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SERIES "HYPOXIA: ERS LUNG SCIENCE CONFERENCE"
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Regulation of alveolar epithelial function by hypoxia

G. Zhou, L.A. Dada and J.I. Sznajder

ABSTRACT: Patients with acute respiratory distress syndrome and high-altitude pulmonary oedema build up excess lung fluid, which leads to alveolar hypoxia. In patients with acute respiratory distress syndrome and hypoxia, there is a decrease in oedema fluid clearance, due in part to the downregulation of plasma membrane sodium–potassium adenosine triphosphatase (Na,K-ATPase).

In alveolar epithelial cells, acute hypoxia promotes Na,K-ATPase endocytosis from the plasma membrane to intracellular compartments, resulting in inhibition of Na,K-ATPase activity. Exposure to prolonged hypoxia leads to degradation of plasma membrane Na,K-ATPase.

The downregulation of plasma membrane Na,K-ATPase reduces adenosine triphosphate demand, as part of a survival mechanism of cellular adaptation to hypoxia. Hypoxia has also been shown to disassemble and degrade the keratin intermediate filament network, a fundamental component of the cell cytoskeleton, affecting epithelial barrier function.

Accordingly, better understanding of the mechanisms regulating cellular adaptation to hypoxia may lead to the development of novel therapeutic strategies for acute respiratory distress syndrome and high-altitude pulmonary oedema patients.

KEYWORDS: Alveolar epithelium, hypoxia, keratin intermediate filament, oedema fluid clearance, reactive oxygen species, sodium–potassium adenosine triphosphatase

The alveolar epithelium contributes to the maintenance of surface tension, basic host defence properties, gas exchange and oedema clearance [1]. It is normally well oxygenated since oxygen is exchanged across the alveolocapillary membrane. However, under a number of conditions, the alveolar epithelium is exposed to low oxygen levels (hypoxia). For example, during ascent to high altitude, the partial pressure of oxygen drops due to the decline in barometric pressure, which can contribute to high-altitude pulmonary oedema (HAPE) [2]. In turn, HAPE exaggerates alveolar hypoxia as a consequence of alveolar flooding [3]. In addition, patients with acute respiratory distress syndrome or congestive heart failure develop pulmonary oedema, resulting in impaired oxygen transfer from the airspaces into the pulmonary circulation [1, 4, 5]. Patients who cannot clear oedema efficiently have worse outcomes, suggesting that hypoxia plays a deleterious role in alveolar epithelial function [1, 6]. The mechanisms contributing to alveolar epithelial dysfunction during hypoxia are not completely understood. Recent advances on this topic are reviewed herein.

ALVEOLAR EPITHELIUM

In the human lungs, the alveolar epithelium is populated by squamous alveolar type (AT)I and cuboidal ATII cells. There are similar numbers of ATI and ATII cells; however, ATI cells are elongated, with long thin cytoplasmic extensions, and cover 95% of the surface area [7, 8]. ATII cells, which produce, secrete and recycle surfactant, cover the remaining 5% of the surface area and are thought to differentiate into ATI cells. Recent studies have demonstrated that both ATI and ATII cells express sodium–potassium adenosine triphosphatase (Na,K-ATPase) and the

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STATEMENT OF INTEREST
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amiloride-sensitive epithelial sodium channel (ENaC), and thus contribute to active Na^+ transport and alveolar fluid resorption [7–10].

Interactions between adjacent epithelial cells through domains such as tight junctions and adherens junctions provide a physical barrier between the alveolar airspaces and the interstitium (fig. 1). Tight junctions surround the cells like rubber O-rings, preventing large molecules from crossing the epithelial layer [11]. The relative impermeability of the alveolar epithelium to paracellular solute diffusion is predominantly regulated by tight junctions [12–14]. Tight junctions consist of integral membrane proteins: occludins, claudins and junctional adhesion molecules [15, 16]. Cytoplasmic plaque proteins transduce signals between tight junctions and cytoplasmic signalling molecules or the actin cytoskeleton [16, 17]. In addition, tight junctions divide the epithelial plasma membrane into the apical and basolateral domains [15, 16]. Ion transporters and other membrane proteins are asymmetrically distributed in these two domains [15, 16].

