IL-1β stimulates alveolar fluid absorption in fetal guinea pig lungs via the hypothalamus-pituitary-adrenal gland axis

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Ye, Xin, Reshma Acharya, Jonathan B. Herbert, Sarah E. Hamilton, and Hans G. Folkesson. IL-1β stimulates alveolar fluid absorption in fetal guinea pig lungs via the hypothalamus-pituitary-adrenal gland axis. Am J Physiol Lung Cell Mol Physiol 286: L756–L766, 2004—We tested the hypothesis that interleukin (IL)–1β–induced cortisol synthesis stimulates alveolar fluid clearance in preterm fetuses. IL-1β was administered subcutaneously daily to timed-pregnant guinea pigs for 3 days with and without simultaneous cortisol synthesis inhibition by metyrapone. Fetuses were obtained by abdominal hysterotomy at 61 and 68 days gestation and instilled with isosmolar 5% albumin in the lungs, and alveolar fluid movement was measured over 1 h from the change in alveolar protein concentration. Alveolar fluid clearance was induced at 61 days gestation and stimulated at 68 days gestation by IL-1β, which both were attenuated by cortisol synthesis inhibition. Plasma ACTH and cortisol concentrations were increased by IL-1β at both gestational ages, and metyrapone reduced cortisol concentrations. IL-1β was mostly low or undetectable in both fetal and maternal blood. Prenatal alveolar fluid clearance, when present as well as IL-1β induced, was always propranolol and amiloride sensitive, suggesting that β-adrenoceptor stimulation and amiloride-sensitive Na\(^+\) channels were critical for fluid absorption. IL-1β increased lung β-adrenoceptor density at gestation day 61, and cortisol synthesis inhibition attenuated the increased β-adrenoceptor density. Epithelial Na\(^+\) channel and Na\(^+\)-K\(^+\)-ATPase subunit expressions were both increased by IL-1β and attenuated by cortisol synthesis inhibition. These results may explain why babies delivered preterm after intrauterine inflammation have a reduced risk of developing severe respiratory distress.

adrenocorticotropic hormone; alveolar epithelium; β-adrenoceptors; cortisol; epithelial sodium channel; epinephrine; interleukin-1β; respiratory distress syndrome; prenatal lung development; sodium transport.

IT WAS ESTABLISHED in the nineteenth century that the fetal lung is fluid filled (34) and that the source of the fluid was the lungs (7). Although this fluid is essential for normal lung development, the continued presence of fluid in the neonatal lung would be life-threatening. Experimental and clinical evidence supports the notion that rapid pre- and postnatal lung fluid clearance is critical for the establishment of normal pulmonary gas exchange immediately after birth (10, 16, 20, 31). It is believed that rising endogenous catecholamine concentrations, especially epinephrine, near term contribute to this decrease in alveolar fluid volume (11, 12, 31). The majority of fetal lung fluid is absorbed by lung epithelia after an osmotic gradient that is generated by active Na\(^+\) absorption (11, 25). Na\(^+\) absorption through alveolar epithelial apical entry pathways, the amiloride-sensitive epithelial Na\(^+\) channel (ENaC) (11), is driven by the activity of alveolar epithelial cell basolateral Na\(^+\)-K\(^+\)-ATPases (29). ENaC is considered important for alveolar fluid absorption at birth because transgenic mice made α-ENaC-deficient die within 40 h after birth, possibly because of insufficient fluid clearance from the airspaces (17).

Cytokines, such as interleukin-1 (IL-1), may be important during pregnancy, because IL-1 may be a signal for parturition onset, since IL-1 can stimulate prostaglandin biosynthesis, which is important for labor onset (37). IL-1 has been detected in the placenta and in the amniotic fluid during the second half of a normal pregnancy (9, 45). During a normal pregnancy, IL-1 is present in amniotic fluid in low concentrations, but in preterm labor high amniotic fluid IL-1 concentrations have been detected, especially after an intra-amniotic infection, where the highest IL-1 activity seems to be attributable to IL-1β (45). Laboratory investigations have provided evidence that systemic IL-1 administration produced preterm labor and delivery in mice (26, 36). A high frequency of preterm infants suffers from infections and the respiratory distress syndrome (RDS), which are important reasons for preterm baby mortality. Recently, it has been suggested that an incomplete transition of lung fluid secretion to lung fluid absorption may be an important cause of RDS (1). Meanwhile, experimental data suggested that proinflammatory cytokines might signal lung maturation (8, 19, 47). Specifically, intra-amniotic IL-1α increases mRNA expression of surfactant proteins SP-A and SP-B, improves lung compliance in fetal rabbits, and alters postnatal adaptation in premature lambs without eliciting a systemic inflammatory response (8, 47).

