Oxytocin-induced labor augments IL-1β-stimulated lung fluid absorption in fetal guinea pig lungs

Prem K. Nair, Tianbo Li, Reshma Bhattacharjee, Xin Ye, and Hans G. Folkesson

Department of Physiology and Pharmacology, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio

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Cytokines, such as interleukin-1 (IL-1), can be important during pregnancy, because IL-1 has been detected in the placenta and amniotic fluid (12, 44). In this regard, interleukins have been proposed as a signal for parturition onset, as cytokines can stimulate prostaglandin biosynthesis (41). During normal pregnancies, IL-1 is present in the amniotic fluid in low concentrations, but during preterm labor much higher amniotic fluid IL-1 concentrations are present (44). Intrauterine infection/inflammation may further elevate cytokine levels, the highest activity being attributable to IL-1β (44). Many prematurely born infants suffer from fetal infection and/or respiratory distress syndrome (RDS), both of which are important reasons for mortality of premature babies. Recent human data suggest that ion transport abnormalities across lung epithelial cells, i.e., an incomplete transition of lung fluid secretion to lung fluid absorption, can contribute to RDS development (1). Factors accelerating ion transport across alveolar cells may thus have benefits for the resolution of RDS. Recent experimental data have suggested that proinflammatory cytokines could also promote lung maturation (9, 14, 26, 47). IL-1α increased mRNA surfactant protein expression and improved lung compliance in fetal rabbits (9). IL-1α also improved postnatal lung function with increased surfactant pool size without eliciting systemic inflammatory responses in lambs (14, 47). We recently reported that IL-1β pretreatment of timed-pregnant guinea pigs induced fluid absorption in fetal lungs, sodium transporter protein, and β-adrenergic expression via hypothalamic-pituitary-adrenal gland axis stimulation (48).

Lung fluid absorption occurs secondarily to transepithelial Na+ absorption (3, 5, 8, 17, 22, 31, 35, 37, 45, 48, 49) and is driven by basolateral Na-K-ATPases (16, 23) and apical epithelial sodium channels (19, 22, 30, 31). Fluid absorption occurs in near-term lungs (6, 17, 35, 37, 45) and is induced/stimulated by cortisol and triiodothyronine (20, 48) and labor-released epinephrine (17, 35, 45).

Our hypothesis was that since the lung matured after IL-1β pretreatment, labor induction would be beneficial to the neonate’s well-being. We used our recently published model (48) of maternal IL-1β injections, which simulates the presence of IL-1β in the maternal blood circulation during intrauterine inflammation, and combined these findings with those in an earlier report on oxytocin induction of labor (35). In this study, we tested whether labor in itself additively stimulated IL-1β-induced lung fluid absorption. Because labor-induced epinephrine release stimulates lung fluid absorption at birth in guinea pigs (17, 35), our first aim was to investigate whether lung fluid absorption becomes more sensitive to endogenous β-adrenergic stimulation after the combined IL-1β and oxytocin treatments. A second aim was to study development of the fractional amiloride sensitivity of lung fluid absorption under normal conditions and after oxytocin induction of labor with and without IL-1β. We also carried out parallel sets of studies after cortisol synthesis inhibition by metyrapone with and without IL-1β- and oxytocin-induced labor. Sodium transporter expression, i.e., the epithelial sodium channel (ENaC) and Na-K-ATPase, was also examined after oxytocin induction of labor with and without IL-1β.

MATERIALS AND METHODS

Animals

Preterm Dunkin-Hartley guinea pigs (Hilltop Lab Animals, Scottdale, PA) were used (n = 351 obtained from 89 litters). Timed-pregnant guinea pigs were maintained on a 12:12-h day-night rhythm and had free access to food (Purina standard guinea pig chow; Copley...
Feed, Copley, OH) and tap water. The Institutional Animal Care and Use Committee at the Northeastern Ohio Universities College of Medicine approved this study.

**Solutions and Chemicals**

**Instillate solutions.** We prepared the 5% albumin instillate by dissolving 50 mg/ml bovine serum albumin (BSA; Calbiochem-Novabiochem, La Jolla, CA) in 0.9% NaCl. In some studies, the sodium channel inhibitor, amiloride hydrochloride (1 mM; MP Biomedicals, Aurora, OH) or the general β-adrenoceptor antagonist, propranolol (0.1 mM; MP Biomedicals), was added to the 5% albumin instillate.

**Injection solutions.** We prepared the IL-1β pretreatment solution by dissolving 10 μg of rat recombinant IL-1β (Sigma Chemical) in 0.1% BSA in 0.9% NaCl. Dissolved IL-1β was aliquoted into vials (each containing 500 ng) and stored frozen at −20°C.

We prepared the 11-β-hydroxylase inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma Chemical) pretreatment solution by dissolving 62.5 mg/ml metyrapone in 24% ethanol in 0.9% NaCl. We have previously demonstrated that injection of 24% ethanol in 0.9% NaCl does not affect lung fluid absorption in adult guinea pigs (33).

