Impairment of cation transport in A549 cells and rat alveolar epithelial cells by hypoxia

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Mairbäurl, Heimo, Ralf Wodopia, Sigrid Eckes, Susanne Schulz, and Peter Bärtsch. Impairment of cation transport in A549 cells and rat alveolar epithelial cells by hypoxia. Am. J. Physiol. 273 (Lung Cel. Mol. Physiol. 17): L797–L806, 1997.—A reduced cation reabsorption across the alveolar epithelium decreases water reabsorption from the alveoli and could diminish clearing accumulated fluid. To test whether hypoxia restricts cation transport in alveolar epithelial cells, cation uptake was measured in rat lung alveolar type II pneumocytes (AII) cells in primary culture and in A549 cells exposed to normoxia and hypoxia. In AII and A549 cells, hypoxia caused a Po2-dependent inhibition of the Na-K pump, of Na-K-2Cl cotransport, and of total and amiloride-sensitive 22Na uptake. Nifedipine failed to prevent hypoxia-induced transport inhibition in both cell types. In A549 cells, the inhibition of the Na-K pump and Na-K-2Cl cotransport occurred within ~30 min of hypoxia, was stable >20 h, and was reversed by 2 h of reoxygenation. There was also a reduction in cell membrane-associated Na-K-ATPase and a decrease in Na-K-2Cl cotransport flux after full activation with calcyulin A, indicating a decreased transport capacity. [14C]serine incorporation into cell proteins was reduced in hypoxic A549 cells, but inhibition of protein synthesis with cycloheximide did not reduce ion transport. In AII and A549 cells, ATP levels decreased slightly, and ADP and the ATP-to-ADP ratio were unchanged after 4 h of hypoxia. In A549 cells, lactate, intracellular Na, and intracellular K were unchanged. These results indicate that hypoxia inhibits apical Na entry pathways and the basolateral Na-K pump in A549 cells and rat AII pneumocytes in culture, indicating a hypoxia-induced reduction of transepithelial Na transport and water reabsorption by alveolar epithelium. If similar changes occur in vivo, the impaired cation transport across alveolar epithelial cells might contribute to the formation of hypoxic pulmonary edema.

IN THE NORMAL LUNG, an equilibrium exists between the entry of fluid into the alveolar space and fluid reabsorption in order to keep the film of alveolar lining fluid thin and to allow an adequate diffusion of respiratory gases. In hypoxia, e.g., at high altitude, this equilibrium can be disturbed by pulmonary edema and alveolar flooding (10, 32) that, by thickening of the diffusion barrier, worsens the degree of hypoxemia. The mechanisms that lead to the accumulation of fluid in the alveolar space in hypoxia can be manifold (32). Hypoxic vasoconstriction of pulmonary blood vessels causes pulmonary hypertension and increases the amount of fluid that leaves the vascular bed by filtration. Nifedipine prevents this effect presumably by reducing the blood pressure in the pulmonary artery (1). Mediators released from lung cells, like alveolar macrophages and endothelial cells, which can be activated by hypoxia, by exercise, or by infections, might alter the permeability of the endothelium and the alveolar barrier, allowing plasma or plasma water to leave the vasculature and to enter the interstitium and alveolar space. Furthermore, cation transport processes across alveolar epithelial cells, which in normoxia mediate Na-coupled water reabsorption in the adult lung (22), might be disturbed, resulting in an impaired clearance of alveolar fluid, and might contribute to edema formation if this transport is reduced in hypoxia. However, the role of ion transport in the removal of accumulated edema fluid in hypoxia is unclear. Evidence demonstrating the significance of alveolar Na reabsorption for fluid reabsorption from the alveolar space comes from studies on the clearance of lung liquid after birth (25). These studies indicate that hormone-activated and amiloride-inhibitable pathways mediate the removal of lung liquid (3, 28). In support of that are observations on a knockout mouse that lacks the α-subunit of the epithelial Na channel (ENaC; see Ref. 11). These animals die within a few hours after birth since they are unable to remove the fluid that is contained in the lung airspace during prenatal development (11). Results by Planes et al. (31) on virus-transformed rat alveolar epithelial cells indicate a reduced activity of the Na-K pump when these cells are exposed to hypoxia for at least 12 h. The basolaterally located Na-K pump mediates the removal of Na that entered the cells across the alveolar interface. Therefore, inhibiting the Na-K pump without reducing apical Na entry would cause the alveolar epithelial cells to swell and lyse. Because there are no reports on the destruction of alveolar epithelium in subjects exposed to hypoxia at high altitude and because cells exposed to hypoxia in tissue culture tolerate hypoxia well, there have to be other mechanisms that preserve the cellular integrity.

