNF-α standard used was ovine recombinant TNF-α, the mean sensitivity over nine assays was 0.13 ng/ml, and the mean intra- and interassay coefficient of variation (CV) was 6.5 and 8.3%, respectively. Total plasma nitrate/nitrite concentrations were measured colorimetrically (Dynatech, Guernsey, UK) using a commercially available kit (catalog no. 780001, Cayman Chemicals). The analytical range was 2.0–80 μM, and the intra- and interassay CV was 2.7 and 3.4%, respectively.

Cerebral endothelial function. Fetal cerebral endothelial function was assessed by assembling dose-response curves for BK (0.001–0.5 μg/kg) before the first LPS infusion, 8–12 h after each LPS infusion, and 24 h after the final (third) infusion. Each BK response was averaged from a minimum of three repeated injections at each dose, allowing a minimum 2-min recovery period between each injection. Absence of tachyphylaxis was confirmed by responses to the repeated injections being maintained.

Histology. At the conclusion of physiological studies, ewes were reanesthetized, and the saline control and LPS-surviving fetuses were perfused transcardially with 2 liters of heparinized saline (10 IU/ml) followed by 2 liters of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4); ewes were then killed using pentobarbital sodium (150 mg/kg iv). Fetal brains were removed and immediately cut into hemispheres at the midsagittal line. One hemisphere was postfixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C before being cut coronally into 10-mm-thick blocks and paraffin embedded. We selected regions topographically using the published nomenclature of sections 800–1,000 for the sheep brain (34). These regions include periventricular white matter and the caudate nucleus, both of which have been shown to be damaged by LPS (31, 43). Paraffin blocks were sectioned at 10 μm, and every 40th section was collected and stained with hematoxylin and eosin. In fetuses that had died in utero, deterioration and liquefaction of the brain prevented examination. Placental cotyledons were also paraffin embedded, sectioned, stained with hematoxylin and eosin, and examined microscopically for evidence of chorioamnionitis based on evidence of placental inflammation (57).

Data Analysis and Statistics

Physiological signals were reviewed to detect and reject artifacts and were then analyzed second to second with average values calculated for every 30 s over each hour of the control, infusion, and postinfusion periods. Cerebral perfusion pressure (CPP) was calculated as ABP – ICP. Cerebral vascular resistance (CVR) was calculated as CPP/CBF. O2 transport was calculated as CBF × arterial O2 content (CaO2), where CaO2 = (1.34 × [Hb] × SaO2/100 + 0.003 × PaO2), where PaO2 is arterial partial pressure of O2.

Physiological data were analyzed using repeated-measures ANOVA. Data from LPS-treated fetuses that survived and those that died were analyzed separately. Dose-response curves for BK (61) were analyzed by comparing responses to baseline control using repeated-measures ANOVA; when data failed normality, nonparametric ANOVA (Friedman repeated-measures ANOVA on ranks) was used after transformation to normal logarithms to improve normality. Differences that were detected by ANOVA were subjected to post hoc analysis using the Student-Newman-Keuls method. BK response differences pre- and post-LPS were compared using the paired Student’s t-test. Data are means ± SE. All tests employed SigmaStat v3.01.0 (SPSS; http://www.spss.com), and P < 0.05 was considered to be significant.

RESULTS

Baseline Values

Baseline (pre-LPS) values of ABP, CBF, and CVR among NS-treated, LPS-treated, and fetal death in utero (FDIU) groups were not different for any of the measurements (Table 1).

