

Effects of β_2 -adrenergic receptor overexpression on alveolar epithelial active transport

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β -Adrenergic receptor (β AR) agonists accelerate the clearance of edema from the alveolar airspace by increasing the function of epithelial transport proteins, including epithelial Na^+ channels and Na,K -adenosinetriphosphatases. To improve our understanding of the role of the β_2 AR in regulating alveolar fluid clearance, we used an adenoviral-mediated gene transfer strategy to effect significant increases in membrane-bound β_2 AR number and function in the alveolar epithelium of normal rats. Alveolar fluid clearance in β_2 AR-overexpressing lungs, measured by means of an isolated lung model in the absence of catecholamine supplementation, was 100% greater than in controls. These findings were associated with significant increases of epithelial Na^+ channel function and Na,K -adenosine triphosphatase function in the peripheral lung. Experiments performed with adrenalectomized rats, a β_2 -agonist (procaterol), and a nonspecific β -antagonist (propranolol) indicate that overexpression maximally up-regulates β_2 -adrenergic-responsive alveolar fluid clearance and improves responsiveness to endogenous catecholamines. Mechanistic studies in human lung epithelial cells (A549) indicate that receptor overexpression prevents homologous receptor desensitization, possibly by overwhelming endogenous regulatory pathways. Our studies demonstrate that overexpression of β_2 AR in lung epithelial cells can be used to study the role and regulation of alveolar β_2 ARs. They also suggest a therapeutic role for the β_2 AR in the treatment of pulmonary edema. (*J Allergy Clin Immunol* 2002;110:S242-6.)

Key words: Alveolar fluid clearance, alveolar epithelium, β_2 -adrenergic receptor, gene transfer, pulmonary edema

Edema fluid is cleared from the alveolar airspace by active Na^+ transport processes that can be accelerated by both β_1 -adrenergic receptors (β_1 ARs) and β_2 ARs.¹⁻⁴ This active transport is accomplished by alveolar epithelial transport proteins, including basolaterally located Na,K -adenosinetriphosphatases (Na,K -ATPases) and apically positioned Na^+ entry pathways such as the amiloride-sensitive epithelial Na^+ channel (ENaC).⁵ These transporters extrude Na^+ from the airspace, generating a

Abbreviations used

ad β_2 AR: Adenovirus containing, in part, a human β_2 AR cDNA
adNull: Adenovirus not containing cDNA
AFC: Alveolar fluid clearance
ATPase: Adenosine triphosphatase
 β AR: β -Adrenergic receptor
cAMP: Cyclic adenosine monophosphate
ENaC: Epithelial Na^+ channel
GRK2: G-protein-coupled receptor kinase 2

transepithelial osmotic gradient that causes movement of fluid from the alveolus through transcellular and paracellular pathways (Fig 1). Data from human studies and experimental models indicate that these transporters may be down-regulated or dysfunctional in the setting of acute lung injury.⁶⁻¹² These findings suggest that pulmonary edema is due not only to increased fluid flux into the airspace but also to impairment of clearance mechanisms. In support of this paradigm, we have reported that adenoviral-mediated overexpression of Na,K -ATPase subunit genes increases active Na^+ transport in human and rat lung epithelial cells^{6,13,14} and mitigates oxidant-mediated decreases in active Na^+ transport in rat fetal distal lung epithelial cells.¹⁵ These findings correlate with significant increases in alveolar fluid clearance (AFC) in normal rats¹³ and in rats with high- and low-pressure pulmonary edema.^{6,16} Other groups have similarly reported that Na,K -ATPase gene transfer attenuates thiourea-induced lung injury in mice.¹⁷ These studies demonstrate that enhancement of alveolar epithelial active Na^+ transport and maintenance of a "dry" alveolus can protect the lung from acute injury.

