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Research Article

Sodium Transport and Alveolar Fluid Clearance in Acute Lung Injury

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Abstract

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) are characterised by pulmonary oedema results from increased vascular permeability. The resolution of pulmonary oedema and ALI depends upon intact Alveolar Fluid Clearance (AFC). Sodium transport Across Alveolar Epithelial Cells (AECs) leads to osmotic alveolar water transport and plays a dominant role in AFC. Sodium transports via apical sodium channels, mainly the Epithelial Sodium Channel (ENaC) and basolateral Sodium-Potassium Adenosine Triphosphatase (Na^+, K^+-ATPase) in the AEC membrane. In ALI/ARDS, the imbalance of oxygen, Reactive Nitrogen and Oxygen Species (RNS and ROS, respectively), and Tumour Necrosis Factor-α (TNF-α) lead to a decrease in AFC, due in part to the down regulation of ENaC and Na^+, K^+-ATPase in the alveolar epithelium.

In ALI, hypoxia inhibits ENaC and Na^+, K^+-ATPase activity through different mechanisms. The definite mechanism of ENaC and Na^+, K^+-ATPase activity regulation by RNS and ROS is unclear. TNF-α, and its lectin-like domain (designated TIP) differentially impact sodium transport across the alveolar epithelium.

In this review, we will discuss the regulatory mechanisms of alveolar sodium transport and AFC for the development of effective therapeutic strategies for ALI/ARDS patients.

Keywords: Epithelial sodium channel; Hypoxia, Reactive nitrogen species; Reactive oxygen species; Sodium-Potassium adenosine triphosphatase; Tumour necrosis factor-α

Abbreviations

AEC: Alveolar Epithelial Cell; AFC: Alveolar Fluid Clearance; ALI: Acute Lung Injury; AMPK: Adenosine Monophosphate-activated Protein Kinase; AP-2: Adaptor Protein-2; ARDS: Acute Respiratory Distress Syndrome; AT I cell: Alveolar Type I cell; AT II cell: Alveolar Type II cell; ATP: Adenosine Triphosphate; cAMP: cyclic AMP; CFTR: Cystic Fibrosis Transmembrane conductance Regulator; cldn: Claudin; Cl⁻: Chlorine; CNG cation channel: Cyclic Nucleotide-Gated cation channel; CRAC channel: Calcium Release-Activated Calcium channel; DEX: dexamethasone; ENaC: Epithelial Sodium Channel; FDLE cell: Foetal Distal Lung Epithelial Cell; GC: Glucocorticoid; GR: GC Receptor; HSC: Highly Selective Cation channel; iNOS: inducible NO Synthase; mROS: mitochondrial Reactive Oxygen Species; NO: Nitric Oxide; Na^+: Sodium; Na^+, K^+-ATPase: Sodium-Potassium Adenosine Triphosphatase; NSC: Nonselective Cation channel; PLC: Phospholipase C; PKCζ: Protein Kinase C; PKA: Protein Kinase A; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; SGK1: Serum and Glucocorticoid-inducible Kinase 1; TJ: Tight Junction; TNF-α: Tumour Necrosis Factor-α; ZO-1: Zonula Occludens-1; ZO-2: Zonula Occludens-2; α-ENaC: ENaC α-subunit; $α_1$-Na^+, K^+-ATPase: $α_1$-isoform of Na^+, K^+-ATPase; $β_2$AR: $β_2$-Adrenergic Receptor

Introduction

ALI and ARDS are life-threatening diseases and remain a significant burden of morbidity and mortality. In the early stages, ALI/ARDS is pathologically characterised by pulmonary oedema results from increased vascular permeability. A higher survival rate for patients with ALI/ARDS is related to maximal AFC, but the majority of patients have impaired AFC [1]. In ALI/ARDS, impaired AFC is mainly due to the down regulation of active sodium (Na^+) transport. In the pulmonary alveoli, the regulation of the fluid balance depends on the active transport of Na^+, via osmotically driven water movement across the alveolar epithelium. In vivo, this active Na^+ transport supports the foetal and adult reabsorption of alveolar fluid, especially when alveolar permeability to plasma proteins is increased [2]. In Alveolar Epithelial Cells (AEC), the major functional proteins that contribute to Na^+ transport involve apical ENaC and basolateral Na^+, K^+-ATPase in the plasma membrane. During ALI/ARDS, changes in oxygen, RNS and ROS, and TNF-α levels result in ENaC and Na^+, K^+-ATPase dysfunction, leading to decreased alveolar Na^+ and fluid transport. However, the mechanisms from this down regulation are not completely understood. The recent advances on this topic are reviewed here.