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First LPS Exposure

After the first dose of LPS in those fetuses that survived (n = 5, Fig. 1A), ABP started to fall 4 h post-LPS (80 ± 6%, P < 0.05) and remained low through to 24 h post-LPS. CVR increased significantly within the first hour (153 ± 10%, P < 0.05), and concomitantly, CBF fell, reaching −33 ± 2% (P < 0.05). Subsequently, there was a significant reduction in CVR (to 63 ± 5%, P < 0.05) at 2 h, which persisted through to 24 h; over this period, CBF rose steadily and plateaued ~6 h after LPS (159 ± 9%, P < 0.05). This higher flow persisted through to 24 h (P < 0.05) despite coincident hypotension. Cerebral O2 transport was unchanged following LPS treatment. In NS-treated animals, ABP, CBF, CVR, and O2 transport did not show significant changes over the equivalent 24-h period. In both LPS- and NS-treated animals, it remained possible that there were undetected changes in the nonsignificant tests, given that the power of ANOVA was low (<0.10). However, the sole trend in the nonsignificant data was a tendency for O2 transport to increase late in the 24-h period in LPS-treated fetuses (Fig. 1A). Because there was no tendency for O2 transport to decrease after LPS, there is little possibility of a type II error in the analysis.

In fetuses that survived (Table 2), arterial pH fell significantly by 1 h post-LPS (7.30 ± 0.02, P < 0.05) accompanied by increased arterial partial pressure of CO2 (PaCO2) after 4 h (52 ± 1 mmHg, P < 0.05), lower SaO2 after 4 h (44 ± 4%, P < 0.05), and lower PaO2 after 2 h (19 ± 1%, P < 0.05). Both pH and PaCO2 began recovery toward normal levels 8 h post-LPS, whereas PaO2 and SaO2 remained low for at least 10–12 h (P < 0.05). Hemoglobin levels increased transiently 2 h post-LPS and then fell between 8 and 24 h, a pattern similar to previous descriptions (8, 56). CaO2 was persistently lower from 4 h post-LPS, in association with lower SaO2 and hemoglobin.

Repeated LPS Exposures

Responses to repeated LPS infusions in fetuses that survived are shown in Fig. 2. ABP remained low 24 h after LPS1 (78 ± 8%) but then recovered to the pre-LPS level after LPS2 and remained at the baseline level after LPS3. The decreased CVR (47 ± 7%) and the associated increase in CBF (178 ± 18%) that were evident 24 h after LPS1 were not changed by repeated LPS infusion. Thus the low CVR and high CBF that

Table 1. Baseline circulatory parameters in fetal sheep

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Values are means ± SE (n = no. of sheep) for fetal sheep treated with normal saline (NS), fetuses surviving LPS (LPS Survived), and LPS-treated fetuses that died in utero (LPS-FDIU).
were induced by the first LPS infusion persisted 24 h after each of the subsequent infusions (Fig. 2). Similarly, cerebral O2 transport was maintained at pre-LPS levels throughout the repeated LPS infusions (Fig. 2).

**CBF vs. SaO2**

Regression of CBF with SaO2 in fetuses that survived for the periods of 0–4 h and 6–12 h after LPS1 and LPS3 are shown in Fig. 3. In the early phase (0–4 h) after LPS1, a significant hyperbolic relationship existed between CBF and SaO2, with CBF increasing as SaO2 fell (Fig. 3A, R = 0.80, P < 0.001). Somewhat later following LPS1 (6–12 h), the relationship between SaO2 and CBF reversed, with CBF now rising as SaO2 rose (Fig. 3B, R = 0.48, P < 0.05). After LPS3, CBF was unrelated to SaO2 (Fig. 3, C and D).

**Plasma Nitrate/Nitrite**

In the fetuses that survived, total plasma nitrate/nitrite increased 4 h after LPS1 (Fig. 4A) and reached significantly elevated levels at 6 h (43 ± 7 μM, P < 0.05). The level of nitrate/nitrite remained high 8 h post-LPS1 (49 ± 8 μM, P < 0.05) but had returned to baseline at 24 h. A lesser, abbreviated nitrate/nitrite increase (P < 0.05) occurred after LPS3 (Fig. 4A).

**Cytokine Responses**

In surviving fetuses, TNF-α quickly reached a peak 1 h after LPS1 and promptly fell below the limits of detection 6 h later (Fig. 4B); TNF-α also increased significantly after LPS3, but the peak concentration reached was 10 times less than that for LPS1 (78 ± 35 vs. 7 ± 5 ng/ml, P < 0.05), suggestive of tolerance having been induced.