This study was performed to investigate effects of hypoxia on ion transport systems in alveolar epithelial cells that are involved in Na entry and Na exit and to evaluate possible mechanisms accounting for the hypoxia-induced changes found in cultured alveolar epithelial cells. Our results of flux measurements on A549 cells, a human lung-derived carcinoma cell line that shows most functions of alveolar type II cells (17), and on rat alveolar type II cells in primary culture indicate that not only the Na-K pump activity but also Na entry via the Na-K-2Cl cotransporter and Na channels is reduced already after short-term exposure to hypoxia in a reversible manner. This reduction in ion transport...
seems to be caused by a decreased transport capacity and appears to be independent of protein synthesis and of the energy status of the cells.

MATERIALS AND METHODS

Reagents

All media were prepared from deionized water and analytical grade reagents. Ouabain, amiloride, trypsin, and soybean trypsin inhibitor as well as various protease inhibitors (see below) were from Sigma Chemical. Phosphate-buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12, penicillin/streptomycin (PenStrep), fetal calf serum (FCS), and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) were from Gibco. Calyculin A and okadaic acid were from Calbiochem. HOE-694 ([3-methylsulphonyl-4-piperidino-benzoyl]guanidine methanesulfonate) was a gift from Hoechst, and bumetanide was a gift from Hofmann LaRoch.

Preparation of Rat Alveolar Type II Cells

Alveolar type II (AII) cells for primary culture were prepared similar to the method described by Richards et al. (33). Briefly, lungs from male Sprague-Dawley rats (100–200 g) were extracted after Trapanal (Byk Gulden) anesthesia and perfusion of the ventilated lung with cold PBS to remove blood cells. The lungs were lavaged five times with PBS, filled with trypsin (0.25% in Hanks’ balanced salt solution) to its maximal capacity, and incubated at 37°C for 20 min. Trypsin digestion was stopped by instillation of trypsin inhibitor in DMEM. Major blood vessels and bronchi were removed, and the tissue was microdissected under a stereomicroscope. The minced tissue was vortexed with deoxyribonuclease for 10 min and was passed through filters of 150 and 17 µm pore size. The cells were washed and preplated (60 min) in AII medium (33). Nonadhering cells were seeded on untreated 24-well plates (Costar) at a density of ~300,000 cells/well. The viability assessed by trypan blue exclusion was usually >90%, and >80% of the cells resembled AII pneumocytes, as judged from tannic acid stain. Experiments were either performed on freshly prepared cells in suspension or on primary cultured cells after reaching confluence (3–4 days after seeding) but before differentiation as indicated by the loss of lamellar bodies (1–2 days after reaching confluence).

A549 Cells

Most of the experiments presented were performed on A549 cells (American Type Culture Collection) that were derived from human lung carcinoma cells and that resemble many features of AII cells (17). The cells were grown on untreated 24- or 96-well plates (Costar) in Ham’s F-12 medium substituted with 7% FCS and PenStrep and buffered with HEPES and sodium bicarbonate. Confluence was reached ~3–4 days after seeding. All experiments were performed on 2- to 8-day-old confluent monolayers.

Tissue Culture, Hypoxia

Both rat AII cells in primary culture and A549 cells were kept in an incubator (Heraeus) under the usual tissue culture conditions at 37°C and 5% CO₂. For exposure to hypoxia, cells in suspension (AII cells) or cells grown to confluence in normoxia in the incubator (AII and A549 cells) were transferred into a thermostatted (37°C) acrylic glass box (volume ~8 liters) that was flushed with sterile-filtered (0.22 µm) humidified gas composed of varying concentrations of O₂, 5% CO₂, and N₂ for varying periods of time. Hypoxia was initiated by replacing the tissue culture medium with one previously equilibrated to the appropriate gas. After an initial flushing with the appropriate gas at a high rate for 5 min, the gas flow was kept constant at a rate of ~0.2 l/min during the incubation. Based on the results by Wolff et al. (41) on O₂-dependent erythropoietin production by hepatocytes which indicate that the oxygen supply to the cultured cells might be limited even in normoxia when no adequate equilibration is guaranteed, shaking of the box appeared necessary. The acrylic glass box was therefore placed on a shaker (12 per min). Experiments to control for the shaking procedure indicated that ion fluxes were the same regardless of whether cells were kept without shaking in an O₂/CO₂ controlled incubator or in the rocked acrylic glass box under normoxic and hypoxic conditions. Therefore, especially for long-term incubations, an O₂/CO₂ controlled incubator (Nunc) was used to expose cells to hypoxia. PO₂ and PCO₂ in the atmosphere of the incubator and the acrylic glass box and in the tissue culture medium supernatant to the cells was checked with a blood gas analyzer (model 278; Corning).

Flux Measurements

The activity of ion transport pathways was determined by unidirectional tracer uptake measurements. The cells were washed two times with washing medium [150 mM NaCl and 2 mM HEPES, pH 7.4 at room temperature (RT)] to remove tissue culture medium and were equilibrated to the flux medium for 15–40 min at RT under either normoxic or hypoxic conditions (CO₂ and bicarbonate free). The flux medium was usually composed of (in mM) 140 NaCl, 5 KCl, 1 Na₃HPO₄, 1 MgCl₂, 0.2 CaCl₂, 10 glucose, and 20 HEPES, pH 7.4, at RT. Both media were equilibrated to the respective gas.

