Hypocapnic but Not Metabolic Alkalosis Impairs Alveolar Fluid Reabsorption

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Acid-base disturbances, such as metabolic or respiratory alkalosis, are relatively common in critically ill patients. We examined the effects of alkalosis (hypocapnic or metabolic alkalosis) on alveolar fluid reabsorption in the isolated and continuously perfused rat lung model. We found that alveolar fluid reabsorption after 1 hour was impaired by low levels of CO₂ partial pressure (Pco₂; 10 and 20 mm Hg) independent of pH levels (7.7 or 7.4). In addition, Pco₂ higher than 30 mm Hg or metabolic alkalosis did not have an effect on this process. The hypocapnia-mediated decrease of alveolar fluid reabsorption was associated with decreased Na,K-ATPase activity and protein abundance at the basolateral membranes of distal airspaces. The effect of low Pco₂ on alveolar fluid reabsorption was reversible because clearance normalized after correcting the Pco₂ back to normal levels. These data suggest that hypocapnic but not metabolic alkalosis impairs alveolar fluid reabsorption. Conceivably, correction of hypocapnic alkalosis in critically ill patients may contribute to the normalization of lung ability to clear edema.

Keywords: alveolar epithelial cells; hypocapnic alkalosis; ion transport; Na,K-ATPase; pulmonary edema

The resolution of pulmonary edema is the result of active sodium transport across the alveolar epithelium (1–5). Sodium enters the alveolar epithelial cells via apically located sodium channels, and it is extruded via basolaterally located sodium pumps (Na,K-ATPases), which causes water to be reabsorbed from the alveolar space (4, 6–10). In experimental models, it has been reported that alveolar fluid reabsorption (AFR) is decreased during acute hyperoxia (11), hypoxia (12), ventilator-associated lung injury (13), increased left atrial pressures (14), infection (15), and scorpion envenomation (16). In some of these models of lung injury, the impairment in AFR has been associated with decreased Na,K-ATPase activity and protein abundance at the basolateral membrane of alveolar epithelial cells (13, 16–18).

Acid-base disturbances and particularly hypocapnia can be observed during hyperventilation because it may occur during air travel, hiking at high altitudes, early asthma attack, diabetic ketoacidosis, or systemic inflammatory response syndrome (19–22). The effect of hypocapnic alkalosis on alveolar fluid reabsorption as an index of alveolar epithelial function has not been eluci-

dated. However, it has been reported that when AFR is impaired in patients with lung dysfunction, it is associated with worst outcomes (23).

The present study examined the effects of hypocapnic alkalosis by changing the levels of CO₂ in the pulmonary circulation of the isolated, perfused, fluid-filled rat lung model. In parallel experiments, we examined the effects of CO₂ partial pressure (Pco₂) changes on Na,K-ATPase activity and protein abundance at the plasma membranes of distal airspaces.

METHODS

Pathogen-free male Sprague-Dawley rats weighing 320 to 350 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were provided food and water *ad libitum* and were maintained on a 12- to 12-hour light-dark cycle. Animals were handled according to National Institutes of Health guidelines and Institutional Animal Care and Use Committee-approved experimental protocols. A total of 46 rat lungs were studied.

Isolated Perfused Lung Model

The isolated lung preparation was performed as previously described (1, 11, 13, 14). Briefly, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight) after which the lungs and heart were removed *en bloc*. The pulmonary artery and left atrium were catheterized and continuously perfused with a solution of 3% bovine serum albumin in buffered physiologic salt solution. Trace amounts of fluorescein isothiocyanate-labeled albumin were also added to the perfusate to check capillary barrier integrity along the experiment. Arterial and venous pressures were set at 12 and 0 cm H₂O, respectively, to avoid high hydrostatic pressure through alveolar-capillary barrier. Lungs were also immersed in a "pleural" bath filled with the same bovine serum albumin solution, and the entire system was maintained at 37°C. The lungs were then instilled via the tracheal cannula with a 5-ml volume of the bovine serum albumin solution containing 0.1 mg/ml Evans blue dye-labeled (Sigma, St. Louis, MO) albumin, 0.02 μ Ci/ml of ²²Na⁺ (DuPont NEN, Boston, MA) and 0.12 μ Ci/ml of [³H]mannitol (DuPont NEN).