Because proinflammatory cytokines, such as IL-1, have been proposed to signal lung maturation (8, 19, 47), this lead us to hypothesize that maternal IL-1β treatment would lead to preterm lung maturation and a more complete transition of lung fluid secretion to lung fluid absorption. Specifically, our aim for this investigation was to determine if 1) maternal IL-1β treatment induces Na\(^+\) and fluid absorption in otherwise fluid-secreting fetal lungs and 2) maternal IL-1β leads to β-adrenoceptor-stimulated alveolar fluid clearance in fetal lungs. We found that maternal IL-1β pretreatment induced (converted secretion into absorption) amiloride- and propranolol-sensitive alveolar fluid clearance in 61-day-gestation fetal guinea pigs and stimulated (increased already present absorption) alveolar fluid clearance in 68-day-gestation fetal guinea pigs. Accordingly, we designed additional experiments to determine if 1)
the IL-1β-induced alveolar fluid clearance was mediated by increased ENaC and Na⁺-K⁺-ATPase expression and 2) the β-adrenoceptor sensitivity after IL-1β pretreatment was the result of increased β-adrenoceptor density. Both ENaC subunit expression, Na⁺-K⁺-ATPase subunit expression, and β-adrenoceptor expression were all elevated after maternal IL-1β pretreatment. Our third set of aims was to investigate potential mechanisms for the IL-1β stimulation of fetal alveolar fluid absorption and its potential for reducing RDS development. Plasma cortisol is important for maintaining alveolar fluid balance in both adult and newborn animals (15, 22, 30), and it has been demonstrated that IL-1β may induce hypothalamic corticotrophin-releasing factor (CRF) release and thereby activate the adrenocortical axis with ACTH secretion from the pituitary gland and eventually plasma cortisol synthesis and secretion from the adrenal glands (39). By this pathway, IL-1β has the potential to induce lung maturation and reduce RDS development. Therefore, we hypothesized that IL-1β mediated its effects on alveolar fluid clearance at least partly via stimulation of maternal and/or fetal plasma cortisol synthesis and release via the adrenocortical axis and thus protects the animal against RDS development. We tested this hypothesis in three ways. First, we pretreated timed-pregnant guinea pigs simultaneously with the 11-β-hydroxylase inhibitor, metyrapone, and IL-1β and measured alveolar fluid clearance with and without β-adrenoceptor inhibition. In addition, we measured β-adrenoceptor density after cortisol synthesis inhibition. Second, we measured alveolar fluid clearance and amiloride sensitivity, ENaC, and Na⁺-K⁺-ATPase expression in fetuses with and without IL-1β pretreatment and with and without cortisol synthesis inhibition. Third, we measured maternal and fetal plasma IL-1β, epinephrine, norepinephrine, ACTH, and cortisol concentrations and correlated those findings to the changes in alveolar fluid clearance, receptor density, and transporter expression.

MATERIALS AND METHODS

Animals

Preterm Dunkin-Hartley guinea pigs (Hilltop Laboratory Animals, Scottsdale, PA) were used (n = 252 divided into 70 litters). Timed-pregnant guinea pigs were kept at a 12:12-h day-night rhythm and had free access to food (Standard guinea pig chow; Purina; 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

Injection solutions. The IL-1β pretreatment solution was prepared by dissolving 10 μg rat recombinant IL-1β (I-2393; Sigma Chemical, St. Louis, MO) in 0.1% BSA in 0.9% NaCl. The dissolved IL-1β was then separated into aliquots into tubes each containing 500 ng and stored frozen at −20°C until used.

The 11-β-hydroxylase inhibitor, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; Sigma Chemical), pretreatment solution was prepared by dissolving 62.5 mg/ml metyrapone in 24% ethanol in 0.9% NaCl. Injection of 24% ethanol in 0.9% NaCl has been demonstrated earlier without effect on alveolar fluid clearance in guinea pigs (30).

Instillation solutions. A 5% albumin solution was prepared by dissolving 50 mg/ml BSA (Calbiochem-Novabiochem, La Jolla, CA) in 0.9% NaCl. In some studies, the Na⁺ channel inhibitor amiloride hydrochloride (10⁻³ M; ICN Biomedicals, Aurora, OH) or the general β-adrenoceptor antagonist propranolol (10⁻⁴ M; P-0084; Sigma Chemical) was added to the instillate solution. To study the direct effects from IL-1β on alveolar fluid clearance, 50 ng IL-1β/ml were added to the instillate solution.

Pretreatment of Timed-pregnant Guinea Pigs with IL-1β

Timed-pregnant guinea pigs of 59 and 66 days gestation were injected subcutaneously on the dorsal neck one time daily with 250 ng/kg body wt IL-1β for 3 days. Control timed-pregnant guinea pigs were given injections of 0.9% NaCl at the same time. The alveolar fluid clearance experiment was carried out on the morning of the last pretreatment day.

Metyrapone. Pretreatment with the 11-β-hydroxylase inhibitor metyrapone was carried out over 3 days simultaneously with IL-1β. Subcutaneous metyrapone injections were given two times daily in the morning and in the evening (25 mg/kg body wt to reach a total daily dose of 50 mg/kg body wt) to guinea pigs of 59 and 66 days gestation. In the morning of the day of the alveolar fluid clearance experiment, one-half of the daily metyrapone dose was given. The metyrapone dose was adopted from its higher ranges of clinical dosage.

Surgical Preparations

Abdominal hysterotomy. Timed-pregnant guinea pigs were anesthetized by intraperitoneal pentobarbital sodium injections (120 mg/kg body wt; Abbott Laboratories, Chicago, IL) and killed by direct intracardiac injections of 60 mg pentobarbital sodium. A laparotomy was rapidly done, and the fetuses were carefully delivered. The umbilical cord was ligated to prevent bleeding. The fetuses were immediately killed by intraperitoneal pentobarbital sodium (12 mg) with 50 IU heparin (Elkins-Sinn, Cherry Hill, NJ).

