Prostaglandins mediate the fetal pulmonary response to intrauterine inflammation

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Westover AJ, Hooper SB, Wallace MJ, Moss TJM. Prostaglandins mediate the fetal pulmonary response to intrauterine inflammation. Am J Physiol Lung Cell Mol Physiol 302: L664–L678, 2012. First published January 27, 2012; doi:10.1152/ajplung.00297.2011.—Intra-amniotic (IA) lipopolysaccharide (LPS) induces intrauterine and fetal lung inflammation and increases lung surfactant and compliance in preterm sheep; however, the mechanisms are unknown. Prostaglandins (PGs) are inflammatory mediators, and PGE2 has established roles in fetal lung surfactant production. The aim of our first study was to determine PGE2 concentrations in response to IA LPS and pulmonary gene expression for PG synthetic (prostaglandin H synthase-2 (PGHS-2) and PGE synthase (PGES)) and PG-metabolizing [prostaglandin dehydrogenase (PGDH)] enzymes and PGE2 receptors. Our second study aimed to block LPS-induced increases in PGE2 with a PGHS-2 inhibitor (nimesulide) and determine lung inflammation and surfactant protein mRNA expression. Pregnant ewes received an IA saline or LPS injection at 118 days of gestation. In study 1, fetal plasma and amniotic fluid were sampled before and at 2, 4, 6, 12, and 24 h after injection and then daily, and fetuses were delivered 2 or 7 days later. Amniotic fluid PGE2 concentrations increased (P < 0.05) 12 h and 3–6 days after LPS. Fetal lung PGHS-2 mRNA and PGES mRNA increased 2 (P = 0.0084) and 7 (P = 0.014) days after LPS, respectively. In study 2, maternal intravenous nimesulide or vehicle infusion began immediately before LPS or saline injection and continued until delivery 2 days later. Nimesulide inhibited LPS-induced increases in PGE2 and decreased fetal lung IL-1β and IL-8 mRNA (P ≤ 0.002) without altering lung inflammation cell infiltration. Nimesulide decreased surfactant protein (SP)-A (P = 0.05), -B (P = 0.05), and -D (P = 0.0015) but increased SP-C mRNA (P = 0.023). Thus PGHS-2 mediates, at least in part, fetal pulmonary responses to inflammation.

preterm lung; surfactant; chorioamnionitis; respiratory distress syndrome; nimesulide

INTRAUTERINE INFLAMMATION is a major contributing factor to many preterm deliveries (16). Preterm birth is typically associated with neonatal lung immaturity and increased risk of respiratory distress syndrome (RDS). However, epidemiological studies (2, 4) show that intrauterine inflammation decreases the risk of RDS. In sheep, intrauterine inflammation induces structural changes in the fetal lung and a profound increase in pulmonary surfactant, resulting in a marked improvement in neonatal lung function in preterm lambs (21, 38, 40). These results demonstrate the likely biological mechanism for protection against RDS in preterm infants after exposure to inflammation in utero.

The underlying mechanisms by which inflammation induces preterm lung “maturatio n” are not known. It has been suggested that inflammation may induce lung maturation by eliciting a fetal stress response, resulting in increased cortisol levels, known to have a critical role in lung maturation (30). However, it appears that inflammation-induced increases in surfactant in the preterm lungs occur via a mechanism independent of glucocorticoid signaling. This is because the characteristics of the fetal lung response to inflammation are different from those of glucocorticoid-induced lung maturation in preterm lambs (20) and the small increase in fetal plasma cortisol induced by intra-amniotic LPS injection is unlikely to be sufficient to induce lung maturation (42). Prostaglandins are critical mediators of inflammation, and some [particularly prostaglandin E2 (PGE2)] are known to increase surfactant production by the preterm fetal lung (1, 36). Prostaglandins are produced from arachidonic acid by isoforms of the enzyme prostaglandin H synthase (PGHS). PGHS-1 is constitutively expressed in most cells of the body and serves a homeostatic role, whereas PGHS-2 is an inducible form of the enzyme responsible for production of prostaglandins in response to physiologic stresses such as inflammation (41, 53). In gestational tissues and the fetus, a major PGHS-2 metabolite is PGE2, which is produced by PGE synthase (PGES). This enzyme is functionally coupled to PGHS-2 (PGES). This enzyme is functionally coupled to PGHS-2 and is induced by inflammation. PGE2 signals via membrane-bound G-protein-coupled prostaglandin receptors that have been classified into four subtypes, EP1–4 (46).

PGE receptors and the principal prostaglandin metabolizing enzyme prostaglandin dehydrogenase (PGDH) are all present in the fetal lung and may mediate normal fetal lung development (10, 36, 46). Furthermore, in human fetal lung explants dilation of terminal air sacs and differentiation of the pulmonary epithelium are accelerated by addition of prostaglandins and inhibited by indomethacin, a nonspecific PGHS inhibitor (19). In vivo, administration of indomethacin to pregnant sheep inhibits fetal pulmonary surfactant production, indicating a critical role for PGs in lung development (8, 28).

We aimed to determine if intrauterine inflammation induced by intra-amniotic LPS injection in pregnant sheep (1) increases PGE2 concentrations in amniotic fluid, fetal lung liquid, and the fetal circulation; and 2) alters mRNA levels of prostaglandin synthetic and metabolic enzymes and prostaglandin receptors in the fetal lungs. In a separate experiment, we aimed to determine if a preferential PGHS-2 inhibitor, nimesulide, could 1) inhibit the inflammation-induced increases in PGE2 concentrations, 2) alter the in-
flammatory response, and 3) alter the fetal lung response to intra-amniotic LPS injection. We hypothesized that exposure of preterm fetal sheep to intra-amniotic LPS would increase PGE\(_2\) levels and alter gene expression of PGHS-2, PGES, PGDH, and the EP receptor subtypes in the preterm fetal lungs, indicating pulmonary PGs may mediate the fetal lung response to intrauterine inflammation. Furthermore, we hypothesized that nimesulide infusion would inhibit LPS-induced PGE\(_2\) production, modulate the fetal pulmonary inflammatory response, and reduce the effect of inflammation on fetal lung development.