**Pretreatment of the Timed-Pregnant Guinea Pigs**

**IL-1β.** Guinea pigs of 59 and 66 days gestation were injected subcutaneously in the dorsal neck daily with 250 ng/kg body wt of IL-1β for 3 days. The IL-1β dose was selected after an initial dose-response study was carried out (data not shown). Control timed-pregnant guinea pigs were given injections of 0.9% NaCl at the same times. The lung fluid absorption experiment was carried out on the morning of the last pretreatment day.

**Metyrapone.** Pretreatment with metyrapone was carried out over 3 days concurrently with IL-1β treatments (33, 48). Subcutaneous metyrapone injections were given twice daily in the morning and evening (25 mg/kg body wt for a total daily dose of 50 mg/kg body wt) to guinea pigs of 59 and 66 days gestation. On the morning of the third day, one-half the daily total dose was given. The metyrapone dose was adapted from its higher ranges of clinical dosage.

**Oxytocin.** Labor was induced by subcutaneous oxytocin injections (1 mg/kg body wt; Phoenix Pharmaceutical, St. Joseph, MO) every 15 min for 45 min (35). Fetuses were delivered by abdominal hysterotomy (see **Surgical Preparations**) after 45 min, if normal vaginal delivery did not occur. Timed-pregnant guinea pigs with 1 day of gestation remaining usually delivered their fetuses vaginally within 45 min after oxytocin, whereas fetuses of timed-pregnant guinea pigs with 8 days gestation remaining were delivered by abdominal hysterotomy. The uterus was significantly contracted upon postmortem examination in the guinea pigs that received the oxytocin without delivery of the fetuses vaginally, i.e., the 61-day gestation animals. This was taken as evidence that oxytocin had induced labor in the 61-day gestation guinea pigs. The oxytocin dose was adapted from the clinical dose for labor induction in humans (7).

**Surgical Preparations**

Timed-pregnant guinea pigs were anesthetized by intraperitoneal (ip) injections of pentobarbital sodium (120 mg/kg body wt; Abbott Laboratories, Chicago, IL) and killed by intracardiac injections of 60 mg of pentobarbital sodium. A laparotomy was rapidly done, and the fetuses were carefully delivered. Amniotic fluid was collected from three 61-day-gestation guinea pigs in each condition to determine whether IL-1β caused inflammatory cell influx. The umbilical cord was ligated to prevent bleeding. The fetuses were immediately killed by pentobarbital sodium (12 mg ip) mixed with 500 IU heparin (Elkins-Sinn, Cherry Hill, NJ). The guinea pig fetuses were weighed immediately after delivery. The 61-day-gestation fetuses weighed 69 ± 12 g, whereas 68-day gestation fetuses weighed 95 ± 21 g. These weights were very similar to previously published fetal weights for the studied gestation ages in the guinea pig (28).

After death, an endotracheal tube (PE-190; Becton Dickinson, Parsippany, NJ) was inserted through a tracheotomy. The endotracheal tube was immediately connected to a constant O2 flow (O2 fraction 1.0; Praxair, Akron, OH), and the lungs were expanded by adjusting the O2 flow to a constant positive airway pressure (CPAP) of 5 cmH2O. The entire surgical procedure after death required 5 min. The animals were placed in between heating pads to maintain body temperature. A temperature probe measured body temperature, and heating was adjusted to maintain body temperature at 37–38°C. Airway pressure was continuously monitored by calibrated pressure transducers, analog-to-digital converters, and amplifiers (ADInstruments, Grand Junction, CO).

**Endothelial and Epithelial Protein Permeability**

Seven newborn guinea pigs were anesthetized and catheterized in the jugular vein via a 0.58-mm (inner diameter) catheter (PE-50, Becton Dickinson). A solution containing 2.5 mg/ml fluorescein-isothiocyanate-(FITC)-conjugated dextran 70,000 (FD70, Sigma) was injected intravenously (iv). The FD70 solution was run through a gel filtration column (D-Salt Excellulose Plastic Desalting Column; Pierce, Rockford, IL) before the iv injection to separate free, unbound FITC from the injected FD70. The guinea pigs were allowed to rest for at least 15 min after the iv injection, and then a 0.5-ml blood sample was obtained via the catheter. Three guinea pigs were then killed and prepared for the 1-h lung fluid absorption experiment. The other four guinea pigs remained anesthetized until the 1-h experimental time had passed and were then killed, and samples were obtained. At this time, a blood sample was obtained for FD70 analysis, and the lungs were collected for determination of extravascular plasma equivalents as previously described (31, 34, 40, 46). Trichloroacetic acid (TCA) precipitation of plasma and urine samples verified that the FITC label (>97%) remained bound to the dextran tracer throughout the experimental time period.