After equilibration, the medium was replaced with fresh flux medium containing either ⁸⁶Rb (used as tracer for K) or ²²Na, both at final activities of 2 µCi/ml. The tracer uptake into the cells, measured at RT, was terminated with six washes with washing medium to remove radioactive contamination. The cells were lysed with 0.1 M NaOH. The radioactivity in the lysate was measured in a β-counter (model TR 2100; Canberra Packard), and the protein content (see below) was used for standardization.

In controls experiments, the tracer uptake was found to be linear over a period of at least 10 min. Therefore, in most experiments, only one 6-min time point was taken unless otherwise stated. All fluxes were measured in duplicate or triplicate on batches of cells from different passages.

The Na/K pump was taken as the ouabain-inhibitable portion of ⁸⁶Rb uptake. Because of the low affinity for ouabain, the concentration used was 3 mM in rat AII pneumocytes but 0.1 mM in the human-derived A549 cells. The Na-K-Cl cotransport was taken as the portion of ⁸⁶Rb or ²²Na uptake inhibited by 50 µM bumetanide. Amiloride inhibition of ²²Na uptake was complete at an inhibitor concentration of 300 µM, but HOE-694 had no effect. The maximal effective concentration of all inhibitors used was verified with dose-response curves only in A549 cells and not in AII cells due to the limited amount of material.

Preparation of Cell Membranes

For the measurement of Na-K-ATPase activity and the electrophoretic separation of membrane proteins, A549 cells grown to confluence in 80-cm² tissue culture flasks in normoxia were exposed to normoxia or hypoxia and then were washed free of culture medium with a lysis medium composed of 250 mM sucrose and 50 mM tris(hydroxymethyl)aminomethane base, pH 7.4 at RT, containing the protease inhibi-
Intracellular Na (Na\textsubscript{i}) and K (K\textsubscript{i}) concentrations were measured using a test kit from Sigma Chemical. Lactate in supernatant tissue culture medium (A549 cells) was determined by liquid scintillation counting.

**Other Measurements**

Membranes prepared from A549 cells as described above were assayed for Na-K-ATPase activity to obtain a measure of Na-K pump capacity. ATPase activity was measured according to Forbush (6). The Na-K-ATPase activity was taken as the activity inhibited by 0.1 mM ouabain.

**Measurements of Cell Metabolites and Cations**

Freshly isolated AII cells kept in suspension and A549 cells grown to confluence in normoxia in 80-cm\textsuperscript{2} culture flasks were exposed to either normoxia or hypoxia as described above. Supernatant culture medium was removed by two washes with ice-cold washing medium. The cells were lysed with ice-cold 0.6 N perchloric acid (PCA). Precipitated protein was removed by centrifugation (3 min, 13,000 \( g \)) in a microfuge. The pH of the supernatant was adjusted to seven with K\textsubscript{2}CO\textsubscript{3}. ATP and ADP were measured by high-performance liquid chromatography according to Weicker et al. (39). Lactate in supernatant tissue culture medium (A549 cells only) was measured using a test kit from Sigma Chemical. Intracellular Na (Na\textsubscript{i}) and K (K\textsubscript{i}) concentrations were measured by flame photometry (model 410; Corning) in PCA extracts of confluent A549 cells.

**Protein Synthesis**

The amount of [\textsuperscript{14}C]serine incorporated into cell protein was determined as a measure of protein synthesis. [\textsuperscript{14}C]serine was added to A549 cells grown to confluence in 3-cm dishes in normoxia and then was exposed to either normoxia or hypoxia. After incubation, the culture medium and contaminating [\textsuperscript{14}C]serine were removed by four washes with washing medium. The cells were lysed with 0.6 N PCA. The precipitated protein was collected on cellulose filters (GF/C, average pore size 0.22 \( \mu \text{m} \); Whatman). The radioactivity on the filters was determined by liquid scintillation counting.

**Other Measurements**

Na\textsubscript{i} and K\textsubscript{i} were measured by flame photometry after washing the cells two times with a medium composed of 150 mM choline chloride and 2 mM HEPES, pH 7.4 at RT, and subsequent lysis with 0.1% lithium dodecyl sulfate in water. The protein concentration was measured according to Bradford using a test kit from Bio-Rad and standards composed of human serum albumin and globulin in saline diluted with 0.1 M NaOH.

**Statistical Evaluation**

All measurements were performed in duplicates to quadruplicates on cells from at least three different passages. Results are presented either as mean values ± SD of repeated measurements from one of several experiments with similar results or as mean values ± SD of several experiments on different batches of cells as indicated. Comparisons were made using Student's t-tests. Dose-response curves and time courses were tested by one-way analysis of variance and linear correlation analysis, respectively. The level of significance was \( P \leq 0.05 \). Graphical and statistical evaluation was performed with the SigmaPlot (Jandel Scientific) and Systat (Systat) software packages.

