Inflammation in utero exacerbates ventilation-induced brain injury in preterm lambs

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Inflammation in utero exacerbates ventilation-induced brain injury in preterm lambs. J Appl Physiol 112: 481–489, 2012. First published November 3, 2011; doi:10.1152/japplphysiol.00995.2011.—Cerebral blood flow disturbance is a major contributor to brain injury in the preterm infant. The initiation of ventilation may be a critical time for cerebral hemodynamic disturbance leading to brain injury in preterm infants, particularly if they are exposed to inflammation in utero. We aimed to determine whether exposure to inflammation in utero alters cardio-pulmonary hemodynamics, resulting in cerebral hemodynamic disturbance and related brain injury during the initiation of ventilation. Furthermore, we aimed to determine whether inflammation in utero alters the cerebral hemodynamic response to challenge induced by high mean airway pressures. Pregnant ewes received intra-amniotic lipopolysaccharide (LPS) or saline either 2 or 4-days before preterm delivery (at 128 ± 1 days of gestation). Lambs were surgically instrumented for assessment of pulmonary and cerebral hemodynamics before delivery and positive pressure ventilation. After 30 min, lambs were challenged hemodynamically by incrementing and decrementing positive end-expiratory pressure. Blood gases, arterial pressures, and blood flows were recorded. The brain was collected for biochemical and histological assessment of inflammation, brain damage, vascular extravasation, hemorrhage, and oxidative injury. Carotid arterial pressure was higher and carotid blood flow was more variable in 2-day LPS lambs than in controls during the initial 15 min of ventilation. All lambs responded similarly to the hemodynamic challenge. Both 2- and 4-day LPS lambs had increased brain interleukin (IL)-1β, IL-6, and IL-8 mRNA expression; increased number of inflammatory cells in the white matter; increased incidence and severity of brain damage; and vascular extravasation relative to controls. Microvascular hemorrhage was increased in 2-day LPS lambs compared with controls. Cerebral oxidative injury was not different between groups. Antenatal inflammation causes adverse cerebral hemodynamics and increases the incidence and severity of brain injury in ventilated preterm lambs.

cerebral injury; periventricular leukomalacia; preterm birth; chorioamnionitis; intraventricular hemorrhage

NEONATAL BRAIN INJURY, PARTICULARLY within the white matter, and both peri- and intraventricular hemorrhage (IVH) are major complications of very preterm births (<28 wk gestation) that are associated with significant rates of mortality and long-term morbidity (46). The incidence and severity of such injury increases with decreasing gestational age and birth weight (13, 28). Whereas immaturity is the primary factor that renders the preterm infant’s brain vulnerable to injury, it is likely that several mechanisms are involved. The principal etiological factors associated with neonatal preterm brain injury are infection/inflammation (both ante- and postnatal) and disrupted cerebral blood flow due to impaired cerebral auto-regulation (9, 26, 29). Resuscitation, mechanical ventilation, rapid volume expansion, and a patent ductus arteriosus are associated with disrupted cerebral hemodynamics in preterm infants (9). The observation that 50% of IVH occurs in the first hours of life, with <5% of significant bleeds occurring after 4-days (47), highlights the immediate neonatal transition of the preterm infant as a particularly vulnerable time for the development of brain injury.

Exposure to inflammation/infection in utero is causally related to preterm birth (39) and increases the risk and severity of neonatal cardiopulmonary disease (42, 43). It also independently increases the incidence and severity of IVH and periventricular leukomalacia in very preterm infants (12). Inflammation in utero is a major risk factor for long-term neurological outcomes such as intellectual impairment and cerebral palsy (14, 52). Previous studies by our group and others have shown that chronic fetal exposure to inflammation per se causes brain inflammation and evidence of brain injury (3, 10, 11, 32, 40, 44). However, the postnatal physiological consequences of exposure to inflammation in utero are incompletely understood but may confer vulnerability to further injury caused by subsequent preterm ventilation. Since chorioamnionitis is also associated with cardiovascular and systemic hemodynamic disturbances in preterm infants <3 h after birth (51), it is possible that preterm infants exposed to inflammation in utero are particularly vulnerable to brain injury in the immediate neonatal period.

