Acute and chronic effects of endotoxin on cerebral circulation in lambs

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Submitted 10 July 2009; accepted in final form 8 January 2010

Feng SY, Samarasinghe T, Phillips DJ, Alexiou T, Hollis JH, Yu YY, Walker AM. Acute and chronic effects of endotoxin on cerebral circulation in lambs. Am J Physiol Regul Integr Comp Physiol 298: R760–R766, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00398.2009.—The impact of endotoxemia on cerebral endothelium and cerebral blood flow (CBF) regulation was studied in conscious newborn lambs. Bacterial endotoxin [LPS, 2 μg/kg iv] was infused on 3 consecutive days. Cerebrovascular function was assessed by monitoring CBF and cerebral vascular resistance (CVR) over 12 h each day and by the endothelium-dependent vasodilator bradykinin (BK) (n = 10). Inflammatory responses were assessed by plasma tumor necrosis factor-α (TNF-α, n = 5). Acutely, LPS disrupted the cerebral circulation within 1 h, with peak cerebral vasoconstriction at 3 h (CBF −28 and CVR +118%, P < 0.05) followed by recovery to baseline by 12 h. TNF-α and body temperature peaked ~1 h post-LPS. BK-induced vasodilatation (CVR −20%, P < 0.05) declined with each LPS infusion, was abolished after 3 days, and remained absent for at least the subsequent 5 days. Histological evidence of brain injury was found in four of five LPS-treated newborns. We conclude that endotoxin impairs cerebral perfusion in newborn lambs via two mechanisms: 1) acute vasoconstriction (over several hours); and 2) persistent endothelial dysfunction (over several days). Endotoxin-induced circulatory impairments may place the newborn brain at prolonged risk of CBF dysregulation and injury as a legacy of endotoxin exposure.

EPIDEMIOLOGICAL DATA point to infection as a major cause of brain injury in the perinatal period (14, 42), although the precise timing and exact mechanisms of infection-induced injury are uncertain. Cerebral hypoperfusion is a potential explanation since bacterial infection is linked to hypotension (43), and impaired cerebral autoregulation exposes the brain to falls in cerebral blood flow (CBF) as arterial pressure falls (38). Although there is evidence of an association between infection, hypotension, and hemodynamic disturbances in early life, potential effects on cerebral perfusion remain uncertain (43).

Apart from hypotension-induced hypoperfusion, cerebral endothelial injury may also represent a critical mechanism linking bacterial infection to impaired cerebral perfusion, since the endothelium is a rich source of vasoregulatory factors that are important for perinatal CBF regulation (45). Moreover, the endothelium is vulnerable to injury and dysfunction during bacterial infection and endotoxia (22). However, although hypotension is an important factor in perinatal infection, whether disturbed cerebral vasoregulation is also involved is an important question that warrants further study (36).

Cerebral circulatory responses to infection and endotoxia in the immature brain appear to vary, because newborn CBF monitored intermittently has been found to decrease (44) or to be unchanged (7). Similarly, fetal CBF was increased (32) or unchanged (2, 3). By contrast, the CBF response in adult brain is a consistent increase (30).

We aimed to clarify cerebral circulatory responses to infection by continuously monitoring CBF and vascular resistance after LPS injection. To assess the potential contribution of endothelial injury to CBF dysregulation, we tested cerebral endothelial function throughout the study period using bradykinin (BK) (10). We also assessed LPS-induced responses of the vasoconstrictor and proinflammatory cytokine tumor necrosis factor-α (TNF-α) to test whether inflammation-induced vascular injury disturbs cerebral vasoregulation. Finally, as systemic and pulmonary circulatory tolerance to endotoxin develops over 2–3 days (5, 41), we monitored the circulatory changes, endothelial function, and TNF-α over several days to test whether tolerance might also develop in cerebral vessels.

METHODS

Thirty newborn lambs (Merino/Border-Leicester cross) were prepared for chronic study (11). All surgical and experimental procedures were approved by the Monash University-Monash Medical Centre Committee on Ethics in Animal Experimentation.

