Alveolar Epithelial Ion and Fluid Transport

Hypoxia decreases active Na transport across primary rat alveolar epithelial cell monolayers

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Mairbäurl, Heimo, Katrin Mayer, Kwang-Jin Kim, Zea Borok, Peter Bärtsch, and Edward D. Crandall. Hypoxia decreases active Na transport across primary rat alveolar epithelial cell monolayers. Am J Physiol Lung Cell Mol Physiol 282: L659–L665, 2002. First published December 14, 2001; 10.1152/ajplung.00355.2001.—Hypoxia has been reported to inhibit activity and expression of ion transporters of alveolar epithelial cells. This study extended those observations by investigating the mechanisms underlying inhibition of active Na transport across primary cultured adult rat alveolar epithelial cell monolayers grown on polycarbonate filters. Cell monolayers were exposed to normoxia and hypoxia (1.5% and 5% O2, 5% CO2), and resultant changes in bioelectric properties [i.e., short-circuit current (Isc) and transepithelial resistance (Rt)] were measured in Ussing chambers. Results showed that Isc decreased with duration of exposure to hypoxia, while relatively little change was observed for Rt. In normoxia, amiloride inhibited ~70% of Isc. The amiloride-sensitive portion of Isc decreased over time of exposure to hypoxia, whereas the magnitude of the amiloride-insensitive portion of Isc was not affected. Na pump capacity measured after permeabilization of the apical plasma membrane with amphotericin B decreased in monolayers exposed to 1.5% O2 for 24 h, as did the capacity of amiloride-sensitive Na uptake measured after imposing an apical to basolateral Na gradient and permeabilization of the basolateral membrane. These results demonstrate that exposure to hypoxia inhibits alveolar epithelial Na reabsorption by reducing the rates of both apical amiloride-sensitive Na entry and basolateral Na extrusion.

alveolar type II cells; Ussing chambers; sodium channels; sodium pump; amphotericin B

VECTORIAL TRANSPORT of Na across the alveolar epithelium is mediated by apical Na entry via amiloride-inhibitable Na channels and Na extrusion by basolateral ouabain-sensitive Na pumps (5, 17, 18). This active Na transport generates an osmotic gradient for fluid movement from the alveolar to the interstitial compartment, which helps maintain the alveolar surface relatively free of fluid and provides a thin diffusion barrier for gas exchange.

Recent findings suggest that transporters involved in active Na transport are rapidly inhibited when cultured alveolar epithelial cells (AEC) are exposed to hypoxia (15, 19, 21, 22). Inhibition of ion transporters might cause a reduced clearance of Na and water from alveolar fluid and, therefore, contribute to the formation of alveolar edema and hypoxemia if these changes in transport activity observed in cultured cells also occur in vivo. In support of this notion are results showing that a reduction of the transepithelial transport capacity is associated with 1) lack of alveolar fluid clearance after birth in transgenic mice lacking the alpha-subunit of the epithelial Na channel (ENaC) (12) and 2) susceptibility to pulmonary edema in the adult mouse lung after rescue with partial ENaC restoration and exposure to hypoxia (14).

While the level of expression of transporters such as the Na pump and ENaC have been found to be reduced following hypoxia in lung tissue, A549 cells, and cultured AEC of rats (15, 20, 21, 23, 30), hypoxia-induced diminution of active Na absorption across alveolar epithelium has not been directly demonstrated. Whether decreases in activities of either Na channel or Na pump (or both) contribute to such diminution of net active ion transport is currently unknown. In this study, we investigated hypoxia-induced changes in active Na transport capacity in cultured alveolar epithelial cells exposed to hypoxia and evaluated the role of amiloride-sensitive Na entry in absorption of Na across the alveolar epithelium.
transport by measuring short-circuit currents ($I_{sc}$) across primary cultured rat AEC monolayers grown on permeable supports (3–5). We also separately determined alterations in rates of apical entry of Na and basolateral extrusion of Na following hypoxia to help identify the mechanism(s) underlying decreased active Na absorption across the alveolar epithelium. Our results indicate that hypoxia leads to inhibition of transepithelial Na transport by primary effects causing decreased rates of both amiloride-sensitive Na entry and ouabain-sensitive Na extrusion.