**METHODS**

**Animal Experimentation**

The relevant Monash University Animal Ethics Committee approved all experimental procedures. All pregnant ewes underwent aseptic surgery at 112 days of gestation (term is \(\sim 147\) days) to have catheters inserted into the fetal trachea, a jugular vein and carotid artery, the amniotic cavity, and a maternal jugular vein.

**Study 1.** On day 118 of gestation, ewes were randomly assigned to receive saline (4 ml; \(n = 12\)) or 20 mg LPS (Escherichia coli 055:B5; Sigma) solubilized in 4 ml saline (\(n = 11\)) into the amniotic cavity via the indwelling catheter. Fetal lung liquid (3 ml), amniotic fluid (3 ml), and arterial blood (3.5 ml) samples were collected immediately before and at 2, 4, 6, 12, 24, and 48 h after saline (\(n = 6\)) or LPS (\(n = 5\)) administration in one (2-day) cohort of sheep, and immediately before and at 2, 4, 6, 12, and 24 h and then daily until 7 days after intra-amniotic injection in another (7-day) cohort (saline, \(n = 6\); LPS \(n = 6\)). Three milliliters of each arterial blood sample were centrifuged at 3,000 rpm for 10 min to isolate plasma; the remaining 0.5 ml were used for measurement of blood gases, electrolytes, and metabolites (Radiometer ABL 500 blood-gas analyzer; Radiometer, Copenhagen, Denmark). Fetal plasma, lung liquid, and amniotic fluid samples were mixed with indomethacin (20 \(\mu\)l) and immediately diluted 1:1 with 0.12 M methyloxyamine hydrochloride (Sigma) in sodium acetate buffer (1 M, pH 5.6) containing 10% ethanol for subsequent PGE\(_2\) measurement. Samples were left overnight at room temperature and then stored at \(-20^\circ\)C until analysis.

At 120 days (2-day cohort) or 125 days (7-day cohort), the ewe and fetus were humanely killed (pentobarbitone, 5 g; injected into the maternal jugular catheter). The fetus was delivered and weighed, and the chest was opened. An endotracheal tube was tied into the fetal trachea, and lung compliance was assessed by inflation with air to 40 cmH\(_2\)O pressure and sequentially lowering the pressure to 20, 10, 5, and 0 cmH\(_2\)O while the corresponding lung volumes were recorded. The lungs were removed from the chest; the left lung was chopped into pieces and were frozen in liquid nitrogen; and the right lung was fixed in paraformaldehyde.

**Study 2.** On day 118 of gestation, ewes were randomly assigned to one of two cohorts. The first received a continuous maternal intravenous infusion of nimesulide (20 mg·kg\(^{-1}\)·day\(^{-1}\)) and a single injection of either saline (4 ml; \(n = 6\)) or LPS (20 mg solubilized in 4 ml saline; \(n = 6\)) into the amniotic sac. In the other cohort, ewes received a control continuous maternal intravenous infusion of the vehicle solution (90% polyethylene glycol 400, 10% ethanol; 1 ml/h) and a single

### Table 1. Oligonucleotide primer sequences

<table>
<thead>
<tr>
<th>Gene Name and Genbank Accession Number</th>
<th>Sequences (5‘-3’)</th>
<th>Quantitative RT-PCR cDNA Concentration, ng/μl</th>
<th>Annealing Temperature, °C</th>
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</thead>
<tbody>
<tr>
<td>18S (X01117)</td>
<td>F: GTC TGT GAT GCC CTT AGA TGT C  R: AAC CTT ATG ACC CGC ACT TAC</td>
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<td>EP(_1) (AF035415)</td>
<td>F: AGT CGT TCG CAC ACT CTG  R: CCC AAG TGC TCC TCG TGG TTA G</td>
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<td>EP(_2) (AJ876410)</td>
<td>F: GCT GGA TCA CTT GAA GTA TGC  R: CTG TCG TTG TCT CCG ATG</td>
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<td>60</td>
</tr>
<tr>
<td>EP(_3) (AF035417)</td>
<td>F: CCG TGT CGT ATG ATG TCG  R: CTG TGT ATG TCT TGC AGT GCT C</td>
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<td>59</td>
</tr>
<tr>
<td>EP(_4) (AF167580)</td>
<td>F: CGA TCG TGG TGG TCT GTA AG  R: CCG CAT ACC AGT GTC TAG AAA G</td>
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<td>59</td>
</tr>
<tr>
<td>IL-1β (NM_001004965)</td>
<td>F: CGA TGA GCT TCT GTG GTA TG  R: CTT TGA GAG GAG GTG GAG AG</td>
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<td>60</td>
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<tr>
<td>IL-6 (NM_001009392)</td>
<td>F: CCA AAG GGT TAT CAT CAT CC  R: CCC AGG AAC TAC CAC AAT CA</td>
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<td>59</td>
</tr>
<tr>
<td>IL-8 (NM_001009401)</td>
<td>F: CCT CAG TAA AGA TGG CAA TGA  R: TGA CAA CCC TAC ACC AGA CC</td>
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<td>59</td>
</tr>
<tr>
<td>PGDH (NM_001034419)</td>
<td>F: GCA CAG CAC CTT GTT TAT TG  R: CAT GAG ATT AGC ACC CAT CG</td>
<td>1,000</td>
<td>60</td>
</tr>
<tr>
<td>PGES (NM_174443)</td>
<td>F: AOT GAG GCC GCA GAA  R: GCA CAT CGT GCT GCT TCC</td>
<td>1,000</td>
<td>60</td>
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<tr>
<td>PGHS-2 (NM_001009432)</td>
<td>F: GGT CCG TGG TGG TGG GAA  R: CTC TGT GCT GTG TGG AAG TGA A</td>
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<tr>
<td>SP-A (AF211856)</td>
<td>F: CAT CAA GTC TCG CAG TCA GA  R: GCC CAT TGG TAG AGA AGA CC</td>
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<td>60</td>
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<tr>
<td>SP-B (AF035417)</td>
<td>F: GTC CTC TGC TGG AGC TCA AGA TG  R: GCC GAG GTC TGG TCT TGT AG</td>
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<td>SP-C (AF076634)</td>
<td>F: GTC AAG ATC AAA CCC CTT TCC  R: TGT GAA GAC CAC TGA GCA</td>
<td>1,000</td>
<td>60</td>
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</table>