Alveolar sodium and fluid transport

Alveolar fluid transport is regulated by the transport of ions, such as Na^+ and Cl⁻, across the alveolar epithelium. This epithelium is composed of squamous Alveolar Type I (AT I) and cuboidal Alveolar Type II (AT II) cells, which line >95% and <5% of the alveolar surface area, respectively [3]. The previous paradigm was that Na^+ and Cl⁻ are mainly transported by AT II cells. As less is known about AT I cells, they were thought to form only a passive barrier for water movement [3,4]. However, the current paradigm is that AT I cells also have a

full complement of ion channels (Figure 1), including the ENaC, Cyclic Nucleotide-Gated (CNG) cation channel and Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), and take part in active ion transport [3,4]. Because AT I cells line most of the surface area in alveoli, they may have a significant potential in alveolar Na$^+$ transport and fluid balance. Expression of ENaC and Na$^+$, K$^+$-ATPase, the main active Na$^+$ transporters, were identified in both AT I and AT II cells *in vitro* and *in situ* [5] and contribute to alveolar transepithelial Na$^+$ transport and AFC.
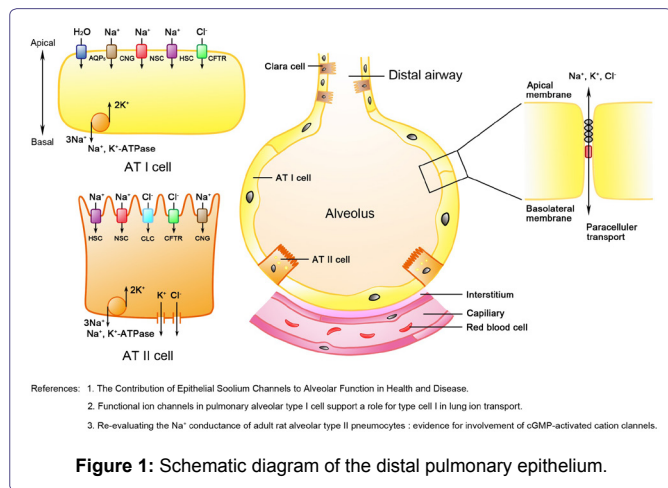

Figure 1: Schematic diagram of the distal pulmonary epithelium.

The alveolar epithelium is composed of squamous Alveolar Type I (AT I) and cuboidal Alveolar Type II (AT II) cells. Both AT I and AT II cells contain two forms of amiloride-sensitive epithelial sodium channels, including the Highly Selective Cation channel (HSC) and Nonselective Cation channel (NSC) as well as the Cyclic Nucleotide-Gated (CNG) cation channel and sodium-potassium adenosine triphosphatase (Na$^+$, K$^+$-ATPase), which are involved in alveolar transepithelial sodium transport. In addition, AT I cells have aquaporin 5, which contributes to either water or gas exchange. AT II cells have the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) and Chlorine (Cl$^-$) channels, members of the CLC family of proteins, which mediate apical Cl$^-$ transport. The tight junctions (a chain in grey between Alveolar Epithelial Cells (AECs)) and adherens junctions (in red between AECs) between adjacent alveolar epithelial cells provide a physical barrier from paracellular solute transport [4,6,7].

Intercellular junctions between adjacent alveolar epithelial cells, such as Tight Junctions (TJs) and adherens junctions, provide a physical barrier for paracellular solute transport. TJ plays a more notable role in regulating paracellular solute transport. The proteins that compose the core TJ protein complex, including tetraspan transmembrane Claudin (cldn), occludin, and cytoplasmic scaffold proteins Zonula Occludens-1 (ZO-1) and ZO-2 [8], intersect one another in a coordinated manner and create junctional proteins strands, which surround the cells like a ring and divide the epithelium into apical and basolateral surfaces. Other proteins, such as cytoskeletal protein actin, are also known components of the TJ. Of these TJ proteins, recent studies have focused more on the cldn family because TJ regulation of paracellular ion transport is directly mediated by the cldn family, which includes nearly two dozen cldns [8]. The distribution of cldns is tissue-specific. In AECs (Figure 2), cldn-18.1 is abundantly expressed by AT I cells, cldn-3 is predominantly expressed by AT II cells, and cldn-4 is subdominantly expressed by AT I and AT II cells [9]. The function of

these three claudins in the alveolar epithelium, especially cldn-3 and cldn-18.1, has not been fully explored. Cldn-4 is specifically upregulated in ALI [10] and increased cldn-4 expression may related to the resolution of pulmonary oedema [9]. Elevated cldn-4 expression can increase alveolar epithelial transepithelial electrical resistance in primary rat AECs [10], while inhibiting cldn-4 function has been shown to lead to decreased transepithelial electrical resistance and air space fluid clearance, thus impacting paracellular transport, AFC and sensitivity to pulmonary oedema in a ventilator-induced lung injury model [11]. Changes in cldn-18 expression in ALI also can be seen. However, the regulation of cldn-4 and cldn-18 is in opposite directions. In experimental bleomycin-induced lung injury, the level of mRNAs encoding tight junction proteins, especially claudin-18, were demonstrated to be down regulated [12]. In addition, increased cldn-3 expression was also shown to decrease paracellular permeability, which was evaluated by transepithelial electrical resistance and dye flux measurements, but cldn-3 regulation in ALI is still not clearly understood.
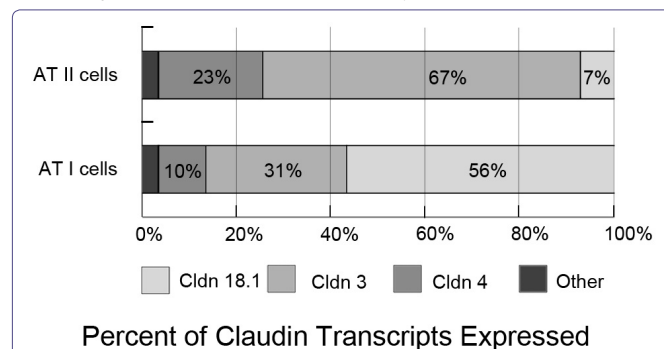
Reference: Keratinocyte growth factor enhances barrier function without altering claudin expression in primary alveolar epithelial cells.

