Role of Ubiquitination in Na,K-ATPase Regulation during Lung Injury

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During acute lung injury edema accumulates in the alveolar space, resulting in hypoxemia due to intrapulmonary shunt. The alveolar Na,K-ATPase, by effecting active Na⁺ transport, is essential for removing edema from the alveolar spaces. However, during hypoxia it is endocytosed and degraded, which results in decreased Na,K-ATPase function and impaired lung edema clearance. Na,K-ATPase endocytosis and degradation require the phosphorylation and subsequent ubiquitination of the Na,K-ATPase. These events are the results of cross-talk between post-translational modifications, and how ubiquitination of a specific protein can result from injurious extracellular stimuli. Here, we review current knowledge on the regulation of Na,K-ATPase activity during lung injury, focusing on the role of Na,K-ATPase ubiquitination during hypoxia. A better understanding of these signaling pathways can be of relevance for the design of novel treatments to ameliorate the deleterious effects of acute lung injury.

Keywords: lysosome; proteasome; protein degradation

LUNG INJURY AND ALVEOLAR EDEMA CLEARANCE

Normal functioning of the alveolar epithelium is required to sustain life, as it provides the entry point into the body for the oxygen required for cellular respiration. The apical surface of the alveolar epithelium is covered with a thin layer of fluid that maintains surface tension, facilitates gas exchange, and also protects against pathogens (1). Underlying the alveolar epithelium basally is the vascular endothelium, and the close proximity of these two tissues promotes gas exchange between the bloodstream and the air space. The alveolar epithelium consists of equal numbers of alveolar type I (ATI) and type II (ATII) cells, with the former accounting for approximately 95% of the surface area (2). Both cell types are polarized, containing well-organized adherens and tight junctions, as well as an asymmetric distribution of ion transporters, including the basolaterally located Na,K-ATPase. Thus, the intact epithelium forms a selectively permeable barrier which is critical for regulating the movement of liquid and proteins between the alveolar and interstitial spaces (1–3).

During acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), there is impairment of the alveolo-capillary barrier (4, 5). Damage to the alveolar epithelium results in accumulation of protein-rich edema fluid from the capillary and interstitial spaces into the alveoli, leading to flooding of the air spaces and impaired gas exchange. Lung edema must be cleared for the restoration of normal lung function and patient survival,

yet alveolar fluid reabsorption is impaired in most patients with ALI/ARDS (4–6). Mortality rates of ALI/ARDS are unacceptably high, with an estimated 190,000 ALI cases in the United States annually (7, 8). The initial injury to the lung that disrupts barrier function can be due to diverse causes (4–8). The injury can be direct (such as during infection) or may be caused indirectly (e.g., by sepsis or trauma). For example, an excessive inflammatory response during these indirect insults can increase the extent of the damage to the lung. Alveolar edema can also occur at high altitude, a condition known as high altitude pulmonary edema (HAPE), which has a high mortality rate in the absence of edema resolution (9).

The major force driving lung edema clearance is active Na⁺ transport across the alveolar epithelium (10) which is effected by the concerted actions of the basolateral Na,K-ATPase and the apical sodium channels and Na⁺-cotransporters (6, 11, 12). The polarized localization of ion transporters creates a Na⁺ gradient across the epithelium, resulting in an osmotic gradient that is critical for allowing the flux of fluid out of the alveolar spaces (5, 13). Because edema clearance is effected by active Na⁺ transport, which is impaired in patients with ALI/ARDS (4), understanding the regulation of the Na,K-ATPase during lung injury is of clinical significance.

