Influence of Inhaled Amiloride on Lung Fluid Clearance in Response to Normobaric Hypoxia in Healthy Individuals

Courtney M. Wheatley,^{1,#} Sarah E. Baker,^{1,*} Bryan J. Taylor,^{2,†} Manda L. Keller-Ross,^{2,‡}
Steven C. Chase,^{2,§} Alex R. Carlson,^{2,§} Robert J. Wentz,^{2,§} Eric M. Snyder,^{1,**} and Bruce D. Johnson^{2,§}

Abstract

Wheatley, Courtney M., Sarah E. Baker, Bryan J. Taylor, Manda L. Keller-Ross, Steven C. Chase, Alex R. Carlson, Robert J. Wentz, Eric M. Snyder, and Bruce D. Johnson. Influence of inhaled amiloride on lung fluid clearance in response to normobaric hypoxia in healthy individuals. *High Alt Med Biol.* 18:343–354, 2017.

Aim: To investigate the role of epithelial sodium channels (ENaC) on lung fluid clearance in response to normobaric hypoxia, 20 healthy subjects were exposed to 15 hours of hypoxia (fraction of inspired oxygen [FiO₂] = 12.5%) on two randomized occasions: (1) inhaled amiloride (A) (1.5 mg/5 mL saline); and (2) inhaled saline placebo (P). Changes in lung fluid were assessed through chest computed tomography (CT) for lung tissue volume (TV), and the diffusion capacity of the lungs for carbon monoxide (DLCO) and nitric oxide (DLNO) for pulmonary capillary blood volume (V_C). Extravascular lung water (EVLW) was derived as TV–V_C and changes in the CT attenuation distribution histograms were reviewed.

Results: Normobaric hypoxia caused (1) a reduction in EVLW (change from baseline for A vs. P, $-8.5\% \pm 3.8\%$ vs. $-7.9\% \pm 5.2\%$, $p < 0.05$), (2) an increase in V_C ($53.6\% \pm 28.9\%$ vs. $53.9\% \pm 52.3\%$, $p < 0.05$), (3) a small increase in DLCO ($9.6\% \pm 29.3\%$ vs. $9.9\% \pm 23.9\%$, $p > 0.05$), and (4) CT attenuation distribution became more negative, leftward skewed, and kurtotic ($p < 0.05$).

Conclusion: Acute normobaric hypoxia caused a reduction in lung fluid that was unaffected by ENaC inhibition through inhaled amiloride. Although possible amiloride-sensitive ENaC may not be necessary to maintain lung fluid balance in response to hypoxia, it is more probable that normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of healthy individuals. The observed reduction in interstitial lung fluid means alveolar fluid clearance may not have been challenged.

Keywords: chest computed tomography (CT); diffusion capacity of the lungs for carbon monoxide and nitric oxide (DLCO/DLNO); epithelial sodium channels (ENaC)

Introduction

PULMONARY EDEMA RESULTS from an imbalance between forces driving fluid into the alveoli, namely Starling's Law of fluid filtration and the integrity of the alveolar–capillary barrier and the biological mechanisms for its removal, primarily active sodium (Na⁺) transport, which osmotically drives water reabsorption from the alveolar space, and lymphatic drainage. Hypoxic pulmonary vasoconstriction plays a role in the development of high-altitude pulmonary edema (HAPE)

(Motley et al., 1947; Sartori et al., 2004; Swenson, 2013). The increase in capillary hydrostatic pressure results in an increase in net filtration of fluid from the capillary to the interstitial space. Fluid that has been filtered, but not reabsorbed from the interstitial space, is then removed by the pulmonary lymphatics.

Stimulation of increased lung lymph flow can occur in response to beta-2 adrenergic receptor stimulation mediated by increases in catecholamines, and increases in ventilation. These events will deform the tissue that lymphatic vessels attach to, and thereby facilitate pumping and production of

¹Department of Pharmaceutical Science, University of Arizona, Tucson, Arizona.

²Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota.

[#]Current affiliation: Department of Cardiovascular Diseases, Mayo Clinic, Scottsdale, Arizona.

^{*}Current affiliation: Department of Anesthesiology, Mayo Clinic, Rochester, Minnesota.

[†]Current affiliation: Faculty of Biological Sciences, School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom.

[‡]Current affiliation: Division of Physical Therapy, University of Minnesota, Minneapolis, Minnesota.

[§]Current affiliation: Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota.

^{**}Current affiliation: Department of Kinesiology, University of Minnesota, Minneapolis, Minnesota.

lymph (Ikomi et al., 1991; Mahe et al., 1991; Pearse et al., 2005; Zawieja, 2009). Previous work by our group demonstrated that exposure to normobaric hypoxia increases ventilation and catecholamines and reduces lung fluid (Snyder et al., 2006; Snyder et al., 2008).

The close proximity of the capillaries to the alveoli allows for optimal gas exchange, but this closeness also subjects the alveoli to potential fluid infiltration in conditions such as higher pulmonary artery pressure and increased pulmonary vascular resistance which can result in fluid accumulation in the interstitial space and if not cleared cause fluid to build up in the alveoli. Because of the inverse and exponential relationship between rate of diffusion and membrane thickness, increases in airway surface liquid (ASL) that are not quickly reabsorbed will increase the distance across the alveolar–capillary membrane and greatly impact rate of diffusion and alveolar–capillary membrane conductance (D_M). Lungs must be kept moist, but not wet for effective and efficient gas diffusion. The principal determinant of ASL depth is the mass of salt on the airway surface (Boucher, 1999).

Active transport of Na^+ from the airspace through epithelial sodium channels (ENaC) and then across the basolateral membrane by Na^+/K^+ ATPase is believed to be the primary determinant of alveolar fluid clearance by creating an osmotic gradient, with ENaC-mediated Na^+ absorption being the rate-limiting step (Matthay et al., 1996; Matthay et al., 2002). The importance of ENaC in keeping the lungs moist, but not wet is supported by evidence which suggests: (1) mice with a nonfunctional ENaC will demonstrate a failure to thrive in neonates due to an inability to clear amniotic fluid from their lungs following birth (Hummler et al., 1996); (2) individuals with pseudohypoaldosteronism, characterized by a loss of ENaC function, have been shown to have excess ASL (Kerem et al., 1999); and (3) the pathologically dry lungs of patients with cystic fibrosis which is partially due to hyperabsorption of Na^+ by ENaC (Mall et al., 2004).

Evidence for ENaC's role in alveolar fluid clearance is abundant, but less clear is the evidence that it is necessary for maintaining lung fluid balance in response to hypoxia, and if impairment of channel function is sufficient to cause pulmonary edema. Furthermore, there is evidence for impairment in Na^+ transport in individuals susceptible to HAPE. For example, Sartori et al. (2004) demonstrated that baseline nasal potential difference (NPD) was lower and the amiloride-sensitive Na^+ transport reduced in mountaineers susceptible to this condition. Sodium transport was shown to be further reduced with altitude exposure in HAPE susceptible individuals, although this reduction with altitude was not in amiloride-sensitive Na^+ flux.

In contrast, amiloride administration in rats caused a decrease in Na^+ transport with no additional decrease with exposure to hypoxia. These data suggest that the reductions in ENaC activity were responsible for the reduction in Na^+ transport with hypoxia (Tomlinson et al., 1999) and this hypoxia-induced reduction in Na^+ transport has been suggested to be mediated by downstream reduction in ENaC channel expression (Gille et al., 2014). Additionally, prophylactically taken beta-2 agonists can prevent HAPE in the susceptible subjects during altitude exposure (Sartori et al., 2002) and stimulate amiloride-dependent lung fluid clearance in hypoxia-exposed rats (Vivona et al., 2001) by reversing the hypoxia-mediated reduction in Na^+ transport.

