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EPITHELIAL SODIUM CHANNEL IN AIRWAY EPITHELIUM OF THE NEWBORN INFANT

IMPLICATIONS FOR POSTNATAL PULMONARY ADAPTATION

BY

OTTO HELVE

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CONTENTS

PREFACE

I wish to express my sincere gratitude to Professors Mikael Knip and Christer Holmberg, the present Heads of the Hospital for Children and Adolescents, to the former Head of the Hospital for Children and Adolescents, Professor Emeritus Jaakko Perheentupa, and to Professor Erkki Savilahti, the Head of its Research Laboratory for providing me with an excellent research atmosphere and facilities.

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ABBREVIATIONS

AQP	Aquaporin
BPD	Bronchopulmonary dysplasia
BW	Birth weight
CAP	Channel-activating protease
CK	Cytokeratin
ENaC	Epithelial sodium channel
GA	Gestational age
LC	Lung compliance
N-PD	Nasal potential difference
PCR	Polymerase chain reaction
RDS	Respiratory distress syndrome
RT-PCR	Reverse-transcriptase polymerase chain reaction
SGK	Serum- and glucocorticoid-induced kinase
TTN	Transient tachypnea of the newborn

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by roman numerals.

I. **Helve O**, Pitkänen OM, Andersson S, O'Brodovich H, Kirjavainen T, Otulakowski G. Low expression of human epithelial Na^+ channel (hENaC) in airway epithelium of preterm infants with respiratory distress. Pediatrics 2004;113:1267-1272

II. **Helve O**, Pitkänen OM, Kirjavainen T, Andersson S. Sodium transport in airway epithelium predicts lung compliance in healthy newborns. J Pediatr 2005;146:273-6

III. **Helve O**, Andersson S, Kirjavainen T, Pitkänen O. Postnatal lung compliance and sodium transport in airway epithelium in term infants. Am J Respir Crit Care Med 2006;173:448-52.

IV. **Helve O,** Janér C, Pitkänen O, Andersson S. Expression of the epithelial sodium channel in airway epithelium of newborn infants depends on gestational age. Pediatrics 2007;120:1311-6

ABSTRACT

The aim of the present thesis was to study the role of the epithelial sodium channel (ENaC) in clearance of fetal lung fluid in the newborn infant by measurement of airway epithelial expression of ENaC, of nasal transepithelial potential difference (N-PD), and of lung compliance (LC). In addition, the effect of postnatal dexamethasone on airway epithelial ENaC expression was measured in preterm infants with bronchopulmonary dysplasia (BPD).

The patient population was formed of selected term newborn infants born in the Department of Obstetrics (Studies II-IV) and selected preterm newborn infants treated in the neonatal intensive care unit of the Hospital for Children and Adolescents (Studies I and IV) of the Helsinki University Central Hospital in Finland. A small population of preterm infants suffering from BPD was included in Study I. Studies I, III, and IV included airway epithelial measurement of ENaC and in Studies II and III, measurement of N-PD and LC. In Study I, ENaC expression analyses were performed in the Research Institute of the Hospital for Sick Children in Toronto, Ontario, Canada. In the following studies, analyses were performed in the Scientific Laboratory of the Hospital for Children and Adolescents. N-PD and LC measurements were performed at bedside in these hospitals.

In term newborn infants, the percentage of amiloride-sensitive N-PD, a surrogate for ENaC activity, measured during the first 4 postnatal hours correlates positively with LC

measured 1 to 2 days postnatally. Preterm infants with BPD had, after a therapeutic dose of dexamethasone, higher airway epithelial ENaC expression than before treatment. These patients were subsequently weaned from mechanical ventilation, probably as a result of the clearance of extra fluid from the alveolar spaces. In addition, we found that in preterm infants ENaC expression increases with gestational age (GA). In preterm infants, ENaC expression in the airway epithelium was lower than in term newborn infants. During the early postnatal period in those born both preterm and term airway epithelial βENaC expression decreased significantly. Term newborn infants delivered vaginally had a significantly smaller airway epithelial expression of αENaC after the first postnatal day than did those delivered by cesarean section. The functional studies showed no difference in N-PD between infants delivered vaginally and by cesarean section.

We therefore conclude that the low airway epithelial expression of ENaC in the preterm infant and the correlation of N-PD with LC in the term infant indicate a role for ENaC in the pathogenesis of perinatal pulmonary adaptation and neonatal respiratory distress. Because dexamethasone raised ENaC expression in preterm infants with BPD, and infants were subsequently weaned from ventilator therapy, we suggest that studies on the treatment of respiratory distress in the preterm infant should include the induction of ENaC activity.

TIIVISTELMÄ

Raskauden aikana sikiön keuhkot ovat täynnä nestettä. Keuhkojen pintasolukko tuottaa nesteen erittämällä osmoottisesti aktiivista kloridia. Vesi seuraa kloridia passiivisesti täyttäen keuhkot. Syntymän yhteydessä keuhkojen tyhjeneminen ylimääräisestä nesteestä on välttämätöntä normaalin kaasujen vaihdon onnistumiseksi. Väitöskirjatutkimuksen tavoitteena oli selvittää epiteliaalisen natriumkanavan (ENaC) merkitystä vastasyntyneen lapsen keuhkonesteen poistossa. Väitöskirjassa selvitettiin myös, miten bronkopulmonaarisesta dysplasiasta (BPD) kärsivälle ennenaikaisesti syntyneelle lapselle annettu deksametasoni vaikuttaa ilmateiden ENaC:n mRNA:n määrään eli ilmentymiseen. Väitöskirjaan sisältyvissä tutkimuksissa on mitattu ilmateiden ENaC:n ilmentymistä sekä välillisesti ENaC:n toimintaa mittaamalla nenän pintasolukon eli epiteelin transepiteliaalista potentiaalieroa (N-PD) ja keuhkojen myötäävyyttä, keuhkokomplianssia (LC).

Potilasaineisto muodostui Naistenklinikalla täysiaikaisina syntyneistä lapsista (tutkimukset I-IV) sekä Lastenklinikan vastasyntyneiden teho-osastolla hoidossa olleista ennenaikaisina syntyneistä lapsista (tutkimukset I ja IV). Tutkimuksessa I oli pieni otos BPD:stä kärsiviä ennenaikaisesti syntyneitä lapsia. Tutkimuksissa I sekä III-IV mittasimme ilmateiden ENaC:n ilmentymistä. Tutkimuksissa II ja III mittasimme N-PD:n sekä LC:n. Tutkimuksen I osalta potilasnäytteiden ENaC:n ilmentyminen mitattiin Hospital for

Sick Children -sairaalan tutkimusinstituutissa Kanadan Ontariossa, Torontossa. Kaikki muut laboratoriotutkimukset toteutettiin Lasten ja nuorten sairaalan Tieteellisessä laboratoriossa. Kaikki N-PD- ja LC-mittaukset suoritettiin joko Naistenklinikan tai Lastenklinikan osastoilla.

Toiminnallisten tutkimusten avulla selvitimme, että ensimmäisten elintuntien aikana mitattu ENaC:n toimintaa kuvaava amiloridiherkän N-PD:n prosenttiosuus oli yhteydessä LC:n arvoon kahden ensimmäisen syntymän jälkeisen päivän aikana. Totesimme, että BPD:stä kärsiville ennenaikaisesti syntyneille lapsille annettuun deksametasonihoitoon liittyi ENaC:n ilmentymisen nopea nousu. Näillä lapsilla taudin oireet lievenivät siten, että hengityskonehoito voitiin lopettaa. Tämä liittyy mahdollisesti ylimääräisen keuhkonesteen poistumiseen hoidon myötä. Totesimme myös, että syntymähetken ilmateiden ENaC:n ilmentyminen oli yhteydessä raskauden kestoon: ennenaikaisesti syntyneillä lapsilla ilmateiden ENaC:n ilmentyminen oli matalampi kuin täysiaikaisilla lapsilla. Ilmateiden βENaC-alayksikön ilmentyminen laski merkitsevästi syntymän jälkeen sekä ennenaikaisesti että täysiaikaisina syntyneillä lapsilla. Alateitse syntyneillä täysiaikaisilla lapsilla ilmateiden αENaC:n ilmentyminen oli merkitsevästi alhaisempi ensimmäisen elinpäivän jälkeen kuin keisarinleikkauksella syntyneillä lapsilla.

Yhteenvetona toteamme, että ENaC saattaa olla merkittävä tekijä täysiaikaisen vastasyntyneen lapsen keuhkojen sopeutumisessa syntymänjälkeiseen ympäristöön, mutta myös ennenaikaisesti syntyneen lapsen hengitystoimintaan liittyvissä ongelmissa.

INTRODUCTION

The newborn infant is faced with the immense challenge required by adaptation from a fluid-filled environment to the breathing of air. Most of us when undergoing this phase rarely encounter obstacles, but the more premature the infant, the more likely he or she is to develop problems during the adaptation process. Related to pulmonary adaptation is respiratory distress syndrome (RDS), an acute lung disease of the newborn preterm infant. It results from lack of surfactant, but also from failure to clear the lung of perinatal fluid. Later on, preterm infants may progress to bronchopulmonary dysplasia, a chronic lung disease. The term newborn may, however, also encounter problems with adaptation. This entity, called transient tachypnea of the newborn or "wet lung" syndrome, is also a possible result of inefficient lung fluid clearance.

The fetal lung is filled with fluid that gives support to the shaping of the lung. Lung fluid secretion is characterized by active chloride transport, and therefore also water, through the airway epithelium into the lung lumen. At birth, with the aid of ion transport through lung epithelial sodium channels, the direction of lung fluid transport changes, and the pulmonary epithelium begins to absorb fluid. For the clearance of fetal lung fluid, this process is crucial.

The following studies further examine the expression and activity of the epithelial sodium channel in the postnatal adaptation process of the newborn infant.

REVIEW OF THE LITERATURE

Lung Development

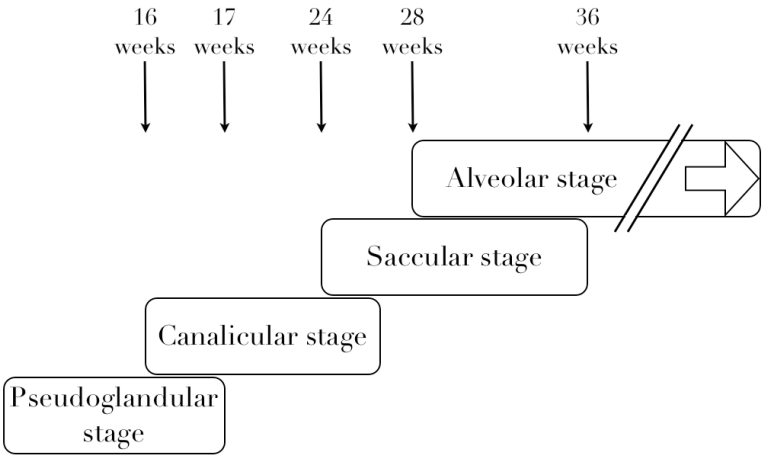
Macroscopic development of the embryonic lung begins from a protrusion of the foregut and proceeds by branching of the lung bud and penetration by the main bronchi into the mesenchyme. Gradually, this development progresses from the proximal towards the distal pulmonary structures (Martin et al. 2006). Differentiation of the lung epithelium follows in a similar pattern: The most distal tubules are lined with undifferentiated cells, with escalating differentiation in the more proximal airways. The mature respiratory epithelium lines the entire respiratory tract from the nostrils to the bronchi. While small histological variation exists, the majority of the airways among it the nasal cavity are lined with ciliated pseudostratified columnar epithelium and goblet cells (Kierszanbaum 2002). Distally, goblet cells become sparser and are replaced by surfactant-producing Clara cells.

The epithelium begins a transformation into simple cuboidal epithelium in the terminal parts of the bronchioles. Towards the distal end of the airways, in the respiratory bronchioles, the low cuboidal epithelium is replaced in part by squamous type I alveolar cells (ATI). Respiratory bronchioles lead to the alveoli, the epithelium of which consists mainly of ATI (> 95% of the internal surface) and the minority of surfactant-secreting type II alveolar cells (ATII) (Kierszanbaum 2002). Type I cells are extremely permeable to water (Dobbs et

al. 1998). Both ATI and ATII contain water-transporting channels, aquaporins (AQP) (Zelenina et al. 2005), epithelial sodium channels, and Na^+K^+ATPase (Yue et al. 1995, Factor et al. 1998b, Johnson et al. 2002, Ridge et al. 2003).

Normal development of the human lung is a process that begins in early gestation and ends in early childhood. Development is divided histologically into five overlapping stages (Martin et al. 2006): the embryonic, pseudoglandular, canalicular, saccular, and alveolar stage (Figure 1). The embryonic stage (from 1 to 7 weeks of gestation) is characterized by the formation of pulmonary veins and the division of the airways into subsegmental branches. The pseudoglandular stage (from 5 to 17 weeks of gestation) ends the formation of airways into the yet-to-be-developed lungs. During the canalicular stage (16-26 weeks of gestation), alveolar type II and subsequently type I cells begin to form in the distal lung, and the lung parenchyma begins to be canalized by capillaries. The saccular stage (from 24 to 36 weeks of gestation) is characterized by the formation of saccules, widened terminal air spaces. This step, septation, results in increased size of the lung parenchyma. During the alveolar stage (starting from 28 weeks of gestation) the alveoli, the main units for gas exchange in the lungs, are formed. It is speculated that up to half the adult alveoli are present at term (Hislop et al. 1986), and that the process of alveolarization may then continue for up to 2 years after birth.

Figure 1
Stages of Lung Development



Fluid is present in the lung lumen throughout fetal lung development, presumably beginning as early as the sixth week of gestation (McCray et al. 1992). Whereas the fluid content of the fetal lung was described earlier, an approximation of the amount of fluid in the fetal mammalian lung was first mentioned in 1923 in studies performed on fetal and newborn lambs (Faure-Fremiet E 1923). Even that early, it was noted that, at birth, the fluid content of the lungs rapidly declines. At that time, however, fetal lung fluid was considered to be aspirated amniotic fluid. Data from studies on humans and animals with congenital atresias of the trachea and larynx and experimentally ligated tracheas in animals show that lungs distal to the occlusion are distended, suggesting a pulmonary origin for lung fluid (Potter et al. 1941, Carmel et al. 1965, Griscom et al. 1969). The concept of the lung's secreting fluid was introduced in 1941 and noted to be "transudation of body fluid through the alveolar wall" (Potter et al. 1941). Eventually, lung fluid composition was demonstrated to differ from that of plasma and therefore was

suggested possibly to be fluid secreted by the pulmonary epithelium, not to be a plasma ultrafiltrate (Adams et al. 1963, Ross 1963, Adamson et al. 1969, Mescher et al. 1975, Alcorn et al. 1977).

