

β_2 -Adrenergic Stimulation Blunts Inhibition of Epithelial Ion Transport by Hypoxia of Rat Alveolar Epithelial Cells

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Key Words

Pulmonary edema • Cl^- transport • ENaC • Na^+/K^+ -ATPase • β_2 -adrenergic signaling

Abstract

Hypoxia impairs alveolar fluid clearance by inhibition of Na^+ reabsorption, and also impairs β_2 adrenergic signaling in alveolar epithelium. Since both are major rescue mechanisms preventing pulmonary edema, we studied whether acute and prolonged treatment with terbutaline would prevent hypoxic inhibition of ion transport. Short circuit currents (ISC) were measured on normoxic and hypoxic (1.5% O_2 ; 24h) primary rat alveolar type II (ATII) cells in absence and presence of terbutaline (1 to 100 μM , 24h). Control and pre-treated cells were stimulated acutely with terbutaline. Transepithelial transport was measured as short circuit current (ISC) in Ussing chambers. Terbutaline induced a rapid decrease ISC (-20%) followed by a slow raise. The transient change in ISC was not inhibited by amiloride but was prevented after Cl^-

depletion indicating a Cl^- current. The slow increase after this transient was amiloride-sensitive indicating a Na^+ current. Total ISC, its amiloride-sensitive component, and the transient decrease upon terbutaline stimulation were decreased by hypoxia. 24h treatment with terbutaline stimulated these currents in normoxia and hypoxia, although stimulation was less in the latter. 24h treatment with terbutaline increased the capacity of Na^+/K^+ -ATPase and ENaC as measured after permeabilization with amphotericin. These changes were not paralleled by altered mRNA expression. Acutely applied terbutaline induced a 4-fold increase in cAMP formation in normoxia; terbutaline-induced cAMP-formation was impaired by hypoxia (-20%). Pre-treatment with terbutaline for 24h decreased terbutaline-induced cAMP formation by 85%. Despite this desensitization, addition of terbutaline to terbutaline pre-treated cells caused a larger increase in Cl^- and Na^+ transport both in normoxia and hypoxia than in non pre-treated cells. These results indicate that β_2 adrenergic stimulation increased Na^+ - and Cl^- transport in ATII cells even in hypoxia thus restoring normal reabsorption.

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Introduction

Active Na⁺ transport is essential for alveolar fluid balance and the clearance of pulmonary edema since it generates the driving force for the reabsorption of water from the alveolar space. Reabsorption is vital for clearing excess fluid that might accumulate when filtration into the alveolar space is increased causing alveolar edema as in ALI, ARDS, and in alveolar hypoxia as at high altitude. In such situations β -agonist therapy has been shown to reduce the severity of pulmonary edema by attenuation of inflammation and by stimulation of alveolar reabsorption [1-3].

Alveolar reabsorption is driven by reabsorption of NaCl. Na⁺ enters cells through apical epithelial Na⁺ channels (ENaC) and the Na⁺/K⁺-ATPase extrudes it across the basolateral membrane (for review see e.g. [4]). Electro-neutrality is warranted by a concomitant vectorial transport of Cl⁻, water follows passively. Reduced alveolar PO₂ and alveolar edema impairs the oxygen supply to alveolar epithelial cells, which decreases alveolar epithelial Na⁺ transport by inhibition of ENaC and Na⁺/K⁺-ATPase as shown in cultured alveolar epithelial cells and in the fluid-instilled lung [5-8]. Inhibition seems to be caused by internalization of ENaC [9] and Na⁺/K⁺-ATPase [10] and by decreasing their expression [5, 6]. An impairment of Cl⁻ transport by hypoxia has not been demonstrated in the lung. In MDCK-cells, improved oxygenation by growing cells in an air-liquid interface or submerged but in a 95% oxygen environment increased CFTR-maturation and/or trafficking, which indirectly points to a decreased activity in a low oxygen environment [11].

β_2 -adrenergic agents are well known stimulators of alveolar Na⁺ transport thus favoring edema clearance (for review see e.g. [12]). Data from knock-out mice indicate that the presence of functional β_2 -receptors (β_2 AR) is essential for the clearance of alveolar edema via Na⁺ channels even without exogenous stimulation [13]. This is in line with results from the BALTI study [14], which shows that in humans with ALI/ARDS sustained treatment with intravenous beta-agonists reduces extra-vascular lung water. Stimulation of alveolar fluid reabsorption by β_2 -agonists is caused by a stimulation of Na⁺ transport [15, 16] and requires the presence of CFTR [17]. Transport stimulation can be explained by insertion of endogenous ENaC [9]. Also stimulation of expression has been reported, which is,

however, a slow process. [18]. Thus it appears that β_2 AR stimulation might prevent hypoxia-effects on alveolar reabsorption.

We have shown recently that hypoxia directly impairs β_2 AR signaling in primary rat alveolar epithelial cells, which was indicated by a decreased formation of cAMP [19]. It was paralleled by a decreased number of β_2 -receptors and stimulatory G α s proteins in the plasma membrane. But the main effect of hypoxia seemed to be a relative increase in the activity of the inhibitory G α_i protein [19]. Functional data also show impaired β_2 AR function in hypoxia as indicated by a decreased degree of stimulation of alveolar fluid clearance and Na⁺/K⁺-ATPase activity by isoproterenol in lungs of hypoxic rats (24h, 8% O₂) [20]. Short term hypoxia of the isolated perfused rat lung decreased tissue cAMP upon stimulation with terbutaline [21]. In line are results obtained on cultured primary rat ATII cells where hypoxia decreased terbutaline-induced cAMP production. In contrast, Vivona et al. [7] showed that in rats exposed to hypoxia (24h, 8% O₂) terbutaline-stimulated fluid reabsorption reached the same level as in normoxic animals, which would point to no impairment of β_2 AR function in hypoxia. Similarly, in mountaineers, inhalation of high doses of the β_2 -stimulator salmeterol reduced the incidence of high altitude pulmonary edema [2]. This has been suggested to result from a stimulation of alveolar reabsorption [2] even though the sympathetic activity is increased [22]. Prolonged increase in sympathetic activity causes desensitization of β_2 -adrenergic receptors, which might decrease the ability to respond to β_2 -agonists [23].

We studied therefore whether acute and prolonged stimulation with terbutaline blunts the hypoxia-induced inhibition of alveolar reabsorption and which transport pathways might be affected. The results should help clarify the discrepancies showing full [7] and impaired [20] β_2 AR stimulation of alveolar fluid clearance in hypoxia, which is an important basis for the prevention and treatment of alveolar edema by β_2 AR agonist application. Results obtained on monolayers of primary rat alveolar epithelial cells cultured in hypoxia indicate that acute application of terbutaline stimulates Na⁺- and Cl⁻ transport, although not to the same extent as in normoxic cells. Prolonged treatment with terbutaline restores normoxic values of transport activity. Preliminary results have been presented as abstracts [24, 25].

Materials and Methods

Reagents

Media were prepared from deionized water (18 MΩcm²) and analytical grade reagents. Na-gluconate, amiloride, terbutaline, isobutyl-1-methylxanthine (IBMX), CGP-20712A (CGP) and ICI-118551 (ICI) were from Sigma-Aldrich (Deisenhofen, Germany). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), gentamycin, neonatal calf serum (NCS), rat IgG were from PAA (Coelbe, Germany), and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) was from Life Technologies (Karlsruhe, Germany). Elastase was from Elastin Products (Owensville, MS).

