Congenital diaphragmatic hernia prevents absorption of distal air space fluid in late-gestation rat fetuses

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Congenital diaphragmatic hernia prevents absorption of distal air space fluid in late-gestation rat fetuses. Am J Physiol Lung Cell Mol Physiol 290: L478–L484, 2006. First published October 7, 2005; doi:10.1152/ajplung.00124.2005.—We hypothesized that congenital diaphragmatic hernia (CDH) may decrease distal air space fluid absorption due to immaturity of alveolar epithelial cells from a loss of the normal epithelial Na+ transport, as assessed by amiloride and epithelial Na+ channel (ENaC) and Na-K-ATPase expression, as well as failure to respond to endogenous epinephrine as assessed by propranolol. Timed-pregnant dams were gavage fed 100 mg of nitrofen at 9.5-day gestation to induce CDH in the fetuses, and distal air space fluid absorption experiments were carried out on 22-day gestation (term) fetuses. Controls were nitrofen-exposed fetuses without CDH induction. Lung fluid absorption in near-term control fetuses, elevated endogenous epinephrine levels, is not functional in CDH fetuses. Administration of the pesticide nitrofen to pregnant rats causes CDH with resultant pulmonary hypoplasia in 60% of the fetuses (2, 3), a condition that resembles CDH in human infants. Thus we decided to use this model to investigate the impact of CDH on absorption of fetal lung fluid at birth. A stimulus to this study was our clinical experience with two infants with severe pulmonary hypoplasia from CDH. The two infants were treated with extracorporeal membrane oxygenation (ECMO) because mechanical ventilation could not maintain adequate oxygenation and ventilation. Measurements showed that lung fluid absorption rates in these infants were far lower than expected based on previous studies in adult human lungs (see Discussion for details).

The first objective of the present study was to investigate distal air space fluid absorption near term (22-day gestation) in rat fetuses with nitrofen-induced CDH. Because it has been shown that catecholamines may be an important stimulus for distal air space fluid absorption at birth (14, 45), our second aim was to investigate whether endogenous β-adrenergic stimulation of absorption of fetal lung fluid was affected by the presence of CDH. Plasma samples were also obtained for measurements of endogenous epinephrine levels. Epithelial Na+ transport via amiloride-sensitive Na+ channels provides the driving force for distal air space fluid reabsorption in the lung (14, 23, 32). Therefore, the third aim was to determine whether the amiloride-sensitive fraction of distal air space fluid absorption in the fetal lungs was affected by CDH. A part of this third aim was to determine whether CDH affected lung ENaC and Na-K-ATPase expression.

METHODS

Animals. Rats of the Sprague-Dawley strain (96 fetuses divided on 8 litters and 8 timed-pregnant rats; Charles River, Hollister, CA) were used in the study. Both male and female rat fetuses were used in the study. The timed-pregnant rats were housed in separate cages in temperature- and humidity-controlled units (20 ± 2°C and 55 ± 10% relative humidity). All studies were approved by the University of California San Francisco Committee on Animal Research.

CDH induction. Nitrofen (2,4-dichloro-4’-nitrodi phenyl ether; Wako Chemicals, Osaka, Japan) was dissolved in 1 ml of olive oil by incubation at 50°C with rotation for 1 h. Timed-pregnant rats were at gestation day 9.5, anesthetized with ketamine (90 mg/kg body wt; Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (1.25 mg/kg body wt; Rompun; Bayer, Pittsburgh, PA), and gavage fed 100 mg of nitrofen. The timed-pregnant rats were then allowed to recover in their
rises and were returned to where they were housed for the remainder of gestation.

Preparation of instillates. The $^{131}$I-labeled 5% albumin instillate was prepared by dissolving 50 mg/ml bovine serum albumin (Sigma, St. Louis, MO) in an isosmolar aqueous solution of 0.9% NaCl with 0.1 $\mu$Ci $^{131}$I-labeled human serum albumin (Frost, Laboratories, Montreal, Canada). For studies of endogenous epinephrine dependence of distal air space fluid absorption, we added the general $\beta$-adrenergic antagonist propranolol ($10^{-4}$ M; Sigma) to the $^{131}$I-labeled 5% albumin instillate. For determination of fractional amiloride inhibition of distal air space fluid absorption, we added amiloride ($10^{-3}$ M; ICN Biochemicals, Costa Mesa, CA) to the $^{131}$I-labeled 5% albumin instillate. We used $10^{-3}$ M amiloride because $\sim 50\%$ of amiloride becomes protein bound and another significant fraction escapes the air spaces, resulting in lower functional concentrations (32, 48).

