Hypoxia reversibly inhibits epithelial sodium transport but does not inhibit lung ENaC or Na-K-ATPase expression

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Carpenter, Todd C., Stacey Schomberg, Christopher Nichols, Kurt R. Stenmark, and John V. Weil. Hypoxia reversibly inhibits epithelial sodium transport but does not inhibit lung ENaC or Na-K-ATPase expression. Am J Physiol Lung Cell Mol Physiol 284: L77–L83, 2003. First published September 6, 2002; 10.1152/ajplung.00181.2002.—Hypoxia reduces alveolar liquid clearance and the nasal potential difference, a marker of airway epithelial sodium transport. The mechanisms underlying this impaired epithelial sodium transport in vivo remain uncertain. We hypothesized that epithelial sodium transport impaired by hypoxia would recover quickly with reoxygenation and that hypoxia decreases the expression of lung epithelial sodium channels and Na,K-ATPases. We studied adult rats exposed to normoxia, hypoxia (FiO2 = 0.1) for 24 h, or hypoxia followed by recovery in normoxia. Nasal potential differences decreased by 40% with hypoxia (P < 0.001), returning to baseline levels with reoxygenation. Lung Na,K-ATPase activity decreased by 40% with hypoxia (P = 0.003), recovering to baseline levels with reoxygenation. Lung expression of mRNA encoding for epithelial sodium channel (ENaC)-α, -β, and -γ or for Na,K-ATPase-α1 did not change significantly with hypoxia or recovery nor did lung expression of ENaC-α, ENaC-β, Na,K-ATPase-α1, or Na,K-ATPase-β protein. We conclude that subacute exposure to moderate hypoxia reversibly impairs airway epithelial sodium transport and lung Na,K-ATPase activity but that those changes are not due to changes in the lung expression of sodium-transporting proteins.

LOW LEVELS OF OXYGEN have profound effects on the physiology of the lung, including in some species promoting the formation of pulmonary edema. One of the primary defense mechanisms in the lung against alveolar edema accumulation is the active transport of sodium out of the air spaces, creating an osmotic gradient that water then follows. Hypoxia has been clearly shown to impair alveolar liquid clearance and may thus contribute to alveolar edema accumulation (16, 18). Whether hypoxia impairs alveolar epithelial sodium transport as well is less certain, although previous work has shown that exposure to hypoxia decreases airway epithelial sodium transport in adult rats as measured by the nasal potential difference (NPD) (17). Such an inhibition has been proposed to contribute to the formation of human high-altitude pulmonary edema, a condition remarkable for its rapid reversibility with descent to lower altitude (14, 15). It is not known whether the hypoxia-induced drop in NPD reverses with reoxygenation in a manner consistent with human high-altitude pulmonary edema nor are the mechanisms by which hypoxia impairs epithelial sodium transport well understood.

One potential mechanism by which hypoxia might alter epithelial sodium transport is by causing alterations in the expression of epithelial proteins involved in sodium transport, in particular the apical epithelial sodium channels (ENaC) and the basolateral Na,K-ATPase proteins. In vitro studies using cultured epithelial cells showed significant decreases in epithelial sodium transport after exposure to profound hypoxia and reversal of those changes with reoxygenation, apparently due to reversible decreases in ENaC and Na,K-ATPase expression (10, 19). The extent to which similar processes occur in vivo remains uncertain, and two recent reports have in fact reached opposite conclusions in this regard (18, 19).

We sought to determine, then, whether the impairment in epithelial sodium transport seen in vivo with hypoxia reverses rapidly with reoxygenation and whether those changes are due to altered expression of epithelial sodium-transporting proteins. To test this hypothesis, we studied the effect of hypoxia and reoxygenation on epithelial sodium transport in the adult rat as measured by the NPD. To begin to understand the mechanisms by which hypoxia might impair epithelial sodium transport in the lung, we also measured lung Na,K-ATPase enzymatic activity as well as lung ENaC and Na,K-ATPase subunit expression in the same animals at both the protein and mRNA levels.

