0031-3998/03/5304-0679
PEDIATRIC RESEARCH

Vol. 53, No. 4, 2003
*Printed in U.S.A.*

# Intraamniotic Endotoxin Increases Lung Antioxidant Enzyme Activity in Preterm Lambs

ILENE R.S. SOSENKO AND ALAN H. JOBE

*Division of Neonatology, Department of Pediatrics, University of Miami School of Medicine, Miami, Florida, U.S.A. [I.R.S.S.], and Cincinnati Children's Hospital Medical Center, Division of Pulmonary Biology, Cincinnati, Ohio, U.S.A. [A.H.J.]*

## ABSTRACT

Proinflammatory stimulation resulting from intraamniotic endotoxin improves lung function, increases surfactant protein mRNA expression and protein content, increases alveolar and lung saturated phosphatidylcholine pools, and accelerates lung morphometric maturation in fetal sheep. The mechanism for induction of lung maturation does not involve an increase in fetal cortisol. The effect of endotoxin on the maturation of a different lung system, the antioxidant enzyme (AOE) system, has not been examined. Therefore, we hypothesized that intraamniotic endotoxin would produce acceleration of AOE activity in fetal sheep at similar doses and schedule of administration to those producing lung functional and surfactant maturation. In a dose-response study, intraamniotic injections of 1, 4, 20, or 100 mg of *Escherichia coli* 055:β5 endotoxin were administered 7 d before preterm delivery of sheep at 125 d gestation. In a study examining time interval of administration before delivery, 20 mg of endotoxin was injected at either 1-, 2-, 4-, 7-, or 15-d intervals before preterm delivery at 125 d. Doses of 1–100 mg of endotoxin produced significant increases in glutathione peroxidase activity; doses of 4–100 mg significantly increased catalase activity, whereas doses of 20–100 mg resulted in significant increases in total superoxide dismutase activity. Glutathione peroxidase activity was elevated within 2 d, whereas superoxide dismutase was increased by 4 d and catalase activity increased by 7 d after endotoxin. No AOE increases were sustained for 15 d. Endotoxin increased fetal lung AOE activity at similar dosing amounts and intervals to those producing maturation of lung function and surfactant. Thus, mechanisms involving proinflammatory stimulation, unrelated to glucocorticoid hormones, can induce maturation of the AOE system of the fetal lung. **(*Pediatr Res* 53: 679–683, 2003)**

**Abbreviations**

**AOE,** antioxidant enzymes
**SOD,** superoxide dismutase
**CAT,** catalase
**GP,** glutathione peroxidase
**PC,** phosphatidylcholine
**SP,** surfactant protein

Chronic infection and chorioamnionitis are associated with a decreased incidence of hyaline membrane disease (1). The mechanism of this apparent acceleration in lung maturation may be related to an increase in endogenous glucocorticoid hormones or to the stimulatory effects of inflammatory cytokines on lung maturation (2, 3). Paradoxically, however, infants exposed to chorioamnionitis may have an increased likelihood of developing chronic lung damage, or bronchopulmonary dysplasia, thought to be related to the initiation and propagation of inflammation as a result of cytokine exposure (1).

Antenatal exposure to the proinflammatory stimulation produced by intraamniotic endotoxin accelerated lung maturation in sheep. Specifically, antenatal exposure to intraamniotic endotoxin improved lung mechanics, increased saturated PC in lung tissue and alveolar pools, increased surfactant protein mRNA and surfactant proteins, and changed lung morphometry, resulting in fewer, larger alveoli with thinner walls and a decreased volume of interstitial tissue (4–8). The lung maturational changes brought about by intraamniotic endotoxin appear not to be mediated by cortisol, as no increases were found in cord plasma cortisol after endotoxin administration (4). In addition, evidence of chorioamnionitis, with increased inflammatory cell infiltration in amnion and chorion, preceded changes in lung function, surfactant, and morphometry (7).