The amount of instilled Evans blue dye albumin remains constant during the experimental protocol, so any change in its concentration at a given time will reflect the change in the airspace volume. Differences in concentration of Evans blue dye albumin among samples taken from the instillate at the beginning and after a determined time reflect the amount of fluid that has been reabsorbed. The fraction of fluorescein isothiocyanate albumin that appears in the alveolar space during the experimental protocol was used to calculate the albumin flux from the pulmonary circulation into the alveolar space.

Perfusate acid-base status was controlled and perfusing solution was monitored in real time during experimental course with a pH electrode located inside the circuit (Semimicro pH electrode, 476346; Corning, Inc., Corning, NY). Additional samples were taken every 15 minutes to measure levels of pH and perfusate gases using a blood gas analyzer (pHOx Plus Analyzer, StatProfile; Nova Biomedical Corp., Waltham, MA). Samples were quickly processed to avoid accidental degassing during measurement maneuvers. Changes on prefixed parameters (pH and/or Pco₂) were immediately corrected by bubbling more or less CO₂ and/or adding NaOH or HCl in the pulmonary circulation according to each experimental condition.

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Additional details on the method for making these measurements are provided in the online supplement.

Specific Protocols

Group A. We conducted experiments to determine baseline AFR over 1 hour in a control group of rat lungs in which the pH was maintained at 7.40 and P_{CO_2} = 40 mm Hg (n = 5).

Group B. Group B had three components as follows:

B1. We investigated the effects of respiratory alkalosis for 1 hour on AFR (first group: pH = 7.50, with $\sim P_{CO_2}$ 30 mm Hg; second group: pH = 7.60, $\sim P_{CO_2}$ 20 mm Hg; third group: pH = 7.70, $\sim P_{CO_2}$ 10 mm Hg; n = 5 in each group).

B2. We studied the effects of hypocapnia ($\sim P_{CO_2}$ = 10, 20, and 30 mm Hg) while maintaining the pH at 7.40 for 1 hour (n = 5 in each group). The pH was maintained at 7.40 by adding HCl (0.1 normality) to the pulmonary circulation perfusate.

B3. We assessed the effects of metabolic alkalosis while maintaining P_{CO_2} at 40 mm Hg and pH = 7.70 (n = 5). The desired P_{CO_2} was achieved by bubbling additional or less 10% CO_2 -21% O_2 mixture in the perfusate as needed, and alkaline pH was achieved with titration of NaOH (0.1 normality) during the experiment.

Group C. We investigated the reversibility of the respiratory alkalosis effect on AFR by conducting 3-hour experiments: the pH and P_{CO_2} were maintained at normal values (pH = 7.40, $\sim P_{CO_2}$ 40 mm Hg) during the first hour. Respiratory alkalosis was established (pH = 7.70, $\sim P_{CO_2}$ 20 mm Hg) during the second hour, and then the P_{CO_2} and pH were returned to normal values (7.40/40) during the third hour (n = 5).

Basolateral Plasma Membrane Isolation and Western Blotting

Basolateral plasma membrane proteins were obtained from tissue collected from the distal 2 to 3 mm of right rat lungs after serial bronchoalveolar lavage and perfusion of the pulmonary artery, as previously described (24). Protein fractions enriched from the basolateral plasma membrane domain were produced from distal lung tissue and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, as previously described (18, 24, 25). Immunodetection was achieved using a monoclonal antirat α_1 Na,K-ATPase (Upstate Biotech, Inc., Uppsala, NY) primary antibody and a chemiluminescent detection system (PerkinElmer Life Sciences, Boston, MA). The bands were quantified by densitometric scanning (ImageJ 1.29X; National Institutes of Health, Bethesda, MD).

