Review

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;25:63-70

Accepted: September 08, 2009

Importance of ENaC-Mediated Sodium Transport in Alveolar Fluid Clearance Using Genetically-Engineered Mice

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Key Words
Epithelial sodium channel • ENaC • CAP1 • Channel-activating protease • Edema • Transgenic mice

Abstract
The lung possesses specific transport systems that intra- and extracellularly maintain salt and fluid balance necessary for its function. At birth, the lungs rapidly transform into a fluid (Na^+)-absorbing organ to enable efficient gas exchange. Alveolar fluid clearance, which mainly depends on sodium transport in alveolar epithelial cells, is an important mechanism by which excess water in the alveoli is reabsorbed during the resolution of pulmonary edema. In this review, we will focus and summarize on the role of ENaC in alveolar lung liquid clearance and discuss recent data from mouse models with altered activity of epithelial sodium channel function in the lung, and more specifically in alveolar fluid clearance. Recent data studying mice with hyperactivity of ENaC or mice with reduced ENaC activity clearly illustrate the impaired lung fluid clearance in these adult mice. Further understanding of the physiological role of ENaC and its regulatory proteins implicated in salt and water balance in the alveolar cells may therefore help to develop new therapeutic strategies to improve gas exchange in pulmonary edema.

Introduction

ENaC is limiting in the control of ionic composition of the extracellular fluid, regulation of blood volume and blood pressure, lung alveolar fluid clearance and airway mucociliary clearance, and skin barrier function [1, 2]. The highly amiloride-sensitive epithelial sodium channel ENaC is encoded by three different genes, *Scnn1a, Scnn1b and Scnn1g*, localized on chromosome 6 (α-ENaC) and 7 (β- and γ-ENaC) in the mouse [3-5] and chromosome 12 (α-ENaC) and 16 (β- and γ-ENaC) in human [6]. Jasti and colleagues proposed a trimeric structure for the chicken asic-sensing ion channel 1 (ASIC1), that belongs to the same ion channel family as ENaC, although these channels are different in their sensitivity and activation/inactivation [7, 8]. In preterm rat and human lung, ENaC messenger RNA transcripts are barely detectable, but expression

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levels increase considerably in the perinatal period and remain high throughout adult life, suggesting a role for ENaC in maintaining a dry alveolar state postnatally [9]. Complete inactivation of all three *Scnn1* genes (encoding for α-ENaC, β-ENaC, and γ-ENaC) leads to early postnatal death [10-12]. Inactivation of the α subunit of ENaC (*Scnn1a*) had clearly demonstrated the crucial role of this channel in lung liquid clearance at birth [10]. Mice deficient for the α-ENaC gene locus (*Scnn1a*^{tm1}) present poor feeding, costal retractions, and cyanosis a few hours after birth. They die within 40 hours after birth with their lungs filled with fluid, demonstrating the importance of the α subunit in the perinatal lung liquid clearance. Measurements of the amiloride-sensitive transepithelial potential differences in lung explants from α-ENaC knockout mice revealed that ENaC activity is completely abolished. We concluded that channels made of β-/γ- subunits do not confer sufficient activity to be the surrogates of the ENaC function in the lung [10]. Mice deficient for either β or γ-ENaC (*Scnn1b*$^{-/-}$ or *Scnn1g*$^{-/-}$) showed increased lung water contents measured as lung wet/dry ratio after birth, but otherwise did not exhibit the symptoms of respiratory distress like α-ENaC knockout mice (*Scnn1a*^{tm1}) [11, 12], and this despite a 6-fold reduction in activity as estimated from measurements of the amiloride-sensitive potential difference in tracheal explants from *Scnn1g*$^{-/-}$ mice [11]. In human, Barker and coworkers suggested that ENaC deficiency in the lung could be a cause of respiratory distress syndrome of very premature infants [13]. However, no case of neonatal respiratory distress syndrome has been reported so far in patients with pseudohypoaldosteronims (PHA-1) who show a reduced ENaC activity [14] (Table 1). In these patients, the lung phenotype manifests later (a few months after birth) and exhibited frequently recurring lower respiratory tract infections, and persistently elevated sweat and saliva electrolyte values have been reported [15-17]. Species-specific differences such as anatomical immaturity of the newborn mouse lung [18] or the presence of a δ-ENaC subunit that has only been further confirmed in human and rabbit, and appears to be present in chimpanzee (GeneBank™ accession number O46547) [19-21] or the mutations involved in human PHA-1 which may confer sufficient residual ENaC activity to rescue or attenuate the human lung phenotype may account for this effect [22].