EP, prostaglandin E receptor; PGDH, prostaglandin dehydrogenase; PGES, prostaglandin E synthase; PGHS, prostaglandin H synthase; SP, surfactant protein; F, forward primer; R, reverse primer. *PCR for 18S rRNA was performed using the same primer and cDNA concentrations as the gene of interest.
injection of either saline (n = 6) or LPS (n = 5), as above, into the amniotic sac. Nimesulide \([N-(4-\text{nitro-2-phenoxypenyl})-\text{methanesulfonylamine}]\) is a nonsteroidal anti-inflammatory drug that preferentially inhibits PGHS-2 relative to PGHS-1 activity to the order of 733 in sheep (22) and 70 in human enzymes (5). Indeed, nimesulide is particularly selective in sheep and has a lower IC50 for ovine (0.03 at \(100 \mu\text{M}\) arachidonic acid; Ref. 22) than human PGHS-2 (1.27 at \(100 \mu\text{M}\) arachidonic acid; Ref. 5). A continuous infusion of nimesulide (20 mg·kg\(^{-1}\)·day\(^{-1}\)) to laboring ewes is known to greatly reduce fetal PGE\(_2\) levels without adverse fetal effects (17) that occur with inhibition of constitutively generated prostaglandins using nonspecific PGHS inhibitors (43). Nimesulide or vehicle infusions commenced 1 h before intra-amniotic LPS or saline injection.

Amniotic fluid (3 ml) and arterial blood (3.5 ml) samples were collected immediately before and at 2, 4, 6, 12, 24, and 48 h after saline or LPS administration. Samples were processed as in study 1 for measurement of blood gases, electrolytes, and PGE\(_2\) concentrations. At 120 days, the ewe and fetus were humanely killed for the same assessments described in study 1.

### PGE\(_2\) Radioimmunoassay

PGE\(_2\) concentrations were measured in amniotic fluid and plasma samples by radioimmunoassay (7, 13). Assay mixtures consisted of samples (0.2 ml) or standard PGE\(_2\) concentrations dissolved in 0.2 ml of charcoal-stripped plasma. All standards and samples were assayed in duplicate after incubation at 4°C overnight with \(^{3}\text{H}-\text{PGE}_2\) tracer (~5,000 cpm; 0.1 ml/tube) and antiserum raised in sheep against the methyl oxime of PGE\(_2\). The cross-reactivities of the antiserum at 50% displacement were 4.9%

### Table 2. Study 1: Fetal blood gases

<table>
<thead>
<tr>
<th>Fetal Arterial Blood Measurement/Cohort/Treatment</th>
<th>pH</th>
<th>(\text{PaCO}_2), mmHg</th>
<th>(\text{PO}_2), mmHg</th>
<th>tHB, g/dl</th>
<th>Glucose, mmol/l</th>
<th>Lactate, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 day</td>
<td>Saline</td>
<td>7.36 ± 0.004</td>
<td>45.66 ± 0.72</td>
<td>10.33 ± 0.10</td>
<td>1.30 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>7.37 ± 0.004</td>
<td>47.48 ± 0.33</td>
<td>10.44 ± 0.16</td>
<td>1.74 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>7 day</td>
<td>Saline</td>
<td>7.36 ± 0.002</td>
<td>51.92 ± 0.49</td>
<td>11.06 ± 0.06</td>
<td>1.41 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>7.37 ± 0.002</td>
<td>50.51 ± 0.30</td>
<td>10.52 ± 0.22</td>
<td>1.43 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE across all time points. tHB, hemoglobin.

Fig. 1. Study 1: effect of intra-amniotic LPS on expression of mRNA for proinflammatory cytokines in the fetal lung. Levels of interleukin (IL)-1\(\beta\) (A), IL-6 (B), and IL-8 (C) mRNA, as determined by quantitative RT-PCR, were highest in fetal lungs from the 2-day LPS group. Data are expressed as means ± SE. *\(P < 0.05\) vs. saline controls in the same cohort.
with PGE₂, 0.47% with 15-keto-PGE₂, 0.15% with 13,14-dihydro-15-keto-PGE₂, 0.11% with PGA₂, and <0.006% with PGD₂, PGF₂α, PGE₂, 6-keto-PGFα, and thromboxane B₂. Intra-amniotic LPS resulted in an increase in the viscosity of lung liquid, rendering volume measurement inaccurate at early time points and thus preventing measurement of PGE₂ concentrations in fetal lung liquid at these times. By 7 days after intra-amniotic LPS, lung liquid viscosity had normalized, allowing a single measurement at this time. In study 1, the sensitivities of the amniotic fluid, lung liquid, and plasma assays were 5.4, 3.2, and 4.4 ng/ml, respectively. In study 2, sensitivities of the amniotic fluid and fetal plasma assays were 1.3 and 1.5 ng/ml, respectively.

**Luong Morphology**

Study 1. Secondary septal crests project out from the primary saccule walls into the lung lumen to form alveoli and can be identified by elastin deposition (12, 14). The relative volume density of secondary septal crests is a relative measure of alveolar number per unit volume of lung. Blocks of lung tissue from the right upper, middle, and lower lung lobes were embedded in paraffin, cut into 5-μm sections, and mounted onto glass slides. Two sections from each of the right lung lobes from each fetus were stained with Hart’s resorcin-fuchsin stain for elastin, incubated for 4 h, and counterstained with tartrazine (0.25%) in saturated picric acid to identify secondary septal crests. Five nonoverlapping fields from each section were captured at ×40 magnification, and a linear point-counting grid (19 × 26) was superimposed to determine relative volume density of secondary septal crests for each lobe; this was expressed relative to the tissue volume. Similarly, a point-counting technique was used to determine the ratio of tissue to air space volumes for each lung lobe. Lung lobes were analyzed separately to identify potential regional differences; none were identified so data were combined to provide a whole lung value. Lung morphometric analysis was not performed for study 2.