Mechanical ventilation adversely affects cardiopulmonary and systemic hemodynamics in preterm lambs, an effect that is further exacerbated by the presence of intrauterine inflammation (35, 36). Furthermore, it is clear that many cardiopulmonary factors are closely associated with cerebral pathology in immature newborns, including resuscitation, mechanical ventilation, rapid volume expansion, and patent ductus arteriosus (8). We aimed to determine whether exposure to inflammation in utero alters cardiopulmonary hemodynamics and results in cerebral hemodynamic disturbance and related brain injury during the initiation of ventilation. Furthermore, we aimed to determine whether a hemodynamic challenge, caused by high

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mean airway pressures, has deleterious effects on cerebral hemodynamics after inflammation in utero. We hypothesized that inflammation in utero would cause adverse cardiopulmonary and cerebral hemodynamics and increase the incidence and severity of brain injury resultant from the initiation of ventilation and experimental hemodynamic challenge.

**MATERIALS AND METHODS**

The experimental protocol was approved by The University of Western Australia, Monash University, and The University of Sydney animal ethics committees.

**Antenatal and postnatal animal care.** Singleton ewes were randomly assigned to receive an ultrasound-guided intra-amniotic injection of lipopolysaccharide (LPS; *E. coli* 055:B5 10 mg; Sigma Aldrich) either 2 (*n* = 7) or 4-days (*n* = 7) before instrumentation and preterm delivery of the lambs at 128 (1) days of gestation (term is ∼150 days). Controls (total *n* = 5) received intra-amniotic injection of saline (2 ml) either 2-days (*n* = 2) or 4-days (*n* = 3) before delivery (total *n* = 5). Intra-amniotic LPS injection elicits a profound inflammatory response in the fetus, which peaks 2-days after injection and results in lung airway and vascular remodeling after 4-days (23, 24, 27).

**Instrumentation.** Surgery was performed at 128 days under general anesthesia (2% isoflurane in oxygen, Bovac Animal Health) for insertion of polyvinyl catheters into the fetal right carotid artery and the main pulmonary artery. Ultrasonic flow transducers (3 or 4 mm; Transonic Systems, Ithaca, NY) were placed around the left main pulmonary artery and left carotid artery as described previously (36, 37). Left main pulmonary arterial blood flow (PBF), carotid arterial blood flow (CBF), and pulmonary (*P*<sub>PA</sub>) and carotid arterial pressures (*P*<sub>CA</sub>) were recorded digitally (1 kHz; Powerlab, ADInstruments, Castle Hill, Australia). The fetal chest was closed, the fetal trachea was intubated orally, and lung liquid was drained passively.

**Preterm delivery and ventilation.** Lambs were delivered, dried, weighed, and placed on positive-pressure ventilation using a rate of 60 breaths/min, a peak inspiratory pressure (PIP) of 30 cmH<sub>2</sub>O, and a positive end-expiratory pressure (PEEP) of 4 cmH<sub>2</sub>O (Babylog 8000+, Dräger, Lübeck, Germany) for 30 min to allow stabilization of pulmonary hemodynamics. PIP was adjusted intermittently to maintain a tidal volume (VT) of ∼7 ml/kg body wt and arterial *P*<sub>CO2</sub> (*P*<sub>CO2</sub>) of 50–60 mmHg; moderate hypercapnia was allowed to reduce barotrauma from the high pressures required to lower *P*<sub>ACO2</sub> in lambs at this developmental stage. The upper limit for PIP was 45 cmH<sub>2</sub>O. Inspired oxygen fraction (*F*<sub>O2</sub>) was adjusted to maintain arterial oxygen saturation (Sao<sub>2</sub>) between 90 and 95%. The equations for calculated variables are listed in the online data supplement (available at the Journal of Applied Physiology website).

**Hemodynamic challenge and ventilation maintenance.** At 30 min, all lambs underwent a hemodynamic challenge consisting of 2-cmH<sub>2</sub>O stepwise increments in PEEP every 10 min to 10 cmH<sub>2</sub>O and subsequent decrements back to baseline (36). We have previously shown that this strategy reduces pulmonary blood flow and left ventricular output, especially in lambs exposed to inflammation in utero (35).

