Alveolar epithelium and Na,K-ATPase in acute lung injury

Abstract  Active transport of sodium across the alveolar epithelium, undertaken in part by the Na,K-adenosine triphosphatase (Na,K-ATPase), is critical for clearance of pulmonary edema fluid and thus the outcome of patients with acute lung injury. Acute lung injury results in disruption of the alveolar epithelial barrier and leads to impaired clearance of edema fluid and altered Na,K-ATPase function. There has been significant progress in the understanding of mechanisms regulating alveolar edema clearance and signaling pathways modulating Na,K-ATPase function during lung injury. The accompanying review by Morty et al. focuses on intact organ and animal models as well as clinical studies assessing alveolar fluid reabsorption in alveolar epithelial injury. Elucidation of the mechanisms underlying regulation of active Na⁺ transport, as well as the pathways by which the Na,K-ATPase regulates epithelial barrier function and edema clearance, are of significance to identify interventional targets to improve outcomes of patients with acute lung injury.

Keywords  Edema clearance · Hypoxia · Coagulation · Catecholamines · Dopamine · Protein trafficking

Alveolar epithelial barrier function in acute lung injury

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are associated with disruption of the alveolo-capillary barrier, which results in edema fluid accumulation and impaired gas exchange [1]. Injury of the alveolo-capillary barrier alters active Na⁺ transport, leading to impaired edema fluid clearance from the alveolar spaces. Failure to return to normal clearance is associated with poor prognosis [2, 3].

The monolayer of the alveolar epithelium consists of squamous type I cells (ATI) and cuboidal type II cells (ATII). While the number of ATI and ATII cells in the alveolar epithelium is equal, ATI cells represent ~95% of the alveolar surface area [4]. Under physiological conditions, the alveolo-capillary barrier is highly impermeable, primarily due to the tight epithelial barrier, while the capillary endothelium is more leaky [5]. Interestingly, there appears to be a cross-talk between these monolayers. In an elegant series of experiments Kuebler and coworkers have demonstrated that inflammatory stimuli to the alveoli initiate rapid alveolo-capillary signaling leading to propagation of the inflammation [6]. Thus the alveolar epithelium might play a central role in modulating endothelial cell biology.

The apical surface of the alveolar epithelium is lined by a thin layer of fluid, which facilitates the maintenance of surface tension, host defense properties, and gas exchange [7]. During lung injury, pulmonary edema accumulates, leading to a life-threatening impairment of gas exchange. Thus, clearing the excess liquid from the alveolar space is of critical importance.
**Active Na\(^+\) transport is the primary force of alveolar fluid reabsorption**

The primary force driving fluid reabsorption from the alveolar space into the interstitium and the pulmonary circulation is active Na\(^+\) transport. Sodium is taken up on the apical surface of the alveolar epithelium by amiloride-sensitive and -insensitive Na\(^+\) channels [8, 9] and is subsequently “pumped” out of the cell by the Na,K-adenosine triphosphatase (Na,K-ATPase) on the basolateral side [10, 11] (Fig. 1). This process generates an osmotic gradient, which drives passive movement of water from the apical side of the epithelium (the alveolar space) to the basolateral side (the interstitium) paracellularly and through aquaporin-5 water channels which are located in ATI cells [12]. Extensive research on human lungs and in animal models has demonstrated that alveolar fluid clearance is inhibited by amiloride, an inhibitor of epithelial Na\(^+\) channels (ENaC), as well as by the Na,K-ATPase inhibitor, ouabain [12]. Moreover, in ENaCa(-/-) mice Na\(^+\) transport was found to be defective, resulting in fatal respiratory distress of the neonates [13]. Furthermore, it has been well established that up-regulation of ENaC and Na,K-ATPase increases active Na\(^+\) transport, leading to increased ability of the lungs to clear edema [14, 15].

**Na,K-ATPase**

While Na\(^+\) uptake is driven by several Na\(^+\) transport mechanisms (apically located amiloride-sensitive and -insensitive sodium channels and cotransporters of Na\(^+\)) in the alveolar epithelium (and notably in many other epithelia as well), the only transporter by which alveolar epithelial cells actively extrude sodium out of the cell is the Na,K-ATPase [4].