Over the past years, we have generated an allelic series of mutations at the ENaC (*Scnn1*) gene loci showing that any modified expression of the ENaC subunits may cause a lung, kidney and skin disease [2, 9, 23-27]. Constitutive gene inactivation of all three subunits revealed that the absolute ENaC expression is essential for survival [10-12]. Removal of the α-ENaC subunit (*Scnn1a*) resulted in completely abolished ENaC activity, whereas inactivation of the β-ENaC (*Scnn1b*) and γ-ENaC (*Scnn1g*) subunit led to reduced ENaC activity. Mice without β-ENaC (*Scnn1b*) and γ-ENaC (*Scnn1g*) develop hyperkalemia and die soon after birth [10]. Failure to thrive and lethargy are associated with urinary Na^+ wasting, K^+ retention, and increased plasma aldosterone concentrations (for review, see ref 26). Mutations that result in hypofunction are expected to induce a salt wasting syndrome in the kidney similar to type 1 pseudohypoaldosteronism (see for review ref [28]), although we recently found that ENaC expression is also required for the postnatal skin function [2]. Mice deficient for ENaC suffer from an increased dehydration [2].

In our laboratory, various mouse lines have been generated in which the ENaC activity ranges from hypoactive channels (5-15% of total ENaC activity) to hyperactive channels (>150% of total ENaC activity) (for review, see ref [29]). Generally, reduced ENaC activity in mice bearing ENaC mutations led to clinical symptoms similar to human pseudohypoaldosteronism type 1 (PHA-1) ranging from mild (e.g. mutation in the β-ENaC gene locus [24]) to severe phenotype (e.g. γ-ENaC knockout mice [11]). We further introduced one of the classical human Liddle mutations (R566STOP) into the mouse β-ENaC (*Scnn1b*) gene locus, thereby generating mice with hyperactivity of ENaC, which reproduce to a large extent the sodium retaining phenotype as seen in Liddle's syndrome [25]. These mice present an impaired ENaC internalization, and exhibit ENaC-mediated transport features that are consistent with an overall increased ENaC activity [30]. Mineralocorticoid-mediated up-regulation of ENaC expression and function is still maintained in these mice with high sensitivity to aldosterone [30-32]. Renal cells from these mice exhibit hyperactive apical vasopressin-regulated CFTR Cl^- conductance [33] that could contribute to the enhanced NaCl reabsorption observed in the distal nephron of patients with Liddle's syndrome (for review, see ref [34]). Finally, by using conditional gene targeting of ENaC subunits, we are now able to induce the knockout of single ENaC subunits in a given tissue [35, 36] or at a given time point.

	Lung phenotype	ENaC function in the lung		Reference
		in vitro	*in vivo*	
human				
PHA-1	excess airway liquid	nd	decreased	[15]
	Recurring lower respiratory tract infections			
	Elevated sweat and saliva electrolyte values	nd	nd	[16,17]
Liddle's syndrome	abnormalities in nasal potential differences	nd	increased	[66]
mouse				
Scnn1a^{tm1}	alveolar perinatal lung clearance failure (RDS) low amiloride-sensitive potential difference in tracheal explants	abolished	abolished	[10]
Scnn1a$^{-/-}$tg	increased severity and delayed resolution of pulmonary edema under hyperoxia	decreased	nd	[47]
		nd	decreased	[48]
Scnn1b$^{-/-}$	delayed perinatal lung liquid clearance	nd	decreased	[12]
Scnn1b$^{neo/neo}$	delayed perinatal lung liquid clearance	decreased	decreased	[24]
	decreased alveolar fluid clearance and predisposition to pulmonary edema	nd	decreased	[55]
Scnn1b$^{Lid/Lid}$	increased alveolar fluid clearance	nd	nd	[25]
	decreased severity of hydrostatic pulmonary edema	nd	increased	[50]
Scnn1b-Tg	cystic fibrosis-like phenotype	increased	increased	[40]
Scnn1g$^{-/-}$	delayed perinatal lung liquid clearance	decreased	decreased	[11]

Table 1. Lung-specific anomalies in human and mice harbouring mutations that modify ENaC function.