Surgery and Experimental Procedures

Each lamb was anesthetized (halothane 1–2%, nitrous oxide 60%, and oxygen 38–39%) and instrumented using sterile techniques. To record CBF, a transit-time ultrasonic flow probe (2-mm diameter, Transonic Systems, Ithaca, NY) was positioned around the superior sagittal sinus for quantitative, beat-by-beat measurement (11). A nonocclusive double-lumen catheter (1.7 mm OD, Argyle, Tyco Healthcare Group, Mansfield, MA) was inserted into the carotid artery for arterial blood pressure (ABP) monitoring, BK injection, and blood sampling, and a nonocclusive single-lumen catheter (0.86 mm ID, 1.52 mm OD) was inserted into the jugular vein for LPS infusion. A catheter (1.57 mm ID, 2.41 mm OD) was positioned under the dura to record intracranial pressure (ICP). Following surgical procedures, animals were treated with antibiotics (Fortum 50 mg/kg, GlaxoSmith-Kline; and gentamicin 2.5 mg/kg, Pharmacia) and analgesics (Fina-dyne, 1 mg/kg, Schering-Plough). At conclusion of experiments lambs were killed (sodium pentobarbitone, 150 mg/kg iv).

Study Conditions

Lambs were studied over 5–9 days after ≥48 h postoperative recovery (11); age at the first study day was 12 ± 2 days (means ± SE, n = 10) for the LPS group, 9 ± 1 days for saline-infused control group (n = 10), and 9 ± 1 days (n = 10) for the cytokine and...
nitrate/nitrite assay group. The flow probe was connected to the flowmeter (model T101 Ultrasonic Blood Flowmeter, Transonic Systems, Ithaca, NY). Catheters were connected to calibrated strain-gauge manometers (Cobe CDX III, Cobe Laboratories; Lakewood, CO) referenced to the midthoracic level. Pressure and flow signals were low-pass filtered at 100 Hz (PowerLab, Chart v5.4.1, ADInstruments, Sydney, Australia).

**Experimental Protocol**

**LPS and cardiorespiratory changes.** LPS 2 μg/kg (Escherichia coli O127-B8, L3129, Sigma) or saline was infused intravenously over 30 min on 3 consecutive days (each n = 10). Circulatory parameters ABP, ICP, and CBF were recorded continuously before and up to 12 h after each LPS infusion, then repeated 24 h post-LPS. Blood samples were collected for arterial blood gas analysis and body temperature measured at 0, 1, 2, 3, and 24 h post-LPS.

**LPS and cerebral endothelium function.** Cerebral endothelial function was assessed by assembling BK dose-response curves (0.001–0.5 μg/kg) before the first LPS infusion, 1–3 h after each LPS infusion, and 24 and 120 h after the final (third) infusion. Each BK response was averaged from three replicates in each animal. A control group (n = 5) received saline intravenously over a 9-day period equivalent to the LPS group.

**Cytokine and nitrate/nitrite analysis.** Body temperature was measured and arterial blood samples were collected at 0, 1, 2, 4, 6, 8, 10, 12, and 24 h post-LPS for the measurement of TNF-α, total nitrate/nitrite, and arterial blood gas and pH; these measurements were performed in an additional group (n = 5), separate from the first group but subject to an identical LPS treatment protocol. TNF-α concentrations in plasma were determined via an immunoassay validated to measure ovine TNF-α (19). The TNF-α standard used was ovine recombinant TNF-α, the mean sensitivity over nine assays was 0.13 ng/ml, and the mean intraassay coefficient and interassay coefficients of variation were 6.5 and 8.3%, respectively. Total plasma nitrate/nitrite concentrations were measured colorimetrically (Dynatech, Guernsey, Channel Islands) using a commercial kit (Cayman Chemicals cat. no. 7800001). The analytical range was 2.0–80 μmol/l, and the intra-assay and interassay coefficient of variation was 2.7 and 3.4%, respectively.