Fetal surgery. After death, an endotracheal tube (PE-190; Clay Adams, Becton-Dickinson, Parsippany, NJ) was inserted through a tracheotomy. The animals were immediately connected to a constant O2 flow (FIO₂ = 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 surgical procedure after death required 5 min. The animals were placed in between heating pads to maintain their body temperature during the experiments. A temperature probe measured incubation temperature, and heating was adjusted to maintain the temperature at 37–38°C. Airway pressure was monitored continuously by calibrated pressure transducers (ADI Instruments, Grand Junction, CO) and analog-to-digital converters and amplifiers (ADInstruments).

Alveolar Fluid Clearance Experiments

After surgery and connection to CPAP, the albumin solution (10 ml/kg body wt) was instilled in the lungs through the endotracheal tube, as follows. First, the animals were briefly disconnected from the CPAP circuit, and then the lungs were deflated by gently aspirating residual air with the instillation syringe. The instillation solution was then instilled in the lungs and withdrawn again. This procedure was repeated four times to allow thorough and adequate mixing of instillate and preexisting fetal lung fluid, and then the fluid was finally instilled. A 0.1-ml sample of the instillation solution-lung fluid mixture (initial solution) was retained in the instillation syringe and was saved for protein measurement. The animals were then reconnected to the CPAP circuit and maintained on CPAP for the 1-h study period. Because the fetal lung is fluid-filled in utero (5), we expected that a certain fraction of fluid would still be present in the lungs at the time of study. This fluid is virtually free of protein and will thus dilute the albumin concentration of the instillate when mixed with the instillate solution. After 1 h, the lungs and heart were carefully removed en bloc through a midline sternotomy, and a sample of the remaining alveolar fluid was collected. Total protein concentrations in instillates and
initial and final solutions were determined spectrophotometrically (Labsystems Multiscan Microplate Reader; Labsystems, Helsinki, Finland) by the method of Lowry et al. (23) adapted for microtitre plates.

Alveolar fluid clearance or alveolar fluid secretion was calculated from the change in protein concentration over 1 h. This was possible because the alveolar epithelium is relatively impermeable to large molecules, such as albumin (mol wt 67,000). Therefore, water movement (absorption or secretion) will change the airspace protein concentration. The preexisting fluid volume calculated from the dilution of the original 5% albumin instillate solution was used to correct the instilled protein concentration by the dilution of the instillate that would occur by the fluid already in the lung before instillation of the 5% albumin solution. This method has been used successfully in our earlier studies (11, 31). Alveolar fluid clearance (AFC) or alveolar fluid secretion (AFS) was then calculated from Eqs. 2 and 3 below

\[
V_{\text{final}} = (V_{\text{instilled}} \times C_{\text{initial}})/C_{\text{final}} \quad \text{and} \quad V_{\text{final}} = (V_{\text{initial}} \times C_{\text{initial}})/C_{\text{final}}
\]

(1)

AFC or AFS = \[\left(\frac{V_{\text{final}} - V_{\text{initial}}}{V_{\text{initial}}}\right) \times 100\] (2)\n
where \(V_{\text{instilled}}, V_{\text{initial}}, V_{\text{final}}, C_{\text{initial}}, C_{\text{instilled}}, C_{\text{final}}\) and \(C_{\text{final}}\) are volumes of instillate, initial, and final solutions, respectively. \(C_{\text{instilled}}, C_{\text{initial}}, C_{\text{final}}\) are protein concentrations in corresponding solutions.

To ascertain that alveolar fluid clearance proceeded at similar rates in our in situ lungs as in vivo lungs, we compared our control alveolar fluid clearance rates with those previously published in ventilated and in CPAP-ventilated anesthetized fetal and newborn guinea pigs according to previous protocols. Alveolar fluid clearances in ventilated (11, 30), earlier in situ CPAP animals (31), and in our in situ CPAP animals were not significantly different (data not shown).

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 the timed-pregnant guinea pigs gave birth to their earlier litter, since guinea pigs enter estrus immediately after birth. All groups contained at least two litters, and all fetuses were surgically prepared as described in Surgical Preparations and studied on 5 cmH2O CPAP for 1 h after fluid instillation. Alveolar fluid clearance was measured as described in Alveolar Fluid Clearance Experiments. Plasma and lung tissue was collected from each group, snap-frozen in liquid nitrogen, and stored at −80°C until used.

IL-1β studies. Preterm guinea pig fetuses of 61 days (n = 6) and 68 days (n = 6) gestation were delivered by abdominal hysterectomy from timed-pregnant guinea pigs that were pretreated with IL-1β. In some studies, 10−4 M propranolol (β-adrenoceptor blocker; n = 6 at each gestation age) was added to the 5% albumin instillate solution. In other studies, 10−3 M amiloride (Na+ channel blocker; n = 6 at each gestation age) was added to the 5% albumin instillate solution. In parallel to the IL-1β studies, preterm guinea pig fetuses of 61 and 68 days gestation were delivered by abdominal hysterectomy from timed-pregnant guinea pigs pretreated with saline injections one time daily and used as control animals without (n = 6 at each gestation age) and with (n = 6 at each gestation age) amiloride and propranolol (n = 6 at each gestation age). Amiloride at the concentration of 10−3 M was used because a large fraction becomes protein bound and another significant fraction rapidly leaves the airspaces because of its low molecular weight (33, 48); therefore, the active amiloride concentration in the alveoli was probably lower. Also, the same amiloride concentration has been used in several earlier studies of alveolar fluid clearance (11, 31).