The lung AOE system is critical in protecting the lung from free radical injury during neonatal adaptation. In a time course similar to the maturation of the surfactant system, fetal lung AOE activity, specifically SOD, CAT, and GP, increases during the final 15–20% of intrauterine life (9, 10). In terms of

Received June 17, 2002; accepted December 18, 2002.
Correspondence: Ilene R.S. Sosenko, M.D., Department of Pediatrics (R-131), University of Miami School of Medicine, PO Box 016960, Miami, FL 33101, U.S.A.; e-mail: isosenko@miami.edu
Supported in part by grant HL-65397 from the National Heart, Lung and Blood Institute.

**DOI: 10.1203/01.PDR.0000055769.19891.C4**

hormonal influences on AOE and surfactant maturation, both systems are accelerated by antenatal glucocorticoids (11, 12), whereas thyroid hormones appear to stimulate surfactant maturation while delaying late gestational AOE activity increases (13).

The dose-response and time course of the effects of intraamniotic endotoxin in fetal sheep on lung function and saturated PC pools have been reported (4). The present study investigates the effects of dose and time course schedules of intraamniotic endotoxin on AOE activities. Because of the similarities in maturational pattern and hormonal stimulation of both the lung surfactant and AOE systems (9–12), it was hypothesized that intraamniotic endotoxin would also promote lung AOE maturation with a similar time course of administration and at similar doses.

## METHODS

***Experimental animals, endotoxin doses, and dosing schedules.*** Approval for these studies was obtained from the appropriate animal care and use committees in Australia and at the Cincinnati Children's Hospital. Date-mated singleton Merino ewes were bred in Western Australia, where the tissue for these studies was collected (4). For dose-response studies, at gestational d 100, ewes were weighed and randomized into different endotoxin dose groups. Specifically 1, 4, 20, or 100 mg of intraamniotic endotoxin or saline (as controls) was administered on gestational d 118, 7 d before preterm delivery at gestational d 125. Similarly, for treatment interval studies, ewes were selected at gestational d 100 for randomization to different intervals from endotoxin injection until preterm delivery. For these studies, 20 mg of intraamniotic endotoxin (or saline) was administered at either 1, 2, 4, 7, or 15 d before preterm delivery at 125 d of gestation.

The endotoxin (*Escherichia coli* 055:$\beta$5; Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in saline at a concentration of 20 mg/mL, filtered, and then diluted as necessary to a final volume of 2 mL (or 5 mL for the 100-mg endotoxin dose). With the ewes gently restrained in the shearing position, and under ultrasound guidance, a 20-gauge spinal needle was inserted transabdominally into the amniotic cavity, and fluid was initially withdrawn and assumed to be amniotic fluid if opalescent and particulate, after which the endotoxin or saline dose was administered. Subsequently, the fluid withdrawn from the ewe was verified to be amniotic fluid rather than allantoic fluid by analyzing $Na^+$ and $Cl^-$ concentrations (4). Only animals that had received verified intraamniotic injections of endotoxin were analyzed for AOE activity.

The investigators delivering and providing postdelivery care of the lambs were blinded to the treatment groups. At gestational d 125, the lambs were delivered by cesarean section and ventilated for 40 min as described previously (4).

***Antioxidant enzyme measurements.*** Lungs then were removed from the chest, and portions of the right lower lobe were excised and frozen in liquid nitrogen. Although no perfusion of the vasculature was performed before obtaining lung samples, the lambs had been bled so that minimal residual blood remained in the lung tissue. Thawed lungs were weighed, homogenized in cold saline (1:10–1:20 wt/vol; Brinkmann Polytron, Westbury, NY, U.S.A.), centrifuged at 15,000 rpm for 45 min, and then assayed by standard spectrophotometric techniques for activities of total SOD (with the xanthine/xanthine oxidase method, whereby SOD activity was assayed by inhibition of the reduction of cytochrome *c* in the xanthine oxidase reaction) (14), of CAT (using the rate of reduction of hydrogen peroxide at 240 nm) (15), and of GP (using the rate of oxidation of NADPH at 340 nm using cumene hydroperoxide as substrate) (16). Lung homogenates were also assayed for total DNA content (17). Purified standards for SOD, CAT, and DNA assays were obtained from Sigma Chemical; GP standard was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

