Modulation of sodium transport in fetal alveolar epithelial cells by oxygen and corticosterone

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Thome, Ulrich H., Ian C. Davis, Susie Vo Nguyen, Brent Jay Shelton, and Sadis Matalon. Modulation of sodium transport in fetal alveolar epithelial cells by oxygen and corticosterone. Am J Physiol Lung Cell Mol Physiol 284: L376–L385, 2003. First published November 15, 2002; 10.1152/ajplung.00218.2002.—Regulation of active Na+ transport across fetal distal lung epithelial cells (FDLE) by corticosterone (CST), corticotropin-releasing hormone (CRH), and oxygen tension may be crucial for postnatal adaptation. FDLE isolated from 19-day rat fetuses (term: 22 days) were grown on permeable supports to confluent monolayers (duo-

SHORTLY AFTER BIRTH, active reabsorption of Na+ across the alveolar epithelium creates an osmotic gradient favoring the reabsorption of fetal lung fluid. Na+ enters the apical membranes of alveolar epithelial cells through amiloride-sensitive epithelial Na+ channels (ENaC) and is extruded across the basolateral membranes by the ouabain-sensitive Na+-K+-ATPase (50). The importance of active Na+ transport in fetal fluid reabsorption was clearly demonstrated by the work of Hummler et al. (23), who showed that newborn mice lacking the α (pore-forming)-subunit of the amiloride-sensitive channel (α-ENaC) failed to clear their lung fluid and died within 40 h from respiratory failure. Additional studies have shown that active Na+ transport plays an important part in decreasing alveolar fluid, thus optimizing gas exchange, in both the neonatal and adult lung (44, 45).

Active alveolar epithelial Na+ transport may be compromised in a number of pathological situations, including prematurity birth and respiratory distress syndrome (4, 7). During lung inflammation, reactive oxygen and nitrogen species, generated by epithelial and inflammatory cells (17, 22, 53), may damage apical and basolateral Na+ transporters. In addition, volutrauma during mechanical ventilation (12) was found to decrease lung liquid clearance (36). Both O2 toxicity and volutrauma contribute to the pathogenesis of respiratory distress syndrome and bronchopulmonary dysplasia. For these reasons, enhancing Na+ reabsorption across the newborn and adult alveolar epithelium may be of significant clinical benefit. In previous studies, we have shown that treatment of fetal distal lung epithelial (FDLE) cells with an adenoviral vector encoding for the β1-subunit of the Na+-K+-ATPase increased vectorial Na+ transport (62).

In addition to their well-known effects on surfactant production, prenatally administered glucocorticoids may also improve lung function in premature infants by stimulating increased pulmonary Na+ absorption (24, 63). Mineralocorticoids and glucocorticoids have been shown to increase both electrogenic Na+ absorption and fluid clearance by enhancing transcription of both ENaC and Na+-K+-ATPase genes (5, 60). Increased amiloride-sensitive short-circuit current (Isc) was found after exposure of fetal alveolar cells to glucocorticoids (11), although it is unclear whether this effect was the result of upregulation of ENaC, Na+-K+-ATPase, or both. The effect of corticosteroids on the ouabain-sensitive current (Iouab), as an indicator of Na+-K+-ATPase activity has not yet been measured. Furthermore, there is significant evidence that both levels of expression of Na+ transporters (ENaC and Na+-K+-ATPase) and levels of Na+ transport across alveolar...
epithelial cells are dependent on O₂ tension (2, 3, 19, 29, 37, 48, 52, 54, 55, 67). Moreover, the interactions between corticosteroids and the increased O₂ concentration, to which the alveoli are exposed after birth, remain undefined.

Corticotropin-releasing hormone (CRH) is a hypothalamic and placental hormone that controls adrenal glucocorticoid production through the release of ACTH from the hypophysis. Importantly, CRH is also abundant in other organs (39) and thought to control the timing of birth (47). Therefore, CRH may also be involved in lung maturation independent of its stimulatory effect on ACTH and corticosterone (CST) release. Direct effects of CRH on fetal alveolar cells are completely unknown.