Lambs were anesthetized with a continuous umbilical venous infusion of remifentanil (0.05 μg·kg<sup>−1</sup>·min<sup>−1</sup>; Abbott Laboratories) and propofol (0.1 mg·kg<sup>−1</sup>·min<sup>−1</sup>; Norbrook Laboratories, Victoria, Australia). Well being was monitored by regular blood gas analysis (Rapidlab 865, Bayer Diagnostics) and postductal transcutaneous oxyhemoglobin saturation (*SpO2*; Nellcor Oximax N65, Tyco Healthcare, Australia). Left ventricular output and ductus arteriosus flow direction were assessed using pulsed Doppler ultrasound (36). At the conclusion of the ventilatory protocol, lambs were humanely killed and weighed, and the lungs and brain were collected.

**Calculations.** Specific dynamic compliance (*C*<sub>dyn,spec</sub>) was calculated as VT/kg/(PIP – PEEP). Ventilatory efficiency index (VEI) was determined as 3,800/(ΔP·f·*P*<sub>ACO2</sub>), where 3,800 is a constant for production of carbon dioxide (ml·cmH<sub>2</sub>O·kg<sup>−1</sup>·min<sup>−1</sup>), ΔP = PIP – PEEP, and f is the respiratory frequency (18). Arterial oxygenation was described using the alveolar-arterial difference in oxygen (A–*A*<sub>DO2</sub>) (38). Oxygenation index was calculated as (*F*<sub>O2</sub> × mean airway pressure × 100)/arterial *P*<sub>O2</sub>.

**Lung processing.** Bronchoalveolar lavage was performed on the left lung for measurement of total protein concentration (30) and differential cell counts (19). Sections of the right lower lobe were frozen in liquid nitrogen for subsequent measurement of interleukin (IL)-1β, IL-6, and IL-8 mRNA expression using quantitative RT-PCR (24). 

**Brain processing.** The brain was removed, and the cerebellum was divided along the vermis. Samples taken from the right hemisphere were frozen in liquid nitrogen for subsequent measurement of IL-1β, IL-6, and IL-8 mRNA expression. The left hemisphere was sectioned into ∼4-mm slices and fixed in 4% paraformaldehyde for morphometric analyses. Serial sections (5 μm) at the level of the lateral ventricle (Fig. 1) were stained with hematoxylin and eosin for assessment of brain damage and Masson’s trichrome for vascular injury. Immunohistochemistry was used to assess the presence of inflammatory cells using peroxidase-labeled lectin (1:200, Sigma), lipid peroxidation [4-hydroxynonenal (4-HNE), 1:1,000, Calbiochem], and blood-brain barrier integrity (serum albumin, 1:1,000, Accurate Chemical & Scientific). Lectin-positive cells were counted in four random non-overlapping high-powered fields in the subcortical and periventricular white matter and expressed as lectin-positive cells/mm<sup>2</sup> (ImageJ, NIH image, Bethesda, MD). Albumin and 4-HNE analysis was conducted using six random high-powered fields per section, using two sections per lamb, and was analyzed (DP2-BSW, Olympus) from the external capsule and deep-, periventricular-, or
indexes of injury. Statistical significance was accepted for method. ANOVA was used to compare biochemical and histological above. Post hoc comparisons were performed using the Holm-Sidak min were separated from the remaining data for separate analyses as
with repeated measures (Sigmastat, version 3.0, SPSS). The first 15
Serial data were compared between groups using two-way ANOVA
Paw, cmH2O 20.3
PaO2 41.2
C
dyn,spec 0.17 ± 0.02
FlO2 0.97 ± 0.04
A-aDO2 539.2 ± 22.4
Values are means of all measurements ± SE over the 90-min ventilation protocol. Vt, tidal volume; MV, minute ventilation; PIP, peak inspiratory pressure; Paw, mean airway pressure; VEI, ventilator efficiency index; C
dyn,spec, dynamic specific compliance; PaO2, arterial PO2; A-aDO2, alveolar-arterial difference in
oxygen. *Significant difference between 4-day LPS and both 2-day LPS and controls (P < 0.05).

RESULTS

Umbilical arterial blood gas and acid/base status at birth were normal for all lambs and not different between groups (data not shown). Fetal body weights were not different between groups (control: 3.4 ± 0.3 kg; 2-day LPS: 3.1 ± 0.1 kg; 4-day LPS: 3.1 ± 0.5 kg).