Cortisol inhibition studies. Preterm guinea pig fetuses with or without IL-1β pretreatment of 61 days (n = 6 in each group) and 68 days (n = 6 in each group) gestation were delivered by abdominal hysterectomy from timed-pregnant guinea pigs pretreated with the 11-β-hydroxylase inhibitor metyrapone for 3 days. In some studies, 10−4 M propranolol (n = 6 at each gestation age and treatment) was added to the 5% albumin instillate, and, in other studies, 10−3 M amiloride (n = 6 at each gestation age and treatment) was added to the 5% albumin instillate.

Direct effects from IL-1β on alveolar fluid clearance. Preterm 68-day-gestation (n = 6) guinea pig fetuses were delivered by abdominal hysterectomy from timed-pregnant guinea pigs. Alveolar fluid clearance was measured after instillation of the 5% albumin solution containing 50 ng IL-1β/ml. Control 68-day-gestation (n = 6) fetuses received an instillation of the 5% albumin solution without IL-1β.

Western Blot Protocols

General protocol. Lung tissue from four fetuses in each experimental group was homogenized in T-PerReagent (Pierce, Rockford, IL) 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 separated into aliquots in multiple tubes for each sample and snap-frozen in liquid nitrogen. One tube 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.

PAGE and transfer to nitrocellulose membrane (Pierce) were carried out using standard protocols. After electrophoresis and transfer, the nitrocellulose membrane was placed in blocking buffer (SuperBlot Dry Blend blocking buffer in Tris-buffered saline (TBS); Pierce), and blocking was carried out overnight.

ENaC subunit detection. The anti-ENaC antibodies used were a gift from Dr. James D. Stockand at 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 (42). 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 membrane was incubated with the primary antibody anti-α-ENaC (rabbit) and anti-β-ENaC (rabbit)) on an orbital shaker for 1 h at room temperature. After incubation, the membrane was washed five times with wash buffer (pH = 7.5; TBS with 0.1% Tween 20). After the wash process, the membrane was incubated with the enzyme-conjugated secondary antibody (goat anti-rabbit IgG) for 1 h at room temperature. After incubation, the membrane was washed again. Next, the substrate solution (SuperSignal West Femto substrate solution; Pierce) was added to the blot 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. The anti-Na+-K+-ATPase antibodies were obtained from Upstate Cell Signaling Solutions (Waltham, MA) and were 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 of the Na+-K+-ATPase) in rats. A rat heart microsomal protein preparation was always run on the gel as a positive control. We tested for cross-reactivity with guinea pig and found similar bands specifically labeled for both subunits. After blocking in SuperBlock the membrane was incubated with primary polyclonal antibodies [anti-α1-Na+-K+-ATPase (rabbit) and anti-β1-Na+-K+-ATPase (rabbit)] on an orbital shaker overnight at +4°C. After incubation, the membrane was washed five times with wash buffer. Next, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit IgG) for 1 h at room temperature. After incubation and wash, the substrate solution (SuperSignal West Femto) was added.

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to the blot and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and densitometrically analyzed using the TotalLab software.

**ELISA Analyses**

Plasma was collected from guinea pigs in all experimental groups and gestational ages (n = 4–5 in each experimental group) as described in *Animals*. After abdominal hysterotomy and delivery of the fetuses, the lungs were removed and snap-frozen in liquid nitrogen. The lungs were stored at −80 °C until the binding assay.

**Lung β-adrenoceptor density analysis by 125I-iodocyanopindolol binding.** The total number of lung β-adrenoceptor sites (cell membrane and internalized β-adrenoceptors) was determined using the lipophilic radioligand 125I-iodocyanopindolol (125I-ICYP), as described by Morgan and colleagues (28). Briefly, 25 mg lung tissue were homogenized with a Polytron for 10–15 s in 250 μl of 50 mM ice-cold Tris buffer (pH 7.4) containing 10 mM MgCl2. The homogenate was centrifuged for 10 min at 40,000 g. The resulting tissue pellet was resuspended in 6 ml Tris buffer (pH 7.4) containing 1 mM EDTA and incubated for 30 min at 36 °C to dissociate away endogenous ligand or competing drug from receptor binding sites. This process was repeated a second time. Binding assays in a total volume of 0.5 ml were conducted in polystyrene assay tubes containing homogenized tissue with the following 125I-ICYP concentrations: 300, 200, 100, 75, 50, 25, 10, and 5 pM. Nonspecific 125I-ICYP binding was defined by the presence of 2 μM (S)-(-)-propranolol. Homogenates were incubated for 60 min at 36 °C. The assays were terminated by a rapid filtration through Whatman GF/B filters previously soaked in 0.05% polyethyleneimine using a Brandel R48 filter manifold. The filters were washed three times with 7 ml ice-cold buffer and counted in a gamma counter. Maximal binding (B max) and dissociation constant (K d) were determined using the LIGAND curve-fitting program (NIH). B max determinations were normalized by expressing the data as a fraction of tissue protein concentration (determined by the Lowry protein assay).

