Oxidative Stress in Fetal Lambs Exposed to Intra-amniotic Endotoxin in a Chorioamnionitis Model

FOOK-CHOE CHEAH, ALAN H. JOBE, TIMOTHY J. MOSS, JOHN P. NEWNHAM, AND SUHAS G. KALLAPUR

Department of Neonatology [F.-C.C., A.H.J., S.G.K.], Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229; Department of Obstetrics and Gynaecology [T.J.M., J.P.N.], The University of Western Australia, King Edward Memorial Hospital, Subiaco, WA 6008, Australia

ABSTRACT: Chorioamnionitis is a risk factor for the development of bronchopulmonary dysplasia. Endotoxin-induced oxidative stress to the fetus in the uniquely hypoxic intrauterine environment has not been reported. Using a model of chorioamnionitis, we measured markers of pulmonary and systemic oxidant exposures in fetal lambs at 124 d gestation (term = 150 d) exposed to 10 mg intra-amniotic endotoxin 2 d ($n = 6$) or 7 d ($n = 6$) before delivery, or saline as controls ($n = 9$). The 7 d endotoxin-exposed animals had 3-fold higher protein carbonyls (0.66 ± 0.46 *versus* 0.23 ± 0.14 nmol/mg protein) and 10-fold greater myeloperoxidase activity (2.38 ± 1.87 *versus* 0.27 ± 0.18 nM) in the bronchoalveolar lavage fluid (BALF), suggestive of neutrophil-derived oxidant activity. However, in the lung tissue, protein carbonyls, superoxide dismutase, and peroxiredoxin 1 were not different between groups. The expression of peroxiredoxin 1 was prominent, primarily in the peri-bronchiolar epithelium. Notably, evidence of oxidant exposure was minimal at 2 d when BALF inflammatory cells, lung IL-1β, and IL-8 were highest. Intra-amniotic endotoxin induced systemic oxidative stress as plasma protein carbonyl was elevated at 7 d (0.14 ± 0.04 nmol/mg protein; $p = 0.005$). Surfactant protein A and B mRNAs were highest at 2 d, suggesting that oxidative stress did not contribute to the lung maturation response. A modest lung oxidative stress in chorioamnionitis could contribute to bronchopulmonary dysplasia. (*Pediatr Res* **63:** 274–279, 2008)

Intrauterine infection causes chorioamnionitis (CAM), an important risk factor for preterm delivery (1). The infectious stimuli that cause CAM induce recruitment of inflammatory cells and cytokine production in the chorioamnion, the amniotic fluid, and fetal lungs (2–4). Fetal exposure to CAM may lead to the development of bronchopulmonary dysplasia (BPD), a lung injury closely associated with inflammation and oxygen toxicity (5,6).

In the fetal lamb model of acute CAM, intra-amniotic *Escherichia coli* endotoxin causes both lung inflammation and early gestational lung maturation (7,8). A 10-mg dose of intra-amniotic endotoxin results in lung inflammation characterized by a predominantly neutrophil recruitment into the air spaces that peaks at about 72 h. The mature neutrophil is a major source of reactive oxygen species (ROS) and there is increasing evidence that ROS-mediated lung injury is involved in the development of

BPD (9,10). The fetal lung could be responding to oxidative stress in CAM, but this possibility has not been well studied. Intra-amniotic endotoxin does induce antioxidant enzymes in the lungs, suggestive of a host response to mitigate oxidative injury (11). Although intra-amniotic endotoxin increased the expression of heat shock protein 70 (HSP 70), the effect was brief and limited (8). Furthermore, the fetus is in a relatively hypoxic intrauterine environment and it is unclear whether the reduced oxygen tension would modify or limit the extent of oxidative injury. In addition to pulmonary changes, a fetal systemic inflammatory response is observed in CAM (12). Whether intra-amniotic endotoxin also induces a systemic reaction by oxidative stress is unknown.