Secretion of fluid into the lung lumen results in increased intrapulmonary pressure. The closed vocal chords, larynx, and nasopharynx constrict the outflow of lung fluid (Brown et al. 1983, Fewell et al. 1983, Fisk et al. 1992); therefore the intrathoracic pressure in fetal sheep is, in the third trimester, higher than is the elastic pressure of the chest wall (Vilos et al. 1982). Because pressure in the fetal lung is crucial for keeping the developing pulmonary structures open, lung development is dependent on a certain amount of lung fluid. Pathological states such as oligohydramnios, in which the formation of normal intrapulmonary fluid pressure is inhibited, result in lungs' being of small volume, or hypoplastic (Moessinger et al. 1986). This can also be the result of pulmonary arterial occlusion, congenital diaphragmatic hernia, skeletal dysplasia, or diaphragmatic paralysis (Wigglesworth 1988, Wallen et al. 1990, 1994), in all of which, intrapulmonary pressure is lower than in the healthy fetus. With a surplus of fetal lung fluid, the lungs become larger than the normally developed lung, or hyperplastic (Alcorn et al. 1977, Wigglesworth et al. 1987, Moessinger et al. 1990).

Fluid Secretion in Fetal Lung

From mid-term towards term, lung fluid secretion increases, but it decreases significantly before labor (Kitterman et al. 1979, Pfister et al. 2001). The actual rate of secretion has remained speculative. A review by Olver suggests that during the third trimester in the fetal sheep, rate of secretion ranges from 3.5 to 5.5 mL/h/kg (Olver et al. 2004). This suggestion was based on an earlier review and on undisclosed data, however (Strang 1991).

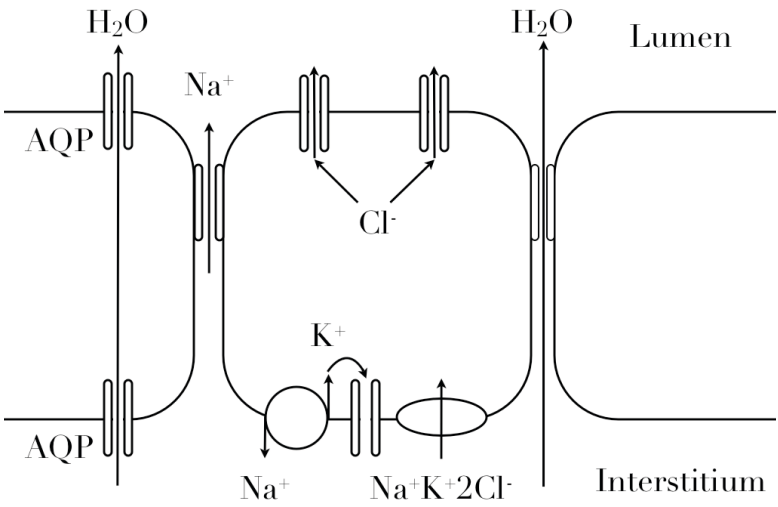
An important step in unveiling the source of lung fluid was a report on the difference in composition between fetal lung fluid and amniotic fluid or plasma (Adamson et al. 1969). In the fetal lamb lung, the transport of chloride ions was noticed to take place against the existing electrochemical gradient (Olver et al. 1974). Further studies in rat alveolar epithelial cells suggested that the epithelium secretes Cl^- and thus drives fluid secretion (Krochmal et al. 1989). In addition, in fetal sheep, the increase in filtration pressure in fetal lungs did lead to an increased secretion rate of lung fluid (Carlton et al. 1992). An alternate mechanism was required.

Chloride Transport

From early gestation, fetal lung fluid is secreted through active chloride secretion (Adams 1966, Adamson et al. 1969, Olver et al. 1974, 1981). Na^+K^+ATPase creates a gradient inside the cell for chloride entry into the cell through the basolateral $Na^+K^+2Cl^-$ cotransporter. Thus, the intracellular Cl^- is raised above its electrochemical gradient, pushing chloride through apical anion-selective Cl^- channels into the developing airways. Water follows passively in the same direction, possibly through a paracellular route or water-specific AQP channels. In the fetal mammal lung epithelium that at least AQP4, a basolaterally located channel, and AQP5, an apically located channel, are present (Liu et al. 2003). In general, towards the end of gestation, the expression of AQPs increases (Ruddy et al. 1998) (Figure 2).

The exact location for Cl^- secretion in the epithelium remains unclear. In animals, fetal distal lung epithelial cells demonstrate the potential for a Cl^--secreting activity (Rao et al. 1991), whereas the epithelial cells in bronchioles have sodium absorptive capacity (Olver et al. 1986b), suggesting a more distal source. However, the cellular origin of fetal chloride secretion between species may differ.

Figure 2.
Mechanisms of Fetal Chloride Secretion



With the aid of Na^+K^+ATPase, the amount of intracellular Cl^- exceeds its electrochemical gradient, pushing Cl^- through apical channels into the developing airways. Water follows in a parallell direction.

Fetal Regulation of Secretion

Because the magnitude of secretion of lung fluid is dependent on gestational age (GA), it is likely to use several different mechanisms for regulation. In animals, fetal administration of prolactin stimulates secretion (Cassin et al. 1982). In the first trimester, prostaglandins and growth factors (McCray et al. 1993, Graeff et al. 1999) also seem to stimulate secretion, but prostaglandin inhibitors decrease secretion (Cassin 1984). The drainage of lung fluid from fetal sheep results in an increase in lung fluid secretion, suggesting that a pressure-sensing mechanism is involved in the regulation of chloride secretion (Nardo et al. 1995).

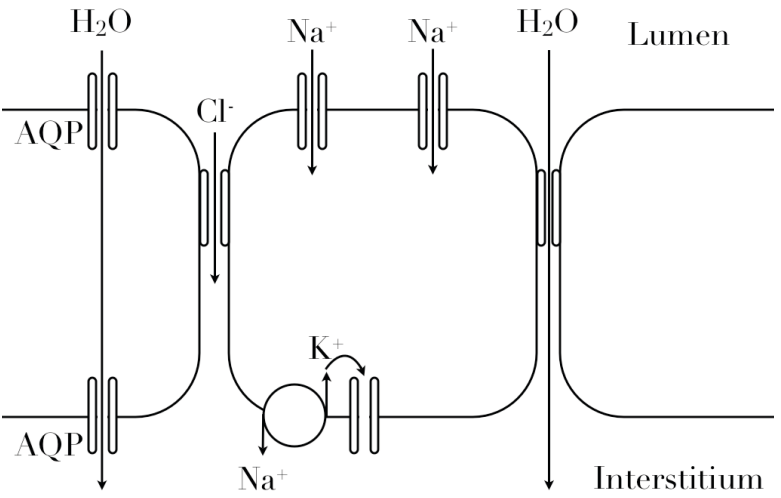
Perinatal Lung Fluid Movement

At birth, because the newborn infant needs to commence respiration the rapid removal of fetal lung fluid is crucial. Early experiments on animals were already showing significant reduction in lung fluid content after birth (Faure-Fremiet E 1923, Aherne et al. 1964). In the term newborn rabbit, lung wet weight decreases substantially during the first 2 hours (Aherne et al. 1964) and up to 24 hours after birth continues to decrease (Bland et al. 1980). Fetal breathing movements may clear some of the fetal lung fluid several days prior to birth (Pfister et al. 2001). In addition, some transient passive movement of lung fluid through the epithelium may occur during the first hours after birth (Egan et al. 1975, Pitkanen et al. 1996). Because ion transport is crucial for lung fluid secretion, this may be a potential mechanism also for its clearance through active sodium absorption (Cotton et al. 1988a, 1988b), but the exact mechanism was for a time unclear.

At least four different means exist for transport of water across cell barriers: by passive diffusion through plasma membranes, through AQPs, paracellularily through tight junctions, or by co-transporters. On the basis of reports confirming the existence of sodium channels and Na^+K^+ATPase in ATII cells, these were considered the main site of alveolar ion transport (Olivera et al. 1994, Voilley et al. 1994, Matalon et al. 1999). ATI cells were described as expressing AQPs and therefore providing a route for water to follow the gradient created by ATII cell ion transport (Nielsen et al. 1997, Dobbs et al. 1998). However, recent reports on patch clamp studies contribute to these findings by depicting highly selective cation channels in ATI consistent with ENaC, but also with Na^+K^+ATPase (Johnson et al. 2002, Johnson et al. 2006). It is thus likely that ATI cells, constituting over 95% of the internal surface of the lung, also play a role in active ion transport across the lung epithelium.

The mechanism for perinatal sodium transport involves the basolateral Na^+K^+ATPase that creates a gradient for sodium entry into the cell. Apical sodium channels are activated, allowing sodium to be transported from the lung lumen into the interstitium. Being an osmotically active molecule, sodium draws water via a paracellular route or through water-specific AQP-channels (Figure 3).

Figure 3.
Mechanisms of Perinatal Sodium Absorption



With the help of basolateral Na^+K^+ATPase, a gradient is formed in the epithelial cell that the apical Na^+ channels activate. This allows Na^+ to be transported from the lung lumen into the interstitium. Being an osmotically active agent, Na^+ draws water in a parallell direction.

In near-term fetal sheep, the addition of amiloride, a sodium channel blocker, results in increased secretion of lung fluid (Olver et al. 1986a), indicating that sodium transport and therefore fluid absorption, is already then a part of net fluid transport.

Several endogenous factors contribute to the switch of the lung epithelium from a secreting to an absorbing organ. During labor, the fetus is subjected to a mechanical stimulus, but also to a surge in hormonal stimuli. Many of these factors are dependent on method of delivery, i.e., whether it is preceded by labor. Therefore, the recognition that animal fetuses born by elective cesarean section without labor had slower lung fluid clearance showed the importance of labor in lung fluid removal (Adams et al. 1971, Bland et al. 1979). Labor was known to be connected to a surge of β-adrenergic stimuli (Lagercrantz et al. 1977), and the discovery of adrenaline's causing rapid clearance of lung fluid resulted in a search for its mechanism (Walters et al. 1978). In addition, lung maturity was shown to be of importance in the conversion from fluid secretion to absorption in response to β-agonists (Brown et al. 1983). However, some changes in lung fluid secretion are apparent even before labor: Animal experiments indicate that during the last days of gestation prior to the onset of labor the lung is already reducing its rate of fluid secretion (Bland et al. 1979, Bland et al. 1982).

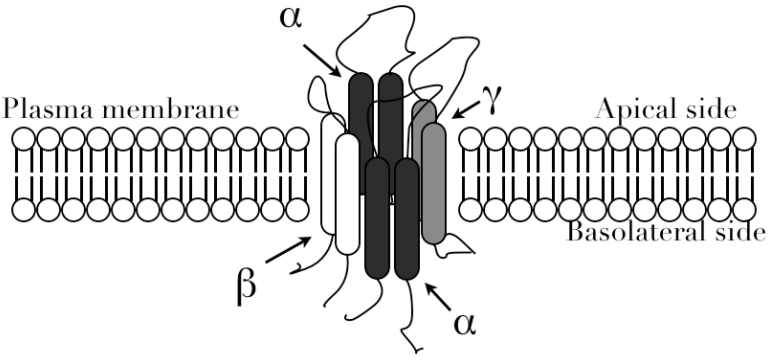
The focus of the majority of studies concerning the regulation of lung fluid clearance has been on the role of catecholamines, glucocorticoids, and thyroid hormone (Baines et al. 2000, Folkesson et al. 2000, Chen et al. 2002, Mustafa et al. 2004), but arginine vasopressin (Perks et al. 1989, Wallace et al. 1990) may also play a part in this perinatal switch. That an increase in oxygen concentration elevates sodium transport in cultured fetal epithelial cells suggests that ambient PO_2 is also important in the clearance of fetal lung fluid (Pitkanen et al. 1996).

Sodium Transport

Amiloride has served as a means of studying sodium transport, due to its inhibitory effect on sodium channels (Kleyman et al. 1988). Thus the finding that amiloride instilled into the

trachea of newborn guinea pigs in the early postnatal period causes respiratory distress (O'Brodovich et al. 1990) led to increased interest in the role of sodium channels in postnatal lung-fluid clearance.

FIGURE 4.
STRUCTURE OF THE EPITHELIAL SODIUM CHANNEL (ENAC)



ENaC is formed of α-, β-, and γ-subunits. A subunit is formed of two transmembrane domains, an extracellular loop, and short N- and C-termini.

Of the ion channels on the apical surface of the airway epithelium, the amiloride-sensitive sodium channel (ENaC) is rate-limiting for the process of lung-fluid transport across the epithelium (Hummler et al. 1996). ENaC, first isolated from the colonic epithelium of rats, was soon shown to be expressed also in the kidney and the lung (Canessa et al. 1993, O'Brodovich et al. 1993, Voilley et al. 1994). In airway epithelium, ENaC is composed of three subunits, the α-, β-, and γ-subunits, each formed of two transmembrane domains, an extracellular loop, and short N- and C-termini (Canessa et al. 1993, 1994) (Figure 4). The three subunits expressed together produce channel activity 100-fold greater than that of αENaC alone (Canessa et al. 1994, Hummler et al. 1996, Fyfe et al. 1998). In addition, in different epithelia, several apical amiloride-sensitive channels

have been characterized by selectivity for Na^+ over K^+ (Sariban-Sohraby et al. 1992, Voilley et al. 1994, Jain et al. 1999, 2001).

The importance of ENaC for postnatal pulmonary adaptation was highlighted in animal experiments when αENaC-knock-out mice, unable to clear their lungs of perinatal fluid, died of respiratory insufficiency (Hummler et al. 1996). In rats, ENaC subunits are not expressed until late in gestation (Tchepichev et al. 1995). In the human fetal lung, expression of ENaC subunits is already present in the earliest stages of lung development (Smith et al. 2000), but fetal expression of ENaC has been lower than in the adult lung (Voilley et al. 1994). In the nasal epithelium of the preterm newborn infant, amiloride-sensitive nasal transepithelial potential difference (N-PD), a surrogate measure for ENaC activity, correlates with GA (Gaillard et al. 2007).

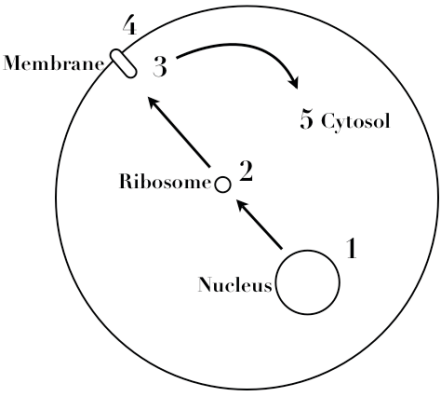
The level of ENaC expression along the respiratory tract does not remain constant. Studies both on animals and in humans have shown increasing β- and γENaC expression in relation to αENaC expression towards the distal lung (Matsushita et al. 1996, Farman et al. 1997, Pitkanen et al. 2001). Potential difference studies have demonstrated a decrease in amiloride-sensitive N-PD from the nasal towards the bronchial epithelium, indicating lower ENaC activity in the distal lung (Knowles et al. 1982, Fajac et al. 1998).

Regulation of Sodium Transport

Regulation of ENaC activity and therefore of sodium transport include several mechanisms, both transcriptional (Figure 5; 1) and post-transcriptional (Figure 5; 2-5). Post-transcriptional mechanisms include translation, assembly, and stability of ENaC subunits (Figure 5; 2), transport of the channel to the cell membrane (Figure 5; 3), control of ENaC function on the membrane (Figure 5; 4), and phosphorylation and degradation of ENaC (Figure 5; 5) (Smith et al. 2000). The main regulatory

mechanisms for ENaC may be those controlling the expression of channels on the cell surfaces (Snyder 2005).