**Statistics**

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

**RESULTS**

**Alveolar Fluid Clearance Experiments**

Alveolar fluid clearance was studied in fetal guinea pigs (61 and 68 days gestation) after IL-1β pretreatment of timed-pregnant guinea pigs. Control 61-day-gestation fetal lungs were still in the fluid secretion state (Fig. 1). Maternal IL-1β injections induced (converted secretion into absorption) alveolar fluid absorption at 61 days gestation (Fig. 1). At 68 days gestation, the control lungs had already begun to absorb alveolar fluid, and IL-1β stimulated (increased absorption that was present already) alveolar fluid clearance at 68 days gestation (Fig. 1). Direct instillation of the IL-1β-containing instillate solution in the lungs of 68-day-gestation fetal guinea pigs did not increase alveolar fluid clearance compared with normal 68-day-gestation control fetal guinea pigs [9 ± 3% (n = 6) and 8 ± 4% (n = 9), respectively; Student’s t-test]. Concomitant IL-1β and metyrapone administration attenuated the IL-1β-induced or IL-1β-stimulated alveolar fluid clearance at both 61 and 68 days gestation (Fig. 2, A and B). In the corresponding 61-day-gestation metyrapone-pretreated control group, no changes were observed compared with untreated controls. Once plasma cortisol already began to rise, i.e., in 68-day-gestation guinea pig fetuses (see below), cortisol synthesis blockade also affected alveolar fluid clearance in unstimulated control fetuses.

We hypothesized that IL-1β increased fetal lung β-adrenoceptor density and stimulation that would help to stimulate the lung to convert from secretion to absorption. We therefore measured β-adrenoceptor density with and without IL-1β pretreatment and with and without metyrapone pretreatment at 61 and 68 days gestation. At 61 days gestation, β-adrenoceptor density was increased compared with control fetuses after IL-1β pretreatment (Fig. 3A). Cortisol synthesis inhibition blocked the increased β-adrenoceptor density induced by IL-1β at 61 days gestation, whereas no effect on β-adrenoceptor density was seen at 68 days gestation (Fig. 3, A and B). Receptor K d values remained unchanged irrespective of treatment.

We then measured β-adrenoceptor stimulation and its effects on fetal alveolar fluid clearance. In 61-day-gestation guinea pig fetuses, IL-1β-induced alveolar fluid clearance was sensitive to propranolol (Fig. 4A). In age-matched control fetuses, there was no sensitivity to propranolol (Fig. 4A). In 68-day-gestation guinea pig fetuses, IL-1β-induced alveolar fluid clearance was sensitive to propranolol (Fig. 4A).
pig fetuses, propranolol inhibited both IL-1\textbeta-stimulated and control alveolar fluid clearance (Fig. 4B). Because epinephrine stimulates alveolar fluid clearance by \beta-adrenoceptor stimulation and cortisol may affect \beta-adrenoceptor activity/density, we used propranolol to investigate if pretreatment by metyrapone altered \beta-adrenoceptor responsiveness. After metyrapone pretreatment, inhibition of alveolar fluid clearance by propranolol was attenuated (Fig. 4, A and B).

Plasma epinephrine concentrations were low at 61 days gestation and began to increase at 68 days of gestation. At both 61 and 68 days gestation, plasma epinephrine concentrations were increased compared with the age-matched control fetuses after IL-1\textbeta pretreatment (Fig. 5A). There were no differences between control and IL-1\textbeta-pretreated fetuses with respect to plasma nor-epinephrine concentrations at either gestational age (Fig. 5B). These results correlated with the propranolol sensitivity of the alveolar fluid clearance.

Alveolar fluid clearance depends on water absorption secondary to Na\textsuperscript+ transport through amiloride-sensitive and -insensitive pathways. Amiloride acts via blocking ENaC and consequently inhibits Na\textsuperscript+ transport. We therefore used amiloride to investigate whether IL-1\textbeta-stimulated alveolar fluid clearance was associated with amiloride-sensitive Na\textsuperscript+ transport. In 61-day-gestation guinea pig fetuses, only IL-1\textbeta-induced alveolar fluid absorption was blocked by amiloride (Fig. 6A), whereas control fluid secretion was unaffected. In 68-day-gestation guinea pig fetuses, both control and IL-1\textbeta-stimulated alveolar fluid absorption were blocked by amiloride (Fig. 6B). After metyrapone pretreatment, the inhibitory effect on alveolar fluid clearance by amiloride was attenuated (Fig. 6, A and B).

**ENaC Expression**

We then investigated if IL-1\textbeta upregulated expression of ENaC subunits in lung epithelia via cortisol. Figure 7, left, shows representative Western blots of the \(\alpha\)-ENaC and \(\beta\)-ENaC subunits. ENaC subunit expression increased with gestational age and lung development (Fig. 7, right). In both age groups, IL-1\textbeta pretreatment significantly increased \(\alpha\)-ENaC and \(\beta\)-ENaC expression compared with age-matched control fetuses (Fig. 7, right). Administration of metyrapone alone had no effects on \(\alpha\)-ENaC and \(\beta\)-ENaC expression in 61-day-gestation control fetuses (Fig. 7, right), whereas administration of metyrapone in combination with IL-1\textbeta attenuated IL-1\textbeta-induced ENaC \(\alpha\)- and \(\beta\)-subunit expression (Fig. 7, right). The results were slightly different in 68-day-gestation fetuses (Fig. 7, right). Here metyrapone administration decreased both \(\alpha\)-ENaC and \(\beta\)-ENaC expression in both control fetuses and after IL-1\textbeta pretreatment (Fig. 7, right). Intra-alveolar propranolol had no effect on ENaC subunit expression at either gestational age (data not shown).