Therefore, the purpose of this study was to determine if ENaC is necessary for lung fluid clearance in response to

normobaric hypoxia in healthy humans. We hypothesized that ENaC inhibition by amiloride would result in greater lung fluid accumulation evidenced by (1) an increase in lung tissue density (measured through computed tomography [CT]) and estimated extravascular lung water (EVLW), (2) an elevation in exhaled breath condensate Na^+ , where an increase in Na^+ would suggest an increase in ASL depth as water follows salt, and (3) a reduction in diffusion capacity of the lungs due to alveolar fluid accumulation increasing the diffusion distance across the alveolar–capillary membrane.

Materials and Methods

Subjects

Twenty-three healthy nonsmoking adults of average fitness ($\text{VO}_{2\text{PEAK}}$ 106% predicted) agreed to participate in this study. The protocol was reviewed and approved by the Mayo Clinic Institutional Review Board, all participants provided written informed consent before study, and all aspects of the study were performed according to the declaration of Helsinki. Exclusion criteria included (1) cardiovascular or pulmonary abnormalities; (2) history of renal disease; (3) obese (body mass index [BMI] >30); (4) pregnancy; (5) hospital contact restrictions or an inability to exercise. Two participants were “screen failures”, one due to illness and the other due to hospital contact restrictions. In addition, one subject was removed from the hypoxic tent after 4 hours due to nausea and general malaise. As such, the data reported reflects the results of the 20 subjects who completed the study.

Protocol

At an initial screening visit, (1) height and weight were measured, (2) a blood draw was taken to rule out anemia and (3) a pregnancy test was completed in female subjects. Next, baseline pulmonary function was measured before each subject performed a maximal exercise capacity test on a cycle ergometer. Those without any of the exclusion criteria returned and were exposed to ~ 15 hours of normobaric hypoxia in a double-blind, crossover, and randomized fashion of two experimental conditions: (1) nebulized amiloride (A) (1.5 mg in 5 mL normal saline), and (2) saline placebo (P). The experimental conditions were performed on different occasions separated by ≥ 3 days. Changes in lung fluid from before to after hypoxic exposure were assessed through chest CT for lung tissue volume (TV), exhaled breath condensate sodium concentration (EBCNa) for ASL sodium flux, and the diffusion capacity of the lungs for carbon monoxide (DLCO) and nitric oxide (DLNO) for the determination of pulmonary capillary blood volume (V_C). EVLW and changes in the CT attenuation distribution histograms were reviewed. A summary of the hypoxic exposure visit is provided in Figure 1.

Normobaric hypoxia exposure

For both treatment visits, subjects arrived at the Mayo Clinic Clinical Research Unit (CRU) at 13:00 and a venous catheter was placed in a brachial or antecubital vein. Following 30 minutes of quiet rest, a baseline blood draw was taken for measurement of complete blood count, serum catecholamines, and plasma sodium and chloride. Baseline measurement of lung fluid and pulmonary arterial pressure (PAP) were taken. Subjects were then transferred to the hypoxic tent (fraction of inspired oxygen [FiO_2] 12.5%, $\text{P}_{\text{A}}\text{O}_2$

FIG. 1. Hypoxia visit schematic. CRU, Clinical Research Unit; CT, computed tomography; DLCO, diffusion capacity of the lungs for carbon monoxide; DLNO, diffusion capacity of the lungs for nitric oxide; SpO₂, peripheral oxygen saturation; PFT, pulmonary function test.

91.5 mmHg) (Colorado Altitude Training, Boulder, CO) at ~16:00. Vital signs, including heart rate (HR), blood pressure, respiratory rate, respiratory sounds, acute mountain sickness symptoms [modified Lake Louise Score (LLS) (Savourey et al., 1995); see Supplementary Data; Supplementary Data are available online at www.liebertpub.com/ham], as well as tent temperature, barometric pressure, and CO₂ level were assessed and recorded every 2 hours by a CRU nurse assigned to the patient.

