Endotoxin Has Acute and Chronic Effects on the Cerebral Circulation of Fetal Sheep

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ABSTRACT

We studied the impact of endotoxemia on cerebral blood flow (CBF), cerebral vascular resistance (CVR) and cerebral oxygen transport (O₂ transport) in fetal sheep. We hypothesized that endotoxemia impairs CBF regulation and O₂ transport, exposing the brain to hypoxic-ischemic injury. Responses to lipopolysaccharide (LPS, 1μg/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 ~ +50%) was followed by vasodilatation maximal at 5-6 h (ΔCVR ~ -50%) when CBF had increased (~ +60%) despite reduced ABP (~ -20%). Decreased CVR and increased CBF persisted 24 h post-LPS, and the two subsequent LPS infusions. Cerebral O₂ transport was sustained, though SaO₂ 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 ~ -20%, P < 0.05) was abolished after LPS. Fetuses which 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 endotoxemia. In contrast, cerebral hypoperfusion and reduced O₂ transport occur in fetuses destined to die, emphasizing the importance of sustaining O₂ transport for survival.

KEYWORDS
Endotoxin, Cerebral Blood Flow, Fetus, O₂ transport
ABBREVIATIONS

ABP = mean arterial blood pressure
BK = bradykinin
CaO₂ = arterial oxygen content
CBF = cerebral blood flow
CVR = cerebral vascular resistance
LPS = lipopolysaccharide
eNOS = endothelial nitric oxide synthase
FDIU = Fetal death in utero
iNOS = inducible nitric oxide synthase
NS = normal saline
ICP = intracranial pressure
O₂ transport = oxygen transport
PAF = amniotic fluid pressure
TNF-α = tumour necrosis factor-α
WMI = white matter injury
INTRODUCTION

Brain injury in the perinatal period remains a major cause of disability, with the numbers of affected infants rising with improvements in perinatal care. Though preterm infants are at particularly high risk (19), more than half cerebral palsy (CP) infants are born at term (23). Intrauterine infection is strongly implicated in the pathogenesis of perinatal brain injury with chorioamnionitis conferring a four-fold increased risk of CP in term infants (71).

Cerebral hypoperfusion has been viewed as a principal cause of infection-induced perinatal brain injury (32). Although hypotension is commonly associated with infection in human neonates (59) and in fetal animal models of endotoxemia (7, 8, 10, 15, 56), it is not clearly associated with cerebral hypoperfusion. Thus, neither chorioamnionitis 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, nor 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). As 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 is normally low in fetal life, and falls further during endotoxemia (7, 8, 10, 15, 56). As a consequence, oxygen transport (O₂ transport) to the brain may be compromised and neuronal injury may result even if CBF were to be maintained. Several studies have described falls in cerebral O₂ transport during endotoxemia (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 O$_2$ transport in endotoxemia, 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. As variability between studies might arise from intermittent measurements, we continuously monitored changes of CBF and O$_2$ transport in fetal sheep exposed to LPS. Also, we repeated the study on successive days, as circulatory tolerance to endotoxin may develop with repeated LPS exposure (10, 70). Additionally, as nitric oxide (NO) synthesis might be critical for sustaining CBF in endotoxemia (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 pro-inflammatory cytokine tumour necrosis factor-$\alpha$ (TNF-$\alpha$) were also measured to assess its role in cerebral regulation in conditions of endotoxemia.
METHODS

Fourteen ewes (Merino/Border-Leicester cross) were brought into the animal house 2 weeks prior to 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 approved by the Monash University-Monash Medical Centre Committee on Ethics in Animal Experimentation.