**Na\textsuperscript+-K\textsuperscript+-ATPase Expression**

We also investigated if IL-1\textbeta upregulated Na\textsuperscript+-K\textsuperscript+-ATPase \(\alpha\)- and \(\beta\)-subunit expression in lung epithelia via cortisol. Figure 8, left, shows representative Western blots of \(\alpha\)-Na\textsuperscript+-K\textsuperscript+-ATPase and \(\beta\)-Na\textsuperscript+-K\textsuperscript+-ATPase subunits. Na\textsuperscript+-K\textsuperscript+-ATPase subunit expression increased with gestational age and...
lung development (Fig. 8, right). In both age groups, IL-1β administration significantly increased α1-Na⁺-K⁺-ATPase and β1-Na⁺-K⁺-ATPase expression compared with age-matched control fetuses (Fig. 8, right). Metyrapone administration alone had no effects on α1-Na⁺-K⁺-ATPase and β1-Na⁺-K⁺-ATPase expression at both gestational ages (Fig. 8, right), whereas metyrapone administration in combination with IL-1β attenuated IL-1β-induced Na⁺-K⁺-ATPase α1- and β1-subunit expression (Fig. 8, right). Intra-alveolar propranolol had no effect on Na⁺-K⁺-ATPase subunit expression at either gestational age (data not shown).

Plasma IL-1β, Cortisol, and ACTH Measurements

Control fetuses had no detectable plasma rat IL-1β. Fetal plasma rat IL-1β concentrations were mostly below assay sensitivity; in a few IL-1β-pretreated fetuses, we could detect rat IL-1β above the sensitivity, albeit in low levels [61 days gestation: 19.4 ± 8.6 (SD) pg/ml (n = 20); 68 days gestation: 26.6 ± 15.9 (SD) pg/ml (n = 17)]. Rat IL-1β was not detected in any maternal plasma, irrespective of experimental condition.

Fetal plasma cortisol concentrations were stimulated after IL-1β pretreatment at 61 days gestation and remained high at 68 days gestation (Fig. 9A). Maternal plasma cortisol concentrations were always lower than fetal cortisol levels in both age-matched groups after IL-1β pretreatment (Fig. 9B). This suggested that fetal plasma cortisol synthesis was stimulated, but not depending on that maternal plasma cortisol crossing the placenta.

Plasma ACTH concentrations were increased by IL-1β pretreatment at both 61 and 68 days gestation compared with age-matched control fetuses (Fig. 10A). ACTH concentrations were also increased by IL-1β in maternal plasma (Fig. 10B). In contrast to plasma cortisol concentrations, maternal plasma ACTH concentrations were higher than those in age-matched fetal groups (Fig. 10, A and B). This suggested that plasma ACTH may have crossed the placenta, and the fetuses therefore may have produced their own cortisol in response to ACTH.

DISCUSSION

The guinea pig lung begins to convert from fluid secretion to fluid absorption 3–5 days before birth (11, 31). Before this time point, the lung epithelium secretes fluid in the airspaces. Several studies have demonstrated that the fetal lung fluid volume decreases toward the end of the gestation (10, 20) and
that this results from fluid absorption across the alveolar epithelium. Alveolar fluid absorption reaches a maximal rate on the day of birth, with this process culminating in "dry" alveoli to ensure efficient gas exchange. The success of this transition is directly related to postnatal lung function. In this regard, it has been hypothesized that preterm infants with RDS might exhibit an immature alveolar epithelial Na\(^+\) reabsorptive capability in addition to a critical surfactant shortage (1).

Our study suggested that maternal IL-1\(\beta\) injection induced fluid absorption at 61 days gestation. The present data thus indicate that IL-1\(\beta\) may accelerate lung maturation in guinea pigs, resulting in an accelerated epithelial conversion to fluid absorption. Fig. 6. Alveolar fluid clearance in guinea pig fetuses of 61 days gestation (A) and 68 days gestation (B) after IL-1\(\beta\) pretreatment with and without cortisol synthesis inhibition by metyrapone and with and without Na\(^+\) channel inhibition by 10\(^{-5}\) M amiloride. *P < 0.05 compared with age-matched control; †P < 0.05 compared with control; ANOVA with Tukey’s test post hoc.

