Vascular changes after intra-amniotic endotoxin in preterm lamb lungs

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Submitted 6 August 2004; accepted in final form 18 August 2004

Kallapur, Suhas G., Cindy J. Bachurski, Timothy D. Le Cras, Shubhada N. Joshi, Machiko Ikegami, and Alan H. Jobe. Vascular changes after intra-amniotic endotoxin in preterm lamb lungs. Am J Physiol Lung Cell Mol Physiol 287: L1178–L1185, 2004.—Chorioamnionitis is associated with preterm delivery and bronchopulmonary dysplasia (BPD), characterized by impaired alveolar and pulmonary vascular development and vascular dysfunction. To study the vascular effects in a model of chorioamnionitis, preterm lambs were exposed to 20 mg of intra-amniotic endotoxin or saline for 1, 2, 4, or 7 days and delivered at 122 days gestational age (term = 150 days). This intra-amniotic endotoxin dose was previously shown to induce lung maturation. The effect of intra-amniotic endotoxin on expression of endothelial proteins was evaluated. Muscularization of the media and collagen deposition in adventitia of small pulmonary arteries was used to assess vascular remodeling. Compared with controls, bronchial pulmonary lavage fluid protein content was increased 6 days after intra-amniotic endotoxin exposure. Vascular endothelial growth factor (VEGF) 165 isoform mRNA decreased 2–4 days after intra-amniotic endotoxin. VEGF, VEGF receptor-2, endothelial nitric oxide synthase (eNOS), platelet endothelial cell adhesion molecule-1, and Tie-2 protein expression in the lung coordinately decreased 1–7 days after intra-amniotic endotoxin. Intra-amniotic endotoxin appeared to selectively decrease eNOS expression in small pulmonary vessels compared with large vessels. Medial smooth muscle hypertrophy and increased adventitial fibrosis were observed 4 and 7 days after intra-amniotic endotoxin. These results demonstrate that, in the preterm lamb lung, antenatal inflammation inhibits endothelial cell protein expression followed by vascular remodeling changes in small pulmonary arteries. Exposure to antenatal inflammation may cause vascular remodeling and contribute to the development of BPD.

BRONCHOPULMONARY DYSPLASIA (BPD) is a frequent problem in preterm infants and occurs in ~50% of infants weighing <1,000 g at birth (30). Pathologically, BPD in these very-low-birth-weight infants is characterized by an arrest of secondary septation at the vascular stage, decreased lung microvascular development, and decreased alveolarization (11, 46). The pathogenesis of BPD is thought to be multifactorial, including antenatal and postnatal insults (20). Clinical and epidemiological studies suggest that chorioamnionitis and antenatal inflammation can increase the risk for developing BPD (47, 49).

Preterm infants with BPD have decreased angiogenic growth factors and endothelial proteins critical for endothelial function (7, 28). In the preterm sheep BPD model induced by exposure to mechanical ventilation, pulmonary edema is observed (8). Similarly, in the preterm baboon mechanical ventilation-induced BPD model, decreased angiogenic growth factors and endothelial proteins and impaired microvascular development are present (34). Pulmonary hypertension is a frequent complication in infants with BPD and can cause significant morbidity and mortality (2, 17, 18). These studies suggest that decreased angiogenic growth factors and reduced endothelial proteins, vascular dysfunction, and remodeling are a central feature of BPD induced by postnatal insults. It is not known whether antenatal inflammation alone without exposure to mechanical ventilation causes pulmonary vascular changes and remodeling.

Vascular endothelial proteins VEGF-R2 and eNOS play an important role in pulmonary vascular development. Inhibition of the vascular endothelial growth factor-receptor 2 (VEGF-R2) signaling (19, 29) or lack of heparin-binding VEGF isoforms VEGF-165 and VEGF-188 in the developing lung of rodents causes decreased pulmonary vascular development and alveolarization (36). Deficiency of eNOS also causes impairment of alveolarization and reduced pulmonary vascular development with exposure to mild hypoxia (6). Decreased alveolar growth, reduced eNOS expression and signaling, as well as abnormal pulmonary vascular development also occur in preterm sheep and baboons exposed to mechanical ventilation and supplemental oxygen for >2 wk (3, 8, 9, 12). Expression of these endothelial proteins after antenatal inflammation is not known.