Although we did not measure urine output as part of the initial protocol, we observed and received feedback from the first six subjects that within a few hours of the initial nebulization the participants seemed to be urinating more frequently on one visit compared with the other visit. Due to the known diuretic effects of oral amiloride in the kidney, and the known short surface half-life and subsequent absorption of nebulized amiloride across the lung epithelium (Mentz et al., 1986; Knowles et al., 1990; Noone et al., 1997), we thought this may have been a sign that the local nebulization amiloride was having quite rapid systemic effects. As such, we modified the protocol to record fluid input and output in all remaining subjects, so net fluid output could be quantified.

Subjects wore a Wrist Pulse Oximeter (Nonin WristOx 3100; Nonin Medical, Inc., Plymouth, MN) to allow for continuous HR and peripheral oxygen saturation (SpO₂) monitoring during their time in the tent. The subjects remained in the tent overnight for a total of 15.3 ± 0.9 hours. If subjects needed to use the rest room, they were fitted with a portable mask connected to a gas reservoir attached to a cylinder of the hypoxic gas (12.5% O₂) until returning to the hypoxic tent. At around 6:00 the following morning (14.6 ± 0.9 hours post-entry to the tent), the blood draw was repeated and followed by exhaled breath condensate (EBC) collection inside the tent. A mask and gas reservoir were used to keep the subjects hypoxic during the CT and DLCO and DLNO measurements.

Hemoglobin and hematocrit measured from the complete blood count completed by the Mayo Clinic Clinical Core Laboratory (Sysmex XE5000) were used to estimate the change in plasma volume (PV) using the following equation (Dill and Costill, 1974):

$$\Delta\%PV = \left(\left(\frac{Hb_{t1}}{Hb_{t2}} \times \frac{100 - Hct_{t2}}{100 - Hct_{t1}} \right) - 1 \right) \times 100$$

Epinephrine (EPI) and norepinephrine (NE) were measured by the Mayo Clinic CRU immunochemical core laboratory using High-Performance Liquid Chromatography.

Drug administration

The randomization and preparation for the administration of nebulized amiloride (1.5 mg in 5 mL saline) and nebulized saline placebo (5 mL saline) was performed by the Mayo Clinic CRU pharmacy ensuring that the study investigators, technicians, nursing staff, and subjects were blinded. Amiloride and saline were nebulized using standard apparatus (ReliaMed) connected to a room air supply flowing at 8 L/min. Each treatment was administered at three time points during exposure to hypoxia: (1) upon entering the tent at 16:00; (2) 21:00; and (3) 4:00 the following morning. The investigators were unblinded after all subjects had completed the study.

Chest CT assessment of lung fluid

Chest CT measurements were performed before and 15.5 ± 0.9 hours following hypoxic exposure for both treatment visits. The CT protocol followed what has been previously used in our laboratory (Snyder et al., 2006; Johnson et al., 2012). Briefly, the same scanner (GE LiteSpeed Spiral CT Scanner, GE Healthcare) was used for all CT scans. A scout scan was performed on the baseline visit of each overnight stay to determine the location and size of the lungs. The noncontrast chest CT scan was obtained with 2.5-mm-thick slices with a 1.2 mm overlap initially and then reconstructed to 1.25 mm with a 0.6 mm overlap.

Before the subject was removed from the scanner, a mark was placed on the subject's skin to designate the anatomical location of the start of the scan and the table height and number of slices were recorded. The baseline scout scan was repeated for each hypoxic exposure visit (placebo and amiloride), but the table height and number of slices was kept consistent with what was done at the first hypoxic exposure visit.