**Immunohistochemistry**

Immunostaining of CD45 (a protein tyrosine phosphatase expressed on the surface of leukocytes; Ref. 52) was performed to assess inflammation. Lung and chorioamnion tissue sections were mounted as above, paraffin was removed using xylene, and sections were rehydrated in graded alcohol. Antigen retrieval was performed by incubation with 0.01 M sodium citrate (pH 6) in a microwave for 20 min at 900 W, and endogenous peroxidase was blocked by incubation with 3% H₂O₂ in distilled water for 20 min. The slides were incubated in 10% normal horse serum for 30 min, and sections were counterstained with hematoxylin. Images from five nonoverlapping fields from each section (2 sections from each of the right lung lobes and 2 sections of chorioamnion per fetus) were viewed under a light microscope and captured at ×40 magnification using a digital camera (Nikon Eclipse 80i; Coherent Scientific). Quantification of CD45 immunohistochemistry was performed using the Image-Pro Plus (Media Cybernetics) software program. For each field of view, the number of CD45-positive cells was counted. Mean values for each tissue specimen were calculated. Lung lobes were analyzed separately, but data were combined to provide a single measure for each subject.

**Real-time PCR**

Total RNA was extracted from frozen portions of the left lung lobe with the RNeasy Maxi kit (75162; Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed on 1 µg of RNA with the Superscript III First Strand synthesis system for real-time PCR kit as specified by the manufacturer (18080–051; Invitrogen).

Quantitative RT-PCR was used to measure gene expression as described by Sozo et al. (49) under optimized primer-specific conditions (Table 1).

**Study 1.** The messenger RNA levels for each fetus were normalized to the 18S rRNA values for that fetus and are expressed relative to the mean mRNA levels for that gene in the saline control fetuses.

**Study 2.** When all samples would not fit onto one PCR plate, amplification of the gene of interest and 18S was performed on a calibration cDNA sample in quintuplicate in each assay to permit comparisons between all groups. The messenger RNA levels for each fetus were normalized to the 18S rRNA values for that fetus and are expressed relative to the mean mRNA levels in vehicle + saline fetuses.

**Statistical Analysis**

Results are presented as means ± SE. Analysis of blood gases and electrolytes, radioimmunoassay data, and lung pressure-volume curves was by two-way repeated measures ANOVA with post hoc comparisons performed using Fisher’s least significance difference (LSD) test. Area under the curve was calculated using the trapezoidal rule (GraphPad Software). Body and organ weights were compared between control and LPS groups by Student’s t-test.

**Study 1.** Analysis of lung morphology was by two-way ANOVA with post hoc comparisons made using Fisher’s LSD test. Comparisons of mRNA levels and day 7 lung liquid PGE₂ concentrations between control and LPS groups were by Student’s t-test. Statistical comparisons between 2-day and 7-day cohorts were not performed.

**Study 2.** Comparisons of mRNA levels between treatment groups and infusion cohorts were by two-way ANOVA with post hoc comparisons performed using the Fisher LSD test.

**RESULTS**

**Study 1**

**Fetal blood gases and organ weights.** Intra-amniotic LPS did not affect fetal pH, PaCO₂, PaO₂, hemoglobin, or fetal blood electrolytes, radioimmunoassay data, and lung pressure-volume curves was by two-way repeated measures ANOVA with post hoc comparisons performed using Fisher’s least significance difference (LSD) test. Area under the curve was calculated using the trapezoidal rule (GraphPad Software). Body and organ weights were compared between control and LPS groups by Student’s t-test. Statistical comparisons between 2-day and 7-day cohorts were not performed.

<table>
<thead>
<tr>
<th>Tissue/ Cohort/Treatment</th>
<th>No. CD45 Cells/Field of View</th>
<th>P Value (Compared with Corresponding Saline Group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorioamnion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 day</td>
<td>Saline</td>
<td>2.30 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>13.87 ± 1.48</td>
</tr>
<tr>
<td>7 day</td>
<td>Saline</td>
<td>0.53 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>10.17 ± 2.19</td>
</tr>
<tr>
<td>Lung</td>
<td>Saline</td>
<td>2.71 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>13.95 ± 0.89</td>
</tr>
</tbody>
</table>

Table 3. Study 1: CD45 expression in the chorioamnion and fetal lung

Source: AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00297.2011 • www.ajplung.org
glucose concentrations (Table 2). Fetal blood lactate concentrations were significantly elevated 12 h after LPS administration (LPS: 2.24 ± 0.27 mmol/l; saline: 1.35 ± 0.076 mmol/l; \( P = 0.007 \)) but returned to control levels by 24 h. There were no statistically significant differences in fetal body weights or ratios in lung, heart, liver, spleen, thymus, or kidney-to-body weight ratios between groups in either cohort. Brain-to-body weight ratios were significantly lower (\( P = 0.013 \)) after intra-amniotic LPS in the 7-day cohort but were not different between groups in the 2-day cohort.

Fig. 2. Study 1: effect of intra-amniotic LPS on preterm lung compliance and surfactant protein mRNA levels. A: deflation limbs of pressure-volume curves show greater lung gas volumes at 7 days but not 2 days after LPS administration. B: there was increased expression of surfactant protein (SP)-A mRNA in the fetal lungs at 2 and 7 days after LPS. C: SP-B mRNA tended to be elevated 2 days after LPS but was not significantly different between groups in either cohort. D: expression of SP-C was elevated at 2 and 7 days after LPS. E: SP-D mRNA was elevated 2 days after LPS. Data are expressed as means ± SE. Data for each cohort are expressed relative to the mean value for the saline group. \( *P < 0.05 \) vs. saline controls in the same cohort.
Inflammation in the lung and chorioamnion. Fetal lung interleukin (IL)-1β mRNA levels were significantly higher 2 days (Fig. 1A; \(P = 0.0092\)) and 7 days (Fig. 1A; \(P = 0.045\)) after LPS, compared with saline groups. IL-6 (Fig. 1B) and IL-8 (Fig. 1C) mRNA levels in fetal lung tissue tended to be higher in LPS groups, but values were not significantly different from saline groups.