Ventilation and oxygenation. Vt, minute ventilation, PIP, and mean airway pressure were not different between control, 2-day LPS, or 4-day LPS lambs throughout the ventilation period (Table 1). PIP decreased in all groups after the hemodynamic challenge (P < 0.001).

Paco2 was not different between groups and did not change during the ventilation period (Table 1). Paco2 was significantly lower in 4-day LPS lambs than controls at 50 and 60 min but variability prevented detection of statistical significance at other time points (Fig. 2A). FlO2, OI and A-aDO2 were lower (i.e., oxygenation was better) in 4-day LPS lambs compared with 2-day LPS and controls (Table 1 and Fig. 2).

C
dyn,spec was not different between groups (Table 1). VEI was higher in 4-day LPS lambs compared with 2-day LPS lambs and controls (Fig. 2D).

Table 1. Ventilation and oxygenation

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>2-Day LPS (n = 7)</th>
<th>4-Day LPS (n = 7)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vt, ml/kg</td>
<td>5.7 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>6.8 ± 0.3</td>
<td>0.328</td>
</tr>
<tr>
<td>MV, 1·min⁻¹·kg⁻¹</td>
<td>0.22 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.343</td>
</tr>
<tr>
<td>PIP, cmH2O</td>
<td>42.1 ± 3.5</td>
<td>39.4 ± 2.6</td>
<td>33.1 ± 2.6</td>
<td>0.252</td>
</tr>
<tr>
<td>Paw, cmH2O</td>
<td>20.3 ± 2.6</td>
<td>20.9 ± 2.1</td>
<td>16.7 ± 2.2</td>
<td>0.272</td>
</tr>
</tbody>
</table>
| C
dyn,spec        | 0.17 ± 0.02    | 0.14 ± 0.02       | 0.28 ± 0.05      | 0.199   |
| PaO2             | 41.2 ± 6.3     | 35.9 ± 8.2        | 50.8 ± 13.2      | 0.242   |
| FlO2             | 0.97 ± 0.04    | 0.96 ± 0.05       | 0.67 ± 0.03*     | 0.025   |
| A-aDO2           | 539.2 ± 22.4   | 523.5 ± 35.4      | 362.1 ± 23.1*    | 0.035   |

Subcortical white matter. Each field was scored by a blinded assessor (A. A. Babaramani) and designated a qualitative score: 0 = none, 1 = mild (<50% of the field of view occupied by positive staining), 2 = severe (>50% of the field of view occupied by positive staining).

Statistical analysis. Data are presented as means ± SE. Group sizes were chosen based on power calculations using an α value of 0.05 and desired power of >0.8 to detect differences in pulmonary and carotid blood flow assuming variation (SD of ~5 ml·kg⁻¹·min⁻¹) and large mean differences (~8 ml·kg⁻¹·min⁻¹) similar to our previous studies. Serial data were compared between groups using two-way ANOVA with repeated measures (Sigmastat, version 3.0, SPSS). The first 15
min were separated from the remaining data for separate analyses as above. Post hoc comparisons were performed using the Holm-Sidak method. ANOVA was used to compare biochemical and histological indexes of injury. Statistical significance was accepted for P < 0.05.