Cell preparation

Experiments were performed on primary cultures of cells of alveolar type II cells (AII) isolated from lungs of normoxic, male rats (Sprague-Dawley; 160-180 g) as previously described [6, 26]. Briefly, lungs from rats anesthetized with 100 mg/kg pentobarbital sodium i.p. (Trapanal®, Byk Gulden, Germany) were perfused with PBS while being ventilated with air. AII cells were isolated by elastase digestion, mincing of lung tissue, filtration, and differential adhesion to IgG-coated plates. Non-adherent cells were suspended in DMEM-N (DMEM supplemented with 10% NCS, 4 mM glutamine, and 50 µg/ml gentamycin), and were plated on tissue culture-treated nuclepore filters (pore-size: 0.4 µm; diameter: 6mm or 12 mm; Transwell; Costar, Cambridge, MA) at a seeding density of 1.5×10^6 cells/cm². Both purity and viability of AII cells were >90%. Cells were maintained in room air supplemented with 5% CO₂ at 37°C and reached confluence, typically on day 3 after plating. Formation of tight monolayers was tested by measuring transepithelial resistance (typically > 1.5kΩ) using a volt-ohmmeter and chopstick electrodes (EVOM; World Precision Instruments, Sarasota, FL). Confluent monolayers were also exposed to severe hypoxia (1.5% O₂, 5% CO₂, rest N₂) for 24 hours in an oxygen-controlled tissue culture incubator (Nunc, Wiesbaden, Germany) and were treated with terbutaline at the indicated concentrations for 24 hours in normoxia and hypoxia prior to measurements.

Ussing chamber measurements

After being mounted in Ussing chambers, monolayers were bathed with media composed of (mM) 141 NaCl, 5.4 KCl, 0.78 NaH₂PO₄, 1.8 CaCl₂, 0.8 MgCl₂, 5 glucose, and 15 HEPES, pH 7.4, at 37°C. When required, a portion of the NaCl was replaced with Na-gluconate to decrease the Cl⁻ concentration to 10 mM. Cell monolayers were kept under open-circuit conditions for about 10 min to equilibrate to the medium. The epithelium was then short-circuited by clamping the transepithelial potential to 0 mV and the short circuit current (ISC) was recorded continuously (W.Nagel, Munich, Germany) and stored on a computer for off-line analysis. The total ISC (ISC_{tot}) of control cells measured in the Ussing chambers was typically in the range between 4 and 7 µA/cm². Terbutaline was added at the indicated concentrations. Amiloride (final concentration 10 µM) was used to inhibit apical Na⁺

channels. Na⁺/K⁺-ATPase was measured after permeabilization of the apical plasma membrane with amphotericin B (5 µM). It is the portion of ISC inhibited with 3mM ouabain (ISC_{Na/K-ATPase}). Since the concentration of intracellular Na⁺ increases after permeabilization of the apical membrane, the activity of Na⁺/K⁺-ATPase measured under these conditions resembles pump-capacity [6, 27]. It is therefore a measure of the amount of active pump protein in the plasma membrane. Asymmetric media were used for the measurement of the capacity of ENaC. Regular bathing medium (high Na⁺) was on the apical side, whereas Na⁺ at the basolateral side was decreased to 25mM by substitution with choline⁺ to generate a driving force for Na⁺ transport across the apical plasma membrane when the Na⁺/K⁺-ATPase is not functioning due to basolateral permeabilization with amphotericin (5 µM). Under these conditions, ENaC-capacity (ISC_{CapAamil}) was the ISC inhibited by amiloride [6, 27].

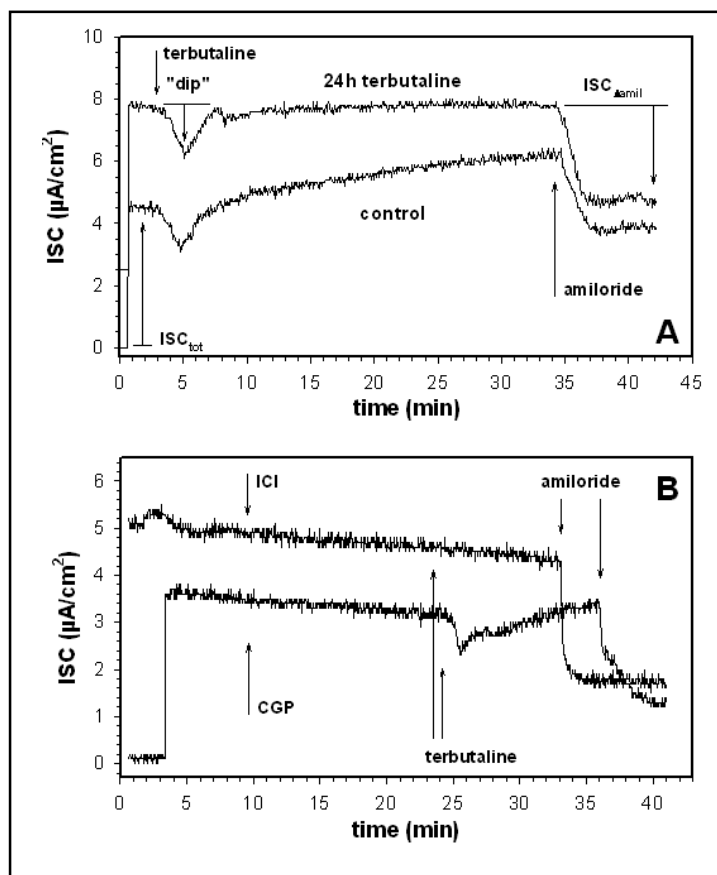
cAMP production

cAMP production was measured in normoxic or hypoxic, confluent AII cells cultured in 96-well plates after incubation in DMEM-N containing phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM) for 30 min at 37°C. β₂AR-mediated cAMP production was measured after acute stimulation with terbutaline (1 µM to 100 mM; 10 min). Reactions were terminated by aspirating the medium and lysing the cells with ice-cold HCL (0.1 N). cAMP was measured by radioimmunoassay with the kit from Immunotech (Kiel, Germany) according to the manufacturer's instructions. Values were normalized to total cell protein.

RNA isolation and RT-PCR

After the 24h-exposure to hypoxia and terbutaline, cells were kept on ice, washed twice with ice cold PBS and lysed with RLT (Qiagen, Hilden, Germany). Total RNA was isolated according to the manufacturer's instructions. RNA (0.1 µg) was transcribed with Superscript II reverse transcriptase (Life technologies, Bochum, Germany) using random hexamere primers (Roche, Mannheim, Germany). Real time quantitative PCR was performed in the Lightcycler® as described earlier [8]. The QuantiTect® SYBR Green PCR kit was used with the commercial primers for 28S rRNA, β1 Na⁺/K⁺-ATPase, and α, β, γ ENaC (QuantiTect®, Qiagen, Hilden, Germany). The primers used for amplification of α1 Na⁺/K⁺-ATPase (sense: TAT GTC TGA CGC TCA CTG CC, antisense: TGG CTG ACG TCT TGT CAA AG) and CFTR (sense: AGG AGA CTG TCC CTG GTT CC, antisense: TGG CAA TTT TAG TGC CAT TG) were from MWG (Germany). In this case the LC Fast Start PCR mix (Roche, Mannheim, Germany) was used for the PCR. Specificity of amplification was tested after separation on agarose gels stained with Gelstar (BMA, ME). Standards for quantification of mRNA expression were prepared from the PCR products after elution from the agarose gel [8]; amplification of these products showed the same efficiency as the samples. 28S rRNA was used to control for differences in the efficacy of reverse transcription. 28S rRNA remained unchanged during exposure to hypoxia and terbutaline (not shown).