***Statistical analysis.*** Results of the AOE activity analyses were calculated as units of enzyme activity per milligram of DNA. Data were expressed as mean ± SEM. Statistical analysis was performed using ANOVA, using the Student-Newman-Keuls method for multiple comparisons, comparing each one of the different dosages in the dose-response study or each of the different treatment intervals with each other in the treatment interval study. For groups that were not normally distributed, the Kruskal-Wallis one-way ANOVA of ranks was performed, with pairwise multiple comparison procedures using Dunn's method. A $p$ value of $<0.05$ was considered statistically significant (18). In addition, individual values for saturated PC reported elsewhere (4) from the dose-response study were compared with individual values for SOD, CAT, and GP activity by linear regression.

## RESULTS

***Dose-response study.*** The three AOE demonstrated quite similar responses to increasing doses of endotoxin, administered 7 d before preterm delivery at 125 d of gestation. Although mean values for total SOD activity increased sequentially after doses of 1 and 4 mg of endotoxin, doses of 20 and 100 mg produced significant increases above control values (Fig. 1*A*). For CAT activity, endotoxin doses of 4, 20, and 100 mg resulted in significant increases above control values (Fig. 1*B*). Unlike the other two AOE studied, GP activity was significantly increased with all doses of endotoxin (Fig. 1*C*).

The pattern of response of the three AOE is shown in Figure 2. SOD and CAT had a similar pattern of response to increasing doses of endotoxin, with sequential (although not significantly different) increases with each dosing increment. GP, however, demonstrated significant increases as a result of all doses from 1 to 100 mg of endotoxin, but these increases did not follow a dose-response pattern.

***Time course study.*** Total SOD activity did not increase when endotoxin was given at 1 or 2 d before preterm delivery at 125 d of gestation. Significant increases in SOD occurred when endotoxin was administered 4 and 7 d before delivery; this significant elevation in SOD activity was not maintained when a 15-d interval elapsed between endotoxin administration and preterm delivery (Fig. 3*A*). No increases in CAT activity occurred when endotoxin was given 1, 2, or 4 d before delivery. A significant increase in CAT activity was seen when