**Cerebral Endothelial Function**

Dose responses to BK in surviving fetuses immediately before LPS1 and 24 h after LPS3 are shown in Fig. 5. Before LPS administrations, BK induced vasodilatation at doses of 0.05–0.1 μg/kg. Maximal vasodilatation occurred at 0.05 μg/kg, where the CVR reduction averaged 18 ± 3% (P < 0.05). Table 3 shows CVR responses to BK (0.05 μg/kg, n = 5) before LPS infusion (pre-LPS), 8–12 h after each LPS infusion (LPS1, LPS2, and LPS3), and 24 h post-LPS3 in surviving fetuses (n = 5). The fall in CVR pre-LPS was abolished with repeated LPS infusion and remained absent 24 h after the final LPS infusion. In a matching series of control infusions of NS (n = 5) over 5 days, the baseline CVR reduction induced by 0.05 μg/kg BK (−18 ± 5%, P < 0.05) remained unchanged (Table 3).

**Histology**

No NS-infused control animals showed structural abnormalities or signs of brain injury (Fig. 6A). In contrast, neurons in all five LPS-treated fetal brains exhibited an irregular cytoplasm lacking any definitive border, indicative of dying cells (Fig. 6B). In addition, brains of the LPS group showed abundant infiltration of macrophages into the parenchyma containing dying neuronal cells (Fig. 6C). In one LPS-treated fetus, a grade IV intracranial hemorrhage extended from the lateral ventricle to occupy parenchyma, including periventricular
animals, however, there was no subsequent cerebral vasodilation in the FDIU group at 0.5 h post-LPS. In contrast with surviving similar to that observed in surviving animals was seen in the initial dose of LPS. Transient cerebral vasoconstriction similar to that observed in surviving animals was seen in the LPS1. CBF remained elevated 24 h after the first LPS infusion (LPS1) but recovered 24 h after the variation in findings, since the fetal sheep used in the study (7, 8, 15) whereas another suggests that there is a delayed cerebral flow increase several hours after LPS (56).

Studies reporting no change in CBF may have employed a period of measurement that was too short to identify the delayed cerebral flow increase that follows LPS exposure. For example, CBF was measured for 1 h (7, 15) or 4 h (13) in previous studies in which CBF was found to be unchanged. In our data, CBF tended to fall soon after LPS but then rose to be significantly elevated by 6 h post-LPS. Subsequently, CBF remained clearly elevated throughout the 24 h post-LPS in our data, in contrast to a previous 24-h study (8). The difference may reflect the single point (microsphere) estimates of blood flow method employed in that study (8), since the onset of the flow increase after LPS can be highly variable (56). Although the cerebrovascular response to LPS can be dose dependent (58), it is unlikely that this variation in the fetus is explained by differing LPS dosages, since the dose used in our study (1 μg/kg) was equal to that used in the study where no flow increase was found (8). Moreover, a much lower LPS dose (0.1 μg/kg) elicited a flow increase (~50%) similar to that which we found (56). It also is unlikely that repetition of LPS administration was a factor. Although one dose of LPS was administered in some studies (7, 8, 13, 15), whereas three were administered in ours, we noted changes in CBF after the first LPS infusion. Also, gestational age differences cannot explain the variation in findings, since the fetal sheep used in the study of Peebles et al. (56), where flow increased, were of equivalent age to those studied by Dalitz et al. (8), in which it did not.

Intense but transient cerebral vasoconstriction, along with a reduction in CBF, was an early response to LPS infusion (Fig. 1A), similar to that seen in the adult brain (14). Increased CVR occurred within the first hour, although ABP remained in the normal range, inferring that the cerebral vasculature is more transport fell markedly after LPS. Moreover, there was no increased CBF related to SaO₂ reduction in the FDIU group (Fig. 1B and Table 2). Total plasma nitrate/nitrite levels were similar to those of the surviving group before LPS (28 ± 4 μM). However, in contrast to the survivors, the plasma nitrate/ nitrite levels did not increase after LPS1. On the other hand, TNF-α responses of FDIU fetuses were similar to those of the surviving group in response to LPS1, with peak values occurring 1–2 h post-LPS1 and averaging 500 ± 50 times the pre-LPS level.