**Surgical and Experimental Procedures**

Ewes were anesthetized at 123 ± 1 d gestation (halothane 1–2%, nitrous oxide 60%, and oxygen 38–39%) and both ewe and fetus were then instrumented for study. A non-occlusive double lumen catheter (1.7mm od, Argyle, Tyco Healthcare Group LP, Mansfield, MA 02048, U.S.A.) was inserted into the fetal carotid artery for ABP monitoring, bradykinin (61) injection, and blood sampling, and a non-occlusive single lumen catheter (0.86 mm id 1.52 mm od) was inserted into the amniotic cavity for measuring PAF. A catheter (1.57 mm id, 2.41 mm od) was also positioned under the dura to record fetal ICP. Non-occlusive jugular venous catheters (1.57 mm id, 2.41 mm od) were inserted in both fetus and ewe for drug administration and blood sampling. To record fetal CBF, a transit-time ultrasonic flow probe (2 mm diameter, Transonic Systems, Ithaca, NY) was positioned around the fetal superior sagittal sinus as previously described (20). In brief, a 2 cm by 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, taking care 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). Used in combination with non-occlusive 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 fetus and ewe were treated with antibiotics (Fortum 50mg/kg, Glaxo SmithKline, Australia; and Gentamicin 2.5mg/kg, Pharmacia, Australia) and analgesic (Finadyne, 1mg/kg, Schering-Plough, Australia).

**Study Conditions**

Fetuses were studied over a 5 d period beginning at a gestational age of 125 ± 1 d (mean ± SE; 0.85 gestation, term at 147 days), after 48 h post-operative recovery. During the study, the ewe’s cage was partitioned to prevent the sheep from turning around 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, Ithaca, NY). 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 (E. coli O127-B8, L3129, Sigma. USA) or normal saline (NS) was infused IV to fetuses by syringe pump (sp 100i infusion pump, WPI, USA) over 30 min on three consecutive days (LPS n=9,
Fetal circulatory 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 9 assays was 0.13 ng/ml, and the mean intra- and inter-assay coefficient of variation (CV) was 6.5 and 8.3%, respectively. Total plasma nitrate/nitrite concentrations were measured colorimetrically (Dynatech, Guernsey, Channel Islands) using a commercially available kit (Cayman Chemicals Cat no. 780001). The analytical range was 2.0 – 80 µmol/L and the intra-assay and inter-assay CVs were 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 3 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 re-anesthetised and the saline control and LPS-surviving fetuses were perfused transcardially with 2 l of heparinised saline (10 I.U./ml) followed by 2 l of 4% paraformaldehyde in 0.1M phosphate buffer-saline (pH 7.4); ewes were then killed using sodium pentobarbitone (150 mg/kg, IV). Fetal brains were removed and immediately cut into
hemispheres at the mid-sagittal line. One hemisphere was post-fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer-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 - 1000 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 then analyzed sec-sec with average values calculated for each 30 sec over each hour of the control, infusion, and post-infusion periods. Cerebral perfusion pressure (CPP) was calculated as (ABP − ICP). CVR was calculated as CPP/CBF. O2 transport was calculated as O2 transport = CBF x CaO2, where CaO2 = (1.34 x Hb x SaO2 / 100 + 0.003 x PaO2).

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 natural 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 presented as mean ± SE. All tests employed Sigma-Stat 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 between NS-treated, LPS-treated and fetal death in utero (FDIU) groups were not different for any of the measurements (Table 1).

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 %, mean ± SE %, $P < 0.05$) 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 non-significant tests as the power of ANOVA was low (< 0.10). However, the sole trend in the non-significant data was a tendency for O₂ transport to increase late in the 24 h period in LPS-treated fetuses (Fig. 1A). As 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 PaCO₂ after 4 h (52 ± 1 mmHg, $P < 0.05$), by lower SaO₂ after 4 h (44 ±
4%, \( P < 0.05 \), and by lower \( \text{PaO}_2 \) after 2 h (19 ± 1 \%, \( P < 0.05 \)). Both \( \text{pH} \) and \( \text{PaCO}_2 \) began recovery toward normal levels 8 h post-LPS, while \( \text{PaO}_2 \) and \( \text{SaO}_2 \) remained low for at least 10 - 12 h (\( P < 0.05 \)). Hemoglobin levels increased transiently 2 h post-LPS, then fell between 8 - 24 h, a pattern similar to previous descriptions (8, 56). Arterial oxygen content (\( \text{CaO}_2 \)) was persistently lower from 4 h post-LPS, in association with lower \( \text{SaO}_2 \) 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 were induced by the first LPS infusion persisted 24 h after each of the subsequent infusions (Fig. 2). Similarly, cerebral \( \text{O}_2 \) transport was maintained at pre-LPS levels throughout the repeated LPS infusions (Fig. 2).