**RESULTS**

**A549 Cells**

Exposure of A549 cells to hypoxia (3% O\textsubscript{2}, \( \sim 21 \) mmHg) for 4 h caused a 14% decrease in the ATP content (Fig. 1). No statistically significant change was found in the ADP content in the cell lysate and the ATP-to-ADP ratio. The lactate concentration in supernatant culture medium was 4.8 ± 0.8 mM in cells cultured in normoxia and 4.7 ± 0.6 mM in cells exposed to hypoxia. The total amount of protein per well was not different between normoxic cells and cells exposed to 3% O\textsubscript{2} for 4 h. Also, the Na and K content of the cells was not different between normoxic cells and cells exposed to 3% O\textsubscript{2} for 4 h. Weibull et al. (40) reported that inhibition of the Na-K pump capacity by 0.1 mM ouabain did not affect the ATP-to-ADP ratio. ATPase activity was measured according to Forbush (6) and according to Forbush (6).

**Fig. 1.** Energy status of alveolar type II (AII) cells and A549 cells in hypoxia. A549 cells (A and B) were grown to confluence in tissue culture flasks, and AII cells (C and D) from rat lungs were used in suspension directly after preparation. Both cell types were exposed to normoxia or hypoxia by replacing the culture medium with one equilibrated to the appropriate normoxic or hypoxic gas mixture and were kept in normoxia (open bars) or hypoxia (3% O\textsubscript{2}, hatched bars) for 4 h. Cells were washed with ice-cold phosphate-buffered saline (PBS) and were lysed with ice-cold 0.6 M perchloric acid (PCA). ATP and ADP concentrations were measured by high-performance liquid chromatography. Results are mean values ± SD (A549 cells: \( n = 5 \) cells from different passages; AII cells: \( n = 5 \) different cell preparations from 2 lungs each). \( *P \leq 0.05 \) compared with normoxic controls.
remained unchanged during hypoxia (results not shown).

Figure 2 shows the time course of changes in the total \(^{86}\)Rb uptake by A549 cells upon exposure to 3% \(O_2\). Figure 2 shows that, within 15 min of hypoxia, the total \(^{86}\)Rb uptake was decreased by \(-40\%\) and that this decrease was maintained for 20 h. Reoxygenation for 2 h of cells exposed to 3% \(O_2\) for 2, 4, and 20 h was sufficient to restore the normal \(^{86}\)Rb uptake almost completely.

In normoxia, \(-40\%\) of the total \(^{86}\)Rb uptake by A549 cells can be attributed to the Na-K pump. Exposure to hypoxia reduced the Na-K pump. This inhibition was seen already after 15 min of exposure to hypoxia (3% \(O_2\)) and was stable for 20 h. A higher degree of hypoxia (1.5% \(O_2\)) caused a further reduction of the pump flux (Fig. 3). Also, the activity of the Na-K-ATPase measured on plasma membranes prepared from cells exposed to 3% \(O_2\) for 4 h decreased by \(-55\%\) (Fig. 4).

About 45% of the total \(^{86}\)Rb uptake was inhibited by 50 \(\mu\)M bumetanide (Fig. 5A). The half-maximal inhibitory concentration (IC\(_{50}\)) for the bumetanide inhibition of \(^{86}\)Rb uptake was 1.1 \pm 0.05 \(\mu\)M. These values are comparable to results obtained on different cell types (9). Bumetanide-sensitive \(^{86}\)Rb uptake and bumetanide-sensitive \(^{22}\)Na uptake (not shown) were about of the same magnitude. Therefore, it appears likely that this flux represents Na-K-2Cl cotransport. Na-K-2Cl cotransport is reduced by \(-40\%\) already within 15 min of exposure of A549 cells to hypoxia (3% \(O_2\); \(n = 2\); not shown). Inhibition (\(-50\%\)) was stable over the period of 4–20 h of hypoxia (Fig. 6). At 1.5% \(O_2\), the inhibition was more pronounced. As with the Na-K pump, upon reoxygenation for 2 h of cells exposed to 3% \(O_2\) for 2, 4, and 20 h was sufficient to restore the normal \(^{86}\)Rb uptake almost completely.

Figure 2. Effects of hypoxia and reoxygenation on total \(^{86}\)Rb uptake by A549 cells. A549 cells grown to confluence in normoxia were exposed to hypoxia (\(\leq 3% O_2\)) at 37°C for the time periods indicated. Time point 0 indicates normoxic controls. Subsets of hypoxia-exposed cells were reoxygenated for 2 h (\(\bullet\)) after replacing the culture medium with one equilibrated to a gas composed of 21% \(O_2\), 5% \(CO_2\), and \(N_2\) (\(\bigcirc\), normoxic controls). Total \(^{86}\)Rb uptake was measured at room temperature (RT) in a flux medium composed of (in mM) 140 NaCl, 5 KCl, 1 Na\(_2\)HPO\(_4\), 1 MgCl\(_2\), 0.2 CaCl\(_2\), 10 glucose, and 20 HEPES, pH 7.4 at RT, which was equilibrated with the appropriate gas (\(CO_2\) free). Results are mean values \pm SD (hypoxia effect: \(n = 12\); reoxygenation: \(n = 3\)). All fluxes were measured in triplicate. *\(P < 0.05\) compared with normoxic controls (time = 0); #\(P < 0.05\) compared with 3% \(O_2\), mgProt, mg protein.