FIGURE 5.
SIMPLIFIED SCHEME OF ENAC REGULATION



	1 Transcription	2 Translation	3 Trafficking	4 Activity	5 Degradation
Glucocortico-steroids	+	+	+	+	-
Beta-agonists	0	0	+	+	0
Oxygen	+	+	0	0	0
Thyroxin	(+)	0	0	0	0

Regulatory mechanisms can be divided into transcriptional (1) and post-transcriptional mechanisms (2-5). Post-transcriptional mechanisms include translation (2), trafficking (3), activity (4), and degradation (5) (Smith et al. 2000). To demonstrate, four regulators and their sites of regulation have been included here along with type of effect. Glucocorticoid affects all sites of regulation (Champigny et al. 1994, Tchepichev et al. 1995, Otulakowski et al. 1999, Sayegh et al. 1999, Debonneville et al. 2001, Itani et al. 2002, Snyder et al. 2002, Boyd et al. 2005), whereas β-agonists affect only trafficking and activity of ENaC (Saldias et al. 1999, Snyder 2000). Increasing oxygen concentration affects both transcription and translation of ENaC (Pitkanen et al. 1996, Baines et al. 2001, Planes et al. 2002, Richard et al. 2003). Thyroid hormone, although affecting transcription, is dependent on simultaneous stimulation with glucocorticosteroids (Champigny et al. 1994, Otulakowski et al. 1999).

In cultures of fetal distal lung epithelium and in experimental animals, glucocorticoid treatment induces fetal lung fluid clearance (Cott et al. 1993, Tchepichev et al. 1995, Folkesson et

al. 2000), but its effect may be tissue-specific (Stokes et al. 1998, Nakamura et al. 2002). Glucocorticoid hormones enhance sodium transport in the fetal lung phenotype through several means. There exists a glucocorticoid-responsive element in the 5' flanking region of the αENaC through which glucocorticoid hormones enhance transcription of αENaC (Champigny et al. 1994, Tchepichev et al. 1995, Chow et al. 1999, Otulakowski et al. 1999, Sayegh et al. 1999). Corticosteroids exert a significant effect on expression of ENaC. All three ENaC subunits are up-regulated by dexamethasone in human fetal lung explant cultures (Venkatesh et al. 1997). However, some cell lines have shown contradictory results (Sayegh et al. 1999, Lazrak et al. 2000). In addition, glucocorticoids elevate ENaC trafficking and retention in the membrane through serum- and glucocorticoid-regulated kinase (SGK) (Itani et al. 2002). Any increase in expression of SGK is associated with an increase in the expression of αENaC (Boyd et al. 2005). SGK also phoshorylates developmentally downregulated protein 4-2 (Nedd4-2) (Debonneville et al. 2001) which, in turn, inhibits Nedd4-2 from binding to ENaC and reduces Nedd4-2 attenuated ENaC degradation (Snyder et al. 2002). Silencing Nedd4-2 causes increased lung ENaC expression in newborn rats (Li et al. 2007a). In the absence of glucocorticoids, very nonselective cation channels are expressed in the ATII cells, which are not likely to transport major quantities of sodium and thus may not noticeably contribute to absorption of fetal lung fluid (Jain et al. 2001).

Among the catecholamines, adrenaline induces a rapid clearance of lung fluid in term fetal lambs, an effect that may be inhibited by propranolol (Walters et al. 1978). This effect is dependent upon GA (Brown et al. 1983). Dopamine also affects lung fluid reabsorption in both fetal guinea pig ATII (Chua et al. 1998, Doe et al. 1998) and rodent ATI cells through the apical D1- and basolateral D2-receptors (Helms et al. 2006).

β-Adrenoceptor agonists that elevate intracellular cAMP levels stimulate lung fluid clearance, possibly through activation of protein kinase A (Chen et al. 2002, Morgan et al. 2003). Once activated it not only increases the localization of Na^+K^+ATPase at the basolateral membrane (Saldias et al. 1999) and the activity of ENaC on the apical membrane, but also the trafficking of ENaC to the apical membrane (Snyder 2000). The effect of β-adrenoceptor agonists is attributeable both to β-1 and β-2 stimulation (Norlin et al. 1998) and is apparent even in ATI cell preparates (Helms et al. 2006).

Thyroid hormones enhance the effect of adrenaline on sodium transport in fetal sheep lungs (Barker et al. 1991). On the other hand, in rat alveolar cells, no increase in ENaC expression occurs after the introduction of thyroid hormones, but thyroid hormones potentiate the effect of glucocorticoids on the expression of ENaC (Champigny et al. 1994, Otulakowski et al. 1999).

After the commencement of breathing at birth, oxygen concentration in the alveoli increases substantially. This change in oxygen concentration in rat fetal distal lung epithelium results in time-dependent changes both in epithelial permeability and sodium transport (Pitkanen et al. 1996, Baines et al. 2001). Increase in αENaC expression is mediated through a redox-sensitive transcription factor, nuclear factor κB (Baines et al. 2002), and its effect in epithelial cells from rat fetal distal lung can be blocked by a superoxide scavenger (Rafii et al. 1998). ENaC activity may be affected through the post-transcriptional effect of oxygen. In rat alveolar cells, hypoxia causes a rapid decrease in sodium transport without a decrease in ENaC expression, but with a smaller amount of ENaC on the apical membrane. In contrast, hyperoxia results in an increase in sodium transport in rat fetal distal lung epithelial cells without any increase in ENaC expression (Planes et al. 2002, Richard et al. 2003). This can be attributed to an increase in Na^+K^+ATPase activity (Baines et al. 2001). Oxygen and

glucocorticoids regulate translation of αENaC (Otulakowski et al. 2006).

Insulin causes an increase in the open probability of ENaC (Tong et al. 2004a) and the number of channels (Blazer-Yost et al. 2003) on the plasma membrane. These effects have recently been demonstrated to be mediated through protein kinase B and SGK, for example by abolishing the effect of Nedd4-2 (Lee et al. 2007).

A multitude of studies focus on enhancing ENaC activity. In a recent study on fragmented interleukin-1, sodium absorption and therefore fluid absorption in guinea pigs was increased, possibly through enhanced cortisol secretion (Li et al. 2007b). Some in vitro studies have demonstrated that interleukin-4 and interferon gamma inhibit ENaC expression, but the addition of tumor necrosis factor causes no modulation of ion transport (Galietta et al. 2000, 2002). However, tumor necrosis factor α also reduces ENaC expression and activity in cultured cells (Dagenais et al. 2004). Epidermal growth factor elevates sodium transport (Borok et al. 1996). In adult rats in vivo, inhibition of tumor necrosis factor α reduces (Borjesson et al. 2000) and administration of transforming growth factor α raises (Folkesson et al. 1996) alveolar fluid clearance. In animal fetuses, vasopressin inhibits lung fluid secretion and facilitates lung fluid absorption through vasopressin V1 receptors (Albuquerque et al. 1998). Somatostatin has an inhibitory effect on sodium transport (Perks et al. 1992), but serotonin reduces lung fluid production in a gestationally-dependent manner (Chua et al. 1999).

Some of these regulators interact with each other; for example, thyroid hormone interacts with glucocorticoid hormone in the fetal lamb lung to potentate the effect of adrenaline on sodium transport (Barker et al. 1990). In rat fetal distal lung epithelial cells, glucocorticoid fails to induce αENaC expression in the presence of fetal oxygen tension, but in

ambient oxygen, αENaC expression increases (Otulakowski et al. 2006).

ENaC is expressed in epithelia that absorb sodium, and studies of ENaC regulation have focused on factors that affect all epithelia. However, several studies have elucidated mechanisms for more local regulation of sodium transport. This is made possible by membrane-bound serine proteases, such as trypsin, that act as channel-activating proteases (CAP) for ENaC (Vallet et al. 1997). In *Xenopus* oocytes, mouse CAP co-expressed with ENaC induces an increase in ENaC mediated current (Vuagniaux et al. 2000). In mammals, CAP1-3 is co-expressed with ENaC in epithelia that transport sodium (Vuagniaux et al. 2002, Verghese et al. 2004), suggesting that endogenous CAPs can act as regulators of sodium transport. A study with cultured mouse alveolar epithelial cells showed that ENaC is constitutively activated in vitro by endogenous serine proteases located on the apical membrane (Planes et al. 2005). In humans, prostasin, a homolog of CAP1, has been considered a possible tissue-specific regulator of sodium channel activity (Yu et al. 1995, Tong et al. 2004b).

Other Factors

Na^+K^+ATPase activity is not the rate-limiting step in sodium transport, but it is crucial in creation of a gradient for sodium entry into the cells. Upregulation of the β1-subunit of Na^+K^+ATPase induces lung fluid clearance in rat airway epithelial monolayers (Factor et al. 1998a). In addition to causing increased ENaC expression and trafficking, glucocorticoids also enhance the expression of both α1- and β1-subunits of the Na^+K^+ATPase (Chalaka et al. 1999). The increased activity has also been attributed to translational regulation (Devarajan et al. 2000). Moreover, in a rat alveolar epithelial cell line, prolonged hypoxia inhibits Na^+K^+ATPase (Planes et al. 1996).

In addition to ENaC, lung fluid transport in some species is also driven by amiloride-insensitive channels (Norlin

et al. 2001). Histologically, less-specific cyclic nucleotide-gated nonselective cation channels have been demonstrated in rat lungs (Ding et al. 1997), but, these channels seem to be efficient only in adult rodents and have not been suggested to play a role in postnatal adaptation (Junor et al. 1999, Kemp et al. 2001).

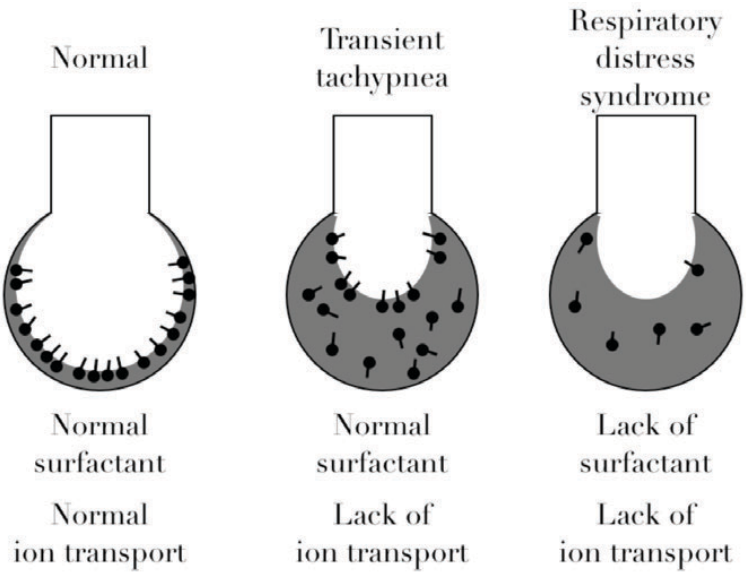
Neonatal Lung Disease

Two main entities of neonatal lung disease have been associated with lung fluid clearance, namely respiratory distress syndrome (RDS) and transient tachypnea of the newborn (TTN) (Figure 6). Recently, through the effect of inflammatory cytokines, decreased ion transport has been suggested to contribute also to the pathogenesis of bronchopulmonary dysplasia (BPD).

Respiratory Distress Syndrome

RDS is a neonatal lung disease characterized by low surfactant production (Martin et al. 2006), excess lung fluid, increased alveolar capillary permeability, and, in the alveoli, the appearance of fibrin and desquamated epithelium plates called hyaline membranes. With the introduction of surfactant treatment, RDS incidence and severity have decreased (Vidyasagar et al. 1987, Jobe 1993). Because preterm RDS infants have increased lung fluid content, RDS could, hypothetically, in part result from inefficient lung fluid clearance (DeSa 1969, Adams et al. 2002). In animals, ENaC expression is lower in the fetal than in the postnatal lung (Tchepichev et al. 1995), and αENaC knock-out mice succumb to respiratory distress rapidly after birth (Hummler et al. 1996). Human preterm infants have significantly lower amiloride-sensitive N-PD a correlate of ENaC activity than do term infants (Barker et al. 1997, Gaillard et al. 2005), suggesting impaired airway epithelial ion transport and therefore inability to clear the lung of fetal fluid.

Figure 6.
Neonatal Lung Disease Hypothesis



In the healthy term newborn infant, a functioning sodium absorption mechanism results in a thin layer of fluid lining the alvoli supporting an adequate amount of surfactant. In the term neonate suffering from transient tachypnea, the amount of surfactant is sufficient, but excess lung fluid may be the result of ineffective sodium transport. Respiratory distress syndrome of the preterm infant is characterized by low surfactant production and inability to clear the lung of perinatal fluid due to defective sodium transport. Modified from O'Brodovich 1996.

Transient Tachypnea of the Newborn

Transient tachypnea of the newborn (TTN) is a self-limiting disorder that typically affects term infants or close-to-term infants. These infants suffer from respiratory distress in the first hours after birth, with symptoms usually disappearing within the first 48 postnatal hours. Based on the typical xray findings of prominent perihilar streaking signifying enlargement of lymphatics, and amiloride-sensitive N-PD lower than in healthy newborn infants, TTN may result from inefficient lung fluid clearance (Gowen et al. 1988, Martin et al. 2006).

Bronchopulmonary Dysplasia

Despite progress made in treating RDS, BPD remains an important disease affecting chiefly very low birth-weight infants (Jobe et al. 2001, Lorenz 2001). This disease is characterized by deficient alveolarization and prolonged inflammation of the lungs (Speer 2004) and is, in extreme cases, treated with dexamethasone (Truffert et al. 2003). BPD is defined as a need for supplemental oxygen for at least 28 days after birth, and its severity by the respiratory support required at 36 weeks of gestation (Jobe et al. 2001). As inflammatory mediators affect ion transport (Borjesson et al. 2000, Galietta et al. 2000, 2002), N-PD measurements have been performed on preterm infants with and without BPD. At 29 days of age infants suffering from BPD have significantly lower N-PD than do age-matched controls without BPD (Gaillard et al. 2007).

OBJECTIVES OF THE INVESTIGATION

I. We evaluated whether expression of ENaC subunits in airway epithelium immediately after birth differs between preterm infants with RDS and healthy term infants. We also studied the effect of dexamethasone treatment on expression of ENaC subunits in airway epithelium in preterm infants with bronchopulmonary dysplasia.

II. We also evaluated the relationship between the ion transport capacity of airway epithelial ENaC measured as transepithelial N-PD and postnatal pulmonary adaptation measured as static lung compliance (LC) within the first 48 postnatal hours in healthy term infants.

III. We further investigated the connection between airway epithelial ENaC subunit expression and functional measurements, N-PD and LC, at three postnatal time-points of sampling in healthy term infants.

IV. In addition, we evaluated whether expression of ENaC subunits in airway epithelium at birth is dependent on newborn infants' gestational age.

METHODS

All studies were performed with the approval of the Ethics Committee of the Hospital for Children and Adolescents, University Central Hospital, Helsinki, Finland, and Study I also with the approval of the Research Ethics Board of the Hospital for Sick Children, Toronto, Canada.