The Na,K-ATPase is a transmembrane protein expressed on the basolateral surface of most mammalian epithelial cells. In an ATP-consuming process, it maintains gradients of Na\(^+\) and K\(^+\) across the plasma membrane by pumping three sodium ions out of the cell in exchange for two ions of potassium into the cell, accounting for up to 40% of a cell’s energy expenditure [10]. The Na,K-ATPase is a heterodimeric holoenzyme composed of an α- and a β-subunit. The catalytic α-subunit is a large polypeptide comprising approximately 1,000 amino acid residues, spanning the plasma membrane 10 times. This subunit contains sites for cleavage of high-energy phosphate bonds, binding of Na\(^+\), K\(^+\) and nucleotides, and binding of the classic Na,K-ATPase inhibitor, ouabain [16]. Four α-isoforms have been identified (α\(_1\) – α\(_4\)), each with a unique tissue distribution [17, 18]. The α\(_1\) and α\(_2\) Na,K-ATPase subunits are expressed in the alveolar epithelium [19–21]. The glycosylated β-subunit, a smaller polypeptide comprising approximately 370 amino acid residues, has a single membrane-spanning domain and regulates the membrane insertion and activity of the enzyme [16, 22]. Four β-isoforms have been reported (β\(_1\) – β\(_4\)), which are also expressed in a tissue- and cell-specific manner [23]. Recently a small, transmembrane protein of the FXYD family (characterized by the short invariant sequence of phenylalanine-X-tyrosine-aspartate) has been identified as a γ-subunit, which associates with the Na,K-ATPase in

**Fig. 1** Schematic representation of the alveolo-capillary barrier and the channels and transporters involved in ion and fluid transport through the alveolar type I (ATI) and alveolar type II (ATII) cells.
some tissues [24]. Though it may have an important role in regulating the affinity of the sodium pump for ATP, recent evidence suggests that this γ-subunit is not an integral part of the holoenzyme complex [25].

### Role of Na,K-ATPase in epithelial barrier integrity

The Na,K-ATPase is known for its function in maintaining a transepithelial osmotic gradient, secondary to the establishment of vectorial sodium transport, and its role in the clearance of lung edema fluid [11]. However, it is less widely recognized for its critical role in epithelial barrier function. In fact, Na,K-ATPase expression and function is intimately coupled to that of junctional complexes, including tight junctions [26]. Through the action of tight junctions, alveolar epithelial cells form a barrier, restricting the passage of lipid-insoluble molecules from the interstitial to the alveolar space (and vice versa). Disruption of this barrier results in altered lung permeability, accumulation of pulmonary edema, and impaired gas exchange [27]. Therefore, through its role in barrier integrity, the Na,K-ATPase is crucial for the maintenance of a selectively permeable epithelium and for the prevention of lung edema formation.

### Structure and function of tight junctions

The relative impermeability of alveolar epithelia to paracellular solute diffusion depends upon tight junctions. These tight junctions have a dual role in regulating the passage of molecules between alveolar and interstitial spaces, and in separating the apical and basolateral surfaces of the epithelium, allowing the cells to polarize [26–28].

Tight junctions form intimate intercellular contacts, which are best viewed by electron microscopy [29]. These tight junctions are comprised of three integral membrane protein types: occludins, claudins, and junctional adhesion molecules [30, 31]. These protein complexes associate with cytoplasmic plaque proteins, zona occludens (ZO-1), which in turn bind cytoplasmic signaling molecules and the actin cytoskeleton [31, 32]. Effective tight junction assembly allows for the polarized phenotype exhibited by normal epithelial cells.

