

The role of hypoxia-induced modulation of alveolar epithelial Na⁺ - transport in hypoxemia at high altitude

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Abstract

Reabsorption of excess alveolar fluid is driven by vectorial Na⁺-transport across alveolar epithelium, which protects from alveolar flooding and facilitates gas exchange. Hypoxia inhibits Na⁺-reabsorption in cultured cells and in-vivo by decreasing activity of epithelial Na⁺-channels (ENaC), which impairs alveolar fluid clearance. Inhibition also occurs during in-vivo hypoxia in humans and laboratory animals. Signaling mechanisms that inhibit alveolar reabsorption are poorly understood. Because cellular adaptation to hypoxia is regulated by hypoxia-inducible transcription factors (HIF), we tested whether HIFs are involved in decreasing Na⁺-transport in hypoxic alveolar epithelium. Expression of HIFs was suppressed in cultured rat primary alveolar epithelial cells (AEC) with shRNAs. Hypoxia (1.5% O₂, 24 h) decreased amiloride-sensitive transepithelial Na⁺-transport, decreased the mRNA expression of α -, β -, and γ -ENaC subunits, and reduced the amount of $\alpha\beta\gamma$ -ENaC subunits in the apical plasma membrane. Silencing HIF-2 α partially prevented impaired fluid reabsorption in hypoxic rats and prevented the hypoxia-induced decrease in α - but not the $\beta\gamma$ -subunits of ENaC protein expression resulting in a less active form of ENaC in hypoxic AEC. Inhibition of alveolar reabsorption also caused pulmonary vasoconstriction in ventilated rats. These results indicate that a HIF-2 α -dependent decrease in Na⁺-transport in hypoxic alveolar epithelium decreases alveolar reabsorption. Because susceptibles to high-altitude pulmonary edema (HAPE) have decreased Na⁺-transport even in normoxia, inhibition of alveolar reabsorption by hypoxia at high altitude might further impair alveolar gas exchange. Thus, aggravated hypoxemia might further enhance hypoxic pulmonary vasoconstriction and might subsequently cause HAPE.

Keywords

hypoxia, alveolar fluid clearance, ENaC, HIF-2 α , high-altitude pulmonary edema

Date received: 26 March 2020; accepted: 2 June 2020

Pulmonary Circulation 2020; 10(S1) 50–58

DOI: 10.1177/2045894020936662

Introduction

The multiple functions of the alveolar epithelium are crucial for oxygen diffusion from inspired air into blood and thus for tissue oxygen supply. Alveolar type II cells secrete surfactant which controls surface tension.¹ Another important function of epithelial type I and type II cells is reabsorption of excess lining fluid from the alveolar surface, where water movement is coupled to the reabsorption of Na⁺ by amiloride-sensitive epithelial Na⁺-channels (ENaC) located in the apical membrane.^{2,3} Na⁺ is then extruded by Na⁺/K⁺-ATPase, which is located in the basolateral membrane.⁴ Vectorial transport of Na⁺ across the alveolar epithelium

by ENaC and Na⁺/K⁺-ATPase generates the osmotic driving force for the removal of excess water from the alveolar surface,⁵ which keeps the layer of the surface fluid film thin. Alveolar reabsorption is of particular importance in hypoxia, when oxygen diffusion is reduced because of decreased

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oxygen partial pressure in inspired and in alveolar air. Impaired reabsorption results in alveolar edema.⁶

In this article, we present evidence for the significance of alveolar reabsorption for acclimatization to high altitude. We also report some new results on possible regulation of alveolar function by hypoxia-inducible factors (HIF), and discuss the relevance of these processes for the pathophysiology of high-altitude pulmonary edema (HAPE).

Alveolar Na⁺-transport in hypoxia: alveolar epithelial cells and animal models

Prolonged exposure of laboratory rats to hypoxia significantly inhibited alveolar fluid reabsorption.⁷⁻⁹ Hypoxic inhibition seems to be caused by reduced activity of Na⁺-reabsorption because no further inhibition has been found in the presence of the ENaC-blocker amiloride.⁷ Measuring ion transport of cultured alveolar epithelium in Ussing chambers confirmed decreased activity of ENaC in primary rat lung alveolar epithelial cells (AEC)¹⁰ and showed further that hypoxia also decreased the capacity of ENaC and also of the Na⁺/K⁺-ATPase.¹⁰ Decreased capacity of transporters is an indicator of decreased number of active transporters inserted into the plasma membrane. Inhibition was paralleled by decreased mRNA expression of ENaC and of Na⁺/K⁺-ATPase.^{8,11,12} Planes et al. showed that hypoxia also decreased the number of ENaC molecules in the apical membrane.¹³ Dada et al. demonstrated removal of the Na⁺/K⁺-ATPase from the basolateral plasma membrane by internalization.¹⁴ A hypoxia-induced increase in mitochondrial reactive oxygen species¹⁵ and protein kinase C¹⁶ mediates internalization of Na⁺/K⁺-ATPase in hypoxia. These results indicate that reduction of alveolar Na⁺- and fluid reabsorption is a controlled process that helps individual cells to survive in a hypoxic environment by conserving energy consumption due to active Na⁺-transport.¹⁷ Mechanisms inducing ENaC inhibition are less well understood.