MATERIALS AND METHODS

Reagents. Media were prepared from deionized water (18 MΩ-cm) and analytical grade reagents. N-methyl-D-glucamine (NMDG), ouabain, amiloride, amphotericin B, and trypsin were from Sigma Chemical (Deisenhofen, Germany). Phosphate-buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, fetal calf serum (FCS), and HEPES were from Life Technologies (Karlsruhe, Germany). Elastase was from Elastin Products (Owensville, MS).

Cell isolation and culture. Experiments were performed on primary cultures of cells resulting from plating of type II pneumocytes isolated from lungs of normoxic male rats (Sprague-Dawley; 150–200 g). The procedure conformed with the guidelines of the ethics committee of the University of Heidelberg. Briefly, lungs from rats anesthetized by intraperitoneal injection with 100 mg/kg pentobarbital sodium (Trapanal, Byk Gulden, Germany) were perfused with PBS while being ventilated with air. Alveolar type II (ATII) cells were isolated by elastase digestion, mincing of lung tissue, filtration, and differential adhesion on IgG-coated plates as previously described (3–5, 15). Nonadherent cells were suspended in DMEM (Sigma, D-5546) supplemented with 10% FCS, glutamine (4 mM), and gentamicin (50 μg/ml), and were plated on tissue culture-treated Nuclepore filters (0.4-μm and 12-mm Transwell; Costar, Cambridge, MA) at a seeding density of 1.5 × 10⁶ cells/cm². Both purity and viability of ATII cells were >85%. Cells were maintained in normoxia (room air-5% CO₂) until they reached confluence (typically on day 3 after plating) (3–5). Formation of tight monolayers was tested by measuring transepithelial resistance ($R_t$) and potential difference ($P_d$) using an epithelial voltohmmeter (EVOM) device and chopstick electrodes (World Precision Instruments, Sarasota, FL). For exposure to hypoxia, confluent monolayers were placed in a glass box that was flushed with gases composed of 1.5% or 5% O₂, containing 5% CO₂, while being ventilated with air. Alveolar type II (ATII) cells were isolated by elastase digestion, mincing of lung tissue, filtration, and differential adhesion on IgG-coated plates as previously described (3–5, 15). Nonadherent cells were suspended in DMEM (Sigma, D-5546) supplemented with 10% FCS, glutamine (4 mM), and gentamicin (50 μg/ml), and were plated on tissue culture-treated Nucleopore filters (0.4-μm and 12-mm Transwell; Costar, Cambridge, MA) at a seeding density of 1.5 × 10⁶ cells/cm². Both purity and viability of ATII cells were >85%. Cells were maintained in normoxia (room air-5% CO₂) until they reached confluence (typically on day 3 after plating) (3–5). Formation of tight monolayers was tested by measuring transepithelial resistance ($R_t$) and potential difference ($P_d$) using an epithelial voltohmmeter (EVOM) device and chopstick electrodes (World Precision Instruments, Sarasota, FL). For exposure to hypoxia, confluent monolayers were placed in a glass box that was flushed with gases composed of 1.5% or 5% O₂, containing 5% CO₂, and the balance N₂, at 37°C. Exposure to hypoxia (1.5% O₂, 24 h) did not affect cell viability, measured by trypan blue exclusion, compared with that observed for normoxic cells.

Ussing chamber measurements. For Ussing chamber studies, cell monolayers were typically used on days 3–5 after plating. After being mounted in modified Ussing chambers, cell monolayers were bathed with media composed of (in 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, Na was replaced with NMDG. During the measurements, the bathing media were equilibrated with humidified room air or with CO₂-free gases containing 1.5 or 5% O₂, balance N₂, when effects of acute hypoxia were studied.

Amiloride (final concentration: 10 μM) was used to inhibit the activity of apical Na channels, and ouabain (final concentration: 3 mM) was added to the basolateral side for inhibition of Na pumps. To measure transport across the apical or basolateral membranes, amphotericin B was added to the opposite compartment at a final concentration of 7.5 μM. Higher concentrations seemed to affect the barrier properties most likely due to rapid permeabilization of both the apical and basolateral plasma membranes.