Alveolar fluid resorption is accomplished through active Na^+ transport across alveolar epithelium [18, 19]. As depicted in figure 1, Na^+ is taken in on the apical surface of alveolar epithelial cells, primarily through ENaC. Subsequently, Na^+ is actively extruded through the basolateral surface into the lung interstitium by the Na,K-ATPase, generating a transepithelial osmotic gradient. Water then follows the osmotic gradient into the interstitial space and pulmonary circulation, leading to the resorption of alveolar fluid [18, 19].

The apical surface of epithelial cells expresses the ENaC, which is a major Na^+ transporter and widely distributed in the lung, kidney and colon [20–23]. It comprises three subunits (α, β and γ) and usually exists as a tetramer made up of two α-, one β- and one γ- subunit or a much larger complex containing three of each subunit [24, 25]. Maximal transport requires the presence of all three subunits. ENaC-mediated ion transport

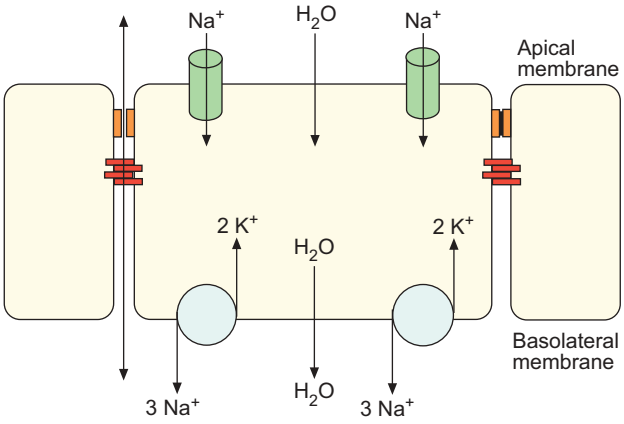

FIGURE 1. Schematic diagram of the alveolar epithelium. Tight (in orange) and adherens junctions (in red) on adjacent epithelial cells provide a restrictive barrier in order to maintain selective permeability. The asymmetrically distributed epithelial sodium channel (in green) and sodium–potassium adenosine triphosphatase (in light blue) confers the vectorial transport of sodium ions, which is crucial for alveolar fluid resorption. ↕: paracellular transport.

is voltage-dependent, activated by calcium ions (at high doses) and inhibited by 1 μM amiloride [26]. α-ENaC knockout mice die due to pulmonary oedema immediately after birth [27], whereas β- or γ-ENaC knockout mice show a compromised rate of fluid clearance [28, 29]. The ENaC inhibitor amiloride does not completely inhibit Na^+ transport in alveolar epithelial cells, suggesting the presence of amiloride-insensitive pathways that contribute to fluid resorption. Among these are cyclic nucleotide-gated cation channels, the Na^+–glucose transporter and other co-transporters (*e.g.* Na^+–amino acid) [25].

The Na,K-ATPase resides in the basolateral membrane of the cells. It utilises the energy released from adenosine triphosphate (ATP) hydrolysis to pump $3Na^+$ out of cells in exchange for $2K^+$ into cells, generating Na^+ and K^+ gradients across the plasma membrane [30]. Na,K-ATPase is a heterodimer of α- and β-subunits, both of which are necessary for its activity [31]. It is believed that the α- and β- subunits are synthesised independently and then assembled into a dimer in the endoplasmic reticulum and delivered to the plasma membrane [31, 32]. The α-subunit is a transmembrane protein that catalyses ATP hydrolysis and contains the binding sites for Na^+, K^+ and the inhibitor ouabain [30]. Four α-subunit isoforms, α1– α4, have been described [31]. These isoforms are highly conserved, each containing a >77% identical primary amino acid sequence. The α1-isoform is found in most tissues, whereas the other isoforms are tissue-specific [30]. Mice with deletion of either the α1- or α2-isoform do not survive, suggesting a fundamental role of the α1- or α2-isoform in organ development [33, 34]. Loss-of-function mutations in the α2-subunit are associated with familial hemiplegic migraine type 2 [35–37], and missense mutation of the α3-subunit causes dystonia-Parkinsonism [38]. The β-subunit has four isoforms and contains three glycosylation sites. It controls heterodimer assembly and insertion into the plasma membrane [30].