**DISCUSSION**

Our study provides new information that clarifies uncertainty regarding the CBF response and the delivery of oxygen to the brain in the fetus surviving exposure to LPS. We also have newly identified substantially altered responses in the fetus destined to die during endotoxemia.

Our data revealed that the cerebral circulatory response to endotoxin has two phases, with an early, transient vasoconstriction followed by a prolonged vasodilatation; during the early vasoconstriction, there was a fall in blood flow (~33%), but in the ensuing period of vasodilatation, the CBF increase was substantial (~60%) and prolonged. Thus our study offers a clarification of the variable findings of several previous examinations, some of which have showed unchanged fetal cerebral flow soon after LPS (7, 8, 13, 15), whereas another suggests that there is a delayed blood flow increase several hours after LPS (56).

Fetal Death In Utero

Baseline values for circulatory parameters for the fetal group that died in utero (FDIU) following LPS treatment (n = 4) are shown in Table 1, and arterial blood gases and pH are shown in Table 2. In the FDIU group, ABP tended to fall progressively after LPS and was below the control level in all animals after 6 h (Fig. 1B). Fetuses were found dead within 8 h of the first (n = 2), second (n = 1) or last dose of LPS (n = 1). By contrast with the surviving animals, CBF did not increase after the initial dose of LPS. Transient cerebral vasoconstriction similar to that observed in surviving animals was seen in the FDIU group at 0.5 h post-LPS. In contrast with surviving animals, however, there was no subsequent cerebral vasodilatation. Again in contrast with surviving animals, cerebral O₂ transport fell markedly after LPS. Moreover, there was no increased CBF related to SaO₂ reduction in the FDIU group (Fig. 1B and Table 2). Total plasma nitrate/nitrite levels were similar to those of the surviving group before LPS (28 ± 4 μM). However, in contrast to the survivors, the plasma nitrate/ nitrite levels did not increase after LPS1. On the other hand, TNF-α responses of FDIU fetuses were similar to those of the surviving group in response to LPS1, with peak values occurring 1–2 h post-LPS1 and averaging 500 ± 50 times the pre-LPS level.

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![Fig. 2. Baseline responses to repeated LPS infusion in surviving fetuses (solid bars, n = 5) and matching saline infusion in controls (normal saline (NS), open bars, n = 5). Values are averages at the 24-h postinfusion time point, expressed as percentages of the preinfusion baseline on day 1. Note that ABP remained low 24 h after the first LPS infusion (LPS1) but recovered 24 h after subsequent LPS infusions (LPS2 and LPS3). CBF remained elevated 24 h after the first LPS infusion and remained high with repeated LPS infusions; the pattern of CVR mirrored the CBF changes. ★ P < 0.05 vs. preinfusion baseline. Note that cerebral O₂ transport was unchanged throughout the study period. No significant changes occurred with NS infusion.](http://ajpregu.physiology.org/DownloadedFrom hp://ajpregu.physiology.org)
sensitive than the systemic (noncerebral) vasculature to vasoconstriction following LPS. Elevation in TNF-α coincided with increased CVR (Fig. 4A), consistent with other findings that TNF-α is a powerful cerebral vasoconstrictor, reducing cerebral blood volume by 15–30% via an endothelin- and TNF-α-type 2 receptor-dependent pathway (60). As one of the important inflammatory markers, TNF-α has been found to be increased in amniotic fluid (75) and in cord plasma (76) of infants suffering from brain injury. TNF-α is also increased in the periventricular leukomalacia (PVL) lesion itself (77), and although it is unclear whether proinflammatory cytokines can cross the blood-brain barrier (BBB), TNF-α disrupts endothelium (9) and increases BBB permeability (5). Whereas a direct neurotoxic effect of proinflammatory cytokines has been reported (1), our data suggest the potential for them to also injure the fetal brain by inducing cerebral vasoconstriction in the fetal cerebral circulation. Enhancing the potential for cerebral vasoconstriction by cytokines, endothelin-1 (16, 62), and COX-2-derived vasoconstrictors (30) are upregulated by LPS, whereas the vasodilator endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) are downregulated (44). In addition to altering cerebral vascular resistance, systemic changes induced by LPS and its vasoactive products may prejudice cerebral perfusion by impairing cardiac function and by inducing systemic vasodilatation and hypotension (28). Further investigation of these systemic mechanisms and other vasoactive stimuli of parenchymal origin is needed to delineate the exact basis of vasoconstriction after LPS.