Figure 2: Claudin mRNA expression profiles for Alveolar Type I (AT I) and Alveolar Type II (AT II) cells.

Claudins-3, -4, and -18 are the primary claudins expressed in freshly isolated AT I and AT II cells (fluorescence-activated, cell-sorted). Cldn-18.1 is the major transcript in AT I cells, while cldn-3 is predominantly expressed by AT II cells. Both cell types express cldn-4. [9].

Pulmonary oedema reabsorption depends on active Na$^+$ transport across the alveolar epithelium [13,14]. Primary Na$^+$ transport via the apical surface of AEC, mainly through ENaC and an amiloride-insensitive pathway, and is subsequently pumped out by Na$^+$, K$^+$-ATPase from the basolateral surface into the lung interstitium to form the transepithelial osmotic gradient. This osmotic gradient drives water movement from the alveolar spaces into the alveolar interstitium [13,14].

Sodium reabsorption mediated by ENaC can be inhibited by amiloride, a pyrazine compound. Single-channel studies have shown that at least two forms of amiloride-sensitive ENaC are present in the apical membrane of AT I, AT II (Figure 1) and Foetal Distal Lung Epithelial (FDLE) cells [2,4,6] : the highly selective cation channel (HSC) and Nonselective Cation channel (NSC). These two channels differ in selectivity for Na$^+$ over K$^+$ (HSC with a Na$^+$/K$^+$ selectivity >40; NSC with a Na$^+$/K$^+$ selectivity of ~1.4), unitary conductance (HSC=4-6 pS and NSC=19-24 pS) and other biophysical characteristics [6].

ENaC is composed of three homologous subunits: α, β, and γ [15], but the total number of subunits for functional channels are uncertain and vary from four (two α-, one β-, and one γ-subunit)

subunits assembled as a tetramer to nine (three α-, three β-, and three γ-) subunits to create a much larger complex [16]. Edith Hummler and colleagues [17] observed that transgenic neonate mice without the ENaC α-subunit (α-ENaC) die immediately after birth due to impaired lung fluid clearance. Nadia Randrianarison and colleagues [18] have reported that disrupting the β-ENaC gene locus in mature mice lead to low expression levels of β-ENaC mRNA and a compensatory increase in α-ENaC and γ-ENaC protein expression, and yet a moderate impairment in baseline AFC was seen. Moreover, sufficient expression of β-ENaC may be essential to β_2-agonist stimulation of AFC. Pierre M. Barker and colleagues [19] discovered that newborn mice without γ-ENaC had a decreased lung liquid clearance rate. These data indicate that the three ENaC subunits are all indispensable for maximal AFC.

The phenomenon that part of lung liquid resorption could not be inhibited by amiloride was discovered in the lungs of several species including sheep, rabbits, guinea pigs, rats, and humans [16]. As liquid resorption follows the transepithelial osmotic gradient produced by active Na^+ transport, there might be an amiloride-insensitive pathway for Na^+ transport across the alveolar epithelium. Subsequent studies have indicated that the amiloride-insensitive channel may be a member of CNG cation channel family. CNG cation channels are gated by cAMP or cGMP but not voltage [20,21]. The amiloride-insensitive fraction of Na^+ transport-dependent AFC may be partially mediated by CNG cation channels [14]. Ion channels that are directly activated by cyclic nucleotides were first found in the plasma membrane of retinal photoreceptors [21], and now CNG channels have been discovered not only in photoreceptors and olfactory sensory neurons but also in other neurons and non-neuronal tissues, including airway epithelial cells and endothelial cells of the pulmonary artery [22]. CNG cation channels are functionally expressed in adult AT II cells [7], and one of the CNG cation channel isoforms CNGA1 is mainly expressed in rat AT I cells [20]. Activating CNG cation channels with a cGMP analogue, 8Br-cGMP (100 μM), resulted in activated whole-cell cation conductance in isolated rat AT II cells and increased lung liquid clearance in situ. Both effects were still apparent in the presence of amiloride [7,20]. Thus, CNG cation channels are activated by cGMP and may contribute to at least part of the amiloride-insensitive fraction of lung liquid clearance. Although less is known about AFC mediated by CNG cation channels, the potential role of CNG cation channels in lung liquid clearance is worth studying for novel therapeutic strategies for pulmonary oedema.