### Regulation of tight junction integrity by Na,K-ATPase

Rajasekaran and others demonstrated an important role for the Na,K-ATPase β1-subunit in the establishment of functional tight junctions and epithelial polarity [26]. Maloney sarcoma virus-transformed Madin–Darby canine kidney (MDCK) cells, which express low levels of E-cadherin (a transmembrane protein central to adherens junctions, thus representing the main mediator of intercellular adhesion) and Na,K-ATPase β1-subunit, were used in their experiments. These cells do not form effective tight junctions, and therefore have lost the polarized phenotype. However, reconstitution of both E-cadherin and β1-subunit induced the formation of tight junctions as well as epithelial polarization. In contrast, overexpression of E-cadherin alone failed to do so, though it did restore the presence of tight junction proteins in the plasma membrane [26]. This finding implicated the Na,K-ATPase in barrier function, separate from its role in vectorial sodium transport.

Effects of Na,K-ATPase on tight junction formation were shown to correlate with the absolute concentration of Na+ in the cell, rather than with the Na+ gradient across the plasma membrane. Removal of extracellular Na+, though it did disturb the transmembrane gradient, did not prevent formation of tight junctions or the establishment of polarity [33]. Therefore, aspects of cell function which depend on a transmembrane Na+ gradient, such as cytoplasmic pH or calcium concentration, are not likely to be involved in Na,K-ATPase regulation of tight junctions. Alternative signaling mechanisms as a modulator of barrier function have been suggested, including normal intracellular Na+ homeostasis, a decrease in cell K+ concentration, depolarization of the plasma membrane potential, and small cell volume changes [32]. Recently, signaling pathways involved in this tight junction regulation have been described. For example, a role for RhoA GTPase, a Ras-related small GTP-binding protein, in the formation of functional tight junctions [29], where overexpression of RhoA GTPase partially interrupted the negative effect of Na,K-ATPase inhibition on tight junction integrity. Conversely, endogenous RhoA activity was found to be significantly decreased in Na,K-ATPase-inhibited cells, suggesting that RhoA GTPase acts downstream of Na,K-ATPase to regulate tight junction formation [33].

A role for RhoA in regulating the assembly of filamentous actin in intestinal epithelial cells, which subsequently influences the integrity of tight junctions, has also been implicated [29]. Transient formation of these bundled stress fibers in MDCK cells correlates with tight junction assembly. Inhibition of Na,K-ATPase activity with ouabain, K+ depletion, or gramicidin prevented the formation of bundled stress fibers [33]. Taken together, these studies suggest that Na,K-ATPase interacts with RhoA GTPase to affect actin polymerization (i.e. the formation of stress fibers), which in turn influences tight junction formation.

### Molecular mechanisms of impaired alveolar edema clearance in ALI

Barrier integrity of the alveolar epithelium, created in large part by tight junctions, is not only required to prevent the formation of pulmonary edema; it is also essential for fluid clearance. Creation of a sodium gradient by vectorial trans-
port (which then allows for passive flux of water across the epithelium) depends upon the integrity of tight junctions, which separate the alveolar and interstitial spaces and form a fence between apical and basolateral domains, allowing for the asymmetric distribution of Na\(^+\) channels and sodium Na\(^+\) pumps to the apical and basolateral membranes, respectively [34].

Effective alveolar fluid reabsorption is impaired in the majority of patients with ALI/ARDS, in part as a consequence of disrupted alveolo-capillary barrier integrity, and is associated with worse clinical outcomes [2]. Interestingly, fluid reabsorption is often altered in models of lung injury, even in the absence of a gross barrier leak [35–39].

Effects of hypoxia on alveolar edema clearance

Alveolar hypoxia commonly develops during ALI/ARDS, when damage to the alveolar-capillary barrier results in airspace flooding. This edema impairs normal oxygen transfer from the alveolar airspaces into the circulation [40]. It is well established that hypoxia significantly reduces active Na\(^+\) transport across the alveolar epithelium [41–43], in a process that is reversible by β-adrenergic stimulation of the Na,K-ATPase [35, 44].