The objectives in this study were to determine the temporal responses to oxidative stress in relation to inflammation in fetal lambs exposed to intra-amniotic endotoxin. Specifically, we measured in bronchoalveolar lavage fluid (BALF), lung tissue, and plasma the oxidant load contributed by the marked cellular inflammatory responses to intra-amniotic endotoxin.

METHODS

Animals and intra-amniotic endotoxin administration. The sheep experiments were performed in Perth, Western Australia, as approved by the Animal Care and Use Committees of the Cincinnati Children's Hospital Medical Center and Western Australia Department of Agriculture. Date-bred Merino ewes were randomized to receive ultrasound guided intra-amniotic injections of 10 mg *E. coli* endotoxin (055-B5 strain) (Sigma Chemical Co., St. Louis, MO) 2 d or 7 d before delivery, or control injections at 2 or 7 d with equal volumes of saline. The 2-d interval is when inflammation induced by intra-amniotic endotoxin is maximal and surfactant protein mRNAs are increased (8). The 7-d interval is optimal for evaluation of induced lung maturation by saturated phosphatidylcholine (Sat PC) measurements and deflation pressure volume curves (13). Lambs were delivered prematurely at 124 d gestational age (term gestation is 150 d).

Delivery and sampling methods. Preterm lambs were surgically delivered, and umbilical artery blood samples were used for blood gas and pH analyses (Rapidlab 860; Bayer Corp., Elkhart, IN) and measurements of protein carbonyl in the plasma. The lambs were killed with intravascular pentobarbital, dried, and weighed. The lambs were then exsanguinated; the chest was opened and a deflation pressure volume curve was measured following inflation of the lungs to a pressure of 40 cm H_2O (13). Each lung was weighed and the left lung was lavaged three times by infusion and withdrawal of a sufficient volume of 4°C normal saline to fill the lung (13). The three BALFs

Abbreviations: BALF, bronchoalveolar lavage fluid; **BPD,** bronchopulmonary dysplasia; **CAM,** chorioamnionitis; **LPS,** lipopolysaccharide; **MPO,** myeloperoxidase; **Prx 1,** peroxiredoxin 1; **ROS,** reactive oxygen species; **Sat PC,** saturated phosphatidylcholine; **SOD,** superoxide dismutase; **SP-,** surfactant protein

were pooled; the total volume was recorded and aliquots were saved for later analysis. The right upper lobe of the lung was inflation fixed with 10% formalin at 30 cm H_2O pressure (8). Tissue from the right lower lobe was snap frozen for mRNA and other analyses. Lung homogenate samples were prepared using a high shear tissue homogenizer just before analysis (Omni TH, Omni International, Marietta, GA).

Inflammatory indices. Differential cell counts of inflammatory cell infiltrates (neutrophils and monocytes) were performed on cytospin preparations of BALF stained with Diff-Quik (Dade Behring, Dudingen, Switzerland). Protein in BALF was quantified with the Lowry assay or the Bradford method adapted for micro-titer plate based assays (13). MPO activity was determined by measuring the oxidation of tetramethylbenzidine (TMB) (14) against standard concentrations of pure MPO (Athens Research, & Technology, Athens, GA) determined on the basis of its $[epsilon]_{430}$ 91,000 M/cm/heme (15). IL-8 was assayed by ELISA using antibodies from Chemicon (Temecula, CA) (7). Recombinant IL-8 protein for standards was a kind contribution from CSIRO (Parkville, Victoria, Australia). The lower detection limit of this assay was 0.5 ng/mL. To quantify for cytokine mRNAs in lung tissue, ribonuclease protection assays were performed using the method previously described (16). Ovine IL-1β and IL-8 RNA transcripts and ovine ribosomal protein L32 as a reference RNA were used.