We have developed fetal preterm lamb models in which intra-amniotic endotoxin injection given as either a single dose or as a continuous infusion causes impaired alveolarization in preterm lambs (35, 50). In these fetal models, reduced alveolarization similar to BPD occurs without exposure to postnatal insults, such as oxygen or mechanical ventilation. Vascular changes have not been studied in this model.

We recently showed that the angiostatic chemokine IP-10 mRNA is induced in the fetal lamb lung 1–4 days after intra-amniotic endotoxin exposure (24). We therefore hypothesized that intra-amnionic endotoxin would inhibit expression of proteins critical for endothelial function followed by vascular remodeling in the preterm lung. We evaluated the time course of expression of endothelial proteins and studied remodeling in the muscular and adventitial compartments in the preterm lung exposed to antenatal endotoxin.

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PECAM-1 cDNA was excised and the plasmid relegated. The result-
-P32]UTP-labeled probes for 16 h at 55°C. Single-stranded RNA
T7 RNA polymerase was used to generate an antisense riboprobe
fragment 198 bp). The plasmid pGEMT-sTie1 was cut with

METHODS

Animals. The animal studies were performed in Western Australia with date-mated Merino ewes with singleton gestations. All studies were approved by the animal care and use committees at the University of Western Australia, Perth, and at Cincinnati Children’s Hospital Medical Center, Ohio. Intra-amniotic injections of saline (control) or 20 mg of Escherichia coli endotoxin (055:B5; Sigma, St. Louis, MO) were given using ultrasound guidance and with electrolyte analysis of amniotic fluid to verify intra-amniotic injection (22). The lambs in this study were previously used to characterize the lung and systemic inflammation and lung maturation (25).

Delivery and bronchoalveolar lavage fluid collection. After intra-
amniotic injection (1–7 days), the ewes were preanesthetized with ketamine and xylazine and received spinal anesthesia for cesarean section delivery of preterm lambs at 119–125 days gestation (term is 150 days). After cesarean section, a cord blood arterial sample was collected followed by injection of pentobarbital sodium (100 mg/kg) via an umbilical vein. Pieces of right lower lobe of lung were snap-frozen for RNA and protein analysis. The right upper lobe of lung was in inflammation and lung maturation (25).

20 mg of

Escherichia coli

was then inactivated, and protected RNA was precipitated using the


DNA isolation and RNAse protection assays. Total RNA was
isolated, and 10 μg of total RNA were used for multiprobe RNase
protection analysis, as described previously (25). A 188-bp fragment
comprising the last two nucleotides of exon 4, exon 5, and exons 7–8 corresponding to the nucleotides 386–573 of the ovine VEGF 165 (Genbank accession no. AF071015) was cloned using PCR. pGEMT-VEGF 165–188 was linearized with SspI and SP6 RNA polymerase was used to generate an antisense riboprobe. This ribo-
probe protects a 188-bp fragment of the VEGF 165 isoform and a
155-bp fragment of the VEGF 188 isoform. An 845-bp fragment of the sheep platelet endothelial cell adhesion molecule-1 (PECAM-1) coding region and a 1-kbp fragment of the sheep Tie-1 coding region were cloned based on homology to the corresponding bovine and mouse sequences using PCR. A BamHI-I-SpeI fragment of the sheep PECAM-1 cDNA was excised and the plasmid relegated. The result-
ing plasmid, pGEMT-PECAM 518, was cut with SpeI, and SP6 RNA polymerase was used to generate an antisense riboprobe (protected fragment 198 bp). The plasmid pGEMT-sTie1 was cut with BsuI and T7 RNA polymerase was used to generate an antisense riboprobe (protected fragment 355 bp).