CBF and CVR are normally closely coupled to cerebral metabolic requirements in the fetus, with resistance increasing and flow falling as metabolic rate declines (36). LPS may have contributed to the early increase in CVR via reduced cerebral metabolic rate since it can suppress neuronal activity in the brain (69). However, the data regarding LPS effects on cerebral
where the CVR reduction averaged /H11002 between CBF and SaO2 was apparent early in the post-LPS period, vasculature of fetal sheep (10). An inverse relationship between circulatory tolerance that develops in the systemic vasculature after twice-repeated LPS exposures (Fig. 2), differing from our model, cerebral vasodilatation was observed after LPS treatment, no cerebral vasodilatation was observed at any BK dose. *P < 0.05 vs. preinfusion baseline. □P < 0.05 vs. preinfusion baseline.

Fig. 5. Bradykinin (BK; 0.001–0.5 μg/kg, intracarotid injection) dose-response curves in the cerebral circulation pre-LPS and 24 h post-LPS3 in surviving fetuses (n = 5). BK induced maximal vasodilatation at 0.05 μg/kg, where the CVR reduction averaged −18 ± 3% (P < 0.05). After LPS treatment, no cerebral vasodilatation was observed at any BK dose. *P < 0.05 vs. preinfusion baseline. □P < 0.05 vs. preinfusion baseline.

Another study in endotoxin-treated fetal sheep has shown that although CBF was not increased, inhibition of NO before LPS halved the CBF response 1 h after LPS infusion (7). A delayed (5–22 h) CBF rise after LPS has been ascribed to NO production (56). Although hypoxemia developed during this period in our study, NO retains the ability to vasodilate the fetal cerebral circulation during hypoxia (46, 54). Constitutive eNOS and nNOS are unlikely sources of NO in the delayed vasodilatation, since their expression is depressed by LPS (44). However, inducible NOS (iNOS) is upregulated in the cerebral circulation during systemic hypotension coincided with the time of iNOS induction 3–5 h after LPS (27, 30, 39, 52) and is a likely source of significant amounts of NO that may underpin the sustained elevation of CBF. In our study, significant cerebral vasodilatation and systemic hypotension coincided with the time of iNOS induction 3–5 h after LPS (27, 30, 39, 52). All observations that plasma nitrate/nitrite levels were increased 6–8 h post-LPS1 indicate that increased iNOS underpinned the prolonged vasodilatation after the first exposure to LPS. iNOS induces widespread vasodilatation in fetuses (4) and adults (39, 52), including the brain vasculature (52). NO is mainly produced by iNOS under conditions of hypoxia (53), and the availability of oxygen limits this form of NO production (12, 65) in keeping with our observation that the later, elevated CBF level was correlated with higher levels of SaO2 (Fig. 3B).