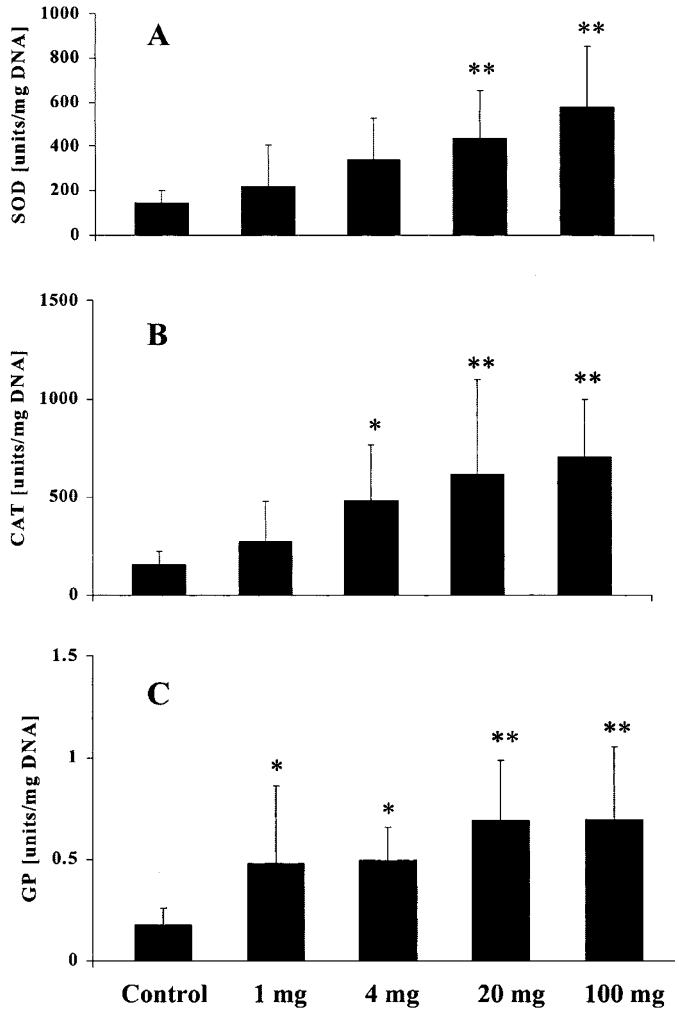


**Figure 1.** *A*, values for total SOD activity (units/mg DNA) after 1, 4, 20, or 100 mg intraamniotic endotoxin or saline (control) injection 7 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 5$–12/group; $^{**}p < 0.01$ *vs* control. *B*, values for CAT activity (units/mg DNA) after 1, 4, 20, or 100 mg intraamniotic endotoxin or saline (control) injection 7 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 5$–12/group; $^{**}p < 0.01$ *vs* control; $^{*}p = 0.053$ *vs* control. *C*, values for GP activity (units/mg DNA) after 1, 4, 20, or 100 mg intraamniotic endotoxin or saline (control) injection 7 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 5$–12/group; $^{*}p < 0.05$ *vs* control, $^{**}p < 0.01$ *vs* control.

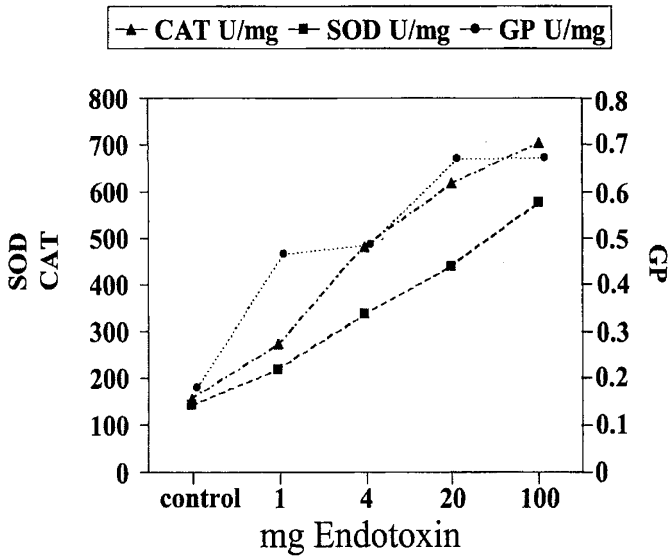


**Figure 2.** Comparative dose-response pattern of 1–100 mg endotoxin intraamniotic injection or saline (control) at 7 d before preterm delivery at 125 d gestation for total SOD, CAT, and GP activity. Mean values of SOD and CAT increased sequentially with each increase in endotoxin dose. Mean values for each dose were not significantly different from each other.

endotoxin was administered 7 d before delivery, but, like SOD, this increase was not maintained at 15 d before delivery (Fig. 3*B*). Finally, significant increases in GP occurred when endotoxin was given as early as 2 d before delivery and was also seen at treatment intervals of 4 and 7 d. Similar to the other two AOE, the significant increase in GP activity seen at the 2-, 4-, and 7-d treatment intervals was not maintained at the 15-d treatment interval before delivery (Fig. 3*C*).

***Correlation of AOE with saturated PC.*** When individual saturated PC values for lung homogenates (4) from the dose-response study were compared by linear regression with individual values for the three AOE, the results demonstrated a significant linear relationship between lung homogenate saturated PC and SOD, CAT, and GP (Fig. 4).

## DISCUSSION

We described the AOE activity response to varying dosages of intraamniotic endotoxin, administered to ewes at 7 d before preterm delivery at 125 d of gestation. In addition, the AOE activity response to one dose (20 mg) of endotoxin injected at different treatment intervals, 1–15 d, before preterm delivery at gestational d 125 was evaluated. These same dose-response and time-interval protocols had produced changes in lung function and surfactant that were similar to the AOE responses (4). Although the lowest dose of 1 mg (as well as each of the higher doses studied) produced similar improvements in lung compliance and ventilation efficiency index, and increased saturated PC in alveolar wash and lung tissue, this low dose resulted in a significant elevation in GP activity only. CAT activity was not increased for an endotoxin dose lower than 4 mg, and SOD activity was not increased for a dose lower than 20 mg. Nevertheless, the AOE values demonstrated significant linear relationships with lung saturated PC (Fig. 4). Thus, functional as well as surfactant indices of lung maturation appear to be more sensitive to effects of endotoxin than two of the three AOE studied.