Fig. 2. Oxygenation and ventilation efficiency index. Arterial Pco2 (Paco2; A), arterial oxygen saturation (SaO2; B), oxygenation index (OI; C), and ventilator efficiency index (VEI; D) in control (●), 2-day LPS (○), and 4-day LPS (shaded triangles) lambs during the ventilation procedure. Shaded region indicates level of positive end-expiratory pressure (PEEP) during the hemodynamic challenge. Data are means ± SE. *Significant difference between 4-day LPS vs. controls and 2-day LPS. #Significant difference between controls and 2-day LPS. ¥ Significant difference between 2-day LPS and both 2-day LPS and controls (P < 0.05).
Cardiopulmonary-cerebral hemodynamics. Pulmonary blood flow (PBF) was not different between groups during the initial 15 min or during the hemodynamic challenge (Fig. 3A). PBF decreased similarly in all groups during the hemodynamic challenge (P < 0.001). There was a trend for higher CBF in 2-day LPS lambs compared with controls and 4-day LPS lambs during the initial 15 min (Fig. 3B). The coefficient of variation of carotid blood flow was significantly higher in 2-day LPS lambs compared with controls (37 ± 4%) during the initial 15 min (P = 0.017; data not shown). Pulmonary arterial pressure was higher in 2-day LPS lambs compared with controls and 2-day LPS lambs during the hemodynamic challenge (P = 0.031; Fig. 3C). Carotid arterial pressure was significantly higher and more variable in 2-day and 4-day LPS lambs compared with controls during the first 15 min but was lower in 4-day LPS lambs compared with controls and 2-day LPS lambs during the hemodynamic challenge (P = 0.031; Fig. 3C). Carotid arterial pressure was significantly higher and more variable in 2-day and 4-day LPS lambs compared with controls during the first 15 min (Fig. 3D). However, carotid arterial pressure was significantly lower in 4-day LPS lambs during the hemodynamic challenge. Left ventricular output was not different between groups at any stage but was significantly decreased in all groups during the hemodynamic challenge (P < 0.001; data not shown). The ratio of left-to-right to right-to-left shunt through the ductus arteriosus after 70 min was significantly lower in 2-day LPS lambs (0.43 ± 0.13) compared with controls (0.7 ± 0.02) and 4-day LPS lambs (0.76 ± 0.16; P = 0.042), indicating a greater proportion of right-to-left shunt across the ductus arteriosus (data not shown).

Lung inflammation. The presence of intrauterine inflammation was confirmed visually by thickened and edematous fetal membranes and/or umbilical cord in all LPS lambs; these indicators were absent in controls. The total number of inflammatory cells in the bronchoalveolar lavage fluid was significantly higher in 2-day LPS lambs (mean over 15 min: 48 ± 2%) compared with controls (37 ± 4%) during the initial 15 min (P = 0.017; data not shown). Pulmonary arterial pressure was higher in 2-day LPS lambs compared with controls and 2-day LPS lambs during the hemodynamic challenge (P = 0.031; Fig. 3C). Carotid arterial pressure was significantly higher and more variable in 2-day and 4-day LPS lambs compared with controls during the first 15 min but was lower in 4-day LPS lambs compared with controls and 2-day LPS lambs during the hemodynamic challenge (P = 0.031; Fig. 3C). Carotid arterial pressure was significantly higher and more variable in 2-day and 4-day LPS lambs compared with controls during the first 15 min (Fig. 3D). However, carotid arterial pressure was significantly lower in 4-day LPS lambs during the hemodynamic challenge. Left ventricular output was not different between groups at any stage but was significantly decreased in all groups during the hemodynamic challenge (P < 0.001; data not shown). The ratio of left-to-right to right-to-left shunt through the ductus arteriosus after 70 min was significantly lower in 2-day LPS lambs (0.43 ± 0.13) compared with controls (0.7 ± 0.02) and 4-day LPS lambs (0.76 ± 0.16; P = 0.042), indicating a greater proportion of right-to-left shunt across the ductus arteriosus (data not shown).

In all groups, the greatest increase of lectin-positive cells was observed in the subcortical white matter (Fig. 5, A–C), with negligible numbers of inflammatory cells observed in other assessed regions of the brain. Within the subcortical white matter, 2-day LPS lambs had significantly more inflammatory cells per tissue area compared with 4-day LPS lambs and control lambs (P = 0.04 and P = 0.037, respectively; Table 2).

Brain damage and hemorrhage. The incidence and severity of brain damage were higher in 4-day LPS lambs, particularly in the subcortical white matter and external capsule, compared with 2-day LPS and controls (Table 2; Fig. 5, D–I). The incidence and severity of brain damage in the deep white matter was higher in both 2-day and 4-day LPS lambs compared with controls (Table 2). The incidence of hemorrhage in the periventricular white matter, evidenced by extravasation of red blood cells, was significantly higher in 2-day LPS lambs compared with controls and 4-day LPS lambs (Table 2). More lambs in the 4-day LPS group showed evidence of extravasation of serum albumin than controls and 2-day LPS lambs (Table 2; Fig. 5, J–L),
indicative of increased propensity of vascular leakage. The proportion of blood vessels with fragmented adventitia, indicative of damage to the surrounding support structure of blood vessels, was significantly increased in 4-day LPS lambs compared with 2-day LPS and control lambs ($P < 0.05$; Table 2). The incidence and severity of lipid peroxidation (4-HNE positive cells), indicative of oxidative injury, was not different between groups (Table 2; Fig. 5, M–O). Importantly, both the cell body and white matter tracts stained positively for 4-HNE (Fig. 5, N and O).

