Integrating acute lung injury and regulation of alveolar fluid clearance

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The acute respiratory distress syndrome (ARDS) is characterized by non-cardiogenic pulmonary edema and flooding of the alveolar air spaces with proteinaceous fluid. ARDS develops in response to inflammatory stresses including sepsis, trauma, and severe pneumonia, and despite aggressive critical care management, it still has a mortality of 30–50%. At the time of its original description in 1967, relatively little was known about the specific mechanisms by which the alveolar epithelium regulated lung fluid balance. Over the last 20 years, substantial advances in our understanding of the alveolar epithelium have provided major new insights into how molecular and cellular mechanisms regulate the active transport of solutes and fluid across the alveolar epithelium under both normal and pathological conditions. Beginning with the elucidation of active sodium transport as a major driving force for the transport of water from the air space to the interstitium, elegant work by multiple investigators has revealed a complex and integrated network of membrane channels and pumps that coordinately regulate sodium, chloride, and water flux in both a cell- and condition-specific manner. At the Experimental Biology Meeting in San Francisco on April 4, 2006, a symposium was held to discuss some of the most recent advances. Although there is still much to learn about the mechanisms that impair normal alveolar fluid clearance under pathological conditions, the compelling experimental findings presented in this symposium raise the prospect that we are now poised to test and develop therapeutic strategies to improve outcome in patients with acute lung injury.

MAJOR PROGRESS HAS BEEN MADE in the last 25 years in understanding the role of active ion transport in removing edema fluid from the distal air spaces of the lung. The general paradigm is that vectorial transport (apical to basal) of sodium and chloride transport creates a miniosmotic that osmotically removes water from the distal air spaces of the lung (18, 26–29, 32). This process can be upregulated by both cation-choline-dependent and -independent mechanisms. In terms of cation-choline-dependent mechanisms, β2-adrenergic agonist therapy upregulates both epithelial Na channel (ENaC)-dependent sodium transport and cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride transport (12, 27, 32). In support of the possible value of β2-adrenergic agonist therapy, one experimental study reported the therapeutic value of β2-adrenergic agonists for reducing edema in the setting of acid-induced lung injury in rats (30). Also, one small placebo-controlled, randomized clinical trial reported that intravenous β2-adrenergic agonist therapy reduced pulmonary edema in patients with acute lung injury (ALI) (34). For these reasons, a large randomized, double-blind phase III trial sponsored by the National Heart, Lung, and Blood Institute will begin later this year to test the potential value of aerosolized β2-adrenergic agonist therapy for improving clinical outcomes in clinical lung injury. In addition to testing the potential clinical value of β2-adrenergic agonist therapy in patients with ALI, several advances in basic science, as illustrated in this symposium, have substantially deepened our understanding of the mechanisms that regulate lung fluid balance and the resolution of alveolar edema.

I. USING SMALL INTERFERING RNA IN WHOLE LUNG: β-ADRENERGIC STIMULATION OF ENaC

Alveolar fluid clearance (AFC) occurs secondary to vectorial sodium transport across the alveolar epithelium where sodium enters alveolar epithelial cells presumably through apical ENaC and is extruded by basolateral Na-K-ATPases (27, 28). The fairly specific ENaC blocker amiloride inhibits a significant fraction of unstimulated and stimulated AFC (27, 28). Also, β-adrenoceptor (βAR) agonists that increase intracellular cAMP levels can stimulate AFC (27, 28). In our studies (25), we determined the involvement of ENaC in both baseline and βAR-stimulated AFC in rats by specific gene knockdown using the novel RNA interference technology. We used small interfering RNA (siRNA)-generating plasmid DNA (pDNA) vectors constructed and generated against rat αENaC to investigate ENaC involvement in AFC. Of the four pDNA vectors we generated and tested, pSi-4 was the most effective at silencing ENaC expression (Fig. 1, A and B). Alveolar epithelial type II (ATII) cells were then isolated 24 h after in vivo pSi-4 pretreatment and αENaC was undetectable; thus ATII cell ENaC expression was attenuated after pSi-4 instillation (Fig. 1C). We determined that pSi-4-pretreatment reduced baseline AFC by ~30%, whereas the βAR agonist terbutaline failed to increase AFC after pSi-4 pretreatment (Fig. 1D). There existed a dose-response relationship that supported a specific effect from pSi-4 pretreatment on αENaC knockdown.
We tested for specificity and found no changes in βENaC expression or α₁-Na-K-ATPase expression, and αENaC knockdown was organ specific. The most remarkable finding of this study was the fact that only terbutaline-stimulated AFC was fully sensitive to αENaC silencing. When using amiloride as an inhibitor of AFC, amiloride typically inhibits 40–70% (13, 27, 28, 33, 36, 43) of both stimulated and baseline AFC. The apparent discrepancy suggests that there are two separate mechanisms: one that may not rely on ENaC entirely, although is amiloride sensitive and responsible for baseline AFC, and a second that relies fully on ENaC and is responsible for terbutaline-stimulated AFC. Another possibility is that amiloride did not inhibit all ENaC due to loss of amiloride from the distal air spaces after instillation, thus generating a potentially low amiloride concentration. We used amiloride at a concentration of 10^{-3} M, which in fact may be considered a fairly high amiloride concentration. Even after significant leak and protein binding (43), the expected active air space amiloride concentration would still be high at ~10^{-4} M. Thus we believe that the vast majority of amiloride-sensitive ENaC would be inhibited by this amiloride concentration. This relationship is further supported by RT-PCR analysis of ENaC mRNA and Western blot analysis of ENaC protein where ENaC expression was attenuated by pSi-4 pretreatment regardless of later terbutaline treatment. Thus βAR stimulation of AFC depends critically on ENaC presence in the lung, whereas baseline AFC seems to be less ENaC dependent.