**CBF versus \( \text{SaO}_2 \)**

Regression of CBF with \( \text{SaO}_2 \) 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 \( \text{SaO}_2 \), with CBF increasing as \( \text{SaO}_2 \) fell (Fig. 3A, \( R = 0.80, P <0.001 \)). Somewhat later following LPS1 (6 - 12 h), the relationship between \( \text{SaO}_2 \) and CBF reversed, with CBF now rising as \( \text{SaO}_2 \) rose (Fig. 3B, \( R= 0.48, P <0.05 \)). After LPS3, CBF was unrelated to \( \text{SaO}_2 \) (Fig. 3C-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 μmol/L, \(P < 0.05\)). The level of nitrate/nitrite remained high 8 h post-LPS1 (49 ± 8 μmol/L, \(P < 0.05\)), 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 for LPS1 (78 ± 35 ng/ml versus 7 ± 5 ng/ml, \(P < 0.05\)), suggestive of tolerance having been induced.

**Cerebral endothelial function**

Dose-responses to BK in surviving fetuses immediately prior to LPS1 and 24 h post-LPS3 are shown in Fig. 5. Prior to 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 (0.05μg/kg, n=5) prior to 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 normal saline (NS, n=5) over 5 d, the baseline CVR reduction induced by 0.05μg/kg BK (-18 ± 5, \(P < 0.05\)) remained unchanged (Table 3).
**Histology**

No normal saline-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 white matter and caudate nucleus. Histological chorioamnionitis was found in the placentas of 3 of 5 LPS-treated animals.

**Fetal death in utero (FDIU)**

Baseline values for circulatory parameters for the fetal group that died in utero following LPS treatment (n=4) are shown in Table 1, and arterial blood gases and pH 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 the 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 prior to LPS (28 ± 4μmol/L). 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 have also 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), while another suggests that there is a delayed blood 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, by 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), as the onset of the flow increase after LPS can be highly variable (56). Though 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, as the dose used in our study (1μg/kg) was equal to that used in the study where no flow increase was found (8). Moreover, a much lower LPS dose (0.1μg/kg) elicited a similar flow increase (~50%) to that which we found (56). It is
also unlikely that repetition of LPS administration was a factor. Though 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. Nor can gestational age differences explain the variation in findings as 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, though arterial blood pressure remained in the normal range, inferring that the cerebral vasculature is more sensitive than the systemic (non-cerebral) vasculature to vasoconstriction following LPS. Elevation in TNF-α coincided with increased CVR (Fig. 4B), 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 PVL lesion itself (77), and while it is unclear whether proinflammatory cytokines can cross the blood-brain barrier (BBB), TNF-α disrupts endothelium (9) and increases BBB permeability (5). While 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 up-regulated by LPS, while the vasodilator endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) are down-regulated (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.

Cerebral blood flow and cerebral vascular resistance 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 as it can suppress neuronal activity in the brain (69). However, the data regarding LPS effects on cerebral metabolic rate are contradictory, with reports of a low (3, 42) or an unchanged rate in human endotoxaemia (48), while some animal studies show an increase (14). In our model, CBF fell transiently after LPS by ~33%. As 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 caused little or no injury (13). Interestingly, prenatal LPS also sensitizes the neonate to hypoxic ischemic injury, though it confers some protection in adulthood by reducing grey matter injury (68).

Significant (50%) cerebral vasodilatation and elevation of CBF occurred despite the development of hypotension between 6 - 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 in order 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 - 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 Fig. 3D).

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 though CBF was not increased, inhibition of NO prior to LPS halved the CBF response one hour after LPS infusion (7). A delayed (5 - 22 h) CBF rise after LPS has been ascribed to NO production (56). Though 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 as their expression is depressed by LPS (44). However, inducible NOS (iNOS) is upregulated in the cerebral circulation ~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). Also, 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).