Surgery. Timed-pregnant rats and their fetuses were anesthetized by maternal intramuscular injections of ketamine (180 mg/kg body wt) mixed with xylazine (2.5 mg/kg body wt). After all fetuses had been delivered, the female rats were euthanized with an overdose of intracardiac pentobarbital sodium (100 mg/kg body wt; Nembutal; Abbott Laboratories, Chicago, IL) followed by bilateral pneumothoraces.

The anesthetized timed-pregnant female rats were placed on a temperature-controlled pad heated to 38°C to maintain their body temperature, an abdominal hysteroscopy was carried out by a midline laparotomy, and the fetuses were delivered one by one as described earlier (15). The success rate for the fetal surgery was 93 $\pm$ 9% (yielding 62 fetuses from 67 surgeries).

General experimental protocol. The 5% albumin instillate with the $^{131}$I-labeled albumin as a distal air space protein tracer was instilled into both lungs over $2-3$ s through the tracheal cannula as described in detail in our earlier study (15). Lungs lacking significant radioactivity, i.e., when the radioactivity left in the lungs was <70% of instilled radioactivity, were discarded with their corresponding distal air space fluid samples because of uncertainty of instillate location in those fetuses. Immediately after the fluid collection, presence of clinical CDH was confirmed by autopsy of the thorax-abdomen. A diaphragm defect and herniation of abdominal viscera into the thorax were taken as evidence of fetal CDH. The fetuses were then grouped as CDH and nitrofen control animals and studied as such. Plasma was collected from littermate fetuses not used for the fluid absorption experiments at the same time as for the fluid absorption studies and measured by a commercially available ELISA (IBL, Hamburg, Germany). The inter-assay and intra-assay coefficients of variation for the ELISA were 6.2% and 15.2%, respectively.

Specific experimental protocols. All rat fetuses used for distal air space fluid absorption studies were studied for 30 min. All experimental groups contained fetuses from at least two different dams. The n values represent individual fetuses. Our previous study confirmed that the rate of alveolar fluid absorption did not differ between anesthetized rats and our in situ rat fetuses (15). Of 62 rat fetuses used for the distal air space fluid absorption studies, 45 fetuses (73% success rate) had >70% of the instilled radioactivity in the lungs and were divided into the experimental groups below.

Group 1: nitrofen control studies (22-day gestation; n = 6). Rat fetuses with no postmortem signs of CDH from two dams were instilled with 10 ml/kg body wt of the $^{131}$I-labeled 5% albumin instillate into the lungs.

Group 2: CDH studies (22-day gestation; n = 6). Rat fetuses with postmortem confirmed CDH from two dams were instilled with 10 ml/kg body wt of the $^{131}$I-labeled 5% albumin instillate into the lungs.

Group 3: $\beta$-adrenoceptor blockade studies (22-day gestation nitrofen control; n = 6, 22-day gestation CDH; n = 15). Rat fetuses from three dams were instilled with 10 ml/kg body wt of the $^{131}$I-labeled 5% albumin instillate containing $10^{-4}$ M propranolol into the lungs. Endogenous epinephrine levels were measured in plasma from littermates to rat fetuses used for distal air space fluid absorption studies in each group (n = 10 nitrofen control; n = 5 CDH; n = 12 dams).

Group 4: amiloride studies (22-day gestation nitrofen control; n = 6, 22-day gestation CDH; n = 6). Rat fetuses from two dams were instilled with 10 ml/kg body wt of the $^{131}$I-labeled 5% albumin instillate containing $10^{-3}$ M amiloride into the lungs.