An automated voltage clamp unit (DVC 100, World Precision Instruments) was used to continuously monitor the transepithelial $P_d$ and the $I_{sc}$ (5, 13). All measurements were made at 37°C. Cell monolayers were kept under open-circuit conditions for about 10 min during equilibration to the medium. The epithelium was then short circuit by clamping the transepithelial potential to 0 mV, and $I_{sc}$ was continuously displayed on a chart recorder, digitized, and stored in a computer for off-line analyses.

Data evaluation and statistical analysis. Each experiment was repeated on several monolayers obtained from at least two different cell preparations. All data are presented as means ± SD of the indicated number of measurements.

![Fig. 1. Effects of hypoxia on transepithelial potential difference (A) and transepithelial resistance (B) of primary cultured rat alveolar epithelial cell (AEC) monolayers. Cells were cultured on 1-cm² Transwell filters (Costar) and were exposed to normoxia and hypoxia. Bioelectrical measurements were performed with an epithelial voltohmmeter. Mean values ± SD of 12 monolayers from 2 different cell preparations for each group are shown. *P < 0.05 compared with monolayers kept in normoxia.](http://ajplung.physiology.org/)
data from more than two experimental groups were analyzed with one-way analysis of variance using Tukey’s post hoc tests to determine the significance of differences among group means. Unpaired, two-tailed Student’s t-tests were used to determine the significance of differences between two group means. Level of significance was \( P < 0.05 \). Graphs were created and statistical analysis was performed using SigmaPlot (version 5) and SigmaStat (version 2) software packages (SPSS Science Software, Erkrath, Germany), respectively.

RESULTS

To screen for differences in transepithelial transport activity between primary cultured rat AEC monolayers cultured in normoxia vs. hypoxia, transepithelial \( P_d \) and \( R_t \) were measured under open-circuit conditions using an EVOM. In normoxia, AEC monolayers on days 3–5 had a \( P_d \) between 20 and 25 mV (apical negative) and an \( R_t \) between 3 and 4 k\( \Omega \) cm\(^2\). Addition of amiloride to the apical fluid bathing the monolayers decreased \( P_d \) by \( \approx 75\% \), while \( R_t \) increased by \( \approx 10\% \).

Figure 1A shows that the \( P_d \) generated was decreased significantly in monolayers that were exposed to hypoxia for 4 and 24 h relative to those cultured in normoxic conditions. The decrease in \( P_d \) was significantly larger after 24 h at 1.5% O\(_2\) (75%) than at 5% O\(_2\) (25%). \( R_t \) (Fig. 1B) was slightly increased after 4 h at 1.5% O\(_2\) and 24 h at 5% O\(_2\) but was decreased by \( \approx 15\% \) after 24 h at 1.5% O\(_2\).

Ussing chamber measurements in the voltage-clamp mode (clamping transmonolayer voltage to 0) were performed to follow rapid time courses of changes in \( I_{sc} \) upon addition of inhibitors and exposure to hypoxia. Figure 2A shows a tracing of \( I_{sc} \) across cell monolayers grown in normoxia. Figure 2B indicates that hypoxia caused an immediate decrease in \( I_{sc} \) when the gas used for oxygenation was switched from room air to a gas mixture containing 1.5% O\(_2\). The rapid decrease in \( I_{sc} \) caused by acute exposure to hypoxia was reversed by reoxygenation (not shown). Figure 2A and B also show that the addition of amiloride (10 \( \mu \)M) to the apical fluid causes a rapid decrease in \( I_{sc} \), indicating that a large fraction (~70%; see also Fig. 3) of \( I_{sc} \) is mediated by amiloride-sensitive apical Na entry into cells. Figure 2C summarizes the results of acute exposure to hypoxia (of up to 30 min) on \( I_{sc} \), along with results obtained on cell monolayers that were exposed for 4 and 24 h to 1.5% O\(_2\) and for 24 h to 5% O\(_2\). These data indicate that exposure to 1.5% O\(_2\) decreases \( I_{sc} \).
and that the degree of inhibition increases with time of exposure from 3% of $I_{sc}$ after 5 min to 68% of $I_{sc}$ after 24 h. For monolayers exposed to 5% O$_2$ for 24 h, the decrease in $I_{sc}$ was somewhat less pronounced (~50%).