Arterial oxygen content was persistently lower from 4 h post-LPS in association with falling SaO₂ and hemoglobin. However, rises in CBF post-LPS were sufficient to prevent any fall in cerebral OT 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 pro-inflammatory 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, as in other studies of endotoxin-induced injury cerebral O₂ transport was not maintained (7, 8, 15). Our new data, taken together with those of Peebles et al. (56), add support to the view that though 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 white matter injury (43).

While 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 prior to LPS tended to be lower than surviving fetuses, but this was not a significant difference (Table 2). Also, no ABP, CBF (Table 1) or BK response differences (results not shown) prior to 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). As TNF-α rose in the FDIU fetuses to levels similar to 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 pro-inflammatory 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 who 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, non-endothelial sources, possibly iNOS induced by LPS in smooth muscle cells (67) or in extra-vascular sites such as perivascular neurons (47) may mediate the later dilatation. A shift in the balance from an endothelial to an extra-vascular 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-\(\alpha\) nor 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). Nor does LPS cross the placenta after administration to the ewe (31). Nevertheless, LPS given to the ewe does lead to fetal inflammatory brain injury, indicating that 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). While intrauterine infection is associated with brain injury in both preterm and term fetuses, causing cerebral palsy 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 endotoxaemia in the sheep reflect the general human pattern of relative risk, in that the preterm is particularly susceptible, though the near-term fetus is also
at increased risk. Thus after endotoxin exposure in the fetal sheep, pathologic changes in the brain developed in 50 percent of the surviving preterm fetuses, and 28 percent of surviving near-term fetuses (66); moreover, 40 percent of preterm fetuses died within 8 h of LPS exposure, versus 22 percent 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, 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). Taken together, these data suggest that the tolerance of the preterm fetus to asphyxial injury might be compromised by exposure to LPS. It is also 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, so 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.

Acknowledgements
This work was supported by the National Health and Medical Research Council of Australia (A.W. and D.P.), and by a Faculty of Medicine International Postgraduate Scholarship, Monash University, Australia (Y.F.). We acknowledge the essential contributions of Ms Thilini Samarasinghe and Dr Lisa Hutton in the histological analyses and interpretation.


TABLE AND FIGURE LEGENDS

Table 1. Values (mean ± SE) are for fetal sheep treated with normal saline (NS), fetuses surviving LPS (LPS-survived), and LPS-treated fetuses that died in utero (LPS-FDIU).

Table 2. Values for carotid arterial samples after treatment with normal saline (NS1, n=5), with LPS (LPS1, n=5), or LPS followed by fetal death in utero (FDIU, n=4). Mean ± SE shown for the first day of treatment. Note that a complete set of FDIU values is available only until 8 h, after which fetuses died. For other abbreviations, see text. * P < 0.05 versus baseline.

Table 3. Values (mean ± SE) are changes from baseline after bradykinin (0.05 µg/kg) in surviving LPS fetuses (LPS) and normal saline-treated fetuses (NS). For other abbreviations see text. * P < 0.05 signifies change from baseline, † P < 0.05 from the Pre-LPS response.

Figure 1A. Time course of circulatory changes after the first LPS infusion in surviving fetuses (●, n=5) and saline-infused controls (○, n=5). Values shown are means plus SE. Asterisks denote significant differences of LPS-treated fetuses versus time zero (★, P < 0.05). Arterial blood pressure (ABP) started to fall 4 h post-LPS and remained low at 24 h. Cerebral vascular resistance (CVR) increased within the first hour but subsequently fell, while CBF fell transiently then rose. Cerebral vasodilation persisted through to 24 h post-LPS, and CBF remained elevated despite persisting hypotension. Cerebral O₂ transport was maintained throughout the post-LPS period despite persistent reductions in SaO₂ and CaO₂ (Table 2). No significant changes occurred with saline infusion.