CELLULAR ADAPTATION TO HYPOXIA

During hypoxia, cells respond to this stress through adaptive mechanisms [39, 40]. One response is to increase the level of expression of genes responsible for angiogenesis, in order to provide more efficient blood flow, and of genes involved in glycolytic pathways [39]. This regulation of gene expression during hypoxia is carried out by turning on the master transcription factor, hypoxia-inducible factor (HIF), a dimer consisting of the subunits HIF-α and -β [41]. HIF-α is a short-lived protein, whereas HIF-β is constitutively expressed [42]. Under normoxic conditions, oxygen- and iron-dependent prolyl hydroxylase hydroxylates HIF at prolines 402 and 564, two highly conserved amino acids within the oxygen-dependent degradation domain of HIF-α [43]. In turn, von Hippel–Lindau protein (pVHL) recognises and binds to the prolyl-hydroxylation sites, targeting HIF-α for ubiquitination and eventual degradation in the proteasome [42, 44–46]. Under hypoxic conditions, prolyl hydroxylase activity is inhibited; therefore, HIF-α is stabilised, since it is neither hydroxylated nor degraded in the proteasome. After stabilisation, HIF translocates into the nucleus and activates downstream genes, such as vascular endothelial growth factor, erythropoietin, glucose transporter 1 and enzymes involved in the glycolytic cascade, to improve delivery of oxygen and glucose to cells [41, 42].

Cells can also adapt to hypoxia by maintaining ATP homeostasis [40]. During hypoxia, insufficient oxygen limits ATP production through mitochondrial oxidative phosphorylation, and thus cellular ATP levels decrease [47, 48]. In order to maintain ATP homeostasis, cells can either increase ATP production *via* anaerobic glycolysis or decrease ATP demands *via* inhibition of ATP-consuming enzymes, such as Na,K-ATPase and the protein synthesis machinery [40]. Na,K-ATPase activity may account for ~40% of ATP consumption in cells, dependant upon cell type [40, 49, 50]. It has been reported that hypoxia decreases ATP demand by reducing the amount of plasma membrane Na,K-ATPase [51, 52].

EFFECTS OF HYPOXIA ON ALVEOLAR FLUID RESORPTION

Hypoxia has been shown to impair alveolar fluid clearance by inhibiting transepithelial active Na^+ transport [53, 54]. It has been demonstrated that exposure of rodents to hypoxia results in a significant decrease in alveolar fluid resorption, which is associated with a decrease in ENaC, as well as Na,K-ATPase, activity [54–56]. In cultured alveolar epithelial cells, hypoxia-mediated downregulation of Na,K-ATPase is time and oxygen concentration-dependent [52, 57, 58]. Short-term exposure to hypoxia decreases Na,K-ATPase activity and its protein abundance at the plasma membrane without significant changes in its total amount, suggesting that endocytosis of Na,K-ATPase occurs during hypoxia [52].

The endocytosis of Na,K-ATPase during hypoxia appears to be mediated by mitochondrial reactive oxygen species (ROS), since ROS scavengers prevented the hypoxia-induced down-regulation of Na,K-ATPase [52]. Moreover, treatment with hydrogen peroxide is sufficient to cause both Na,K-ATPase endocytosis from plasma membrane and a decrease in Na,K-ATPase activity [52]. The source of ROS in this process was further assessed in mitochondrial-DNA-depleted (ρ^0) A549 cells [59]. These cells lack a competent electron transport chain and are thus incapable of generating ROS during hypoxia [60]. In ρ^0 A549 cells, hypoxia failed to induce Na,K-ATPase endocytosis and decrease Na,K-ATPase activity. Furthermore, in rodents exposed to hypoxia, overexpression of manganese superoxide dismutase inhibited mitochondrial ROS production and blocked the decrease in Na,K-ATPase abundance and alveolar fluid resorption [54]. Together, these studies suggest a role for mitochondrial ROS in the hypoxia-mediated endocytosis of Na,K-ATPase.

During hypoxia, mitochondrial ROS activate protein kinase Cζ, leading to Na, K-ATPase α1-subunit phosphorylation at serine 18 [52]. In addition, plasma membrane Na,K-ATPase was ubiquitinated; however, mutation of the four lysines surrounding serine 18 to arginine prevented Na,K-ATPase ubiquitination, implying that these are the ubiquitination sites [61]. Mutation of serine 18 to alanine prevented ubiquitination and endocytosis of the Na,K-ATPase α1-subunit, suggesting that serine 18 phosphorylation is a prerequisite for these processes (fig. 2) [52, 61].