Mechanisms of hypoxia-induced inhibition of Na\(^+\) transport depend on the duration and severity of the hypoxic exposure. Transepithelial Na\(^+\) transport is inhibited in both A549 human epithelial adenocarcinoma cells and in primary rat epithelial ATII cells upon exposure to hypoxia (3% O\(_2\)) [43]. Short-term exposure of alveolar epithelial cells (as early as 15 min) to severe hypoxia (1.5% O\(_2\)) induced a time-dependent decrease in the number of Na,K-ATPase molecules at the plasma membrane of alveolar epithelial cells. Hypoxia caused a rapid increase in levels of mitochondrial reactive oxygen species (ROS), leading to activation and translocation of PKC-ζ from cytosolic to membrane compartments of the cells. PKC-ζ directly phosphorylated the Na,K-ATPase α1-subunit at the Ser-18 residue, thereby promoting Na,K-ATPase endocytosis [45]. In contrast, long-term exposure to severe hypoxia down-regulated both ENaC and Na,K-ATPase at both mRNA and protein levels [43, 46]. However, a recent report suggested that prolonged exposure of alveolar epithelial cells to severe hypoxia (1.5% O\(_2\)) resulted in preferential degradation of the plasma membrane Na,K-ATPase, via the ubiquitin-conjugating system. This degradation led to decreases in Na\(^+\) pump activity and oxygen consumption [47]. Plasma membrane-associated Na,K-ATPase, which represents the active, ATP-consuming Na\(^+\) pump population, was most vulnerable to hypoxic effects. In contrast, intracellular pools, representing an inactive form of the Na,K-ATPase, required 24–30 h exposure to hypoxia before degradation was observed. These findings suggested that cellular adaptation to hypoxia involves decreased ATP consumption, particularly by conserving ATP utilization by the Na,K-ATPase. More detailed reviews on hypoxia-mediated effects of alveolar fluid reabsorption are available [27, 48, 49].

Effects of coagulation on alveolar fluid reabsorption

Coagulation is an emerging area of interest in the pathogenesis of ALI/ARDS. In particular, concentrations of the edemagenic coagulation factor thrombin are elevated in plasma and broncho-alveolar lavage fluids of patients with ALI/ARDS [50]. Elevated levels of thrombin in the pulmonary circulation significantly reduced active Na\(^+\) transport and fluid clearance in the lung. Thrombin decreased Na,K-ATPase activity by promoting its endocytosis from the plasma membrane, which interestingly, just as in hypoxic exposure, was mediated by ROS and PKC-ζ [51], suggesting a central role for these signaling pathways in the regulation of edema clearance. Understanding the role of signaling induced by elements of the enhanced coagulation cascade in ALI/ARDS has yielded, and will likely continue to yield, new therapeutic approaches, including antithrombin III, thrombomodulin, heparin, activated protein C and tissue factor pathway inhibitor. More information regarding these therapeutic approaches is provided in the accompanying review by Morty et al.

Ventilator-induced lung injury

Morbidity and mortality associated with respiratory failure is in part iatrogenic. While mechanical ventilation must be used to support severely ill patients with ALI/ARDS, deleterious physiologic and morphologic alterations may occur as a consequence of mechanical ventilation with high tidal volumes [52]. The resulting alveolo-capillary barrier damage, altered lung permeability, and pulmonary edema are defined as ventilator-induced lung injury (VILI) [52]. However, VILI not only increases lung permeability to small and large solutes, but also decreases Na,K-ATPase activity and active Na\(^+\) transport, and therefore lung edema clearance [53]. Furthermore, Frank and coworkers demonstrated that ventilation with high tidal volumes for 2 h resulted in inhibition of cAMP-dependent alveolar fluid reabsorption, through activation of nitric oxide synthase 2 and increased formation of reactive nitrogen species [54]. Thus ventilation with high tidal volume not only promotes the formation of alveolar edema but also impairs its resolution.