Fig. 1. β_2 -adrenergic stimulation induces a transient decrease of ISC. Confluent ATII cell monolayers were cultured in normoxia and treated with terbutaline (1 μ M) for 24h before being mounted into Ussing chambers. Cells were bathed in a medium composed of (mM) 141 NaCl, 5.4 KCl, 0.78 NaH_2PO_4 , 1.8 CaCl_2 , 0.8 MgCl_2 , 5 glucose, and 15 HEPES, pH 7.4, at 37°C. (A) Typical recording showing that stimulation with terbutaline (1 μ M) caused a rapid transient decrease in ISC (“dip”) in control cells and in cells pretreated with terbutaline (1 μ M) for 24h. Inhibition of Na^+ channels with amiloride (10 μ M) resulted in a decrease in ISC. (B) Terbutaline mediated changes in ISC are specific for stimulation of β_2 -receptors: Pre-treatment with the β_2 -specific inhibitor ICI-118551 (1 μ M) prevented ISC-changes upon stimulation with terbutaline (100 μ M), whereas the β_1 -antagonist CGP-20712A (0.1 μ M) did not. ISC_{tot} denotes to the basal ISC before acute stimulation with terbutaline and in absence of amiloride, ISC_{dip} is the maximal, rapid decrease in ISC observed after acute stimulation with terbutaline, and $\text{ISC}_{\Delta\text{amil}}$ is the amiloride-inhibitable portion of ISC ($\Delta\text{amiloride}$).



Data evaluation and statistical analysis

Experiments were repeated on several monolayers of ATII cells from at least two different cell preparations. Results are presented as means \pm SD of the indicated number of measurements. All short circuit currents are shown as positive values. ISC_{tot} denotes to the short circuit current (ISC) in absence of amiloride, $\text{ISC}_{\Delta\text{amil}}$ is the portion of ISC_{tot} inhibited by amiloride. ISC_{dip} is the decrease in ISC_{tot} immediately after addition of terbutaline (Fig. 1A). Student's t-test was used to compare individual group means. Multiple comparisons were performed by one-way ANOVA followed by Fisher LSD posthoc testing. Level of significance was defined by a $P = 0.05$. Statistical analyses were performed using Sigma Stat (version 3, Erkrath, Germany).

Results

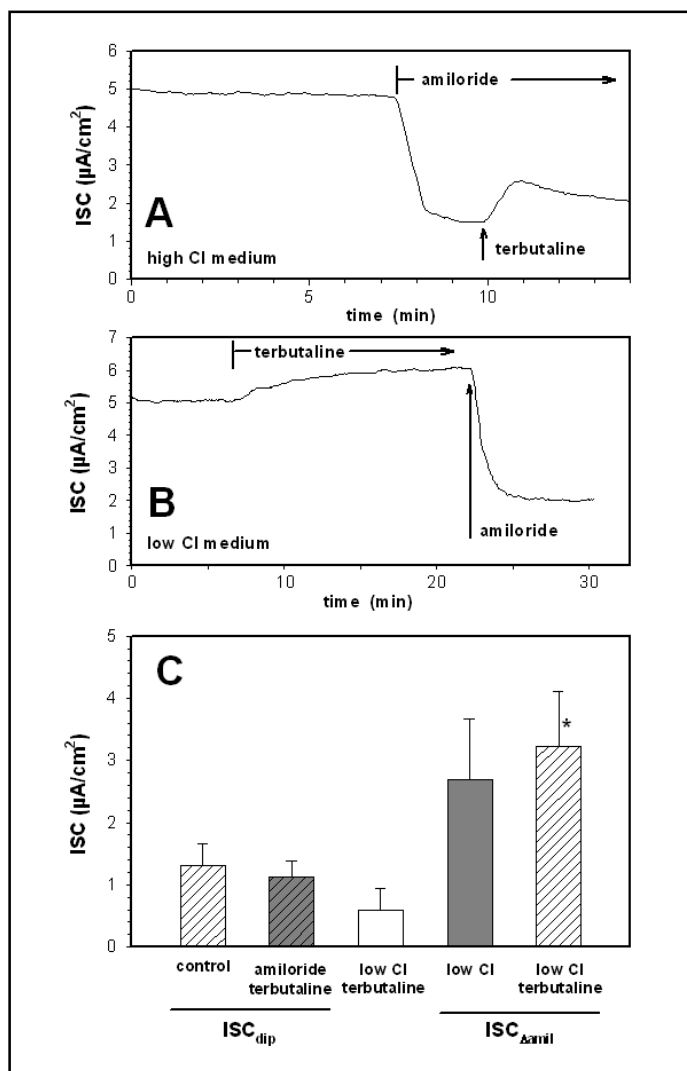
In this study we wanted to test whether acute and prolonged β_2 AR stimulation of transepithelial transport across ATII cell monolayers can prevent the transport inhibition by hypoxia [28–30], and, if so, which ion currents are affected. Our results indicate that hypoxia decreases not only basal but also the terbutaline-induced ISC, where it decreases both components, Cl^- and Na^+ transport.

Indeed, we could observe a stimulation of ISC by terbutaline not only in normoxic but also in hypoxia-exposed ATII cell monolayers.

Ionic currents involved in the acute terbutaline response

As depicted in Fig. 1A, addition of terbutaline to monolayers mounted in the Ussing chamber rapidly and transiently decreased ISC reaching a minimum after about 1 min (we called the decrease ISC_{dip}), after which ISC increased again. This response to terbutaline is specific for β_2 AR-mediated signaling since it was prevented by the specific antagonist ICI-118551 but not by the β_1 AR inhibitor CGP-20712A (Fig. 1B). We wanted to investigate which ionic currents caused these acute changes in ISC upon acute stimulation with terbutaline. In normoxic control cells ISC_{dip} amounted to approximately 1.3 $\mu\text{A}/\text{cm}^2$ when cells were stimulated with 1 μM terbutaline (Fig. 2C, control bar). It was not increased further when higher terbutaline concentrations were used (not shown). Fig. 2A shows that the acute, transient decrease in ISC was not dependent on Na^+ channels as it was still present after addition of amiloride. However, in contrast to experiments

Fig. 2. The transient decrease in ISC by terbutaline represents Cl^- transport, the subsequent increase is Na^+ transport. ISC was measured on ATII cell monolayers mounted in Ussing chambers as described in legend to Fig. 1. (A) Na^+ channels were inhibited by amiloride (10 μM) before acute stimulation of ATII cells by addition of terbutaline (1 μM), which rapidly increased ISC. (B) Cl^- in the medium was decreased to 10mM by substitution with gluconate. Acute stimulation with terbutaline (1 μM) induced a slow increase in ISC. (C) Summary of results of experiments to characterize the fast transient decrease in ISC upon acute terbutaline stimulation (ISC_{dip}) and the increase following the “dip” as shown in Figures 1 and 2: ISC_{dip} , control: After acute stimulation with terbutaline (1 μM) of cells bathed in high Cl^- medium ; ISC_{dip} , amiloride/terbutaline: increase in ISC when terbutaline was added after inhibition of Na^+ channels with amiloride (10 μM); low Cl^- /terbutaline: increase in ISC_{tot} 20 min after acute stimulation with terbutaline (1 μM) in low Cl^- medium; $\text{ISC}_{\Delta\text{amil}}$, low Cl^- : amiloride-sensitive component of ISC measured in low Cl^- medium without acute terbutaline stimulation; $\text{ISC}_{\Delta\text{amil}}$, low Cl^- /terbutaline: amiloride-sensitive component of ISC 20min after stimulation with terbutaline (1 μM); the last two series of experiments were performed on separate filters with cells from the same preparation. Mean values \pm SD of changes in ISC from 5 to 8 measurements on at least 2 preparations of ATII cells. *indicates $P < 0.05$ between $\text{ISC}_{\Delta\text{amil}}$ low Cl^- and low Cl^- /terbutaline.



in absence of amiloride, now the direction of this fast response had changed from a decrease in ISC to a transient increase of about 1.2 $\mu\text{A}/\text{cm}^2$ (Fig. 2C 2nd bar from the left). Such an effect is typically found in epithelial cells with a combined Na^+ and Cl^- -conductance [31] where the Na^+ current determines the direction of the Cl^- current (thus determining between secretion and reabsorption). We therefore hypothesized that the “dip” of ISC was dependent on the presence of Cl^- . In fact, when chloride in the medium was decreased by substitution with gluconate, no rapid change of ISC was seen upon terbutaline-stimulation (Fig. 2B).