Figure 3 shows that in cells cultured in normoxia and measured in Ussing chambers in normoxia, ~70% of $I_{sc}$ is inhibited by 10 μM amiloride. Immediate exposure to hypoxia (1.5% O$_2$) has only small effects on the amiloride-inhibitable portion of $I_{sc}$. However, after 4 and 24 h of exposure to 1.5% O$_2$, the amiloride-inhibitable portion of $I_{sc}$ decreased to ~40% and 10%, respectively, compared with $I_{sc}$ observed in normoxic monolayers. Hypoxia of 5% O$_2$ for 24 h reduced the amiloride-inhibitable portion to ~55% (not shown). The magnitude of the amiloride-insensitive portion of $I_{sc}$ remained unchanged regardless of the level of hypoxia and the duration of hypoxic exposure (Fig. 3).

We next studied whether the decrease in transepithelial Na transport induced by hypoxia is associated with a decrease in transport capacity (i.e., maximal activity) of amiloride-sensitive apical Na entry pathways and/or ouabain-sensitive basolateral Na pumps. This appears likely, since recent results indicate a hypoxia-induced decrease in the amounts of Na transport-related proteins of AEC cultured on impermeable supports (21, 30). Figure 4A shows the recording of a typical experiment in which monolayers were bathed with the usual high-Na medium. It shows the inhibition of $I_{sc}$ upon addition of apical amiloride and an increase in the current upon addition of amphotericin B, which reaches a plateau after a few minutes, similar to results reported by Guo et al. (10). This increase was fully prevented by 3 mM ouabain added basolaterally immediately following the apical amiloride treatment (not shown), indicating that this current is generated by Na transport mediated by the Na pump. The pump current is also much smaller when monolayers are bathed on both sides with a medium containing only 5 mM Na, thus indicating activation of the Na pump by high Na at the cytosolic side of the pump (not shown).

Figure 4A also shows that the Na pump current at high cytosolic Na exceeds the $I_{sc}$ of the nonpermeabilized monolayer in the absence of amiloride, indicating that the Na pump current generated at the high cytosolic Na concentration represents a measure of the capacity (i.e., maximal activity) of the Na pump. The results of
several such experiments performed on AEC monolayers kept in normoxia and at 1.5% O₂ for 24 h are summarized in Fig. 4B. In monolayers exposed to hypoxia, both the Iₘ of the nonpermeabilized epithelium and the Na pump-mediated current are decreased by ~40%.

The capacity of the amiloride-inhibitable portion of apical Na entry was estimated after lowering the basolateral Na concentration to 25 mM to generate a driving force for Na movement from the apical to the basolateral side, followed by permeabilization of the basolateral membranes of monolayers with 7.5 μM amphotericin B to avoid possible limitation of Na transport by the activity of the Na pump. Figure 5A shows that upon addition of basolateral amphotericin B, Iₛccoli increases and reaches a plateau after a few minutes (Iₘ). Approximately 80% of Iₘ is inhibited when amiloride is added to the apical side of the monolayer. When symmetrical Na concentrations are used, no increase in Iₛccoli is observed upon basolateral permeabilization (not shown). Results of these experiments obtained on monolayers exposed to normoxia and 1.5% O₂ for 24 h are summarized in Fig. 5, B and C. Both the maximal current achieved after basolateral permeabilization and the amiloride-inhibitable portion of Iₘ decrease significantly in hypoxia-exposed monolayers.

DISCUSSION

The present study shows that hypoxia inhibits transepithelial Na transport across primary cultured rat AEC monolayers in a time- and dose-dependent manner by inhibition of both apical amiloride-sensitive Na entry and ouabain-sensitive basolateral Na extrusion. Transport inhibition is associated with a decreased capacity (i.e., maximal activity) of apical Na entry pathways and basolateral Na pumps. The degree of hypoxic inhibition of Na transport across AEC monolayers increases with the duration of hypoxic exposure.