Oxidative markers. Protein carbonyl was used as the primary oxidative marker because it is a reliable indicator of oxidative injury (15,17). The assay for protein carbonyl in BALF, plasma, and lung tissue was based on derivatization of samples with dinitrophenylhydrazine (DNP) and measurement by ELISA with the detection system consisting of a biotinylated anti-DNP primary antibody, streptavidin-horseradish peroxidase conjugated secondary antibody, and a TMB-based chromogen substrate complex system (Sigma Chemical Co., St. Louis, MO) (15). Lung tissue was homogenized in 5 vol of ice-cold 50 mM Tris-HCL buffer, pH 7.5, and 0.1 mM EDTA. Homogenates were centrifuged at 3000 rpm for 20 min at 4°C. The supernatants were used for protein assays, measurements for SOD activity, protein carbonyls, and Western blotting (WB) for peroxiredoxin 1 (Prx 1). One of the first-line antioxidant defense enzymes, SOD was assessed in lung tissue as a reflection of enzymatic response to the endotoxin-induced oxidative stress. SOD activity was determined based on 50% inhibition of the rate of formation of a highly water-soluble formazan dye produced by oxidation of the tetrazolium salt, WST-1 (Dojindo Laboratories, Kumamoto, Japan) by superoxide generated from xanthine oxidase/hypoxanthine (Sigma Chemical Co.) (18). As a major regulator of hydrogen peroxide source of oxidative stress and signaling molecule in cellular processes, we measured the ubiquitous Prx 1 antioxidant enzyme (19). To quantify Prx 1 using WB, 40 μg proteins were denatured, electrophoresed on a 10-20% Tris-glycine gel (Novex, Invitrogen, Carlsbad, CA), and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with a rabbit polyclonal anti-Prx 1 antibody (GeneTex, San Antonio, TX) diluted at 1:10,000 in TBS-T (10 mM Tris-HCL, pH 8, 150 mM NaCl, 0.1% vol/vol Tween-20) and 5% nonfat milk. The membrane was washed with TBS-T and incubated for 1 h in room temperature with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Calbiochem, La Jolla, CA) diluted at 1:20,000 in TBS-T and 5% nonfat milk. Detection by chemiluminescence was performed using ECL reagents (GE Healthcare, Piscataway, NJ). A positive control was included using lysates from a lung carcinoma cell line (A549) known to over-express Prx 1 (20). Beta-actin (C4) (mouse MAb against chicken gizzard actin) as a primary antibody diluted at 1:2000 and a secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) were used to re-probe the blots as a loading control.

Immunohistochemistry for Prx 1. To localize Prx 1 expression, formalin-fixed, paraffin-embedded lung tissue cut in 5-μm sections were deparaffinized, quenched of endogenous peroxidase activity with hydrogen peroxide, and subjected to a citrate buffer antigen unmasking process. Nonspecific binding sites were blocked with 5% goat serum before overnight incubation at 4°C with anti-Prx 1 antibody (1:1000). The sections were washed followed by incubation at room temperature for 1 h with a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted at 1:200. Immunoreactivity was determined by the diaminobenzidine method. The tissues were counterstained with nuclear fast red.

Indicators of lung maturation. The lung tissue mRNA for surfactant proteins A and B (SP-A and SP-B) were measured using S1 nuclease protection assays as previously described (21). Sat PC was isolated from chloroform-methanol (2:1) extracts of BALF by neutral alumina column chromatography after exposure of lipid extracts to osmium tetroxide (13). Sat PC was quantified by phosphorus assay (13).

Statistics. Statistical analyses were performed using SigmaStat v.1.0 (Jandel Corporation, San Rafael, CA). All data are presented as mean ± SD unless otherwise indicated. For comparisons of more than two groups, ANOVA followed by Student-Newman-Keuls tests for post hoc analyses were used. Selective comparisons of two groups were made using unpaired *t* tests or Mann-Whitney *U* tests as indicated. Significance was accepted as $p < 0.05$.