Distal air space fluid absorption calculation. Distal air space fluid absorption or secretion was measured by the increase or decrease in distal air space protein concentration of the instillate over 30 min as previously reported (15). The increase in protein concentration due to removal of water from the air spaces is a direct reflection of distal air space fluid absorption, as demonstrated previously (7, 17, 25, 29). Data are presented as distal air space fluid absorption as percent of instilled volume. Net distal air space fluid absorption (DFA) was calculated from:

$$DFA = \frac{[V_f - V_i]V_I}{100}$$

where $V_I$ is instilled volume (ml) corrected for preexisting lung fluid volume as described previously (15), and $V_f$ is final distal air space volume (ml) calculated from the $^{131}$I-labeled albumin concentrations in instilled and final distal air space fluids.

Western blot protocols: general protocol. Lung tissue from eight CDH and six nitrofen control fetuses was pooled by group and homogenized in T-Per reagent (Pierce, Rockford, IL) containing the protease inhibitors aprotinin (30 $\mu$g/ml, Sigma) and leupeptin (1 $\mu$g/ml, Sigma) with a Tissue Tearor homogenizer on ice. The tissue homogenate was centrifuged at 13,000 g for 5 min at $+4^\circ$C. The supernatant (membrane and cytosol fraction) was collected and aliquoted in multiple vials for each 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 $-80^\circ$C until analysis.

Polyacrylamide gel electrophoresis and transfer onto nitrocellulose membrane (Pierce) were carried out using standard protocols. After electrophoresis and transfer, the nitrocellulose membrane was placed in blocking buffer [SuperBlock Dry Blend blocking buffer in Tris-buffered saline (TBS, Pierce) for 1 h. Primary antibody incubations were carried out overnight at $+4^\circ$C on an orbital shaker.

ENaC. The anti-ENaC antibodies used were a gift from Dr. James D. Stockand at University of Texas Health Science Center and were directed against residues 137–161 of Xenopus laevis $\alpha$-ENaC and residues 624–647 of $\beta$-ENaC (42). These antibodies specifically recognize membrane proteins of appropriate sizes (85–90 kDa for $\alpha$-ENaC and 90–95 kDa for $\beta$-ENaC) in rats. After blocking, the membranes were incubated with the primary antibody [anti-$\alpha$-ENaC (rabbit) and anti-ENaC (rabbit), respectively, diluted 1:1,000]. After incubation, the membranes were washed 5× with wash buffer (PH 7.5, TBS with 0.1% Tween 20). After the washing process, the membranes were incubated with the enzyme-conjugated secondary antibody (goat anti-rabbit IgG, diluted 1:1,000) for 1 h at room temperature. After incubation, the membranes were washed again. Then, 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 TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom).

Na-K-ATPase. The anti-Na-K-ATPase antibody was obtained from Upstate Cell Signaling Solutions (Waltham, MA) and was directed against residues 338–518 of the $\alpha_1$-subunit of the Na-K-ATPase. The antibody specifically recognizes a membrane protein of appropriate size ($\sim$95 kDa for the $\alpha_1$-subunit of the Na-K-ATPase) in rats. A rat heart microsomal protein preparation was run on the gel as a positive control. After blocking, the membrane was incubated with the primary polyclonal antibody [anti-$\alpha_1$, Na$^+$/K$^-$-ATPase (rabbit), diluted 1:1,000]. After incubation, the membrane was washed 5×10 min in wash buffer. Then, the membrane was incubated with horseradish-
in the current study, the incidence of CDH after the nitrofen exposure was 48 ± 21% based on 96 fetuses and 8 litters. This CDH incidence was similar to previously published data (4).

Distal air space fluid absorption in CDH and nitrofen control fetal lungs. Distal air space fluid absorption in nitrofen-exposed control rat fetuses was normal (Fig. 1) and not different from the previously published distal air space fluid absorption of 18 ± 8% in normal 22-day gestation rat fetuses (15). Distal air space fluid absorption in our earlier study (15) was not different from that in the adult rat when extrapolated to 1 h (16 ± 2%) in the 40-h-old rats (data from Ref. 15) and compared with that in adult rats (17 ± 2%; data from Ref. 16). In CDH fetuses, there was no net distal air space fluid absorption; in fact, the lungs remained in the fluid secretory state with a net distal air space fluid secretion (Fig. 1).