The number of CD45-positive cells per field of view in the chorioamnion and the fetal lungs was higher in the 2-day and 7-day LPS groups compared with corresponding saline groups (Table 3; \(P < 0.02\)).

Fig. 3. Study 1: effect of intra-amniotic LPS on lung morphometry. Secondary septal crest densities were not different between saline and LPS groups in the 2-day cohort. However, there were significantly more septal crests in the lungs 7 days after LPS than in the respective saline group, and a significantly greater septal crest density in lungs from the 7-day cohort than the 2-day cohort (A). Tissue-to-air space ratio was not different between groups in the 2-day cohort and increased with increasing gestational age in both 7-day groups (B). However, LPS significantly reduced the normal gestational-age related increase. Data are expressed as means ± SE. *\(P < 0.05\) vs. saline.

Fig. 4. Study 1: effect of intra-amniotic LPS on expression of prostaglandin synthesizing and metabolizing enzymes in the fetal lungs. Relative abundance of prostaglandin H synthase type-2 (PGHS-2; A) mRNA was higher than control 2 days after LPS and prostaglandin E synthase (PGES; B) mRNA was elevated 7 days after LPS administration. C: expression of prostaglandin dehydrogenase (PGDH) mRNA in the fetal lung was not different between groups. Data for each cohort (means ± SE) are expressed relative to the mean value for the saline group. *\(P < 0.05\) vs. saline controls in the same cohort.
Lung compliance and surfactant protein mRNA. Deflation limbs of pressure-volume curves showed greater lung gas volumes in the LPS group than saline group in the 7-day cohort ($P < 0.002$), but no difference between LPS and saline groups 2 days after intra-amniotic injection (Fig. 2A). Surfactant protein (SP)-A (Fig. 2B; 2-day cohort: $P = 0.028$, 7-day cohort: $P = 0.033$) and SP-C (Fig. 2D; 2-day cohort: $P = 0.034$, 7-day cohort: $P = 0.021$) mRNA levels were higher in 2-day and 7-day LPS groups than controls. SP-B (Fig. 2C) mRNA levels tended to be higher 2 days after LPS ($P = 0.058$) but were not significantly different between groups in either cohort. SP-D (Fig. 2E; $P = 0.0004$) mRNA levels were higher 2 days, but not 7 days, after LPS than in the corresponding saline group.

Lung morphometry. The relative volume density of secondary septal crests in the fetal lungs was significantly higher in the 7-day LPS group than in the 7-day saline group (Fig. 3A; $P = 0.027$). Values were not different between groups in the 2-day cohort, but there were significantly more septal crests in fetal lungs from the 7-day cohort than the 2-day cohort (Fig. 3A; $P < 0.0001$). The tissue-to-air space volume ratio was not different between saline and LPS groups in the 2-day cohort (Fig. 3B). The tissue-to-air space ratio was significantly lower after LPS in the 7-day cohort (Fig. 3B; $P < 0.0001$) and was greater in the 7-day cohort than the 2-day cohort (Fig. 3B; $P < 0.0001$).

Prostaglandin-metabolizing enzymes. The relative abundance of PGHS-2 mRNA in the lungs was significantly higher in the LPS group than in the saline group for the 2-day cohort (Fig. 4A; $P = 0.0084$). PGES gene expression in the lung was significantly higher in the 7-day LPS group than the 7-day saline group (Fig. 4B; $P = 0.014$). No significant differences in PGDH expression were observed between LPS and saline groups in either cohort (Fig. 4C).

PGE receptor subtypes. Expression of mRNA for the PGE receptors EP$_{1-3}$ (Figs. 5, A–C) in the fetal lung was not significantly different between LPS and saline groups in either the 2-day or 7-day cohort. However, EP$_4$ receptor mRNA expression in the fetal lung was significantly higher in the 2-day LPS group than the corresponding saline group (Fig. 5D; $P = 0.011$); EP$_4$ mRNA levels tended to be higher 7 days after LPS, although expression was not significantly different between groups ($P = 0.11$).

PGE$_2$ concentrations. Mean amniotic fluid PGE$_2$ concentrations increased slightly but not significantly within the first 24 h after LPS but were significantly higher in the 7-day LPS...
group than in the saline group at 2–6 days after intra-amniotic injection (Fig. 6A; \( P < 0.05 \)). The cumulative PGE\(_2\) response (area under the curve) was significantly higher (\( P = 0.039 \)) in the 7-day LPS group (3,726 ± 854 nM/168 h) than in the 7-day saline group (1,170 ± 476 nM/168 h).

Fetal plasma PGE\(_2\) concentrations tended to be higher in 7-day LPS-exposed fetuses, compared with the 7-day saline group, from 12 h to 2 days after intra-amniotic injection but values were not significantly different between groups at any of the time points (Fig. 6B). Area under the curve was not significantly different between 7-day saline (160 ± 59 nM/168 h) and LPS groups (361 ± 151 nM/168 h).

The PGE\(_2\) concentration in fetal lung fluid 7 days after intra-amniotic LPS (3.03 ± 0.43 nM) was higher than in the 7-day saline group (1.58 ± 0.85 nM), but values were not significantly different (\( P = 0.081 \)).

Study 2

**Fetal blood gases and organ weights.** Intra-amniotic LPS or maternal nimesulide infusion did not affect fetal pH, PaCO\(_2\), or fetal blood hemoglobin or glucose concentrations (Table 4). In the vehicle cohort, fetal blood lactate concentrations were significantly elevated at 6 h (LPS: 2.40 ± 0.49 mmol/l; saline: 1.08 ± 0.11 mmol/l; \( P = 0.021 \)) and 12 h (LPS: 1.93 ± 0.24; saline: 1.07 ± 0.033 mmol/l; \( P = 0.023 \)) after LPS administration. There were no statistically significant treatment or infusion effects on fetal body weights or on lung, heart, liver, spleen, or kidney-to-body weight ratios; however, nimesulide caused a significant increase (\( P = 0.045 \)) in thymus-to-body weight ratios.