Expression of Epithelial Sodium Channel Subunits

In Studies I, III, and IV, the expression of ENaC subunits was quantified in scrape samples from the nasal epithelium of newborn infants. The ENaC expression of each sample was normalized against that of cytokeratin 18 (CK18), which served as an epithelial marker (ENaC: CK18, attomole (amol) per femtomole (fmol)).

Since the respiratory epithelium extends from the nasal introitus to the bronchi, the nasal epithelium served as a target for airway expression of ENaC subunits. During the sampling for Study I, an independent pathologist subjected the scrape samples from three additional newborn infants to histologic analysis for classification of epithelium type. The findings were typical of the location and similar to findings in adults (Otulakowski et al. 1998).

Sample Collection and Storage

Prior to sampling, all infants received glycerol per os as an anesthetic. Then the nasal samples were collected under direct vision by scraping the nasal epithelium of both nostrils with a Rhino-Probe (Arlington Scientific, Springville, UT, USA). In preterm infants, the samples were gathered from the nostril contralateral to the nostril with the nasotracheal intubation tube. Scraping was performed along the floor of the nose. A commercially available purification kit was used for total RNA preparation (RNeasy Kit, Qiagen, Valencia, CA, USA). The samples were placed on ice and immediately dispersed with an insulin syringe into a lysis buffer containing 10 µL beta-mercaptoethanol per 1 mL of buffer and were stored at -80°C. The following total RNA purification steps for samples in Studies III and IV were performed as sets of 5 to 8 samples as described by the manufacturer.

Quantification of mRNA

For the preterm infants receiving dexamethasone treatment in Study I and for infants in Studies III and IV, the total RNA quantitation of the samples was performed with a commercially available kit including RiboGreen quantitation reagent (RiboGreen RNA Quantitation Kit, Molecular Probes, Eugene, OR, USA) and a preweighed standard RNA preparation. After excitation at 480 nm by a spectrofluorometer, the emission at 520 nm of the adducts was measured (LS50B, Perkin Elmer, Shelton, CT, USA), and the sample RNA contents were deduced from the standard plot.

The purified total RNA preparations in the rest of the patients in Study I were quantified by slot blot analysis, diluted to 2.4 ng/µL, and stored at -80°C in single-use aliquots.

Quantitative Competitive Reverse-Transcriptase PCR

In Study I, samples for competitive quantitative reverse-transcriptase-polymerase chain reaction (QRT-PCR) were, after storage at -70°C if necessary, diluted to give a series that

extended from 24 to 0.375 ng of total RNA. Reaction mixtures contained a constant amount of the truncated α-, β-, γENaC, or CK18 cRNA used as a competitive internal standard. The sequence for the CK18 probe was CTT CAC CAC TCG CTC C, for the forward primer TCT CCC CGG ACA GCA TGA, and for the reverse primer GCA CCG GTA GTT GGT GGA GAA. The primers used for ENaC subunits have been described separately (Otulakowski et al. 1998). Products from the PCR reaction were separated by electrophoresis, stained with ethidium bromide, and quantitated with a CCD camera and SCION Image software. ENaC and CK18 mRNA concentrations were calculated (in attomoles per μg total RNA) from these results. QRT-PCR was done in duplicate for each target mRNA in each sample. Assay results were averaged, with the mean as a single datum.

Real-Time PCR

In Studies III and IV, real-time PCR allowed a greater throughput of specimens. Reverse transcription of RNA to cDNA was performed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 50 μL according to manufacturer's instructions (Loffing et al. 2001). Real-time PCR was performed with specific pre-developed primers and probes for ENaC subunits (SCNN1A for αENaC; SCNN1B for βENaC; SCNN1G for γENaC; Applied Biosystems). Primer express software (Applied Biosystems) was used to design primers, and probes for CK18. PCR reactions were run as singleplex in duplicate wells in a reaction volume of 25 μL. Tissue excised, during rhinoplasty, from a healthy turbinate served as a known standard after determination by quantitative competitive RT PCR as described in *Quantitative Competitive Reverse Transcpritase PCR*. A dilution series of the known standard was included with each reaction plate for quantification of expression.

Transepithelial Nasal Potential Difference

In Studies II and III, sodium transport in the airway epithelium was quantified as potential difference across the nasal epithelium. Ion transport across the cell membrane forms an electrochemical gradient on the luminal side of the epithelium that is dependent on changes in ion transport. This gradient is a relative correlate measurable as a potential difference in comparison to another epithelial membrane. The N-PD measurement was introduced for diagnostic purposes in cystic fibrosis (Knowles et al. 1981a, Knowles et al. 1981b, Alton et al. 1990). As this method includes the focal addition of amiloride on the epithelium to further study sodium transport, especially the activity of ENaC, such experiments were soon performed on newborn infants with postnatal pulmonary problems (Gowen et al. 1988, Barker et al. 1991). The method was, however, not originally designed for the study of infants, and several variations of the apparatus have emerged.

Based on methods described earlier, a novel method was developed for N-PD measurement to better suit newborn infants (Knowles et al. 1981b, Fajac et al. 1998, Southern et al. 2001). A silver-wire electrode was inserted into a 3-lumen central catheter (23 G, COOK, Bjaeverskov, Denmark) to allow accurate administration of the perfusion fluids. The system for N-PD measurements included a voltmeter and a device for recording and saving data (Logan Research Ltd, Maidstone, UK). The circuit was checked prior to measurement, by confirming that the negative potential difference of the exocrine glands on the infant's skin was 35 mV or less. If the readings did not reach 30 mV or fell below 60 mV, the electrodes were replaced or the circuit re-checked. Measurements were performed on the floor of the nose. Before perfusion of physiological saline, the N-PD of both nostrils was recorded and the maximal stabile N-PD measured for 10 seconds. The function of the amiloride-sensitive sodium channel was

determined by inhibition with amiloride (10^{-4} M). In attempt to measure chloride transport, the perfusion was continued with a chloride-free perfusion solution that included 10^{-4} M amiloride. Perfusion was continued for 2 minutes for each solution, during which a stable N-PD for 10 to 20 seconds was achieved. Only measurements during which amiloride sensitivity was achieved were considered successful. The measurement was discontinued if the infant became restless or if the electrode showed an obvious change of position. N-PD measured during perfusion with physiological saline served as a reference in calculating effects of amiloride and chloride-free perfusions.

To ensure the stability and compatibility of readings from the silver-wire exploring electrode, separate experiments were performed on five adult volunteers and tested against a conventional method with an agar-bridge electrode (Alton et al. 1990). The baseline current, determined with the present method versus the conventional method, was -16.0 (± 3.6) and -16.2 (± 3.5) mV (n.s.), and the amiloride-sensitive current -6.4 (± 1.1) and -6.8 (± 3.3) mV (n.s.).

Static Lung Compliance

To assess the functional correlate of the lung mechanics in newborn infants in Studies II and III, LC was measured, a sign of the elasticity of the lungs calculated from the change in volume per unit change in pressure. This was done through induction of the Hering Breuer inflation reflex of infants (Rabbette et al. 1994); we applied the double occlusion technique using a computerized pulmonary function-testing device with an automated occlusion valve (Labmanager 4.52i, Erich Jaeger GmbH, Hoechberg, Germany) (Fletcher et al. 1996). During each sudden, short interruption of breathing, the pressure in the respiratory system equilibrates. The change in pressure and volume from each occlusion throughout

expiration allows calculation of static LC (Chernick et al. 2002). This testing was performed according to standards set by the combined Task Force of the European Respiratory Society and the American Thoracic Society (Gappa et al. 2001).

To ensure that LC measurements were performed during quiet non-REM sleep, a subset of patients in Studies II and III were studied during polysomnographic follow-up. This included two electroencephalograms (C3A2, O2A1), two electro-oculograms, chin electromyogram, and airflow and pulse oximetry recordings. In the remaining subjects, quiet sleep was determined by direct observation of the absence of eye and body movements, use of accessory respiratory muscles (i.e. absence of the abdominal breathing characteristic of REM sleep), and regular breathing shown by an airflow signal. Airflow and LC measurements were taken with a pneumotachometer connected to a full-face mask. No significant difference in LC appeared between infants studied under polysomnography and those studied under visual observation (19.7 ± 2.5 and 19.9 ± 1.6 mL/kPa/kg, n.s.).

Statistics

In all studies, patient data are given as mean ± standard deviation. In Study I, data are mean ± standard deviation, and comparisons between preterm and term groups were by the Mann-Whitney U-test. In Studies II to IV, study data are mean ± standard error of means. In Study II, comparisons were performed with the Mann-Whitney U-test or the paired t-test; the Pearson test served for correlations. In Study III, comparisons were performed with the Friedman-Dunn test or the Mann-Whitney U-test, which was corrected for multiple measurements; the Spearman test served for correlations. In Study IV, comparisons were performed with the Wilcoxon matched pairs test or the Mann-Whitney U-test, correlations

were performed with the Spearman test. All tests were performed with GraphPad Prism version 4.00 for Windows and 4.0b for Mac (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

PATIENTS

Recruitment

All mothers of newborn infants were recruited at the Department of Obstetrics several hours prior to giving birth. Recruitment was performed when mothers were admitted to the hospital. In the case of premature infants treated with dexamethasone, patients were recruited and informed consent was obtained from the parents at the Neonatal Intensive Care Unit of the Hospital for Children and Adolescents.

Study I

Twelve newborn infants were studied for airway epithelial ENaC subunit expression within 5 hours after birth (Table 1). All seven control term infants were healthy, whereas the five preterm infants had RDS. From 18 days to 24 h before delivery, the mothers had received ante partum betamethasone treatment as a total of one to four doses of 12 mg intramuscularly. Ante partum terbutaline had been given to two mothers: One received an infusion of 1 mg/h for 6 hours which was discontinued 5 hours before delivery; the second received 5 mg/h for 48 hours until delivery. None of the mothers had preeclampsia, and none had diabetes or any other chronic conditions imposing additional risks to pregnancy. The five preterm infants received an average of two doses of surfactant

(Curosurf ®, Chiesi, Parma, Italy) and their initial arterial-to-alveolar oxygen tension-ratio of was 0.05 to 0.8.

Table 1.
Clinical Characteristics of Newborn Infants

Term subjects	Study I *(n=7)*	Study II *(n=20)*	Study III *(n=41)*	Study IV *(n=61)*
Male/Female	2/5	10/10	19/22	27/34
Vaginal delivery/Cesarian section	6/1	13/7	19/22	28/33
Gestational age (weeks)	39.3±0.9	39.6±1.5	39.5±1.4	39.6±1.0
Birth weight (kg)	3.72±0.38	3.7±0.6	3.67±0.46	3.66±0.37
Cord Artery Blood pH	7.29±0.1		7.29±0.06	7.30±0.05
APGAR 1 min (mean)	9	9	9	9
Preterm subjects	*(n=5)*			*(n=29)*
Male/Female	3/2			14/15
Vaginal delivery/Cesarian section	4/1			3/26
Gestational age (weeks)	27.2±0.9			31.1±3.5
Birth weight (kg)	0.9±0.28			1.83±1.16
Cord Artery Blood pH	7.26±0.1			7.29±0.06
APGAR 1 min (mean)	6			7
Preterm subjects on DEX	*(n=4)*			
Male/Female	3/1			
Vaginal delivery/Cesarian section	3/1			
Gestational age (weeks)	24.9±0.2			
Birth weight (kg)	0.76±0.18			
Postnatal age at treatment (days)	43±6			
Postnatal weight at treatment (kg)	1.21±0.26			
Days to extubation after DEX	2-5			

Four additional preterm infants suffering prolonged respiratory distress were studied for airway epithelial ENaC expression (Table 1). Treatment with dexamethasone was started at 43 ± 6 postnatal day (GA = 24.9 ± 0.2 wk, BW = 764 ± 178 g) to achieve extubation. These infants received dexamethasone at a dose of 0.2 mg/kg per day as two daily doses for 3 days, and thereafter at 0.1 for 3 and 0.05 mg/kg per day for 5 days. Initial samples were gathered prior to the commencement of dexamethasone

treatment. Subsequently, samples were gathered in these patients at 7 to 20 hours after the initial dose. All four infants were successfully weaned from the ventilator at 2 to 5 days after initiation of treatment. The FIO_2 of the infants studied fell from 0.6 ± 0.18 to 0.34 ± 0.05 during the first 3 days of dexamethasone treatment.

Study II

In Study II, N-PD and LC were measured on 20 healthy term newborn infants at 1 to 4 and again at 21 to 24 hours after birth (Table 1). All mothers were healthy and all pregnancies uneventful. Breech presentation or previous cesarean section were indications for an elective cesarean section.

Between the two groups, the clinical characteristics of infants born vaginally and by cesarian section showed no significant difference.

Study III

Subjects in Study III comprised 41 healthy term newborn infants, of whom 17 were included in Study II (Table 1). These infants were studied at 1 to 4, 21 to 27, and 45 to 50 hours after birth. These mothers were healthy, and all pregnancies were uneventful. Of these 41, 19 infants were delivered vaginally and 22 by cesarean section. Indications for an elective cesarean section were breech presentation or previous cesarean section.

Study IV

We included a total of 90 newborn infants for Study IV (Table 1), and studied them at 1 to 5 and 22 to 28 hours after birth for

airway epithelial ENaC subunit expression. Of these, 29 infants were born preterm (GA < 37 weeks). Of the mothers of preterm infants, 24 had received antenatal betamethasone, 4 had preeclampsia, and one had chorioamnionitis. In four cases, delivery followed premature rupture of the membranes for more than 24 hours previously. One infant developed sepsis. RDS was diagnosed by an attending clinician and BPD according to approved diagnostic criteria (Jobe et al. 2001).

Ethical Considerations

The Helsinki University Hospital Ethics Committee for Obstetrics and Gynecology approved all study protocols. All parents gave their informed consent.

EVALUATION OF THE RESULTS

ENaC Expression Studies

Airway epithelial ENaC expression was studied in Studies I, III, and IV. Expression of α-, β-, γENaC, or CK18 occurred in all nasal epithelial samples.

In Study I, expression of all ENaC subunits in the nasal epithelium at 1 to 5 hours after birth was significantly lower in preterm infants who had RDS than in healthy term infants (preterm vs. term infants: α- and βENaC: $P < 0.05$; γENaC: $P < 0.005$; Table 2; Study I: Figure 1). We concluded that in newborn preterm infants low airway αENaC expression may be linked with RDS. In Study IV, in infants born at a GA of 25 weeks and 5 days to 42 weeks, a significant correlation existed between the expression of α- and βENaC and GA at 1 to 5 hours after birth (αENaC: $n = 89$, $r = 0.418$, $P < 0.0001$; Study IV: Figure 1; βENaC: $n = 82$, $r = 0.338$, $P < 0.005$). Between γENaC and GA no significant correlation existed. At 1 to 5 hours after birth, expressions of α-, β- and γENaC subunits were significantly lower in preterm infants (GA < 37 weeks) than in term infants (αENaC: $P < 0.0001$; βENaC: $P < 0.005$; γENaC: $P < 0.01$; Table 2; Study IV: Figure 2). Differences in levels of expression of all ENaC subunits between the two groups were no longer significant at 22 to 28 hours after birth.