Na^+, K^+-ATPase is essential to transepithelial active Na^+ transport and exists in the basolateral cell membrane. Na^+, K^+-ATPase pumps three sodium ions out of the cell and exchanges two potassium ions into the cell [23]. This process consumes 20-30% of Adenosine Tri Phosphate (ATP) at rest to form Na^+ and K^+ gradients across the cell membrane [24]. The Na^+ and K^+ gradients are essential for maintaining membrane potentials, cell volume and the active transport of other solutes [24]. Four isoforms of the α-subunit and five isoforms of the β-subunit of Na^+, K^+-ATPase have been identified [13]. The minimal functional Na^+, K^+-ATPase is a heterodimer of a single α- and β-subunit. The α-subunit hydrolyses ATP and provides binding sites for cations. The β-subunit is necessary for the stability and trafficking of this combination [23]. The α_1-, α_2-, β_1-isoform of Na^+, K^+-ATPase (α_1-, α_2-, β_1-Na^+, K^+-ATPase) has been discovered in lung epithelial cells [25]. Additionally, α_1-Na^+, K^+-ATPase has been reported to be expressed by AT II cells, α_1- and α_2-Na^+, K^+-ATPase can be found on AT I cells [26]. Moreover, α_2-Na^+, K^+-ATPase in AT I cells

mediates most of the active Na^+ transport and basal lung liquid reabsorption seen in isolated, ouabain-perfused rat lungs [26]. The activity of α_1- or α_2-Na^+, K^+-ATPase in these isolated lungs can be selectively inhibited by ouabain at a specific concentration, and the selective inhibitions were used to determine the differential contribution of α_1- or α_2-Na^+, K^+-ATPase to AFC ouabain ouabain [26]. In transgenic mice, a 50% decrease in protein expression of either α_1- or α_2 -Na^+, K^+-ATPase does not affect basal or stimulated AFC, while a 50% protein loss in both α_1 and α_2 -Na^+, K^+-ATPase produces a submaximal cAMP-stimulated AFC without affecting basal AFC [27]. These results indicate that α_1- and α_2-Na^+, K^+-ATPase regulate cAMP-stimulated AFC in a coordinated manner.

Regulation of alveolar sodium and fluid transport during ALI

Alveolar fluid secretion and reabsorption are precisely balanced by several regulatory mechanisms, including active Na^+ transport. During ALI/ARDS, a variety of agents or changes, including hypoxia, RNS and ROS, and TNF-**α**, influence the activity or expression of ENaC and Na^+, K^+-ATPase, alveolar Na^+ transport and AFC. In addition, an influenza viral infection has been reported to be able to influence lung liquid clearance. The influenza virus strain A/PR/8/34 reduced the open probability of single ENaC channels in apical cell-attached patches and rapidly inhibited amiloride-sensitive lung fluid transport *in vivo*. As inhibitors of Phospholipase C (PLC) and cytosolic tyrosine kinase Src could block the inhibition of ENaC by the influenza virus, this inhibition might be mediated via PLC-induced activation of PKC [28]. We focused on the secondary changes in the course of ALI, which play a regulatory role in alveolar Na^+ fluid transport. The detailed mechanisms involved in these effects are described below.

Effects of hypoxia on alveolar sodium and fluid transport during ALI

The amount of alveolar fluid affects the thin liquid layer lining the alveolar epithelium and thus influences gas exchange. Patients with ALI/ARDS clinically manifest with hypoxaemia, due to increased oedema and inflammatory cellular infiltrates in the lungs. Meanwhile, alveolar hypoxia leads to decreased sodium-dependent oedema clearance, at least in part, by reducing the activity of amiloride-sensitive ENaC and Na^+, K^+-ATPase in the plasma membrane.

In hypoxic rat AT II cells, hypoxia reduces the transepithelial Na^+ current and activity of the amiloride-sensitive Na^+ channel, which is related to the hypoxia-induced decrease (quantified by biotinylation) in ENaC subunits, especially the β- and γ-subunits, in the apical membrane, but hypoxia does not decrease mRNA or protein expression of the ENaC subunits [29]. The β_2-agonist terbutaline reverses the hypoxia-induced down regulation of transepithelial Na^+ transport by stimulating Na^+ channel activity and increasing the insertion of ENaC subunits into the membrane of hypoxic AECs via cAMP stimulation [29]. In summary, hypoxia inhibits amiloride-sensitive Na^+ channel activity by decreasing the apical expression of ENaC subunits, and this inhibition can be reversed by a β_2-agonist. More importantly, terbutaline increases Na^+, Cl^- transport in AT II cells during normoxia and hypoxia [30]. However, in isolated rat AT II cells, hypoxia impairs β_2-Adrenergic Receptor (β_2AR) signalling. Although hypoxia decreases terbutaline-stimulated cAMP production and β_2AR density, the potency of terbutaline and

affinity between terbutaline and β_2AR are not impacted, and the decrease in cyclic AMP (cAMP) production can be reversed by reoxygenation [31]. β_2AR belongs to a family of G protein-coupled receptors, and the hypoxia-induced downregulation results from increased $G_{i/o}$ activity [31]. Subsequent research has indicated that prolonged hypoxia impairs β_2AR signalling in alveolar epithelia and whole lungs, but this impairment is no longer aggravated [32]. In addition, alveolar sodium transport under hypoxic conditions can be ameliorated by Dexamethasone (DEX) [33,34].