CΔO2 was persistently lower from 4 h post-LPS in association with falling SaO2 and hemoglobin. However, rises in CBF metabolic rate are contradictory, with reports of a low (3, 42) or an unchanged rate in human endotoxemia (48), whereas some animal studies show an increase (14). In our model, CBF fell transiently after LPS by 33%. Because such a fall in CBF is normally well compensated by increased oxygen extraction in the term and preterm brain (22), the flow decrement itself would not be expected to disrupt oxidative metabolism and not to be injurious. However, LPS dramatically increases the vulnerability of the neonatal rat brain to short hypoxic-ischemic insults that by themselves cause little or no injury (13). Interestingly, prenatal LPS also sensitizes the neonate to hypoxic ischemic injury, although it confers some protection in adulthood by reducing gray matter injury (68).

Significant (50%) cerebral vasodilatation and elevation of CBF occurred despite the development of hypotension between 6 and 24 h after LPS (Fig. 1A). This vasodilatation was sustained after twice-repeated LPS exposures (Fig. 2), differing from the circulatory tolerance that develops in the systemic vasculature of fetal sheep (10). An inverse relationship between CBF and SaO2 was apparent early in the post-LPS period that is typical of the acute cerebral circulatory response to hypoxia (Fig. 3A). Typically, CBF increases hyperbolically as SaO2 falls to maintain O2 transport to the fetal brain at a level sufficient to meet metabolic needs (36). The presence of this relationship suggests that the depressed SaO2 level that followed LPS was the principal factor responsible for the early phase of the CBF increase. Subsequently, the CBF–SaO2 relationship was altered from the inverse hyperbolic form that typifies the acute hypoxic response. Between 6 and 12 h post-LPS, CBF exhibited a positive correlation with SaO2, increasing as SaO2 rose (Fig. 3B). Later, after LPS3 when CBF was at a sustained high level, it did not correlate at all with changes in SaO2 (Fig. 3C and D).

NO has often been implicated in the response to LPS, and NO arising from constitutive NOS may have counteracted vasoconstrictor influences and limited the early (~1 h) fall in CBF that we observed. Another study in endotoxin-treated fetal sheep has shown that although CBF was not increased, inhibition of NO before LPS halved the CBF response 1 h after LPS infusion (7). A delayed (5–22 h) CBF rise after LPS has been ascribed to NO production (56). Although hypoxemia developed during this period in our study, NO retains the ability to vasodilate the fetal cerebral circulation during hypoxia (46, 54). Constitutive eNOS and nNOS are unlikely sources of NO in the delayed vasodilatation, since their expression is depressed by LPS (44). However, inducible NOS (iNOS) is upregulated in the cerebral circulation during systemic hypotension coincided with the time of iNOS induction 3–5 h after LPS (27, 30, 39, 52) and is a likely source of significant amounts of NO that may underpin the sustained elevation of CBF. In our study, significant cerebral vasodilatation and systemic hypotension coincided with the time of iNOS induction 3–5 h after LPS (27, 30, 39, 52). All our observations that plasma nitrate/nitrite levels were increased 6–8 h post-LPS1 indicate that increased iNOS underpinned the prolonged vasodilatation after the first exposure to LPS. iNOS induces widespread vasodilatation in fetuses (4) and adults (39, 52), including the brain vasculature (52). NO is mainly produced by iNOS under conditions of hypoxia (53), and the availability of oxygen limits this form of NO production (12, 65) in keeping with our observation that the later, elevated CBF level was correlated with higher levels of SaO2 (Fig. 3B).

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Table 3. CVR responses to bradykinin in fetal sheep

<table>
<thead>
<tr>
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<th>ΔCVR, %</th>
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<tbody>
<tr>
<td><strong>LPS-Survived Fetuses</strong></td>
<td></td>
</tr>
<tr>
<td>n Pre-LPS</td>
<td>−18 ± 3*</td>
</tr>
<tr>
<td>LPS1</td>
<td>16 ± 9</td>
</tr>
<tr>
<td>LPS2</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>LPS3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>24 h post-LPS3</td>
<td>5 ± 3</td>
</tr>
<tr>
<td><strong>NS-Treated Fetuses</strong></td>
<td></td>
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</tbody>
</table>
| n Pre-NS         | −12 ± 5*
| NS1              | 12 ± 4  |
| NS2              | −17 ± 5*|
| NS3              | −16 ± 6*|
| 24 h post-NS3    | −19 ± 4*|