Cellular adjustments to hypoxia are often controlled by HIF¹⁸⁻²⁰ which are the key oxygen sensors of the cells regulating responses and adaptation to low oxygen tension. Briefly, HIF- α -subunits are constitutively expressed but are degraded upon hydroxylation by prolyl-hydroxylases (PHD) in the presence of oxygen, i.e. in a normoxic environment (for review, see Rocha¹⁹). Decreased oxygen levels prevent hydroxylation resulting in stabilized α -subunits. HIF- α subunits then form hetero-dimers with HIF- β , which is constitutively expressed. Dimers bind to hypoxia-response elements (HRE) on DNA to control transcription.¹⁹ Three HIF- α subunits have been identified, of which HIF-1 α and HIF-2 α are the best characterized. They have unique, non-redundant functions.

The role of HIFs in the control of epithelial Na⁺-transport is not well understood. HIF-1 α seems to play a role in stabilizing the activity of Na⁺/K⁺-ATPase in hypoxia by causing degradation of protein kinase C zeta (PKC- ζ).²¹

PKC- ζ phosphorylates the α 1-subunit of Na⁺/K⁺-ATPase, which results in internalization and thus inactivation of Na⁺-extrusion from the cells. Thus, HIF-1 α protects from total loss of Na⁺/K⁺-ATPase, which would eventually result in cell swelling and death. In fact, Na⁺/K⁺-ATPase is among the best preserved enzymatic processes in hypoxic cells.²²

It is not known whether HIFs are involved in the control of other Na⁺-transporters in hypoxia such as ENaC in alveolar epithelium. To address this question, we prepared primary rat lung AECs^{10,23} and cultured them in normoxia and hypoxia (1.5% oxygen) for the measurement of Na⁺-transport activity in Ussing chambers.^{10,24} Sequences encoding for specific short hair-pin RNAs were introduced by use of adenoviral infection of AECs (see supplement for methods), which decreased mRNA-expression of HIF-1 α and HIF-2 α by >50% (supplemental Table S1A) and the expression of HIF-1 α protein >60% and HIF-2 α >40% (supplemental Fig. S1).

Fig. 1a shows that hypoxia decreased ENaC-mediated transport across AEC monolayers by \sim 50%. This confirmed previous findings.^{9,10} Fig. 1a further shows that silencing HIF-2 α restored a small portion of ENaC-mediated Na⁺-transport, whereas silencing HIF-1 α had no effect (Fig. 1a). There was also pronounced inhibition of Na⁺/K⁺-ATPase by hypoxia; silencing HIF caused some recovery of its activity (P = 0.066; Fig. 1b).

It has been shown that hypoxia decreases the mRNA expression of all three ENaC-subunits.^{8,12} The supplemental Table S1B shows that decreased ENaC-mRNA was not affected by silencing HIFs, whereas silencing both HIFs completely prevented the hypoxia-induced decrease in the mRNA expression of the α 1-subunit of the Na⁺/K⁺-ATPase and partially upregulated β 1Na⁺/K⁺-ATPase mRNA expression. Planes et al. showed that hypoxia decreased the amounts of β - and γ -subunits of ENaC in apical membrane of AECs, which indicates that decreased activity is caused by a decreased number of active transporters in the plasma membrane.¹³ Figs 2 and 3 show that not only membrane-contained ENaC (Fig. 2), but also the amount of intracellular α -, β -, and γ -ENaC (Fig. 3) decreased in hypoxic alveolar epithelium (see supplement for methods). Figs 2 and 3 also show that silencing HIF-2 α fully restored the amount of α -ENaC protein in the apical plasma membrane and in the intracellular compartment of hypoxia-exposed AECs, whereas the amounts of intracellular β - and γ -ENaC remained decreased. Together, these results indicate that only the internalization of the α -ENaC subunit depends on HIF-2 α but not internalization of β - and γ -ENaC. This effect seems not due to increased activity of Nedd4-2 (Supplementary Fig. 2). This enzyme ubiquitinates ENaC, which then leads to its internalization and degradation.²⁵

The relative amounts of the α : β : γ -ENaC subunits is essential for optimal Na⁺-channel activity. α -ENaC is required for the formation of Na⁺-conducting pores.