Fig. 7. Western blots of α-epithelial Na\(^+\) channel (ENaC) and β-ENaC. Left, typical Western blots of both ENaC subunits. Right, summary analysis of the optical density (OD) for 3 gels (samples) from each condition. *P < 0.05 compared with age-matched control; †P < 0.05 compared with IL-1\(\beta\) (61-day fetuses); ‡P < 0.05 compared with IL-1\(\beta\) (68-day fetuses); ANOVA with Tukey’s test post hoc.
absorption at 61 days gestation. By 68 days gestation, the fetal lung has converted from a secreting to a fluid-absorbing organ. Maternal IL-1β injection also stimulated alveolar fluid clearance in the 68-day-gestation fetal lungs. Thus maternal IL-1β injection could induce alveolar fluid absorption in 61 days gestation and stimulate alveolar fluid absorption in 68 days gestation. Several studies have also suggested a role for IL-1 in modulation of lung maturation (8, 19, 47). Our study provides novel information that IL-1β modulates lung maturation by induction/stimulation of alveolar fluid absorption in the fetal guinea pig lung.

Molecular mechanisms for fetal alveolar fluid clearance have been suggested to be apical ENaCs; this channel is sensitive to amiloride inhibition (12, 14, 25, 33) and basolateral Na⁺-K⁺-ATPases (18, 44). In our study, we found a significant increase in α- and β-ENaC subunit expression after IL-1β administration at both 61 and 68 days gestation. Paralleling these increases were increases in expression of both Na⁺-K⁺-ATPase α₁- and β₁-subunits. The molecular evidence correlated well with alveolar fluid clearance induction/stimulation and further supported our hypothesis that IL-1β could upregulate lung epithelial fluid transport. Functional ENaCs were thus evaluated by amiloride in the animal studies. Previous studies (11, 31) demonstrated that, before birth, amiloride sensitivity is close to 100% because there seem to be few or no other pathways for Na⁺ absorption besides amiloride-sensitive pathways. Simultaneous with birth, an alternative amiloride-insensitive pathway (possibly the cyclic nucleotide gated channel) may develop (32). Both types of channels are suggested to remain in postnatal lungs. Amiloride did not affect alveolar fluid secretion at 61 days gestation but completely inhibited the induced alveolar fluid absorption after maternal IL-1β pretreatment. This may be explained by the fact that amiloride blocked IL-1β-induced Na⁺ transport. Supporting that hypothesis, native 61-day-gestation fetuses have little or no ENaC expressed in the lung, but ENaC expression was upregulated significantly after IL-1β pretreatment. Similar results were found at 68 days gestation. The stimulated alveolar fluid clearance in this age group was totally inhibited by amiloride. At this gestational age, amiloride may inhibit all Na⁺ channels expressed, both those developed normally and those induced by IL-1β. Thus molecular evidence correlated well with changes in alveolar fluid clearance and further supported our hypothesis that IL-1β could upregulate ion transport across lung epithelia.
expression study like this one, it is not possible to deduce whether IL-1β could increase the open probability of ENaC and/or increase the activity of the Na⁺-K⁺-ATPase. However, the apparent upregulation of ENaC and Na⁺-K⁺-ATPase protein provided evidence that IL-1β could at least induce maturation of ENaC and Na⁺-K⁺-ATPase expression.

Plasma epinephrine is an important regulator of alveolar fluid transport (25, 46) because it can enhance active Na⁺ transport and accelerate alveolar fluid clearance (11, 46). β-Adrenoceptor stimulation is thought to increase transepithelial Na⁺ transport by stimulating ENaC at the apical membranes of alveolar epithelial type II cells (2) and possibly in the membranes of the type I cells as well (6) and by increasing the number and activity of Na⁺-K⁺-ATPases at the basolateral membranes of alveolar epithelial type II cells (38). Consequently, endogenous β-adrenoceptor stimulation by labor-released epinephrine may accelerate alveolar fluid transport near term (11, 12). The effect of endogenous β-adrenoceptor stimulation on alveolar fluid clearance during normal gestation can be studied by propranolol and measurement of plasma catecholamines. We therefore added propranolol to the instillate to investigate if endogenous β-adrenoceptor stimulation affected alveolar fluid clearance after IL-1β. In 61-day-gestation fetuses, IL-1β-induced alveolar fluid clearance was sensitive to propranolol, whereas control alveolar fluid secretion was insensitive to propranolol. In 68-day-gestation fetuses, propranolol inhibited both control and IL-1β-stimulated alveolar fluid clearance. We also measured fetal plasma epinephrine and norepinephrine concentrations with or without IL-1β pretreatment at 61 and 68 days gestation. In both age groups, plasma epinephrine concentrations were moderately but significantly increased with IL-1β pretreatment, whereas there were no differences in plasma norepinephrine concentrations. Plasma epinephrine concentrations in the 61-day- and 68-day-gestation control fetuses were similar to that previously reported in guinea pigs (31). Because IL-1 has been proposed as a signal for the onset of parturition and because it has been demonstrated to stimulate prostaglandin biosynthesis, which is important for labor onset (37), the IL-1β-stimulated plasma epinephrine concentrations might have resulted from an earlier onset of asymptomatic preterm labor. Thus IL-1β may affect β-adrenoceptor sensitivity and/or density on alveolar epithelial cells. To test the hypothesis that IL-1β sensitized the lung
by upregulating β-adrenoceptor density on lung epithelial cells, we determined total β-adrenoceptor density. At 61 days gestation, β-adrenoceptor density was significantly increased after IL-1β pretreatment, although no increase was seen at 68 days gestation. In an earlier study (41), IL-1 was demonstrated to synergistically with cortisol increase β-adrenoceptor density on lung cells. There are also multiple studies displaying either an upregulation of β-adrenoceptors (4, 43), a downregulation (21, 24, 27), or no change (40, 43) in expression. Because epinephrine stimulates alveolar fluid clearance by β-adrenoceptor stimulation and cortisol may affect β-adrenoceptor activity/density, we tested this in two ways. First, we added propranolol to the instillate after metyrapone and found that propranolol sensitivity of alveolar fluid clearance was attenuated, indicating that cortisol may modulate β-adrenoceptor function. Second, we measured β-adrenoceptor density after metyrapone and discovered that the increased β-adrenoceptor density after IL-1β was attenuated by metyrapone in 61-day-gestation fetal lungs, thus suggesting that cortisol may be responsible for upregulation of lung β-adrenoceptor density in the 61-day fetuses.