Determination of Na,K-ATPase Activity

Twenty micrograms of basolateral membrane protein was resuspended in 100 μ l of a high (Na^+)/low (K^+) reaction buffer (in mmol/L: 50 Tris-HCl, pH 7.4; 50 NaCl; 5 KCl; 10 Mg_2Cl ; 1 mmol/L ethyleneglycol-bis-[β -aminoethyl ether]- N,N' -tetraacetic acid, 10 mmol/L Na_2ATP , with [γ - ^{32}P]-ATP [3.3 nCi/ μ l]). Triplicate samples were placed at $-20^\circ C$ for 15 minutes before incubation for 15 minutes at $37^\circ C$. The reaction was terminated by addition of 5% trichloroacetic acid/10% charcoal and cooling to $4^\circ C$. The charcoal phase containing unhydrolyzed nucleotide was separated by centrifugation ($12,000 \times g$ for 5 minutes) and the liberated ^{32}P quantified. Na,K-ATPase activity was calculated as the difference between the test samples (total ATPase activity) and samples assayed in reaction buffer with 2.5 mmol/L ouabain but devoid of Na^+ and K^+ (nonspecific ATPase activity). Results are expressed as nmol of P/mg of protein/minute.

Data Analysis

Data are expressed as means $\pm$ SEM; n represents the number of animals in each group. Data were compared using analysis of variance adjusted for multiple comparisons by the Tukey test. A p value of 0.05 or less was considered significant.

Figure 1. (A) Alveolar fluid reabsorption (AFR) decreased during progressive hypocapnic alkalosis (pH 7.50, 7.60, and 7.70). (B) AFR was also impaired by effect of low partial pressure CO_2 (P_{CO_2} ; $\sim$ 10, $\sim$ 20, and $\sim$ 30 mm Hg, and pH 7.40). (C) Metabolic alkalosis (pH 7.70; P_{CO_2} , $\sim$ 40 mm Hg) did not affect AFR. Bars represent mean $\pm$ SEM. * p < 0.05; ** p < 0.01; n = 5.

RESULTS

AFR

As shown in Figure 1A, AFR was impaired when P_{CO_2} levels were decreased, with corresponding increase in pH. The P_{CO_2} levels were 40.8 ± 1.9 mm Hg for a pH of 7.40, 32.1 ± 1.4 mm Hg for a pH of 7.50, 25.2 ± 0.5 mm Hg for a pH of 7.60, and 14.6 ± 0.7 mm Hg for a pH of 7.70. To determine whether hypocapnia and/or pH caused the observed AFR impairment, additional experiments were conducted. As showed in Figure 1B, P_{CO_2} was decreased, whereas the pH was maintained at 7.40 (P_{CO_2} = 29.6 ± 1.5 , 19.8 ± 2.0 , and 13.2 ± 0.7 mm Hg). The AFR was impaired only when P_{CO_2} was decreased to 13.2 and 19.8 mm Hg (but not 29.6 mm Hg). In contrast, in Figure 1C, in another set of experiments, the P_{CO_2} was maintained at normal range (38.7 ± 4.8 mm Hg), whereas the pH was increased to 7.70 and the AFR was not impaired. However, when the P_{CO_2} was reduced (14.6 ± 0.7 mm Hg) and pH maintained at 7.70, the AFR was reduced by 50%. We found a linear correlation between decreasing P_{CO_2} levels and decreased AFR: r^2 = 0.929 (Figure 2). As depicted in Figure 3, the effect of hypocapnia on AFR was reversible and, when P_{CO_2} and pH were restored to normal values on the third hour of the experimental protocol, the AFR reverted to normal values (first hour: pH = 7.40, P_{CO_2} = 41.67 ± 1.03 mm Hg; second hour:

Figure 2. AFR was plotted against P_{CO_2} . There was a significant correlation between them ($r^2 = 0.961$, $p < 0.001$). Values are mean $\pm$ SEM, $n = 5$.

pH = 7.70, $P_{CO_2} = 14.8 \pm 0.44$ mm Hg; and third hour: pH = 7.40, $P_{CO_2} = 39.47 \pm 0.35$ mm Hg).