REGULATION OF THE Na,K-ATPase

The Na,K-ATPase, a member of the P-type ATPase superfamily, is thought of as a heterodimer of a catalytic α - and a regulatory β -subunit with a 1:1 stoichiometry (14–16). To date, four α - and β -subunit isoforms have been identified in mammals, which are expressed in a tissue-specific manner (17). A γ -subunit can provide further tissue-specific regulation (18, 19). In the lung, the Na,K-ATPase localizes at basolateral surface of ATI and ATII cells (20), moving three Na⁺ ions out of the cell and two K⁺ ions into the cell per ATP molecule consumed. The main function of the Na⁺-pump is the regulation of intracellular Na⁺ concentration, which with other transporters regulates cell volume, as well as glucose and amino acid transport (21). It has been proposed that the Na,K-ATPase has other evolutionarily conserved physiological functions beyond ion transport. Particularly interesting is the requirement of the Na,K-ATPase in the formation and maintenance of intercellular junctions, where it can act as a signaling and scaffolding center (16–18).

Given the ubiquitous distribution and essential functions of the Na,K-ATPase, it must be able to adapt to diverse (patho)-physiologic stimuli. For example, short-term regulation can be due to changes in Na,K-ATPase activity via conformational modification of the enzyme turnover rate or change in the total number of functioning pumps (5, 22). Long-term regulation occurs via transcriptional and post-transcriptional regulation of the Na,K-ATPase (5, 15, 23–25). Importantly, down-regulation of the Na,K-ATPase has been reported in several ALI models (5, 10, 22), and the impairment in alveolar fluid clearance observed in these models can be reversed increasing Na,K-ATPase function, either by the effects of catecholamines (9, 26–31) or its overexpression (30–34). Lung injury can lead to

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Na,K-ATPase ubiquitination, and we will focus on hypoxia as a model stimulus of the Na,K-ATPase ubiquitination, endocytosis, and degradation.

UBIQUITINATION AS A TRAFFICKING SIGNAL

Protein turnover is crucial for cell health, and up to 30 years ago it was thought that protein degradation occurred exclusively in the lysosome. However, experiments conducted by Drs. Ciechanover, Hershko, and Rose in reticulocytes that lack lysosomes were able to degrade proteins, indicating that other pathways also existed (35). In addition, it was particularly difficult to explain the spectrum of half-lives exhibited by different proteins within a cell, ranging from a few minutes to several days, using only the lysosomal model of protein degradation. These investigations suggested the existence of an alternative method of protein degradation that required adenosine triphosphate (ATP), and that such process in reticulocyte cell extracts consisted of at least two separate components, one of which was later identified as ubiquitin (36, 37). The 2004 Nobel Prize in Chemistry was awarded in recognition of this discovery (38).

In addition to regulating protein stability, ubiquitination has been reported to participate in cell signaling events (39–42). Ubiquitin is a highly conserved 76-amino acid protein, which can be covalently linked via its C-terminal glycine to target proteins at the ϵ -amino group of their lysine residues. Ubiquitin was the first protein shown to covalently modify other proteins, but several other such ubiquitin-like proteins have since been identified, including SUMO and NEDD8 (43). The functional outcome of ubiquitination (degradation or traffic) is determined by the number and topology of ubiquitin molecules attached to the target protein, as well as by the subcellular localization where the process occurs (44). A single ubiquitin can attach to one lysine (monoubiquitination) or to multiple lysine residues on the target (multi-monoubiquitination), or a ubiquitin chain can build at a single lysine residue (polyubiquitination). Furthermore, ubiquitin itself contains seven lysine residues that can provide binding sites for other ubiquitin molecules, forming branched ubiquitin chains, the specific organization of which determines functional outcome (45).

Conjugation of ubiquitin to a target protein occurs via three stepwise and hierarchical enzymatic reactions (46). As illustrated in Figure 1, ubiquitin is first activated by an E1 ubiquitin-activating enzyme by the formation of a high-energy thiolester bond between the enzyme and ubiquitin, requiring ATP. The majority of eukaryotes have only one E1 enzyme, with humans having around ten (45, 47). The ubiquitin is then transferred via a transthiolester reaction to an E2 ubiquitin-conjugating enzyme, which in turn transfers ubiquitin onto the target protein. This conjugation step often occurs in conjunction with the activity of E3 ubiquitin ligases, which are critical regulators of the ubiquitination process as they specify the target proteins selected for ubiquitination, as well as the timing of this post-translational modification (48). The number of E3s in humans is thought to be in excess of 1,000 (45), with a single E3 recognizing a distinct set of substrates and interacting with one, or a few of the an different E2 enzymes. This hierarchy means that regulation at the level of E3s allows for greater specificity, and, given the multitude of different E3s expressed within one cell type and the ever-increasing number of processes in which ubiquitination is implicated, E3 ligases are now more widely being thought to be prevalent and very important in cell signaling events as for example kinases (49).