After the considerations outlined above, we hypothesized that Na⁺ transport through rat fetal alveolar cells is 1) higher in cells grown at ambient O₂ tension than in cells grown at reduced O₂ tension, 2) increased by CST, the major glucocorticoid in the rat, and 3) modulated by CRH. We investigated these hypotheses by measuring Iₑ across alveolar cell monolayers in Ussing chambers. Using selective antagonists, we further examined whether current changes were attributable to changes in function of ENaC, Na⁺-K⁺-ATPase, or both. We also measured ENaC and Na⁺-K⁺-ATPase subunit protein expression by Western blotting.

**METHODS**

**FDLE cell isolation and culture.** The procedure for isolating FDLE cells has been described previously (27). In brief, lungs of 19- to 20-day gestation fetal rats (term = 22 days) were digested in a solution containing 0.125% trypsin and 0.4 mg/ml DNase in Eagle’s minimal essential medium (MEM) for 10 min. Digestion was stopped by the addition of MEM containing 10% FBS. Cells were collected by centrifugation and resuspended in 15 ml of MEM containing 0.1% collagenase and DNase. This solution was incubated for 15 min at 37°C. Collagenase activity was then neutralized by the addi-
that had flown off. Filters grown under identical conditions were pooled with their fluid in a single tube for each condition. The tubes were sonicated for 30 s in ice water and then centrifuged at 500 g and 4°C for 10 min to accumulate the fluid at the bottom of the tubes. The almost dry filters were discarded, leaving the protein-rich lysate. Protein content was measured using a standard protein assay (BCA, no. 23223; Pierce, Rockford, IL). Proteins were denatured by 10 min of incubation with 10% 2-mercaptoethanol at 95°C or, for Na⁺-K⁺-ATPase, at 37°C for 20 min and separated by SDS-PAGE through a 10% gel. Proteins were then transferred to a prewetted polyvinylidene difluoride membrane. Blots were probed by overnight incubation at 4°C with rabbit antibodies against α-ENaC, γ-ENaC (Alpha Diagnostics, San Antonio, TX), β-ENaC (gift from the laboratory of Douglas C. Eaton, Emory University, Atlanta, GA), α₁-Na⁺-K⁺-ATPase (Research Diagnostics, Flanders, NJ), or β₁-Na⁺-K⁺-ATPase (gift from the laboratory of Phillip Factor, Northwestern University, Chicago, IL), all diluted 1:1,000 in 50 mM Tris, 0.9% NaCl, and 0.1% Tween 20 (TBST) containing 3% nonfat dry milk. Blots were washed in TBST, and bound primary antibody was detected by incubation for 1 h at room temperature with a goat anti-rabbit polyclonal antibody, conjugated to horseradish peroxidase (diluted 1:10,000 in TBST-3% milk). Blots were washed again, and horseradish peroxidase activity was detected by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ). Band intensity was measured by densitometry using FluorChem software on an Alpha Innotech Imager. In some experiments, primary antibodies were preincubated with blocking peptides (derived from the same sources as each antibody; concentration of peptide to antibodies 10:1) for 30 min before probing of blots.

Statistical analysis. Significant differences among group means and interactions were determined by three-way ANOVA and Tukey’s post hoc test, using SAS software (SAS Institute, Cary, NC). P < 0.05 was considered significant.

RESULTS

Electrophysiological studies. All FDLE cells used in the electrophysiological studies were obtained from 11 different cell isolations. The mean value for transepithelial resistance for all monolayers was 0.55 ± 0.28 kΩ·cm² (mean ± SD, n = 250).