In cells bathed in high Cl^- -medium ISC increased after this initial dip and reached pre-stimulation values after about 15 to 20 min (Fig. 1A), which, in some instances only exceeded pre-stimulation values. Mean values of ISC 20 min after stimulation were not significantly different from pre-stimulation values ($P=0.58$). As mentioned above, there was no

transient decrease in ISC upon acute stimulation with terbutaline, when cells were bathed in low Cl^- medium. However, in these cells acute addition of terbutaline induced a slow increase in ISC. As shown in the 3rd bar from the left in Fig. 2C this increase amounted to about 0.6 $\mu\text{A}/\text{cm}^2$. The two bars at the right of Fig. 2C show the amiloride-inhibitable portion of ISC measured in low Cl^- medium before and after stimulation with terbutaline, which were obtained in different sets of cells, one stimulated and one not. It shows that terbutaline significantly increased $\text{ISC}_{\Delta\text{amil}}$ by about 0.6 $\mu\text{A}/\text{cm}^2$ ($P=0.047$). The similarity between these two values implies that the increase in ISC after the initial dip represents an amiloride-sensitive Na^+ current. Taken together these results indicate that acute stimulation with terbutaline induces a fast Cl^- current and a slow increase in a Na^+ current. In cells bathed in medium containing the normal Cl^- concentration it might be masked by the terbutaline-induced Cl^- current.

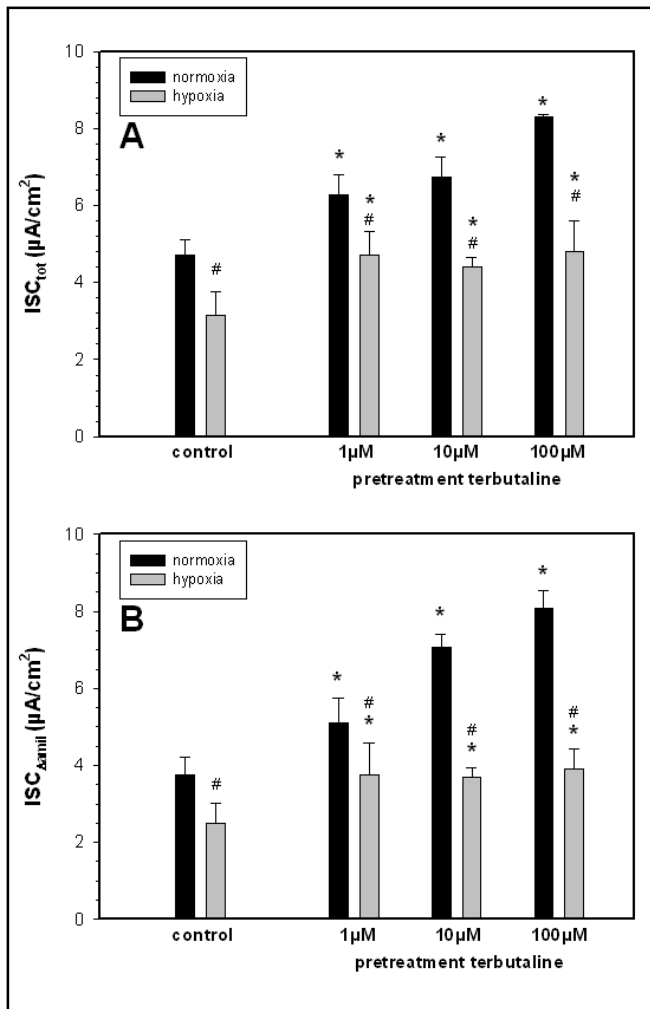


Fig. 3. Pre-treatment with terbutaline for 24h increases total and amiloride sensitive ISC in normoxia and hypoxia. ATII cell monolayers were measured as described in legend to Fig. 1. (A) Cells were kept in hypoxia (1.5% O_2) in absence (control) and presence of terbutaline (indicated concentrations) for 24h. After recording ISC_{tot} (A), amiloride (10 μM) was added to determine $\text{ISC}_{\Delta\text{amil}}$ (B). Mean values \pm SD from 5 to 10 monolayers from at least 3 different preparations of ATII cells for each experimental condition. * $P < 0.05$ effect of terbutaline relative to controls at the respective oxygenation level; # $P < 0.05$ effect of hypoxia at the respective treatment.

Prolonged terbutaline treatment

As shown previously, 24h exposure to hypoxia decreased ISC in ATII cells (Fig. 3A) [6]. Hypoxic cells were still responsive to terbutaline showing the same pattern of changes in ISC as normoxic control cells. However, both $\text{ISC}_{\Delta\text{amil}}$ before terbutaline-stimulation ($P = 0.035$; Fig. 3B) and ISC_{dip} ($P = 0.023$; Fig. 4) were significantly decreased. The increase in ISC_{dip} (Fig. 4)

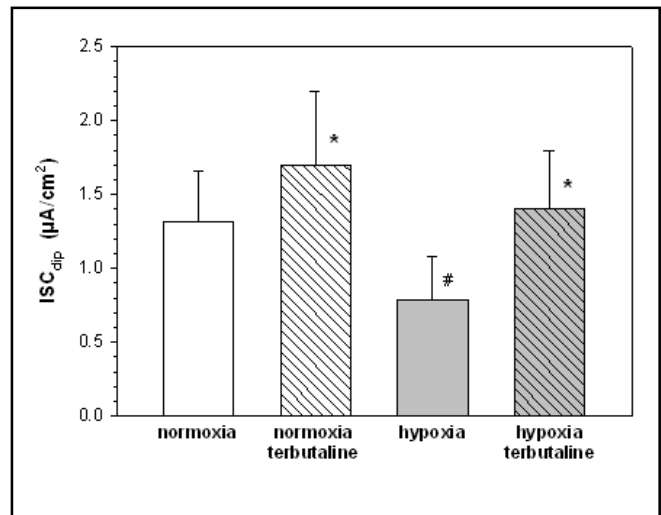


Fig. 4. ISC_{dip} is decreased by hypoxia but increased by 24h pre-treatment with terbutaline. ATII cells were kept in normoxia and hypoxia (1.5% O_2 , 24h) in absence and presence of terbutaline (1 μM). Monolayers were mounted in Ussing chambers and stimulated with terbutaline (1 μM). ISC_{dip} represents the maximal, rapid decrease in ISC after stimulation with terbutaline (Fig. 1). Mean values \pm SD from 5 to 10 monolayers from at least 3 different ATII cell preparations for each experimental condition. # $P < 0.05$ effect of hypoxia in control and terbutaline-pre-treated cells; * $P < 0.05$ effect of 24h pre-treatment with terbutaline.

by acute stimulation of cells that were pretreated with terbutaline for 24h indicates that terbutaline not only increased Na- but also Cl-currents. It was not associated with altered CFTR mRNA expression. In contrast, hypoxia decreased CFTR mRNA levels significantly (Table 1).