RESULTS

Description of the lambs. A total of 21 preterm 124 d gestation lambs were studied. There were six lambs each in the groups exposed to intra-amniotic endotoxin 2 d or 7 d before delivery and nine lambs in the saline control group that had received intra-amniotic saline 2 or 7 d before delivery (Table 1). The results for the control lambs were similar and these animals were combined into one group for analysis. There were no differences in the mean birth weights between groups. All lambs had normal cord blood pH and P_{CO_2} values at delivery.

Acute lung inflammation. Neutrophils and monocytes were increased more than 100-fold in the BALF at 2 d or 7 d after endotoxin exposure (Table 1). The increase in neutrophils was about 2-fold greater than monocytes. The recruitment of these cells was highest at 2 d, consistent with results from our previous study (7). Endotoxin increased the total protein in BALF at 2 d and 7 d (Table 1). The neutrophil chemoattractant IL-8 was highest in BALF at 2 d and remained elevated at 7 d (Fig. 1A). In contrast, MPO, a measure of neutrophil activity was not elevated in BALF 2-d exposure but was significantly higher than controls at 7d (Fig. 1B). Intra-amniotic endotoxin greatly increased IL-1β (36-fold) and IL-8 mRNA (18-fold) in the lung parenchyma at 2 d but not at 7 d (Fig. 1, *C* and *D*).

Oxidative stress markers. Protein carbonyl concentrations in the BALF were increased about 3-fold in the 7 d endotoxin-exposed group (Fig. 2A). Systemically, protein carbonyl concentrations were significantly elevated in plasma samples from the 7 d endotoxin group relative to controls or the 2 d group (Fig. 2B). However, there were no significant differences in protein carbonyl levels in lung tissue between groups ($p = 0.13$) (Fig. 2C). SOD activity was also not different between the control and endotoxin-exposed groups ($p = 0.14$) (Fig. 2D).

Table 1. *Characteristics of fetal lambs and inflammatory changes in BALF with exposure to intra-amniotic endotoxin*

Treatment groups (*n*)	Saline control (9)	2 d Endotoxin (6)	7 d Endotoxin (6)
Weight (kg)	2.8 ± 0.3	2.5 ± 0.4	2.7 ± 0.4
pH in cord blood	7.37 ± 0.03	7.33 ± 0.06	7.37 ± 0.02
P_{CO_2} (mm Hg) in cord blood	60 ± 7	65 ± 12	57 ± 3
BALF**			
Neutrophils ($\times 10^6$/kg body wt)	0.3 ± 0.5	87.0 ± 54.8*	65.7 ± 63.4*
Monocytes ($\times 10^6$/kg body wt)	0.5 ± 0.7	66.2 ± 39.6*	49.5 ± 34.6*
Protein (mg/kg body wt)	15.6 ± 6.7	39.7 ± 14.9*	32.2 ± 13.6*

* Statistically significant *vs* control by ANOVA.

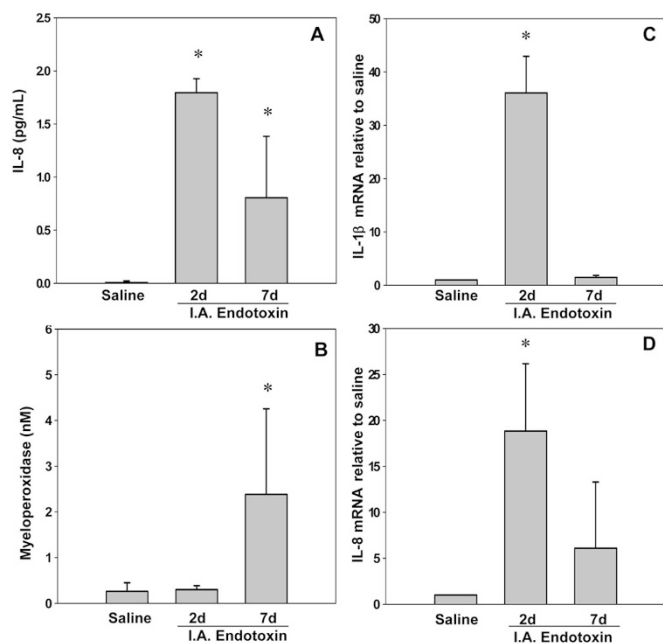** 2 d endotoxin group (*n* = 4); 7 d endotoxin group (*n* = 5).