While hypoxia, coagulation defects, and VILI have been included as insults relevant to patients with ALI/ARDS, additional pathophysiologic perturbations resulting in altered Na,K-ATPase function in ALI are numerous and may represent potential therapeutic targets as the signaling pathways become elucidated. These
Mechanisms that lead to endocytosis of Na,K-ATPase pump [60]. Thus, mechanisms that phosphorylate intermediate proteins, such as α- and β-subunits of Na,K-ATPase, induced lung injury and the Na,K-ATPase pump activity and thus alveolar fluid clearance during acute lung injury. These pathways include, though they are certainly not limited to, alveolar hypoxia (O$_2$↓), elevated levels of the coagulation factor thrombin (THR), ventilator-induced lung injury (VILI), hypoxic alkalosis (CO$_2$↑), hypercapnia (CO$_2$↑) and ischemia/reperfusion injury (I/R)

Fig. 2 Mechanisms that lead to endocytosis of Na,K-ATPase from the cell surface to intracellular compartments alter sodium pump activity and thus alveolar fluid clearance in acute lung injury [70, 71]. Treatment of alveolar epithelial cells with dopamine for only minutes results in increased Na,K-ATPase activity [66]. The signaling mechanisms in this post-transcriptional regulation of Na,K-ATPase translation, which involves activation of the protein S6 kinase (p70$^S6k$) enzymatic complex [68]. Thus, both short- and long-term β-adrenergic stimulation up-regulate Na,K-ATPase function, representing a potential therapy in the resolution of pulmonary edema.

Dopamine-mediated regulation of Na,K-ATPase

Dopamine increases fluid reabsorption by up-regulating both the apical Na$^+$ channels [69] and Na,K-ATPase in the alveolar epithelium [70, 71]. Treatment of alveolar epithelial cells with dopamine for only minutes results in translocation of Na,K-ATPase from intracellular pools to the basolateral membrane of the cell. This short-term effect is mediated by D$_1$ receptors and activation of protein phosphatase 2A (PP2A), a serine/threonine protein phosphatase that plays an important role in the trafficking of transporters [72]. PP2A was suggested to be involved in dephosphorylation of the sodium pump, an initial signal in its trafficking [73]. Recently, it has been reported that PP2A directly interacts with the N-terminus of the Na,K-ATPase α$_1$-subunit, leading to its dephosphorylation at the Ser-18 residue and its recruitment to the plasma membrane from intracellular compartments in alveolar epithelial cells [74]. It has also been shown that PKC-δ and PKC-ε were essential for dopamine-induced Na,K-ATPase exocytosis and increased activity [75]. These PKCs are differentially compartmentalized, and activation of PKC-δ precedes that of PKC-ε. Whether PKC-δ and -ε phosphorylate intermediate proteins, such as actin or other adapter proteins, that subsequently regulate Na,K-ATPase function in the lung remains unresolved.
Signaling pathways resulting in recruitment of Na,K-ATPase molecules from intracellular pools to the basolateral membrane of the alveolar epithelium enhance Na,K-ATPase function and thus alveolar edema clearance and might represent new therapeutic approaches in acute lung injury. In particular, β-adrenergic receptor (β-AR) agonists, dopamine, mineralocorticoids and growth factors have been implicated.

The MAPK/ERK cascade is an important signaling system by which cells transduce extracellular signals into intracellular responses. Activation of ERK classically involves ligand binding to a receptor tyrosine kinase, although ERK proteins can also be activated by G protein-coupled receptors, such as the dopaminergic receptors. Treatment of alveolar epithelial cells with dopamine for long periods of time (24 h) activated the MAPK pathway via D2 receptors, resulting in increased Na,K-ATPase activity. This effect is mediated by Ras, which associates with the serine/threonine kinase Raf-1. Raf-1 subsequently phosphorylates and activates the downstream kinase MAPK kinase MEK1, the direct activator of ERK proteins [76]. This dopamine-induced activation of ERK leads to a significant increases in Na,K-ATPase β1-subunit mRNA and protein levels and thus increases abundance of functional Na,K-ATPase molecules in the plasma membrane [77]. Therefore, application of dopamine (both short- and long-term) stimulates Na,K-ATPase function and appears to be beneficial in the resolution of alveolar edema.