In terms of time interval from endotoxin treatment to delivery, GP activity increased significantly as early as 2 d before delivery, similar to increases in the mRNAs for the surfactant proteins, but earlier than changes in lung function. SOD activity was increased after a 4-d interval after endotoxin, later than PC changes but similar to lung functional maturational effects. Finally, CAT activity increased by 7 d after endotoxin injection, later than the changes in lung compliance, ventilation efficiency index, and surfactant PC. None of the AOE activities remained elevated by 15 d after endotoxin injection, although all functional and surfactant PC measurements remained significantly increased at 15 d.

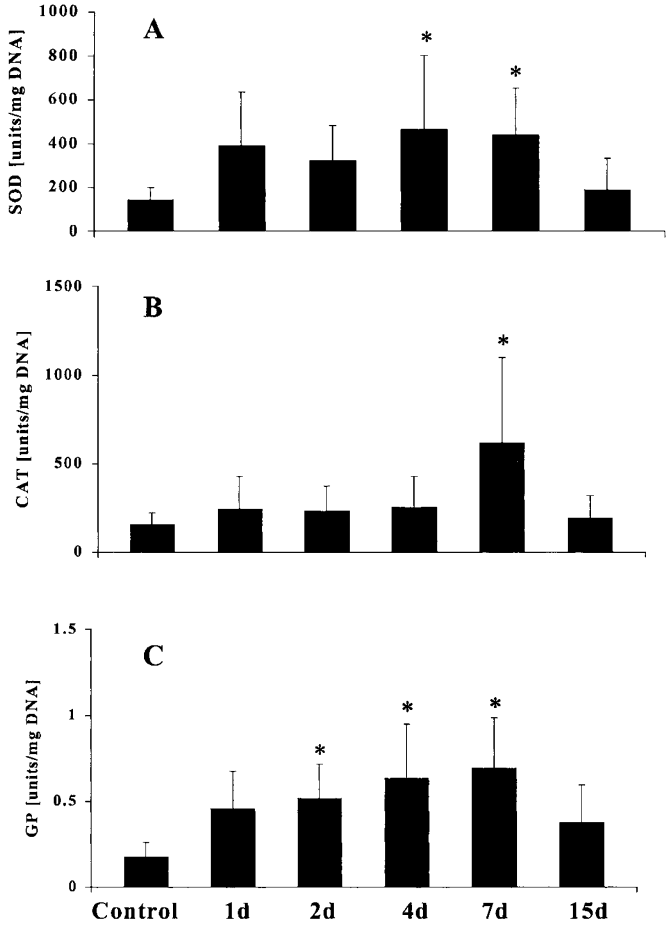


**Figure 3.** *A*, values for total SOD activity (units/mg DNA) after 20 mg intraamniotic endotoxin or saline (control) injection at 1, 2, 4, 7, or 15 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 6$–12/group; $*p < 0.05$ *vs* control. *B*, values for CAT activity (units/mg DNA) after 20 mg intraamniotic endotoxin or saline (control) injection at 1, 2, 4, 7, or 15 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 6$–12/group; $*p < 0.05$ *vs* control. *C*, values for GP activity (units/mg DNA) after 20 mg intraamniotic endotoxin or saline (control) injection at 1, 2, 4, 7, or 15 d before preterm delivery at 125 d gestation. Mean ± SEM; $n = 6$–12/group; $*p < 0.05$ *vs* control.