To estimate the clearance of the vascular tracer FD70 into the lung extravascular compartments (interstitium and air spaces), total extravascular FD70 accumulation in plasma, alveolar fluid, and lung homogenate was measured spectrophotofluorometrically (Fluoroscan FL; Labsystems, Helsinki, Finland). Passage of FD70 molecules across the endothelial-epithelial barriers was considered to be equal to that of albumin, since they have similar molecular masses (70 vs. 68 kDa). The theory and method behind the plasma equivalent determination have been described in multiple published reports and in various animal species (31, 34, 40, 46). We calculated endothelial protein leak using the FD70 concentrations in the different compartments and applying them in the equation:

\[
FD70_{(extravascular, lung)} = FD70_{(total, lung)} - FD70_{(vascular space, lung)}
\]

To calculate FD70_{(vascular space, lung)} in the lungs, the averaged FD70 measurements in the plasma samples were multiplied by the blood volume in the lungs. The blood volume (Q_B) in the lungs at the end of the experiment was calculated from the following relation:

\[
Q_B = 1.039 \times (Q_H \times F_{W_H} \times H_{B_H})/(F_{W_S} \times H_{B_S})
\]

where Q_H is the lung homogenate weight, F_{W_H} is the lung water fraction, H_{B_H} and F_{W_S} are the hemoglobin concentration and water fraction, respectively, in supernatants obtained after lung homogenate centrifugation, and H_{B_S} is the hemoglobin concentration of the last blood sample. The lung water fraction was obtained by gravimetric measurements of the lung as previously described (5, 31, 34). The density of blood was considered to be 1.039 g/ml.
Lung Fluid Absorption Measurements

After surgery and connection to CPAP, the 5% albumin instillate (10 ml/kg body wt) was instilled into the lungs through the endotracheal tube, as follows. First, the fetuses were briefly disconnected from the CPAP circuit, and the lungs were deflated by gently aspirating residual air with the instillation syringe. The instillate was then instilled into the lungs and withdrawn. This procedure was repeated four times to allow thorough mixing of the instillate and preexisting fetal lung fluid. After the procedure, the mixed fluid was finally instilled. The fetuses were then reconnected to the CPAP and maintained on CPAP for the 1-h study period. A 0.1-ml sample of instillate-lung fluid mixture (initial solution) was retained in the syringe for protein measurement. After 1 h, a blood sample was obtained with an air-tight syringe by transversing the thoracic aorta in six fetuses for blood gas measurement. The final blood had a pH of 6.95 ± 0.11, a P02 of 97 ± 10 Torr, and a Pco2 of 65 ± 7 Torr. Plasma collected after centrifugation of the blood at 3,000 g was aliquoted in three separate vials, snap-frozen in liquid nitrogen, and stored at −80°C until used for hormone analyses. The lungs and heart were carefully removed en bloc through a midline sternotomy, and a sample of the remaining lung fluid was collected. We also differentially counted cells in alveolar fluid samples to assess whether IL-1β produced an inflammatory response in fetal lungs of six 61-day-gestation fetuses. After fluid collection, the lungs were snap-frozen in liquid nitrogen and stored at −80°C until used for further analyses. Total protein concentrations in instillates, initial, and final solutions were determined spectrophotometrically (Multiscan Microplate Reader, Labsystems) by the Lowry method (29) adapted for microtiter plates.

Lung fluid absorption or lung fluid secretion was calculated from the change in protein concentration over 1 h. This is possible because the lung epithelium is relatively impermeable to large molecules, such as albumin (molecular wt 67,000). Therefore, water movement (absorption or secretion) will result in a change in air space protein concentration. Because fetal lungs are fluid-filled in utero (6, 19), we expected that a certain fraction of fluid would still be present in the lungs at the time of experiment. This fluid is virtually protein-free and will not add protein to the instilled albumin concentration. Instead, it will dilute the instillate protein concentration and thereby influence lung fluid absorption calculations depending on the volume of endogenous fluid present at the different gestation ages. The preexisting fluid volume was used to correct the instilled protein concentrations by the dilution of the instillate that would occur if there would be fluid already in the lung before instillation of the 5% albumin instillate. Lung fluid absorption (LFA) or lung fluid secretion (LFS) was calculated from these equations:

\[
V_{\text{initial}} = \frac{(V_{\text{instilled}} \times C_{\text{instilled}})}{C_{\text{initial}}}
\]

\[
V_{\text{final}} = \frac{(V_{\text{initial}} \times C_{\text{final}})}{C_{\text{final}}}
\]

\[
\text{LFA or LFS} = \frac{[V_{\text{initial}} - V_{\text{final}}]}{V_{\text{initial}}} \times 100
\]

where \(V_{\text{instilled}}, V_{\text{initial}}, V_{\text{final}}\) are the volumes of instillate, initial, and final solutions, respectively. \(C_{\text{instilled}}, C_{\text{initial}}, C_{\text{final}}\) are the protein concentrations in the corresponding solutions.

Specific Protocols

Guinea pig fetuses at 61 and 68 days gestation (term = 69 days gestation) were studied. Day of conception was set to the day when timed-pregnant guinea pigs gave birth to their earlier litter, since guinea pigs enter estrus immediately after birth. All groups contained fetuses from at least two litters and all fetuses were surgically prepared as described in the Surgical Preparations section. All fetuses were studied on 5 cmH2O CPAP for 1 h following fluid instillation. Lung fluid absorption was measured as described in Lung Fluid Absorption Measurements.