**Histology and postmortem procedures.** At the conclusion of physiological studies, saline control and LPS lambs (each n = 5) were reanesthetized and perfused transcardially with 2 liters of heparinized saline (10 IU/ml) followed by 2 l of 4% paraformaldehyde in 0.1 M phosphate buffer-saline (pH 7.4); lambs were then killed using sodium pentobarbitone (150 mg/kg iv). Lamb 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 buffer-saline (pH 7.4) at 4°C before being cut coronally into 10-mm-thick blocks and paraffin embedded. Regions were selected topographically by using the published nomenclature of sections 800–1,000 for the sheep brain (18). These regions include periventricular white matter and the caudate nucleus of the thalamus, both of which have been shown to be damaged by LPS (17, 26). Paraffin blocks were sectioned at 10 μm, and every 40th section was collected and stained with hematoxylin and eosin. The number of resting and active macrophages within the caudate nucleus and periventricular white matter was determined within one field of view (×200 magnification) from three separate tissue sections, 400 μm apart. Macrophages were defined as either resting or active depending on morphological characteristics; resting macrophages lacked granularity and typically had a single nucleus, whereas active macrophages contained granular deposits and typically were multinuclear (17). For each lamb the numbers of resting and active macrophages from each section and within each brain region were summed prior to statistical analysis.

**Data analysis and statistics.** Physiological signals were analyzed second by second, then averaged for each 30 s over each h of control, infusion, and postinfusion periods. Cerebral perfusion pressure (CPP) was calculated as ABP−ICP and cerebral vascular resistance (CVR) as CPP/CBF.

Physiological data and dose-response curves for BK (expressed as means ± SE) were analyzed by repeated-measures ANOVA and Dunnett’s post hoc analysis to identify changes from pre-LPS values. When data failed normality, nonparametric analysis (Friedman repeated-measures ANOVA) was used. BK response differences pre- and post-LPS were compared by a paired Student’s t-test. Quantitative histology comparisons were made by use of two-way ANOVA with one repeated measure and a Student-Newman-Keuls post hoc test. Tests employed SigmaStat v3 (SPSS, //www.spss.com) and P < 0.05 was considered significant.

**RESULTS**

**LPS and Circulatory Changes**

**Baseline.** Baseline (pre-LPS) circulatory parameters and arterial blood gases (Table 1) are similar to those for healthy lambs (11). Although baseline values of ABP, ICP, CBF, and CVR changed within the 24-h period post-LPS, there were no persisting baseline differences at 24 h. Arterial blood gases and hemoglobin were also unchanged from baseline over the 12-h period following each LPS infusion and at 24 h on successive days.

**LPS infusions.** Circulatory responses (ABP, ICP, CBF, and CVR) within the 24-h period following LPS infusion are illustrated in Fig. 1. After the first injection of LPS (LPS1) there was no hypotension, but within 2 h CBF fell and CVR rose. Peak cerebral vasconstriction occurred 3 h post-LPS when CBF had fallen to 72 ± 4% (means ± SE, P < 0.05) and CVR had risen to 218 ± 37% (P < 0.05) of baseline. After reaching its minimum, CBF recovered to baseline at ~5 h. After its initial rise, CVR recovered to baseline at ~5 h and remained at baseline thereafter.

Overall, similar patterns of circulatory disruption were evident following the second (LPS2) and third (LPS3) infusions, with early depression of CVR (~70% of baseline) arising from cerebral vasconstriction (~200% of baseline), as blood pressure rose slightly.

No arterial blood gas or pH changes occurred within each 12-h period. Body temperature peaked 1–2 h after each of the three LPS infusions (Fig. 2A). Peak body temperature was greatest after LPS1 (41.0 ± 0.2°C vs. 40.1 ± 0.2°C for LPS2 and 40.1 ± 0.2°C for LPS3, respectively, P < 0.05). TNF-α
quickly reached a peak 1 h post-LPS1 and promptly fell below the limits of detection established in saline control studies (Fig. 2B); TNF-α also increased after LPS3, but the peak concentration was lower than with LPS1 (75 ± 100 ng/ml vs. 6 ± 4 ng/ml, P < 0.05).