Solution hybridization was performed using a molar excess of
[α-32P]UTP-labeled probes for 16 h at 55°C. Single-stranded RNA
was digested with RNase A/T1 (Pharmingen, San Diego, CA). RNAse
was then inactivated, and protected RNA was precipitated using the
RPA III inactivation/precipitation buffer (Ambion, Austin, TX). The ribosomal protein mRNA L32 was used as an internal control. The protected fragments were resolved on 6% polyacrylamide (8 mol/l urea) gels, visualized by autoradiography, and quantified on a Phos-
phorImager using ImageQuant version 1.2 software (Molecular Dy-
namics, Sunnyvale, CA).

Protein analysis. Frozen lung samples were homogenized in ice-
cold buffer containing 50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail (complete minitables; Roche, Mannheim, Germany) supplemented with phenylmethylsulfonyl flu-
oride (1 mM; Sigma). The samples were sonicated and then centrif-
fuged at 500 g for 20 min at 4°C to remove cellular debris. Protein
content in the supernatant was determined by the bicinchoninic acid
(BCA) method, using BSA as the standard. Protein sample (50 μg/lane) was resolved by 3–8% Tris-acetate gel electrophoresis
(Novex precast gels; Invitrogen, Carlsbad, CA), and proteins from the gel were transferred to a polyvinylidene difluoride membrane by electroblocting (Invitrogen). Blots were blocked 1 h in 5% nonfat dry milk in TBS with 0.1% Tween 20. These blots were incubated with the primary antibody overnight at 4°C using one of the following antibodies diluted in blocking buffer: mouse monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-VEGF (sc-152), mouse monoclonal anti-VEGF (sc-7269), goat polyclonal anti-PECAM-1 antibody (sc-1505), rabbit polyclonal anti-Tie-2 antibody (sc-324), or rabbit polyclonal anti-VEGF-R2 an-
tibody (sc-504), all from Santa Cruz Biotechnology. Blots were incubated for 1 h at room temperature with the appropriate conjugated secondary antibody. After being washed, bands were visualized by chemiluminescence (ECL kit; Amersham PharmaciaBiotek, Buck-
inghamshire, UK) and radiographed. The autoradiographs were
scanned at high resolution, and images were acquired using Adobe photoshop software. The densitometric quantitation was performed using ImageQuant version 1.2 software (Molecular Dynamics). To ensure meaningful comparisons between the large number of blots, the same set of controls was used for densitometric comparison.

Immunohistochemistry. The immunostaining methods were per-
formed as described (48). Paraffin sections (5 μm) of formalin-fixed tissue on polylysine-coated slides were deparaffinized and rehydrated. Antigen retrieval was performed using citrate buffer, pH 6.0, boiled, and pretreated with 0.5% hydrogen peroxide to destroy endogenous peroxidase. Sections were incubated with monoclonal anti-eNOS antibody (1:25; Transduction Laboratories) or anti-α-smooth muscle actin (1:5,000; Sigma) at 4°C overnight followed by incubation with goat anti-mouse IgG-biotin (Santa Cruz Biotechnology) for 1 h at room temperature. Nonspecific interactions were inhibited by using 2% goat serum during both primary and secondary antibody incubation. Immunostaining was visualized by the Vectastain ABC peroxi-
dase Elite kit to detect the antigen-antibody complexes (Vector Laboratories). The antigen detection was enhanced with nickel-dia-
minorbenzidine for eNOS staining, followed by incubation with Tris-
cobalt to give a black precipitate. The nuclei were counterstained with Nuclear Fast Red for photomicroscopy.

Vascular morphometry. Measurements of arteriolar wall thickness were made using α-smooth muscle actin (SMA) immunostaining to demarcate the muscularis media. Ten arterioles per lamb with measure-
ments of <50 μm external diameter accompanying the terminal bronchioles (identified by morphologic criteria) were measured by a blinded observer, and three to four lambs were evaluated per group. Only transversely sectioned airways were evaluated to minimize distortion of arteriolar muscularis media. Morphometric measure-
ments included thickness of the muscularis media, external vessel diameter, external vessel area, and area of smooth muscle (vessel lumen external area-internal area) and were analyzed using Meta-
morph version 6.1 software (Universal Imaging, Downingtown, PA) on digitally acquired images. The wall thickness was expressed as [(2 x medial wall thickness)/(external diameter)] x 100.