Sensitivity to endogenous β-adrenergic stimulation. Circulating epinephrine levels were elevated in 22-day gestation rat fetuses [normal range in adult rats 10–100 pg/ml (10, 36, 37)] and unaffected by the presence of CDH (Fig. 2). The plasma from the dams displayed equally high circulating plasma epinephrine levels as the rat fetuses.

Because endogenous plasma epinephrine levels in near-term rat fetuses (22-day gestation) were high and alveolar fluid absorption in adult rats can be stimulated by exogenous administration of β-adrenergic agonists (21), we studied whether distal air space fluid absorption would be sensitive to inhibition by the β-adrenergic antagonist propranolol and if such a sensitivity would vary with the presence of CDH. Addition of propranolol significantly inhibited distal air space fluid absorption in 22-day gestation nitrofen control rat fetuses but had no effect in rat fetuses with postmortem-confirmed CDH (Fig. 1).

Involvement of amiloride-sensitive Na⁺ channels. We used amiloride to determine the contribution of the amiloride-sensitive Na⁺ channels for distal air space fluid absorption in 22-day gestation nitrofen control and CDH-positive rat fetuses. Addition of amiloride to the 131I-labeled 5% albumin instillate decreased distal air space fluid absorption in the 22-day gestation nitrofen control rat fetuses but had no effect in rat fetuses with postmortem-confirmed CDH (Fig. 1).

Expression of α1-ENaC, β-ENaC, and the α1-Na-K-ATPase subunit. ENaC and Na-K-ATPase expression were investigated in nitrofen-exposed control and CDH rat fetal lungs by Western blot. Both the α-ENaC and β-ENaC subunits were significantly decreased in expression in the CDH-positive fetal lungs (Fig. 3). The optical density (OD) values were normalized to the nitrofen control OD values. In fact, as seen in the figure, it is difficult to see a positive band in either of the CDH-positive fetal lungs on the displayed representative blot.

In contrast, the expression of the α1-Na-K-ATPase subunit was unchanged between the nitrofen-exposed control rat fetal lungs and the CDH-positive lungs (Fig. 4). The OD values were normalized to the nitrofen control OD values.

DISCUSSION

The majority of infants make the transition from intrauterine life to postnatal life without complications, but only hours before birth, the lungs are filled with an essentially protein-free isosmolar solution that has been actively secreted by the pulmonary epithelium. Complications such as CDH may have a serious impact on the ability and rate of fluid removal from the distal air spaces at birth and drastically affect the ability to oxygenate the newborn. We therefore determined the rate of distal air space fluid absorption in developing rat fetuses with and without nitrofen-induced confirmed CDH. Normally, fetal rat lungs of 22-day gestation have the ability to rapidly clear the distal air spaces from the fetal lung fluid, a fluid absorption that depends on plasma epinephrine and β-adrenoeceptor stim-
ulation and lung epithelial Na\textsuperscript{+} absorption (15). The nitrofen-exposed control rats in the current study displayed alveolar fluid absorption rates indistinguishable from those previously published in normal healthy rat fetuses of the same gestational age (15). Moreover, the responses to propranolol and amiloride were identical in the nitrofen-exposed control rat fetuses and normal healthy rat fetuses (15). Importantly, rat fetuses with confirmed postmortem CDH completely lacked the ability to clear the fetal distal air spaces from fluid. These lungs remained in the fetal fluid secretory stage, in sharp contrast to their nitrofen-exposed control littermates, in which the distal air spaces cleared the fetal distal air space fluid at a normal rate. Moreover, the CDH fetal lungs were nonresponsive to \( \beta \)-adrenoceptor stimulation by the elevated fetal plasma epinephrine levels, plasma levels that were no different than in nitrofen-exposed control fetal lungs. Also, amiloride had no effect on the fluid movement across the distal air space epithelia in the CDH-positive fetal lungs.