Basal Isc values were significantly higher in monolayers grown in 20% O₂ than in those grown at lower O₂ tensions (P < 0.0001; Fig. 1A). However, addition of CST to the culture media did not alter Isc at any O₂ tension. When similar studies were performed in Cl⁻-free solutions, baseline Isc was reduced slightly (Fig. 1B) and inhibited completely by the addition of 10 μM amiloride to the apical compartment (Fig. 2A). These findings indicate that Isc resulted mainly from transepithelial movement of Na⁺. In contrast to currents measured in solutions containing normal levels of Cl⁻, both baseline Isc (Fig. 1B) and amiloride-sensitive Isc (Fig. 2A) in Cl⁻-free solutions depended not only on O₂ (P < 0.001) but also on CST (P < 0.001). In additional experiments, the Iosch (the difference in Isc measured in the presence and absence of 1 mM ouabain in the basolateral compartment) was dependent on the O₂ concentration during culture (P < 0.001) but not on CST in either normal (Fig. 2B) or Cl⁻-free (data not shown) solutions.

The Iosch may not reflect maximal Na⁺-K⁺-ATPase capability because Na⁺ entry in the cells, through amiloride-sensitive Na⁺ channels, may be limiting total Na⁺ transepithelial transport. We therefore permeabilized the apical membrane of FDLE cell monolayers with amphotericin B (32), which elicited a marked increase of Isc (Fig. 3A). Similar to findings in nonpermeabilized cells, ouabmax was higher in monolayers grown in 20% O₂ than in those grown at 2.5–5 or 12% O₂ (P < 0.001, Fig. 3B). Furthermore, ouabmax was significantly lower if CST had been present in the cell culture (P < 0.05).

Apical Na⁺ permeability was measured as the Iamil in the presence of both an apical-to-basolateral Na⁺ gradient of 145:5 mM and a permeabilized basolateral membrane (Fig. 4A). The Iamil under these conditions...
was significantly higher in monolayers cultured in the presence of CST ($P < 0.0001$, Fig. 4B). Furthermore, amiloramine was influenced by the O₂ concentration during culture, but to a lesser extent.

Exposure to 3 nM CRH during culture did not have any significant effect on baseline $I_{sc}$ in the presence or absence of Cl$^{-}$ (Fig. 5). Likewise, amiloride- and ouabain-sensitive $I_{sc}$ values, measured across intact and permeabilized monolayers, were not different from their corresponding values in the absence of CRH (data not shown).

**Protein expression.** FDLE cells used for Western blotting were obtained from four different cell isolations. All three subunits of ENaC were detected in the cell lysates. The $\alpha$-, $\beta$-, and $\gamma$-subunits formed bands at 150, 130, and 160 kDa, respectively (Fig. 6A). An additional $\beta$-ENaC band of $\sim$100 kDa molecular mass was detected only in cells cultured in hypoxia in the presence of CST. Blots in which binding of primary antibody was inhibited by the presence of blocking peptides showed no immunoreactivity, thus confirming specificity of the $\alpha$- and $\gamma$-antibodies (data not shown). Expression of all three subunits was reduced significantly only by culturing cells in the presence of hypoxia and CST (Fig. 6B, $P < 0.01$, $n = 5$ individual blots analyzed for each subunit). In the absence of CST, O₂ tension did not have a significant influence on ENaC subunit expression, and, in the presence of room air, CST did not influence ENaC expression (Fig. 6B).

The $\alpha_{1}$- and $\beta_{1}$-subunits of the Na$^{+}$-K$^{+}$-ATPase formed bands at 100 and 45 kDa, respectively (Fig. 7A). Similar to ENaC subunits, $\alpha_{1}$-Na$^{+}$-K$^{+}$-ATPase expres-
The amiloride-sensitive component of $I_{sc}$ was $\sim70\%$ of total $I_{sc}$ which was slightly higher than in a previous study (35). Furthermore, herein we observed that removal of Cl⁻ had little effect on $I_{sc}$ while previously we reported that $\sim40\%$ of $I_{sc}$ was the result of secretion of anions (Cl⁻ and HCO₃⁻; see Ref. 35). These differences most likely resulted from culturing fetal cells in serum-free media (this study) vs. 10% FBS (35).