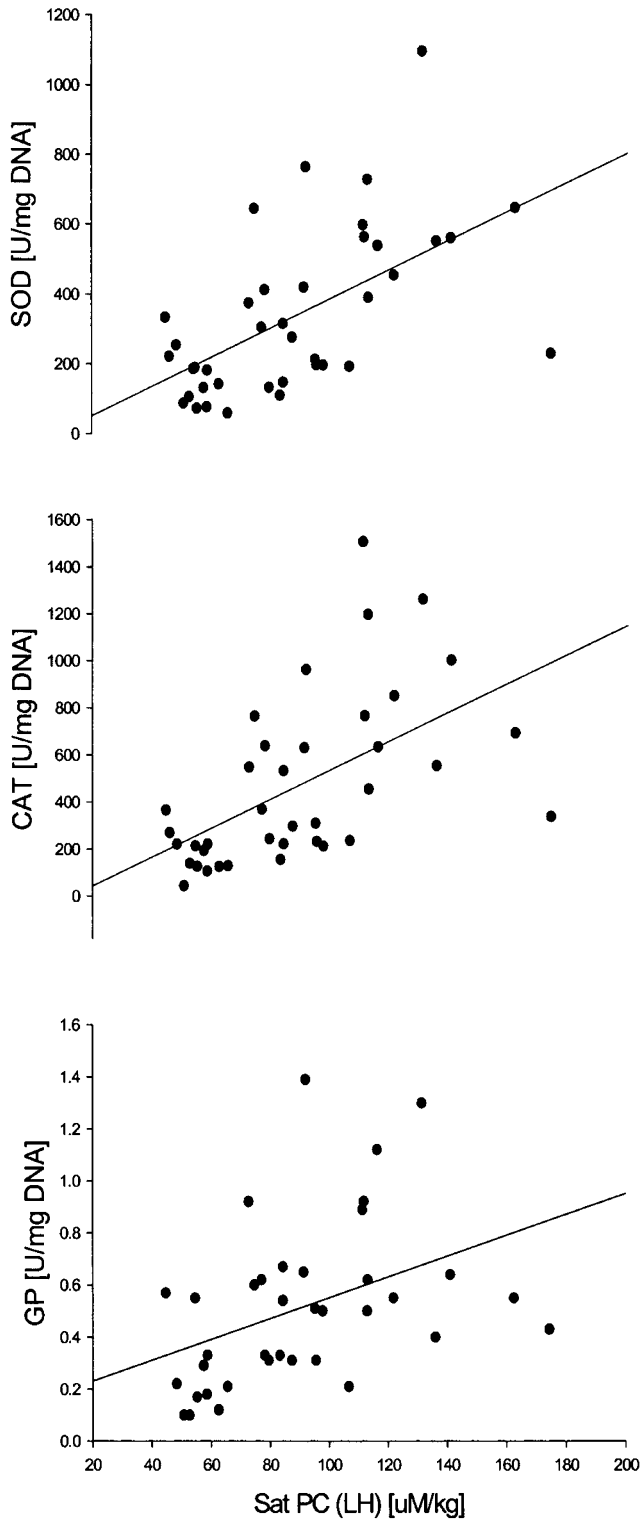


**Figure 4.** Linear regression analysis of individual lung homogenate (*LH*) saturated PC (*Sat PC*) values in μM/kg [reported in Jobe *et al.* (4)] *vs* total SOD (*Top*), CAT (*Middle*), and GP (*Bottom*) activity in units/mg DNA for dose-response study. Regression for Sat PC *vs* SOD: $r^2 = 0.58$, $p < 0.001$; Sat PC *vs* CAT: $r^2 = 0.558$, $p < 0.001$; Sat PC *vs* GP: $r^2 = 0.404$, $p = 0.013$.

By 2 d after endotoxin injection, mRNAs for SP-A, SP-B, and SP-C were found to be maximally induced; SP-D mRNA was increased 4-fold 1 d after endotoxin administration. These SP mRNA elevations preceded increases in SP content in the alveolar pool as well as processing of SP-B and improvements in lung function (6). Although mRNA for the AOE were not examined in the present investigation, the elevation in GP activity by d 2 after endotoxin and SOD by d 4 suggest that increases in AOE mRNA occurred by 1–2 d after endotoxin injection, in an analogous time course to the SP mRNAs.