We had hypothesized that lung fluid absorption was decreased in CDH based on our previous experience with two infants with CDH in 1996 and 1998. Because they had severe pulmonary hypoplasia due to CDH (2, 18, 26, 40, 41), mechanical ventilation was not sufficient for adequate oxygenation and ventilation; thus both infants were treated with ECMO. Based on studies in experimental animals (1, 47), we attempted to stimulate lung growth in these infants by filling their lungs and airways with fluid at a constant positive pressure of 12 cmH\textsubscript{2}O. This was accomplished by attaching a tube to the endotracheal tube and instilling a protein-free, isosmolar fluid into the lungs via the tube. The pressure was maintained for 48 h by adding fluid as needed to keep the meniscus of the fluid at a level of 12 cm above the infants’ midtorax. We recorded the amount of fluid needed to maintain the pressure. We assumed that the rate at which we added fluid represented the rate of fluid absorption by the lungs. Based on fluid absorption studies from adult lungs, we ex-

Fig. 3. Expression of \( \alpha \)-epithelial Na\textsuperscript{+} channel (\( \alpha \)-ENaC) and \( \beta \)-ENaC subunits in lung tissue from CDH-positive fetal lungs and nitrofen-exposed fetal control (NC) lungs. Left: representative Western blots of \( \alpha \)-ENaC (top) and \( \beta \)-ENaC (bottom). Right: summary graphs of the optical density (OD) analyses of the Western blots. The OD graphs were normalized to the OD of the nitrofen control fetal lungs. Values are means \( \pm \) SD, \( n = 6 \) nitrofen control rat fetuses, \( n = 8 \) CDH rat fetuses for each marker. \(*P < 0.05\) compared with 22-day gestation nitrofen control rat fetuses (ANOVA with Tukey’s test as post hoc). MW, molecular weight.

Fig. 4. Expression of the \( \alpha_{1} \)-Na-K-ATPase subunit in lung tissue from CDH-positive fetal lungs and nitrofen-exposed fetal control lungs. Left: representative Western blot of the \( \alpha_{1} \)-Na-K-ATPase. Right: summary graph of the OD analysis of the Western blot. The OD graph was normalized to the OD of the nitrofen control fetal lungs. Values are means \( \pm \) SD, \( n = 6 \) nitrofen control rat fetuses, \( n = 8 \) CDH rat fetuses for each marker. H, positive control sample for the \( \alpha_{1} \)-Na-K-ATPase subunit from heart cell membrane microsomes.
expected a lung fluid absorption rate between 2 and 9 ml/h, representing 11–55%/h (11, 24, 38, 46). Similar approaches have been subsequently reported using perfluorocarbon filling of the developing air spaces to promote lung growth ex utero (13, 19, 44). To our surprise, fluid was absorbed at an initial rate of only 0.8 ml/h, which later slowed down to ~0.3 ml/h, representing 2%/h (Fig. 5), thus suggesting a significant abnormality in the normal perinatal processes of conversion of the fetal lungs from being a fluid-secreting organ to becoming one of fluid absorption. Some of the initial fluid absorption rate could have been caused by an initial distention of the lung air spaces as the fluid is instilled. In addition, it is possible that some fluid leaked from the air spaces of the damaged lungs. In either case, it would result in an overestimation of lung fluid absorption, thus further strengthening our observation of the subnormal distal lung fluid absorption rate in these lungs made hypoplastic by the CDH. This very low rate of fluid absorption may contribute to the respiratory insufficiency seen clinically in infants with CDH.