Cells react to hypoxia through adaptive mechanisms by increasing the expression of genes involved in angiogenesis and glycolytic pathways and maintaining cell ATP homeostasis [35]. As ALI/ARDS is clinically characterised by the abrupt onset of hypoxaemia, about half of patients with ALI/ARDS have died or have given up treatment in 7-10 days [36]. Decreasing the processes that consume ATP, such as Na^+, K^+-ATPase, is more important for compensation. The exposure of AECs to hypoxia leads to a time-dependent decrease in Na^+, K^+-ATPase activity via its endocytosis from the plasma membrane into intracellular compartments. Therefore, the amount of α_1-Na^+, K^+-ATPase in the basal cell membrane is decreased, but the total abundance of cellular protein is not [37]. Hypoxia increases the mitochondrial Reactive Oxygen Species (mROS) levels via a capable electron transport chain. Mitochondrial DNA-deficient (ρ^0) A549 cells in the absence of the cytochrome oxidase subunit II could not generate hypoxia-induced mROS production and Na^+, K^+-ATPase endocytosis and decreased Na^+, K^+-ATPase activity [37]. Meanwhile, antioxidants can inhibit this hypoxia-induced enhancement of mROS and downregulation of Na^+, K^+-ATPase [37]. Therefore, these results suggest that the endocytosis of Na^+, K^+-ATPase during hypoxia is mediated by mROS.

In rat AECs, hypoxia-generated mROS leads to the phosphorylation/activation of Adenosine Monophosphate-activated Protein Kinase (AMPK) at the Thr172 residue. The activated AMPK α subunit binds and directly phosphorylates Protein Kinase C (PKCζ) at the Thr410 within the PKCζ activation loop [38]. Moreover, phosphorylation of PKCζ at the Thr410 is required for the hypoxia-induced Na^+, K^+-ATPase endocytosis. Small interfering RNA knockdown of AMPK α_1 but not α_2 suggests that PKCζ is specifically activated by AMPK α_1 isoform [38]. Activated PKCζ in turn mediates the phosphorylation of Na^+, K^+-ATPase at Ser18 in the α_1-subunit and Na^+, K^+-ATPase endocytosis but without significant changes in total cell protein abundance [37]. Meanwhile, mROS increases the degradation of Na^+, K^+-ATPase, which is mediated by the ubiquitin-conjugating system [39]. The connection between the endocytosis and ubiquitination of Na^+, K^+-ATPase has been described. During hypoxia, ubiquitination of α_1-Na^+, K^+-ATPase has been discovered at the basolateral membrane of AEC and plays an important role in the endocytosis of Na^+, K^+-ATPase [40]. Both the endocytosis and ubiquitination of α_1-Na^+, K^+-ATPase are prevented when the Ser18 residue in the N-terminus of the α_1-subunit (PKCζ phosphorylation motif) is mutated to alanine, suggesting that Ser18 phosphorylation is necessary for these two processes [40]. In addition, mutation of the four lysine residues (K16, K17, K19, K20) adjacent to Ser18 to arginine inhibits the endocytosis and ubiquitination of Na^+, K^+-ATPase during hypoxia [40]. In conclusion (Figure 3), exposure of AECs to hypoxia leads to AMPK α_1 activation by mROS, leading to direct PKCζ phosphorylation at Thr410. Activated PKCζ phosphorylates Na^+, K^+-ATPase at Ser18 in the α_1 subunit, which

causes ubiquitination at the sequence KKSKK [four lysine residues (K16, K17, K19, K20) surrounding Ser 18] of α_1-Na^+, K^+-ATPase, leading Na^+, K^+-ATPase endocytosis. Subsequently, further studies on hypoxia-generated downregulation of Na^+, K^+-ATPase activity were performed. During hypoxia, mROS also leads to calcium entry through Calcium Release-Activated Calcium (CRAC) channels, which transport stores-operated calcium to replenish endoplasmic reticulum Ca^{2+} stores in nonexcitable cells [41]. This calcium entry induces Na^+, K^+-ATPase endocytosis and the phosphorylation/activation of AMPK via Calmodulin-dependent kinase β (CaMKKβ) [41]. Moreover, hypoxia-induced impairment of AFC is prevented when CRAC channel function is inhibited [41]. Thus, during hypoxia, the calcium entry via CRAC channels induces CaMKKβ/AMPK activation and Na^+, K^+-ATPase endocytosis. Additionally, mROS has been shown to possibly play an upstream role in calcium signalling; thus, the calcium flux mediated by CRAC channels may be an intermediary process between mROS-induced AMPK α_1 activation and CaMKKβ/AMPK activation. Moreover, in H441 human airway epithelial cells, AMPK activation with the AMP mimetic AICAR leads to the inhibition of amiloride-sensitive HSC and NSC, which is associated with a decreased channel open probability [42]. Recent research has shown that hypoxia also activates an AMPK-independent pathway in H441 cells, modification of AMPK avtivity by Lentiviral prevented the effect of hypoxia on Na^+, K^+-ATPase but not apical amiloride-sensitive Na^+ conductance, which different from the regulation of Na^+, K^+-ATPase and ENaC activity via the above AMPK-dependent pathway [43].
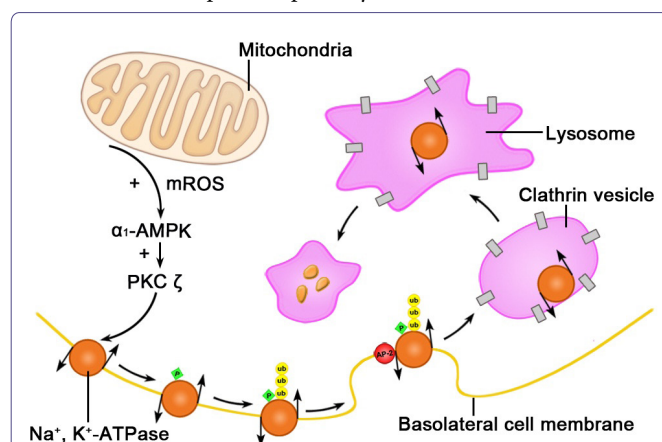