Total plasma nitrate/nitrite in the LPS-treated lambs increased 4 h after LPS1 (Fig. 2C) and reached significant elevation at 6 h (11 ± 3 μmol/l, P < 0.05). Nitrate/nitrite remained elevated until 12 h post-LPS1 (10 ± 1 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. 2C).

Endothelial function tests. BK dose-responses pre-LPS1 and 24 h post-LPS3 are shown in Fig. 3. Pre-LPS, BK induced vasodilatation between 0.05–0.1 μg/kg. Maximal vasodilatation occurred at 0.05 μg/kg where CVR was reduced by −20 ± 4% (P < 0.05). At 24 h following LPS3, no cerebral vasodilatation was observed at any dose (Fig. 3). Daily CVR responses to BK (0.05 μg/kg) after LPS1, LPS2, and LPS3, 24 h post-LPS3, and 120 h post-LPS3 are illustrated in Fig. 4. BK-induced vasodilatation diminished progressively with repeated LPS administration and remained abolished 120 h after the final LPS infusion. In saline-infused control lambs, vasodilatory responses to BK (0.05 μg/kg) were unchanged over this period, with a preinfusion CVR reduction of −20 ± 3% and a postinfusion CVR reduction of −19 ± 5% (n = 5, P > 0.05).

Histology

LPS-treated lambs had an abundant infiltration of macrophages into the parenchyma of the brain that exhibited evidence of brain injury (Fig. 5). Compared with saline-treated control lambs, LPS-treated lambs had an approximate twofold increase in the number of active macrophages within the caudate nucleus and periventricular white matter of the thalamus (Table 2, P < 0.001). In addition, the number of active macrophages significantly exceeded resting macrophages in both regions (P < 0.001 and P < 0.005, respectively).
were no differences in the number of resting macrophages between LPS-treated and saline-treated lambs, nor were there any differences between the number of active and resting macrophages in the saline-treated lambs. We found evidence of cellular injury in the caudate nucleus and periventricular white matter of the thalamus in four of five LPS-treated newborns. Cells in the LPS-treated injured brains exhibited an irregular cytoplasm lacking any definitive border, indicative of dying cells. No abnormality was evident in the saline-treated controls. In the single LPS-treated lamb that did not show cellular injury, the extent of macrophage infiltration was less than in the injured lambs; active macrophages exceeded resting macrophages in the caudate nucleus (28 vs. 22 cells/field, respectively), but not in periventricular white matter (14 cells/field for both).

DISCUSSION

Our study in newborn lambs confirms that cerebral hypoperfusion is a feature of the response to LPS and identified a complex pattern of cerebral circulatory dysfunction following LPS that was typified acutely by transient CBF reductions and chronically by persisting cerebral endothelial dysfunction. Previous conflicting studies reporting varied CBF responses, or no responses, may have failed to identify these transient CBF changes. Moreover, ours is the first study to identify persisting LPS-induced endothelial dysfunction in the cerebral circulation in newborn lambs.

Acute Phase

Continuous monitoring revealed large CBF reductions of $\sim 30\%$ 1–3 h after LPS administration, but this has not been a consistent finding in other perinatal studies. In newborn models, CBF appeared only marginally affected by LPS when measured intermittently by radiographic or microsphere methods. CBF was reduced in periventricular and occipital white matter in newborn dogs soon after $E.\ coli$ endotoxin; however, in gray matter CBF was unchanged (44). In the immature newborn rat exposed to hypoxic ischemia, whole brain CBF was unchanged by prior exposure LPS although it tended to be lower by $\sim 5$–10% (7). It is possible that with continuous CBF measurement significant CBF changes, such as those we found, may have been evident in these earlier studies. Nonetheless, on a cautionary note, global measurements of blood flow such as those we used may not detect regional ischemia sufficient to produce focal white matter injury (26, 44). In the fetus, hypoxemia after LPS may have contributed to variable CBF (3), since it is a powerful cerebral vasodilator (20). Our studies found no hypoxemia or hypercapnia that limited the observed CBF reduction.