II. ION CHANNELS IN ATI AND ATII CELLS: A NEW PARADIGM FOR ALVEOLAR FLUID CLEARANCE

ALL presents a unique physiological challenge to the alveolar epithelium. To facilitate optimal gas exchange, the epithelium must rapidly remove any excess fluid that accumulates in its alveolar spaces (19, 32). The alveolar flooding and compromise of gas exchange that ensue in conditions such as ARDS represent a pathological extreme of a failure to maintain proper airway fluid balance and account for a significant
burden of morbidity and mortality worldwide (29). Extensive research done in the last two decades has shown that precise regulation of the alveolar surface fluid layer is achieved through vectorial transport of solutes between the alveolar surface and interstitial spaces, with active sodium transport across the alveolar epithelium generating the osmotic force for movement of water (26). However, gaps still exist in our understanding of how this fine balance is achieved in vivo. In particular, ATI cells constitute ~95% of the alveolar surface area, but their role in ion transport is not completely clear (24). If there are significant differences between the transport characteristics of ATI cells and ATII cells, the current paradigm describing alveolar fluid clearance will need to be modified (5, 18, 22, 37). We have recently identified a number of ion transport pathways in ATI cells (Fig. 2) making it reasonable to propose that the entire alveolar epithelium composed of ATI and ATII cells participates in alveolar fluid homeostasis (21). ATI cells have similar numbers of highly selective channels and nonselective channels and many more cyclic nucleotide-gated (CNG) channels per patch compared with ATII cells. However, since the cell surface area of ATI to ATII cells is of the order of >40:1, ATII cells possess many more sodium channels than ATII cells. The significant number of functional CNG channels that can be detected in ATII cells, but not in ATI cells, may account for some if not all of amiloride-insensitive alveolar salt and water transport. However, CFTR channels in ATI cells are seen much less frequently than in ATII cells, and they represent the rate-limiting step for augmented salt transport by these cells. The precise role of chloride channels and potassium channels observed in ATII cells but not in ATII cells needs to be determined. Overall, based on the complement of functional channels we can identify in the two cell types, we believe that they play a complementary role in alveolar fluid clearance, with ATI cells mediating basal salt and fluid clearance and ATII cells responsible for augmented transport. In addition, significant differences exist in how salt transport is regulated in the two cell types, and the response to various endogenous and exogenous agents known to alter ion transport may be determined primarily by the complement of receptors each cell expresses and the intracellular signaling pathways activated by the interaction of these receptors with appropriate agonists (16). Ultimately, the ability of the alveolar epithelium to respond to sudden changes in fluid flux such as those accompanying ALI will be contingent on the coordinated response of ATI and ATII cells.

III. EFFECTS OF HYPOXIA ON ALVEOLAR EPITHELIUM

Patients with ALI usually have pulmonary edema leading to gas exchange impairment, which results in significant morbidity and mortality. Starling forces regulate edema formation during lung injury; however, the lung ability to clear edema is dependent on active sodium transport where sodium enters the cell via apical ENaC and is extruded by the basolateral Na-K-ATPase, with water following the sodium gradient (32). The Na-K-ATPase, a membrane protein critically important for the maintenance of the ion gradients required for cell homeostasis, consists of a catalytic α-subunit and a regulatory β-subunit. Active sodium and potassium transport by this protein is responsible for ~20–80% of the cell’s resting metabolic rate and ~30% of cellular ATP consumption. Exposure of the alveolar epithelium to hypoxic conditions has significant adverse effects on epithelial function (20). Important observations have been made over the recent past on the effects of acute hypoxia on alveolar epithelial function. We propose that during hypoxia, the cell senses the lack of oxygen, and via a complex signaling process involving mitochondrial reactive oxygen species (ROS) and the ubiquitin system, regulates its adaptive response by stabilizing hypoxia-inducible factor-1 (6, 17, 38). This in turn leads to activation of transcription factors