Compared with lower O₂ concentrations, room air (20% O₂) profoundly increased baseline $I_{sc}$ and its amiloride-sensitive and ouabain-sensitive components in intact monolayers. Likewise, experiments with permeabilized monolayers showed an increase in both amiloride-sensitive and ouabain-sensitive components.

The differences in total $I_{sc}$ and amiloride-sensitive and ouabain-sensitive $I_{sc}$ were not quite reflected in the measured protein expression. For example, we found that the combination of hypoxia and CST treatment resulted in a marked downregulation of ENaC subunit expression, which is in accordance with the lower $I_{sc}$ found in these monolayers. However, hypoxia without CST treatment induced a similar reduction in $I_{sc}$ but without reducing ENaC subunit protein expression. This may be explained by two facts. First, protein expression was evaluated in whole cell lysates, which contain both cytoplasmic and membrane-associated proteins. It is therefore impossible to determine how much functional ENaC or Na⁺-$K^+$-ATPase protein was actually inserted in the membranes and in functional conditions. Western blot analysis of preparations of isolated apical and basolateral membranes would be
necessary to clarify this point, but such samples are technically very difficult to obtain from cells grown on permeable supports. Second, multiple intracellular regulatory systems, such as protein kinases, modulate the functional activity of ion transport proteins. Therefore, even when isolated cell membranes are used, measurement of expressed protein may not reflect actual activity. Thus our data also show that measurement of protein expression is no substitute for actual measurement of ion transport.

The molecular masses of ENaC α- and γ-subunits were somewhat higher than those found by others in adult alveolar type II cells (26). However, we confirmed specificity of the antibodies by repeating the binding in the presence of blocking peptides, which blocked binding completely. Furthermore, older studies have also found higher molecular masses, i.e., between 135 and 150 kDa, depending on cell type and culture conditions (20, 42, 43, 51, 66). We speculate that differences in glycosylation, as well as the length of the actual protein molecule between species and cell lines, may be responsible for different molecular masses of the isolated proteins. Such differences may even be modulated by culture conditions, as shown by the lower molecular mass band that appeared in the β-ENaC blots when cells were cultured in hypoxia and in the presence of CST.

Few previous studies have addressed the effect of O2 on Na+ transport. Hyperoxia resulted in increased expression and activity of ENaC and Na+/K+-ATPase (29, 48, 52, 67). A similar dependence was described in the lower PO2 range, in accordance with our results. FDLE cell monolayers responded with increased amil-[426x112]ride- and ouabain-sensitive $I_{Na}$ after atmospheric PO2 was increased from 23 to 100 mmHg (3, 55). In another study using FDLE cells, increased ENaC expression, total current, and $I_{osm}$ were found after raising the PO2 from 23 to 160 and 380 mmHg (54), but Na+-K+-ATPase activity was not assessed in this study. Furthermore, the expression of ENaC and Na+-K+- ATPase subunits was increased with increasing PO2 in A549 cells (65) and alveolar cells from adult rats (18, 37, 38). Live animals kept in hypoxia showed similar effects (65). We chose to test several hypoxic O2 concentrations because the real PO2 at the cellular level was likely to be lower than the O2 concentration in the atmosphere around the cell culture. PO2 depends on the balance between diffusion of O2 through the culture media and the metabolic rate of the cells (59, 64). The latter of these parameters was unknown, and the testing of several PO2 values in the range of 19–152 mmHg therefore increased the likelihood that some culture conditions resemble fetal conditions, which are characterized by PO2 values between 16 mmHg (tissue) and 20–25 mmHg (arterial; see Refs. 1 and 61). It turned out that results were similar after cell culture in 2.5, 5, or 12% O2, whereas only cells grown at 20% O2 had distinctively higher currents, suggesting that PO2 needs to exceed a certain threshold to affect ion transport. Therefore, cells grown with 12% O2 appeared to add little information and were not included in all experiments. During the actual measurements in Ussing chambers, hypoxia was not continued because this would have been technically difficult, and the duration of measurements was much shorter than the...
4 h that is the minimum exposure needed to alter epithelial properties (54).