Figure 3: Hypoxia induces the endocytosis and degradation of basolateral membrane sodium-potassium adenosine triphosphatase (Na^+, K^+-ATPase) in alveolar epithelial cells exposed to hypoxia.

Hypoxia-generated mitochondrial reactive oxygen species activate the α subunit of adenosine monophosphate-activated protein kinase (α_1-AMPK). Activated α_1-AMPK increases protein kinase C activity, which phosphorylates basolateral membrane Na^+, K^+-ATPase, leading to its ubiquitination. This process leads to the recognition of Na^+, K^+-ATPase by the Adaptor Protein-2 (AP-2) and subsequent Na^+, K^+-ATPase endocytosis via clathrin-coated vesicles, which traffic Na^+, K^+-ATPase to lysosomes for degradation. [35,44,45].

In AECs, hypoxia-induced Na^+, K^+-ATPase endocytosis requires the binding of Adaptor Protein (AP)-2 to the tyrosine-based motif (Tyr-537) in the α_1-subunit of Na^+, K^+-ATPase, leading to the incorporation of Na^+, K^+-ATPase into clathrin-coated vesicles [44]. Subsequently, phosphorylated and ubiquitinated Na^+, K^+-ATPase is degraded in the lysosomes in AECs [45]. Hypoxia-induced degradation of Na^+, K^+-ATPase is prevented by lysosome and proteasome inhibitors [39].

In addition, mild and severe hypoxia has been shown to lead to a disruption in the cytoskeleton in primary rat AECs, including disorganisation of actin and α-spectrin; moreover, exposure of AECs to mild and severe hypoxia results in the mislocalisation of occludin from the tight junction to the cytoplasm and reduced ZO-1 protein expression [46]. The hypoxia-induced reduction in occludin abundance in the AEC plasma membrane is mediated by PKCζ and protein phosphatase 2A [47]. This downregulation may decrease alveolar Na⁺ transport through the paracellular pathway.

Regulation of alveolar sodium and fluid transport by RNS and ROS during ALI

Inflammatory mediators, which play a role in the pathophysiological changes in ALI, also induce the production of ROS by the membrane-bound enzyme complex NADPH oxidase and RNS through calcium-insensitive inducible NO Synthase (iNOS) in lung alveolar cells, endothelial cells, and airway epithelial cells, activated alveolar macrophages and neutrophils [16,48]. ROS and RNS lead to impaired Na⁺ transport across the alveolar epithelium, resulting in alveolar epithelial dysfunction.

Nitric Oxide (NO) modulates lung Na⁺ transport under both basal conditions and inflammation during ALI [49-53]. Both cGMP-dependent and -independent mechanisms are involved in the regulation of lung Na⁺ transport by NO. NO produced by iNOS under basal conditions is required to regulate amiloride-sensitive Na⁺ transport via ENaC in the lung epithelium [54,55] because iNOS knockout mice lack amiloride-sensitive Na⁺ transport across the alveolar and airway epithelia [54]. The specific iNOS inhibitor (1400W) injected intraperitoneally in C57BL/6 mice prevented Na⁺-dependent amiloride-sensitive AFC [55]. Endogenous NO under basal conditions regulates by the amount of α and γ-ENaC via post-transcriptional, cGMP-independent mechanisms [55]. However, in the H441 human bronchiolar epithelial cell line, using two NO donors, NO was demonstrated to reduce Na⁺ reabsorption by inhibiting the activity of HSC and Na⁺, K⁺-ATPase [53]. In AT II cells, using a NO donor, NO has been suggested to reduce the activity of a predominant apical Na⁺-permanent cation channel with a conductance of 20.6 ± 1.1 (SE) pS and Na⁺/K⁺ selectivity of 0.97 ± 0.07 (possible NSC), and this inhibition is mediated by a cGMP-dependent protein kinase [50]. NO reacts with superoxide to form other reactive species, leading to different effects on Na⁺ transport in lung epithelial cells. These effects may partially be responsible for the diverse effects of NO and NO donors on ENaC activity.

In vitro and *in vivo* results from H441 cells have indicated that PKGII in the NO/cGMP/PKG pathway activates ENaC [56]. Additionally, cGMP has been shown to activate either PKG I or PKGII in cells, and 8-pCPT-cGMP, a PKGII activator, increased amiloride-sensitive short-circuit current (I_{sc}) and whole-cell currents in H441 cells *in vitro*. These upregulations could be inhibited by Rp-8-pCPT-cGMP, a specific PKGII inhibitor. Consistently, 8-pCPT-cGMP improved amiloride-sensitive AFC *in vivo*. In addition, Na⁺/K⁺-ATPase downregulation by NO or peroxynitrite has also been discovered in the liver, kidneys and lungs [53,57,58].