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**Fig. 2. The new paradigm for alveolar salt transport.** Sodium (Na\(^+\)) is absorbed from the apical surface of both ATI and ATII cells via ENaC, both highly selective channels (HSC) and nonselective channels (NSC channels), and via cyclic nucleotide-gated channels (CNGC; seen only in ATI cells). Electroneutrality is conserved with chloride (Cl\(^-\)) movement through the cystic fibrosis transmembrane conductance regulator (CFTR) or through chloride channels (CLC) in ATI and ATII cells and/or paracellularly through tight junctions. In ATII cells, β-adrenergic agonists activate both ENaC and CFTR; in contrast, ENaC is activated in ATI and ATII cells by dopamine, but CFTR is not. Purines inhibit ENaC in ATI cells, but their effect on ENaC in ATII cells is yet to be determined. Na\(^+\) is transported from the basal surface of both cell types into the interstitial space by Na\(^+\)-K\(^+\)-ATPase. Potassium (K\(^+\)) may be transported from alveolar epithelial cells via K\(^+\) channels located on the apical surface of ATI cells or through potential-dependent basolateral K\(^+\) channels in ATII cells. CNGC are alternative pathways for the movement of Na\(^+\) that would be amiloride insensitive; functional CNGC can be found only in ATII cells and could also serve as a pathway for calcium (Ca\(^{2+}\)) entry into the cells. If the net ion movement is from the apical surface to the interstitium, an osmotic gradient would be created, which would in turn direct water transport in the same direction, either through aquaporins (AQP) or by diffusion.
that ultimately induce protection against hypoxia. In parallel, the ubiquitin system regulates a decrease in cellular Na-K-ATPase activity during hypoxia as generation of mitochondrial ROS activate PKC-ζ and consequent phosphorylation and ubiquitination of the Na pump (9, 10). These steps ultimately promote endocytosis of the pump from the plasma membrane into intracellular pools, and if hypoxia persists, the degradation of the pump at the plasma membrane (Fig. 3). Further work is needed to better understand the effects of hypoxia on alveolar epithelial function and to design optimal therapeutic strategies to prevent these deleterious effects.

IV. TRANSFORMING GROWTH FACTOR-β AND ALI

The cytokine transforming growth factor-β1 (TGF-β1) belongs to a family of proteins that includes three members: TGF-β1, TGF-β2, and TGF-β3. Stored as a nonactive protein in the extracellular space by its binding to latent-associated peptide (LAP), TGF-β1 can be activated by mild protein denaturation, oxidants, several classes of proteases, or thrombospondin-1. Furthermore, two integrins have been shown to activate TGF-β1 in the lung. The αvβ5-integrin activates TGF-β1 via an inside-out signal that results in the presentation of active TGF-β1 to its receptors in a paracrine fashion without the release of free active TGF-β1. In contrast, the αvβ6-integrin activates TGF-β1 by presenting LAP to metalloproteinases that degrade LAP and release free active TGF-β1 (reviewed in Ref. 39).

TGF-β1 has multiple roles in the lung that include, but are not limited to, development, cell proliferation, immunity, and cancer. After ALI, TGF-β1 has been most thoroughly evaluated during the late phases of tissue repair where it plays a critical role in the development of pulmonary fibrosis (40). However, recent experimental evidence indicates that the expression levels of several TGF-β1-inducible genes are dramatically increased before the development of alveolar flooding caused by exposure to bleomycin (23) or nickel (42). In vitro, TGF-β1 directly increases the permeability of lung endothelial and epithelial monolayers (3, 8, 35). In vivo, active TGF-β1 is a critical mediator of alveolar edema induced by bleomycin, Escherichia coli endotoxin (35), or nickel (42). Furthermore, TGF-β1 inhibits alveolar epithelial sodium and fluid transport, both in vitro and in vivo, via an ERK1/2-dependent repression of αENaC mRNA and protein expression (13). Finally, TGF-β1 potentiates Fas-induced lung epithelial cell apoptosis (15), inhibits surfactant release (2), and induces the expression of the plasminogen activator inhibitor by distal epithelial cells (4), three important mechanisms associated with lung epithelial dysfunction in ALI (41).