METHODS

Experimental animals. Experimental animals were adult male Sprague-Dawley rats, weighing 250–350 g, purchased from a commercial vendor (Harlan Sprague Dawley, Indiana...
Hypoxia does not inhibit lung Na,K-ATPase expression

All animals were allowed to adjust to Denver altitude (5,280 feet above sea level) for at least 1 wk before being studied. The animals were allowed standard chow and water ad libitum, and they were subjected to similar day-night light cycles. After the completion of the planned studies, rats were killed with an intraperitoneal injection of pentobarbital sodium (80 mg/kg).

Animals were exposed to normobaric hypoxia (FiO2 = 0.1) in a ventilated chamber for 24 h. Animals recovering from hypoxia were removed from the chamber after 24 h and remained at Denver altitude for the remainder of the study.

Measurement of NPD. As a marker of epithelial sodium transport, the transepithelial NPD was measured as previously described (17). Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). The NPD was then measured between a 24-gauge catheter filled with Ringer solution placed in contact with the nasal epithelium via a nostril and a butterfly needle filled with 1% Ringer agar inserted subcutaneously into the abdominal wall. Measurements were made using a high-impedance voltmeter and recorded on a computer using BioPac software. Transepithelial potentials were normalized to baseline NPD and was then exposed to normobaric hypoxia for later analysis. Membrane isolation and Na,K-ATPase activity were taken as the molybdate reaction. Na,K-ATPase activity was taken as the previously published method (5) based on the malachite green-nitrogen for later analysis. Membrane isolation and Na,K-ATPase activity assays.

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Effect of hypoxia and recovery on the expression of Na,K-ATPase.

To assess whether the changes in measured NPD and Na,K-ATPase activity correlated with changes in the expression of mRNA coding for proteins involved in lung epithelial sodium transport, RNA was extracted from lung tissue obtained as described above. The RNA was extracted using a commercial kit (RNaseasy, Qiagen, Valencia, CA) following the manufacturer’s instructions and then quantitated by UV spectrophotometry. One microgram of total RNA per sample was primed with oligo dT and reverse transcribed to cDNA using a commercial kit (Superscript, Invitrogen, Carlsbad, CA). The relative expression level of various mRNAs was then assayed by real-time PCR in a GeneAmp 5700 machine (Applied Biosystems, Foster City, CA). The formation of PCR product was monitored by fluorescence of Sybr Green dye and level of expression of target genes was determined using comparative Ct analysis. Primers used in the PCR reaction were as follows: ENaC-α, 5′-GGA CCA AGG AAC AAA TAG AAC AGC-3′ (2,473–2,496 bp), 5′-TCA AGG AGA GGA GCA GAC ATC AG-3′ (2,577–2,555 bp), ENaC-β, 5′-AGG CTC TCT GTT TGG AAC GGA G-3′ (807–828 bp), 5′-CCA GTT GAA GAT GTA GCA GTT GCC-3′ (897–874 bp); ENaC-γ, 5′-TTG GAC AGT GAG ACA AAA CAG GC-3′ (401–423 bp), 5′-TTG AAG AAT CTG GTG GTT GTG C-3′ (519–498 bp); Na,K-ATPase-α1, 5′-GCC AGT GTT TCA GGC TAA CAA GCA G-3′ (1,550–1,571 bp), 5′-TTC TCC CTC ATC TCC TCC ATC ACG G-3′ (1,678–1,657 bp); Na,K-ATPase-β1, 5′-TCT TCA TCG GGA CCA TCC AAG-3′ (605–625 bp), 5′-AAT CTG TGT TCA TGG CGG-3′ (699–769 bp); β-actin, 5′-TAT TGG CAA CGG GCG GTT CC-3′ (669–688 bp); 5′-GCC ATA GAG GT GTC TTT ACG GAT GTC-3′ (807–784 bp). Each sample was assayed in triplicate in each of two separate experiments.

Effect of hypoxia and recovery on the expression of lung sodium transport proteins.