Figure 1. Inflammatory markers in fetal lambs exposed to intra-amniotic endotoxin. The graphs represent mean $\pm$ SD values of (*A*) IL-8 protein and (*B*) myeloperoxidase in BALF sampled from saline controls ($n = 9$), 2 d (endotoxin given 2 d before delivery) ($n = 6$), and 7 d (endotoxin given 7 d before delivery) ($n = 6$). For IL-8 measurements, $n = 4$ (group 2 d) and $n = 5$ (group 7 d). (*C*) IL-1β mRNA and (*D*) IL-8 mRNA represent mean $\pm$ SD values of cytokine mRNA measured by ribonuclease protection assays. Values were internally normalized to L32 (ribosomal protein mRNA). Results were expressed as fold-increases above values of controls normalized to 1. The sample numbers for groups saline, 2 d and 7 d are 6, 4, and 5, respectively. *Statistically significant ($p < 0.05$) against controls by ANOVA.

Figure 2. Oxidative markers in fetal lambs after exposure to intra-amniotic endotoxin. Measurements (mean $\pm$ SD) for oxidative markers in BALF, plasma, and lung tissue homogenate samples from three groups of fetal lambs: saline controls ($n = 9$) and intra-amniotic endotoxin given at 2 d ($n = 6$) or 7 d ($n = 6$) before delivery. (*A*) BALF protein carbonyl and (*B*) plasma protein carbonyl. The average protein content in plasma samples (20 g/L) of the lambs were not significantly different between groups (data not shown). (*C*) Lung tissue protein carbonyl and (*D*) superoxide dismutase activity in lung tissue homogenates. *Statistically significant ($p < 0.05$) against controls by ANOVA.

Prx 1 was expressed in the lungs of all the fetal lambs, but there were no significant differences in the levels of expression with exposure to endotoxin (Fig. 3). Expression of Prx 1 was localized primarily to the peri-bronchiolar epithelium at 124d gestational age (Fig. 3, *B–D*), and a similar pattern was noted in sections from term lambs (data not shown).

Lung maturation. Lambs at 124 d gestation have very low amounts of SP-A and SP-B mRNA and the earliest indicator of induced lung maturation is the increase in the mRNA for surfactant proteins. Intra-amniotic endotoxin increased SP-A and SP-B mRNA by about 10-fold and 3.5-fold, respectively (Fig. 4, *A* and *B*). The amount of Sat PC recovered in BALF was similar to control values for the 2 d endotoxin group. The Sat PC in BALF increased about 10-fold in the 7 d endotoxin-exposed lambs (Fig. 4*C*). The pattern of responses was similar for the lung gas volumes measured at 40 cm H_2O pressure, with a more than 2.5-fold increased values for the 7 d endotoxin group compared with the control (Fig. 4*D*).