TABLE 2.
RESULTS OF AIRWAY EPITHELIAL ENAC EXPRESSION EXPERIMENTS

	Study I[1]		Study III	Study IV	
Measurement	Preterm	Term	Term	Preterm	Term
ENaC[1], ≤5 h[2]					
αENaC	5.4±2.0 *n=5*	9.1±2.3 *n=7*	8.1±0.7 *n=35*	4.7±0.6 *n=29*	9.5±0.7[¶] *n=60*
βENaC	2.4±1.4 *n=5*	4.3±1.1 *n=7*	12.0±1.4[¶§] *n=34*	8.3±1.5[§] *n=26*	14.7±1.5[*] *n=56*
γENaC	2.4±0.1 *n=5*	6.8±3.2 *n=7*	19.1±5.9[¶] *n=35*	3.8±1.7 *n=20*	14.3±3.9[§] *n=54*
ENaC, ≥21 h[3]					
αENaC			7.7±1.2 *n=29*	4.1±1.3 *n=17*	7.1±1.0[¶] *n=45*
βENaC			4.0±0.8[¶] *n=27*	3.2±0.7[§] *n=17*	4.7±1.0[*] *n=41*
γENaC			8.7±4.5[¶] *n=27*	1.1±0.4 *n=11*	4.7±2.8[§] *n=40*
ENaC, 45-50 h					
αENaC			7.5±1.1 *n=27*		
βENaC			2.6±0.7[§] *n=26*		
γENaC			2.2±0.7[¶] *n=24*		

Statistical comparisons were performed within the subject category
[1] Data are mean ± standard deviation
[2] *ENaC*, airway epithelial expression of the Epithelial Sodium Channel (amol/fmol CK18)
[3] 1 to 5 hours after birth in Studies I and IV; 1 to 4 hours after birth in Study III
[4] 21 to 27 hours after birth in Study III; 22 to 28 hours after birth in Study IV
[*] $P < 0.0001$
[§] $P < 0.005$
[¶] $P < 0.05$

In Study IV, in infants developing RDS, airway epithelial ENaC expression did not significantly differ in those surviving without RDS. It is possible that the samples were gathered too soon after birth and not during the phase of severe RDS. In addition, amount of expression is not always reflected in amount of functional protein on the plasma membrane. The level of

immediate postnatal expression may have been maximal due to early response to respiratory distress.

In the four preterm infants sampled after several weeks of assisted ventilation in Study I, expression of α- and βENaC (amol/fmol CK18) increased markedly after commencement of dexamethasone treatment. Initial αENaC was 47.9 ± 26.1, βENaC was 12.4 ± 7.3, and γENaC was 2.9 ± 1.3. Twenty hours after treatment with dexamethasone, an increase occurred of 143 ± 155% from basal level in the expression of αENaC (amol/fmol CK18) and an increase of 195 ± 130% in the expression of βENaC (amol/fmol CK18). In three patients, γENaC expression (amol/fmol CK18) increased slightly, whereas in one patient, expression decreased (Study 1: Figure 2). As these infants were subjected to constant irritation of the nasal epithelium due to nasogastric feeding tubes and intubation tubes, we analyzed the expression of CK18 to rule out the possibility of damage to the epithelium. However, CK18 expression levels were similar to those in newborn preterm infants.

In Study III, no significant change emerged in the expression of αENaC in the serial samples of term newborn infants. Expressions of β- and γENaC, however, decreased significantly, expression of βENaC already during the first 21 to 27 hours after birth (Table 2; Study III: Figure 2). In preterm infants in Study IV, αENaC expression did not change significantly during the study period. In term infants, αENaC expression was significantly lower at 22 to 28 than at 1 to 5 hours after birth (Table 2). In both groups, βENaC expression was significantly lower at 22 to 28 than at 1 to 5 hours (Table 2; Study IV: Figure 2). In term infants, γENaC expression was significantly lower at 22 to 28 than at 1 to 5 hours after birth (Table 2).

We showed that βENaC expression is highest close to birth and decreases drastically during the first postnatal day. Further, expression of γENaC after birth decreases, but with a great variation in its level of expression. Contrary to the initial

hypothesis, however, we saw no postnatal change in the expression of αENaC in Study III. In the larger population in Study IV, only a small decrease in αENaC expression occurred during the first day of life in the airway epithelium of healthy term infants. This indicates that either the expression of αENaC had already peaked prior to sampling or that in the healthy term infant αENaC expression level is sufficient for eventual channel assembly and function even prior to birth. Whereas αENaC is considered crucial for successful postnatal adaptation, it is apparent that in the healthy term infant, postnatal changes in airway epithelial expression of the other two subunits are more profound, which emphasizes their individual roles. Preterm newborn infants seem to show a slightly different expression pattern.

Since in Study III, the infants delivered by cesarean section showed a tendency to greater increase in LC (Table 4; Study III: Figure 1), albeit not statistically significantly, we quantified the difference in ENaC subunit expression between the two groups. A significant difference appeared in the expression of αENaC between infants born vaginally (5.0 ± 1.6 amol/fmol CK18, n = 12) and infants born by cesarian section at 21 to 27 hours after birth (9.7 ± 1.6 amol/fmol CK18, n = 17, $P < 0.05$; Study III: Figure 3). Moreover, at 21 to 27 hours after birth, the expression of γENaC was lower in infants born vaginally (0.5 ± 0.2 amol/fmol CK18, n = 11) than in infants born by cesarean section (14.3 ± 7.4 amol/fmol CK18, n = 16, $P < 0.05$). In Study IV, lower expression of α-, β-, and γENaC in term infants born vaginally than in those born by cesarean section occurred at 22 to 28 hours after birth, but this was of only significant in β- and γENaC (vaginal delivery (amol/fmol CK18): αENaC: 5.1 ± 1.2; βENaC: 3.7 ± 1.3; γENaC: 0.8 ± 0.3; cesarean delivery: αENaC: 8.7 ± 1.4; βENaC: 5.5 ± 1.3; γENaC: 7.9 ± 4.9; $P = 0.06$, $P = 0.04$, $P < 0.01$, respectively). In conclusion, it seems that airway epithelial ENaC expression remains high in term

infants born by cesarean section, possibly related to a higher requirement of eventual sodium transport.

The mothers of 24 preterm infants in Study IV had received antenatal betamethasone. The GA of these infants was 30.1 ± 2.8 weeks, whereas the GA of the preterm infants whose mothers had not received betamethasone was 36.1 ± 0.5 weeks ($P < 0.005$). The time between the last dose of antenatal steroids and the first sampling (330 ± 70 hours) did not correlate with ENaC subunit expression. Of the 29 preterm infants, 15 were diagnosed with RDS (GA 28.8 ± 2.6 weeks vs. 33.5 ± 2.5 weeks in preterm infants without RDS; $P < 0.0005$), and 6 developed BPD (GA 27.6 ± 1.6 weeks vs. 32.0 ± 3.2 weeks in preterm infants without BPD (n=23); $P < 0.005$). Expression of ENaC subunits in infants with RDS did not differ from expression in those without RDS. Expression of αENaC in the BPD group at 1 to 5 hours after birth was lower than in preterm infants who did not develop BPD (2.4 ± 0.9 amol/fmol CK18 vs. 5.3 ± 0.7 amol/fmol CK18, respectively; $P < 0.05$). In recent in vivo studies, the N-PD of preterm infants with BPD has differed from that of unaffected preterm infants; the amiloride-sensitive component of N-PD is lower during the first postnatal days (Thome et al. 2006), or at 29 days after birth (Gaillard et al. 2007). However, age-matched infants in Gaillard's study all had similar N-PDs at birth. It is possible that a low level of αENaC expression may be linked to later BPD, characterized by lower N-PD values.

The commonly accepted paradigm of the importance of αENaC for postnatal lung fluid clearance is based mainly on experiments performed in animal models (O'Brodovich et al. 1991, Hummler et al. 1996). It is also possible that there exists a species-specific mechanism; this is suggested by our finding of GA-dependent ENaC expression in humans in contrast to no expression in mice until day 16 (Talbot et al. 1999). That the expression of subunits of ENaC in our studies was differentially regulated gives rise to questions about the differing functions of subunits. γENaC knock-out mice require more time for

postnatal pulmonary adaptation (Barker et al. 1998). The γ-subunit is also important for ENaC trafficking (Konstas et al. 2003). Recently, the role of β- and γENaC has been highlighted in several experiments, both in animals and humans. Elias et al. (2007) have shown that in cultured fetal rat lung explants, absorption of the additional edema fluid is markedly decreased in explants from β- and γENaC knock-out mice, while no difference from wild-type mice appears in explants from αENaC knock-out mice. The over-expression of βENaC, but not of α- or γENaC, results in cystic-fibrosis-like excessive absorption of sodium by the respiratory epithelia (Mall et al. 2004). A possible regulatory role has also been suggested for γENaC: The inferior human nasal turbinate shows an inverse correlation with γENaC expression and amiloride-sensitive N-PD (Otulakowski et al. 1998). No such relation emerged in Study III of healthy term newborn infants, thus highlighting the importance of further clinical function studies.

In humans, little data exists as to the respiratory status of newborn infants with pseudohypoaldosteronism type I, a rare genetic disease in which the expression of ENaC subunits is decreased (Bonny et al. 1999). Reports have ranged from ones describing infants with severe RDS postnatally (Malagon-Rogers 1999, Akcay et al. 2002) to those describing infants without respiratory distress (Kerem et al. 1999). During the first years of life, respiratory diseases are common in children with systemic pseudohypoaldosteronism due to an excess volume of airway surface fluid, reflecting defective sodium transport (Kerem et al. 1999). Low-level ENaC activity seems sufficient for immediate postnatal adaptation in some phenotypes (Bonny et al. 1999). This suggests that low airway epithelial expression of ENaC in prematurely born infants may, however small, be sufficient for minimum ENaC activity. In regard to our studies on ENaC expression, it is likely that the potential for higher sodium channel activity is apparent in late gestation.

Nasal Potential Difference Measurements

N-PD was measured from healthy term newborn infants in Studies II and III. There occurred no significant change in N-PD of infants between 1 to 4 hours and 21 to 48 hours after birth (Table 3). In Study III, N-PD at 1 to 4 hours, 21 to 27 hours, and 45 to 50 hours after birth between infants born vaginally or by cesarean section did not differ significantly (Table 3). This finding contradicts an earlier report in which the N-PD of term infants born by elective cesarean section had significantly higher basal N-PD and a lower response to amiloride than did term infants born vaginally (Gowen et al. 1988), but it agrees with a more recent one (Gaillard et al. 2003). This is interesting, since we demonstrated a higher increase in LC in infants born by cesarean section during the first 21 to 27 hours after birth, suggesting more efficient lung fluid removal in such infants.

In several earlier studies the amiloride-sensitive component of N-PD has been over 50% of total N-PD, higher than in our Studies II and III. In one term population an over 50% amiloride-sensitive component appeared (Gaillard et al. 2003). There are clear explanations for this discrepancy: Our first measurements were performed very early, when the epithelial sodium transport had possibly not yet reached its peak; measurement protocols in these studies differed somewhat from each other, our studies having been made with a slightly altered version of a protocol including a silver-wire exploring electrode (Fajac et al. 1998).

TABLE 3.
RESULTS OF NASAL POTENTIAL DIFFERENCE EXPERIMENTS

	Study II			Study III		
Measurement	All	Vaginal delivery	Cesarian section	All	Vaginal delivery	Cesarian section
N-PD, 1-4 h *(n)*	*20*	*13*	*7*	*34*	*15*	*19*
Basal, mV	-14.3±1.9*	-14.8±2.8*	-13.3±2.2*	-17.2±1.6*	-15.2±2.3*	-18.7±2.1§
Residual, mV	-8.8±1.7*	-8.8±2.3*	-8.7±2.4*	-10.5±1.5*	-8.4±2.0*	-11.4±2.3§
Amiloride, %	44.0±4.2	46.5±4.8	39.4±8.4	39.9±3.2	46.1±5.1	35.1±3.9
N-PD, ≥21 h[1] *(n)*	*16*	*10*	*6*	*22*	*12*	*10*
Basal, mV	-14.6±2.3*	-13.9±3.0*	-15.7±3.8§	-16.7±2.5§	-17.3±3.6¶	-16.1±3.8§
Residual, mV	-9.0±1.9*	-8.5±2.5*	-10.0±3.0§	-11.8±2.2§	-14.5±3.2¶	-8.7±2.6§
Amiloride, %	43.4±3.7	45.4±4.9	40.2±6.0	37.5±4.7	33.3±7.3	42.3±5.7
N-PD, 45-50 h *(n)*				*21*	*7*	*14*
Basal, mV				-16.7±2.2*	-13.0±3.0¶	-18.9±2.8*
Residual, mV				-10.2±1.6*	-7.4±2.3¶	-11.6±2.0*
Amiloride, %				42.6±4.0	49.0±6.0	39.4±5.1

Statistical comparisons were performed within the subject category
N-PD, transepithelial nasal potential difference
Amiloride, %, percentage of amiloride-inhibition of current at flush
[1]21 to 48 hours after birth in Study II; 21 to 27 hours after birth in Study III
* $P < 0.0001$
§ $P < 0.005$
¶ $P < 0.05$

LUNG COMPLIANCE MEASUREMENTS

LC was measured from healthy term newborn infants in Studies II and III. Because respiratory morbidity is higher in term infants born by elective cesarean section (Jain et al. 2006), special attention was paid to the method of delivery. In both Studies, a significant increase in LC was found during the study period in both the vaginally born infants and the infants born by cesarean section (Table 4). Comparison of LC values within each time period revealed no significant differences dependent on method of delivery.

The results in Table 4 for LC are somewhat higher than in reference data, but because the respiratory system's

resistance is dependent on, for example, age and body size, limits of normality remain to be established (Gappa et al. 2001). In our studies, however, we have paid special attention to sleep-stage analysis to ensure that the measurements were performed at non-REM sleep. The sleep stage at which LC is measured may affect the Hering Breuer inflation reflex and, therefore, also the results. The reflex is most reliably induced in non-REM sleep.

Table 4.
Results of Lung Compliance Experiments

	Study II			Study III		
Measurement	All	Vaginal delivery	Cesarian section	All	Vaginal delivery	Cesarian section
LC, 1-4 h *(n)*	*19*	*12*	*6*	*32*	*15*	*17*
mL/kPa/kg	17.3±1.4§	15.9±1.3	18.0±2.8	16.3±1.1¶	15.0±1.2¶	17.4±1.8¶
LC, ≥21 h[1] *(n)*	*20*	*13*	*7*	*22*	*10*	*12*
mL/kPa/kg	22.8±2.2§	23.1±2.4	22.1±4.6	21.8±1.8	22.2±3.3	21.5±2.1
LC, 45-50 h *(n)*				*21*	*10*	*11*
mL/kPa/kg				24.1±1.7¶	21.7±1.9¶	26.4±2.7¶

Statistical comparisons were performed within the subject category
LC, static lung compliance
[1]21 to 48 hours after birth in Study II; 21 to 27 hours after birth in Study III
§ $P < 0.005$
¶ $P < 0.05$

Correlations

N-PD and LC measurements were performed in Study II. ENaC expression, N-PD, and LC were measured in Study III.