**DISCUSSION**

We have shown that inflammation in utero results in instability in cerebral hemodynamics and brain injury after the initiation of ventilation and an experimental-induced hemodynamic challenge. The initiation of ventilation in controls caused instability in cerebral blood flow and carotid arterial pressure, particularly during the initial 15 min of ventilation, with evidence of mild pathological brain injury present in the white matter. Antenatal inflammation amplified cerebral hemodynamic instability and exacerbated brain inflammation and injury in the periventricular and subcortical white matter. Furthermore, an increased incidence of microvessel leakage and bleeding was also observed in lambs exposed to inflammation in utero.

Exposure to inflammation in utero increases the risk and severity of neonatal cardiopulmonary disease (42, 43) and brain injury in very preterm infants (12). The fetal pulmonary response to inflammation induced by LPS has been well characterized (23, 24, 27). Intra-amniotic LPS results in an acute inflammatory response in the lung, indicated by increased inflammatory cytokines and white blood cell recruitment that peaks 1–2 days after exposure. In this study, pro-inflammatory cytokine mRNA expression and vascular protein leakage in the lungs were not different between groups. Four days after intra-amniotic LPS administration, there are structural effects evident in the lung, including thinning of the interstitial tissue, increased surfactant pool size, and the presence of fewer, larger alveoli (20, 33, 48). Consequently, we observed short-term improvements in respiratory indexes and reduced requirement for ventilatory support after a 4-day exposure to LPS compared with control and 2-day LPS lambs (Table 1; Fig. 2). The lack of difference between groups in lung pro-inflammatory cytokines and vascular leakage is likely due to the increased ventilatory requirements of control and 2-day LPS lambs, resulting in ventilator-induced lung injury, thus masking the antenatal effects. However, the total number of inflammatory cells in the bronchoalveolar lavage fluid was increased 2 and 4 days after LPS compared with controls, consistent with an intrauterine pulmonary inflammatory response to antenatal inflammation (24).

In this study, we demonstrated an upregulation of inflammatory cytokines and white matter injury after intra-amniotic LPS, suggesting that ventilation after intrauterine inflammation exacerbated inflammation in the brain above that observed in controls. Furthermore, we showed an increase in the number of lectin-positive stained cells in 2-day LPS lambs within the subcortical white matter, similar to that of previous findings (3, 10, 11, 32, 40, 44) and mirroring the pattern of inflammatory cytokine mRNA expression. Lectin-positive staining is indicative of both infiltrating macrophages and activated microglia, which are present in the white matter during development (25) and are associated with maturation of myelination (16). Although these can be classified by morphology (17), only the presence of positive staining was assessed in this study. Intrauterine inflammation is known to be one of the principal initiating pathogenic factors in periventricular leukomalacia (26). Previous studies demonstrated cytokine-mediated white matter injury in the premature brain, with increases in TNF-α and IL-1β (21, 25). Similarly, gross anatomical pathology, seen as parenchymal abnormalities in white matter analogous
to periventricular leukomalacia, was increased in lambs that had been exposed to LPS. We showed previously that mechanical ventilation of preterm lambs initiates a systemic inflammatory cascade, which is enhanced by exposure to intratracheal LPS (34). Thus the local immune-mediated cytokine response in the brain that we observed is likely exacerbated by the systemic inflammatory cascade initiated by injurious ventilation, resulting in increased incidence of PVL. Our findings suggest that acutely inflamed lambs are more vulnerable to an increased cerebral inflammatory response resulting from non-protective ventilation, perhaps supporting the increased risk for adverse neurodevelopmental outcomes in babies born after premature rupture of membranes where infection/inflammation likely occurred close to delivery (6). Regardless, our findings provide strong evidence for the requirement of gentle resuscitation/ventilation strategies immediately after delivery for preterm infants, particularly if chorioamnionitis is evident.