Peroxynitrite anion (ONOO⁻), is a reactive oxidant produced from a reaction between NO and superoxide as a response to certain biomolecules, including proteins, lipids and DNA [59]. Physiological concentrations of peroxynitrite reduce AT II cell Na⁺ transport by impairing apical amiloride-sensitive Na⁺ channels [59].

In FDLEs, the switch from foetal (3%) to postnatal (21%) O₂ concentrations increases ENaC mRNA expression and amiloride-sensitive Na⁺ transport, and additionally induce Nuclear Factor-κB (NF-κB) [60]. NF-κB and AP-1 response elements have been found in the promoter regions of ENaC subunits [60]. There may be a connection between the upregulation of ENaC gene expression and the O₂-induced transcription factor NF-κB. Changes in oxygen concentration also lead to changes in ROS. Because the O₂-induced enhancement of ENaC gene expression can be inhibited by the cell-permeable superoxide scavenger tetramethylpiperidine-N-oxyl, the ROS superoxide has been suggested to participate in ENaC regulation [60]. *In vivo*, using two novel whole animal imaging approaches, ROS were shown to increase ENaC activity and activate lung fluid clearance [61]. Moreover, the balance between superoxide and NO may contribute to alveolar fluid homeostasis. Increasing endogenous superoxide levels using a superoxide dismutase inhibitor (Ethiolat) has been shown to prevent NO inhibition of ENaC activity, which was examined using a single channel patch clamp in AT II cells [62].

Regulation of alveolar sodium and fluid transport by TNF-α during ALI

TNF-**α**, a proinflammatory cytokine, plays an important role in ALI and ENaC regulation. The increase in TNF-**α** in blood and bronchoalveolar lavage parallels the decrease in mRNA expression of the three subunits of ENaC in the whole lung tissue during the development of pulmonary oedema. Direct exposure of rat AT II cells to TNF-**α** significantly inhibited mRNA expression of **α** and γ subunits of ENaC and ENaC function [63]. This TNF-induced downregulation of ENaC expression and activity can be alleviated by dexamethasone [64]. However, TNF-**α** can also enhance AFC by an amiloride-sensitive, cAMP-independent mechanism via either a TNF-**α** receptor-dependent or -independent effect. The latter effect stimulates amiloride-sensitive Na⁺ transport via the lectin-like domain of TNF-**α** [65]. In experimental injury of rabbit lungs, aerosolised lectin-like domain of TNF-**α** (designated TIP, a scrambled peptide that corresponds to residues 99-115 of murine TNF-**α**) can improve fluid balance by reducing vascular permeability and increasing Na⁺, K⁺-ATPase exocytosis to the AEC surface, amiloride-sensitive sodium absorption, and AFC [65-67]. Therefore, the role of TNF-**α** in AFC and active sodium transport is still controversial.

Therapies for improving alveolar sodium and fluid transport during ALI

Several mechanisms influence alveolar active Na⁺ transport, thus contributing to the maladjustment of AFC in patients with ALI/ARDS. The major contributors to alveolar active Na⁺ transport are apical ENaC and basal Na⁺, K⁺-ATPase in the alveolar epithelium; thus, upregulation of alveolar active Na⁺ transport, especially through ENaC and Na⁺, K⁺-ATPase, may be essential for better clinical outcomes.

Overexpression of the Na⁺, K⁺-ATPase subunits via gene transfer shows a potential therapeutic role in improving AFC and epithelial function. Adenovirus-induced gene transfer of the α₂-Na⁺, K⁺-ATPase gene leads to protein overexpression of α₂-Na⁺, K⁺-ATPase and increased Na⁺, K⁺-ATPase activity in rat AECs and human A549 cells and enhanced α₂-Na⁺, K⁺-ATPase protein expression *in vivo* and in ventilation-induced injured lungs, thus improving the basal lung fluid clearance rate [26,68]. β₁-Na⁺, K⁺-ATPase over expression via

adenovirus-mediated gene transfer has been demonstrated to be efficient for augmenting lung liquid clearance in normal and injured rat lungs [69,70]. By using electroporation, this non viral gene transfer of β_1-Na$^+$, K$^+$-ATPase improves Na$^+$, K$^+$-ATPase activity and AFC [71]. The similar results were observed when using the same experimental technique in lung contusion-induced lung injury. Gene transfer of the α and β subunits of Na$^+$, K$^+$-ATPase led to improved recovery from ALI [72]. Above all, upregulation of Na$^+$, K$^+$-ATPase via gene transfer of its subunit genes (α_2- or β_1-Na$^+$, K$^+$-ATPase) has proven to be useful, even for improving AFC in normal and injured rat lungs.