β -Agonists increase active Na^+ transport in alveolar epithelial cells,^{2,18} normal animal lungs,^{4,19,20} and injured animal lungs¹⁰ by up-regulating both apical Na^+ entry pathways (including ENaCs)²¹ and basally positioned Na,K -ATPases. This up-regulation can be effected by both β_1 ARs and β_2 ARs^{3,22} and is due to new message transcription, enhanced trafficking of transport proteins to the cell membrane, and increased activity of solute transporters in the cell membrane.^{4,23,24} Recently, it has been reported that subcutaneous infusion of a nonspecific β -agonist (isoproterenol) for 48 hours significantly diminishes lung β AR density and β_2 AR-sensitive edema clearance in rat lungs.²⁵ The attributes of β -agonists

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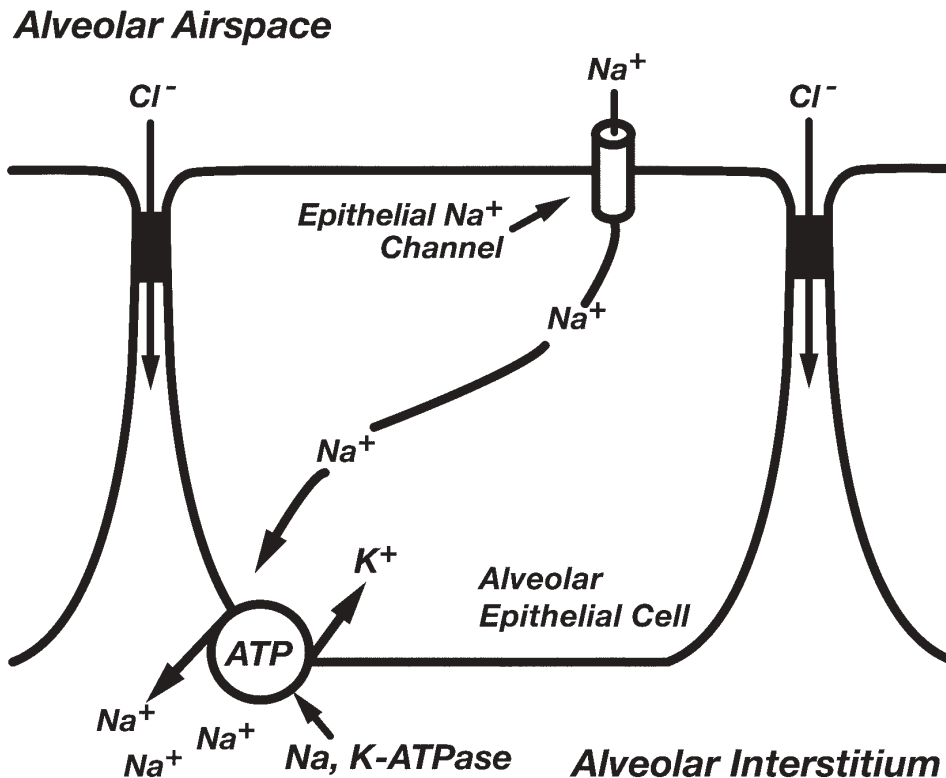


FIG 1. Clearance of pulmonary edema by active Na^+ transport. Alveolar epithelial transport proteins generate a transepithelial osmotic gradient that causes fluid to move out of the airspace by transcellular and paracellular pathways.

combined with the propensity of alveolar $\beta_2\text{AR}$ s for homologous desensitization prompted us to hypothesize that overexpression of a $\beta_2\text{AR}$ in the alveolar epithelium of normal rats could improve $\beta_2\text{AR}$ function, alter receptor desensitization patterns, and enhance catecholamine-responsive active Na^+ transport.

To test our hypothesis, we used a surfactant-based delivery system to spontaneously infect breathing adult, male Sprague-Dawley rats with E1a-/E3- adenoviruses that contain expression cassettes with a human cytomegalovirus immediate-early gene promoter-enhanced element and either a human $\beta_2\text{AR}$ cDNA (ad $\beta_2\text{AR}$) or no cDNA (adNull).²⁶ Rats were infected with 4×10^9 pfu of adenovirus and then were allowed 7 days of recovery to allow vector-induced host responses to subside. We have previously reported that this method yields widespread gene transfer that is confined to the alveolar epithelium in normal and injured rat lungs.^{6,13,27} To confirm that our strategy effected efficient gene transfer, total RNA and protein were prepared from whole-lung homogenates for reverse transcriptase-polymerase chain reaction and Western analyses with human $\beta_2\text{AR}$ -specific primers and antibodies. These studies demonstrated high-level expression of human $\beta_2\text{AR}$ mRNA and protein in ad $\beta_2\text{AR}$ -infected lungs. Immunofluorescent staining localized the transgene to the alveolar epitheli-