All lambs were subjected to a hemodynamic challenge (a ramp increase and decrease in PEEP), which we showed previously to have adverse consequences on PBF and left ventricular output, further exacerbated by intra-amniotic LPS 7 days before delivery (35). However, PBF and left ventricular output were not different between groups at any stage of this study. These different responses are therefore a consequence of different durations of exposure to LPS, reflecting increased resistance arteriole medial thickening and adventitial fibrosis observed at 7 days (23), which would increase vessel stiffness and pulmonary vascular resistance greater than that observed in this study. Conversely, the more advanced structural lung airway maturation evident 7 days after LPS may impede PBF at high PEEP than in an atelectatic lung due to the direct compression of pulmonary capillaries.

Inflammation may initiate downstream mechanisms that can contribute to the pathogenesis of periventricular leukomalacia, such as generation of reactive oxygen species, which can contribute to the pathogenesis of periventricular leukomalacia. We measured 4-HNE, a marker of lipid peroxidation and oxidative stress, and found no difference between groups. We demonstrated previously that inflammation did not alter cerebral oxygen delivery or extraction in these lambs, although a trend emerged for lower cerebral oxygen delivery in 4-day LPS lambs (1). Given the increase in periventricular leukomalacia-type injury in LPS lambs, it is possible that the postnatal assessment of oxidative stress, after ventilation, may have confounded its assessment. Indeed, we showed that protein carbonyl, a marker of systemic oxidative stress, was increased in plasma 7 days after intra-amniotic LPS in nonventilated preterm lambs (5). This finding suggests that LPS-exposed lambs have more oxidative injury than controls. Oxidative injury from reactive oxygen species may result in death of pre-oligodendrocytes (15), but this was not addressed as we considered that the short timeframe of our study would not allow such an effect to manifest.

An association between resuscitation, mechanical ventilation, the presence of cerebral hemodynamic disturbance, and later brain pathology has been postulated (9). More than half of IVH occurs in the first hours of life (47), suggesting that resuscitation and the initiation of ventilator support may precipitate cerebral injury in preterm neonates. Cerebral hemodynamics was significantly altered during the initial 15 min after

### Table 2. Assessment of brain injury

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5), %</th>
<th>2-Day LPS (n = 7), %</th>
<th>4-Day LPS (n = 7), %</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Mild</td>
<td>Severe</td>
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<tr>
<td>Gross anatomical injury</td>
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<tr>
<td>Periventricular white matter</td>
<td>60</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Subcortical white matter</td>
<td>20</td>
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<td>20</td>
</tr>
<tr>
<td>White matter</td>
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<td>0</td>
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<tr>
<td>External capsule</td>
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<tr>
<td>Lipid Peroxidation</td>
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<td>Periventricular white matter</td>
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<td>60</td>
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<td>Subcortical white matter</td>
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<td>40</td>
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<tr>
<td>Vascular protein extravasation</td>
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<td>PVWM and SCXWM</td>
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<tr>
<td>Vascular RBC extravasation</td>
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<tr>
<td>Periventricular white matter</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Infiltrating inflammatory cells</td>
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<td></td>
</tr>
<tr>
<td>Periventricular white matter, cells/mm²</td>
<td>137 ± 29</td>
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<tr>
<td>Subcortical white matter, cells/mm²</td>
<td>218 ± 64</td>
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<td>Fragmented adventitia</td>
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<td>Periventricular white matter, %</td>
<td>34.1 ± 7.6</td>
<td></td>
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</table>