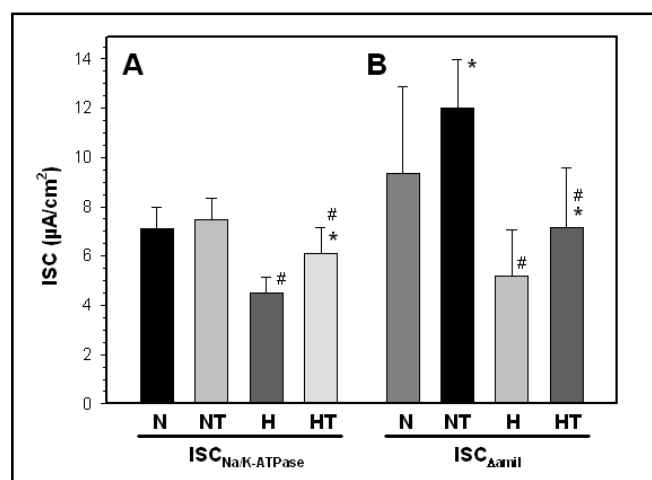
Fig. 3A shows that pre-treatment with terbutaline (1, 10 and 100 μM) for 24h increased ISC_{tot} . This increase in ISC_{tot} was attributable to an increase in the amiloride-sensitive portion of ISC ($\text{ISC}_{\Delta\text{amil}}$; Fig. 3B). Fig. 3A and B also show that hypoxia decreased ISC_{tot} and $\text{ISC}_{\Delta\text{amil}}$ in cells without and with terbutaline-pretreatment for 24h. However, in the terbutaline treated, hypoxic cells ISC_{tot} and $\text{ISC}_{\Delta\text{amil}}$ were higher than in the cells without pretreatment, and ISC reached values found in normoxic control cells. A dose-dependency for the stimulation of ISC_{tot} and $\text{ISC}_{\Delta\text{amil}}$ with terbutaline above 1 μM was only found in normoxia but not in hypoxia pointing to a limitative factor for maximal stimulation.

We tested then, whether the increased ISC_{tot} and $\text{ISC}_{\Delta\text{amil}}$ in cells pre-treated with terbutaline was caused

Table 1. Effects of with hypoxia and pre-treatment with terbutaline on mRNA expression of Na⁺/K⁺-ATPase, ENaC and CFTR. ATII cells were grown on 6-well plates and exposed to hypoxia (1.5% O₂, 24 h) in absence and presence of terbutaline (25 μM, 24 h) before preparation of RNA. 28S rRNA was used as housekeeping gene. Mean values ± SD, n = 4. *P<0.05 effect of hypoxia.

	normoxia	normoxia terbutaline	hypoxia	hypoxia terbutaline
α1-Na ⁺ /K ⁺ -ATPase (fg/ng 28S)	51.8 ± 9.1	66.3 ± 28.3	50.8 ± 6.1	69.7 ± 5.3
β1-Na ⁺ /K ⁺ -ATPase (fg/ng 28S)	18.9 ± 3.7	25.0 ± 8.6	18.6 ± 1.9	27.1 ± 1.9
α-ENaC (pg/ng 28S)	23.6 ± 6.5	34.4 ± 5.3	29.8 ± 4.4	37.5 ± 5.2
β-ENaC (fg/ng 28S)	3.8 ± 1.2	3.5 ± 1.4	4.5 ± 1.1	5.2 ± 0.3
γ-ENaC (fg/ng 28S)	13.3 ± 5.8	15.9 ± 8.2	11.9 ± 3.9	20.7 ± 4.7
CFTR (fg/ng 28S)	2.37 ± 0.39	1.69 ± 0.80	0.86 ± 0.22*	0.74 ± 0.17*

Fig. 5. Terbutaline blunts hypoxia mediated inhibition of Na⁺/K⁺-ATPase and ENaC capacity. ATII cell monolayers were exposed to normoxia (N) and hypoxia (1.5% O₂, 24h) (H) in absence and presence of 1 μM terbutaline (NT, HT). (A) The capacity of Na⁺/K⁺-ATPase [6] was measured after permeabilization of the apical membrane with amphotericin B (5 μM) in symmetric high Na⁺ media (as in legend to Fig. 1). (B) ENaC-capacity (ISC_{CapAamil}) was measured in presence of an apical-to-basolateral Na⁺ concentration gradient (apical: 141mM NaCl; basolateral: 25mM NaCl, 116mM choline-Cl) after permeabilizing the basolateral membrane with amphotericin B (5 μM). Mean values ± SD of 7 experiments from 3 different preparations. * P < 0.05 significant effect of terbutaline at the respective oxygenation level; #P < 0.05 significant effect of hypoxia at the respective treatment.

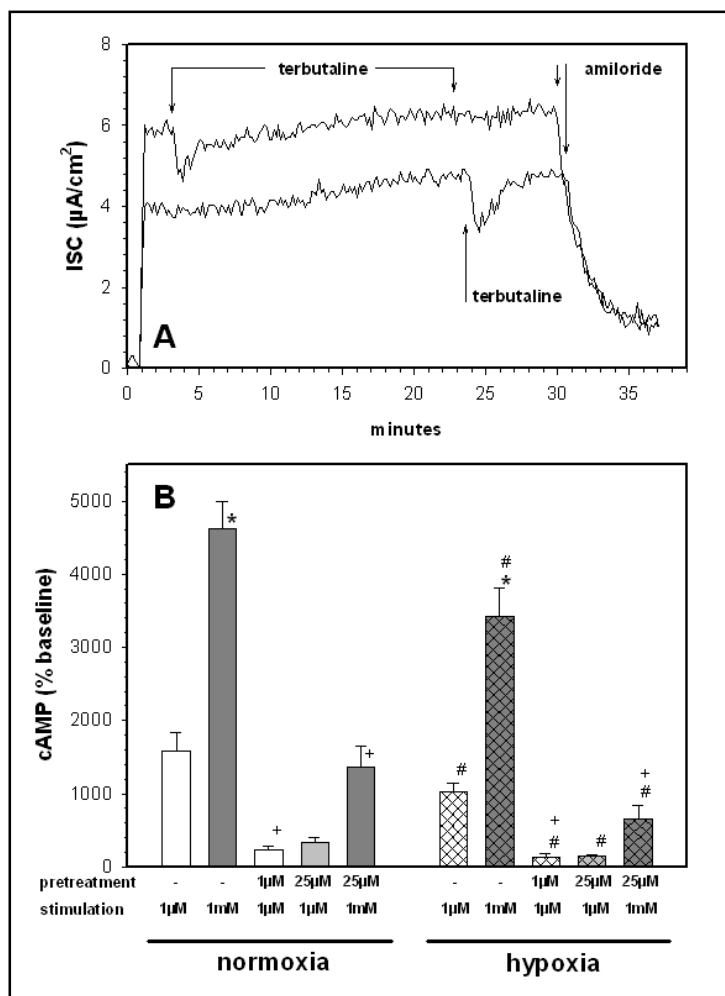


by a change in Na⁺/K⁺-ATPase and ENaC capacity. Fig. 5A shows that hypoxia decreased ISC_{Na/K-ATPase} (P=0.001) thus confirming earlier results [6]. Hypoxia also decreased ISC_{Na/K-ATPase} in cells pre-treated with terbutaline (P=0.013). 24h treatment with terbutaline in normoxia did not increase ISC_{Na/K-ATPase} (P=0.467), whereas a significant increase was found in hypoxia (P=0.005). Hypoxia also significantly decreased the capacity of the amiloride-sensitive component of ISC measured in presence of an apical-to-basolateral Na⁺ concentration gradient (ISC_{CapAamil}; Fig. 5B), as we reported previously [6]. It also decreased ISC_{CapAamil} in cells pre-treated with terbutaline (P=0.045). At either oxygenation level 24h treatment with terbutaline significantly increased ISC_{CapAamil} (P=0.05). The increased capacity of Na⁺/K⁺-ATPase and ENaC was not attributable to terbutaline-induced stimulation of gene expression as assessed by real time PCR, since table 1 shows that terbutaline did not affect the mRNA expression of subunits of Na⁺/K⁺-ATPase and ENaC.