Interestingly, exposure to 1 μM of the glucocorticoid CST did not change baseline $I_{sc}$ in our experiments and brought only an increase in $I_{amil}$ but not in the $I_{ouab}$. Likewise, the $I_{amil}$ in experiments involving permeabilization of the basolateral membrane (amilmax) showed a marked increase after exposure to CST in culture. In contrast, $I_{ouab,max}$ was even slightly reduced after CST exposure. Thus CST appears to upregulate only the function of ENaC and not the Na+-K+-ATPase if used at physiological concentrations. This is further supported by the observation that, in contrast to normal Cl⁻-based solution, baseline $I_{sc}$ was increased after CST exposure if measured in Cl⁻-free conditions (Fig. 2). In normal Cl⁻ solution, Cl⁻ secretion serves as a potential additional pathway for ion movement and may have contributed to the total $I_{sc}$ to a varying extent, compensating for changes in ENaC activity. In Cl⁻-free solution, Na⁺ absorption remains the only source of current. Therefore, changes in Na⁺ channel activity elicited by CST may only become apparent in Cl⁻-free solution, whereas, in normal solution, total current may be more dependent on the Na⁺-K⁺-ATPase activity, which drives both Na⁺ absorption and Cl⁻ secretion.

Corticosteroids have been found to increase amiloride-sensitive Na⁺ transport and Na⁺ channel mRNA transcription in pulmonary epithelia (1, 10, 25). Furthermore, pretreatment with hydrocortisone enhanced a terbutaline-induced, amiloride-sensitive potential increase across cultured fetal lung buds (33) and a terbutaline-induced increase of amiloride-sensitive $I_{sc}$ across rat FDLE monolayers (11). Prenatal administration of dexamethasone to pregnant rat fetuses, dexamethasone treatment mainly increased expression of the $\alpha$-ENaC subunit but not the $\beta$- and $\gamma$-subunits (60). In a human cancer cell line (A549 cells), dexamethasone treatment mainly increased expression of the $\beta$- and $\gamma$-subunits of ENaC, which profoundly changed the biophysical properties of the channels formed (34). However, most previous studies used dexamethasone, a much more potent corticosteroid than CST, and it was given in pharmacological doses to malignant cell lines. Our results presented here demonstrate distinctly different actions of a physiological steroid used in physiological concentrations on native cells (6, 8, 9, 21).

Very limited data regarding the effect of corticosteroids on lung epithelial Na⁺-K⁺-ATPase are available. Prenatal administration of dexamethasone to pregnant rats did not change fetal expression of the $\alpha_1$- and $\beta_1$-subunits of the Na⁺-K⁺-ATPase (60), although no measurement of $I_{ouab}$ was performed in that study. Our study provides the first data on Na⁺-K⁺-ATPase function after corticosteroid treatment of lung epithelia and supports the notion that corticosteroids do not increase Na⁺-K⁺-ATPase expression and activity in fetal lungs. We also show that the action of corticosteroids in physiological doses is dependent on ambient O₂ concentration.

CRH levels in normal fetal rats have not been reported. In umbilical venous blood of normal human fetuses, CRH levels were between 15 and 63 pmol/l (13, 49, 56, 57). However, it is possible that fetal concentrations are much higher under pathological conditions, which are associated with increased maternal CRH levels (14–16, 56). We used 3 nM CRH in this study, which is ~50 times higher than in normal human fetuses and comparable to the highest concentrations achieved in humans at birth (13).