The adventitial fibrosis was evaluated by Masson’s trichrome
staining. Ten arterioles per lamb with measurements of <50 μm
external diameter accompanying the terminal bronchioles were scored
by a blinded observer for collagen staining, and three to four lambs
were evaluated per group. A qualitative scoring system was used to
evaluate adventitial fibrosis: score 0 = none, score 1 = mild, score 2 = moderate, and score 3 = severe.

Statistics. The RNA, protein quantitation, and vessel morphometric and scoring results were expressed as means ± SE. Comparisons
between endotoxin-exposed animals and controls were made with

two-tailed unpaired t-tests, two-tailed Mann-Whitney nonparametric tests, or two-way Kruskal-Wallis nonparametric ANOVA, as appro-
priate. Significance was accepted at P < 0.05.

AJP-Lung Cell Mol Physiol • VOL 287 • DECEMBER 2004 • www.ajplung.org
**RESULTS**

_Cord blood hematocrit and blood gas._ The cord blood hematocrit, PO2, Pco2, and pH were similar between control and endotoxin-exposed preterm lambs. There were no differences in the cord blood hematocrit, PO2, Pco2, and pH values within the different endotoxin-exposed subgroups. The cord blood PO2 level was 13.8 ± 0.9 mmHg in controls and 14.4 ± 0.7 mmHg in endotoxin-exposed animals. The cord blood hematocrit was 38 ± 1 in controls and 34.5 ± 0.6 in endotoxin-exposed animals. There were no fetal deaths in this study, either in the control or the endotoxin-exposed groups.

_Intra-amniotic endotoxin increases BALF protein._ Protein concentration was measured in the BALF (Fig. 1). BALF recovery volumes and the body weights were similar in control and endotoxin-exposed preterm lambs (267 ± 17 ml and 2.4 ± 0.1 kg vs. 258 ± 12 ml and 2.3 ± 0.1 kg, respectively, not statistically different). BALF protein content increased by 2.3-fold 2 days after intra-amniotic endotoxin exposure and returned to control levels at 4 days.

_Intra-amniotic endotoxin decreases eNOS protein expression._ eNOS plays a critical role in regulating pulmonary vascular tone and modulating pulmonary vascular development (6, 13). Western blot analysis showed that eNOS protein expression in lung homogenates decreased 2–4 days after intra-amniotic endotoxin with a maximum decrease to 40% of the control value at 4 days (Fig. 2A). At 7 days after intra-amniotic endotoxin exposure, lung eNOS protein levels approached control levels. The cellular localization of eNOS protein expression was evaluated by immunohistology. In the control preterm lambs, eNOS immunostaining was detected in lung vascular endothelial cells (Fig. 2B). Compared with controls, eNOS staining was faint in the endothelial cells of the small pulmonary vessels at 1, 4, and 7 days after intra-amniotic endotoxin exposure (Fig. 2, C–E). Interestingly, eNOS staining in the endothelium of the large vessels was similar between the controls and the endotoxin-exposed lambs (Fig. 2, B–E, Insets).

_Intra-amniotic endotoxin downregulates VEGF expression._ VEGF signaling is known to regulate the activity of eNOS (51). We therefore evaluated the expression of VEGF mRNA in the lung by RNase protection assay (RPA; Fig. 3A). The antisense VEGF RPA probe was designed to differentially detect two splice forms (VEGF-165 and VEGF-188). VEGF-165 mRNA expression decreased 2 and 4 days after intra-amniotic endotoxin exposure to 55% of the control levels (Fig. 3B). The expression of VEGF-188 mRNA paralleled the decrease in VEGF-165 mRNA expression. VEGF protein levels were evaluated by Western blot analysis using two different antibodies (Fig. 3C). In preliminary experiments using sheep lung homogenates, we found that the rabbit polyclonal antibody (sc-152) detected the dimeric VEGF (42 kDa) but did not detect the VEGF monomer. The mouse monoclonal antibody (sc-7269) detected only the monomeric VEGF (21 kDa). However, a high concentration of 4.4% β-mercaptoethanol only incompletely reduced the sheep lung VEGF disulfide bonds.