β_2ARs are distributed throughout the lung [73] and mediate β-adrenergic stimulation of Na$^+$ and fluid transports [74]. There is no significant difference in the total water content between mice without β_1AR or β_2AR and wild-type controls. β_2AR signalling has been suggested not to be required for basal AFC. However, for physiological adaptive responses, such as hyperoxia, β_2AR signalling is needed for AFC in normal and injured lungs [75]. β_2AR stimulation can increase the activity, expression, and membrane insertion of ENaC and Na$^+$, K$^+$-ATPase and activate CNG cation channels in the alveolar epithelium by increasing intracellular cAMP and cGMP, thus improving AFC [14,31,76]. Moreover, β_2AR-mediated stimulation of Cl$^-$ transport via CFTR also contributes to increased alveolar fluid transport [73]. Both AT I and AT II cells express CFTR and are capable of Cl$^-$uptake [77]. This interdependency between CFTR and β_2AR is required for β_2AR-stimulated alveolar Na$^+$ and fluid transport [78]. In AECs, β_2-agonists stimulate adenylyl cyclase and subsequently increase intracellular cAMP and activate protein kinase A (PKA). PKA augments the number of HSCs, increases NSC activity, and upregulates Na$^+$, K$^+$-ATPase and CFTR [73,79]. However, FDLEs exposed to the β_2-adrenergic agonist terbutaline have increased Na$^+$ transport via a post-transcriptional increase in α_1-Na$^+$, K$^+$-ATPase protein expression, without significant effects on β_1-Na$^+$, K$^+$-ATPase and ENaC [25], suggesting that β_2-adrenergic agonists play different roles in ion transport in adult and foetal distal lung epithelium. Furthermore, prolonged treatment with terbutaline impairs β_2AR signalling in the alveolar epithelia and whole lungs [32].

In addition to, other catecholamine, such as dopamine and isoproterenol, have been discovered to potentially upregulate AFC [75,80,81]. Increasing endogenous and clinically administered dopamine improve AFC by upregulating the alveolar epithelial Na$^+$, K$^+$-ATPase [80,81]. Dopamine increases both ENaC and Na$^+$, K$^+$-ATPase activity via different mechanisms [80,82,83]. The former is induced by a cAMP-mediated, alternative signalling pathway [82], and the latter is mediated by Na$^+$, K$^+$-ATPase exocytosis from late endosomes merged into the basolateral membrane of AECs [83].

Although the use of a high-dose Glucocorticoid (GC) for ALI patients remains controversial, DEX enhances the expression of ENaC and Na$^+$, K$^+$-ATPase during both normoxia and hypoxia in rats and primary alveolar AT II cells [33]. GC signalling is mediated by the GC Receptor (GR). Mice with a GR deficiency in all tissues are born dead due to respiratory failure. In lung epithelium-specific GR knockout mice, a reduction in mRNA expression for surfactant proteins, transepithelial Na$^+$ transport, and fluid clearance at birth is seen, which may be involved in decreased viability [84]. Glucocorticoids increase the surface abundance of the ENaC subunits (α-, β- and γ-ENaC) via a mechanism that may dependent on the activation of Serum and Glucocorticoid-inducible Kinase 1 (SGK1). However, brief but not prolonged exposure of H441 human airway epithelial cells to dexamethasone activates SGK1 and leads to an SGK1-dependent

increase in the surface abundance of the ENaC subunits. This upregulation could not explain the persistent activation of ENaC [85]. Even so, SGK1 plays an important role in activating lung ENaC [85,86]. These findings suggest that Na$^+$ transport stimulated by catecholamines and glucocorticoids strengthens AFC and may related to better clinical outcomes for ALI patients.

Summary

Increased pulmonary oedema formation occurs in ALI/ARDS. Patients who failed to remove alveolar oedema fluid rapidly have worse clinical outcomes. AFC depends on vectorial active Na$^+$ transport via apical Na$^+$ channels and basolateral Na$^+$, K$^+$-ATPase and paracellular Na$^+$ transport. Hypoxia in the course of ALI leads to impaired AFC via downregulation of ENaC, Na$^+$, K$^+$-ATPase, and intercellular junctions. The inflammation during ALI contributes to increased RNS, ROS and TNF-α level, which result in different regulation pattern of lung Na$^+$ transport across the alveolar epithelium. However, although these agents play a notable role in ALI, the regulatory mechanisms for alveolar Na$^+$ and fluid transport in ALI are still undefined. Catecholamines and glucocorticoids stimulate Na$^+$ transport and AFC; in particular, β_2-agonists have been shown to play a remarkable role in limiting alveolar oedema in preclinical experimental studies. A single-centre, placebo-controlled small clinical trial reported that sustained treatment with intravenous β_2-agonists reduces extravascular lung fluid in patients with ALI [87]. However, two recent multicentre, placebo-controlled large clinical trials have reported that both intravenous and aerosolised β_2-agonists therapies did not improve clinical outcomes in patients with ALI [88,89]. Routine use of β_2-agonist treatment in mechanically ventilated patients with ALI cannot be recommended. Intravenous β_2-agonists could increase mortality in patients with early ARDS, but the underlying mechanisms of this increase remain unclear. The results of these large controlled clinical trials are more convincing; however, the cause of the different results between preclinical experimental studies or the small controlled clinical trial and the large controlled clinical trials need to be explained.

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