Corticosteroids and growth factors in Na,K-ATPase regulation

Since inflammation is one of the hallmarks of ALI/ARDS, corticosteroids have been proposed as a potential anti-inflammatory therapeutic modality. Furthermore, it has been reported that a single dexamethasone injection increased alveolar fluid reabsorption in rats by 48 h [78]. The Na,K-ATPase has been shown to be modulated by corticosteroids in different organs. Dexamethasone increased Na,K-ATPase β1 mRNA transcript levels after 6 h of incubation, resulting in elevated Na,K-ATPase protein abundance in alveolar epithelial cells and β- and γ-ENaC mRNA and protein levels without affecting the expression of the α-subunit of the channel [79–81].

As ALI/ARDS is often associated with alveolar epithelial cell death, potential new therapeutic approaches to restore the alveolo-capillary barrier integrity focused on growth factors. Exposing alveolar epithelial monolayers to keratinocyte growth factor (KGF) resulted in a sustained increase of active transcellular Na+ transport [82]. Similarly, epidermal growth factor (EGF) also increased lung liquid clearance [83]. While EGF increased both Na,K-ATPase α1- and β1-subunit mRNA and protein levels it did not enhance ENaC expression [84], but induced non-selective cation channels in ATII cells [85]. Fibroblast growth factor-10 (FGF-10), a protein structurally similar to KGF, is also a potent mitogen that promotes epithelial cell differentiation and wound healing [86]. Treatment of ATII cells with FGF-10 increased Na,K-ATPase activity and membrane protein abundance within 30 min without changing the Na,K-ATPase α1-subunit protein levels in total cell lysates (in contrast to the long-term effects of KGF or EGF), thus demonstrating recruitment of Na,K-ATPase from intracellular pools [87]. FGF-10 effects were prevented by the MAPK kinase inhibitor U0126, suggesting that the FGF-10-induced activation of the Na+ pump was mediated by ERK-1/2 [87]. Thus, growth factors not only facilitate re-epithelialization of the alveolar barrier but may also have beneficial effects in edema resolution. A schematic representation of mechanisms up-regulating cell surface abundance of Na,K-ATPase, and thus alveolar edema clearance, is depicted in Fig. 3.

Gene therapy to overexpress Na,K-ATPase increases alveolar edema clearance

In normal adult rats, overexpression of the β1-subunit gene by utilizing a replication-incompetent human type 5 adenovirus expressing Na,K-ATPase β1-subunit cDNA increased alveolar edema clearance over twofold compared with controls [14]. Similarly, gene transfer of the Na,K-ATPase β1-subunit using electroporation increased alveolar fluid reabsorption [88]. Furthermore, while rats exposed to 100% oxygen develop ALI and impaired alveolar fluid clearance; overexpression of the Na,K-ATPase β1-subunit in the alveolar epithelium of rats increased lung liquid clearance and, most importantly, overexpression of the Na,K-ATPase β1-subunit resulted in 100% survival over 14 days of hyperoxia (compared with 25–31% survival in the non-treated or null virus-treated control groups) [55]. Also, overexpression of the β2-adrenergic receptor leads to increased alveolar fluid clearance in rats by increasing both membrane-bound amiloride-sensitive Na+ channel expression and Na,K-ATPase function, probably by enhancing responsiveness to endogenous catecholamines in the alveolar epithelium [89].
Since oxidative stress has been accredited with a key role in hypoxemic respiratory failure, antioxidant therapy can have a role in the management of ALI/ARDS. Most recently, it has been reported that adenoviral overexpression of the ROS scavenger superoxide dismutase might be protective and prevents alteration in alveolar fluid reabsorption in rats exposed to prolonged hypoxia [35].

Summary

Acute lung injury is characterized by the disruption of the alveolar epithelial barrier, which results in increased edema formation, as well as impaired edema clearance. Impairment of Na,K-ATPase function appears to be a hallmark during lung injury even in a preclinical stage. Therefore, elucidation of the mechanisms by which lung injury alters Na,K-ATPase activity may provide novel therapeutic targets and warrants further studies.

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