Control. Preterm guinea pig fetuses of 61 and 68 days gestation were delivered by abdominal hysterotomy from timed-pregnant guinea pigs given daily saline injections. Control fetuses (n = 6 at each gestation age) received the 5% albumin instillate. In some studies, 10⁻⁴ M propranolol (β-adrenoceptor blocker, n = 6 at each gestation age) was added to the 5% albumin instillate to investigate the role of endogenous epinephrine. In other studies, we added 10⁻³ M amiloride (sodium channel blocker, n = 11 at 61 days gestation and n = 6 at 68 days gestation) to the 5% albumin instillate to investigate the role of Na⁺ transport. Amiloride at a concentration of 1 mM was used because a large fraction becomes protein bound and a significant fraction rapidly leaves the air spaces due to its low molecular weight (37, 49); therefore, the active alveolar concentration was likely lower. This amiloride concentration has been used in earlier studies (17, 34, 48).

IL-1β. Preterm guinea pig fetuses of 61 and 68 days gestation were delivered by abdominal hysterotomy from timed-pregnant guinea pigs pretreated with daily IL-1β injections (250 ng/kg body wt) for 3 days. Control fetuses (n = 6 at 61 and n = 8 at 68 days gestation) received the 5% albumin instillate. In some studies, 10⁻⁴ M propranolol (n = 10 at 61 and n = 7 at 68 days gestation) was added to the 5% albumin instillate. In other studies, 10⁻³ M amiloride (n = 6 at 61 and n = 7 at 68 days gestation) was added to the 5% albumin instillate.

Oxytocin. Guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 and 68 days gestation from both control (n = 6 at each gestation age) and IL-1β (n = 9 at 61 and n = 15 at 68 days gestation)-pretreated animals were given the 5% albumin instillate. Labor was induced by giving timed-pregnant guinea pigs oxytocin as described in Pretreatment of the Timed-Pregnant Guinea Pigs. In some studies, 10⁻³ M propranolol (control, n = 6 at each gestation age; IL-1β, n = 5 at each gestation age) was added to the 5% albumin instillate. In other studies, 10⁻³ M amiloride (control, n = 6 at each gestation age; IL-1β: n = 8 at 61 and n = 5 at 68 days gestation) was added to the 5% albumin instillate. Separate sets of guinea pig fetuses at 61 and 68 days gestation (n = 4 at 61 and n = 7 at 68 days gestation) were obtained from dams injected with vehicle, 0.9% NaCl, every 15 min during 45 min to control for possible stress from the injections per se, and then received the 5% albumin instillate. Guinea pig fetuses from NaCl-injected timed-pregnant guinea pigs of 61 (n = 4) and 68 (n = 4) days gestation were also surgically prepared and instilled with a 5% albumin instillate containing 10⁻⁴ M oxytocin to control for direct oxytocin effects.

Cortisol synthesis inhibition. Preterm guinea pig fetuses with or without IL-1β pretreatment and with and without oxytocin induction of labor of 61 (control, n = 6; IL-1β, n = 6; oxytocin, n = 6; IL-1β and oxytocin, n = 4) and 68 (control, n = 6; IL-1β, n = 6; oxytocin, n = 8; IL-1β and oxytocin, n = 8) days gestation were delivered by abdominal hysterotomy from timed-pregnant guinea pigs pretreated with metyrapone for 3 days. In some studies, 10⁻⁴ M propranolol (control, n = 6; IL-1β, n = 6; oxytocin, n = 6; IL-1β and oxytocin, n = 4 at each gestational age) was added to the 5% albumin instillate. In other studies, 10⁻³ M amiloride (control, n = 6; IL-1β, n = 6; oxytocin, n = 6; IL-1β and oxytocin, n = 7 at each gestational age) was added to the 5% albumin instillate.

Western Blot Protocols

General protocol. Lung tissue from four fetuses in each experiment group was homogenized in T-Per Reagent (Pierce) containing protease inhibitors, aprotinin (30 μg/ml, Sigma Chemical) and leupeptin (1 μg/ml, Sigma Chemical), with a homogenizer (Tissue Tearor) on ice. The tissue homogenate was centrifuged at 13,000 g for 5 min at +4°C. The supernatant (membrane and cytosol fraction) was collected and aliquoted in multiple vials per sample and snap-frozen in liquid nitrogen. One vial was designated to be used for determining total protein concentration of the sample to ensure equal loading of the electrophoresis gel. Aliquots were stored at −80°C until analysis.
Polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes (Pierce) were carried out by standard protocols. After electrophoresis and transfer, the nitrocellulose membranes were blocked [SuperBlock Dry Blend blocking buffer (TBS); Pierce] for 1 h. Primary antibody incubations were always carried out overnight at 4°C on an orbital shaker.