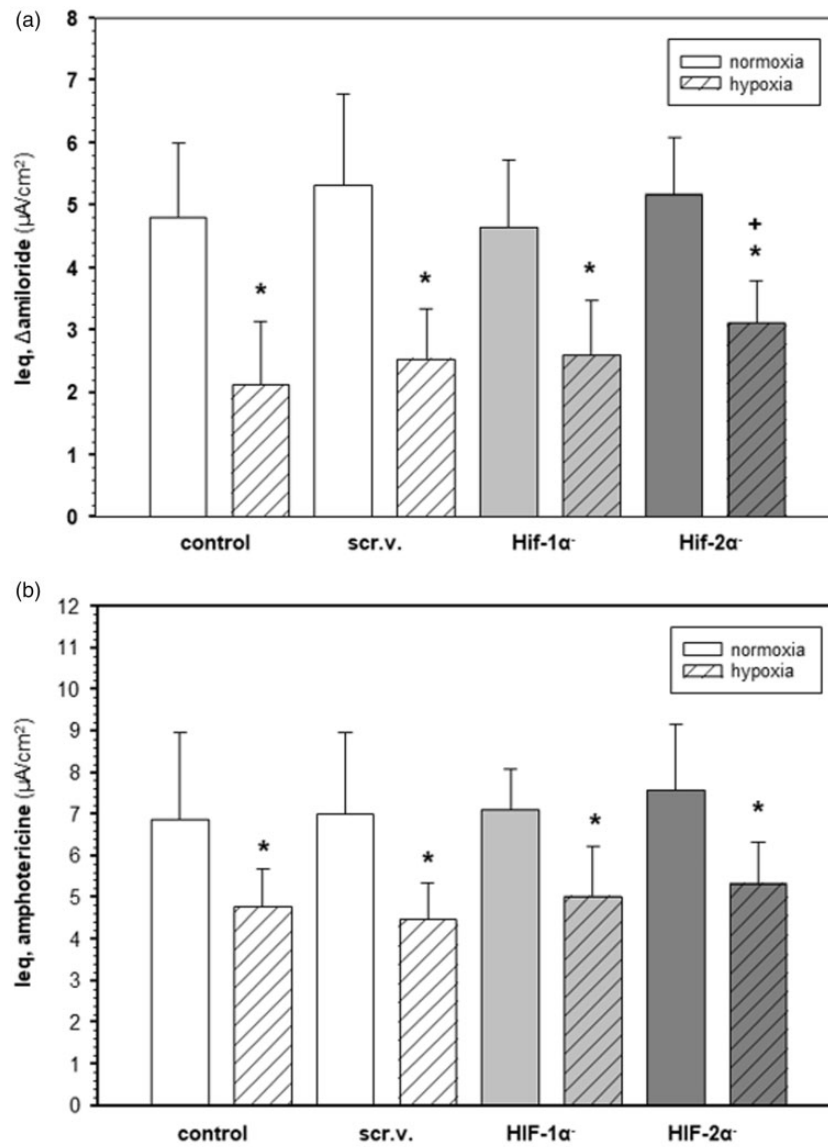


Fig. 1. Effects of hypoxia and HIF silencing on ENaC and Na/K-ATPase activity. Primary rat AEC were treated with adenoviruses to express a scr. or shRNAs to silence the expression of HIF-1 α and HIF-2 α before being exposed to hypoxia (1.5% O₂) for 24 h. ENaC activity was measured in Ussing chambers as the component of leq inhibited by amiloride (10 μM) (a); the capacity of Na/K-ATPase was measured after permeabilizing the apical membrane with amphotericine A (5 μM) in the presence of amiloride (b). Mean values \pm SD from five to eight filters from three different preparations of AEC. *Difference between hypoxia and normoxia; +Effect of silencing HIF-2 α in hypoxia exposed cells compared to hypoxic control ($P < 0.05$).

AEC: alveolar epithelial cells; HIF-1 α ⁻: HIF-1 α silenced; HIF-2 α ⁻: HIF-2 α silenced; scr: scrambled sequence.

However, channels consisting only of α -subunits have a low Na⁺-conductance. Increased amounts of the β - and γ -ENaC subunits serve as stimulators of Na⁺-transport.²⁶ Therefore, the lack of full recovery of ENaC-activity observed after silencing HIF-2 α in hypoxia-exposed AECs might be explained by a relative lack of these stimulatory β - and γ -ENaC subunits.