To assess whether the changes in measured NPD and Na,K-ATPase activity correlated with changes in the expression of proteins involved in lung epithelial sodium transport, the lung tissue of the same rats used for Na,K-ATPase assay was used for the protein studies described below. For protein studies, lung tissue was homogenized in a Tris-sucrose buffer including protease inhibitors by differential centrifugation. Membrane or cytosolic fraction proteins were isolated with changes in the expression of mRNA coding for proteins involved in lung epithelial sodium transport, RNA was extracted from lung tissue obtained as described above. The RNA was extracted using a commercial kit (RNaseasy, Qiagen, Valencia, CA) following the manufacturer’s instructions and then quantitated by UV spectrophotometry. One microgram of total RNA per sample was primed with oligo dT and reverse transcribed to cDNA using a commercial kit (Superscript, Invitrogen, Carlsbad, CA). The relative expression level of various mRNAs was then assayed by real-time PCR in a GeneAmp 5700 machine (Applied Biosystems, Foster City, CA). The formation of PCR product was monitored by fluorescence of Sybr Green dye and level of expression of target genes was determined using comparative Ct analysis. Primers used in the PCR reaction were as follows: ENaC-α, 5′-GGA CCA AGG AAC AAA TAG AAC AGC-3′ (2,473–2,496 bp), 5′-TCA AGG AGA GGA GCA GAC ATC AG-3′ (2,577–2,555 bp), ENaC-β, 5′-AGG CTC TCT GTT TGG AAC GGA G-3′ (807–828 bp), 5′-CCA GTT GAA GAT GTA GCA GTT GCC-3′ (897–874 bp); ENaC-γ, 5′-TTG GAC AGT GAG ACA AAA CAG GC-3′ (401–423 bp), 5′-TTG AAG AAT CTG GTG GTT GTG C-3′ (519–498 bp); Na,K-ATPase-α1, 5′-GCC AGT GTT TCA GGC TAA CAA GCA G-3′ (1,550–1,571 bp), 5′-TTC TCC CTC ATC TCC TCC ATC ACG G-3′ (1,678–1,657 bp); Na,K-ATPase-β1, 5′-TCT TCA TCG GGA CCA TCC AAG-3′ (605–625 bp), 5′-AAT CTG TGT TCA TGG CGG-3′ (699–769 bp); β-actin, 5′-TAT TGG CAA CGG GCG GTT CC-3′ (669–688 bp); 5′-GCC ATA GAG GT GTC TTT ACG GAT GTC-3′ (807–784 bp). Each sample was assayed in triplicate in each of two separate experiments.

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and each Western blotting experiment was repeated in duplicate with similar results.

**Statistical analysis.** Results are given as means ± SE. Comparisons of serial NPD measurements were made using a paired t-test. Multiple group comparisons were made using a two-way ANOVA with Fisher’s protected least squares method posttesting. A P value of <0.05 was considered significant.

**RESULTS**

**Effects of hypoxia and recovery on the NPD.** To show that normobaric hypoxia decreases the measured nasal epithelial potential, a group of rats underwent NPD measurement before and after exposure to normobaric hypoxia for 24 h. The average maximum NPD under normoxic conditions was $-22.6 ± 0.4$ mV. As has been shown for hypobaric hypoxia, exposure to normobaric hypoxia for 24 h led to a significant decrease in NPD, to $-15.4 ± 0.6$ mV ($n = 10, P = 0.0001$).

To assess whether and how rapidly the changes in NPD seen with hypoxia reversed with reoxygenation, a cohort of animals ($n = 6$) had serial NPD measurements made at 1, 24, and 48 h after return to normoxia (Fig. 1). After 1 h of reoxygenation, the mean NPD had actually decreased a small amount from the values obtained immediately after reoxygenation ($-16.3 ± 0.8$ vs. $-15.5 ± 0.9$ mV, $P = 0.03$). By 24 h after reoxygenation, NPD values were significantly higher than after hypoxia, although still slightly below their baseline values ($24$-h reoxygenation $-20 ± 1.2$ mV vs. hypoxia $-16.3 ± 0.8$ mV, $n = 6, P = 0.04$). Three animals had an additional measurement made at 48 h of reoxygenation, and this group was nearly identical to their baseline values (baseline $-21.5 ± 0.8$ vs. 48-h reoxygenation $-22 ± 0.9$ mV, $n = 3, P = 0.09$). To confirm that the recovery in the measured NPD seen with returning to normoxia was due to reoxygenation as opposed to time alone, a group of animals ($n = 4$) was exposed to hypoxia for 48 h and then underwent repeat measurement of the NPD. The NPD in the animals exposed to hypoxia for 48 h was $-13.7 ± 0.7$ mV, which was not significantly different from the NPD in animals exposed to hypoxia for 24 h ($P = 0.12$).