Down-regulation of β₂AR-signaling

It was surprising to see that ATII cells that were pre-treated with terbutaline for 24h still responded to acute stimulation in the Ussing chamber (Fig. 1A). Although prolonged treatment with terbutaline down-regulates β₂AR, ISC_{dip} was even larger in the cells pre-treated with terbutaline than in untreated cells (Fig. 4). Therefore the terbutaline-induced production of cAMP was measured in normoxic and hypoxic control cells and after pre-treatment with terbutaline for 24h. We used 1 μM and 25 μM terbutaline for the 24h pre-treatment. 25 μM is approximately 10-times higher than the EC-50 of terbutaline-stimulated cAMP production and was shown to cause desensitization of the β₂AR signaling system in primary rat ATII cells [19]. Higher concentrations might cause non-specific effects. Results summarized in Fig. 6 show that acute stimulation with 1 μM and 1 mM (maximal stimulation of cAMP production in normoxic ATII cells; [19]) significantly increased cAMP production in normoxic and hypoxic ATII cells, when cells were not pre-treated

Fig. 6. Application of terbutaline desensitizes β_2 AR and decreases terbutaline induced cAMP production in normoxia and hypoxia. (A) Typical recording showing that a 2nd stimulation with terbutaline 20min after a first stimulation does not cause the typical pattern of change of ISC. The second tracing shows a time-control. (B) 24h pre-treatment with terbutaline and hypoxia decreases the terbutaline-induced increase in cAMP. AII cells grown in 24-well plates were exposed to normoxia and hypoxia (1.5% O₂; 24h) in absence and presences of terbutaline at the indicated concentration (pre-treatment). Cells were then treated with IBMX (1mM, 30min) and cAMP production was measured 10min after stimulation with terbutaline. Mean values (% of baseline cAMP) \pm SD from 6 monolayers from 2 independent preparations. *P<0.05 between acute stimulation with 1 μ M and 1mM terbutaline at the respective oxygenation level in cells not pretreated with terbutaline. +P<0.05 between control and 24h pre-treatment with terbutaline at the indicated concentration in cells acutely stimulated with 1 μ M and 1mM terbutaline. # P<0.05 between normoxia and hypoxia at the respective treatment.



with terbutaline. Stimulation of cAMP production was also seen in the cells pre-treated with terbutaline for 24h. However, in those cells the stimulation of cAMP production was significantly lower than in the non pretreated cells.

In contrast to prolonged pre-treatment, exposure of AII cells to terbutaline for 20min resulted in a loss of the ability to respond to terbutaline (Fig. 6A). This indicates a refractory time period for re-stimulation, which, interestingly, is not present after long-term pretreatment.

Discussion

We show here that despite a hypoxia-induced inhibition of alveolar transepithelial transport of Na⁺ and Cl⁻ and impaired β_2 AR signaling, 24h pre-treatment with the β_2 AR agonist terbutaline stimulates alveolar ion transport even in hypoxia, but less than in normoxia. Stimulation is due to increased Cl⁻ transport and increased

Na⁺ transport mediated by ENaC and Na⁺/K⁺-ATPase. Thus, our results indicate that β_2 AR stimulation blunts hypoxic inhibition of alveolar reabsorption in hypoxia thus maintaining a major defense mechanism protecting from alveolar edema.

Acute terbutaline-effects on ISC

In primary rat alveolar epithelial cells, terbutaline induces a rapid, transient decrease in ISC followed by an increase [28, 30, 32], which is a specific response to β_2 -adrenergic signaling. We found that a rapid change in ISC can also be seen in presence of amiloride to block Na⁺ channels, and that it is absent after decreasing Cl⁻. Together with results showing that also complete removal of Cl⁻ [33] and inhibition with Cl⁻ channel blockers [33] prevented this response, our results are clear evidence for a terbutaline-stimulated increase in the Cl⁻-conductance. It is most likely mediated by CFTR, which is present in AII cells [34], and which has been shown to be required for terbutaline-stimulated alveolar fluid clearance [35]. Interestingly, results from

the literature indicate that no such transient change in ISC_{tot} was seen, when cAMP was increased by other stimulators such as adenosine [36], forskolin [37], or 8Br-cAMP [29]. These results might point to a β_2 AR-specific, compartmentalized intracellular signaling pathway which stimulates Cl^- - and Na^+ transport.

After the initial drop due to Cl^- transport, ISC increases slowly reaching a plateau. Several reports indicate an increase in ISC above initial values about 30 to 60 min after terbutaline application [28-30]. Amiloride has been shown to prevent this component of ISC [33] indicating that this current is mediated by epithelial Na^+ channels (ENaC). However, we found no significant increase in $ISC_{\Delta amil}$ within this time period, even when the terbutaline concentration used for stimulation was increased to 100 μM (not shown). This discrepancy has been pointed out in the literature as well (summary in [38]), its reason is unclear. Differences in the composition of culture media and addition of supplements such as dexamethasone [30] are a most likely explanation, since dexamethasone increases the expression of Na^+ channels [18].

It has been proposed that stimulation of Cl^- currents is required for activation of Na^+ transport by β_2 -adrenergic receptor agonists [33]. Here we show that the increase in ISC upon stimulation with terbutaline is still seen (Fig. 2B) when the initial Cl^- current has been prevented by substituting Cl^- with gluconate. This indicates that stimulation of Cl^- transport is not required for activation of Na^+ transport and that both transport systems are stimulated simultaneously but independently.

Effects of pre-treatment with terbutaline on ISC

ISC and its amiloride-sensitive component were increased significantly, when cells were pre-treated with terbutaline for 24h in normoxia and hypoxia. The effect tended to be more pronounced at higher concentrations of terbutaline. It is most likely due to increased membrane surface expression of ENaC [9, 39-41] as indicated by an increase in its capacity measured after permeabilization of the basolateral membrane [6, 27]. Altered gene expression seems not to account for the increased capacity since terbutaline did not affect ENaC mRNA levels. There are reports on a stimulation of gene expression of ENaC and Na^+/K^+ -ATPase by beta adrenergic stimulation [18]. However, elevation of mRNA and protein levels required several days of pre-treatment with 100 μM β_2 AR-agonist or with a high concentration of db-cAMP [18, 42].

Surprisingly, ATII cells pre-treated for 24h with terbutaline still respond to acutely applied terbutaline with a rapid decrease in ISC followed by a slow increase, as it was seen in control cells. Use of receptor antagonists prevented the change in ISC indicating that this response was specific for β_2 AR stimulation. This response was seen despite the fact that in pre-treated cells the increase in cAMP upon acute stimulation with terbutaline was decreased by 90% (Fig. 6). However, we have shown previously, that after 20min of desensitization terbutaline still causes a significant increase in cAMP [19], a process that has been observed in many different cell types and tissues [43]. In contrast, when ATII cells mounted in the Ussing chamber were treated with terbutaline for 20min, a second stimulation with terbutaline did not cause the typical biphasic response of ISC (Fig. 6A) despite an impaired but still significant increase in cAMP [19]. Thus there is dissociation between the terbutaline-effect on cAMP and on Na^+ transport. Down-regulation of β_2 AR has also been shown in the lung in vivo upon prolonged isoproterenol-infusion as indicated by a decrease in receptor density [23]. *In vivo*, therapeutically relevant concentrations of β_2 AR agonists stimulated alveolar fluid clearance despite a reduced receptor number upon down-regulation [23]. Taken together these results point out that, although β_2 AR are down-regulated by prolonged stimulation, transepithelial transport can still be stimulated and seems to reach an even higher activity than in cells that were not pre-treated with terbutaline. This implies that, although receptor stimulation is required for stimulation of transport, there might not be a direct relation between the magnitude of cAMP production and the activity of ion transport.