The majority of E3s fall into two mechanistic classes: zinc finger-containing *really interesting new gene* (RING) domain

ligases (50), and homologous to E6-AP carboxyl terminus (HECT) domain ligases (51). Ligases of the RING domain class bind the E2 enzyme and target protein at the same time, and therefore are not true enzymes but rather act as adaptors or scaffolds to facilitate the more efficient transfer of ubiquitin from the E2 enzyme to the substrate. In contrast, HECT domain E3 ligases have catalytic activity and can directly bind ubiquitin, transferring it from an E2 to the substrate. RING domain E3 ligases can be further divided into two subclasses: single peptide E3s, and multiprotein E3 complexes (52). More recently a new family of E3 ligases, the U box ubiquitin ligases, has also been described (53).

Protein ubiquitination leads to its degradation in the ATP-dependent multi-subunit 26S proteasome (54), which commonly results from K48-linked polyubiquitination (55). Many soluble cytosolic and nuclear proteins are regulated in this way, for example mitotic cyclins, which undergo degradation in a cell cycle-dependent manner (56). Ubiquitination also has an important role in the intracellular trafficking of proteins between membrane compartments (40, 48, 57). Ubiquitination functions at two distinct steps in the secretory/endocytic pathways: it serves as a signal for endocytosis as well as a signal for entry into the multivesicular body (MVB) pathway (41). Addition of one or a few ubiquitin molecules, or a K63-linked polyubiquitin chain, onto a cell surface protein can serve as triggers for endocytosis (40). K63-linked chains have also been implicated in other nonproteolytic functions such as DNA repair (58) and protein kinase activation (59). Less typical ubiquitin chains, such as those linked at the other five lysine residues in ubiquitin, mixed-linkage chains using different lysines to connect subsequent ubiquitin molecules, and heterologous chains containing both ubiquitin and other ubiquitin-like molecules such as SUMO have all been observed *in vivo*, but their physiological significance has not been fully elucidated (60).

The endocytic pathway can be influenced by ubiquitination in one of two ways. When attached to membrane proteins, ubiquitin can serve as a signal for their entry into vesicles at either the plasma membrane or at the late endosome; alternatively, ubiquitin can alter the trafficking machinery (40). The sorting of plasma membrane proteins into endocytic vesicles and away from the cell surface is a critical mechanism of protein function down-regulation. At the early endosome, after internalization, cargo can either be recycled back to the plasma membrane, or ubiquitin can serve as a further signal for sorting into MVBs and proteolysis by the lysosome (61). Multiple classes of proteins whose endocytosis is regulated by ubiquitination have been identified, including receptors of signal transduction cascades, cell-junctional molecules, and transporters and channels. Well-known examples of each class in mammals include several receptor tyrosine kinases (62–64), which are subsequently degraded in the lysosome, E-cadherin (65), and the ENaC sodium channel (66). Recent work suggests that the Na,K-ATPase is also ubiquitinated, as we discuss in the following section.

Interestingly, the internalization signal in the ubiquitin molecule is provided by its three-dimensional structure. This signal consists of two hydrophobic areas on different surfaces of the ubiquitin molecule (67). Ubiquitin conjugation to a protein is sufficient to cause its endocytosis (68), and, apart from the amino group onto which ubiquitin binds, is independent of any other sequence information on the target protein. An important aspect of decoding the information contained in a ubiquitin conjugates is via proteins that bear ubiquitin-binding modular domains (UBDs) (42, 69). A number of these sequence motifs are known to bind monoubiquitin, while others recognize specific types of branches on polyubiquitin chains (39). UBD-containing proteins

Figure 1. Ubiquitination cascade leading to degradation of proteins.

can function at both the internalization and later sorting steps, and many E2 and E3 enzymes contain these motifs.