We also tested whether HIF-dependent mechanisms play a role in alveolar fluid reabsorption in hypoxia-exposed rats in vivo. HIF-2 α was silenced by intra-tracheal application of the respective adenoviruses using a micro-sprayer after

depleting alveolar macrophages with clodronate²⁷ to minimize a possible inflammatory response subsequent to adenovirus application (supplementary Fig. 3). Rats were then exposed to hypoxia (8% O₂, 24 h). Fluid reabsorption was measured after intra-tracheal instillation of Krebs-Ringer buffer using bovine serum albumin as a volume marker.⁸ Results confirmed earlier findings of an approximately 50% inhibition of alveolar fluid reabsorption upon exposure to hypoxia.⁷⁻⁹ Our results also indicate that silencing HIF-2 α restored a small portion of normal reabsorption capacity (Fig. 3). These results were in accordance with the findings

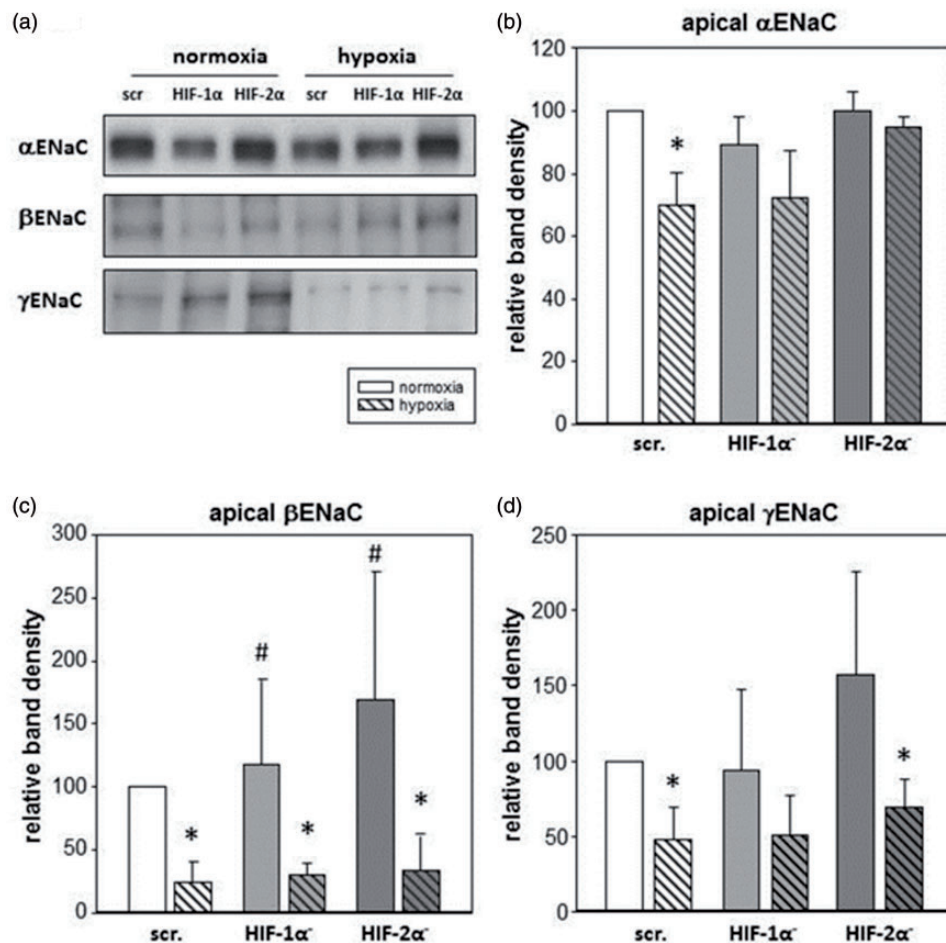


Fig. 2. Effect of hypoxia and HIF silencing on ENaC abundance in apical membranes of AEC. Treatment of cells was as described in legend to Fig. 1. Apical membrane proteins were biotinylated for Western blot analysis of ENaC subunit protein expression. Values are normalized to normoxic cells treated with scr. Representative immunoblots (a) and surface expression of α -ENaC (b), β -ENaC (c), and γ -ENaC (d). Results are means \pm SD of three to five independent cell preparations. *Effect of hypoxia at the respective treatment; #Effect of HIF silencing in normoxia ($P < 0.05$).