Early reductions of CBF after LPS (Fig. 1) were due entirely to cerebral vasoconstriction, as in adult animals (8, 31). A large CVR increment of $+118\%$ was reached 3 h after LPS infusion, and at this time there was no hypotension evident. CBF and CVR are normally closely coupled to cerebral metabolic requirements, with resistance increasing and flow falling as metabolic rate declines (20). Reduced cerebral metabolism...
may have contributed to the early increase in CVR since LPS can suppress neuronal activity (40) and cerebral metabolic rate (25). However, a specific newborn study would be required to examine this possibility.

Among many factors that may have caused early vasoconstriction post-LPS, raised TNF-α (Fig. 2B) is likely to be important because it has powerful vasoactive effects on cerebral vessels (34). In our study TNF-α rose promptly after LPS and reached peak values at 1 h, similar to previous findings (23). Although TNF-α vasodilates superficial cerebral arteries via the NO pathway (11), it also vasostricts intra- and subependymal cerebral vessels (34). In our model, the elevated level of TNF-α occurred at the same time as CBF decreased and CVR increased, suggesting that the vasoconstrictor actions of TNF-α outweighed the vasodilator actions. Dominance of vasoconstriction could have arisen if LPS had impaired endothelium-dependent vasodilator pathways (34), and this is consistent with the reduced BK responses after LPS (Fig. 4). This link between the appearance of TNF-α and cerebral hemodynamic changes suggests that it mediates the cerebral vasoconstriction soon after LPS infusions, possibly in combination with vasoconstrictor prostanoids (16) and endothelin-1 (35), both of which are elevated by LPS. Involvement of vasoconstrictors other than TNF-α is likely because the early reduction of CBF and increased CVR persisted well beyond the time when TNF-α level had returned to baseline in our study. Moreover, as discussed below (Chronic Phase), although TNF-α was attenuated after the third LPS exposure, a CVR increase remained evident.

Coincident with the early cerebral vasoconstriction, large increases in body temperature reaching ~41°C occurred (Fig. 2A). During fever, systemic vasoconstriction occurs to prevent heat loss but whether vasomotor changes occur in the brain remains unclear. Fever is triggered by endogenous pyrogens, including TNF-α (21), mediated by cyclooxygenase-2 (COX-2) that enhances cerebral vasoconstriction (33). The time course of COX-2 upregulation in cerebral arteries is similar to the CVR and body temperature increases that we observed, increasing 1 h post-LPS and persisting for 5 h (16), strongly suggesting a role for COX-2 in the vasoconstriction.

With repeated LPS infusion, the early fall and recovery of CBF remained similar to the first exposure (Fig. 1). Potentially, CBF falls could arise from hypotension, which is found in fetal studies at low endotoxin doses (5) and in adult studies at substantially higher doses than we used (22). However, in our study arterial pressure fell only marginally after LPS1 and not at all after LPS3. Generally, inducible NO synthase (iNOS) is associated with widespread vasodilatation, including the brain (30), and with counteraction of cerebral vasoconstrictors (16), so that iNOS-derived NO may have counteracted the early vasoconstrictor response and contributed to the restoration of CBF in our study. LPS-induced iNOS appears after ~5 h in plasma (23) and brain (16, 30), consistent with elevation of plasma nitrate/nitrite that we found beginning ~6 h after LPS1. The pattern of CBF recovery was similar after LPS3, despite the lesser plasma nitrate/nitrite increases that occurred (Fig. 2C). Possibly, iNOS in smooth muscle cells (37) or in extravascular sites including perivascular neurons (29) rather than the endothelium may have mediated that dilatation. Such a shift in the balance from an endothelial to an extravascular origin of NO is consistent with the reduced plasma nitrate/nitrite response we observed with repeated LPS injection (Fig. 2C).