Role of alveolar ENaC-mediated sodium transport in the pathophysiology of pulmonary edema

In the lung, ENaC is expressed in proximal and distal airways as well as in pulmonary alveoli [37] and is under glucocorticoid control [38]. In the airways, ENaC participates in the control of the extracellular fluid at the air-cell interface by regulating the volume of the airway surface liquid (ASL) and mucociliary clearance [39]. In humans, this imbalance of ENaC activity in the airways leads to a large variety of pathologies. Abnormal increase in ENaC activity contributes to airway mucus dehydration and decreased mucociliary transport as observed in cystic fibrosis [40]. Indeed, in transgenic mice specifically overexpressing β-ENaC (Scnn1b-Tg) in the airways, increased ENaC activity resulted in increased airway Na^+ absorption and initiated a cystic fibrosis-like phenotype, whereas this was not observed in α- and γ-ENaC overexpressing mice [40, 41]. An abnormal decrease in ENaC activity as encountered in PHA-1 patients may lead to airway mucus hyperhydration and iterative bronchial infections [15]. In pulmonary alveoli, active transcellular sodium transport by alveolar epithelial cells (AEC) is a driving force for the absorption of fluid from the alveolar space, and represents the primary mechanism for the resolution of alveolar edema [42-45]. Sodium entry at the apical side of AEC is usually considered as the rate-limiting step for this process. The three ENaC subunits are expressed in both type I and type II AEC, and there is abundant evidence that ENaC channels represent the major pathway for apical Na^+ entry [10, 44, 46], although other transporters such as the Na^+-glucose cotransporter or cyclic nucleotide-gated (CNG) channels might also be involved [42, 46]. Lung epithelial fluid transport may thus be important in the resolution of pulmonary edema [44].

Mice with reduced activity of ENaC are predisposed to develop pulmonary edema

We thus became interested to know whether ENaC may also control airway mucociliary clearance and alveolar fluid clearance, and begun to study the role of sodium and water transport across the alveolar epithelium in the pathogenesis of pulmonary edema. We propose that ENaC dysfunction in adult lung is associ-

ated with augmented susceptibility to pulmonary edema. First experiments using transgenic mice confirmed this correlation and demonstrated the importance of precise regulation of ENaC *in vivo* [23, 47, 48]. We rescued the α-ENaC knockout phenotype by introducing a transgene [23]. ENaC activity was decreased, but this reduced ENaC activity does not lead to neonatal respiratory problems and was sufficient to alleviate the respiratory distress syndrome under normoxic conditions [23] (Table 1).

Using primary tracheal cell cultures from ENaC mutant mice, we found that *in vitro* exposure of lung epithelial cells to acute hypoxia, a condition frequently associated with lung pathology, further decreased ENaC-mediated Na^+ transport, suggesting that the combined effect of low level of ENaC expression in α-ENaC transgenic mice and additional stress could lead to failure of airway fluid clearance and impair the resolution of pulmonary edema [47]. We further confirmed this hypothesis *in vivo* by challenging these α-ENaC mutant mice by experimentally inducing pulmonary edema. Such mice showed a normal lung fluid balance at baseline, despite a 50% decrease in Na^+-driven alveolar fluid clearance (AFC). However, they develop more severe pulmonary edema than control mice when treated with thiourea, a drug increasing air-blood barrier permeability, or when exposed to prolonged hyperoxia [48]. Thus, diminished basal Na^+ transport *per se* is not sufficient to induce alveolar edema, but presents a predisposing factor for edema formation. In these α-ENaC rescue mice exhibiting an estimated overall activity of about 15% of wildtype, an additional stress to the system seems to be necessary for edema accumulation, either to increase alveolar flooding as is the case in toxic or hydrostatic edema [44], or to further reduce the sodium transport capacity such as under hypoxia [49].

Hyperactivity of ENaC may protect mice from developing pulmonary edema

We asked the question whether *a contrario* hyperactivity of ENaC in the distal lung could improve AFC and facilitate the resolution of pulmonary edema. We reasoned that if ENaC is truly rate-limiting for alveolar Na^+ transport, then ENaC hyperactivity in AEC should increase AFC, and possibly decrease the severity of alveolar edema. We therefore studied AFC and lung fluid balance at baseline and under conditions of hydrostatic pulmonary edema in transgenic mice with hyperactivity of ENaC, i.e. in mice that harbour a gain-of-function mutation (R566stop) within the *Scnn1b* gene locus [25]. These mice recapitulate most of the features of Liddle's syndrome and exhibit constitutively increased ENaC activity [25, 30, 33].