To determine whether the recovery in NPD seen with reoxygenation was due to amiloride-sensitive apical sodium transport, NPD measurements were made in animals ($n = 4$) before and after administration of nebulized amiloride at normoxia, after exposure to hypoxia, and after exposure to hypoxia followed by 24 h of recovery in normoxia. Differences in the amiloride-sensitive component of the NPD were significantly different among the three groups (ANOVA $P = 0.003$). The normoxic baseline NPD was $-22.4 ± 0.9$ mV, similar to the initial cohort of animals studied, and the amiloride-sensitive component of the NPD was $-9.2 ± 0.5$ mV. After hypoxia, the measured NPD had diminished to $-14.8 ± 0.8$ mV, and the amiloride-sensitive component had decreased to $-2.8 ± 0.5$ mV ($P = 0.002$ vs. normoxia). After 24 h of recovery in normoxia, the NPD had recovered to $-21.6 ± 0.6$ mV, and the amiloride-sensitive component had also recovered to $-8.7 ± 1.4$ mV ($P = 0.005$ vs. hypoxia).

**Hypoxia decreases lung Na,K-ATPase activity.** To determine whether exposure to hypoxia leads to decreases in lung Na,K-ATPase activity, Na,K-ATPase enzymatic activity was measured on membrane preparations isolated from the lung tissue of normoxic, hypoxic, and recovered rats. Lung Na,K-ATPase activity was decreased significantly in the hypoxic animals compared with the normoxic controls, but Na,K-ATPase activity had returned to baseline levels after 24 h of recovery from hypoxia ($n = 4$ group, ANOVA $P = 0.003$, normoxia vs. hypoxia multiple comparison posttesting $P = 0.006$; Fig. 2).

**Expression of mRNA for lung sodium transport proteins after exposure to hypoxia.** To determine if the changes in NPD and Na,K-ATPase activity seen during and after exposure to hypoxia were associated with changes in the expression of genes encoding sodium transport proteins, the lung level of mRNA expression for the three ENaC subunits and two Na,K-ATPase subunits was assayed by real-time relative PCR. As shown in Fig. 3, ENaC-α mRNA expression did not change across the three groups ($P = 0.96$). Although
nor ENaC-\(\beta\) mRNA expression changed with hypoxia, both ENaC-\(\beta\) mRNA [ANOVA \(P = 0.17\), normoxia (N) vs. hypoxia (H) posttesting \(P = 0.23\)] and ENaC-\(\gamma\) mRNA (ANOVA \(P = 0.13\), N vs. H posttesting \(P = 0.06\)) showed a trend toward increased expression in the animals recovered from hypoxia. The expression of mRNA for the Na,K-ATPase-\(\alpha_1\) subunit did not change significantly with hypoxia or recovery, although a trend toward increased expression with hypoxia was seen (ANOVA \(P = 0.19\), N vs. H posttesting \(P = 0.08\)). A significant increase in the expression of mRNA for the Na,K-ATPase-\(\beta_1\) subunit was seen in the hypoxic animals (ANOVA \(P = 0.04\), N vs. H posttesting \(P = 0.01\)).

Expression of lung sodium transport proteins after exposure to hypoxia. To determine if the changes in NPD and in Na,K-ATPase activity seen during and after exposure to hypoxia were associated with changes in the lung expression of sodium transport proteins, the level of expression of two ENaC subunits and two Na,K-ATPase subunits was assayed by Western blotting of membrane preparations from the lung tissue of normoxic, hypoxic, and recovered rats.