um. Confirmation of appropriate trafficking of the transgenic $\beta_2\text{AR}$ to the cell membrane was obtained by Western analysis of whole-cell membrane fractions isolated from the peripheral 2 to 3 mm of the lung. Membrane-bound human $\beta_2\text{AR}$ was noted only in ad $\beta_2\text{AR}$ -infected lungs. Transgene function was assayed by measuring cyclic adenosine monophosphate (cAMP) production by cell membranes from the peripheral lung in the presence and absence of the β_2 -agonist procaterol (10^{-8} mol/L $\times$ 30 minutes). $\beta_2\text{AR}$ function in receptor-overexpressing lungs was increased up to 5-fold as compared with membranes from controls. These experiments indicate that adenoviral-mediated gene transfer can produce overexpression of a functional human $\beta_2\text{AR}$ in the alveolar epithelium of normal rats.

To test whether alveolar $\beta_2\text{AR}$ overexpression has a physiologically relevant effect on alveolar active Na^+ transport, we infected normal rats with ad $\beta_2\text{AR}$ or adNull as described above and measured AFC by using a fluid-filled, perfused, isolated lung model that allows for concurrent measurement of edema clearance and alveolar permeability.^{6,13} Clearance in these $\beta_2\text{AR}$ -overexpressing lungs was 100% greater than in sham-infected and adNull-infected controls ($n = 5$ per group) and ~40% greater than in control lungs treated with high-dose procaterol (10^{-6} mol/L, via the alveolar instillate and vascu-

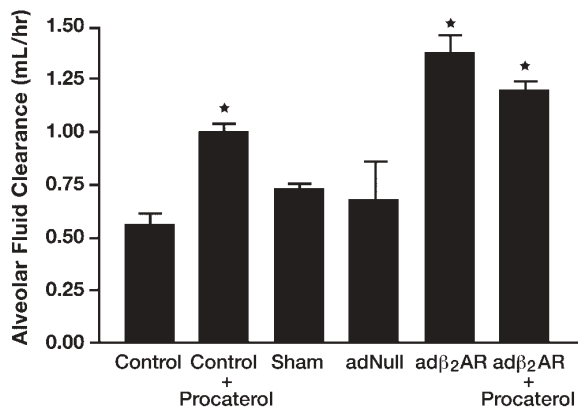


FIG 2. Alveolar fluid clearance in isolated rat lungs. Alveolar fluid clearance in uninfected rat lungs with and without addition of the β_2 AR-specific agonist procaterol (10^{-6} mol/L) (control and control plus procaterol) and lungs 7 days after ad β_2 AR, adNull, or sham infection. Addition of procaterol to the alveolar instillate and vascular perfusate of ad β_2 AR-infected lungs did not increase alveolar fluid clearance more than β_2 AR overexpression alone (rightmost bar). Data are mean $\pm$ SD. * $P < .01$ vs uninfected controls. (From Dumasius V, Sznajder JI, Azzam ZS, et al. β_2 -Adrenergic receptor overexpression increases alveolar fluid clearance and responsiveness to endogenous catecholamines in rats. *Circ Res* 2001;89:907-14. With permission).

lar perfusate) during the clearance measurements (Fig 2). Treatment of β_2 AR-overexpressing lungs with procaterol during clearance measurements did not increase clearance further, suggesting that receptor overexpression maximally up-regulates β_2 AR-sensitive AFC.