ENaC: epithelial Na^+ -channels; HIF-1 α $^-$: HIF-1 α silenced; HIF-2 α $^-$: HIF-2 α silenced; scr: scrambled sequence.

on cultured primary rat alveolar epithelium and indicate that HIFs play a role in the control of lung fluid balance in hypoxia.

High-altitude pulmonary edema

HAPE is a non-cardiogenic edema that occurs typically 24–72 h after rapid ascent of nonacclimatized individuals to altitudes higher than 3000 m²⁸; the incidence is ~5%.^{29,30} Prevalence is highly increased in individuals with previous HAPE, which indicates individual susceptibility.³¹

Typical symptoms of HAPE are dyspnea during exercise and also at rest as well as hypoxemia. Infiltrates are visible in thorax X-ray images.³² Invasive³³ and Doppler-echocardiographic analyses (e.g. Bärtsch et al.³⁴) indicate pulmonary hypertension that is higher than the physiological response.³⁵ Increased pulmonary capillary pressure³³ leads

to alveolar infiltration of plasma proteins and blood cells.³⁶ Interestingly, there are also individuals who do not develop HAPE despite exaggerated pulmonary arterial hypertension at high altitude.^{37,38} Even in HAPE-susceptible individuals, HAPE is prevented by slow ascent³⁹ but also by prophylactic intake of medication preventing the increase in pulmonary arterial pressure such as nifedipine,³⁴ tadalafil,⁴⁰ and dexamethasone.⁴⁰ Descent, inhaled oxygen, and nifedipine are the first choice in the treatment of HAPE.²⁸

Does HIF play a role in HAPE?

Hypoxemia at high altitude, indicated by decreased arterial oxygen saturation, is more pronounced in individuals suffering from HAPE than in those who tolerate the altitude well.³⁹ Because cellular responses to hypoxia depend on HIF-mediated gene expression, it is possible that the higher degree of hypoxemia observed in HAPE