What may regulate distal air space fluid absorption at birth? Endogenous epinephrine can stimulate reabsorption of fetal lung fluid near term (8, 9, 12, 14, 45), levels that also have been shown to increase during labor and delivery (9). We demonstrated in our previous study that plasma epinephrine levels near term stimulated the rate of fluid absorption in fetal rats (15). Therefore, the involvement of endogenous epinephrine in regulating distal air space fluid absorption in CDH fetuses near term was determined in two ways. First, endogenous epinephrine plasma levels were measured in the nitrofen control and CDH rat fetuses and correlated with the ability to clear excess distal air space fluid in nitrofen-exposed control and CDH rat fetuses. Second, functional inhibition of β-adrenergic receptors sympathetic stimulation by addition of propranolol to the instilled fluid determined the extent of receptor stimulation. Endogenous epinephrine levels were significantly elevated in the 22-day gestation nitrofen control and CDH rat fetuses (Figs. 1 and 2) at the time when distal air space fluid absorption normally was at its greatest (15). However, plasma epinephrine failed to stimulate distal air space fluid absorption in CDH-positive fetal lungs. As a confirmation of the importance of epinephrine and β-adrenergic receptors for absorption of fetal lung fluid at birth, distal air space fluid absorption in 22-day gestation nitrofen control rat lungs was highly sensitive to propranolol inhibition, but CDH-positive lungs were insensitive to propranolol inhibition (Fig. 1). All of the results suggest that the defect in β-adrenergic receptor stimulation of distal air space fluid absorption in the CDH-positive fetal lungs resides either at the β-adrenergic receptor level, at the β-adrenergic receptor signaling level, and/or at the Na+/K-transport protein level. Although not investigated in the current study, β-adrenergic receptor downregulation has been demonstrated to be important in impairing distal lung fluid absorption in adult rats and mice (27, 39), and deficiencies in β-adrenergic receptor signaling may also be involved (28). Another possible explanation may reside within the mesenchyme. Pinter and colleagues (34) demonstrated that when, as in fetuses from diabetic mothers, there were more mesenchymal cells present, the expression of the α1-Na-K-ATPase decreased. However, for this to be solely the mechanism, one would expect that the expression of both transporters studied to change in a similar way, i.e., decrease. This was not the case in our studies, where ENaC expression decreased and α1-Na-K-ATPase expression was unchanged after CDH induction. Our results do not exclude any of the above mechanisms and thus do not permit us to make a definite conclusion, but the amiloride data and the ENaC protein expression data suggest that the principal mechanism for the attenuated distal air space fluid absorption in the CDH rat fetuses resides at the level of the apical Na⁺ uptake transport proteins.

In adult lungs, active Na⁺ uptake through apical amiloride-sensitive ENaC channels and basolateral Na-K-ATPases is a
The key mechanism for generating the driving force for alveolar epithelial water reabsorption (for review see Refs. 22 and 24). Amiloride inhibits ENaC and impairs reabsorption of fetal lung fluid in several species (14, 32, 33). Therefore, we investigated whether inhibition of distal air space fluid absorption by amiloride differed with and without nitrofen-induced CDH. Interestingly, the results showed that CDH-positive lungs completely lacked amiloride sensitivity, whereas normal amiloride sensitivity was observed in nitrofen-exposed control lungs (Fig. 1). We also investigated potential variations in α-ENaC and β-ENaC subunit expression in fetal lungs with and without confirmed CDH and found a significant decrease in lung expression of both ENaC subunits in CDH-positive lungs compared with nitrofen-exposed control fetal lungs. These data suggest that the principal impairment in CDH-positive lungs may reside at the level of ENaC expression and at the apical epithelial cell membrane. To further investigate the involvement of the Na+/K+ transport machinery, we studied the expression of the basolaterally expressed Na-K-ATPase, specifically the α1-Na-K-ATPase subunit. Interestingly, the expression of the α1-Na-K-ATPase subunit was unchanged between CDH-positive lungs and nitrofen-exposed control lungs, further suggesting that the defect of distal fluid absorption seen in CDH critically depends on ENaC expression and not on Na-K-ATPase expression. However, since we did not study Na-K-ATPase activity, we cannot exclude that the actual activity of the basolateral Na-K-ATPase was altered by CDH.

In conclusion, our data demonstrate that the presence of anatomically confirmed CDH attenuates normally developing Na+ uptake and absorption of fetal lung fluid in preparation for air-breathing postnatal life. Our data also suggest that the principal defect in the mechanism responsible for distal lung fluid absorption, i.e., the transepithelial Na+ transport, resides in the apical cell membrane and the ENaC channel. This conclusion is also functionally supported by the distal lung fluid absorption studies with amiloride showing no inhibition in CDH-positive lungs. Our findings suggest that, in addition to the well-recognized problems of pulmonary hypoplasia and pulmonary hypertension, human infants with CDH may have decreased development of the normal fluid-absorptive function of the lungs that may contribute to a potentially fatal respiratory insufficiency after birth.

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