The potential clinical relevance of these recent observations is beginning to come into focus. Earlier clinical studies had shown that the TGF-β1-inducible gene, procollagen type III, is one of the earliest and best predictors of the severity of ALI in humans (7). A recent study including a small number of patients showed the presence of functionally active TGF-β1 in the bronchoalveolar lavage fluid from patients within 24 h of the diagnosis of ALI, whereas none was detected in control subjects (11). Investigators at Emory University have provided strong experimental and clinical evidence supporting a pathophysiological role for TGF-β1 in alcoholics. Specifically, these investigators have identified that alcohol abuse increases the risk for developing ALI approximately fourfold after sepsis (31). In experimental models they have shown that alcohol-mediated susceptibility to ALI is related to increased lung endothelial and epithelial permeability and a severe reduction in the level of glutathione in the air spaces. Recent findings from these models implicate angiotensin II-induced expression of latent TGF-β1 in the lungs that, in response to a second insult such as sepsis, is rapidly activated and released into the alveolar space where it promotes permeability of the alveolar-capillary barrier (1). Figure 4 shows a proposed scheme

Fig. 3. Proposed schema by which hypoxia leads to internalization and degradation of the alveolar epithelial Na-K-ATPase pump. Reactive oxygen species (ROS) generated by mitochondria in response to hypoxia activate PKC-ζ, which phosphorylates the pump and thereby initiates a series of steps that ultimately leads to internalization and degradation of the pump by the ubiquitin system.

Fig. 4. Proposed hypothetical scheme by which activation of latent transforming growth factor (TGF)-β1 within the lung contributes to the pathophysiology of acute respiratory distress syndrome (ARDS) and how chronic alcohol abuse amplifies this sequence. TGF-β1 is normally bound in cells or within the extracellular matrix by a latent-associated peptide that keeps it inactive (see text for details). During acute inflammatory stresses such as sepsis, TGF-β1 is released and activated into the alveolar space where it contributes to acute epithelial barrier dysfunction, which is a cardinal feature of ARDS. Chronic alcohol abuse, via the sequential actions of angiotensin II and the consequent redox stress, increases both the lung-specific expression of latent TGF-β1 as well as its activation and release into the alveolar space, mechanisms that may contribute to the ~4-fold increased risk of ARDS observed in otherwise healthy alcoholics in response to sepsis.

The cytokine transforming growth factor (TGF)-β1 within the lung contributes to the pathophysiology of acute respiratory distress syndrome (ARDS) and how chronic alcohol abuse amplifies this sequence. TGF-β1 is normally bound in cells or within the extracellular matrix by a latent-associated peptide that keeps it inactive (see text for details). During acute inflammatory stresses such as sepsis, TGF-β1 is released and activated into the alveolar space where it contributes to acute epithelial barrier dysfunction, which is a cardinal feature of ARDS. Chronic alcohol abuse, via the sequential actions of angiotensin II and the consequent redox stress, increases both the lung-specific expression of latent TGF-β1 as well as its activation and release into the alveolar space, mechanisms that may contribute to the ~4-fold increased risk of ARDS observed in otherwise healthy alcoholics in response to sepsis.
by which activation of latent TGF-β1 contributes to ALI and how both lung-specific expression of TGF-β1 and its activation and release into the airway are amplified by chronic alcohol abuse. This conceptual framework provides the basis for investigating other conditions that cause chronic redox stress and therefore would be predicted to increase TGF-β1 expression and activation. Together, these experimental and clinical results indicate that the activation of TGF-β1 could play an important role not only in the development of lung fibrosis associated with ALI but also in the early exudative phase of this syndrome in humans.

SUMMARY

Alveolar fluid clearance is a complex process that requires the coordinated transport of sodium, chloride, water, and other molecules across the normally tight epithelium barrier into the interstitial space. Although it is clear that the active transport of sodium drives a significant fraction of overall fluid clearance, it is clear that other mechanisms, including chloride transport, are involved. Recent studies that have taken an integrative physiological approach in both experimental models and in clinical studies are beginning to elucidate the critical role of alveolar fluid clearance in both health and in the response to ALI. Studies of isolated ATII cells and, more recently, ATI cells have enabled investigators to identify common as well as cell-specific mechanisms by which the lung removes excess alveolar fluid from the distal air spaces. In addition, the application of powerful techniques such as RNA interference and single-cell patch clamping in intact lung slices has advanced the field rapidly. Recent studies, including those highlighted in the four presentations in this symposium, are revealing the complex mechanisms that maintain this important and unique microenvironment.

REFERENCES


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