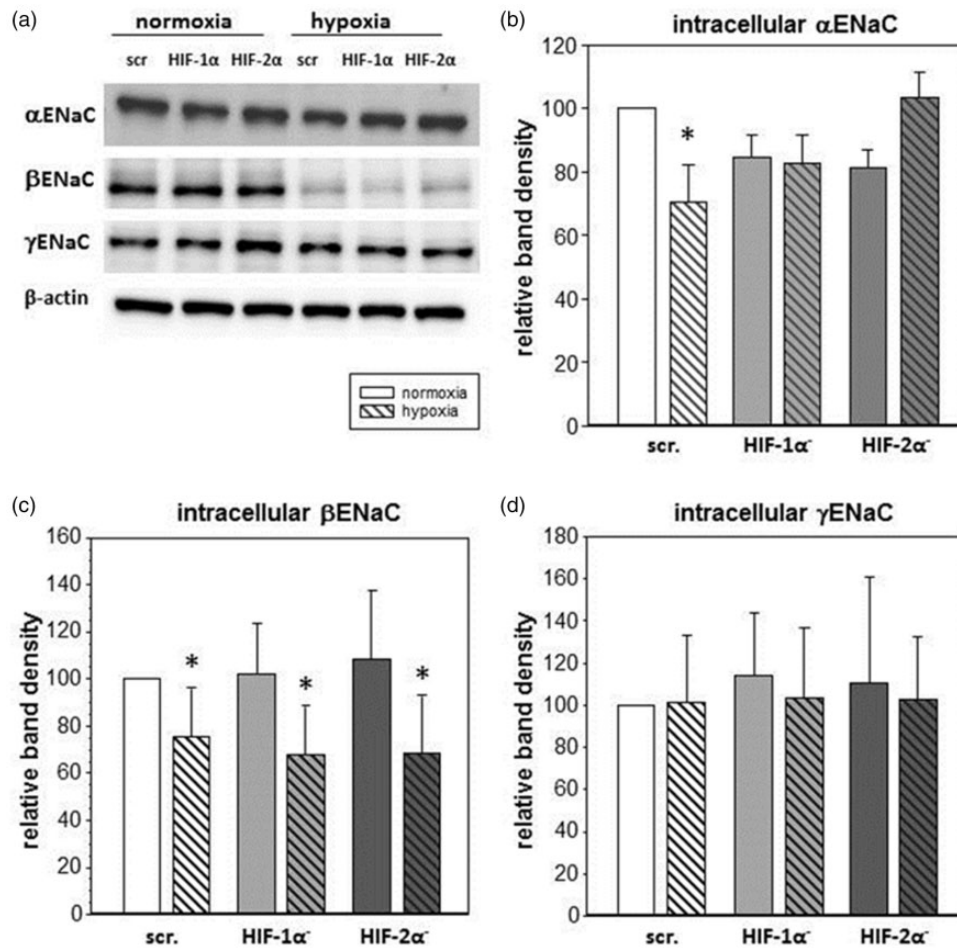


Fig. 3. Effect of hypoxia and HIF silencing on intracellular ENaC protein of AEC. Treatment of cells was as described in legend to Fig. 1. Samples from the non-biotinylated fraction of cell lysates were analyzed by Western blot. Values are normalized to normoxic cells treated with scr. Representative immunoblots (a) and intracellular α -ENaC (b), β -ENaC (c), and γ -ENaC (d). Results are means \pm SD of three to five independent cell preparations. *Effect of hypoxia at the respective treatment ($P < 0.05$). ENaC: epithelial Na^+ -channels; HIF-1 α^- : HIF-1 α silenced; HIF-2 α^- : HIF-2 α silenced; scr: scrambled sequence.

may even enhance HIF-dependent signaling. In agreement with this assumption, we have previously shown that the mRNA expression of HIF-1 α and of the glycolytic enzyme glyceraldehyde dehydrogenase (GAPD), whose expression depends on HIF activation, tended to be elevated in leukocytes from individuals with HAPE, whereas GAPDH expression was not changed in healthy individuals.^{41,42}

Similarly, in whole blood, Yuhong et al. showed increased expression of typical HIF-1 α target genes such as VEGFA and ANGPTL4 in patients with HAPE.⁴³ Also, HAPE has been associated with a genetic variation in the EGLN1 gene,⁴⁴ and elevated expression of EGLN1 in blood cells collected in hypoxia.⁴⁵ Elevated EGLN1, encoding for HIF-prolyl-hydroxylase 2 (PHD2), might indicate increased PHD2 activity and thus accelerated breakdown of newly synthesized HIF- α subunits. This might indicate decreased activity of HIF in HAPE-susceptible individuals. Together, these results seem to point to a role of HIFs in HAPE pathology.

HAPE-susceptibility and alveolar Na^+ -transport

Because alveolar Na^+ -reabsorption controls lung fluid balance, it is conceivable that impaired reabsorption increases the amount of alveolar fluid and thus causes the formation of alveolar edema. This is of particular significance when the filtration of fluid is enhanced such as by the exaggerated pulmonary hypertension and the increased pulmonary capillary pressure found in HAPE in comparison to healthy individuals at high altitude.³³ Unfortunately, neither alveolar fluid reabsorption nor alveolar Na^+ -transport can be measured directly in human alveoli in vivo. Measurement of Na^+ -transport is possible only in the nasal epithelium. This technique has been used to evaluate the activity of Na^+ - and Cl^- -transport in patients suffering from cystic fibrosis⁴⁶ to detect functional consequences of genetic variations of the cystic fibrosis transmembrane regulator. Interestingly, Sartori et al. have shown a decreased nasal

potential difference (NPD) in HAPE-susceptibles even in normoxia,⁴⁷ which we later confirmed.⁴⁸ We also showed that HAPE-susceptibles had lower ENaC activity in normoxia,⁴⁹ and that ENaC activity was decreased at high altitude.⁴⁸ These results indicate that HAPE-susceptible individuals might have a low Na⁺-transport activity in the lung, which would make them more vulnerable to edema formation at high altitude, where exaggerated pulmonary vasoconstriction increases fluid secretion.⁶ It is of importance to note that any association between the occurrence of HAPE and altered nasal epithelial Na⁺-transport remains speculative because it is unclear, whether Na⁺-transport in the nasal epithelium reflects Na⁺-transport in the alveolar epithelium.

A decreased activity of ENaC in HAPE-susceptibles in normoxia might be caused by genetic variations in one of the ENaC subunits or in enzymes involved in the signaling cascade controlling ENaC activity. There is some evidence that lung diffusion capacity might be affected by dysfunctional ENaC: respiratory distress associated with pulmonary edema has been found in pseudo-hypoaldosteronism type 1 due to mutations in ENaC resulting in decreased Na⁺-reabsorption.^{50,51} A genetic variation in the α -subunit of ENaC at amino acid 663 (α A663T) has been shown to affect lung diffusion capacity in otherwise healthy individuals during exercise.⁵² However, genetic variations have not yet been demonstrated in HAPE-susceptibles or in association with altered NPD.

Treatment studies might provide evidence for a potential role of alveolar reabsorption in HAPE.