**Inflammation in the lung and chorioamnion.** Fetal lung IL-1\( \beta \) (\( P = 0.002 \); Fig. 7A) and IL-8 (\( P = 0.0017 \); Fig. 7C) mRNA levels were significantly lower in the nimesulide cohort, for both saline and LPS groups, compared with the vehicle cohort. IL-6 (Fig. 7B) mRNA levels in fetal lung tissue were not significantly different between saline and LPS groups in either vehicle or nimesulide cohorts, or between cohorts.

The number of CD45-positive cells per field of view was significantly higher (\( P = 0.0008 \)) in the chorioamnion after LPS in the vehicle cohort but not after maternal nimesulide

### Table 4. Study 2: Fetal blood gases

<table>
<thead>
<tr>
<th>Fetal Arterial Measurement/Cohort/Treatment</th>
<th>Vehicle</th>
<th>Saline</th>
<th>LPS</th>
<th>Nimesulide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>7.37 ± 0.003</td>
<td>7.34 ± 0.01</td>
<td>7.31 ± 0.007</td>
<td>7.38 ± 0.005</td>
</tr>
<tr>
<td><strong>PaO(_2), mmHg</strong></td>
<td>41.43 ± 1.34</td>
<td>42.23 ± 0.84</td>
<td>45.91 ± 1.06</td>
<td>46.24 ± 1.00</td>
</tr>
<tr>
<td><strong>tHb, g/dl</strong></td>
<td>10.34 ± 0.18</td>
<td>11.92 ± 0.30</td>
<td>10.80 ± 0.28</td>
<td>10.01 ± 0.22</td>
</tr>
<tr>
<td><strong>Glucose, mmol/l</strong></td>
<td>0.77 ± 0.03</td>
<td>0.90 ± 0.04</td>
<td>0.88 ± 0.04</td>
<td>0.76 ± 0.04</td>
</tr>
<tr>
<td><strong>Lactate, mmol/l</strong></td>
<td>1.06 ± 0.03</td>
<td>1.88 ± 0.90</td>
<td>1.61 ± 0.11</td>
<td>2.10 ± 0.31</td>
</tr>
</tbody>
</table>

Values are means ± SE across all time points.
infusion (Table 5). In the fetal lungs, LPS significantly increased \(P < 0.0001\) the number of CD45-positive cells per field of view in both vehicle and nimesulide cohorts (Table 5).

**Surfactant protein mRNA.** Intra-amniotic LPS significantly increased SP-A (Fig. 8A; \(P = 0.013\)) and SP-B (Fig. 8B; \(P = 0.031\)) mRNA levels in the vehicle and nimesulide cohorts, but there was a strong trend for levels to be lower after nimesulide infusion than vehicle (\(P = 0.05\) for SP-A and SP-B). SP-C (Fig. 8C) and SP-D (Fig. 8D) mRNA levels were not different between saline and LPS groups in either cohort. However, nimesulide significantly increased SP-C (\(P = 0.023\)) gene expression but caused a profound reduction in SP-D mRNA expression (\(P = 0.0015\)) compared with vehicle.

**Prostaglandin-metabolizing enzymes.** PGHS-2 mRNA levels were similar in LPS and saline groups in the nimesulide cohort but were 2.4-fold higher in the LPS group compared with saline in the vehicle cohort, although this difference was not statistically significant (Fig. 9A). PGES gene expression in the lung was significantly higher (\(P = 0.049\)) after LPS in both the vehicle and nimesulide cohorts but was not significantly different between cohorts (Fig. 9B). PGDH mRNA levels were not different between LPS and saline groups in either cohort, but nimesulide significantly reduced (\(P = 0.008\)) PGDH mRNA levels compared with vehicle (Fig. 9C).

**PGE receptor subtypes.** Expression of mRNA for the PGE receptors EP1-4 in the fetal lung was not significantly different between LPS and saline groups in either the vehicle or nimesulide cohort (Fig. 10, A-D). However, nimesulide significantly reduced mRNA levels of EP1 (\(P = 0.002\)), EP2 (\(P = 0.014\)), and EP3 (\(P = 0.005\)), but not EP4, compared with vehicle.

**PGE2 concentrations.** In the vehicle cohort, mean amniotic fluid PGE2 concentrations were significantly higher at 24 (\(P = 0.023\)) and 48 (\(P = 0.013\)) h after intra-amniotic LPS, compared with the saline group (Fig. 11A). Amniotic fluid PGE2 concentrations at 24 h (\(P = 0.018\)) and 48 h (\(P = 0.0002\)) after LPS were significantly lower in the nimesulide cohort than in the vehicle cohort. The area under the curve was not significantly different between groups (vehicle + saline: 122 ± 20 nM/48 h; vehicle + LPS: 386 ± 116 nM/48 h; nimesulide + saline: 166 ± 41 nM/48 h; nimesulide + LPS: 201 ± 85 nM/48 h).

### Table 5. Study 2: CD45 expression in the chorioamnion and the fetal lung

<table>
<thead>
<tr>
<th>Tissue/Infusion/Treatment</th>
<th>No. CD45 Cells/Field of View</th>
<th>(P) Value (Infusion)</th>
<th>(P) Value (Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chorioamnion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle Saline</td>
<td>1.15 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>9.94 ± 2.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nimesulide Saline</td>
<td>3.57 ± 1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>4.95 ± 1.28</td>
<td>0.33</td>
<td>0.0008</td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle Saline</td>
<td>1.54 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>6.4 ± 2.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nimesulide Saline</td>
<td>3.21 ± 1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>6.98 ± 0.58</td>
<td>0.35</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 7. Study 2: effect of nimesulide infusion on inflammation-induced expression of mRNA for proinflammatory cytokines in the fetal lungs. Levels of IL-1β (A), IL-6 (B), and IL-8 (C) mRNA in the fetal lungs were not different between saline and LPS groups. However, there was a significant decrease in IL-1β and IL-8 gene expression in the nimesulide cohort compared with the vehicle cohort. Data are expressed as means ± SE. **\(P < 0.01\).**
Fetal plasma PGE$_2$ concentrations were not significantly different between saline and LPS groups in either cohort; however, nimesulide significantly reduced fetal plasma PGE$_2$ concentrations from vehicle levels at 4 ($P = 0.044$), 6 ($P = 0.017$), and 12 ($P = 0.048$) h after LPS (Fig. 11 B). Intra-amniotic LPS did not significantly alter the area under the curve in either cohort, but nimesulide significantly reduced ($P = 0.024$) the area under the curve compared with levels in the vehicle groups (vehicle + saline: 51 ± 17 nM/48 h; vehicle + LPS: 163 ± 66 nM/48 h; nimesulide + saline: 5 ± 5 nM/48 h; nimesulide + LPS: 1 ± 1 nM/48 h).