Values are means ± SE. Incidence and severity of gross anatomical brain injury and infiltrating inflammatory cells are expressed as % of the lambs displaying evidence of mild or severe injury. RBC, red blood cell. †Significant difference between 4-day LPS and both 2-day LPS and controls (P < 0.05). *Significant difference between 2-day LPS and both 4-day LPS and controls (P < 0.05).
delivery in LPS-exposed lambs. There was large variability in 
CBF, particularly in 2-day LPS lambs, as indicated by the 
increased coefficient of variation. This suggests a reduced 
ability to maintain near-constant cerebral blood flow during the 
initiation of ventilation. Cerebral autoregulation is impaired by 
20–50% during the first 5 postnatal days in preterm infants <1,500 g (41). Impaired cerebral autoregulation resulting in 
cerebral blood flow instability is one of the most significant 
contributors to perterm brain injury, particularly in the occur-
rence of IVH (26). We observed increased red blood cell 
evastasation in 2-day LPS lambs and increased albumin ex-
vasion in 2- and 4-day LPS lambs compared with 
controls. Albumin is the most abundant plasma protein and is 
not found outside of cerebral blood vessels unless the blood-
brain barrier has been compromised. Given that brain histology 
changes after LPS are related to vascular damage (2), it is 
likely that compromise of the blood-brain barrier occurs fol-
lowing LPS in fetal sheep (17, 50). However, it is not clear 
from our study whether the increased extravasation resulted 
from LPS-induced damage to cerebral blood vessels per se, the 
large CBF fluctuations, or both. Whatever the mechanism, our 
study support the contention that inflammation in utero in-
creases the risk and severity of hemorrhage in the preterm 
brain.

Interestingly, 4-day LPS lambs had significantly lower ven-
tilatory pressures and improved oxygenation than the other 
groups, yet brain inflammation and injury were increased 
compared with controls. The histological changes in the brain 
caused by ventilation were different between the LPS groups, 
with increased brain damage in 4-day LPS lambs, and greater 
cerebral hemorrhage was apparent in 2-day LPS lambs. Thus 
the timing of exposure to inflammation in utero before preterm 
delivery and ventilation determines the type and severity of 
brain injury. However, we observed significant hypercapnia in 
control and 2-day LPS lambs as a consequence of adherence to the 
PIP limitation imposed by our experimental protocol. It is 
therefore possible that increasing the pressure and volume in 
control and 2-day LPS lambs to mitigate the hypercapnia may 
have resulted in similar brain damage to 4-day LPS lambs. 
Carbon dioxide is a potent vasodilator that increases cerebral 
blood flow. Clinically, hypercapnia increases the risk of grade 
III and IV IVH in very low birth weight preterm infants (22, 
45), thus the increased incidence of cerebral hemorrhage ob-
served in 2-day LPS lambs may have resulted from hypercap-
nia. Interestingly, controls had similar levels of hypercapnia as 
2-day LPS lambs, which suggests that inflammation in utero in-
creases the susceptibility of hemorrhage related to hy-
percapnia. The experimental constraints, particularly the PIP 
imposed, and that of the preterm lamb model prevented normal-
ization of $PaCO_2$, without causing significant lung injury and 
plethorax. Further studies are required to adequately 
address the correlation between hypercapnia and brain injury in 
the presence of inflammation.

We did not administer antenatal corticosteroids, which are 
used routinely in human obstetric practice for women at risk of 
preterm birth. Antenatal glucocorticoids may reduce brain hemorrhage 
and brain damage in this study in LPS and control groups, but 
this association warrants further investigation. We did not 
administer prophylactic surfactant to the lambs since it does not 
alter cardiopulmonary hemodynamics in response to ven-
tilation in preterm lambs (7). However, the reduced require-
ment for intensive ventilatory support (lower PIP and mean 
airway pressures) may reduce cardiopulmonary and cerebral 
blood flow instability during the initial 15 min and, therefore, 
be brain protective. The relationship between prophylactic 
surfactant administration, chorioamnionitis, and preterm brain 
innjury is not known and requires further investigation.

In conclusion, inflammation in utero increases cerebral 
blood flow instability during the initiation of ventilation and 
increases the incidence and severity of microvessel bleeding 
and brain damage in the white matter. Investigation of early 
resuscitation strategies aimed at protecting the cardiopulmo-
nary-cerebral circulation may help reduce the incidence of 
hemodynamic-related brain injury in neonates born after ex-
posure to intrauterine inflammation.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

conception and design of research; G.R.P., I.N., A.A.B., K.J.C., M.K.S., 
J.J.P., S.B.H., and M.K. interpreted results of experiments; G.R.P. and A.A.B. 
prepared figures; G.R.P. and M.K.S. drafted the manuscript; G.R.P., I.N., 
revised the manuscript; G.R.P., I.N., A.A.B., K.J.C., M.K.S., A.W.G., B.J.A., 
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