Values are means ± SE and represent changes from baseline after bradykinin (BK; 0.05 μg/kg) in fetuses surviving LPS and NS-treated fetuses, LPS1–LPS3, sequential LPS infusions; NS1–NS3, sequential NS infusions. *P < 0.05 vs. baseline. †P < 0.05 vs. pre-LPS response.
post-LPS were sufficient to prevent any fall in cerebral O2 despite the development of hypoxemia (Fig. 1A). Our data confirm an earlier suggestion based on measurements of carotid blood flow that O2 transport is maintained to the fetal brain after LPS (56). Both infection and hypoxia can induce elevations in proinflammatory cytokines and potentially cause inflammation-induced brain injury (55). We observed cellular injury in the caudate nucleus, similar to previous studies (43, 56), despite O2 transport being sustained. We also observed extensive macrophage infiltration consistent with inflammatory injury (31). Brain injury also occurred in an earlier study even though cerebral perfusion was sustained, and it was suggested that cerebral hypoxia-ischemia was not the primary etiological factor (56). Previously, this issue was in doubt, because in other studies of endotoxin-induced injury, cerebral O2 transport was not maintained (7, 8, 15). Our new data, together with those of Peebles et al. (56), add support to the view that although LPS induces significant arterial hypoxemia, cerebral hypoxia appears not to be the principal factor in the induction of injury. Nonetheless, on a cautionary note, global measurements of blood flow and O2 transport such as those we used may not detect regional hypoxia-ischemia sufficient to produce focal WMI (32, 43).

Although high CBF after LPS appears beneficial in sustaining O2 transport, it may also be injurious to the fetal brain by promoting oxidative injury in a fashion similar to ischemia-reperfusion injury (33). Persistently high CBF after LPS may also contribute to the pathogenesis of intracranial hemorrhage in infection, a lesion that is recognized in the infant soon after birth but may in some cases have its origin in fetal life (40, 41). We found intraparenchymal hemorrhage in white matter and caudate nucleus in one of five LPS-surviving fetal sheep, similar to a previous observation (43).

In the group that subsequently died in utero, baseline arterial oxygenation before LPS tended to be lower than in surviving fetuses, but this was not a significant difference (Table 2). Also, no ABP, CBF (Table 1), or BK response differences (results not shown) before LPS predicted death in these fetuses. Similar to the surviving group, LPS induced an early (1 h) cerebral vasoconstriction and reduction of CBF in FDIU fetuses (Fig. 1B). Given that TNF-α rose in the FDIU fetuses to levels similar to those in the surviving group, vasoconstriction by TNF-α may explain the early CBF reduction in both. By contrast, in fetuses due to die, there was no early cerebral dilatory response to hypoxemia and no delayed LPS-induced cerebral vasodilatation and increase in CBF, as was evident in surviving fetuses (Fig. 1B). Furthermore, there was no change of nitrate in the FDIU fetuses (results not shown). Thus a failure to increase CBF and NO production, and to sustain cerebral O2 transport after LPS, was a defining feature of these animals, possibly due to greater sensitivity to proinflammatory cytokines.

The endothelium is a rich source of vasoregulatory factors, which are important for fetal CBF regulation (6, 38), and it is vulnerable to injury during bacterial infection and endotoxemia (73) with as many as 25% of endothelial cells lost from the aorta after a single dose of LPS (37). In keeping with prior pharmacological evidence of LPS-induced endothelial dysfunction in damaged abdominal aorta (37) and coronary vessels (28), cerebral vasodilatory responses to BK were abolished after LPS in our study in both surviving fetuses and those that died. Lost BK responsiveness post-LPS was accompanied by substantial basal vasodilatation in surviving fetuses (Fig. 2), so it is possible that vessels were near maximal dilatation, rendering BK ineffective. However, BK responsiveness was also lost in FDIU fetuses, which did not have basal cerebral vasodilatation; thus lost responsiveness is more likely to have arisen from endothelial damage and associated dysfunction (37).