**DISCUSSION**

Fetal plasma PGE$_2$ concentrations were not significantly different between saline and LPS groups in either cohort; however, nimesulide significantly reduced fetal plasma PGE$_2$ concentrations from vehicle levels at 4 ($P = 0.044$), 6 ($P = 0.017$), and 12 ($P = 0.048$) h after LPS (Fig. 11 B). Intra-amniotic LPS did not significantly alter the area under the curve in either cohort, but nimesulide significantly reduced ($P = 0.024$) the area under the curve compared with levels in the vehicle groups (vehicle + saline: 51 ± 17 nM/48 h; vehicle + LPS: 163 ± 66 nM/48 h; nimesulide + saline: 5 ± 5 nM/48 h; nimesulide + LPS: 1 ± 1 nM/48 h).

***Fig. 8.*** **Study 2:** effect of nimesulide infusion on inflammation-induced expression of surfactant proteins in the fetal lungs. LPS increased expression of SP-A (A) and SP-B (B) mRNA in the fetal lungs, but mRNA levels were lower in the nimesulide groups ($P = 0.05$). Nimesulide infusion significantly increased SP-C (C) mRNA levels in fetal lungs and significantly reduced SP-D (D) gene expression from responses in the vehicle cohort. Data are expressed as means ± SE. Data are expressed relative to the mean value for the vehicle + saline group. *$P < 0.05$; **$P < 0.001$.

Similarly, the changes we observed in pressure-volume curves and surfactant protein mRNA in *study 1* reflect the functional “maturation” of the lungs demonstrated in these previous studies (26, 29), which is consistent with a reduced risk of respiratory distress syndrome in preterm infants born after exposure to inflammation in utero (2, 31).

Disparate studies have shown that prostaglandins have a role in regulating pulmonary surfactant synthesis and secretion. Human fetal lung epithelial cells spontaneously synthesize SP-A in vitro, and its mRNA levels can be decreased by incubation with the PGHS inhibitor indomethacin and increased by PGE$_2$ (1). Surfactant lipid secretion in newborn rabbit lung slices is also inhibited by indomethacin and stimulated by PGE$_2$ (34). In vivo, administration of indomethacin to pregnant rabbits (8) or sheep (28) appears to inhibit fetal pulmonary surfactant production, as indicated by decreased saturated phosphatidylcholine concentrations. We aimed to inhibit LPS-induced PGE$_2$ production to elucidate the role of prostaglandins in the fetal lung’s response to inflammation. The increase in PGE$_2$ concentrations in the amniotic fluid 48 h after LPS was inhibited by nimesulide, and circulating PGE$_2$ concentrations were reduced to low levels 4--12 h after LPS injection and remained lower than vehicle responses for the duration of the maternal nimesulide infusion.
The 2-day experimental timeline of study 2 allowed us to investigate events when intra-amniotic LPS-induced increases in PGE₂ concentrations first occur in the amniotic fluid and when the inflammatory response to intra-amniotic LPS is greatest (29). In study 2, intra-amniotic LPS did not induce an increase in IL-1β, IL-6, or IL-8 mRNA levels in the fetal lungs, in contrast to the increase in IL-1β and IL-8 mRNA levels 2 days after LPS in study 1. It is possible that an increase in basal levels of IL-1β and IL-8 mRNA levels in the vehicle cohort masked an LPS-induced increase in cytokine expression because PEG-400 has been observed to stimulate cytokine expression (47). Regardless, nimesulide infusion abolished IL-1β gene expression and significantly reduced IL-8 mRNA levels compared with vehicle-infused animals. This is consistent with studies (32) in nimesulide-treated human macrophages, which demonstrated attenuated cytokine expression in response to influenza virus. Our observed decrease in IL-1β and IL-8 mRNA levels in nimesulide-treated saline and LPS groups indicates a reduction in the basal inflammatory state of the fetal lungs when PGHS-2 is inhibited. We observed equivalent pulmonary inflammatory cell recruitment after LPS exposure in vehicle and nimesulide groups, despite different levels of mRNA for the chemoattractant cytokine IL-8. This is consistent with previous studies (24) in sheep demonstrating that fetal pulmonary inflammatory cell recruitment is not IL-8 dependent. Additionally, lung inflammatory cell recruitment is elevated in adult PGHS-2 knockout mice (relative to wild type) in response to allergen exposure (9, 15), demonstrating a role for PGHS-2 in pulmonary inflammatory cell recruitment. There are likely differences between pharmacological PGHS-2 inhibition in fetal sheep and gene deletion in adult mice, but our data suggest that inflammatory cell recruitment in the lungs of fetal sheep is not mediated by PGHS-2, suggesting differences between fetal and adult animals in this respect.