Inhalation of high doses of the beta-adrenergic agonist salmeterol reduced the incidence of HAPE in HAPE-susceptible individuals at high altitude (4559 m). Lack of effects on systolic pulmonary arterial pressure let the authors to conclude that the beneficial effect may be based on stimulation of alveolar Na⁺-transport⁴⁷ because of the known stimulation of ENaC by beta-adrenergic agents.⁵³ Intake of dexamethasone, another agent known to stimulate activity and expression of Na⁺/K⁺-ATPase and ENaC in lung alveolar epithelium^{54,55} and to increase alveolar Na⁺- and fluid reabsorption even in hypoxic AECs and hypoxic animals,⁸ fully prevented HAPE in susceptible individuals at high altitude.⁴⁰ However, no evidence could be presented to demonstrate altered Na⁺-transport, as ENaC mRNA expression in leukocytes and nasal potential differences were not affected by dexamethasone intake.⁴⁰ Thus, the exact mechanism by which dexamethasone prevented HAPE remains elusive.

Does impaired alveolar Na⁺-transport affect pulmonary vascular resistance?

Alveolar edema impairs oxygen diffusion and causes hypoxic at pulmonary vascular smooth muscle cells, which might cause "hypoxic pulmonary vasoconstriction" even in normoxia.

Therefore, alveolar edema at high altitude might enhance the physiologic hypoxia pulmonary vasoconstriction.⁵⁶ Hence, it is conceivable that decreased Na⁺-transport in HAPE-susceptibles contributes to exaggerated pulmonary arterial hypertension. One experiment to test this hypothesis focused on nasal epithelial Na⁺-transport in individuals with exaggerated hypoxic pulmonary vasoconstriction but without HAPE and compared them with HAPE-susceptibles.³⁷ Unfortunately, the result was not conclusive because, although the individuals with aggravated hypoxic pulmonary vasoconstriction but without HAPE showed a tendency toward slightly higher NPD (and, presumably, alveolar Na⁺-transport) than the HAPE-susceptibles, and the difference was statistically not different.⁴⁹

Another experiment showed that alveolar application of the Na⁺-channel blocker amiloride to anaesthetized rats, which were ventilated with normoxic gas, increased lung water content and impaired alveolar oxygen diffusion indicated by a decrease in arterial SO₂. Interestingly, in this situation, an increase in right ventricular pressure had been observed that was similar to ventilation of rats with hypoxic gas.⁵⁷ However, hypoxia did not enhance the increase in right ventricular pressure induced by amiloride. Lack of additivity weakens the argument of a role of impaired Na⁺-reabsorption as a cause of exaggerated hypoxic pulmonary vasoconstriction,⁵⁶ which one would expect if this model adequately reflected HAPE.

Conclusions

Results from studies on prevention and treatment of HAPE indicate that decreasing pulmonary arterial systolic pressure successfully prevents HAPE in susceptible individuals.^{34,40} It appears therefore likely that exaggerated pulmonary vasoconstriction and subsequent filtration of fluid into the alveolar space is the main effector causing HAPE (Fig. 4). However, the exact mechanisms causing increased susceptibility are not fully understood. Alterations in the control of the membrane potential of lung vascular smooth muscle cells, e.g. by altered K⁺-channel function, but also impaired formation of NO,⁵⁸⁻⁶⁰ and dominance of vasoconstrictors such as endothelin⁶¹ might lead to exaggerated hypoxic pulmonary vasoconstriction. Although experimental evidence is only indirect and not entirely conclusive, a role of alveolar Na⁺- and fluid reabsorption in the formation of alveolar edema in hypoxia and even in the exaggerated pressure response cannot be entirely excluded (Fig. 5). A decreased activity of alveolar Na⁺-reabsorption might be sufficient to increase alveolar fluid volume even at moderate filtration pressures (Fig. 5), which might then result in exaggerated hypoxic pulmonary vasoconstriction.⁵⁷ On the other hand, increased alveolar Na⁺-reabsorption might protect from enhanced fluid filtration due to exaggerated hypoxic vasoconstriction.⁴⁹ Unfortunately, results on the use of β -adrenergic agonists and steroids, which are known stimulators of epithelial Na⁺-reabsorption,^{40, 47} are not conclusive