**ENaC**. Anti-ENaC antibodies were a gift from Dr. James D. Stockand of the University of Texas Health Science Center and were directed against residues 137–161 of Xenopus laevis α-ENaC and residues 624–647 of β-ENaC (43). These antibodies specifically recognize membrane proteins of appropriate sizes (85–90 kDa for α-ENaC and 90–95 kDa for β-ENaC) in rats. We tested for cross-reactivity with guinea pig and found similar bands specifically labeled for both subunits. After blocking, the membranes were incubated with primary antibodies [anti-α-ENaC (rabbit) and anti-β-ENaC (rabbit), respectively]. After incubation, the membranes were washed 5 × 10 min (pH 7.5, TBS with 0.1% Tween 20). After the wash, the membranes were incubated with enzyme-conjugated secondary antibodies (goat-anti-rabbit IgG) for 1 h at room temperature. After incubation, the membranes were washed again. Then, the substrate solution (SuperSignal West Fermo substrate solution; Pierce) was added and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and analyzed densitometrically using the TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

**Na-K-ATPase**. Anti-Na-K-ATPase antibodies were obtained from Upstate Cell Signaling Solutions (Waltham, MA) and directed against residues 338–518 of the α1-subunit and residues 152–340 of the β1-subunit of the Na-K-ATPase. These antibodies specifically recognize membrane proteins of appropriate sizes (~95 kDa for the α1-subunit and ~55 kDa for the β1-subunit) in rats. A rat heart microsomal protein preparation was always run as a positive control. We tested for cross-reactivity with guinea pigs and found similar bands specifically labeled for both subunits. After blocking, the membranes were incubated with primary polyclonal antibodies [anti-α1-Na-K-ATPase (rabbit) and anti-β1-Na-K-ATPase (rabbit), respectively]. After incubation, the membranes were washed 5 × 10 min. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat-anti-rabbit IgG) for 1 h at room temperature. After incubation and wash, the substrate solution (SuperSignal West Fermo) was added and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and densitometrically analyzed using the TotalLab software.

**Biochemical Analyses**

Plasma epinephrine and norepinephrine levels were measured by commercially available ELISA (CatCombi; IBL, Hamburg, Germany). The assays had a sensitivity of 12 pg/ml and intra- and interassay variabilities of 5 and 12%, respectively.

We measured endogenous guinea pig serum protein in 96 randomly selected air space samples from these animals by a luminescence microplate assay. Air space fluid samples were applied to a microplate with nitrocellulose filter well bottoms (0.45 μm; Whatman, Clifton, NJ). Addition of sterile 0.9% NaCl to one to three wells per plate served as negative control and always demonstrated no protein binding. We also ran a standard of dilutions of guinea pig serum and found reproducible binding at concentrations of >100 ng protein/ml. Samples were allowed to bind to the nitrocellulose membrane for 1 h at room temperature, the plate was then placed on a vacuum manifold (Whatman), and vacuum was applied to draw the fluid through the membrane. The plate was then washed three times with wash buffer (pH 7.5, TBS with 0.1% Tween 20) using the vacuum manifold. After washing, the plate was incubated with anti-guinea pig serum antibody (Rockland, Gilbertsville, PA) for 1 h at room temperature. The plate was then washed as above and incubated with the HRP-labeled secondary antibody (goat-anti-rabbit IgG) for 1 h at room temperature. After washing, the substrate solution (SuperSignal West Fermo) was added and incubated for 5 min at room temperature and evacuated by vacuum, and the membranes were read for luminescence in a luminometer plate reader (Fluoroscan FL, Labsystems). Luminescence signal was reported as arbitrary luminescence units, which when increased above baseline (0.9% NaCl) was taken as evidence for endogenous guinea pig serum in the air spaces and analyzed for each experimental group. Concentrations were then calculated from the assayed protein dilutions.

**Statistics**

Values are presented as means ± SD. Statistical analysis was carried out by one-way analysis of variance with Tukey’s test as post hoc or Student’s t-test where appropriate. Differences were considered statistically significant when \( P < 0.05 \).

**RESULTS**

**Assessment of Fetal and Maternal Inflammatory Responses After IL-1β Pretreatment and Lung Endothelial and Epithelial Permeability**

Amniotic fluid cell counts showed no differences in total cell numbers or in the different cell types counted (neutrophils and macrophages) between IL-1β-injected guinea pigs and control guinea pigs at 61 or 68 days gestation (Table 1). In fetal lung fluid there were no differences in total cell numbers or in differential cell numbers (Table 1). These data are in agreement with our earlier published report, where we found that IL-1β plasma levels were generally undetectable in fetal and maternal blood (48). To further assess a potential lung inflammatory response, we measured endogenous guinea pig serum proteins in collected distal lung air space fluid samples. Low concentrations of guinea pig serum proteins were uniformly present, but no differences between the experimental groups or gestation ages were observed (Table 1).

Endothelial protein leak, when calculated as extravascular plasma equivalents, was low in the three examined newborn

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<th>Table 1. Inflammatory parameters in amniotic fluid and fetal alveolar fluid in IL-1β-pretreated and normal control guinea pig fetuses</th>
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Values are means ± SD; n = 6 fetuses at each gestation age and treatment. Mo, macrophages; PMN, polymorphonuclear neutrophils.
guinea pigs in which lung fluid absorption was measured. An extravascular plasma equivalent of $0.26 \pm 0.07$ ml was detected in these guinea pigs after the 1-h lung fluid absorption experiment was carried out. In the corresponding four guinea pigs that were anesthetized, the extravascular plasma equivalents were equally low at $0.23 \pm 0.09$ ml.