A member of the study team was with the subject in the scanning room, and instructed the subject to take a maximal inhalation and hold their breath at the total lung capacity. At this time, the study team signaled the radiology technician to complete the scan, and once through the scanner the subject was told they could relax and return to normal breathing. Although a gated spirometer was not used to control lung volumes, the difference between baseline and postexposure to hypoxia CT-derived air volumes was on average <5%.

The CT images were then analyzed using custom image analysis software (Apollo, VIDA Diagnostics). Analyses were completed by a laboratory member blinded to the condition of the subject's CT scan. The software segments the image to separate lung tissue from surrounding structures. In each picture

element, the lung density was assumed to be a linear combination of air which has a Hounsfield units (HU) = -1000, and lung tissue which has the density of water, HU = 0. As such, an element at -600 HU represents 40% tissue, and -300 HU would represent 70% tissue. A histogram analysis of the picture elements within the lung tissue area was performed to obtain a mean lung density in HU and a TV by summation of all the elements in the lung fields. The density and TV can also be determined for individual lobes of the lung.

Two different methods were used to assess lung water from the CT. First, an estimation of EVLW. Since the TV measured from the CT scan consists of lung tissue, blood, and water, we subtracted the pulmonary capillary blood volume obtained from the DLCO and DLNO measures to remove the blood component ($EVLW = TV - V_C$). If we then assume TV remains relatively constant between the pre and postscans, any change in the EVLW describes changes in lung fluid.

Second, differences in EVLW between study conditions were estimated using a histogram analysis approach. Lung interstitial tissue was segmented from surrounding tissue, large airways, and blood vessels using segmentation algorithms built in MATLAB (MathWorks, Inc., Natick, MA). CT attenuation distributions were generated from the segmented images. Mean, skew, kurtosis, and full-width half-max were calculated from these distributions (Chase et al., 2016). When the attenuation decreases or becomes more negative, more skewed to the left, and/or more kurtotic, this collectively suggest less fluid as the attenuation distribution is becoming less dense, and shifting away from water's attenuation of 0 HU. Previous work has demonstrated a strong positive correlation between attenuation and EVLW (Scillia et al., 1999; Shaker et al., 2004).

Measurement of DLCO, DLNO, and assessment of cardiac output

Before and 16.0 ± 0.9 hours following hypoxic exposure for both treatment visits, DLCO and DLNO and cardiac output (Q) were measured simultaneously with the subjects in an upright seated position using the rebreathing technique with a 5-L anesthesia bag containing 0.7% acetylene, 9% helium, 0.3% carbon monoxide (C₁₈O), 40 PPM NO (diluted immediately before the test in the bag from an 800 PPM gas mixture), and 35% O₂, at a respiratory rate of 32 breaths/minute as described previously (Hsia et al., 1995; Snyder et al., 2005; Snyder et al., 2006; Wheatley et al., 2011a; Wheatley et al., 2011b; Wheatley et al., 2013; Wheatley et al., 2015). The volume of gas placed in the bag was a standardized volume of 1575 mL for all resting measures to ensure the bag did not collapse during inhalation, but also did not cause an unnecessary excess of gas in the bag during the maneuver. Bag volume was reduced to 1050 mL in one subject. At the end of a normal expiration (functional residual capacity), the subjects were switched into the rebreath bag and instructed to nearly empty the bag with each breath for 8–10 consecutive breaths. The maneuver was performed in triplicate before and after hypoxic exposure (performed immediately following completion of the CT scan).

The rate of disappearance of acetylene from the exhaled gas mixture during rebreathing is used to assess pulmonary blood flow. Since acetylene does not bind to hemoglobin, the rate of disappearance of acetylene is limited primarily by the rate at which a new volume of blood is transported through

the lungs. Because all the blood in the pulmonary circulation per minute is equal to the volume of blood in the systemic circulation per minute, the measure of the disappearance of acetylene provides a reliable measure of cardiac output and has previously been validated in our laboratory using direct Fick during exercise (Liu et al., 1997; Johnson et al., 2000).