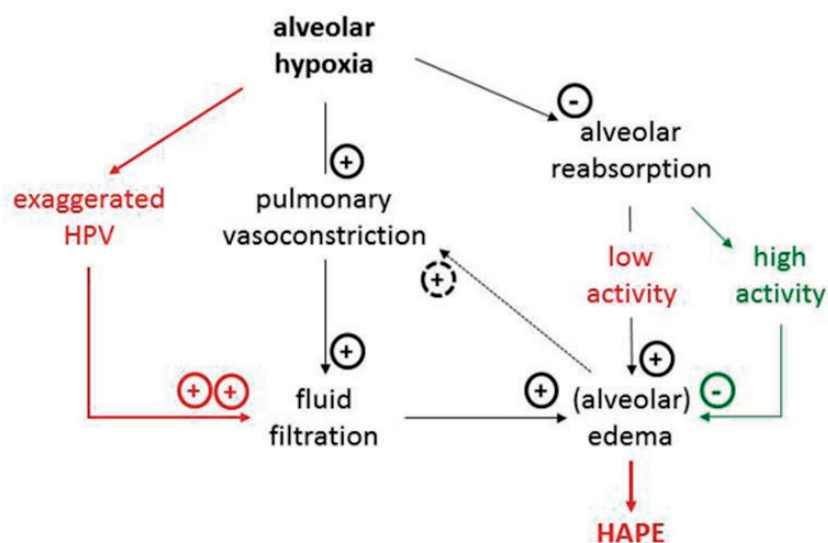


Fig. 4. Impairment of alveolar fluid balance in hypoxia. Schematic presentation of interactions between hypoxia, pulmonary vasoconstriction, and fluid filtration, and the possible role of alveolar reabsorption that might result in HAPE (see text for details). HAPE: high-altitude pulmonary edema; HPV: hypoxic pulmonary vasoconstriction.

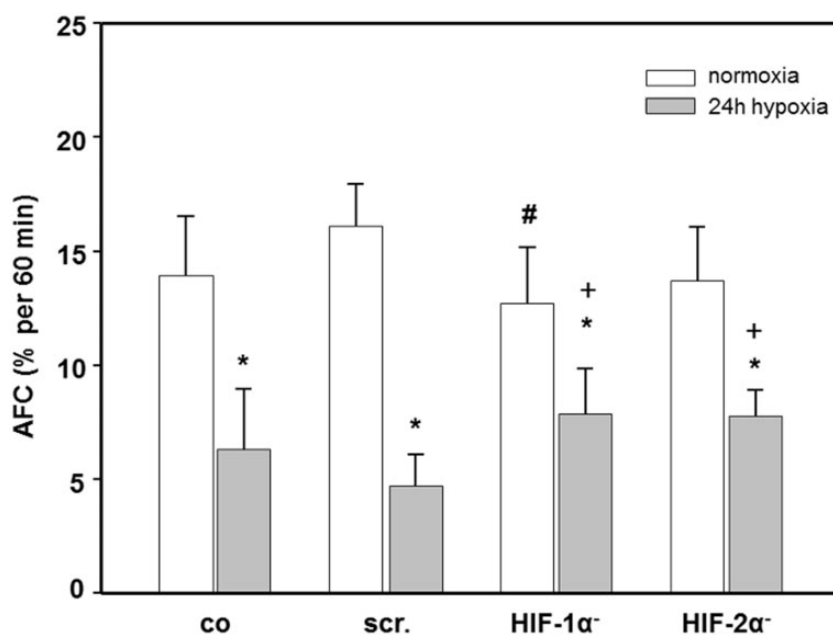


Fig. 5. Effects of in vivo hypoxia and silencing HIFs on alveolar fluid reabsorption in rats. Lung fluid reabsorption was measured in rats after depleting alveolar macrophages with clodronate, application of adenoviruses for introducing scrambled sequence (scr.) and shRNAs for silencing HIFs, and exposure to hypoxia as described in the supplement. Mean values \pm SD of three to eight experiments per condition. *Significant effect of hypoxia; ⁺Significant difference between co and HIF-silencing in hypoxia; [#]Effect of HIF-1 α silencing in normoxia; P < 0.05. HIF-1 α ⁻: HIF-1 α silenced; HIF-2 α ⁻: HIF-2 α silenced; scr: scrambled sequence.

because of non-specific drug actions. Thus, unless a specific stimulator of Na⁺-reabsorption becomes available that can be applied to the alveolar surface, e.g. by inhalation, which does not have direct effects on pulmonary vascular tone, the role of alveolar reabsorption in HAPE remains elusive.

Acknowledgements

We thank Mrs Christiane Herth and Mrs Sonja Engelhardt for excellent technical assistance.

Conflict of interest


The author(s) declare that there is no conflict of interest.

Funding

This work was supported by a grant from the German Research Foundation (DFG; Ma 1503/28-2) to HM and by the Translational Lung Research Center Heidelberg (TLRC-H), which is part of German Center for Lung Research (DZL).

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