Lung Epithelial Fluxes of Solutes during Alkalosis

As shown in Table 1, hypocapnic alkalosis had a tendency to increase (but not significantly) passive mannitol and sodium fluxes. Neither hypocapnic nor metabolic alkalosis increased albumin flux across the lung epithelium.

Na,K-ATPase Activity and Protein Abundance at the Basolateral Membranes

Na,K-ATPase activity in lungs with hypocapnic alkalosis ($P_{CO_2} \sim 14$ mm Hg, pH = 7.70) decreased as compared with control values ($P_{CO_2} \sim 40$ mm Hg and pH = 7.40; Figure 4A). The decreased Na,K-ATPase activity correlated with a decrease in the Na,K-ATPase $\alpha 1$ -subunit protein abundance at the basolateral membrane of hypocapnic alkalosis peripheral lung tissue, without changes in the total amount of protein (Figures 4B and 4C).

DISCUSSION

The clearance of pulmonary edema occurs via active sodium transport across the alveolar epithelial cells via apically located sodium channels and basolateral Na,K-ATPases, with water following isoosmotically the Na^+ gradients (1–5). It has been reported that AFR is an important factor contributing to the outcome of patients with respiratory failure (23, 26). Respiratory and metabolic alkalosis occurs in critically ill patients with different disorders, such as dehydration and early asthma, and in patients with central nervous system disorders (20–22).

Hypocapnia has been associated with adverse outcomes in mechanically ventilated patients with acute lung injury (20, 22). The injurious effects of hypocapnic alkalosis include bronchospasm, increased airway permeability, dysfunctional surfactant, and worsening intrapulmonary shunt (21, 27, 28). In the isolated

Figure 3. AFR was restored to normal levels after correcting the hypocapnia from low to normal pH values in 3-hour experiments: normal pH (7.40) during the first hour, low P_{CO_2} to a pH of 7.70 during the second hour, and back to normal P_{CO_2} and pH during the third hour. Bars represent mean $\pm$ SEM. ** $p < 0.01$, $n = 5$.

TABLE 1. ALBUMIN-FLUORESCHEIN ISOTHIOCYANATE, $^{22}Na^+$, AND $[^3H]$ MANNITOL FLUXES ACROSS ALVEOLAR-CAPILLARY BARRIER DID NOT CHANGE DURING THE DIFFERENT CONDITIONS OF THE EXPERIMENTAL PROTOCOL

Group pH/ P_{CO_2} (mm Hg)	FITC Flux (ml/h)	^{22}Na Flux (ml/h)	$[^3H]$ Mannitol Flux (ml/h)
7.40/40.8	0.020 ± 0.004	1.06 ± 0.11	0.55 ± 0.03
7.50/32.1	0.010 ± 0.006	1.84 ± 0.38	1.17 ± 0.18
7.60/25.2	0.020 ± 0.003	2.29 ± 0.40	1.36 ± 0.40
7.70/14.6	0.015 ± 0.007	2.10 ± 0.17	1.35 ± 0.17
7.40/29.6	0.026 ± 0.002	1.25 ± 0.31	0.89 ± 0.15
7.40/19.8	0.017 ± 0.005	1.26 ± 0.44	0.58 ± 0.28
7.40/13.2	0.020 ± 0.010	1.29 ± 0.4	0.61 ± 0.1
7.70/38.7	0.02 ± 0.008	1.07 ± 0.25	0.42 ± 0.08

Definition of abbreviation: FITC = fluorescein isothiocyanate.

Values represent mean $\pm$ SEM. P_{CO_2} levels as described in RESULTS.

lung model, hypocapnia caused lung injury during mechanical ventilation and after ischemia-reperfusion (28). Furthermore, in that study, hypocapnia resulted in increased capillary permeability ($K_{f,c}$) and lung weight because of increased fluid accumulation during hypocapnia (28). Our data provide further mechanistic

Figure 4. (A) Na,K-ATPase activity (assessed as ouabain-inhibitable hydrolysis of ^{32}P -ATP) decreased at the basolateral membranes (BLM) of peripheral lung tissues during hypocapnic alkalosis as compared with control animals. (B) Na,K-ATPase $\alpha 1$ -subunit protein abundance at the BLM of peripheral lung tissues was decreased during hypocapnic alkalosis as compared with control animals. (C) Representative Western blot. Graphs represent the mean $\pm$ SEM. * $p < 0.05$, $n = 3$. TM = total membranes.