Expression of ENaC subunits in Studies II and III did not correlate with LC or N-PD. This finding can be attributed to the fact that the functional measurements were performed on term newborn infants only. We show that ENaC expression is gestational-age dependent; the level of expression may therefore be more important for preterm than for healthy term newborn

infants in whom amount of plasma membrane ENaC may significantly exceed the level needed for sufficient amiloride-sensitive N-PD. It is also possible that additional mechanisms contribute to transport of fluid through the respiratory epithelium. For example, cyclic nucleotide-gated channels responsible for amiloride-insensitive sodium transport exist in adult rat lung epithelium (Norlin et al. 2001), although they are considered unlikely to play a role in perinatal pulmonary adaptation (Junor et al. 1999, Kemp et al. 2001, Rafii et al. 2002).

In Studies II and III, a significant correlation existed between amiloride-sensitive N-PD, a correlate of sodium transport, at 1 to 4 hours and LC at 21 to 48 hours after birth ($r^2 = 0.40$; $P < 0.003$; Study II: Figure). A correlation appeared between initial amiloride-sensitive N-PD and change in LC between the two time-points ($r^2 = 0.31$; $P < 0.05$; $n = 19$). We confirmed in Study III a positive correlation between amiloride-sensitive N-PD measured at 1 to 4 hours and LC measured at 21 to 27 hours after birth ($r = 0.478$, $P < 0.05$, $n = 18$). No such correlation appeared with earlier or later compliance measurements.

In the functional studies of ion transport in term infants, we demonstrated that amiloride-sensitive N-PD remained unchanged, whereas LC increases during the early postnatal period. This could be explained by a very rapid increase in sodium transport activity prior to the first N-PD measurement.

Newborn preterm infants (≤ 31 weeks of gestation) with RDS have a lower baseline N-PD than do non-affected age-matched infants (Barker et al. 1997). Contradictory to this, in a study on moderately preterm infants (29 – 36 weeks of gestation) N-PD was positively associated with maturity and mechanical ventilation, but not with need for supplemental oxygen, and showed a very high N-PD response to amiloride (Gaillard et al. 2005). However, no one of these infants was suffering from severe RDS, and they were already breathing room air on the

third postnatal day. Recent reports on newborn preterm infants have shown that N-PD increases with GA and that even in a very premature infant a large proportion of the current is amiloride-sensitive, suggesting that an absorptive mechanism is already in place (Thome et al. 2006, Gaillard et al. 2007). Our studies failed to reveal any significant difference in airway epithelial expression of ENaC among preterm infants with or without RDS. However, our epithelial scrape samples were gathered at a much earlier time-point than were the measurements of N-PD of these other studies.

FUTURE PROSPECTS

The aim of this work was to elucidate how ion transport in the airway epithelium takes place during the postnatal adaptation and how it affects postnatal pulmonary adaptation. In newborn infants no previous data exist on airway epithelial ENaC expression. These measurements coupled with functional measurements performed at an early postnatal period allowed investigation of the role of ENaC in postnatal pulmonary adaptation.

Preterm infants are more likely to suffer from RDS than are term infants. We showed that preterm birth is associated with low airway epithelial expression of ENaC subunits and that α- and βENaC expression correlated with GA. Functional measurements could not be performed on preterm infants. Low airway epithelial ENaC expression in the preterm infant may result in a lower amount of sodium transport from the perinatal lung lumen, leading to inefficient lung fluid clearance. Study IV showed no significant difference in ENaC expression between preterm infants without RDS and those suffering from the disease. This is a finding from a preterm population limited in size, and it calls for more focused studies.

We also demonstrated in both preterm and term newborn infants that expression of individual ENaC subunits in the airway epithelium differ at birth and that a significant decrease occurs in the expression of βENaC after birth in both groups. Varying subunit combinations of ENaC differ in their activity (Eaton et al. 2004), and the early change in expression

may reflect the requirement for rapid fluid removal and, within days, the maintenance of low amounts of fluid lining the alveolar walls.

Future studies on postnatal pulmonary adaptation should include a quest for some channel expression or function inductor. Knowledge of subunit-specific individual expression profiles may be useful in these studies.

The finding of N-PD, a surrogate measurement of sodium transport, correlating with LC underscores the importance of sodium transport in postnatal lung fluid clearance. No correlations emerged between ENaC expression and functional measurements in term newborn infants. In the term infant, ENaC expression, related to ENaC activity, is likely to be sufficient for normal pulmonary adaptation and may not be the rate-limiting step. In the preterm infant, however, its correlation with functional measurements remains unclear.

CONCLUSIONS

The fetal lung is filled with fluid which provides support for normal lung growth. At birth, the newborn infant must rapidly clear its lungs of this excess fluid to be able to commence breathing. In experimental animals the most crucial element of this pulmonary adaptation at birth is sodium transport from the lungs through the pulmonary epithelium. ENaC is the rate-limiting pathway in sodium transport, resulting in passive movement of water in the same direction.

The studies presented here show that expression of the airway epithelial sodium channel is dependent on GA, and that in preterm infants, administration of glucocorticosteroids induces expression of epithelial sodium channels. It may be suggested that the lack of functional airway epithelial sodium channel contributes to respiratory distress of the preterm infant and that induction of channel expression by glucocorticosteroids should attenuate the disease. In addition, these studies depict a correlation between the epithelial sodium channel activity, amiloride-inhibited N-PD, and LC, indicating that trans-epithelial sodium transport is important in the postnatal reduction of lung liquid.

Since epithelial sodium channel expression is gestational age- dependent, we conjecture that low amounts of ENaC contribute importantly to RDS in the preterm infant.

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REFERENCES

ADAMS EW, COUNSELL SJ, HAJNAL JV, COX PN, KENNEA NL, THORNTON AS, BRYAN AC, AND EDWARDS AD. Magnetic resonance imaging of lung water content and distribution in term and preterm infants. *Am J Respir Crit Care Med* 166: 397-402, 2002.

ADAMS FH, FUJIWARA T, AND ROWSHAN G. The nature and origin of the fluid in the fetal lamb lung. *J Pediatr* 63: 881-888, 1963.

Adams FH. Functional development of the fetal lung. *J Pediatr* 68: 794-801, 1966.

ADAMS FH, YANAGISAWA M, KUZELA D, AND MARTINEK H. The disappearance of fetal lung fluid following birth. *J Pediatr* 78: 837-843, 1971.

ADAMSON TM, BOYD RD, PLATT HS, AND STRANG LB. Composition of alveolar liquid in the foetal lamb. *J Physiol* 204: 159-168, 1969.

AHERNE W AND DAWKINS MJ. The removal of fluid from the pulmonary airways after birth in the rabbit, and the effect on this of prematurity and pre-natal hypoxia. *Biol Neonat* 78: 214-229, 1964.

AKCAY A, YAVUZ T, SEMIZ S, BUNDAK R, AND DEMIRDOVEN M. Pseudohypoaldosteronism type 1 and respiratory distress syndrome. *J Pediatr Endocrinol Metab* 15: 1557-1561, 2002.

ALBUQUERQUE CA, NIJLAND MJ, AND ROSS MG. Mechanism of arginine vasopressin suppression of ovine fetal lung fluid secretion: lack of V2-receptor effect. *J Matern Fetal Med* 7: 177-182, 1998.

ALCORN D, ADAMSON TM, LAMBERT TF, MALONEY JE, RITCHIE BC, AND ROBINSON PM. Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. *J Anat* 123: 649-660, 1977.

ALTON EW, CURRIE D, LOGAN-SINCLAIR R, WARNER JO, HODSON ME, AND GEDDES DM. Nasal potential difference: a clinical diagnostic test for cystic fibrosis. *Eur Respir J* 3: 922-926, 1990.

BAINES DL, FOLKESSON HG, NORLIN A, BINGLE CD, YUAN HT, AND OLVER RE. The influence of mode of delivery, hormonal status and postnatal O2

environment on epithelial sodium channel (ENaC) expression in perinatal guinea-pig lung. *J Physiol* 522 Pt 1: 147-157, 2000.

Baines DL, Ramminger SJ, Collett A, Haddad JJ, Best OG, Land SC, Olver RE, and Wilson SM. Oxygen-evoked Na+ transport in rat fetal distal lung epithelial cells. *J Physiol* 532: 105-113, 2001.

Baines DL, Janes M, Newman DJ, and Best OG. Oxygen-evoked changes in transcriptional activity of the 5'-flanking region of the human amiloride-sensitive sodium channel (alphaENaC) gene: role of nuclear factor kappaB. *Biochem J* 364: 537-545, 2002.

Barker PM, Markiewicz M, Parker KA, Walters DV, and Strang LB. Synergistic action of triiodothyronine and hydrocortisone on epinephrine-induced reabsorption of fetal lung liquid. *Pediatr Res* 27: 588-591, 1990.

Barker PM, Walters DV, Markiewicz M, and Strang LB. Development of the lung liquid reabsorptive mechanism in fetal sheep: synergism of triiodothyronine and hydrocortisone. *J Physiol* 433: 435-449, 1991.

Barker PM, Gowen CW, Lawson EE, and Knowles MR. Decreased sodium ion absorption across nasal epithelium of very premature infants with respiratory distress syndrome. *J Pediatr* 130: 373-377, 1997.

Barker PM, Nguyen MS, Gatzy JT, Grubb B, Norman H, Hummler E, Rossier B, Boucher RC, and Koller B. Role of gammaENaC subunit in lung liquid clearance and electrolyte balance in newborn mice. Insights into perinatal adaptation and pseudohypo-aldosteronism. *J Clin Invest* 102: 1634-1640, 1998.

Bland RD, Bressack MA, and McMillan DD. Labor decreases the lung water content of newborn rabbits. *Am J Obstet Gynecol* 135: 364-367, 1979.

Bland RD, McMillan DD, Bressack MA, and Dong L. Clearance of liquid from lungs of newborn rabbits. *J Appl Physiol* 49: 171-177, 1980.

Bland RD, Hansen TN, Haberkern CM, Bressack MA, Hazinski TA, Raj JU, and Goldberg RB. Lung fluid balance in lambs before and after birth. *J Appl Physiol* 53: 992-1004, 1982.

Blazer-Yost BL, Esterman MA, and Vlahos CJ. Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity. *Am J Physiol Cell Physiol* 284: C1645-1653, 2003.

Bonny O, Chraibi A, Loffing J, Jaeger NF, Grunder S, Horisberger JD, and Rossier BC. Functional expression of a pseudohypoaldosteronism type I mutated epithelial Na+ channel lacking the pore-forming region of its alpha subunit. *J Clin Invest* 104: 967-974, 1999.

Borjesson A, Norlin A, Wang X, Andersson R, and Folkesson HG. TNF-alpha stimulates alveolar liquid clearance during intestinal ischemia-reperfusion in rats. *Am J Physiol Lung Cell Mol Physiol* 278: L3-12, 2000.

Borok Z, Hami A, Danto SI, Lubman RL, Kim KJ, and Crandall ED. Effects of EGF on alveolar epithelial junctional permeability and active sodium transport. *Am J Physiol* 270: L559-565, 1996.

Boyd C and Naray-Fejes-Toth A. Gene regulation of ENaC subunits by serum- and glucocorticoid-inducible kinase-1. *Am J Physiol Renal Physiol* 288: F505-512, 2005.

Brown MJ, Olver RE, Ramsden CA, Strang LB, and Walters DV. Effects of adrenaline and of spontaneous labour on the secretion and absorption of lung liquid in the fetal lamb. *J Physiol* 344: 137-152, 1983.

Canessa CM, Horisberger JD, and Rossier BC. Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* 361: 467-470, 1993.

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, and Rossier BC. Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. *Nature* 367: 463-467, 1994.

Carlton DP, Cummings JJ, Poulain FR, and Bland RD. Increased pulmonary vascular filtration pressure does not alter lung liquid secretion in fetal sheep. *J Appl Physiol* 72: 650-655, 1992.

Carmel JA, Friedman F, and Adams FH. Fetal tracheal ligation and lung development. *Am J Dis Child* 109: 452-456, 1965.

Cassin S and Perks AM. Studies of factors which stimulate lung fluid secretion in fetal goats. *J Dev Physiol* 4: 311-325, 1982.

Cassin S. Effect of indomethacin on fetal lung liquid formation. *Can J Physiol Pharmacol* 62: 157-159, 1984.

Chalaka S, Ingbar DH, Sharma R, Zhau Z, and Wendt CH. Na(+)-K(+)-ATPase gene regulation by glucocorticoids in a fetal lung epithelial cell line. *Am J Physiol* 277: L197-203, 1999.

Champigny G, Voilley N, Lingueglia E, Friend V, Barbry P, and Lazdunski M. Regulation of expression of the lung amiloride-sensitive Na+ channel by steroid hormones. *Embo J* 13: 2177-2181, 1994.

Chen XJ, Eaton DC, and Jain L. Beta-adrenergic regulation of amiloride-sensitive lung sodium channels. *Am J Physiol Lung Cell Mol Physiol* 282: L609-620, 2002.

Chernick V and Mellins RB. Respiratory function in infants.ed. BC Decker, Hamilton, 2002

Chow YH, Wang Y, Plumb J, O'Brodovich H, and Hu J. Hormonal regulation and genomic organization of the human amiloride-sensitive epithelial sodium channel alpha subunit gene. *Pediatr Res* 46: 208-214, 1999.

Chua BA and Perks AM. The effect of dopamine on lung liquid production by in vitro lungs from fetal guinea-pigs. *J Physiol* 513 (Pt 1): 283-294, 1998.

Chua BA and Perks AM. The pulmonary neuroendocrine system and drainage of the fetal lung: effects of serotonin. *Gen Comp Endocrinol* 113: 374-387, 1999.

Cott GR and Rao AK. Hydrocortisone promotes the maturation of Na(+)-dependent ion transport across the fetal pulmonary epithelium. *Am J Respir Cell Mol Biol* 9: 166-171, 1993.

Cotton CU, Boucher RC, and Gatzy JT. Paths of ion transport across canine fetal tracheal epithelium. *J Appl Physiol* 65: 2376-2382, 1988a.

Cotton CU, Boucher RC, and Gatzy JT. Bioelectric properties and ion transport across excised canine fetal and neonatal airways. *J Appl Physiol* 65: 2367-2375, 1988b.

Dagenais A, Frechette R, Yamagata Y, Yamagata T, Carmel JF, Clermont ME, Brochiero E, Masse C, and Berthiaume Y. Downregulation of ENaC activity and expression by TNF-alpha in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 286: L301-311, 2004.

Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, and Staub O. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. *Embo J* 20: 7052-7059, 2001.

DeSa DJ. Pulmonary fluid content in infants with respiratory distress. *J Pathol* 97: 469-478, 1969.

Devarajan P and Benz EJ, Jr. Translational regulation of Na-K-ATPase subunit mRNAs by glucocorticoids. *Am J Physiol Renal Physiol* 279: F1132-1138, 2000.

Ding C, Potter ED, Qiu W, Coon SL, Levine MA, and Guggino SE. Cloning and widespread distribution of the rat rod-type cyclic nucleotide-gated cation channel. *Am J Physiol* 272: C1335-1344, 1997.