To eliminate variability resulting from differential reductions between the groups and to accurately define the VEGF species, VEGF immunoblot densitometry was performed initially with the monoclonal anti-VEGF antibody followed by the polyclonal anti-VEGF antibody using recombinant mouse and human VEGF standards. Consistent with the decreased VEGF-165 mRNA 2 and 4 days after intra-amniotic endotoxin exposure, both the dimeric and the monomeric VEGF protein were decreased 4 and 7 days after intra-amniotic endotoxin exposure (Fig. 3D). VEGF protein levels were maximally decreased to 40% of control levels 7 days after intra-amniotic endotoxin.

_Intra-amniotic endotoxin decreases endothelial protein expression._ PECAM-1 (CD31), Tie-2, and VEGF-R2 are important for endothelial cell development and function (41, 42). We therefore evaluated the expression of these proteins in the preterm lung after intra-amniotic endotoxin exposure (Fig. 4A). PECAM-1 protein levels decreased to 50% of the control levels at 2 and 4 days after intra-amniotic endotoxin (Fig. 4B). The Tie-2 protein levels decreased to 45% of control levels at 2 days and 75% of control levels at 4 days after intra-amniotic endotoxin. VEGF-R2 protein decreased to 35–40% of the control values at 1–4 days after intra-amniotic endotoxin.

PECAM, Tie-2, and VEGF-R2 protein levels approached control values by 7 days after intra-amniotic endotoxin. Consistent with the decreased protein expression, PECAM mRNA and Tie-1 mRNA also decreased to 60 and 52% of the control value, respectively (data not shown).

_Intra-amniotic endotoxin causes arteriolar smooth muscle hypertrophy and increases in the vascular adventitia._ To determine whether intra-amniotic endotoxin increased muscularization of small pulmonary arteries, the medial wall thickness was measured by morphometry. Resistance arterioles (<50 μm external diameter) accompanying terminal bronchioles of preterm lambs were identified using α-smooth muscle actin immunostaining. The median arteriolar external diameter was 25 μm (range 15–45 μm) in both controls and endotoxin-exposed lambs. Arteriolar smooth muscle thickness increased from 39% in controls to 47% 4 days after intra-amniotic endotoxin and to 53% 7 days after intra-amniotic endotoxin (Fig. 5A, P < 0.05). Consistent with increased smooth muscle wall thickness, the area occupied by smooth muscle/total arteriolar area also increased from 0.62 in controls to 0.74 4 days after intra-amniotic endotoxin and 0.77 7 days after intra-amniotic endotoxin (Fig. 5B, P < 0.05). In addition to the increased arteriolar smooth muscle thickness after intra-amniotic endotoxin, increased adventitial layer fibrosis and cellularity was seen in sections of preterm lamb lungs 7 days after intra-amniotic endotoxin compared with controls (Fig. 5, C–E). To further characterize changes in the vascular adventitia,
Masson’s trichrome staining for detection of collagen deposition was performed (Fig. 5, F-H). The adventitial fibrosis (blue staining) score for control lambs was 0.8 ± 0.1. The adventitial fibrosis score increased 4 days after intra-amniotic endotoxin to 1.63 ± 0.16 and 7 days after intra-amniotic endotoxin to 2.1 ± 0.14 (P < 0.01 for both endotoxin-exposed groups vs. controls).

**DISCUSSION**

These results are the first to demonstrate the effects of antenatal inflammation alone without mechanical ventilation or oxygen exposure on the fetal pulmonary vasculature. Expression of VEGF, eNOS, PECAM-1, Tie-2, and VEGF-R2 was reduced for up to 7 days after intra-amniotic endotoxin. Vascular remodeling, as demonstrated by smooth muscle hypertrophy and increased adventitial collagen deposition in resistance arterioles, occurred 4 and 7 days after intra-amniotic endotoxin exposure.