Intra-amniotic LPS injection increased PGHS-2 mRNA 3-fold in fetal lung tissue after 2 days, which returned near to control levels by 7 days after treatment. This time course is consistent with the acute inflammatory response of the fetal sheep lungs to intra-amniotic injection of LPS (23) and the established role of PGHS-2 in inflammation (11, 56). Our observation confirms previous data from fetal sheep showing a similar time course of elevation in pulmonary PGHS-2 mRNA levels within the first 3 days after intra-amniotic LPS (39). In that study, the levels of PGHS-2 protein were significantly greater than control 5 h after intra-amniotic LPS and tended to be higher 24 h after treatment before returning to baseline levels 3 days after endotoxin exposure (39). We did not measure PGHS-2 protein levels because of this previously demonstrated discordance between mRNA and protein levels. PGHS-2 undergoes rapid protein degradation as a consequence of suicide inactivation (48, 55); thus, protein levels may not accurately reflect enzyme activity and local tissue prostaglandin production. The increased PGE₂ synthetic capacity of fetal lung tissue is indicated by elevated PGHS-2 mRNA levels and is supported by our demonstrated increase in PGES mRNA levels 7 days after intra-amniotic injection of LPS. Fetal lung prostaglandin metabolism is unlikely to be altered by inflammation since PGDH mRNA levels were not changed by intra-amniotic LPS. We did not attempt to measure lung tissue PGE₂ levels because such measurements cannot be simply performed in tissue homogenates. Tissue disruption in-and-of-itself would...
liberate significant amounts of prostaglandins, and their lability in vivo means that concentrations measured in such an assay would unlikely reflect actual fetal lung levels. Rather than performing tissue analyses to investigate the role of prostaglandins in the fetal lung response to inflammation, we aimed to inhibit prostaglandin production in vivo and assess effects on fetal lung inflammation and development. Although nimesulide selectively inhibits PGHS-2 relative to PGHS-1, and this selectivity is greater in sheep than humans (5, 22), there are effects of the drug not mediated by PGHS inhibition (6). Thus the effects in our experiment cannot be solely attributed to PGHS-2 inhibition; non-PGHS-mediated effects may therefore account for at least some of the differences between vehicle and nimesulide cohorts.

Nimesulide tended to reduce LPS-induced increases in PGHS-2 and PGES gene expression, but this effect was not statistically significant. Gene expression of PGDH was significantly lower after maternal nimesulide infusion, consistent with observations in neonatal rat lungs in response to indomethacin (50), indicating that pulmonary prostaglandin catabolism is not affected by LPS, but is dependent on PGHS activity.

Of the four prostaglandin receptor subtypes, only mRNA for EP4 increased in response to LPS, consistent with previous studies of EP4 expression in response to LPS (in murine fibroblast and macrophage-like cells lines; Ref. 3) and PGE2 (in rat bone marrow in vivo; Ref. 54). This is interesting, given that PGE2 stimulates SP-A mRNA expression in human fetal lung tissue in vitro in association with cAMP formation (1), a consequence of EP2 and EP4 activation. Nimesulide infusion reduced mRNA levels of EP1–3 but not EP4. The effect on EP1 expression is interesting given its reported role in mediating PGE2-induced surfactant production by rat alveolar epithelial cells in vitro (36).

Nimesulide infusion resulted in a strong trend for a reduction in SP-A and SP-B expression and a significant reduction in SP-D mRNA levels, and LPS-induced increases in expression of these SPs were also inhibited, suggesting a critical role for inducible prostaglandins in fetal lung development. Our observations are consistent with a high rate of neonatal death described for PGHS-2 knockout mice, which was attributed to patency of the ductus arteriosus (33); however, examination of the pulmonary surfactant system in these animals has not been reported. The effect of nimesulide on SP-C gene expression was the opposite to that observed for the other surfactant proteins, suggesting independent regulation by prostaglandins. The longer term outcomes of PGHS-2 inhibition on surfactant production and lung compliance remain unknown but deserve further investigation.
Nimesulide’s preference for PGHS-2 reduces disruption to homeostatic functions and thereby reduces, but does not abolish, adverse effects. Indeed, there is a possibility for nimesulide to be acting via a mechanism independent to prostaglandins since PGHS-2 gene expression in saline groups is the same between cohorts, but mRNA levels of IL-1β, IL-8, SP-C, SP-D, and EP1–3 are significantly altered after saline in the nimesulide cohort. While most research has focused on the prostaglandin pathway, nimesulide can also induce glucocorticoid receptor phosphorylation (51), suggesting that some of the anti-inflammatory effects of nimesulide may be due to glucocorticoid signaling.

We did not measure mRNA levels of prostaglandin synthetic and metabolizing enzymes in the chorioamnion, as we were unable to recover sufficient RNA from these samples.

However, it has been shown that expression of both PGHS-2 (18, 27) and PGES (45) increases in the fetal membranes in response to inflammation. The fetal membranes produce prostaglandins and the human amnion specifically produces significantly more PGE2 compared with the chorion, decidua, and placenta (35). This indicates that the fetal membranes and, in particular, the amnion, are the likely source of the LPS-induced increase we observed in PGE2 concentrations in the amniotic fluid. In addition to potential local production of prostaglandins by the fetal lungs (indicated by our studies), it is possible that PGE2 generated by the amnion reaches the fetal respiratory system to influence lung development.

The relative volume density of secondary septal crests was greater in preterm lambs 7 days after exposure to LPS, which may potentially represent a rebound in alveolarization. Initially, inflammation decreases the number of distal air spaces in preterm lambs, but there is recovery of alveolar number by later in gestation (25), which persists postnatally (37, 38). It is also possible that the increase in relative septal crest density after LPS may be due to the overall reduction in tissue-to-air space ratio, rather than an increase in absolute number of secondary septal crests. This would be consistent with trends observed previously by us (37, 44), whereby alveolar wall thickness was reduced by LPS. Lung structural effects of intra-amniotic LPS take longer than 2 days to manifest, so we did not assess lung structure after nimesulide infusion.

In summary, we found that PGE2 concentrations increase in the amniotic fluid between 2 and 6 days after intra-amniotic LPS administration in sheep. Increases in fetal lung PGHS-2, PGES, and EP4 gene expression were observed in association with the changes in lung development that accompanied the inflammatory response. Maternal nimesulide infusion inhibited increases in PGE2 concentrations in response to LPS, modulated the inflammatory response in the fetal lungs and altered surfactant protein mRNA levels. These data suggest that prostaglandins mediate inflammation-induced changes in lung development. However, the precise degree to which prostaglandins contribute to inflammation-induced fetal lung maturation remains unknown, as does the underlying mechanism, and warrants further investigation.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