**Effect of IL-1β and Oxytocin on Lung Fluid Absorption**

Lung fluid absorption was measured in fetal guinea pigs (61 and 68 days gestation) after IL-1β pretreatment of the timed-pregnant guinea pigs with and without induction of labor by oxytocin. Control 61-day-gestation fetal lungs were still secreting fluid into the air spaces (Fig. 1), and maternal IL-1β injections induced lung fluid absorption (Fig. 1). IL-1β stimulated existing lung fluid absorption at 68 days gestation (Fig. 1). Oxytocin alone converted 61-day-gestation fetal lungs from fluid secretion to fluid absorption (Fig. 1) and had a tendency to stimulate lung fluid absorption at 68 days gestation (not significant). Oxytocin-induced labor in both 61-day- and 68-day-gestation IL-1β-exposed guinea pigs additively increased lung fluid absorption (Fig. 1). Oxytocin when instilled directly into fetal lungs lacked a stimulatory effect in any condition ($-11 \pm 3\%$ (61 days) and $4 \pm 4\%$ (68 days)). Injections of 0.9% NaCl at the same intervals as oxytocin did not affect fetal lung fluid absorption at any gestation age (data not shown).

**Effect of Amiloride on Lung Fluid Absorption**

Lung fluid absorption depends on water absorption secondary to Na\(^+\) transport via amiloride-sensitive and -insensitive pathways (19, 36). We therefore used amiloride to investigate whether IL-1β-stimulated lung fluid absorption was associated with amiloride-sensitive Na\(^+\) transport. In 61-day-gestation guinea pig fetuses, baseline lung fluid secretion was insensitive to amiloride inhibition (Fig. 2A). IL-1β-induced and oxytocin-induced lung fluid absorption in both control and IL-1β-treated 61-day-gestation guinea pig fetuses was blocked by amiloride (Fig. 2A). In 68-day-gestation guinea pig fetuses, lung fluid absorption in all experimental conditions was blocked by amiloride (Fig. 2B).

**β-Adrenoceptor Stimulation**

In 61-day-gestation guinea pig fetuses, IL-1β-induced lung fluid absorption was sensitive to propranolol inhibition (Fig. 3A). In age-matched control fetuses, no sensitivity to propranolol was observed (Fig. 3A). Oxytocin induction of labor in both control and IL-1β-treated 61-day-gestation fetuses also resulted in propranolol inhibition of lung fluid absorption (Fig. 3A). In 68-day-gestation guinea pig fetuses, propranolol inhibited both IL-1β-stimulated and control lung fluid absorption with and without oxytocin-induced labor (Fig. 3B).

We measured plasma epinephrine concentrations at both 61 and 68 days gestation. Plasma epinephrine concentrations were low at 61 days gestation and began to increase at 68 days of gestation (Fig. 4). At both 61 and 68 days gestation, a small but significant increase in plasma epinephrine concentrations was observed after IL-1β pretreatment (Fig. 4). Oxytocin induction of labor significantly increased fetal plasma epinephrine concentrations to similar degrees at both ages with and without
IL-1β treatment (Fig. 4). These results correlated with the propranolol sensitivity of lung fluid absorption.

Cortisol Synthesis Inhibition and IL-1β-Oxytocin Induction of Lung Fluid Absorption

Lung fluid absorption was studied in fetal guinea pigs after maternal IL-1β pretreatment with and without labor induction by oxytocin and with and without cortisol synthesis blockade by metyrapone. Metyrapone did not affect fluid secretion in control 61-day-gestation fetal lungs (Fig. 5A). However, IL-1β-induced lung fluid absorption was attenuated by metyrapone (Fig. 5A). Metyrapone did not affect the oxytocin-converted 61-day-gestation fetal lung fluid absorption (Fig. 5A). On the other hand, metyrapone partially inhibited oxytocin-induced lung fluid absorption in oxytocin-exposed IL-1β-exposed guinea pig fetuses at 61 days gestation (Fig. 5A). In 68-day-gestation guinea pig fetuses, lung fluid absorption was again partially inhibited by metyrapone (Fig. 5B).

ENaC Expression

We studied if oxytocin and/or IL-1β upregulated ENaC subunit expression in lung epithelia. Figure 6, A and B, bottom, shows representative Western blots of α-ENaC and β-ENaC. ENaC subunit expression in general increased with gestation and lung development (Fig. 6, A and B). In both age groups, IL-1β pretreatment significantly increased α-ENaC and β-ENaC expression compared with age-matched control fetuses (Fig. 6, A and B). However, there were no effects on α-ENaC and β-ENaC expression from oxytocin induction of labor, either with or without IL-1β (Fig. 6, A and B).