The effects of intra-amniotic endotoxin are different from systemic (intramuscular or intravenous) endotoxin exposure. Compared with the 20-mg intra-amniotic endotoxin dose used in this study, systemic injection of far lower doses (~0.05 mg) increases lung vascular permeability in adult sheep and causes death in preterm lambs (10, 23). This animal model differs from infants exposed to chorioamnionitis in the lack of exposure to live replicating bacteria. We have previously reported minimal systemic inflammation and no placental inflammation after 20 mg of intra-amniotic endotoxin (25). In this study, no fetal acidosis or fetal deaths were observed after intra-amniotic endotoxin. In previous studies, a lower intra-amniotic endotoxin dose of 0.1 or 1 mg inconsistently induced lung inflammation and surfactant synthesis (22, 27). Larger doses of 100 mg of intra-amniotic endotoxin caused comparable lung maturational responses without inducing fetal death, decreasing fetal growth, or causing miscarriage in the ewe (22). The mixing of the endotoxin with a relatively large volume (~0.5
liters) of amniotic fluid upon injection and the likely differences between the respiratory epithelial vs. endothelial responsiveness to endotoxin probably contribute to the differences between the intra-amniotic vs. systemic endotoxin exposure. These results suggest that the vascular effects observed in this study occurred as a result of the endotoxin-induced fetal lung inflammation and not because of direct endotoxin effects on the vascular endothelium.

In the preterm lamb model used in this study, maximum influx of activated inflammatory cells and induction of proinflammatory cytokines and the angiostatic interferon inducible chemokine (interferon inducible protein-10 and monokine induced by gamma interferon) mRNAs in the lung occurs 1–2 days after intra-amniotic endotoxin [24, 25, 27]. Airway surfactant proteins and lipids were increased 7 days after intra-amniotic endotoxin [25]. Among the endothelial proteins, striking reductions in the expression of eNOS and VEGF-R2 were observed in this study as early as 1 day after endotoxin exposure. We previously reported no significant change in numbers of alveolar type II cells after intra-amniotic endotoxin in this model [26]. One possible explanation for reduced endothelial proteins is reduced epithelial and vascular surface area after endotoxin exposure. We have previously reported decreased alveolarization at 7 days after intra-amniotic endotoxin exposure [50]. Taken together, these data are consistent with the hypothesis that intra-amniotic endotoxin-induced inflammatory changes and/or the induction of angiostatic chemokines may decrease expression of proteins important for endothelial cell function. The vascular changes along with the fetal lung inflammation may contribute to decreased alveolarization.

In the present study, eNOS protein content in the lung was decreased to approximately 50% of the control levels 1–4 days after exposure to endotoxin (Fig. 4). Decreased endothelial proteins in the lung. A: representative platelet endothelial cell adhesion molecule-1 (PECAM-1), Tie-2, and VEGF-receptor 2 (R2) immunoblots using 50 μg of total protein in each lane from lung homogenate. B: protein densitometry was internally normalized to β-actin, and the mean normalized VEGF densitometry value for controls was assigned a value of 1. The densitometry values from treated lambs were expressed relative to controls. IA endotoxin exposure decreased PECAM-1, Tie-2, and VEGF-R2 expression 1–4 days after IA exposure; n = 4 animals in each group/time point. *P < 0.05 vs. controls.