Fig. 3. Effect of hypoxia on the Na-K pump of A549 cells. A549 cells grown to confluence in normoxia were exposed to hypoxia (1.5% (\(\bigtriangleup\)) and 3% (\(\bullet\)) \(O_2\)) for the time indicated. Fluxes were measured as described in legend to Fig. 2. Na-K pump activity was taken as the portion of \(^{86}\)Rb uptake inhibited by 0.1 mM ouabain. Results are mean values \pm SD (\(n = 12\)). All fluxes were measured in triplicate. *\(P < 0.05\) compared with normoxic controls (time = 0); #\(P < 0.05\) compared with 3% \(O_2\), mgProt, mg protein.

Fig. 4. Effect of hypoxia on Na-K-ATPase activity of A549 cells. Cells grown to confluence in normoxia were exposed to normoxia or hypoxia for 4 h after replacing the culture medium with one equilibrated with gases containing either 21 or 3% \(O_2\). For measurement of the activity of membrane-associated Na-K-ATPase, plasma membranes were prepared by differential centrifugation after mechanical disruption of A549 cells. Results are mean values \pm SD from 3 experiments on different batches of cells. *\(P < 0.05\) compared with normoxia.
generation for 2 h, the inhibition of cotransport was reversed (not shown). As a measure for cotransport capacity, $^{86}$Rb uptake was measured in A549 cells treated with 0.1 µM calyculin A before the flux measurement but after exposure to hypoxia. Thereby, the exposure to calyculin A was limited in time, which was important, since it is not clear whether calyculin A affects the cells response to hypoxia. This experiment is based on the observation that Na-K-2Cl cotransport is active in a phosphorylated state (18) and that, with maximal phosphorylation by inhibition of its dephosphorylation with inhibitors of the protein phosphatases PP1 and/or PP2a, also the cotransport flux reaches its maximal activity (30). Control experiments on normoxic cells indicated that it was necessary to apply calyculin A at least 10 min before the flux measurement to achieve complete activation of the cotransport flux (Fig. 5B). The IC$_{50}$ for calyculin A activation of $^{86}$Rb uptake was 10^{-3} nM, and this value was not changed by hypoxia. Values reported on the Na-K-2Cl cotransport activation by calyculin A obtained on endothelial cells are in the same range (15) and are similar to values for inhibition of type I and IIa protein phosphatases measured in cell-free systems (13). Figure 6 shows that the cotransport activity measured after treatment with calyculin A was inhibited when A549 cells were exposed to hypoxia for 4 and 20 h. As in untreated cells, this inhibition was more pronounced when the PO$_2$ was decreased from 21 to 12 mmHg.

Nifedipine at a concentration of 10 µM reduced total $^{86}$Rb uptake in normoxic and hypoxic A549 cells by ~10% and did not prevent the hypoxia-induced inhibition of transport (Fig. 7A). It did not affect the activity of the Na-K pump.

The uptake of $^{22}$Na was measured to assess the activity of transport systems in which Na transport is not coupled to the transport of K. In normoxia, ~25% of the $^{22}$Na uptake into A549 cells was inhibited by bumetanide. The hypoxia effect on Na-K-2Cl cotransport measured as $^{22}$Na uptake was the same as for $^{86}$Rb uptake (result not shown). There was also a significant portion of $^{22}$Na uptake in normoxic cells that was inhibited by amiloride. However, this fraction varied quite widely from 25 to 65% of the total $^{22}$Na uptake.
Because amiloride inhibits Na-H exchange and Na channels (16), it was important to determine which of these transport systems mediated the amiloride-sensitive \(^{22}\)Na uptake in A549 cells and how they were affected by hypoxia. In normoxia, \(\approx 90\%\) of the rate of protein synthesis is inhibited by 50 \(\mu\)M of cycloheximide (IC\(_{50} = 0.42 \pm 0.03 \mu\)M). Figure 9A shows that the amount of \(^{14}\)C-serine incorporated into protein was reduced after 4 h of hypoxia at 3% \(O_2\) but that the cycloheximide-insensitive portion remained unchanged. Figure 9B shows the results of flux measurements of cells exposed to normoxia and 3% \(O_2\) for 4 h in the absence or presence of 50 \(\mu\)M cycloheximide. Cycloheximide inhibition of protein synthesis did not affect \(^{86}\)Rb uptake in cells exposed to normoxia or hypoxia.