To develop an understanding of how β_2 AR overexpression increases active solute transport, we assayed apical Na^+ channel function by instilling the Na^+ channel blocker amiloride (10^{-6} mol/L, $n = 4/\text{group}$) into the alveolar airspace during the isolated lung studies and observed that β_2 AR-overexpressing lungs were 100% more sensitive to amiloride than were similarly treated sham-infected and adNull-infected controls. Western analysis of apical cell membrane fractions isolated from the peripheral lung showed a 100% increase in membrane-bound expression of the α -subunit of the ENaC only in ad β_2 AR-infected lungs. In parallel studies, we measured ouabain-sensitive ATP hydrolysis by basolateral cell membranes isolated from the peripheral lung. Doing so in the presence of high $[\text{Na}^+]$ and low $[\text{K}^+]$ produces an index of functional, membrane-bound Na,K -ATPase number. These experiments revealed that receptor overexpression increases basolateral membrane Na,K -ATPase abundance by $>300\%$. These data indicate that augmentation of alveolar epithelial β_2 AR function up-regulates both apical Na^+ entry pathways (ie, αENaC) and basolateral Na,K -ATPases.

The observed increases in AFC in the absence of catecholamine supplementation raises concerns of consti-

tutive receptor activity. Thus, we measured the effects of β_2 AR overexpression on AFC in rats with reduced serum catecholamine levels after adrenalectomy and in rats treated with the nonspecific β -antagonist propranolol (1 mg/kg TID $\times$ 5 days). β_2 AR overexpression did not increase AFC in these lungs to the same degree as that seen in normal rats, indicating that receptor overexpression improves responsiveness to endogenous catecholamines rather than causing constitutive receptor activity.

An alternative explanation for the finding of increased AFC in the absence of exogenous agonists might be that receptor overexpression alters receptor-regulatory pathways that affect sensitivity to β -agonists. To test this hypothesis, we overexpressed the β_2 AR in human lung epithelial cells (A549) by using a previously established adenoviral-mediated gene transfer strategy.¹⁴ Infection of these cells with 50 pfu/cell of ad β_2 AR significantly increased β_2 AR procaterol-sensitive cAMP levels in whole-cell homogenates. To pursue how overexpression affects β_2 AR desensitization patterns, ad β_2 AR-infected, adNull-infected, and sham-infected control cells were treated with procaterol (10^{-6} mol/L) in the presence of isobutylmethylxanthine [10^{-4} mol/L] for up to 60 minutes beginning 48 hours after infection. cAMP production in cells infected with adNull rose by $>300\%$ within 5 minutes and promptly tapered off to a level similar to that of baseline within 7 minutes. Conversely, in β_2 AR-infected cells, cAMP production increased by $>500\%$ from baseline. No significant diminution in cAMP levels was observed in ad β_2 AR-infected cells, suggesting that receptor overexpression attenuates homologous desensitization in this model. To gauge receptor regulation in the setting of overexpression, we measured membrane-associated levels of G-protein-coupled receptor kinase 2 (GRK2) by Western analysis. β_2 AR-overexpressing cells demonstrated progressive decreases of GRK2 levels, whereas adNull-infected controls showed a progressive increase. In light of this early data, we hypothesize that receptor overexpression in lung epithelial cells may not be associated with the compensatory increase in receptor phosphorylation that would be expected in the presence of high levels of intracellular cAMP.

CONCLUSIONS

Our study demonstrates that overexpression of a β_2 AR gene in the alveolar epithelium can augment alveolar β_2 AR function and positively affect AFC. These effects are mediated by up-regulation of solute transport proteins on both the apical and basolateral cell membrane domains through enhanced responsiveness to endogenous catecholamines and possibly through alteration of receptor phosphorylation patterns.

DISCUSSION SESSION

Question: Have you studied the other downstream effects of β_2 -receptor overexpression?

Dr Factor: We have just started to study downstream effects. It appears at this point that β -receptor overexpression reduces indices of apoptosis in lungs with injury due to hyperoxia. However, the mechanism is unknown. We recently reported that the adenovirus very efficiently transduces the injured lung. We actually took rats with severe pulmonary edema and lung injury due to hyperoxia, and after the induction of injury we were able to transduce the alveolar epithelium with an efficiency that was equal to or perhaps even greater than that seen in normal lungs. By improving or increasing permeability of the alveolar epithelium, we provide the virus increased access to receptors it might not otherwise have. It works very well in pulmonary edema.

Question: My own experience with adenovirus is that there is some trouble getting through tight junctions. On the other hand, because you use CMV promoters, they get into any cell. Have you found expression in blood vessels, epithelial cells, or mucous cells?