Dobbs LG, Gonzalez R, Matthay MA, Carter EP, Allen L, and Verkman AS. Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. *Proc Natl Acad Sci U S A* 95: 2991-2996, 1998.

Doe S and Perks AM. Alpha-adrenoreceptor influences on liquid movements by in vitro lungs from fetal guinea pigs. *J Appl Physiol* 84: 746-753, 1998.

Eaton DC, Chen J, Ramosevac S, Matalon S, and Jain L. Regulation of Na+ channels in lung alveolar type II epithelial cells. *Proc Am Thorac Soc* 1: 10-16, 2004.

Egan EA, Olver RE, and Strang LB. Changes in non-electrolyte permeability of alveoli and the absorption of lung liquid at the start of breathing in the lamb. *J Physiol* 244: 161-179, 1975.

Elias N, Rafii B, Rahman M, Otulakowski G, Cutz E, and O'Brodovich H M. The Role of {alpha}, {beta}, and {gamma}-EN{alpha}C Subunits in Distal Lung Epithelial Fluid Absorption Induced by Pulmonary Edema Fluid. *Am J Physiol Lung Cell Mol Physiol*, 2007.

Factor P, Saldias F, Ridge K, Dumasius V, Zabner J, Jaffe HA, Blanco G, Barnard M, Mercer R, Perrin R, and Sznajder JI. Augmentation of lung

liquid clearance via adenovirus-mediated transfer of a Na,K-ATPase beta1 subunit gene. *J Clin Invest* 102: 1421-1430, 1998a.

FACTOR P, SENNE C, DUMASIUS V, RIDGE K, JAFFE HA, UHAL B, GAO Z, AND SZNAJDER JI. Overexpression of the Na+,K+-ATPase alpha1 subunit increases Na+,K+-ATPase function in A549 cells. *Am J Respir Cell Mol Biol* 18: 741-749, 1998b.

FAJAC I, LACRONIQUE J, LOCKHART A, DALL'AVA-SANTUCCI J, AND DUSSER DJ. Silver/silver chloride electrodes for measurement of potential difference in human bronchi. *Thorax* 53: 879-881, 1998.

FARMAN N, TALBOT CR, BOUCHER R, FAY M, CANESSA C, ROSSIER B, AND BONVALET JP. Noncoordinated expression of alpha-, beta-, and gamma-subunit mRNAs of epithelial Na+ channel along rat respiratory tract. *Am J Physiol* 272: C131-141, 1997.

FAURE-FREMIET E DJ. Le developpement du poumon foetal chez le mouton. *Arch Anat Micr* 19: 411-474, 1923.

FEWELL JE AND JOHNSON P. Upper airway dynamics during breathing and during apnoea in fetal lambs. *J Physiol* 339: 495-504, 1983.

FISK NM, PARKES MJ, MOORE PJ, HANSON MA, WIGGLESWORTH J, AND RODECK CH. Mimicking low amniotic pressure by chronic pharyngeal drainage does not impair lung development in fetal sheep. *Am J Obstet Gynecol* 166: 991-996, 1992.

FLETCHER ME, BARALDI E, AND STEINBRUGGER B, *Passive respiratory mechanics*, in *Infant Respiratory Function Testing*, STOCKS J, SLY PD, TEPPER RS, AND MORGAN WJ, Editors. 1996, Wiley-Liss: New York. p. 283-327.

FOLKESSON HG, PITTET JF, NITENBERG G, AND MATTHAY MA. Transforming growth factor-alpha increases alveolar liquid clearance in anesthetized ventilated rats. *Am J Physiol* 271: L236-244, 1996.

FOLKESSON HG, NORLIN A, WANG Y, ABEDINPOUR P, AND MATTHAY MA. Dexamethasone and thyroid hormone pretreatment upregulate alveolar epithelial fluid clearance in adult rats. *J Appl Physiol* 88: 416-424, 2000.

FYFE GK AND CANESSA CM. Subunit composition determines the single channel kinetics of the epithelial sodium channel. *J Gen Physiol* 112: 423-432, 1998.

GAILLARD EA, SHAW NJ, WALLACE HL, SUBHEDAR NV, AND SOUTHERN KW. Airway ion transport on the first postnatal day in infants delivered vaginally or by elective cesarean section. *Pediatr Res* 54: 58-63, 2003.

GAILLARD EA, SHAW NJ, WALLACE HL, SUBHEDAR NV, AND SOUTHERN KW. Nasal potential difference increases with gestation in moderately preterm neonates on the first postnatal day. *Arch Dis Child Fetal Neonatal Ed* 90: F172-173, 2005.

GAILLARD EA, SHAW NJ, WALLACE HL, VINCE G, AND SOUTHERN KW. Electrical potential difference across the nasal epithelium is reduced in premature infants

with chronic lung disease but is not associated with lower airway inflammation. *Pediatr Res* 61: 77-82, 2007.

Galietta LJ, Folli C, Marchetti C, Romano L, Carpani D, Conese M, and Zegarra-Moran O. Modification of transepithelial ion transport in human cultured bronchial epithelial cells by interferon-gamma. *Am J Physiol Lung Cell Mol Physiol* 278: L1186-1194, 2000.

Galietta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, and Zegarra-Moran O. IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J Immunol* 168: 839-845, 2002.

Gappa M, Colin AA, Goetz I, and Stocks J. Passive respiratory mechanics: the occlusion techniques. *Eur Respir J* 17: 141-148, 2001.

Gowen CW, Jr., Lawson EE, Gingras J, Boucher RC, Gatzy JT, and Knowles MR. Electrical potential difference and ion transport across nasal epithelium of term neonates: correlation with mode of delivery, transient tachypnea of the newborn, and respiratory rate. *J Pediatr* 113: 121-127, 1988.

Graeff RW, Wang G, and McCray PB, Jr. KGF and FGF-10 stimulate liquid secretion in human fetal lung. *Pediatr Res* 46: 523-529, 1999.

Griscom NT, Harris GB, Wohl ME, Vawter GF, and Eraklis AJ. Fluid-filled lung due to airway obstruction in the newborn. *Pediatrics* 43: 383-390, 1969.

Helms MN, Self J, Bao HF, Job LC, Jain L, and Eaton DC. Dopamine activates amiloride-sensitive sodium channels in alveolar type I cells in lung slice preparations. *Am J Physiol Lung Cell Mol Physiol* 291: L610-618, 2006.

Hislop AA, Wigglesworth JS, and Desai R. Alveolar development in the human fetus and infant. *Early Hum Dev* 13: 1-11, 1986.

Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R, and Rossier BC. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet* 12: 325-328, 1996.

Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, and Thomas CP. Glucocorticoid-stimulated lung epithelial Na(+) transport is associated with regulated ENaC and sgk1 expression. *Am J Physiol Lung Cell Mol Physiol* 282: L631-641, 2002.

Jain L, Chen XJ, Malik B, Al-Khalili O, and Eaton DC. Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity. *Am J Physiol* 276: L1046-1051, 1999.

Jain L, Chen XJ, Ramosevac S, Brown LA, and Eaton DC. Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. *Am J Physiol Lung Cell Mol Physiol* 280: L646-658, 2001.

Jain L and Dudell GG. Respiratory transition in infants delivered by cesarean section. *Semin Perinatol* 30: 296-304, 2006.

Jobe AH. Pulmonary surfactant therapy. *N Engl J Med* 328: 861-868, 1993.

JOBE AH AND BANCALARI E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 163: 1723-1729, 2001.

JOHNSON MD, WIDDICOMBE JH, ALLEN L, BARBRY P, AND DOBBS LG. Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. *Proc Natl Acad Sci U S A* 99: 1966-1971, 2002.

JOHNSON MD, BAO HF, HELMS MN, CHEN XJ, TIGUE Z, JAIN L, DOBBS LG, AND EATON DC. Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport. *Proc Natl Acad Sci U S A* 103: 4964-4969, 2006.

JUNOR RW, BENJAMIN AR, ALEXANDROU D, GUGGINO SE, AND WALTERS DV. A novel role for cyclic nucleotide-gated cation channels in lung liquid homeostasis in sheep. *J Physiol* 520 Pt 1: 255-260, 1999.

KEMP PJ, KIM KJ, BOROK Z, AND CRANDALL ED. Re-evaluating the Na(+) conductance of adult rat alveolar type II pneumocytes: evidence for the involvement of cGMP-activated cation channels. *J Physiol* 536: 693-701, 2001.

KEREM E, BISTRITZER T, HANUKOGLU A, HOFMANN T, ZHOU Z, BENNETT W, MACLAUGHLIN E, BARKER P, NASH M, QUITTELL L, BOUCHER R, AND KNOWLES MR. Pulmonary epithelial sodium-channel dysfunction and excess airway liquid in pseudohypo-aldosteronism. *N Engl J Med* 341: 156-162, 1999.

KIERSZANBAUM AL. Respiratory system. KIERSZANBAUM AL (ed.): Histology and Cell Biology - An introduction to pathology, 1 ed. Mosby, St. Louis, 2002

KITTERMAN JA, BALLARD PL, CLEMENTS JA, MESCHER EJ, AND TOOLEY WH. Tracheal fluid in fetal lambs: spontaneous decrease prior to birth. *J Appl Physiol* 47: 985-989, 1979.

KLEYMAN TR AND CRAGOE EJ, JR. Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* 105: 1-21, 1988.

KNOWLES M, GATZY J, AND BOUCHER R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N Engl J Med* 305: 1489-1495, 1981a.

KNOWLES MR, CARSON JL, COLLIER AM, GATZY JT, AND BOUCHER RC. Measurements of nasal transepithelial electric potential differences in normal human subjects in vivo. *Am Rev Respir Dis* 124: 484-490, 1981b.

KNOWLES MR, BUNTIN WH, BROMBERG PA, GATZY JT, AND BOUCHER RC. Measurements of transepithelial electric potential differences in the trachea and bronchi of human subjects in vivo. *Am Rev Respir Dis* 126: 108-112, 1982.

KONSTAS AA AND KORBMACHER C. The gamma-subunit of ENaC is more important for channel surface expression than the beta-subunit. *Am J Physiol Cell Physiol* 284: C447-456, 2003.

KROCHMAL EM, BALLARD ST, YANKASKAS JR, BOUCHER RC, AND GATZY JT. Volume and ion transport by fetal rat alveolar and tracheal epithelia in submersion culture. *Am J Physiol* 256: F397-407, 1989.

LAGERCRANTZ H AND BISTOLETTI P. Catecholamine release in the newborn infant at birth. *Pediatr Res* 11: 889-893, 1977.

LAZRAK A, SAMANTA A, VENETSANOU K, BARBRY P, AND MATALON S. Modification of biophysical properties of lung epithelial Na(+) channels by dexamethasone. *Am J Physiol Cell Physiol* 279: C762-770, 2000.

LEE IH, DINUDOM A, SANCHEZ-PEREZ A, KUMAR S, AND COOK DI. Akt mediates the effect of insulin on epithelial sodium channels by inhibiting Nedd4-2. *J Biol Chem* 282: 29866-29873, 2007.

LI T, KOSHY S, AND FOLKESSON HG. Involvement of {alpha}ENaC and Nedd4-2 in the conversion from lung fluid secretion to fluid absorption at birth in the rat as assayed by RNA interference analysis. *Am J Physiol Lung Cell Mol Physiol* 293: L1069-1078, 2007a.

LI T, VARADARAJULU S, BEARD LL, YUN J, AND FOLKESSON HG. A noninflammatory interleukin-1beta fragment stimulates fetal lung fluid absorption in Guinea pigs. *J Pharmacol Exp Ther* 320: 877-884, 2007b.

LIU H, HOOPER SB, ARMUGAM A, DAWSON N, FERRARO T, JEYASEELAN K, THIEL A, KOUKOULAS I, AND WINTOUR EM. Aquaporin gene expression and regulation in the ovine fetal lung. *J Physiol* 551: 503-514, 2003.

LOFFING J, ZECEVIC M, FERAILLE E, KAISSLING B, ASHER C, ROSSIER BC, FIRESTONE GL, PEARCE D, AND VERREY F. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol* 280: F675-682, 2001.

LORENZ JM. The outcome of extreme prematurity. *Semin Perinatol* 25: 348-359, 2001.

MALAGON-ROGERS M. A patient with pseudohypoaldosteronism type 1 and respiratory distress syndrome. *Pediatr Nephrol* 13: 484-486, 1999.

MALL M, GRUBB BR, HARKEMA JR, O'NEAL WK, AND BOUCHER RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 10: 487-493, 2004.

MARTIN R, FANAROFF A, AND WALSH M. Fanaroff and Martin's Neonatal-Perinatal Medicine, Vol. 8ed. Mosby Elsevier, Philadelphia, 2006

MATALON S AND O'BRODOVICH H. Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. *Annu Rev Physiol* 61: 627-661, 1999.

MATSUSHITA K, MCCRAY PB, JR., SIGMUND RD, WELSH MJ, AND STOKES JB. Localization of epithelial sodium channel subunit mRNAs in adult rat lung by in situ hybridization. *Am J Physiol* 271: L332-339, 1996.

McCray PB, Jr., Bettencourt JD, and Bastacky J. Developing bronchopulmonary epithelium of the human fetus secretes fluid. *Am J Physiol* 262: L270-279, 1992.

McCray PB, Jr. and Bettencourt JD. Prostaglandins stimulate fluid secretion in human fetal lung. *J Dev Physiol* 19: 29-36, 1993.

Mescher EJ, Platzker AC, Ballard PL, Kitterman JA, Clements JA, and Tooley WH. Ontogeny of tracheal fluid, pulmonary surfactant, and plasma corticoids in the fetal lamb. *J Appl Physiol* 39: 1017-1021, 1975.

Moessinger AC, Collins MH, Blanc WA, Rey HR, and James LS. Oligohydramnios-induced lung hypoplasia: the influence of timing and duration in gestation. *Pediatr Res* 20: 951-954, 1986.

Moessinger AC, Harding R, Adamson TM, Singh M, and Kiu GT. Role of lung fluid volume in growth and maturation of the fetal sheep lung. *J Clin Invest* 86: 1270-1277, 1990.

Morgan EE, Stader SM, Hodnichak CM, Mavrich KE, Folkesson HG, and Maron MB. Postreceptor defects in alveolar epithelial beta-adrenergic signaling after prolonged isoproterenol infusion. *Am J Physiol Lung Cell Mol Physiol* 285: L578-583, 2003.

Mustafa SB, DiGeronimo RJ, Petershack JA, Alcorn JL, and Seidner SR. Postnatal glucocorticoids induce alpha-ENaC formation and regulate glucocorticoid receptors in the preterm rabbit lung. *Am J Physiol Lung Cell Mol Physiol* 286: L73-80, 2004.

Nakamura K, Stokes JB, and McCray PB, Jr. Endogenous and exogenous glucocorticoid regulation of ENaC mRNA expression in developing kidney and lung. *Am J Physiol Cell Physiol* 283: C762-772, 2002.

Nardo L, Hooper SB, and Harding R. Lung hypoplasia can be reversed by short-term obstruction of the trachea in fetal sheep. *Pediatr Res* 38: 690-696, 1995.