Na-K-ATPase Expression

We also investigated whether oxytocin and/or IL-1β upregulated Na-K-ATPase α1- and β1-subunit expression in lung epithelia. Figure 7, A and B, bottom, shows representative Western blots of α1-Na-K-ATPase and β1-Na-K-ATPase subunits. Na-K-ATPase subunit expression increased with gestation age and lung development (Fig. 7, A and B). In both age groups, IL-1β administration significantly increased α1-Na-K-ATPase and β1-Na-K-ATPase subunit expression compared with age-matched control fetuses (Fig. 7, A and B). However, oxytocin induction of labor did not affect α1-Na-K-ATPase or β1-Na-K-ATPase subunit expression either with or without IL-1β (Fig. 7, A and B).

DISCUSSION

This paper expands on two of our earlier studies, one in which we found that IL-1β induced lung maturation via a mechanism involving cortisol over a relatively long time frame (2 days) (48) and the other in which oxytocin resulted in a rapidly occurring lung maturation (within 45–60 min) involving elevated plasma epinephrine levels (35). IL-1β and oxytocin induced lung maturation by two different sets of mechanisms acting over different time frames. Our earlier study (48) demonstrated that IL-1β stimulated cortisol synthesis and release, which increased Na+ transport protein expression and β-adrenoceptor density in the lung, thus providing a favorable

Fig. 3. Propranolol inhibition of lung fluid absorption in guinea pig fetuses of 61 days gestation (A) and 68 days gestation (B) after IL-1β pretreatment with and without labor induction by oxytocin. *P < 0.05 compared with control; ANOVA, Tukey’s post hoc test.

Fig. 4. Plasma epinephrine concentrations in 61-day- and 68-day-gestation guinea pig fetuses with and without maternal IL-1β pretreatment with and without labor induction by oxytocin (OT).
environment for labor-released epinephrine to have a greater effect. The principal finding in the oxytocin study (35) was that the significant induced elevations in plasma epinephrine levels rapidly stimulated the fetal lung to absorb the fetal lung fluid. In this study, we asked the question “Do these two stimuli acting in concert produce an additive effect on lung maturation and fetal lung fluid absorption?” This in fact occurred and was the principal new finding of the study. Because preterm babies may suffer from respiratory distress partly related to immaturities in lung ion transport (1), we hypothesized that since the lung seemed more mature after IL-1β pretreatment, labor, if delivery is nonpreventable, could be beneficial to the outcome. In this study, oxytocin induction of labor dramatically improved the IL-1β-induced/-stimulated fluid-absorptive capacity of the fetal lung, thus providing an opportunity to clear fetal preterm lungs from lung fluid rapidly at delivery. One possible mechanism that IL-1β may utilize to induce lung fluid absorption at 61 days gestation may be via inhibition of fluid secretion alone.

Another potential mechanism may be related to rendering the lung more sensitive to β-adrenoceptor stimulation, therefore increasing the sensitivity to the oxytocin-induced labor release of epinephrine. Catecholamine stimulation has previously been linked to stimulation of fetal and adult lung fluid absorption (5, 6, 17, 35, 38, 45). In fact, maternal IL-1β pretreatment increased β-adrenoceptor expression in fetal lungs, as well as increasing propranolol sensitivity (48). All of this prepared the lungs for labor induction, having an additive stimulatory effect on lung fluid absorption after IL-1β stimulation and preterm delivery. It has also been demonstrated (3, 5, 6, 19, 31) that lung fluid is reabsorbed secondary to Na+ absorption and that this Na+ absorption can be stimulated by agonist binding to β-adrenoceptors. Consequently, more β-adrenoceptors and higher β-adrenoceptor stimulation may lead to amiloride inhibition, it is unlikely that the primary mechanism would be inhibition of fluid secretion alone.

Fig. 5. Lung fluid absorption in guinea pig fetuses of 61 days gestation (A) and 68 days gestation (B) after IL-1β pretreatment with and without cortisol synthesis inhibition by metyrapone and with and without labor induction by oxytocin. *P < 0.05 compared with control; ANOVA, Tukey’s post hoc test.

Fig. 6. Western blots of α-epithelial sodium channel (ENaC, A) and β-ENaC (B). Bottom panels: typical Western blots of both ENaC subunits. Top panels: summary analyses of optical density (OD) for 3 gels (samples) from each condition. *P < 0.05 compared with 61-day-gestation control; †P < 0.05 compared with 68-day-gestation control; ‡P < 0.05 compared with age-matched control; ANOVA, Tukey’s post hoc test.
transport proteins as we measured their expression in lung homogenates. To address this issue, we carried out a study where amiloride was given, since if ENaC were in the membrane, they would likely be inhibited by amiloride and lung fluid absorption would decrease. This effect would be expected to be greatest at 61 days gestation, as normal 61-day-gestation lungs did not absorb fluid and IL-1β-exposed lungs did absorb lung fluid. We found a complete amiloride inhibition in IL-1β-exposed 61-day-gestation fetuses. With respect to the Na-K-ATPase, although we did not carry out corresponding studies, we believe the same to be true for this transporter protein, since it is the Na-K-ATPase that drives Na⁺ into the cell via the apical ENaC channel.