Dr Factor: In normal rats, we found very little expression outside of the alveolar epithelium. Occasionally, we have seen an endothelial cell that is positive. Other groups have reported better expression in endothelial cells and other organs with alveolar delivery. When the lungs are injured and permeability is increased, we see significant increases in endothelial cell expression of the marker genes.

Question: I was intrigued by the changes in GRK levels that you noted. Did you assess total levels in addition to membrane-associated GRK2 levels?

Dr Factor: We looked at whole-cell homogenates, and they do not seem to change. We are beginning nuclear run-on experiments to see if message levels also change. It seems as if the cell does not up-regulate GRK2, despite very significant increases in total cell cAMP levels.

Question: You mentioned that the fluid is pumped into the interstitial space. Where does it go from there? Since vascular pressures are higher, it will not go into the vasculature.

Dr Factor: It is not known. The fluid is thought to be drained through the lymphatic system. Occasionally the fluid is found in the pleural space. A significant portion of it is taken back into the vasculature. From a clinical perspective, we work to keep the capillary pressures as low as possible. If the capillary pressures are too high, no matter how much pump activity there is, pulmonary edema will occur. If the pressures are too high, there will probably be no net clearance. We reported that with overexpression of a sodium potassium ATPase gene in a model of acute left heart failure, if the left atrial pressure is elevated high enough, there is no clearance of pulmonary edema.

Question: How does overexpression of the β_2 -receptor affect the kinetics of surfactant production, particularly the phospholipid component?

Dr Factor: We have not studied this.

Question: How does acute lung injury down-regulate the function of the responsiveness of the β -receptor? Are there ways to modulate that pathway?

Dr Factor: The effects of lung injury on the β -receptor are complex. Although it is desensitized, net cAMP production, at least in hyperoxic lungs, is increased. We have measured receptor number and receptor coupling to adenylate cyclase and observed that total lung cAMP is slightly increased in hyperoxia. Similarly, the number of membrane-bound receptors increased by 50%. So, when you actually look at cAMP per receptor, however, the receptors in hyperoxic lungs produce no more cAMP per receptor than a normal lung. When we looked at adenylate cyclase production, it increased by 500% or 600%. This suggests that the membrane capacity to produce cAMP is dramatically increased and that there is an unused reserve of cAMP production. When we calculate a

receptor coupling index, we find that normal lungs can produce about 80% of their maximum cAMP; conversely, hyperoxic lungs can produce about 20% of the maximal cAMP. This is associated with failure or lack of up-regulation of GRK2. There appears to be an unmet need for cAMP production that for some reason does not occur with injured lungs.