DISCUSSION

Our results provide new insights into oxidant effects of endotoxin-induced CAM on the fetal lung. We found evidence of oxidant activity as indicated by increased protein carbonyls in endotoxin-exposed fetal lambs primarily in the fluid from
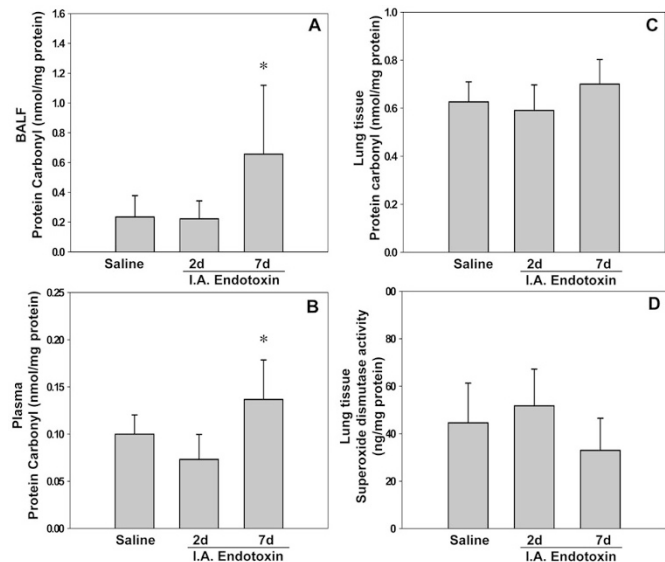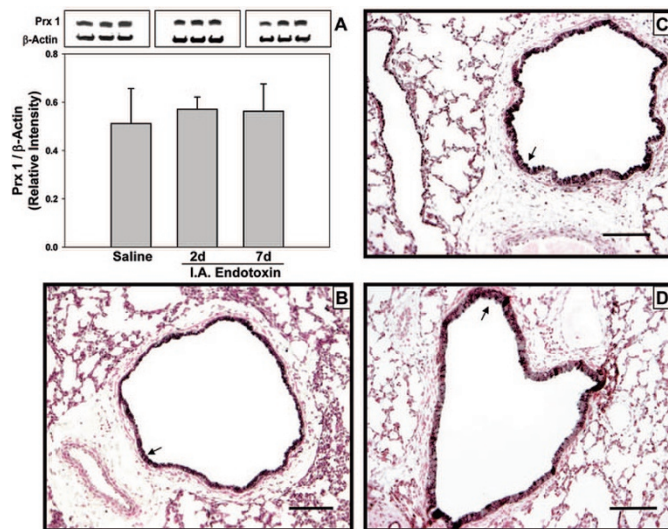

Figure 3. Prx 1 in lung tissue of fetal lambs after exposure to intra-amniotic endotoxin. (*A*) Representative results of Prx 1 measured by Western blotting in lung homogenates from fetal lambs given saline, intra-amniotic endotoxin (2 d) and (7 d) before premature delivery at 124 d gestation. Forty micrograms of protein for each sample was loaded and immunoblots were probed with anti-Prx 1 antibody and β-actin antibody sequentially. Molecular weights (kD) for Prx 1 and β-actin are 26 and 43, respectively. The graphs depict values (mean $\pm$ SD) of relative intensity of the Prx 1 bands normalized to β-actin values in saline control ($n = 9$), 2 d endotoxin ($n = 6$), and 7 d endotoxin ($n = 6$) exposed groups. (*B–D*) Photomicrographs of representative histologic staining for Prx 1 in paraffin-embedded lung tissue from each of the above three groups of fetal lambs using the diaminobenzidine method. Positively stained cells appear as dark brown to black shown primarily in the peri-bronchiolar epithelium (*arrow*). Magnification bar = 100 μm.
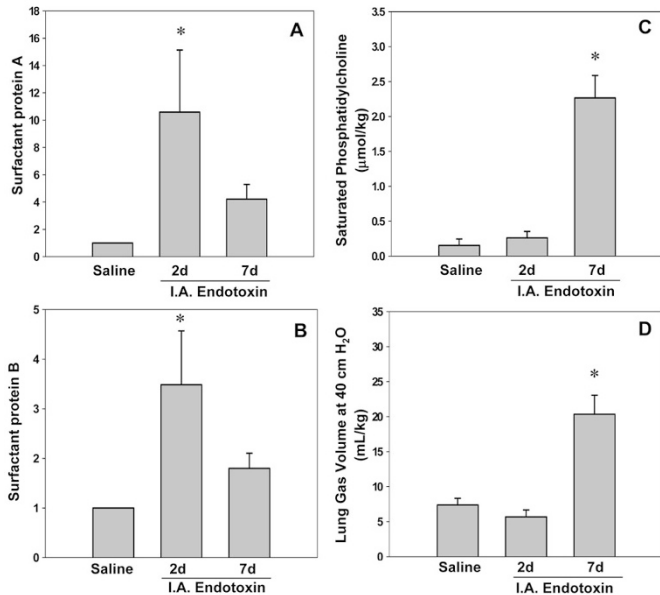
Figure 4. Indicators of lung maturation in fetal lambs after exposure to intra-amniotic endotoxin. (*A* and *B*) SP-A and -B mRNAs as measured by S1 nuclease protection assays in lung homogenate samples of saline controls ($n = 6$), 2 d ($n = 4$) or 7 d ($n = 5$) endotoxin-exposed animals. All values were internally normalized to L32 (ribosomal protein mRNA) before expressed as fold-increases above controls. (*C*) Saturated phosphatidylcholine concentrations in bronchoalveolar lavage fluid as measured by phosphorus assay and (*D*) lung gas volumes of pressure-volume deflation curves at 40 cm H_2O. Values are expressed as mean ± SD. *Statistically significant ($p < 0.05$) against controls by ANOVA.