Our data on hypoxia-induced decreases in Iₛccoli are consistent with results of Planes et al. (21, 22), who found significant inhibition of ²²Na and ⁸⁶Rb uptake of ~30% after 3 h and 50–60% inhibition after 12–18 h of hypoxic exposure of rat AEC cultured on plastic. Acute exposure to hypoxia might inhibit transport by causing small changes in mechanisms that regulate the activity of Na transport-related proteins localized at cell membranes. The relatively delayed response of primary cultured AEC monolayers to hypoxia contrasts with results obtained on A549 cells (15), which showed that significant inhibition of the activity of ouabain-sensitive ⁸⁶Rb uptake occurs within ~30 min of exposure to 3% O₂.

Hypoxic inhibition of Na entry into AEC is brought about by decreasing the amiloride-sensitive component of apical Na uptake, without affecting the amiloride-insensitive portion (Fig. 3). In normoxic primary cultured AEC monolayers, ~70% of Iₛccoli is inhibited by amiloride. This portion decreases to ~10% of Iₛccoli in cell monolayers exposed to 1.5% O₂ for 24 h compared with Iₛccoli in normoxic monolayers. Consistent with these results are findings of a decrease in total and amiloride-sensitive ²²Na uptake of A549 cells after 4 h of hypoxia, which does not seem to involve Na/H exchange (15), and a decrease in amiloride-sensitive ²²Na uptake into primary rat AEC cultured on plastic of ~60% after 18 h of hypoxia (21). In the latter work, Planes et al. (21) also presented evidence that inhibition of Na transport is accompanied by a decrease in expression of ENaC. Decreased amounts of ENaC were also seen by Western blot analysis of membrane fractions of A549 cells after 24 h of exposure to 3% O₂ (30). Our Ussing

Fig. 5. Capacity of apical amiloride-sensitive Na entry of primary cultured rat AEC monolayers. Cells were cultured and handled as described for Fig. 2. For hypoxic exposure, cells were cultured at 1.5% O₂, 5% CO₂, and balance N₂ for 24 h before being mounted into the Ussing chambers. Measurements of hypoxic cells were performed after equilibration of the bathing media to the corresponding hypoxic gas. A: a typical recording to demonstrate the experimental procedure. On the apical side, cell monolayers were bathed with a Ringer medium composed of (in 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. The medium on the basolateral side contained (in mM) 25 NaCl, 116 N-methyl-d-glucamine (NMDG)-Cl, 5.4 KCl, 0.78 NaH₂PO₄, 1.8 CaCl₂, 0.8 MgCl₂, 5 glucose, and 15 HEPES, pH 7.4, at 37°C. After equilibration to this condition (control), amphotericin B (7.5 μM) was added to permeabilize the basolateral plasma membrane. After a stable maximal transmonolayer current (Iₘ) was reached, 10 μM amiloride were added to the apical bathing medium. B and C: summary of results of measurements of Iₘ and the percentage of Iₘ that was inhibited by amiloride (Δamiloride). Mean values ± SD of 13 monolayers from 2 different cell preparations for each group are shown. *P < 0.05 for comparisons between normoxia and hypoxia by unpaired, two-tailed Student’s t-tests.
chamber measurements on primary rat AEC monolayers cultured on permeable filters indicate a decreased capacity of Na transport, since the current \( I_{\text{sc}} \) generated by a Na gradient (apical: 141 mM Na, basolateral: 25 mM Na) after permeabilization of the basolateral plasma membrane with amphotericin B is decreased in cells exposed to hypoxia. In normoxic and hypoxic cell monolayers, ~60% and 45% of \( I_{\text{sc}} \) are inhibited by amiloride, respectively. Together, these results are consistent with the hypothesis that hypoxia-induced inhibition of Na entry across the apical membrane of AEC is due to a decreased number of apical amiloride-sensitive Na entry pathways (21, 30).