Combined effects of hypoxia and terbutaline

It is well established that hypoxia inhibits alveolar fluid reabsorption and Na^+ transport in cultured alveolar epithelial cells [5, 6, 8, 44] and in the fluid instilled lung [7, 8]. Inhibition is associated with a decrease in the expression [5, 45] and membrane insertion of Na^+/K^+ -ATPase [10] and ENaC [9]. Here we show that hypoxia also decreased the acute response of ISC to terbutaline, which constitutes Cl^- transport. Since hypoxia also decreased the amount of mRNA of CFTR this result indicates that the decreased Cl^- current in hypoxia might be due to decreased CFTR expression. The mechanism of decreased CFTR expression has not been further evaluated. Oxygen sensitivity of CFTR has been shown in MDCK cells, where the increased oxygen

availability in an air liquid interface increased CFTR abundance and mRNA, and where decreasing oxygen also decreased forskolin-stimulated ISC [11]. Also, when cells were pre-treated with terbutaline for 24h, ISC_{tot} and its amiloride-sensitive component were decreased indicating that hypoxia also decreased terbutaline-stimulated Na^+ transport. Decreased stimulation of transepithelial transport goes hand in hand with decreased activity of the β_2AR signaling system in hypoxia in AII cells [19]. Recently we have shown that this was due to a relative increase in G_i protein activity together with a decreased receptor number and a decrease in stimulatory G_s protein [19].

Transport in hypoxic cells pre-treated with terbutaline for 24 h was higher than in cells exposed to hypoxia alone indicating that terbutaline was able to stimulate transport also in hypoxia. These results on cultured AII cells are in line with functional data on the fluid instilled lung of hypoxic rats, where Litvan et al. [20] found that, although isoproterenol stimulated alveolar reabsorption in lungs from hypoxic rats, stimulated reabsorption was lower in hypoxic than in normoxic lungs [20]. However, results are contradictory since in a similar experimental setting Vivona et al. [7] found that after stimulation with terbutaline the same maximal level of alveolar reabsorption was achieved in lungs from normoxic and hypoxic rats. This latter result suggests no impairment of stimulation of reabsorption by β_2AR stimulators in hypoxia. The reason for the discrepancy is unclear.

However, with this study we demonstrate that not only time of exposure to terbutaline but also dosing seems crucial for the effects of β_2AR stimulation in hypoxia. In contrast to normoxia we did not see a dose-dependence

of stimulation of Na^+ transport above 1 μM terbutaline in hypoxia. This effect was not attributable to the limitation of cAMP-formation in hypoxia. Therefore a factor distal to cAMP in the signaling cascade must be limiting the stimulation of Na^+ transport by β_2 adrenergic agents in hypoxia. One possibility is a limitation to recruit Na^+ transporters from endogenous stores in hypoxia. Hence, not all of the endogenous transporters might be accessible for re-insertion upon β_2 adrenergic stimulation thus decreasing terbutaline-stimulated transport.

Taken together our results indicate that hypoxia impairs terbutaline-stimulated transport of Na^+ and Cl^- , and that part of this inhibition might be blunted by prolonged pre-treatment of cells with terbutaline. Thus, our results presented here, indicate that despite impaired β_2AR signaling and impaired β_2AR -mediated stimulation of reabsorption, β_2 -agonists can restore normal fluid clearance rates in the hypoxic lung. In pathological situations associated with alveolar edema, prolonged β_2AR -stimulation might therefore prevent a vicious cycle, where hypoxia-induced formation of alveolar edema might exaggerate, when a thickened layer of alveolar lining fluid further decreases oxygen supply to the alveolar epithelium and thus inhibits alveolar reabsorption, which subsequently may cause tissue damage [46].

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References

- Matthay MA, Abraham E: Beta-adrenergic agonist therapy as a potential treatment for acute lung injury. *Am J Respir Crit Care Med* 2006;173:254-255.
- Sartori C, Allemann Y, Duplain H, Lepori M, Egli M, Lipp E, Hutter D, Turini P, Hugli O, Cook S, Nicod P, Scherrer U: Salmeterol for the prevention of high-altitude pulmonary edema. *N Engl J Med* 2002;346:1631-1636.
- 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;163:1376-1383.
- Matalon S, O'Brodovich H: Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. *Ann Rev Physiol* 1999;61:627-661.