**Rat AII Pneumocytes**

A549 cells appear to have many functions of AII cells but are derived from carcinogenous tissue and were passaged many times. It was therefore unclear whether ion transport processes studied on these cells reflect those of the lung alveolar epithelium. To test if A549 cells can be used as a model cell for AII cells, thus substituting for animal experiments, subsets of the experiments shown before were also performed on rat AII cells. These cells were used for experiments either freshly after isolation or after growing to confluence in normoxia under the usual tissue culture conditions.

**Fig. 7. Effect of nifedipine on \(^{86}\)Rb uptake by AII cells and A549 cells in normoxia and hypoxia.** AII cells were prepared as described in MATERIALS AND METHODS and were used in suspension directly after isolation. A549 cells were plated on 24-well plates and were grown to confluence. Both cells were exposed to normoxia and hypoxia by replacing culture media with ones containing 10 \(\mu\)M nifedipine or equivalent volumes of ethanol that were equilibrated to the appropriate gas mixture. Cells were kept for 4 h in the normoxic (N) or hypoxic (H; 3% \(O_2\)) incubator. Fluxes, in presence or absence of fresh nifedipine, were measured as described previously. Results are presented in percent (mean values ± SD; \(n = 5\)) of normoxic controls.

*\(P < 0.05\) compared with normoxia; #\(P < 0.05\) between hypoxia and hypoxia plus nifedipine.

**Fig. 8. Effect of hypoxia on \(^{22}\)Na uptake by A549 cells.** Cells grown to confluence in normoxia were exposed to hypoxia (3% \(O_2\)) for 4 h. Fluxes were measured in presence of 0.1 mM ouabain with and without HOE-694 (10 \(\mu\)M) and amiloride (0.2 mM) as indicated. Flux medium was the same as in the legend to Fig. 2. Mean values ± SD from triplicate flux measurements of 1 of 3 experiments with similar results. *\(P < 0.05\) compared with normoxia.
As with A549 cells, AII cells also decreased their ATP content upon 4 h of exposure to hypoxia (3% $O_2$). The ATP concentration of freshly isolated AII cells that were kept suspended in normoxia- or hypoxia-equilibrated media was ~20% lower in the hypoxic AII cells than in normoxic controls (Fig. 1). No change in AII cell ADP was found, but the ATP/ADP fell significantly by ~25%.

The results of measurements of the Na-K pump activity and Na-K-2Cl cotransport as ouabain-sensitive and bumetanide-sensitive $^{86}$Rb uptake, respectively, and as amiloride-sensitive $^{22}$Na uptake are shown in Table 1. In normoxia, the Na-K pump mediated ~65% of the total $^{86}$Rb uptake, and ~12% was mediated by Na-K-2Cl cotransport. Amiloride (0.2 mM) inhibited ~50% of the total $^{22}$Na uptake measured in the high-Na flux medium. The total $^{86}$Rb uptake was significantly higher in AII cells grown to confluence than in freshly isolated cells; the Na-K pump was 6-fold and the Na-K-2Cl cotransport was 20-fold increased in confluent AII cells. In both freshly isolated and confluent AII cells, all flux components were about one order of magnitude lower than in confluent A549 cells. As in A549 cells and in AII cells, the Na-K pump, Na-K-2Cl cotransport, and the amiloride-inhibitable portion of $^{22}$Na uptake were reduced after 4 h of hypoxia at 3% $O_2$. The percent change in $^{86}$Rb uptake in normoxia was about the same in both cell types, whereas the hypoxia-induced decrease in the amiloride-inhibitable portion of $^{22}$Na uptake was greater in AII cells.

Figure 7 shows that, similar to the results obtained from A549 cells, the hypoxia (3% $O_2$, 4 h)-induced inhibition of $^{86}$Rb uptake by freshly isolated AII cells could not be prevented by pretreatment with nifedipine. On the contrary, when nifedipine was present over the 4-h incubation period, it caused a slight inhibition of transport in both the normoxic and hypoxic cells. In contrast to A549 cells, nifedipine inhibited the Na-K pump in normoxic AII cells.

**DISCUSSION**

The results indicate that the activity of transport pathways involved in the transepithelial transport of Na$^+$ is reduced in both a human lung carcinoma cell line derived from alveolar epithelium and rat AII cells when these cells were exposed to hypoxia under tissue...
culture conditions. The Na⁺ transported by these mecha-
isms is assumed to contribute to the generation of an
electrochemical and osmotic gradient that can be uti-
лизов for the reabsorption of water across the alveolar
epithelium. Any reduction in Na transport can there-
fore be taken as an indication of an impaired water
reabsorption from the alveolar space that might cause
an accumulation of water in the alveoli and therefore
contribute to the formation of lung edema in hypoxia.

In the adult lung, AII pneumocytes are thought to be
the cells responsible for keeping the water film in the
subphase between the surfactant layer and the epithe-
lium thin by controlling cation and water reabsorption
(22). The basis for this assumption is 1) the observation
of active transport in AII cells (7, 21) and 2) the
observation that alveolar type I cells, despite the fact
that they cover ~90% of the alveolar surface but
represent only ~10% of all lung cells, do not contribute
to Na and water reabsorption across the alveolar
epithelium (22), probably because of a very low density
of Na-K pumps (12). However, direct proof for the
exclusive role of either of the two cell types in fluid
reabsorption is lacking.