Lung fluid absorption may be inhibited by amiloride differentially at different pre- and postnatal ages due to differences in sodium channel expression (35). Previous studies (17, 34, 35) demonstrated that before birth, preterm amiloride sensitivity is close to 100% because there seem to exist few or no other pathways for Na⁺ absorption. During normal lung development, amiloride does not affect lung fluid secretion at 61 days gestation. However, amiloride addition to the instillate completely inhibited IL-1β-induced lung fluid absorption, irrespectively of whether or not labor was induced by oxytocin. In 68-day-gestation fetuses, the situation was somewhat different. Amiloride only inhibited a fraction of the fluid absorption when oxytocin was administered, while completely inhibiting fluid absorption otherwise. This may indicate that amiloride-insensitive Na⁺ transport pathways were activated by the high plasma epinephrine levels observed after oxytocin-induced labor. One such potential pathway could be cyclic nucleotide-gated channels, which have been found in developing and adult lungs (25, 36). Maternal IL-1β injections increased ENaC and Na-K-ATPase expression in fetal lungs (48), thus providing an environment that favored a stimulatory effect by labor induction from oxytocin. As both β-adrenoceptors and sodium transport proteins were upregulated by maternal IL-1β, the lung appeared sensitized to the epinephrine surge associated with labor.

Enhorning and colleagues (15) discovered that β-adrenoceptor agonist injection to pregnant rabbits reduced fetal lung water. Intravenous epinephrine also decreased lung fluid secretion in late-gestation fetal sheep, an effect inhibited by β-adrenoceptor antagonists (45). A positive correlation between plasma epinephrine levels and fetal lung fluid reabsorption has been demonstrated in near-term lambs (8) and guinea pigs (17, 35). However, there are some data available suggesting that plasma epinephrine may not be necessary for fluid absorption at the time of birth (11, 32). In our studies, however, paralleling the appearance of β-adrenoceptor stimulation of lung fluid absorption, plasma epinephrine levels increased, suggesting that this catecholamine was important for the conversion of the lung epithelium from secretion to absorption. Moreover, IL-1β pretreatment resulted in more β-adrenoceptors and sodium transport proteins were upregulated by maternal IL-1β, the lung appeared sensitized to the epinephrine surge associated with labor.
binding sites for labor-released plasma epinephrine was a key mechanism. Fluid secretion into fetal lamb and guinea pig lungs decreases before birth (13, 27, 39, 48) simultaneously with the appearance of endogenous catecholamines and stress hormones. β-Adrenoceptor agonist stimulation is thought to increase transepithelial Na⁺ transport by increasing ENaC activity at the apical side of alveolar epithelial type II cell (42) and by increasing the number and activity of the Na-K-ATPases in the basal membrane of the alveolar epithelial type II cell (42). As a significant portion of the IL-1β stimulatory effects occurred from sensitization of the lung to β-adrenoceptor agonists, such as labor-released epinephrine, it is possible that other agents that can activate or enhance β-adrenoceptor signaling could have similar effects.

Did nonspecific effects such as inflammation, low blood pH, barrier protein leak, and cellular infiltration into the lung air spaces affect the results? This is probably unlikely for several reasons. First, IL-1β was not present in the majority of maternal and fetal plasma samples assayed for IL-1β (48). Second, IL-1β exerts its effects via activation of the hypothalamic-pituitary-adrenal gland axis, which results in ACTH release and increased fetal cortisol synthesis/release (48). Third, although the final blood pH was abnormally low, distal lung fluid absorption proceeded at rates similar to those in anesthetized and ventilated guinea pigs and was even able to be stimulated and inhibited, suggesting that pH may have been of minor importance. In fact, pH being low may not be an issue since fetal lung fluid normally has a pH of ~6.3. Fourth, from our data there seemed to be little or no increase in barrier leak, if any, as there were no differences in guinea pig serum levels in the analyzed air space samples (Table 1). Moreover, the low extravascular plasma equivalents in the lung support the assumption of an uninjured lung. Fifth, since IL-1β was not expected to have any direct effects on the fetal lungs (48), we did not expect to see a high degree of inflammatory cell infiltration in these fetal lungs nor in the amniotic fluid (Table 1). Thus it is unlikely that nonspecific effects related to the model significantly affected the outcome of the study.

In this study, it was demonstrated that maternal IL-1β may participate in the modulation of lung maturation. It is clear that maternal IL-1β may have benefits for lung maturation, especially in premature infants. However, even though IL-1β did not cause fetal lung inflammation, IL-1β itself would probably have limited clinical usage due to potentially severe side effects. The beneficial dose range for IL-1β is likely very narrow, and giving excess IL-1β may affect brain development and may cause cerebral palsy (24). We are not at this stage proposing that IL-1β should be utilized clinically. Because IL-1β releases cortisol, which is maturation promoting, i.e., increases the number of available β-adrenoceptors (48) and the expression of the sodium transport proteins in the lung, lung maturation may be influenced. This may also partially explain why the lung responded additively to IL-1β pretreatment and oxytocin-induced labor and may suggest why babies with infection seem to be relatively resistant to developing RDS.

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