Fig. 3. Decreased vascular endothelial growth factor (VEGF) in the lung. A: representative RNase protection assay of VEGF-165 and -185 isoforms using 10 μg of total RNA from lungs in control and endotoxin-exposed animals. B: quantification of VEGF isoforms at time points indicated after normalizing to L32 (ribosomal protein mRNA). The mean mRNA value in control animals was given a value of 1, and levels at each time point were expressed relative to the mean control value. IA endotoxin exposure decreased VEGF-165 isoform mRNA expression at 2 and 4 days after IA endotoxin; n = 3–6 animals in each group/time point. *P < 0.05 vs. controls. C: representative VEGF immunoblots for the monomeric and the dimeric form of VEGF-165 isoform using 50 μg of total protein in each lane from lung homogenate. D: VEGF densitometry was internally normalized to β-actin, and the mean normalized VEGF densitometry value for controls was assigned a value of 1. The densitometry values from treated lambs were expressed relative to controls. IA endotoxin exposure decreased VEGF protein levels at 4 and 7 days after IA exposure; n = 4 animals in each group/time point. *P < 0.05 vs. controls.
to intra-amniotic endotoxin. By immunostaining, eNOS expression appeared to be selectively reduced in the small pulmonary arteries compared with large conducting vessels. Reductions in lung eNOS protein content and decreased responsiveness to inhaled nitric oxide have been reported after 3 wk of chronic mechanical ventilation in preterm lambs (9, 33). Mice homozygous for the targeted deletion of eNOS have decreased postnatal survival, impaired alveolarization, and severe pulmonary hypertension after exposure to mild hypoxia, suggesting a critical requirement of eNOS for postnatal survival (6, 13, 14, 37). Taken together, these studies along with the results of the present study suggest that eNOS deficiency may contribute to reduced alveolarization and remodeling changes after exposure to intra-amniotic endotoxin.

VEGF signaling is essential for endothelial cell proliferation, survival, and angiogenesis (15). A downstream target of VEGF signaling is nitric oxide and nitric oxide synthase expression and activity (51). Inhibition of VEGF or the VEGF receptor signaling causes reduced alveolarization, decreased pulmonary vascular development, and pulmonary hypertension.

**Fig. 5.** Distal pulmonary arteriolar smooth muscle hypertrophy and adventitial fibrosis. A: arteriolar smooth muscle wall thickness (% external diameter). B: smooth muscle area relative to total area in controls and endotoxin-exposed animals. Data are means ± SE of 33–47 observations/group in A and mean arteriolar area in individual animals in B, along with the median in each group (n = 3–4/group). *P < 0.05 vs. controls. Representative α-smooth muscle actin-immunostained lung sections used for arteriolar morphometry showing arteriolar smooth muscle staining (arrow) from control (C), 4 days after IA endotoxin (D), and 7 days after IA endotoxin (E). Exposure to IA endotoxin caused pulmonary arteriolar smooth muscle hypertrophy at 4 and 7 days. Representative Trichrome stained lung sections showing arteriolar adventitial collagen blue staining (arrow) and scoring in control (score = 1; F), 4 days after intra-amniotic endotoxin (score = 2; G), and 7 days (score = 3) after intra-amniotic endotoxin (H). Exposure to IA endotoxin increased adventitial collagen at 4 and 7 days. Bars represent 50 μm.
in the developing lung (16, 19, 29). VEGF mRNA expression in the fetal lambs at the gestation used in this study is largely localized to type II pneumocytes (data not shown). After intra-amniotic endotoxin, expression of surfactant proteins B and C, also products of type II pneumocytes, is markedly induced (4) at the same time that VEGF mRNA and protein are decreased. This result suggests that the effects of antenatal inflammation on VEGF expression are the result of specific gene regulation. In the present study, although VEGF mRNA levels did not decrease for 2 days, the VEGF-R2 expression decreased 1 day after intra-amniotic endotoxin. Consistent with the published literature, our data suggest that decreased VEGF signaling (VEGF-R2 expression) may contribute to reduced eNOS expression.

Preterm baboons exposed to mechanical ventilation and oxygen have decreased lung PECAM-1, VEGF, and VEGF-R1 levels did not decrease for 2 days, the VEGF-R2 expression in inflammation on VEGF expression are the result of specific decreased. This result suggests that the effects of antenatal induction (4) at the same time that VEGF mRNA and protein are severe bronchopulmonary dysplasia. Pediatrics 75: 80–84, 1985.


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