Water reabsorption by AII cells is, as in other reab-
sorptive epithelia, coupled to the transepithelial trans-
port of Na, which is driven by a basolateral Na-K pump
and apical pathways for Na entry into the cell. The
latter ones shown to be present in AII cells are ENaC
(23), Na-H exchange, Na-K-2Cl cotransport, and prob-
ably also Na-coupled glucose and amino acid transport-
ners (24, 35). The rate-limiting steps are assumed to be
the Na entry pathways, whereas the Na-K pump seems
to operate at a high rate. Our own results obtained on
rat AII cells in primary culture and on A549 cells
indicate that up to ~80 or 90% of the Na entry into the
cell is mediated by amiloride- and bumetanide-sensi-
tive pathways. Based on the observation that the
bumetanide-sensitive portion of Na and Rb uptake are
of the same size and that the IC₅₀ for bumetanide is in
the range of 1 µM, it is reasonable to assume that this
transport resembles Na-K-2Cl cotransport. Evidence
for the presence of Na-K-2Cl cotransport in A549 cells
comes also from preliminary experiments in which the
presence of an Na-K-2Cl cotransport protein with a
molecular weight of ~156,000 was identified (not shown)
with the T4 antibody (gift of C. Lytle, University of
California, Riverside, CA) directed against the cotrans-
port protein of T84 cells (19). The portion of Na uptake
inhibited by a high concentration of amiloride (0.2 mM)
appears to be mediated by Na channels rather than by
Na-H exchange. In A549 cells, evidence for Na channel-
methylmediated Na uptake comes from the IC₅₀ for amiloride
inhibition of ~1.2 µM, which is lower than the IC₅₀ for
Na-H exchange, and the lack of effects of dimethylamiloride
and HOE-694, which are both specific inhibitors of
Na-H exchange (16, 37). Further evidence comes from
results showing that about the same portion of Na
uptake that is inhibited by amiloride can also be
inhibited with benzamil (40) and the presence of ENaC
proteins in plasma membranes of A549 cells (40) de-
tected with polyclonal antibodies against ENaC sub-
units α, β, and γ (collaboration with C. Canessa, Dept.
Cellular and Molecular Physiology, Yale University; see
Ref. 2).

However, additional work needs to be done to
specify the type of Na channel. The presence of apical
Na channels in AII cells has been shown by various
authors (26, 27, 29) but has not been demonstrated in
A549 cells before. The significance of amiloride-
sensitive pathways and of the Na-K pump for lung
liquid reabsorption has been demonstrated in studies
on the removal of fluid instilled into the intact lung by
Ussing chamber measurements on AII cells in primary
culture (24, 35) and in a knockout mouse in which the
α-subunit of ENaC has been deleted (11). The contribu-
tion of other Na-coupled transport systems to the
removal of alveolar fluid is less clear.

Whereas it is evident from the above-mentioned
reports that transepithelial ion transport is involved
in lung liquid clearance and that, in alveolar epithelial
cells, hypoxia seems to impair ion transport, there are
no reports that, in hypoxia, an impairment of lung
liquid clearance by reducing transalveolar ion transport
contributes to the formation of hypoxic pulmonary
edema. This issue appears rather controversial. In
vitro, Planes et al. (31) demonstrated a reduction in
Na-K pump activity in an established cell line of SV40
virus-transformed rat AII cells after exposing these
cells to hypoxia for at least 12 h. Inhibition appears to
be independent of the degree of hypoxia (31). In A549
cells, a human lung carcinoma cell line resembling
several functions of AII cells, we show a rapid inhibition
of several cation uptake systems that varies with the
degree of hypoxia (see Figs. 2, 3, and 6) and that is
reversible upon reoxygenation. We also report that, in
rat AII cells in primary culture, the Na-K pump,
Na-K-2Cl cotransport, and (presumably) Na channel-
mediated flux are reduced significantly after 4 h of
hypoxia (Table 1). Although we cannot rule out some
damage and therefore alterations in function of rat
primary AII cells in culture, these results are in agree-
ment with reports on a reduced dome formation by
confluent rat AII cells upon treatment with metabolic
inhibitors (8). Information obtained on intact lungs is
sparse and less clear (32). Any maneuver known to
stimulate transepithelial ion transport in vitro also
enhances the reabsorption of liquid instilled into lungs
(35). However, the absence of blood flow and ventilation
did not prevent the clearance of instilled serum in
sheep lungs (34), but ouabain still inhibited all fluid
reabsorption (34). Although no pulmonary edema was
observed in isolated, perfused rat lungs, inhibitors of
aerobic metabolism decreased the rate of reabsorption of
Na⁺ and instilled fluid (36). Furthermore, it has
never been shown that inhibition of ion transport by
applying blockers of cation transport systems to the
intact lung causes pulmonary alveolar edema. The
α-ENaC knockout mouse model (11) provides some
evidence but has not been studied enough to character-
ize effects that might occur secondary to the deletion.