Na,K-ATPase UBIQUITINATION

Ubiquitination of the Na,K-ATPase was first proposed in 1997 by Coppi and Guidotti (70). These authors reported that both the α_1 - and α_2 -subunits of the Na,K-ATPase can be polyubiquitinated in the COS-7 cells. Although the physiological significance of the modification was not elucidated, it was proposed

that polyubiquitination could play a role in the degradation of misassembled subunits at the endoplasmic reticulum, as well as the internalization and degradation of plasma membrane Na,K-ATPase. Thevenod and Friedmann (71) later showed that oxidative stress caused by cadmium exposure leads to degradation of the Na,K-ATPase, which could be prevented by proteasomal or lysosomal inhibitors.

A physiological role for ubiquitination of the Na,K-ATPase was first shown in alveolar epithelial cells during hypoxia, where severe short-term hypoxia led to Na,K-ATPase endocytosis and

Figure 2. Pathway for the hypoxia-induced Na,K-ATPase, phosphorylation ubiquitination, recognition, endocytosis, and degradation (PURED). During hypoxia the Na,K-ATPase α_1 -subunit is phosphorylated at Ser18 with the consequent ubiquitination; these post-translational modifications make the Na,K-ATPase available for recognition by the endocytic machinery, and subsequent degradation in the lysosome.

degradation in alveolar epithelial cells. We and others (72, 73) have described that PKC- ζ directly phosphorylates the Na,K-ATPase α_1 -subunit at Ser-18. In hypoxic conditions the activation of PKC- ζ requires reactive oxygen species (ROS), which alone are sufficient to trigger endocytosis (72). A subsequent study reported that hypoxia leads to phosphorylation of the central metabolic regulator AMP-activated protein kinase (AMPK) at Thr-172, and that the ROS are of mitochondrial origin (74). AMPK directly binds and activates PKC- ζ by phosphorylating it at Thr-410, and PKC- ζ is translocated to the plasma membrane, where it phosphorylates the Na,K-ATPase α -subunit. Acute hypoxia does not change the total level of Na,K-ATPase protein, indicating that a smaller amount of its membrane abundance is altered (72). However, long-term exposure of alveolar epithelial cells to hypoxia leads to decreased total Na,K-ATPase levels (75). Na,K-ATPase degradation could be prevented by proteasomal and lysosomal inhibitors, as well as with a defective E1 enzyme, suggesting that the Na,K-ATPase is ubiquitinated, but that degradation occurs in the lysosome instead of the proteasome (75). Therefore the Na,K-ATPase became a likely candidate for ubiquitin-dependent intracellular trafficking. A follow-up study confirmed that, indeed, Na,K-ATPase was not endocytosed during hypoxia if the four lysine residues immediately flanking the Ser-18 (KK¹⁸SKK) on the α_1 -subunit N-terminus were mutated to arginine, preventing ubiquitination (76). Isolation of basolateral membranes revealed Na,K-ATPase-ubiquitin conjugates, suggesting that ubiquitination occurs at the plasma membrane which was prevented when Ser-18 was mutated to alanine, indicating that phosphorylation is necessary for ubiquitination. Therefore, regulation of the Na,K-ATPase during hypoxia occurs via a the "P-U-R-E-D" (phosphorylation-ubiquitination-recognition-endocytosis-degradation) mechanism (22), where phosphorylation acts as a signal for ubiquitination, and these provide the "green light" for endocytosis and degradation to occur, as depicted in Figure 2.