Hence, we provide clear *in vivo* evidence that constitutive activation of ENaC in the distal lung is responsible for increased water reabsorption from the alveolar space and thus, confirm the prominent role of ENaC in transepithelial alveolar Na^+ and fluid transport [50]. Our mice with hyperactivity of ENaC clearly escape to develop alveolar edema under acute volume overload, most likely due to an increased ability to remove fluid from their lungs [50]. In line with our findings, it has been previously shown that most patients with hydrostatic pulmonary edema have submaximal or maximal alveolar fluid clearance, whereas the majority of patients with acute lung injury or respiratory distress syndrome have impaired alveolar fluid clearance [45, 51].

In conclusion, these studies highlight the crucial role of ENaC in transepithelial alveolar Na^+ transport and alveolar fluid balance. Indeed, our finding that ENaC hyperactivity in the distal lung results *in vivo* in increased AFC under physiological conditions, and reduced severity of pulmonary edema following acute volume-overload suggests that new therapies targeted at enhancing ENaC activity and AFC could hasten the resolution of hydrostatic pulmonary edema in patients. Accordingly, recent clinical studies in human suggest that β-agonist treatment might represent a potential therapeutic strategy to decrease pulmonary edema and improve gas exchange [52].

New insight into the role of ENaC subunits in the distal lung

On the other side, transgenic models with low expression of ENaC subunits will provide information whether these mice are more predisposed to develop edema and/or on the respective role of α, β and γ-ENaC subunits in distal lung function and regulation. Former patch-clamp studies have identified two different forms of amiloride-sensitive Na^+ channels in both type I and type II AEC: the highly selective cation (HSC) channels which are the most numerous, and the non selective cation (NSC) channels [46, 53]. HSC channels have a unit conductance of 4-6 pS with high selectivity for Na^+ over K^+, and high sensitivity of amiloride, and present thus the same properties of ENaC channels [46]. By contrast,

NSC channels have a unit conductance of 19-21 pS, equal selectivity to Na^+ and K^+, and a lower sensitivity to amiloride [38, 46, 53]. Studies using antisense oligonucleotides to any of the three genes encoding the ENaC subunits strongly suggested that these NSC channels represent "atypical ENaC channels" made of α-ENaC subunits alone, whereas HSC channels are typical ENaC channels made of the three subunits α, β and γ [53, 54]. Consistent with this hypothesis, transgenic models with complete or partial inactivation of *Scnn1a* gene have clearly confirmed the prominent role of the α-ENaC subunit in the lung fluid balance at birth, but also during adulthood [10-12, 23]. By contrast, the relative importance of β or γ-ENaC subunits in the distal lung function is less well established. Complete inactivation of either *Scnn1b* (β-ENaC) [12] or *Scnn1g* (γ-ENaC) [11] genes in the mouse only mildly delayed fetal lung fluid clearance at birth, indicating that β and γ-ENaC subunits are not essential for the transition from fluid-filled to air-filled lung at birth. Due to the premature death of these animals from renal dysfunction, their lung phenotype during adulthood could not be studied. It seems likely however that β or γ-ENaC expression levels influence ENaC stoichiometry and function in AEC.

To better define the role of β-ENaC in the adult mouse distal lung, we studied alveolar Na^+ and water transport in mice with low expression of β-ENaC [24]. In a transgenic mouse model, previous disruption of the *Scnn1b* gene locus resulted in a more than 90% decrease in β-ENaC mRNA levels in all organs tested including the lung, kidney and colon [24]. Homozygous β-ENaC mutant (m/m) mice only show a small delay in lung fluid clearance at birth, but grow normally when fed with normal salt diet. However, they exhibit a severe pseudohypoaldosteronism type 1 phenotype with renal salt loss on low-salt diet [24]. We investigated whether low expression of β-ENaC subunit in adult mutant (m/m) mice would affect lung fluid balance and Na^+-driven AFC under basal and β_2-agonist-stimulated conditions. Low expression of the β-ENaC subunit, as evidenced by the absence of any detectable β-ENaC protein expression in distal lung homogenates, impairs total and ENaC-mediated AFC without affecting distal lung fluid homeostasis at baseline [55]. The decrease in β-ENaC expression is associated with a compensatory increase in α- and γ-ENaC subunit proteins in distal lung epithelial cells, and with a decrease in alveolar sodium channel sensitivity to amiloride [55]. Taken together, these data underline the complexity of ENaC channel stoichiometry in the distal lung. In mutant mice, low expression of the β-ENaC subunit most likely decreases the number of HSC channels at the surface of AEC, and could lead to a compensatory increase in the number of NSC channels (made of α-ENaC alone or in combination with γ-ENaC) less sensitive to amiloride. At least, the increase in α-ENaC and γ-ENaC protein expression observed in (m/m) mouse distal lung cells supports this hypothesis.