Endocytosis of Na,K-ATPase has been reported to be clathrin-dependent [62]. In alveolar epithelial cells, hypoxia-induced endocytosis of Na,K-ATPase requires the binding of adaptor protein 2 to the tyrosine-based motif (tyrosine 537) located in the Na,K-ATPase α1-subunit, leading to the incorporation of Na,K-ATPase into clathrin vesicles [63]. Trafficking of clathrin vesicles requires the actin cytoskeleton in mammalian cells [64]. Activation of Rho GTPase leads to rearrangement of the actin cytoskeleton, thus regulating trafficking of clathrin vesicles [64]. Rho GTPases can activate two types of actin nucleators that directly stimulate actin polymerisation and also regulate cofilin to affect actin reorganisation *via* Rho-associated protein kinase (ROCK)/p21-activated kinase/LIM domain kinase signalling [64]. More recently, it has been suggested that hypoxia-mediated endocytosis of Na,K-ATPase is dependent upon the activation of RhoA/ROCK signalling and actin stress fibre formation [65]. During hypoxia, mitochondrial ROS activate the small GTPase RhoA, leading to the formation of actin stress fibres in alveolar epithelial cells [65], and dominant negative RhoA and ROCK inhibitor prevent the hypoxia-mediated Na,K-ATPase endocytosis [65].

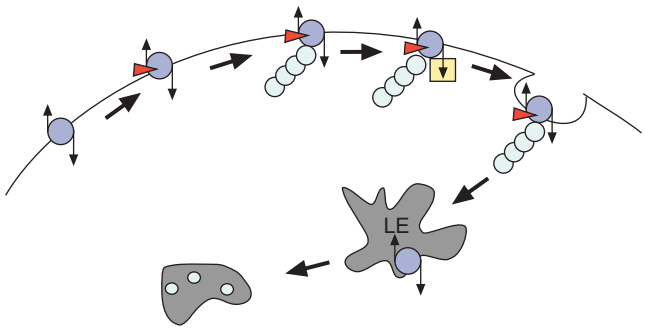

FIGURE 2. Hypoxia induces endocytosis and degradation of plasma membrane sodium–potassium adenosine triphosphatase (Na,K-ATPase; in purple). In alveolar epithelial cells exposed to hypoxia, generation of mitochondrial reactive oxygen species activates protein kinase Cζ, which phosphorylates (in red) plasma membrane Na,K-ATPase, leading to its ubiquitination (in light blue). This series of events triggers Na,K-ATPase endocytosis *via* clathrin (in yellow)-coated vesicles. Na,K-ATPase-containing endosomal vesicles merge to form the late endosome (LE) and Na,K-ATPase is degraded.

Prolonged hypoxia leads to the mitochondrial ROS-dependent degradation of plasma membrane Na,K-ATPase, and both the proteasome and the lysosome are involved in this process [51, 54]. Since the ubiquitination of plasma membrane Na,K-ATPase is required for its endocytosis [61], it is likely that Na,K-ATPase ubiquitination acts as a signal for its endocytosis, stimulating its merger with the proteasome and/or the lysosome for degradation. In contrast, hypoxia did not alter the half-life of total pool Na,K-ATPase, suggesting that degradation of total pool Na,K-ATPase is not affected by hypoxia [51]. A recent study reported that loss of pVHL prevented hypoxia-mediated degradation of plasma membrane Na,K-ATPase [66]. Although this process required the pVHL E3 ligase activity, HIF stabilisation was not required, indicating a role for pVHL in hypoxia-mediated Na,K-ATPase degradation independent of HIF [66]. This study suggests that pVHL may play a dual function during hypoxia: 1) hypoxia inhibits pVHL–HIF interaction, resulting in stabilisation of HIF and upregulation of HIF-responsive genes for glycolytic ATP production and better oxygen delivery; and 2) pVHL facilitates plasma membrane Na,K-ATPase degradation to decrease ATP demands through an HIF-independent mechanism. Taken

together, these data suggest that hypoxia increases mitochondrial ROS generation and induces endocytosis and degradation of plasma membrane Na,K-ATPase, resulting in the inhibition of Na,K-ATPase activity and impaired alveolar fluid clearance.