Figure 1B. Circulatory changes after the first LPS infusion in fetuses subsequently dying in utero. Thick lines with ● symbol are the mean values plus SE for n=4, with SE shown only until 8 h, after
which individual animals died; thin lines indicate responses of individual animals. Asterisks denote significant differences versus time zero (★, P < 0.05). ABP fell after LPS, but there was no compensatory fall in CVR despite hypotension and reduced SaO₂ and CaO₂ (Table 2); as a consequence CBF and cerebral O₂ transport were not maintained. Note that the pattern differs substantially from that of survived fetuses in which CBF rose and O₂ transport was sustained at the pre-LPS baseline level.

Figure 2. Baseline responses to repeated LPS infusion in surviving fetuses (■, n=5) and matching saline infusion in controls (□, n=5). Values represent averages at the 24 h post-infusion time point expressed as percent of the pre-infusion baseline on day one. Note that ABP remained low 24 h after the first LPS infusion, but recovered 24 h after subsequent LPS infusions. 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. Asterisks denote significant differences versus pre-infusion baseline (★, P < 0.05). Note that cerebral O₂ transport was unchanged throughout the study period. No significant changes occurred with saline infusion. Abbreviations as in Fig. 1.

Figure 3. Regression of CBF and carotid arterial SaO₂ in LPS-surviving fetuses (n=5) after the first dose of LPS (LPS1, A and B) and the third dose (LPS3, C and D). CBF values are normalized to the pre-LPS values for each day; CBF averaged 9 ± 1 ml/min prior to LPS1 and 18 ± 2 prior to LPS3 (P < 0.05). In the first 4 h post-LPS1 (A) CBF significantly increased as SaO₂ fell. At 6-12 h post-LPS (B), the correlation of CBF with SaO₂ reversed, with CBF now positively correlated with SaO₂. After the third dose of LPS, CBF did not correlate with SaO₂ (C and D).
Figure 4A. Plasma nitrate/nitrite level in those fetuses that survived LPS treatment (n=5). Note the prolonged nitrate/nitrite increase after LPS1.

Figure 4B. Fetal TNF-α responses to LPS1 and LPS3 in those fetuses that survived LPS treatment (n=5). TNF-α peaked 1 h after each LPS infusion. Note that the scale for TNF-α concentration is 10-fold less for LPS3 than LPS1.

Figure 5. Bradykinin (BK, 0.001-0.5 µg/kg, intra-carotid 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 BK 0.05 µg/kg, where the CVR reduction averaged -18 ± 3% (P < 0.05). Following LPS treatment, no cerebral vasodilatation was observed at any BK dose.

Figure 6. Hematoxylin and eosin stained sections from the caudate nucleus in saline-treated control fetuses (A) and LPS-treated fetuses (B,C). Cells in the control fetus are healthy, indicated by a regular cytoplasm with a definitive border (A). In contrast, cells in the LPS-treated fetuses have an abnormal, irregular cytoplasm indicative of dying cells (B). Note extensive infiltration of macrophages (C, black arrows, same fetus as that depicted in B) which can be seen engulfing cells (white arrow).
Table 1. *Baseline circulatory parameters in fetal sheep*

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Table 2. *Arterial blood gases in fetal sheep*

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Table 3. CVR responses to bradykinin in fetal sheep

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Figure 1.
Figure 2.
Figure 3.

A  0-4 h LPS1

B  6-12 h LPS1

C  0-4 h LPS3

D  6-12 h LPS3

SaO₂, %

CBF, %

R = 0.80
P < 0.001

R = 0.48
P < 0.05

R = 0.13
P = NS

R = 0.24
P = NS
Figure 4.

A. Nitrate / Nitrite, μmol/L

B. TNF-α, % (LPS)

LPS1  LPS3

P < 0.05 versus baseline (0 h)  * LPS1
* LPS3

0 h  1 h  2 h  4 h  6 h  8 h  10 h  12 h  24 h
Figure 5.

![Graph showing changes in ABP, CBF, and CVR in response to BK administration with pre-LPS and post-LPS conditions. The graph indicates significant differences with *P < 0.05 versus baseline.](image-url)
Figure 6.