insights on the effects of hypocapnia on alveolar epithelial function. We propose that persistence of the pulmonary edema could also be caused by hypocapnia (and not metabolic alkalosis)-mediated impairment in the lung ability to clear edema. We provide evidence that PCO_2 -mediated alkalosis impaired the lung ability to clear alveolar epithelial fluid, probably by inhibiting the Na,K-ATPase , and that these effects were reversible on normalization of the PCO_2 (see Figures 1 and 3). We found that mild decreases in PCO_2 to 30 mm Hg had no negative effects on AFR, whereas more significant hypocapnia (20 and 10 mm Hg) was deleterious to the alveolar epithelial function. Furthermore, alveolar epithelial function (assessed as fluid clearance from the lungs) was not altered by metabolic alkalosis (see Figure 1).

It is yet unclear what mechanisms regulate how hypocapnia inhibits the Na^+ pump and alveolar fluid clearance. There are several intracellular pathways that can be linked with CO_2 metabolism that could have a role. One possible explanation could be from changes in intracellular pH, as has been previously suggested (29). For example, hypocarbic alkalization in neutrophils requires HCO_3^- influx as well as proton efflux through ZnCl_2^- -sensitive proton channels. In alveolar epithelial cells, net sodium movement from the apical epithelium to the interstitium and vascular space requires Na^+-H^+ exchange in the apical surface, and this step could be impaired if intracellular pH is alkaline (30). In the same context, $\text{Cl}^-/\text{HCO}_3^-$ exchange is activated during recovery from alkaline loads and may help maintain intracellular pH under normal conditions (31). Bicarbonate, also, has been involved as a modulator of soluble adenylyl cyclases, acting as an intracellular signaling molecule. This modulation has been described in nuclear processes, but the same rationale could be applied to other cAMP-mediated process (32) and $\text{HCO}_3^-/\text{CO}_2$ is a well recognized physiologic buffer system. Also, carbonic anhydrase, which is expressed in alveolar epithelial cells, could participate because of its action in CO_2 hydration and alveolar elimination (33, 34).

Hypocapnia can modify vascular reactivity (35), but it is not clear whether these effects may modulate AFR. For example, endothelin 1 (which can modify vascular reactivity) increases pulmonary edema formation and was associated with an impairment in alveolar fluid clearance and Na,K-ATPase activity (36). Moreover, ET-B receptor-deficient rats develop lung edema (37).

We did not find changes in small or large solutes fluxes during hypocapnia or metabolic alkalosis as compared with control animals, suggesting that there were no large changes in alveolar-capillary barrier during the relatively short term (60 minutes) of the experimental protocol. These results contrast to the report by Laffey and colleagues (28), who found an increase in microvascular permeability. However, these changes in permeability were found only after 3 hours of hypocapnic alkalosis.

Hypocapnia is relatively common during hyperventilation, diabetic acidosis (38), or central nervous system disorders (39), and we believe that it is of relevance to understand that respiratory alkalosis can be harmful to the alveolar epithelium. Na,K-ATPase activity inhibition by G-protein coupled receptors, hypoxia, and models of lung injury has been reported to be because of its endocytosis from the plasma membrane to intracellular compartments (17, 18, 40). The present report provides evidence that hypocapnic alkalosis impairs AFR and inhibits Na,K-ATPase activity by promoting its endocytosis from the basolateral membrane into intracellular compartments.

We provide evidence that severe hypocapnia of up to 20 mm Hg, but not metabolic alkalosis, impairs the function of the alveolar epithelial cell Na,K-ATPase . We reason that hyperventilation-hypocapnia may impair lung edema clearance and have deleterious effects on critically ill patients. Conceivably, correc-

tion of pH by normalizing CO_2 might be a simple but significant supportive measure for patients with hypocapnia.

Conflict of Interest Statement: P.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; V.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.H.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.M.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.J.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.I.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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