the airspaces and in the fetal plasma. The oxidative stress was modest in contrast to the robust and more extensive inflammatory responses that were incited.

Bacterial lipopolysaccharide (LPS) induces chemokines, such as IL-8, that amplify the inflammatory cascade by recruiting neutrophils (22). Cytokines and LPS are also known to prime the increase in oxidative burst of neutrophils through activation of NADPH oxidase (23,24). Therefore, inflammation may lead to an increase in oxidant effects. However, there has been no direct evidence to date demonstrating oxidative responses in the fetus exposed to CAM. Although we found that significantly elevated BALF protein carbonyl concentrations were present in endotoxin-exposed lambs, the carbonyls were increased at 7 d but not at 2 d, when cytokine levels were very high. The maximal carbonyls were concomitant with the rise in MPO activity at 7 d, suggestive of a predominantly neutrophil-mediated oxidative response (15).

In many systems of oxidative injury, the markers of oxidative stress increase before cytokine increase suggesting that ROS signals augment the release of cytokines (25). In our CAM model, IL-1β, IL-8 mRNAs, and IL-8 protein were much higher at 2 d than at 7 d, indicating that the oxidant activity occurred after the primary inflammatory response. Cytokines can prime neutrophils *in vitro* but there is minimal increase in oxidative burst until these cells are subjected to further stimulation (23,24). Another possible explanation for the observed lag in oxidative response is that the inflammation matured neutrophil function. Even though these cells were shown to be functionally competent (8), they may not have

achieved the total potential for oxidant generation in the fetus. We recently reported that intra-amniotic endotoxin matured lung monocytes to macrophages and induced the expression of PU.1 transcription factor in lung-associated neutrophils at 2 d in fetal lambs (26). Another explanation could be a failure in the homeostatic response to counterbalance effects of oxidants at the point when antioxidant capacity of the immature fetus has been exhausted. Inflammatory cells recruited to the fetal lung by endotoxin exposure were still activated at 7 d (27), which may contribute to a persistence in oxidant activity. Moreover, based on our previous study that showed the peak in apoptosis and necrosis of alveolar inflammatory cells occurring between d 5 and 7 (8), a delayed clearance of these cells could result in cellular release of hydrolytic enzymes and oxidants. These events may explain our observation of a simultaneous increase in MPO activity and protein carbonyls in BALF at 7 d.