We have reported that the E2 ubiquitin-conjugating enzyme Ubc5 is required for the Na,K-ATPase endocytosis process (77). It was also shown that the E3 ligase pVHL (von Hippel Lindau protein), of which Ubc5 is known to be the upstream E2, is required for degradation of the Na⁺-pump during hypoxia (77). However, pVHL does not act as a substrate-recognition E3 ubiquitin ligase of the Na,K-ATPase directly, since pVHL and Na,K-ATPase do not co-immunoprecipitate, nor was the Na⁺ pump ubiquitinated by pVHL. A more likely candidate for the direct E3 ligase of the pump is the multi-enzyme SCF (Skp1, cullin and F-box) complex (78), because, like the Na,K-ATPase, most known substrates of the SCF complex are phosphorylated before ubiquitination (49, 79). Endocytosis of the Na,K-ATPase occurs via clathrin-coated vesicles, and binding of the μ 2 subunit of adaptor protein 2 (AP-2) to the Na,K-ATPase is required for endocytosis during hypoxia (80). However, AP-2 is not known to contain a UBDs, and therefore recognition of the ubiquitinated Na⁺-pump is most likely mediated by UBD-containing proteins that frequently localize to clathrin-coated pits (41).

REGULATION OF UBIQUITINATION AND SIGNALING CROSS-TALK

Post-translational modifications (PTMs), which can be reversible, are used by cells to regulate protein function. Many PTMs themselves are highly regulated in response to extracellular stimuli to allow cells to rapidly adapt to a changing environment (47). However, the amount of regulatory information contained in PTMs is significantly increased if multiple PTMs act combinatorially (49). Historically, phosphorylation, largely due to its

relative ease of detection, has been given most attention. As described above, ubiquitination in particular has emerged as a major cell signaling PTM (39, 41, 42), which can be regulated by extracellular stimuli.

Phosphorylation and ubiquitination require ATP and are similar modifications in that they are catalyzed and reversed by a large number of enzymes and are involved in multiple cellular processes, meaning that the majority of proteins in a cell can be targeted by at least one of these modifications. Because preventing the phosphorylation of Na,K-ATPase by PKC- ζ at Ser-18 during hypoxia prevents ubiquitination, the ubiquitination is phosphorylation-dependent and is a prime example of cross-talk between PTMs.

In which ways can phosphorylation regulate ubiquitination of a protein? Phosphorylation can alter subcellular localization of proteins and therefore regulate availability of interaction between a target protein and an E3 ligase (49). Alternatively, phosphorylation can regulate activity of an E3 ligase or a deubiquitinating enzyme, or it can promote recognition of a substrate by an E3 ligase.

CONCLUSIONS

Over the last decade we have learned much about the signaling events that translate an injurious extracellular stimulus, such as for example hypoxia, leading to down-regulation of the Na,K-ATPase, possibly as an adaptive response to conserve energy in oxygen-deprived conditions (74, 75). These signaling cascades appear to start with hypoxia increasing mitochondrial ROS leading to the Na,K-ATPase endocytosis and degradation, most likely in the lysosome. We have learned that phosphorylation-dependent ubiquitination of the Na,K-ATPase is necessary for its endocytosis from the cell surface, yet many of the steps of the ubiquitination process remain unknown. What is the nature of the ubiquitination modification on the Na,K-ATPase at the plasma membrane? Is the trafficking of the Na,K-ATPase regulated by ubiquitination of the endocytic machinery or regulation of E3 ligase activity? The observation that pVHL is required for degradation, but does not act on the Na,K-ATPase, suggests that such mechanisms may exist (77). Here, we have reviewed our current understanding of the internalization of the Na,K-ATPase during hypoxia leading alveolar epithelial dysfunction, which results in decreased alveolar fluid clearance. It is known that an intact epithelial barrier is necessary for alveolar reabsorption to proceed effectively (5), and the Na,K-ATPase can associate with junctional complexes that are required for the maintenance of barrier integrity. An important unanswered question remains whether Na,K-ATPase endocytosis via ubiquitination can directly contribute to the impairment of the epithelial barrier. Despite the current unanswered questions, it is clear that the Na,K-ATPase remains a potential target for developing treatments for ALI.

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