EFFECTS OF HYPOXIA ON KERATIN INTERMEDIATE FILAMENTS

Keratin intermediate filaments (IFs) are the major cytoskeletal component of epithelial cells and play a crucial role in maintaining the structural integrity of cells [67]. Although it was originally thought that the IF is a static structure, accumulating data suggest that keratin IFs can undergo rapid deformation/displacement in epithelial cells in response to stress, suggesting that the IF cytoskeleton transmits mechanical signals from the cell surface to all regions of the cytoplasm [68].

Keratin IFs are assembled as heteropolymers of type I and type II IF proteins. Keratins consist of a conserved central α-helical domain, a non-α-helical N-terminal head and a C-terminal tail domain containing all of the known phosphorylation sites [69]. Lung alveolar epithelial cells primarily express keratins K8 and K18 in equal amounts [70, 71]. Phosphorylation of K8 and K18 promotes their depolymerisation and redistribution *in vitro* [72].

Exposure to hypoxia caused a time-dependent disassembly of K8 and K18, which was associated with an increase in phosphorylation of K8 [73]. In alveolar epithelial cells, the hyperphosphorylation and disassembly of keratin during hypoxia was mediated by mitochondrial ROS, which activate PKC and phosphorylate keratin, leading to disassembly of the keratin IF network [73].

In rats exposed to hypoxia, there was a significant decrease in keratin in alveolar epithelial cells compared to normoxic rats, due to degradation of keratin IFs [73, 74]. Keratin was degraded in alveolar epithelial cells exposed to hydrogen peroxide, suggesting that ROS are necessary for the degradation of keratin [74]. The proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) prevented the degradation of keratin IFs in alveolar epithelial cells exposed to hypoxia, suggesting that keratin degradation was mediated *via* the ubiquitin/proteasome pathway [74]. These data suggest that keratin IF dynamics may play a role in adaptation to hypoxia in alveolar epithelial cells.

POTENTIAL THERAPIES FOR EPITHELIAL FUNCTION IMPROVEMENT DURING HYPOXIA

Since downregulation of Na,K-ATPase contributes to alveolar epithelial dysfunction, its upregulation may improve epithelial function during hypoxia or other pathological conditions. Indeed, overexpression of the Na,K-ATPase β1-subunit *via* viral and nonviral gene transfer resulted in an increase in alveolar fluid resorption in normal and injured rat lungs [75–79]. These studies suggest a potential therapeutic application of Na,K-ATPase gene transfer in the improvement of epithelial function and lung oedema clearance [80, 81].

Administration of catecholamines, such as dopamine, terbutaline and isoproterenol, has been shown to be effective in increasing Na,K-ATPase activity and alveolar fluid resorption. Parallel to this, treatment with dopamine and isoproterenol was associated with increases in Na,K-ATPase protein abundance at the cell basolateral membrane [82]. This increase in protein abundance was mediated by exocytosis of Na,K-ATPase from endosomal compartments into the basolateral membrane [83]. More importantly, the impaired alveolar function of injured lungs can be reversed by administration of dopamine, terbutaline and isoproterenol in animal models of hypoxia, hyperoxia, increased left atrial pressure or ventilation-induced lung injury [53, 54, 84, 85]. The data from these animal model studies led to the development of a clinical trial, in which patients receiving salbutamol, a β-adrenergic agonist, showed a significant reduction in extravascular lung water [86]. Moreover, inhalation of salmeterol has been shown to prevent HAPE in susceptible subjects [87]. These findings support the hypothesis that β-adrenergic agonists accelerate the resolution of alveolar oedema and may improve survival.

SUMMARY

Epithelial dysfunction, in acute respiratory distress syndrome and high-altitude pulmonary oedema patients, is associated with increased oedema formation, and results in a hypoxic environment. Hypoxia contributes to the impaired lung fluid clearance *via* the downregulation of sodium–potassium adenosine triphosphatase, which regulates active sodium ion transport and alveolar fluid clearance. Hypoxia also results in disassembly and degradation of the keratin intermediate filament network, which may aggravate the epithelial dysfunction. β-Adrenergic agonists have been demonstrated to increase active sodium ion transport, suggesting that it may form part of the therapeutic armamentarium for acute respiratory distress syndrome and high-altitude pulmonary oedema patients.

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