Similar to the results of the mRNA studies, the expression of the ENaC-\(\alpha\) subunit protein did not change significantly with hypoxia or recovery, although there was a trend toward increased expression with hypoxia (ANOVA \(P = 0.15\), N vs. H posttesting \(P = 0.2\); Fig. 4). The expression of the ENaC-\(\beta\) subunit protein also did not change significantly with hypoxia or recovery, although multiple comparison posttesting revealed an increase in expression with hypoxia that was largely maintained in the recovered animals (ANOVA \(P = 0.09\), N vs. H posttesting \(P = 0.04\), N vs. recovery (R) posttesting \(P = 0.10\); Fig. 4).

The expression of the Na,K-ATPase protein subunits also generally paralleled the expression of mRNA encoding those proteins across the three experimental groups. The expression of the Na,K-ATPase-\(\alpha_1\) subunit protein did not change significantly with exposure to or recovery from hypoxia (ANOVA \(P = 0.44\); Fig. 5). The expression of the Na,K-ATPase-\(\beta_1\) subunit protein also did not change significantly with hypoxia or recovery.

Fig. 3. Exposure to hypoxia and recovery to normoxia do not change lung expression of mRNA for epithelial sodium channel (ENaC) subunits. A: ENaC-\(\alpha\) mRNA expression. B: ENaC-\(\beta\) mRNA expression. C: ENaC-\(\gamma\) mRNA expression.

Fig. 4. Exposure to normoxia and recovery to normoxia do not change lung expression of ENaC subunit protein. Top: representative lanes of Western blot for ENaC-\(\alpha\) (A) or ENaC-\(\beta\) (B) subunit protein. Bottom: densitometric analysis of ENaC bands normalized to \(\beta\)-actin expression, expressed as arbitrary units; \(n = 4\)/group. N, normoxia; H, hypoxia; R, recovered from hypoxia.
thought to be a critical factor in the clearance of fluids in the lung epithelium at detectable decreases in the expression of the major sodium-transporting proteins of the lung epithelium. Furthermore, those functional changes occurred without any detectable decreases in the expression of the major sodium-transporting proteins of the lung epithelium at either the mRNA or protein level. Sodium transport across the alveolar epithelium is thought to be a critical factor in the clearance of fluid from the air spaces, and impairment of epithelial sodium transport could thus contribute to the accumulation of pulmonary edema (6). Previous work clearly showed that exposure to hypoxia can reduce the ability of the lung to clear liquid from the air spaces, although the mechanisms by which this impairment in liquid clearance occurs remain uncertain (16, 18). Hypoxia has also been shown previously to reduce the NPD, an indicator of epithelial sodium transport (17). This study confirmed and extended those findings, showing not only that exposure to hypoxia decreases the NPD but also that the NPD recovers back to baseline levels within 24 to 48 h of returning to normoxia. These changes in NPD with hypoxia and recovery in normoxia were largely due to alterations in the amiloride-sensitive component of the measured potential difference, a finding that suggests that the changes in potential difference represent changes in epithelial sodium transport. In addition, time control animals studied after 48 h of hypoxic exposure showed similar NPDs to animals studied after 24 h of hypoxia, suggesting that the hypoxia-induced decrease in NPD was not a transient phenomenon and that the recovery in potential difference in reoxygenated animals was, in fact, due to the return to normal oxygen levels. These findings support the hypothesis that hypoxia impairs epithelial sodium transport in vivo acutely and reversibly, and these results are also compatible with previous studies showing that hypoxic impairment of alveolar liquid clearance recovers on a similar time course following a return to normal oxygen levels (18).