REFERENCES

1. Matthay M, Landolt C, Staub N. Differential liquid and protein clearance from the alveoli of anesthetized sheep. *J Appl Physiol* 1982;53:96-104.
2. Crandall ED, Heming TA, Palombo RL, et al. Effects of terbutaline on sodium transport in isolated perfused rat lung. *J Appl Physiol* 1986;60:289-94.
3. Sakuma T, Tuchiha C, Ishigaki M, et al. Denopamine, a β_1 -adrenergic agonist, increases alveolar fluid clearance in ex vivo rat and guinea pig lungs. *J Appl Physiol* 2001;90:10-6.
4. Berthiaume Y, Staub NC, Matthay MA. Beta-adrenergic agonists increase lung liquid clearance in anesthetized sheep. *J Clin Invest* 1987;79:335-43.
5. Factor P. Role and regulation of lung Na,K-ATPase. *Cell Mol Biol (Noisy-le-grand)* 2001;47:347-61.
6. Factor P, Dumasius V, Saldias F, et al. Adenovirus-mediated transfer of an Na⁺/K⁺-ATPase β_1 subunit gene improves alveolar fluid clearance and survival in hyperoxic rats. *Hum Gene Ther* 2000;11:2231-42.
7. Matthay MA, Wiener-Kronish JP. Intact epithelial barrier function is critical for the resolution of alveolar edema in humans. *Am Rev Respir Dis* 1990;142:1250-7.
8. Verghese GM, Ware LB, Matthay BA, et al. Alveolar epithelial fluid transport and the resolution of clinically severe hydrostatic pulmonary edema. *J Appl Physiol* 1999;87:1301-12.
9. Azzam ZS, Saldias FJ, Comellas A, et al. Catecholamines increase lung edema clearance in rats with increased left atrial pressure. *J Appl Physiol* 2001;90:1088-94.
10. Saldias FJ, Comellas A, Ridge KM, et al. Isoproterenol improves ability of lung to clear edema in rats exposed to hyperoxia. *J Appl Physiol* 1999;87:30-5.
11. Carter EP, Wangenstein OD, Dunitz J, et al. Hyperoxic effects on alveolar sodium resorption and lung Na-K-ATPase. *Am J Physiol* 1997;273:L1191-202.
12. Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001;163:1376-83.
13. Factor P, Saldias F, Ridge K, et al. Augmentation of lung liquid clearance via adenovirus-mediated transfer of a Na,K-ATPase β_1 subunit gene. *J Clin Invest* 1998;102:1142-50.
14. Factor P, Senne C, Dumasius V, et al. Overexpression of the Na⁺,K⁺-ATPase α_1 subunit increases Na⁺,K⁺-ATPase function in A549 cells. *Am J Respir Cell Mol Biol* 1998;18:741-9.
15. Thome U, Chen L, Factor P, et al. Na,K-ATPase gene transfer mitigates an oxidant-induced decrease of active sodium transport in rat fetal ATRII cells. *Am J Respir Cell Mol Biol* 2001;24:245-52.
16. Azzam ZS, Dumasius V, Saldias FJ, et al. Na,K-ATPase overexpression improves alveolar fluid clearance in a rat model of elevated left atrial pressure. *Circulation* 2002;105:497-501.
17. Stern M, Ulrich K, Robinson C, et al. Pretreatment with cationic lipid-mediated transfer of the Na⁺K⁺-ATPase pump in a mouse model *in vivo* augments resolution of high permeability pulmonary oedema. *Gene Ther* 2000;7:960-6.
18. Cott GR, Sugahara K, Mason RJ. Stimulation of net active ion transport across alveolar type II cell monolayers. *Am J Physiol Cell Physiol* 1986;250:C222-7.
19. Saldias F, Lecuona E, Friedman E, et al. Modulation of lung liquid clearance by isoproterenol in rat lungs. *Am J Physiol Lung Cell Mol Physiol* 1998;274:L694-701.
20. Lasnier JM, Wangenstein OD, Schmitz LS, et al. Terbutaline stimulates alveolar fluid resorption in hyperoxic lung injury. *J Appl Physiol* 1996;81:1723-9.
21. Matalon S, O'Brodovich H. Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. *Annu Rev Physiol* 1999;61:627-61.

22. Tibayan FA, Chesnutt AN, Folkesson HG, et al. Dobutamine increases alveolar liquid clearance in ventilated rats by beta-2 receptor stimulation. *Am J Respir Crit Care Med* 1997;156:438-44.
23. Matalon S, Benos DJ, Jackson RM. Biophysical and molecular properties of amiloride-inhibitable Na⁺ channels in alveolar epithelial cells. *Am J Physiol* 1996;271:L1-22.
24. Bertorello AM, Ridge KM, Chibalin AV, et al. Isoproterenol increases Na⁺-K⁺-ATPase activity by membrane insertion of α -subunits in lung alveolar cells. *Am J Physiol Lung Cell Mol Physiol* 1999; 276:L20-7.
25. Morgan EE, Hodnichak CM, Stader SM, et al. Alveolar epithelial ion and fluid transport: prolonged isoproterenol infusion impairs the ability of β_2 -agonists to increase alveolar liquid clearance. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L666-74.
26. Dumasius V, Sznajder JI, Azzam ZS, et al. β_2 -Adrenergic receptor over-expression increases alveolar fluid clearance and responsiveness to endogenous catecholamines in rats. *Circ Res* 2001;89:907-14.
27. Factor P, Mendez M, Mutlu GM, et al. Acute hyperoxic lung injury does not impede adenoviral-mediated alveolar gene transfer. *Am J Respir Crit Care Med* 2002;165:521-6.