A number of findings indicated that CRH, a hypothalamic hormone that controls adrenal glucocorticoid production through the release of ACTH from the hypophysis, may have additional effects independent of CST, which may affect maturation of fetal tissues and thus alveolar Na⁺ transport. It is well known that postnatal glucocorticoid administration, no matter how early, is not nearly as effective as prenatal administration in improving lung function of preterm infants (58). After showing that fetal growth and lung function after premature birth in lambs was more strongly influenced if glucocorticoids were given to the ewe rather than directly to the fetus, Jobe et al. (28) speculated that part of the glucocorticoid effect may be mediated through another, as yet unidentified, factor. CRH was a potential candidate for this factor for several reasons. First, it is made not only in the hypothalamus but also in a number of other tissues, including the placenta and the lung (39), but the biological reason for this production has never been elucidated. Second, CRH shares homology to some intestinal signaling peptides. Third, CRH is thought to control the timing of birth (40). CRH levels rise steadily throughout pregnancy. High levels early in pregnancy have been associated with premature birth, whereas low levels are associated with postterm delivery (40, 46, 47). Fourth, there is a positive feedback between fetal glucocorticoids and CRH levels, with glucocorticoids stimulating placental release of CRH (30, 31, 41). Finally, increased levels of CRH have been observed in pathological situations, such as pregnancy-induced hypertension (14–16, 56). One could speculate that either increased prenatal levels of CRH or withdrawal of placental CRH at birth might enhance maturational responses in alveolar cells.

Contrary to our hypothesis, exposure to CRH during culture did not significantly alter Na⁺ transport. The small trends observed after exposure to CRH may become statistically significant with a higher number of experiments; however, differences that small would not be physiologically meaningful. It is unlikely that our CRH concentration was too low. In preliminary experiments using culture media containing serum, a 10-fold higher CRH concentration (30 nM) did not make any difference either. However, our study does not exclude that CRH may have other nonelectrophysiological effects on fetal alveolar cells, such as increasing surfactant production and maturation, which were beyond the scope of this study. Finally, CRH may not have the same effects in rats as it may have in sheep or humans, where most of the research regarding its influence on the timing of birth was done. In vivo, CRH
will also have indirect effects through CST release from the adrenals. Despite permeabilization, which effectively removed the apical barrier for Na\(^+\) entry, ouab\(_{\text{max}}\) was not much higher than the \(I_{\text{amil}}\) in intact monolayers. However, in intact monolayers, the K\(^+\) that is exchanged for Na\(^+\) by the basolateral Na\(^+-K\(^+-\)ATPase leaves the cell through basolateral K\(^+\) channels. Therefore, K\(^+\) remains on the basolateral side and does not contribute to net charge movement, which is being measured. With a permeabilized apical membrane, K\(^+\) is likely to leave the cell through the apical membrane in the negatively charged apical compartment, thus diminishing the measured net charge movement. Given the stoichiometry of the Na\(^+-K\(^+-\)ATPase (3 Na\(^+\) for 2 K\(^+\)) one can therefore assume that the true extrusion rate of Na\(^+\) is three times higher than the measured ouab\(_{\text{max}}\). It is very unlikely, but possible, that a small amount of apically instilled amphotericin B may have gained access to the basolateral membrane. The pores that would then form in the basolateral membrane would have decreased measurable Na\(^+-K\(^+-\)ATPase activity. In this case, our measurements of the ouab\(_{\text{max}}\) would have underestimated the true Na\(^+-K\(^+-\)ATPase activity, but this does not invalidate our conclusions. On the other hand, when permeabilizing the basolateral membrane in the presence of an apical-to-basolateral Na\(^+\) gradient, any amphotericin B that gets to the apical membrane would open further pathways for the movement of Na\(^+\) down the concentration gradient and thus lead to an overestimation of apical membrane Na\(^+\) permeability. Such pores, however, are insensitive to amiloride, and possible confounding effects can thus be eliminated by evaluating only the \(I_{\text{amil}}\).

In conclusion, our data indicate that ENaC function in rat FDLE cells is increased by exposure to increased O\(_2\) concentration or corticosteroids. In contrast, the Na\(^+-K\(^+-\)ATPase function is increased by exposure to increased O\(_2\) concentration but not by corticosteroids. CRH did not have detectable electrophysiological effects on FDLE cells.

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REFERENCES


52. Thomé U, Chen L, Factor P, Dumasius V, Freeman B, Sznajder JI, and Matalon S. Na,K-ATPase gene transfer mit-