The similar time course and dose response of AOE activity, surfactant phospholipids and proteins, and functional indices of lung maturation to intraamniotic endotoxin were consistent with our hypothesis. The late gestational increase in AOE activity during the final 15–20% of gestation in parallel with the maturational pattern of surfactant has been reported for several species, including the lamb (9, 10). In terms of response to hormones, antenatal administration of dexamethasone to pregnant rats resulted in an acceleration of both surfactant and AOE system maturation, whereas blockade of endogenous glucocorticoids with metyrapone produced delays in both surfactant and AOE system maturation (11, 12). When

a single dose of betamethasone was injected intramuscularly into preterm lambs *in utero*, stimulation of AOE activity was seen within 24 h of injection and persisted for 7 d, and was associated with a decrease in evidence of oxidative stress as measured by lipid hydroperoxide formation (19). The time course response of AOE activity to intraamniotic endotoxin differed somewhat from what was seen for intramuscular fetal injection of steroid, because no AOE activity was increased within 24 h, and only GP activity was elevated by d 2. However, the magnitude of elevation of AOE in response to endotoxin was greater than that reported for glucocorticoids (increases in AOE within 40–60% range) (19). Endotoxin induced increases in SOD activity as high as 3-fold (for 100-mg endotoxin dose), increases in CAT greater than 2-fold (for 20-mg dose, 7-d time interval), and in GP greater than 3-fold (for 100-mg endotoxin dose), suggesting a more potent maturational effect for endotoxin on AOE, as well as on lung function, saturated PC, and SPs, compared with the effect of glucocorticoids (5). Interestingly, both antenatal glucocorticoid exposure and endotoxin administration resulted in changes in lung morphometry that were comparable both in nature and magnitude, consisting of fewer and larger alveoli with thinner walls and a decreased volume of interstitial tissue (8).

In this model of intraamniotic endotoxin administration, no increases in fetal cortisol were found in response to endotoxin exposure, indicating that endotoxin was not stimulating AOE activity via a glucocorticoid mechanism (4). Instead, evidence of inflammation after intraamniotic endotoxin occurred early, with an increase in inflammatory cell infiltrate seen in the amnion or chorion within 5 h of endotoxin administration, which persisted for at least 25 d. In association with these early signs of inflammation after endotoxin, elevations were found in mRNAs for IL-1$\beta$, IL-6, IL-8, and tumor necrosis factor-$\alpha$ in amnion or chorion and in the lung (7). Because in this model of intraamniotic endotoxin injection, inflammation and increases in cytokine mRNAs were produced before changes in lung maturation, including the AOE maturation reported here, a logical hypothesis is that the proinflammatory cytokines are the mediators for the acceleration of lung maturation. In both rabbits (3) and lambs (20) intraamniotic IL-1$\alpha$ can produce improved postnatal lung function after preterm delivery. In a recent study, the effects of intraamniotic IL-1$\alpha$ and IL-1$\beta$ were compared with the effects of intraamniotic endotoxin, each administered 7 d before preterm delivery of lambs at gestational d 125. Both cytokines produced significant increases in inflammation, lung compliance and lung gas volumes, alveolar saturated PC pool size, and SP mRNA expression (21). It is presently unknown whether IL-1 will produce similar effects compared with endotoxin on AOE maturation as well. It is also unknown whether the increases in AOE activity found in response to endotoxin represent increased AOE synthesis or activation secondary to inflammatory stimuli. Another possible mechanism for stimulation of AOE activity by antenatal endotoxin may relate to its ability to produce oxidant lung injury. Within 5 h of endotoxin administration in sheep, heat shock protein expression was found in fetal lung epithelial cells and large airways, which may represent evidence of oxidant injury (22).

## CONCLUSIONS

The results of this study demonstrate that the AOE, in a fashion analogous to lung functional maturation, surfactant system maturation, and morphometric maturation, is positively influenced by antenatal exposure to endotoxin. The signaling for early maturation of this crucial lung system can be positively influenced by the proinflammatory stimulation of endotoxin.

***Acknowledgments.*** The authors thank Machiko Ikegami, John Newnham, and Timothy Moss for helping with the management of the animals and for collecting the tissue for these studies.

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