Chronic Phase

Early cerebral vasoconstriction and CBF reductions were all reversed in the 24 h following LPS (Fig. 1). The pattern of circulatory changes was essentially identical for each of the repeated LPS exposures; by contrast, TNF-α and temperature responses were attenuated. After the third LPS exposure, TNF-α reached a peak that was ~10% of the first response; the peak temperature was also less than during LPS1 (Fig. 2). TNF-α attenuation is found with repeated LPS exposures (27),
quently, BK response was abolished for at least 5 days after the CVR had recovered to the baseline level (Fig. 1). Subsequently, BK response remained reduced 24 h post-LPS3, at which time occlusion 1–3 h post-LPS may have been due to the vasoconstriction of the BK response began after the first LPS dose and the peak reduction in CVR averaging after LPS infusion.

We identified significant BK-induced vasodilatation, with the peak reduction in CVR averaging ~20% (Fig. 3). Diminution of the BK response began after the first LPS dose and was completely abolished after the third LPS dose (Fig. 4). Possibly, the early reduction in vasodilator responses to BK occurring 1–3 h post-LPS may have been due to the vasoconstriction occurring at this time (Fig. 1). However, the BK response remained reduced 24 h post-LPS3, at which time CVR had recovered to the baseline level (Fig. 1). Subsequently, BK response was abolished for at least 5 days after the last LPS dose (Fig. 4). As circulatory parameters, TNF-α and body temperature had all recovered to baseline 24 h after each LPS exposure, prolonged reduction of BK responses signified the presence of an underlying, prolonged abnormality of cerebral endothelium. The mechanism underlying the prolonged endothelial dysfunction is likely to involve frank loss of endothelial cells as well as cellular injury arising from LPS exposure. The endothelium is vulnerable to prolonged injury, with as many as 25% of endothelial cells lost from the aorta for at least 5 days after a single dose of LPS (22). Clinically, the identification of persisting cerebral vascular dysfunction may require endothelial markers, such as von Willebrand factor (24), to complement circulatory assessment.

Brain Injury

We found evidence of brain injury and extensive infiltration of active macrophages in the periventricular white matter and the caudate nucleus in the LPS-treated newborn brains. Similar brain lesions occurred in the endotoxin-exposed fetal lamb (9) although there was no apparent deficit in cerebral oxygenation. In the present study, there was a period of hypoperfusion although increased fractional oxygen extraction is likely to have preserved cerebral oxygenation and cerebral metabolic rate. Thus, in the short term, no cerebral hypoxia was likely after LPS. Therefore, endotoxin-induced brain injury may occur independently without cerebral hypoperfusion or hypoxemia in the newborn, as noted for the earlier gestation fetus (6). However, there is a sensitization of the brain to hypoxic-ischemic injury that is immediate (7) and persisting (39) that underlines the accentuated risk of LPS associated with hypoxia. Thus the short period of hypoperfusion may have contributed to the brain injury we observed in these lambs.

Perspectives and Significance

The newborn lamb model we used corresponds in brain maturity to a mature infant or young child, since human brain development at term corresponds to 0.9 gestation in the fetal lamb (14). It is unlikely that the extent of CBF decrement that we produced in this model is itself damaging to the brain, since reductions of that order (~30%) are compensated by oxygen extraction increases that sustain cerebral oxidative metabolism (12). Nevertheless, although LPS caused no CBF change in neonatal rats, it dramatically increased the vulnerability of the brain to injury by subsequent hypoxic-ischemic insults (7). On the basis that fundamental mechanisms of protection and injury are likely to be similar at all ages, our study suggests that change of endothelial function and associated loss of protective cerebrocircular responses may underpin this exaggerated risk of hypoxic-ischemic injury that is the legacy of LPS exposure in early life. Speculatively, neonatal infection, first by initiating cerebral vasoconstriction via upregulating cytokine-induced vasoconstrictor pathways and subsequently by damaging the cerebral endothelium and impairing endothelium-dependent vasoregulatory pathways, places the brain at prolonged risk of CBF dysregulation and potentially of hypoperfusion and neuronal damage.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