One mechanism by which hypoxia might alter epithelial sodium transport is by causing decreases in the level of expression of airway epithelial proteins involved in sodium transport, particularly the apical ENaC and the basolateral Na,K-ATPases. Studies of cultured airway epithelial cells have suggested that severe hypoxia (0–3% oxygen) impairs sodium transport in those cells by triggering reductions in ENaC and Na,K-ATPase expression (10, 19). We hypothesized that similar alterations in ENaC or Na,K-ATPase expression might also occur under more moderately hypoxic conditions in vivo. Contrary to our expectations, we found no evidence that hypoxia decreases whole lung ENaC or Na,K-ATPase expression at either the mRNA or protein level, and when a trend existed it was generally toward increased expression of these proteins. These findings are quite consistent with those of a recently published study of ENaC and Na,K-ATPase expression in type II alveolar epithelial cells freshly isolated from the lungs of rats exposed to moderate hypoxia (18). Of note, however, another recent study of the effects of hypoxia on lung sodium-transporting proteins found, in contrast to our results, that hypoxia decreases Na,K-ATPase protein expression and ENaC mRNA expression (19). The reasons for the differences between our results and those of this latter study are not immediately obvious, although differences in the techniques used may have contributed.

In addition to altered expression of sodium-transporting proteins, another mechanism by which hypoxia might act to alter epithelial sodium transport is via impairment of the enzymatic activity of the basolateral Na,K-ATPases, thus reducing the transeptithelial sodium gradient generated by those pumps. Support for this concept comes from previous in vitro studies of cultured airway epithelial cells, which have found that exposure to hypoxia can reduce Na,K-ATPase activity in those cells (9, 10, 19). Consistent with those earlier studies, we found that hypoxia decreases lung Na,K-
ATPase activity by 40%, and we also found that Na,K-ATPase activity returns to baseline within 24 h, paralleling the changes seen in the NPD. These results are consistent with a previous report assessing the effect of hypoxia on Na,K-ATPase activity in the whole lung (16). Although our results do not prove that Na,K-ATPase impairment reduces in vivo epithelial sodium transport, the enzyme activity results taken together with the nasal potential measurements suggest that this is the case. In addition, previous work has suggested that, at least under normoxic conditions, Na,K-ATPase expression may be the rate-limiting step in alveolar liquid clearance in the rat lung (4), a finding that lends physiological relevance to our results.

The mechanism by which hypoxia impairs lung Na,K-ATPase activity is not certain. Our findings of diminished lung Na,K-ATPase enzymatic activity, taken together with our finding that hypoxia does not diminish lung Na,K-ATPase protein content, suggest that hypoxia exerts its effect on Na,K-ATPase activity by mechanisms other than regulation of Na,K-ATPase subunit protein expression. Several factors have been shown to inhibit Na,K-ATPase activity in other settings and could play a role in this circumstance. For example, cultured airway epithelial cells exposed to hypoxia have been described to secrete a soluble factor capable of inhibiting Na,K-ATPase activity (11), and exposure to reactive nitrogen species has been shown to decrease the activity of brain Na,K-ATPase (12). Either of these factors could modify or bind to membrane-associated proteins in vivo in such a way as to also alter in vitro enzyme activity as measured on isolated membrane preparations, such as was performed in this study. Internalization and recycling of the protein from the membrane have also been suggested to regulate Na,K-ATPase activity (2). Interestingly, some of these same factors have also been reported to affect ENaC function as well, suggesting that common mechanisms might explain functional changes of both ENaC and Na,K-ATPase proteins in the lung (3, 13).

The tissue preparations used in the present study also deserve comment. Previous work studying in vivo expression of sodium transporters in the lung has generally focused on isolated type II alveolar cells. Interestingly, recent studies have shown that type I alveolar epithelial cells also express ENaC and Na,K-ATPase protein and transport sodium (1, 7). Given the predominance of the type I cell in the lung, studies of membranes from whole lung preparations such as were used in the present study may better reflect conditions in the distal lung than preparations of type II cells alone, further supporting the physiological relevance of our findings.

In conclusion, we showed that subacute exposure to moderate hypoxia reversibly decreases the measured NPD, an indicator of airway epithelial sodium transport, with recovery occurring over a 24- to 48-h time period. Exposure to hypoxia also impaired lung Na,K-ATPase activity by a similar amount, with recovery occurring over a similar time frame. We also showed that these changes occur without detectable decreases in the expression of lung sodium-transporting proteins. Further work is needed to clarify the precise mechanisms underlying the hypoxia-induced changes in epithelial sodium transport and the relevance of these findings to human illness.

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