Nielsen S, King LS, Christensen BM, and Agre P. Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. *Am J Physiol* 273: C1549-1561, 1997.

Norlin A, Finley N, Abedinpour P, and Folkesson HG. Alveolar liquid clearance in the anesthetized ventilated guinea pig. *Am J Physiol* 274: L235-243, 1998.

Norlin A, Lu LN, Guggino SE, Matthay MA, and Folkesson HG. Contribution of amiloride-insensitive pathways to alveolar fluid clearance in adult rats. *J Appl Physiol* 90: 1489-1496, 2001.

O'Brodovich H, Hannam V, Seear M, and Mullen JB. Amiloride impairs lung water clearance in newborn guinea pigs. *J Appl Physiol* 68: 1758-1762, 1990.

O'Brodovich H, Hannam V, and Rafii B. Sodium channel but neither Na(+)-H+ nor Na-glucose symport inhibitors slow neonatal lung water clearance. *Am J Respir Cell Mol Biol* 5: 377-384, 1991.

O'Brodovich H, Canessa C, Ueda J, Rafii B, Rossier BC, and Edelson J. Expression of the epithelial Na+ channel in the developing rat lung. *Am J Physiol* 265: C491-496, 1993.

O'Brodovich HM. Immature epithelial Na+ channel expression is one of the pathogenetic mechanisms leading to human neonatal respiratory distress syndrome. *Proc Assoc Am Physicians* 108: 345-355, 1996.

Olivera W, Ridge K, Wood LD, and Sznajder JI. Active sodium transport and alveolar epithelial Na-K-ATPase increase during subacute hyperoxia in rats. *Am J Physiol* 266: L577-584, 1994.

Olver RE and Strang LB. Ion fluxes across the pulmonary epithelium and the secretion of lung liquid in the foetal lamb. *J Physiol* 241: 327-357, 1974.

Olver RE, Schneeberger EE, and Walters DV. Epithelial solute permeability, ion transport and tight junction morphology in the developing lung of the fetal lamb. *J Physiol* 315: 395-412, 1981.

Olver RE, Ramsden CA, Strang LB, and Walters DV. The role of amiloride-blockable sodium transport in adrenaline-induced lung liquid reabsorption in the fetal lamb. *J Physiol* 376: 321-340, 1986a.

Olver RE and Robinson EJ. Sodium and chloride transport by the tracheal epithelium of fetal, new-born and adult sheep. *J Physiol* 375: 377-390, 1986b.

Olver RE, Walters DV, and Wilson SM. Developmental regulation of lung liquid transport. *Annu Rev Physiol* 66: 77-101, 2004.

Otulakowski G, Flueckiger-Staub S, Ellis L, Ramlall K, Staub O, Smith D, Durie P, and O'Brodovich H. Relation between alpha, beta, and gamma human amiloride- sensitive epithelial Na+ channel mRNA levels and nasal epithelial potential difference in healthy men. *Am J Respir Crit Care Med* 158: 1213-1220, 1998.

Otulakowski G, Rafii B, Bremner HR, and O'Brodovich H. Structure and hormone responsiveness of the gene encoding the alpha-subunit of the rat amiloride-sensitive epithelial sodium channel. *Am J Respir Cell Mol Biol* 20: 1028-1040, 1999.

Otulakowski G, Rafii B, Harris M, and O'Brodovich H. Oxygen and glucocorticoids modulate alphaENaC mRNA translation in fetal distal lung epithelium. *Am J Respir Cell Mol Biol* 34: 204-212, 2006.

Perks AM and Cassin S. The effects of arginine vasopressin and epinephrine on lung liquid production in fetal goats. *Can J Physiol Pharmacol* 67: 491-498, 1989.

Perks AM, Kwok YN, McIntosh CH, Ruiz T, and Kindler PM. Changes in somatostatin-like immunoreactivity in lungs from perinatal guinea pigs and the

effects of somatostatin-14 on lung liquid production. *J Dev Physiol* 18: 151-159, 1992.

PFISTER RE, RAMSDEN CA, NEIL HL, KYRIAKIDES MA, AND BERGER PJ. Volume and secretion rate of lung liquid in the final days of gestation and labour in the fetal sheep. *J Physiol* 535: 889-899, 2001.

PITKANEN O, TRANSWELL AK, DOWNEY G, AND O'BRODOVICH H. Increased Po2 alters the bioelectric properties of fetal distal lung epithelium. *Am J Physiol* 270: L1060-1066, 1996.

PITKANEN OM, SMITH D, O'BRODOVICH H, AND OTULAKOWSKI G. Expression of alpha-, beta-, and gamma-hENaC mRNA in the human nasal, bronchial, and distal lung epithelium. *Am J Respir Crit Care Med* 163: 273-276, 2001.

PLANES C, FRIEDLANDER G, LOISEAU A, AMIEL C, AND CLERICI C. Inhibition of Na-K-ATPase activity after prolonged hypoxia in an alveolar epithelial cell line. *Am J Physiol* 271: L70-78, 1996.

PLANES C, BLOT-CHABAUD M, MATTHAY MA, COUETTE S, UCHIDA T, AND CLERICI C. Hypoxia and beta 2-agonists regulate cell surface expression of the epithelial sodium channel in native alveolar epithelial cells. *J Biol Chem* 277: 47318-47324, 2002.

PLANES C, LEYVRAZ C, UCHIDA T, ANGELOVA MA, VUAGNIAUX G, HUMMLER E, MATTHAY M, CLERICI C, AND ROSSIER B. In vitro and in vivo regulation of transepithelial lung alveolar sodium transport by serine proteases. *Am J Physiol Lung Cell Mol Physiol* 288: L1099-1109, 2005.

POTTER E AND BOHLENDER G. Intrauterine respiration in relation to development of the fetal lung. *Am J Obstet Gynecol*: 14-22, 1941.

RABBETTE PS, FLETCHER ME, DEZATEUX CA, SORIANO-BRUCHER H, AND STOCKS J. Hering-Breuer reflex and respiratory system compliance in the first year of life: a longitudinal study. *J Appl Physiol* 76: 650-656, 1994.

RAFII B, TANSWELL AK, OTULAKOWSKI G, PITKANEN O, BELCASTRO-TAYLOR R, AND O'BRODOVICH H. O2-induced ENaC expression is associated with NF-kappaB activation and blocked by superoxide scavenger. *Am J Physiol* 275: L764-770, 1998.

RAFII B, GILLIE DJ, SULOWSKI C, HANNAM V, CHEUNG T, OTULAKOWSKI G, BARKER PM, AND O'BRODOVICH H. Pulmonary oedema fluid induces non-alpha-ENaC-dependent Na(+) transport and fluid absorption in the distal lung. *J Physiol* 544: 537-548, 2002.

RAO AK AND COTT GR. Ontogeny of ion transport across fetal pulmonary epithelial cells in monolayer culture. *Am J Physiol* 261: L178-187, 1991.

RICHARD K, RAMMINGER SJ, INGLIS SK, OLVER RE, LAND SC, AND WILSON SM. O2 can raise fetal pneumocyte Na+ conductance without affecting ENaC mRNA abundance. *Biochem Biophys Res Commun* 305: 671-676, 2003.

RIDGE KM, OLIVERA WG, SALDIAS F, AZZAM Z, HOROWITZ S, RUTSCHMAN DH, DUMASIUS V, FACTOR P, AND SZNAJDER JI. Alveolar type 1 cells express the alpha2 Na,K-ATPase, which contributes to lung liquid clearance. *Circ Res* 92: 453-460, 2003.

ROSS BB. Comparison of foetal pulmonary fluid with foetal plasma and amniotic fluid. *Nature* 199: 1100, 1963.

RUDDY MK, DRAZEN JM, PITKANEN OM, RAFII B, O'BRODOVICH HM, AND HARRIS HW. Modulation of aquaporin 4 and the amiloride-inhibitable sodium channel in perinatal rat lung epithelial cells. *Am J Physiol* 274: L1066-1072, 1998.

SALDIAS FJ, COMELLAS A, RIDGE KM, LECUONA E, AND SZNAJDER JI. Isoproterenol improves ability of lung to clear edema in rats exposed to hyperoxia. *J Appl Physiol* 87: 30-35, 1999.

SARIBAN-SOHRABY S, ABRAMOW M, AND FISHER RS. Single-channel behavior of a purified epithelial Na+ channel subunit that binds amiloride. *Am J Physiol* 263: C1111-1117, 1992.

SAYEGH R, AUERBACH SD, LI X, LOFTUS RW, HUSTED RF, STOKES JB, AND THOMAS CP. Glucocorticoid induction of epithelial sodium channel expression in lung and renal epithelia occurs via trans-activation of a hormone response element in the 5'-flanking region of the human epithelial sodium channel alpha subunit gene. *J Biol Chem* 274: 12431-12437, 1999.

SMITH DE, OTULAKOWSKI G, YEGER H, POST M, CUTZ E, AND O'BRODOVICH HM. Epithelial Na(+) channel (ENaC) expression in the developing normal and abnormal human perinatal lung. *Am J Respir Crit Care Med* 161: 1322-1331, 2000.

SNYDER PM. Liddle's syndrome mutations disrupt cAMP-mediated translocation of the epithelial Na(+) channel to the cell surface. *J Clin Invest* 105: 45-53, 2000.

SNYDER PM, OLSON DR, AND THOMAS BC. Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na+ channel. *J Biol Chem* 277: 5-8, 2002.

SNYDER PM. Minireview: regulation of epithelial Na+ channel trafficking. *Endocrinology* 146: 5079-5085, 2005.

SOUTHERN KW, NOONE PG, BOSWORTH DG, LEGRYS VA, KNOWLES MR, AND BARKER PM. A modified technique for measurement of nasal transepithelial potential difference in infants. *J Pediatr* 139: 353-358, 2001.

SPEER CP. Pre- and postnatal inflammatory mechanisms in chronic lung disease of preterm infants. *Paediatr Respir Rev* 5 Suppl A: S241-244, 2004.

STOKES JB AND SIGMUND RD. Regulation of rENaC mRNA by dietary NaCl and steroids: organ, tissue, and steroid heterogeneity. *Am J Physiol* 274: C1699-1707, 1998.

STRANG LB. Fetal lung liquid: secretion and reabsorption. *Physiol Rev* 71: 991-1016, 1991.

Talbot CL, Bosworth DG, Briley EL, Fenstermacher DA, Boucher RC, Gabriel SE, and Barker PM. Quantitation and localization of ENaC subunit expression in fetal, newborn, and adult mouse lung. *Am J Respir Cell Mol Biol* 20: 398-406, 1999.

Tchepichev S, Ueda J, Canessa C, Rossier BC, and O'Brodovich H. Lung epithelial Na channel subunits are differentially regulated during development and by steroids. *Am J Physiol* 269: C805-812, 1995.

Thome UH, Bischoff A, Maier L, Pohlandt F, and Trotter A. Amiloride-sensitive nasal potential difference is not changed by estradiol and progesterone replacement but relates to BPD or death in a randomized trial on preterm infants. *Pediatr Res* 60: 619-623, 2006.

Tong Q, Gamper N, Medina JL, Shapiro MS, and Stockand JD. Direct activation of the epithelial Na(+) channel by phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate produced by phosphoinositide 3-OH kinase. *J Biol Chem* 279: 22654-22663, 2004a.

Tong Z, Illek B, Bhagwandin VJ, Verghese GM, and Caughey GH. Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 287: L928-935, 2004b.

Truffert P, Empana JP, Breart G, Saugstad OD, Goelz R, Halliday HL, and Anceschi M. Treatment strategies for bronchopulmonary dysplasia with postnatal corticosteroids in Europe: the EURAIL survey. *Acta Paediatr* 92: 948-951, 2003.

Wallace MJ, Hooper SB, and Harding R. Regulation of lung liquid secretion by arginine vasopressin in fetal sheep. *Am J Physiol* 258: R104-111, 1990.

Wallen LD, Perry SF, Alston JT, and Maloney JE. Morphometric study of the role of pulmonary arterial flow in fetal lung growth in sheep. *Pediatr Res* 27: 122-127, 1990.

Wallen LD, Perry SF, Alston JT, and Maloney JE. Fetal lung growth. Influence of pulmonary arterial flow and surgery in sheep. *Am J Respir Crit Care Med* 149: 1005-1011, 1994.

Vallet V, Chraibi A, Gaeggeler HP, Horisberger JD, and Rossier BC. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature* 389: 607-610, 1997.

Walters DV and Olver RE. The role of catecholamines in lung liquid absorption at birth. *Pediatr Res* 12: 239-242, 1978.

Venkatesh VC and Katzberg HD. Glucocorticoid regulation of epithelial sodium channel genes in human fetal lung. *Am J Physiol* 273: L227-233, 1997.

Verghese GM, Tong ZY, Bhagwandin V, and Caughey GH. Mouse prostasin gene structure, promoter analysis, and restricted expression in lung and kidney. *Am J Respir Cell Mol Biol* 30: 519-529, 2004.

VIDYASAGAR D AND SHIMADA S. Pulmonary surfactant replacement in respiratory distress syndrome. *Clin Perinatol* 14: 991-1015, 1987.

WIGGLESWORTH JS, DESAI R, AND HISLOP AA. Fetal lung growth in congenital laryngeal atresia. *Pediatr Pathol* 7: 515-525, 1987.

WIGGLESWORTH JS. Lung development in the second trimester. *Br Med Bull* 44: 894-908, 1988.

VILOS GA AND LIGGINS GC. Intrathoracic pressures in fetal sheep. *J Dev Physiol* 4: 247-256, 1982.

VOILLEY N, LINGUEGLIA E, CHAMPIGNY G, MATTEI MG, WALDMANN R, LAZDUNSKI M, AND BARBRY P. The lung amiloride-sensitive Na+ channel: biophysical properties, pharmacology, ontogenesis, and molecular cloning. *Proc Natl Acad Sci U S A* 91: 247-251, 1994.

VUAGNIAUX G, VALLET V, JAEGER NF, PFISTER C, BENS M, FARMAN N, COURTOIS-COUTRY N, VANDEWALLE A, ROSSIER BC, AND HUMMLER E. Activation of the amiloride-sensitive epithelial sodium channel by the serine protease mCAP1 expressed in a mouse cortical collecting duct cell line. *J Am Soc Nephrol* 11: 828-834, 2000.

VUAGNIAUX G, VALLET V, JAEGER NF, HUMMLER E, AND ROSSIER BC. Synergistic activation of ENaC by three membrane-bound channel-activating serine proteases (mCAP1, mCAP2, and mCAP3) and serum- and glucocorticoid-regulated kinase (Sgk1) in Xenopus Oocytes. *J Gen Physiol* 120: 191-201, 2002.

YU JX, CHAO L, AND CHAO J. Molecular cloning, tissue-specific expression, and cellular localization of human prostasin mRNA. *J Biol Chem* 270: 13483-13489, 1995.

YUE G, RUSSELL WJ, BENOS DJ, JACKSON RM, OLMAN MA, AND MATALON S. Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats. *Proc Natl Acad Sci U S A* 92: 8418-8422, 1995.

ZELENINA M, ZELENIN S, AND APERIA A. Water channels (aquaporins) and their role for postnatal adaptation. *Pediatr Res* 57: 47R-53R, 2005.