We also found that the stimulation of AFC by terbutaline is abolished in mutant mice with low β-ENaC expression [55]. Stimulation of β_2-adrenergic receptors, either by endogenous catecholamines released in case of stress or by β_2-agonist drugs, induces a marked increase in alveolar Na^+ transport and fluid clearance *in vivo* in most mammalian species [44]. *In vitro*, cAMP-agonists have been shown to augment ENaC activity and transepithelial Na^+ transport in various cell types including native AEC, mostly by increasing the turnover and promoting the delivery of ENaC subunits to the cell surface [49, 53, 56]. It was demonstrated that β-adrenergic agonists stimulate Na^+ channels in native rat AEC through a dual effect, by increasing the number of HSC channels at the cell surface and by increasing the open probability of NSC channels [53]. Indeed, we previously reported that terbutaline increased cell surface expression of ENaC subunits in cultured rat AEC, especially that of β- and γ-ENaC subunits, consistent with an increase in the number of HSC channels [49]. Our data in ENaC-modified mice with low level of β- subunit expression [55] provide additional evidence for the functional importance of ENaC in β_2-agonist-stimulated AFC. One explanation for the lack of response to terbutaline in β-ENaC mutant mice may be the compromised recruitment of new β-ENaC subunits at the cell surface, and as a result, the decreased number of HSC channels. Therefore, appropriate expression of β-ENaC allowing the formation of new HSC channels at the surface of AEC appears to be critical in AFC stimulation by β_2-agonists. This may be important in pathophysiology in as much as endogenous catecholamine release in case of pulmonary edema is expected to increase AFC and therefore to hasten the reabsorption of alveolar edema fluid [57].

Conclusions and Perspectives

In summary, the study of mouse models mimicking the salt-losing syndrome (PHA-1) [23] or salt-sensitive hypertension (Liddle's syndrome) [25] have established the epithelial sodium channel ENaC as a limiting factor in

the control of ionic composition of the extracellular fluid and lung alveolar clearance. Transepithelial alveolar sodium (Na^+) transport mediated by the amiloride-sensitive epithelial sodium channel (ENaC) constitutes the driving force for removal of fluid from the alveolar space, and thus, ENaC activity itself, but also factors regulating ENaC may affect pathophysiology of acute lung injury and pulmonary edema through the inhibition of alveolar liquid clearance and sodium transport [44]. Recently, using a doxycycline-inducible (Tet-ON system), combined with the Cre-*lox*P-mediated recombination, we generated alveolar epithelial-deficient serine protease CAP1/*Prss8* [58-62] mice and found that ENaC-mediated lung fluid clearance was markedly reduced and delayed the resolution of hydrostatic pulmonary edema [63], underlining the importance of activators or inhibitors of ENaC function *in vivo*. The analysis of genetically-modified animals presents thus a powerful tool for our understanding of the *in vivo* relevance of these genes and proteins within the whole organism. In particular, since inactivation of all three *Scnn1* genes result in perinatal lethality, tissue-specific, time-dependent and inducible gene expression requires the generation of the corresponding floxed alleles of the gene of interest (e.g., available now for all three ENaC genes [36, 64]) and mice expressing the Cre recombinase under a tissue-specific promoter [65].

Taken all these data together, in the future, there may be a need to develop ENaC blockers, like amiloride analogues or serine protease inhibitor to increase mucociliary clearance for the treatment of cystic fibrosis and chronic bronchitis and ENaC activators to increase alveolar fluid clearance for lung edema treatment.

Acknowledgements

Thanks to all members of the laboratory for contributing to the reseach projects. Work in the laboratory of EH is supported by grants from the Swiss National Science Foundation. Work in the laboratory of CP is supported by INSERM and by Chancellerie des Universités de Paris.

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