Significantly, large CBF responses sustained cerebral O2 transport after LPS in surviving fetuses despite pharmacological evidence for LPS-induced endothelial injury (Fig. 5). Moreover, CBF increases were present for over 72 h, despite the abolition of BK responses as early as 8 h after LPS (Table 3) and also despite the lesser nitrite/nitrate increases that occurred after LPS3 (Fig. 4A). Thus endothelium-derived vasodilators important for normal regulation of the cerebral vessels appear not to be responsible for the later, persisting vasodilatation after LPS; these would include eNOS-derived NO, which normally mediates BK-induced vasodilatation (18). Rather, nonendothelial sources, possibly iNOS induced by LPS in smooth muscle cells (67) or in extravascular sites such as perivascular neurons (47), may mediate the later dilatation. A shift in the balance from an endothelial to an extravascular origin of NO is in keeping with the lower plasma nitrates we observed with repeated LPS (Fig. 4A).

We found no changes in maternal TNF-α or body temperature (data not shown) after LPS was given to the fetus, suggesting that there was no significant cytokine passage.
across the placental barrier from fetus to mother, just as there appears to be no passage from mother to fetus (29, 45). LPS does not cross the placenta after administration to the ewe (31). Nevertheless, LPS given to the ewe does lead to fetal inflammatory brain injury, indicating that the placenta may release a substance that is injurious to the fetal brain (31).

LPS endotoxin exists in all gram-negative bacilli, with E. coli being the most common placental pathogen involved in chorioamnionitis (15). We found histological evidence of chorioamnionitis, the hallmark of intrauterine inflammation, in three of five LPS-surviving fetuses (2, 17, 78). Although intrauterine infection is associated with brain injury in both preterm and term fetuses, causing CP and varying degrees of neurodevelopmental delay (23, 64, 66), the association between infection/inflammation and CP appears stronger in term infants (24, 49, 50, 72).

**Perspectives and Significance**

We chose LPS and a near-term fetal model for their relevance to human fetal infection-induced brain injury. The maturity of the fetal sheep at 0.85 gestation corresponds to human brain development to the end of the third trimester (26). Responses to endotoxemia in the sheep reflect the general human pattern of relative risk, in that the preterm is particularly susceptible, although the near-term fetus is also at increased risk. Thus, after endotoxin exposure in the fetal sheep, pathological changes in the brain developed in 50% of the surviving preterm fetuses and in 28% of surviving near-term fetuses (66); moreover, 40% of preterm fetuses died within 8 h of LPS exposure compared with 22% in the near-term group. At present, the exact basis for the greater risk of infection-induced injury in the preterm remains uncertain, particularly in view of the greater cardiovascular tolerance to asphyxia in early gestation (25) and evidence that LPS can produce neuronal injury in the absence of systemic changes such as hypotension, hypoxemia, or hypercapnia (11). One possible explanation is that LPS can sensitize the immature brain to hypoxic-ischemic injury (13). Another explanation, in keeping with our finding of prolonged impairment of endothelial function after LPS, is that prenatal exposure to LPS worsens hypoxia-ischemic injury in the neonate (68). Together, these data suggest that the tolerance of the preterm fetus to asphyxial injury might be compromised by exposure to LPS. It also is possible that immaturity of NOS expression in the preterm brain may compromise blood flow and oxygenation in infection (51). NOS is necessary for the cerebral blood flow increase in hypoxemia in near-term fetal sheep (21) and, as our data suggest, NO may play a significant role in preventing cerebral hypoxia in infection, thereby limiting the extent of brain injury. Notably, in contrast to the survivors, NO production was evidently deficient and cerebral O₂ transport fell in those fetuses destined to die in our study. Further mechanistic studies of the role of the endothelium and NOS isoforms in regulating cerebral blood flow and preserving cerebral oxygenation may shed light on the exaggerated risk of neuronal injury and death in preterm fetuses.

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