In CAM, the fetal responses to oxidative stress in endotoxin-induced lung inflammation were measurable in the fluid from the airspaces. Any oxidant effects were much less evident in the lung parenchyma as there were no significant increases in markers of oxidative injury in endotoxin-exposed fetal lambs compared with controls. Specifically, there were no changes in lung tissue protein carbonyl content, SOD activity, and Prx 1 expression (Figs. 2 and 3). Higher doses of intra-amniotic endotoxin caused increased expression of anti-oxidant enzymes, indicating that there may be dose-dependent effects that influence the extent of lung oxidative injury (11). Furthermore, another study using HSP 70 as an indicator of oxidative injury showed very early (5 h) and transient increased expression only in conducting airway epithelia (8). The lack of change in Prx 1 is an important finding that has not been shown before in fetal lungs in response to endotoxin. Prx 1 is one of six peroxiredoxins, a ubiquitous family of thiol-specific enzymes, involved in detoxifying hydrogen peroxide and organic hydroperoxides (19,28). Only high concentrations of hydrogen peroxide cause an increase in the expression of Prx 1, suggesting that Prx 1 is not significantly modulated by mild oxidative stress (29). Therefore, oxidative stress was minimal in the fetal lungs in response to CAM. Besides scavenging for ROS, peroxiredoxins are also crucial in mediating oxidant signaling of various cellular functions of proliferation, differentiation, and apoptosis (19,28). The distribution of Prx 1 primarily in the peri-bronchiolar epithelia (Fig. 3) is similar to that of the human lung (30). The appearance of this enzyme already at 124 d gestation with a similar pattern to that of the term lamb (data not shown), implies that it may have a critical role in lung development before birth. However, it does not seem to be directly involved in the endotoxin-induced lung maturational process (Fig. 3). Our study emphasized the detection of protein oxidation markers because they have been shown to be reliable indicators of oxidative stress (15,17) but evaluation for lipid peroxidation and comparing its extent with protein oxidation and nitration in chorioamnionitis would be useful in future experiments.

A unique feature of fetal inflammation in CAM is that the oxidative process occurs in a relatively hypoxic intrauterine environment. Not much information is available on fetal responses to oxidative stress at lower oxygen states. For fetal

lambs, the mean oxygen tension at which Hb is half saturated approximates 14 mm Hg, which gradually increases to 19 mm Hg at term gestation (31). The Michaelis-Menten constant of NADPH oxidase for oxygen is 15 mm Hg (32), which suggests that generation of ROS can occur *in utero*, and we found direct evidence of oxidant activity in fetal alveolar and systemic compartments. It is plausible that reduced oxygen tension may limit oxidative stress by generating less ROS. In addition, lower oxygen tension affects neutrophil response to stimulation by cytokines (33). The minimal oxidative injury caused by intra-amniotic endotoxin in the fetus may be reflective of the modulatory effects of the lower oxygen in the intrauterine environment.

In some developing systems, antioxidant enzymes increase in parallel to the surfactant system during late gestation suggesting that oxidative stress may be involved in fetal lung maturation (34). Furthermore, in response to hyperoxia, the enzymes and surfactant increase in the presence of a growth factor (35). The role of ROS as a mediator in signaling this maturational process is still conjectural. Our results demonstrate that maximal surfactant mRNA induction occurs before BALF carbonyls increase and there are insignificant changes in lung tissue oxidative markers. Thus, oxidants are not major contributors in mediating the early gestational lung maturation induced by endotoxin in CAM.

Intrauterine inflammation is associated with both local and systemic inflammatory responses in the fetus (2–4,12). Intratracheal endotoxin given to mechanically ventilated animals induced pulmonary and systemic cytokines as well as expression of acute phase reactant proteins (36,37). In this study, we demonstrate for the first time that a systemic oxidative stress response may be initiated by intra-amniotic endotoxin.

We demonstrated modest fetal responses to oxidative stress induced by bacterial endotoxin. Many *in vitro* studies showed that phagocytes when primed by LPS or cytokines become activated but did not mount a substantial increase in oxidative burst until further stimulated (23,24). Conceivably, a greater oxidant load would be detectable if the fetus were exposed again. Recently, Van Marter *et al.* (38) reported that infants with prior exposure to CAM, who had repeated infections postnatally, had a much greater risk of developing BPD.

In summary, our experiments showed evidence of fetal responses to oxidative stress in both alveolar and systemic compartments, following endotoxin-induced lung inflammation in CAM. The oxidant load was modest and pulmonary involvement less extensive when compared with marked lung inflammation and early gestational lung maturation.

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