The mechanisms causing the hypoxia-induced inhibi-
tion of ion transport in alveolar epithelial cells in tissue
culture are not understood. The reduction in ATP in the
SV40 transformed cells points to a metabolic limitation of active transport in hypoxia in this cell type (31), which agrees with the previously mentioned reduction of dome formation by metabolic inhibitors (8). In primary cultured AII cells and A549 cells, hypoxia decreased cellular ATP levels by 20% or less, which appears too small to cause inhibition of active transport and points to a mechanism that might be directly dependent on the level of oxygen. Metabolic inhibition might therefore have synergistic effects. A decrease in ATP would primarily affect active ion transport systems. An inhibition of the Na-K pump would cause Na\textsuperscript{+} and cell volume to increase and, subsequently, would lead to cell lysis. The lack of increase in Na\textsuperscript{+} in A549 cells implies therefore a coordinate inhibition of both apical Na entry and basolateral Na-K pumps. This might be a protective mechanism to conserve energy and to prevent cell destruction in hypoxia.

The hypoxia-induced reduction in the activity of the transporters studied might be due to inactivation or reduction of the number of active transporters in the plasma membrane. However, signaling pathways are unclear. Planes et al. (31) found that inhibiting Ca entry with nifedipine also prevents the hypoxia-induced inhibition of Na-K pumps in SV40 virus-transformed alveolar epithelial cells. In contrast, in primary cultured rat AII cells and in the human lung A549 cell, nifedipine did not prevent the inhibition of \textsuperscript{86}Rb uptake caused by hypoxia (Fig. 7). Rather, nifedipine reduced transport in both normoxic and hypoxic cells, indicating some Ca-dependent regulation of ion transport but a lack of involvement of nifedipine-sensitive Ca entry in the inhibition of transport by hypoxia.

Our results indicate that the transport capacity, a measure of the number of active transporters in the plasma membrane, is reduced in A549 cells made hypoxic. The reduction in the number of Na-K pumps and Na-K-2Cl cotransporters in the plasma membrane can be caused by internalization of active transporters into vesicles, an increased rate of degradation, and a reduced synthesis and/or translocation to the membrane. We measured protein synthesis and effects of protein synthesis inhibitors on the ion transport of A549 cells in hypoxia. The result of a reduced amount of protein containing \textsuperscript{14}C-serine indicates that hypoxia causes an inhibition of the overall protein synthesis in A549 cells. Other reports also indicate a reduction of protein synthesis by hypoxia (14), but there is also upregulation of the transcription of certain proteins like the hypoxia-inducible factor or hypoxia-associated proteins (4). However, the application of cycloheximide, which inhibits \textapprox 90% of the protein synthesis in A549 cells, did not alter the total Na-K pump and Na-K-2Cl cotransport-mediated \textsuperscript{86}Rb uptake by A549 cells, neither in normoxia nor in hypoxia. The turnover of the studied transporters appears to be too slow to be affected by a 4-h inhibition of protein synthesis. Therefore, it appear unlikely that a reduced rate of protein synthesis causes the effect of hypoxia on ion transport activity. The failure to prevent the hypoxia-induced reduction of ion transport with cycloheximide indicates further that a factor regulated by transcription, as in the case in the production of erythropoietin (38), is not involved in transducing a signal that mediates the inhibition of ion transport in hypoxia. It also indicates that hypoxia does not accelerate the degradation of transport proteins.

Taken together, these results show that hypoxia affects ion transport in lung alveolar epithelial cells of different origin. The mechanisms causing this effect are unclear and appear to differ depending on the system studied. There are distinct differences in the response to hypoxia between SV40 virus-transformed rat alveolar epithelial cells (31) and primary culture rat AII cells or human A549 lung carcinoma cells, raising the question of which cell type is the better model. Differences include the time course of inhibition of ion transport by hypoxia, which appears much slower in SV40-transformed cells, indicating a greater resistance to oxygen deprivation, as well as the dependency of this inhibition on extracellular Ca and the involvement of nifedipine-sensitive Ca channels in Ca entry in hypoxia. The latter aspect points to differences in the regulation of transport by Ca between these cells. Nevertheless, comparative studies on cultured cells and animal models are required to test whether cell lines can be used as a model system to simulate and study appropriately hypoxia effects on lung alveolar epithelium.

In conclusion, our results obtained in cultured alveolar epithelial cells indicate a hypoxia-induced reduction in the activity of apical Na entry and basolateral Na exit pathways that usually generate the gradients required for the reabsorption of excess water accumulated in the alveolar space. If under hypoxia similar changes occur in vivo, an impaired reabsorption of fluid accumulated in the alveolar space may be another pathophysiological factor that contributes to the formation of pulmonary edema when the capillary pressure and/or the capillary permeability are increased as in high altitude pulmonary edema or adult respiratory distress syndrome.

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