Permeabilization of the apical plasma membrane allowed us to study the activity/capacity of Na pumps located in the basolateral plasma membrane of AEC. Our results on \( I_{\text{sc}} \) shown in Fig. 4B indicate that hypoxic exposure for 24 h reduces the Na pump current of rat AEC monolayers by ~40%. This finding is consistent with earlier data (15, 22) that hypoxia decreases ouabain-sensitive \(^{86}\)Rb uptake (i.e., Na-K-ATPase activity) and the number of copies of basolateral Na pumps. Pump inhibition by hypoxia seems to occur more rapidly in A549 cells (15) than in primary rat AEC cultured on plastic (22). In A549 cells, the activity of Na pumps decreases early upon exposure to hypoxia, which seems to be associated with internalization of Na pump \( \alpha_1 \)-subunits (9). In all cell types studied, prolonged exposure to hypoxia causes a decrease in the amount of Na pump protein measured by Western blot analysis (22, 30).

Hypoxia induces reduction in both Na entry via apical amiloride-sensitive Na channels and basolateral Na extrusion via Na pumps. However, Na entry via amiloride-insensitive pathways is relatively unchanged. This latter result indicates that the Na gradient across the apical cell membrane following hypoxia must have been about the same as that in normoxic cells. This can be possible only if the Na extrusion rate is decreased by hypoxia simultaneously with lowered rates of Na entry due to hypoxia-induced inhibition of apical amiloride-sensitive pathways, thereby keeping intracellular Na concentration approximately unchanged and maintaining the Na gradient across the apical cell membrane.

Results of experiments on different types of AEC show that hypoxic inhibition of both Na transport and the expression of Na transport proteins can be reversed by reoxygenation (15, 21, 24), similar to results of ion transport studies performed on cultured fetal AEC (20, 25). When these cells are cultured at uterine oxygenation conditions, which is at low \( P_{O_2} \), and then cultured at normoxia for up to 48 h, transepithelial Na transport is activated by increasing the expression of ENaC (1, 20) and Na pumps (25), resulting in an increase in \( I_{\text{sc}} \) and in its amiloride-sensitive component of fetal distal lung epithelial cell monolayers. However, the increase in transport activity was transient, indicating regulatory adjustments that might be required to switch these cells from Cl secretion in the fetal period to Na reabsorption when the lungs breathe air.

There is clear evidence that limiting apical Na entry and inhibiting basolateral Na extrusion reduces transepithelial Na movement, thus also reducing the rate of reabsorption of water (6, 8, 16, 26). When the expression of apical amiloride-sensitive epithelial Na channels (e.g., ENaC) in mice is reduced or even prevented, these animals show a reduced rate or even lack, respectively, of alveolar fluid clearance after birth (12). Although partial rescue restores the ENaC phenotype, lungs of these mice have an increased lung water content upon exposure to hypoxia (14). Hypoxic exposure of rats also decreases the number of Na pumps of the whole lung and AEC (30), paralleling the reduction in alveolar fluid clearance (28).

Another consequence of hypoxia for lung function is pulmonary vasoconstriction that leads to pulmonary hypertension (11), altered distribution of lung blood flow, and an increase in the rate of filtration of plasma-water into the alveolar space (7). In mountaineers, this might cause high altitude pulmonary edema and alveolar flooding (2) when the rate of fluid filtration exceeds the rate of its reabsorption (8). Clinical evidence for a possible defect in Na reabsorption was presented by Scherrer et al. (27), who reported that subjects susceptible to high altitude pulmonary edema also had decreased transepithelial nasal \( P_d \) and decreased amiloride-inhibitable component of the nasal \( P_d \) in normoxia relative to nonsusceptible control subjects. Other preliminary data indicate a decrease in the amiloride-inhibitable portion of the nasal potential difference when subjects are exposed to high altitude hypoxia (29).

Results from this study indicate that hypoxia decreases \( I_{\text{sc}} \) across rat alveolar epithelium by inhibiting both apical Na entry and basolateral Na extrusion. Because the amiloride-insensitive portion of \( I_{\text{sc}} \) did not change following hypoxia, the effects on Na transport are due to simultaneous inhibition of both the apical amiloride-sensitive Na entry pathway and Na extrusion via basolateral Na pumps. Overall, the combination of effects of hypoxia on Na entry and Na extrusion pathways are manifested by inhibition of active transepithelial Na transport.

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