- 5 Planes C, Escoubet B, BlotChabaud M, Friedlander G, Farman N, Clerici C: Hypoxia downregulates expression and activity of epithelial sodium channels in rat alveolar epithelial cells. *Am J Respir Cell Molec Biol* 1997;17:508-518.
- 6 Mairbäurl H, Mayer K, Kim KJ, Borok Z, Bärtsch P, Crandall ED: Hypoxia decreases active Na transport across primary rat alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Molec Physiol* 2002;282:L659-L665.
- 7 Vivona ML, Matthay MA, Chabaud MB, Friedlander G, Clerici C: Hypoxia reduces alveolar epithelial sodium and fluid transport in rats: reversal by beta-adrenergic agonist treatment. *Am J Respir Cell Molec Biol* 2001;25:554-561.
- 8 Guney S, Schuler A, Ott A, Höschle S, Baloglu E, Bärtsch P, Mairbäurl H: Dexamethasone prevents transport inhibition by hypoxia in rat lung and alveolar epithelial cells by stimulating activity and expression of Na/K-ATPase and ENaC. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1332-L1338.
- 9 Planes C, BlotChabaud M, Matthay MA, Couette S, Uchida T, Clerici C: Hypoxia and beta(2)-agonists regulate cell surface expression of the epithelial sodium channel in native alveolar epithelial cells. *J Biol Chem* 2002;277:47318-47324.
- 10 Dada LA, Chandel NS, Ridge KM, Pedemonte C, Bertorello AM, Sznajder JI: Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-zeta. *J Clin Invest* 2003;111:1057-1064.
- 11 Bebök Z, Tousson A, Schwiebert LM, Venglarik CJ: Improved oxygenation promotes CFTR maturation and trafficking in MDCK monolayers. *Am J Physiol* 2001;280:C135-C145.
- 12 Matthay MA, Folkesson HG, Clerici C: Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev* 2002;82:569-600.
- 13 Mutlu GM, Dumasius V, Burhop J, McShane PJ, Meng FJ, Welch L, Dumasius A, Mohebahmadi N, Thakuria G, Hardiman K, Matalon S, Hollenberg S, Factor P: Upregulation of alveolar epithelial active Na⁺ transport is dependent on beta(2)-adrenergic receptor signaling. *Circ Res* 2004;94:1091-1100.
- 14 Perkins GD, McAuley DF, Thickett DR, Gao F: The beta-agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. *Am J Respir Crit Care Med* 2006;173:281-287.
- 15 Frank JA, Wang Y, Osorio O, Matthay MA: Beta-adrenergic agonist therapy accelerates the resolution of hydrostatic pulmonary edema in sheep and rats. *J Appl Physiol* 2000;89:1255-1265.
- 16 Tibayan FA, Chesnutt AN, Folkesson HG, Eandi J, Matthay MA: Dobutamine increases alveolar liquid clearance in ventilated rats by beta-2 receptor stimulation. *Am J Respir Crit Care Med* 1997;156:438-444.
- 17 Fang XH, Song YL, Hirsch J, Galiotta LJV, Pedemonte N, Zemans RL, Dolganov G, Verkman AS, Matthay MA: Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells. *Amer J Physiol Lung Cell M Ph* 2006;290:L242-L249.
- 18 Dagenais A, Denis C, Vives MF, Girouard S, Masse C, Nguyen T, Yamagata T, Grygorczyk C, Kothary R, Berthiaume Y: Modulation of alpha-ENaC and alpha₁-Na⁺-K⁺-ATPase by cAMP and dexamethasone in alveolar epithelial cells. *Am J Physiol* 2001;281:L217-L230.
- 19 Baloglu E, Ke A, Abu-Taha IH, Bärtsch P, Mairbäurl H: In vitro hypoxia impairs beta 2 adrenergic signaling in primary rat alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L500-L509.
- 20 Litvan J, Briva A, Wilson MS, Budinger GR, Sznajder JI, Ridge KM: Beta-adrenergic receptor stimulation and adenoviral overexpression of superoxide dismutase prevent the hypoxia-mediated decrease in Na,K-ATPase and alveolar fluid reabsorption. *J Biol Chem* 2006;281:19892-19898.
- 21 Dehler M, Zessin E, Bärtsch P, Mairbäurl H: Hypoxia causes permeability edema in the constant-pressure perfused rat lung. *Eur Respir J* 2006;27:600-606.
- 22 Duplain H, Vollenweider L, Delabays A, Nicod P, Bartsch P, Scherrer U: Augmented sympathetic activation during short-term hypoxia and high-altitude exposure in subjects susceptible to high-altitude pulmonary edema. *Circulation* 1999;99:1713-1718.
- 23 Morgan EE, Hodnichak CM, Stader SM, Maender KC, Boja JW, Folkesson HG, Maron MB: Prolonged isoproterenol infusion impairs the ability of b₂-agonists to increase alveolar liquid clearance. *Am J Physiol Lung Cell Molec Physiol* 2002;282:L666-L674.
- 24 Loeh B, Bärtsch P, Mairbäurl H: Downregulation of beta receptor signalling in alveolar epithelial cells in hypoxia. *Acta Physiologica* 186, 68/OM06-32. 2006.
- 25 Loeh B, Bärtsch P, Mairbäurl H: Pretreatment with beta-agonists stimulates alveolar Na-transport in normoxia and hypoxia. *Faseb J* 2005;19:A173.
- 26 Dobbs LG: Isolation and culture of alveolar type II cells. *Am J Physiol* 1990;258:L134-L147.
- 27 Guo Y, Duvall MD, Crow JP, Matalon S: Nitric oxide inhibits Na⁺ absorption across alveolar type II monolayers. *Am J Physiol* 1998;274:L369-L377.
- 28 Mason RJ, Williams MC, Widdicombe JH, Sanders MJ, Misfeldt DS, Berry LC Jr: Transepithelial transport by pulmonary alveolar type II cells in primary culture. *Proc Natl Acad Sci USA* 1982;79:6033-6037.
- 29 Cott GR, Sugahara K, Mason RJ: Stimulation of net ion transport across alveolar type II cell monolayers. *Am J Physiol* 1986;250:C222-C227.
- 30 Cheek JM, Kim KJ, Crandall ED: Tight monolayers of rat alveolar epithelial cells: bioelectric properties and active sodium transport. *Am J Physiol* 1989;256:C688-C693.
- 31 Blaug S, Hybiske K, Cohn J, Firestone GL, Machen TE, Miller SS: ENaC- and CFTR-dependent ion and fluid transport in mammary epithelia. *Am J Physiol Cell Physiol* 2001;281:C633-C648.
- 32 Jiang XP, Ingbar DH, OGrady SM: Adrenergic stimulation of Na⁺ transport across alveolar epithelial cells involves activation of apical Cl⁻ channels. *Am J Physiol Cell Physiol* 1998;44:C1610-C1620.
- 33 O'Grady SM, Jiang X, Ingbar DH: Cl⁻ channel activation is necessary for stimulation of Na transport in adult alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L239-L244.
- 34 Brochiero E, Dagenais A, Prive A, Berthiaume Y, Grygorczyk R: Evidence of a functional CFTR Cl⁻ channel in adult alveolar epithelial cells. *Am J Physiol Lung Cell Molec Physiol* 2004;287:L382-L392.
- 35 Fang X, Fukuda N, Barbry P, Sartori C, Verkman AS, Matthay MA: Novel role for CFTR in fluid absorption from the distal airspaces of the lung. *J Gen Physiol* 2002;119:199-207.
- 36 Factor P, Mutlu GM, Chen L, Mohameed J, Akhmedov AT, Meng FJ, Jilling T, Lewis ER, Johnson MD, Xu A, Kass D, Martino JM, Bellmeyer A, Albazi JS, Emala C, Lee HT, Dobbs LG, Matalon S: Adenosine regulation of alveolar fluid clearance. *Proc Natl Acad Sci U S A* 2007;104:4083-4088.
- 37 Nielsen VG, Duvall MD, Baird MS, Matalon S: cAMP activation of chloride and fluid secretion across the rabbit alveolar epithelium. *Am J Physiol Lung Cell Molec Physiol* 1998;19:L1127-L1133.

- 38 Widdcombe JH: How does cAMP increase active Na absorption across alveolar epithelium? *Am J Physiol Lung Cell Molec Physiol* 2000;278:L231-L232.
- 39 Kleyman TR, Zuckerman JB, Middleton P, McNulty KA, Hu BF, Su XF, An B, Eaton DC, Smith PR: Cell surface expression and turnover of the alpha-subunit of the epithelial sodium channel. *Am J Physiol* 2001;281:F213-F221.
- 40 Ito Y, Niisato N, O'Brodovich H, Marunaka Y: The effect of brefeldin A on terbutaline-induced sodium absorption in fetal rat distal lung epithelium. *Pflügers Arch Eur J Physiol* 1997;434:492-494.
- 41 Bertorello AM, Ridge KM, Chibalin AV, Katz AI, Sznajder JI: Isoproterenol increases Na⁺-K⁺-ATPase activity by membrane insertion of α -subunits in lung alveolar cells. *Am J Physiol Lung Cell Molec Physiol* 1999;20:L20-L27.
- 42 Minakata Y, Suzuki S, Grygorczyk C, Dagenais A, Berthiaume Y: Impact of beta-adrenergic agonist on Na⁺ channel and Na⁺-K⁺ -ATPase expression in alveolar type II cells. *Am J Physiol* 1998;275:L414-L422.
- 43 Strosberg AD: Structure, function, and regulation of adrenergic receptors. *Protein Sci* 1993;2:1198-1209.
- 44 Mairbäurl H, Wodopia R, Eckes S, Schulz S, Bärtsch P: Impairment of cation transport in A549 cells and rat alveolar epithelial cells by hypoxia. *Am J Physiol* 1997;273:L797-L806.
- 45 Wodopia R, Ko HS, Billian J, Wiesner R, Bärtsch P, Mairbäurl H: Hypoxia decreases proteins involved in transepithelial electrolyte transport of A549 cells and rat lung. *Am J Physiol* 2000;279:L1110-L1119.
- 46 Krick S, Eul BG, Hanze J, Savai R, Grimminger F, Seeger W, Rose F: Role of hypoxia-inducible factor-1 α in hypoxia-induced apoptosis of primary alveolar epithelial type II cells. *Amer J Respir Cell Molec Biol* 2005;32:395-403.