Maternal IL-1β injection could thus induce/stimulate alveolar fluid absorption, and it was associated with amiloride-sensitive Na⁺ transport, increased ENaC expression, increased Na⁺–K⁺-ATPase expression, and increased propranolol sensitivity associated with increased β-adrenoceptor density in the lung. Because cytokines often act as classic hormones, they bind to specific receptors on target cells and exert biological effects via second messengers. However, because the scenario in our study is the transfer of the signal from mother to offspring, there also has to be an extracellular “second messenger.” So, what might be the extracellular mediator or messenger between mother and offspring for IL-1β to trigger the downstream cascade events in the fetal lungs? It may of course be IL-1β itself if it crossed the placental barriers. However, our measurements of maternal and fetal plasma IL-1β suggested that only minute amounts of administered IL-1β actually crossed the placental barriers into the fetal blood circulation. Thus it was not very likely that IL-1β itself was the extracellular messenger. It has been reported earlier that IL-1β acts directly on the hypothalamus to stimulate release of the hypothalamic CRF and thus activates the hypothalamus-pituitary-adrenal gland axis with release of ACTH and plasma cortisol (39). Because cortisol is a well established lung maturation factor and can stimulate fluid absorption, plasma cortisol was hypothesized to be a likely target candidate for our investigation. Thus we hypothesized that IL-1β mediates its effects on alveolar fluid clearance at least partly via stimulation of the hypothalamus-pituitary-adrenal gland axis and plasma cortisol synthesis and release in maternal animals and/or fetuses. We therefore measured fetal and maternal plasma ACTH and cortisol concentrations at both gestational ages and found that both hormones were stimulated by IL-1β at both 61 and 68 days gestation. ACTH concentrations were higher in maternal plasma than in fetal plasma, whereas cortisol concentrations were higher in fetal plasma than in maternal plasma, thus suggesting that ACTH crossed the placenta into the fetal blood circulation and stimulated fetal cortisol synthesis. Thus, because IL-1β may activate the hypothalamus-pituitary-adrenal gland or the pituitary gland directly (3), ACTH would be stimulated in both cases. Because we did not measure CRF, we cannot tell which exact pathway was activated.

How important was plasma cortisol for IL-1β induction/stimulation of alveolar fluid clearance? To answer this question, we carried out a study where we inhibited plasma cortisol synthesis by 11β-hydroxylase inhibition by metyrapone to test if blocking cortisol synthesis would attenuate the IL-1β induction/stimulation on alveolar fluid clearance and transporter expression. In a previous study (30), it has been shown that 2 days of metyrapone pretreatment could significantly inhibit cortisol synthesis and that this affected baseline alveolar fluid clearance in adult guinea pigs. In this study, metyrapone administration significantly reduced fetal plasma cortisol concentrations simultaneously with attenuating amiloride and propranolol sensitivity of the induced/stimulated fetal alveolar fluid clearance. Moreover, metyrapone pretreatment significantly reduced the IL-1β-increased ENaC and Na⁺–K⁺-ATPase expression. These results demonstrate a central and important role of plasma cortisol for IL-1β modulation of lung maturation. Previous studies have demonstrated important roles of plasma cortisol for modulation of lung maturation with respect to surfactant synthesis (15, 22) and provided evidence that epithelial Na⁺ channels possess glucocorticoid regulatory elements (35) and that dexamethasone can stimulate alveolar fluid clearance (13). It has also been demonstrated that exogenous glucocorticoids can accelerate Na⁺–K⁺-ATPase synthesis (18). Our results demonstrate that endogenous plasma cortisol also modulates α- and β-ENaC and α₁- and β₁-Na⁺–K⁺-ATPase expression in the fetal lungs after maternal IL-1β pretreatment. Because both ENaC and the Na⁺–K⁺-ATPase are necessary for transepithelial Na⁺ transport, increasing expression/function of one often leads to a stimulation of the expression/function of the other.

In this study, it was demonstrated that maternal IL-1β participates in modulation of lung maturation by positively affecting multiple cellular and organism systems responsible for stimulating removal of fetal lung fluid near term. Our results indicate that the synthesis and release of ACTH and cortisol induced by IL-1β during intrauterine infections/inflammations, such as chorioamnionitis, may be the reason why babies delivered preterm from these mothers often seem to have a better resistance against developing severe RDS than normal preterm delivered babies. Because IL-1β releases cortisol, which is anti-inflammatory and maturation promoting, and that this stimulates resorption of the fetal lung fluid, it may explain why these babies are relatively resistant to developing RDS.

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