The diffusing capacity of the lungs for carbon monoxide is based on the contribution of both the membrane conductance and the hemoglobin binding and described by the equation developed by Roughton and Forester (Tamhane et al., 2001).

$$\frac{1}{DLCO} = \frac{1}{DM_{CO}} + \frac{1}{\theta_{CO} \cdot V_C}$$

The rate of disappearance of the gases with each breath is calculated from the slope of the exponential disappearance for each gas with respect to helium using custom software (Snyder et al., 2005). Unlike DLCO, DLNO is theoretically based solely on membrane conductance as nitric oxide is scavenged 8000 times faster by hemoglobin than O₂ so its uptake into the blood is nearly instantaneous. Although currently being debated, DLNO has been considered a relatively direct measure of membrane conductance (D_{MNO}) as the diffusion resistance of the blood is trivial (Roughton and Forster, 1957; Tamhane et al., 2001; Hsia, 2002; Hsia and Raskin, 2005), but not infinite, and for our purposes of comparing change in response to a stimulus gives reliable results (Coffman et al., 2016). Using this assumption, the D_{MNO} value is used to calculate the D_M for carbon monoxide (D_{MCO}) by adjusting for differences in diffusion constants based on molecular weight and solubility between the two gases as described previously using an alpha ratio of 2.2 (Tamhane et al., 2001; Wheatley et al., 2011b).

Pulmonary capillary blood volume (V_C) is then calculated from the DL_{CO} measured by subtracting the resistance to diffusion associated with alveolar-capillary barrier (D_{MCO}) and correcting for differences in the rate of uptake and binding to hemoglobin (1/θ) due to differences in hemoglobin concentrations and the alveolar pressure of oxygen as described previously using the Roughton and Forester 2.5 θ_{CO} equation (Roughton and Forster, 1957; Tamhane et al., 2001; Wheatley et al., 2011b). This technique has been validated in our laboratory and used extensively for studies in other clinical populations (Olson et al., 2006; Snyder et al., 2006; Snyder et al., 2008; Wheatley et al., 2011a, 2011b).

Pulmonary function testing

Baseline spirometry was assessed on the screening visit according to American Thoracic Society guidelines (Medical Graphics CPXD, Minneapolis, MN) to determine forced vital capacity (FVC), forced expiratory volume in 1 second of the FVC (FEV₁), and forced expiratory flow at 25%–75% of the FVC (FEF_{25–75}). Before and after hypoxic exposure on visits two and three, subjects repeated FVC maneuvers following each of the diffusion capacity measurements (Miller et al., 2005). Predicted values for all pulmonary function measures were based on predicted equations from NHANES III (Hankinson et al., 1999).

Exhaled breath condensate

EBC samples were collected using a Jaeger EcoScreen cooling unit (Cardinal Health, Yorba Linda, CA) as we have

TABLE 1. POPULATION DEMOGRAPHICS

	All	Male	Female
<i>n</i>	20	11	9
Age (years)	27±5	29±6	25±3
Height (cm)	172±8	176±7	166±4 ^a
Weight (kg)	71±13	80±8	58±6 ^a
BMI (kg/m ²)	24±3	26±3	21±3 ^a
BSA (m ²)	1.8±0.2	2.0±0.1	1.6±0.1 ^a
VO ₂ PEAK (% predicted)	106±19	102±20	111±17
FVC (% predicted)	104±15	106±15	102±16
FEV ₁ (% predicted)	102±15	104±17	99±14
FEF _{25–75} (% predicted)	97±22	101±25	93±18
Hemoglobin (g/dL)	14.0±1.2	15.0±0.8	13.1±0.8 ^a

Data are presented as mean ± SD.

^a*p* < 0.05 versus males.

BMI, body mass index; BSA, body surface area; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 second of FVC; FEF_{25–75}, forced expiratory flow at 25%–75% of the FVC.

previously described (Wheatley et al., 2010). During the 20-minute collections, subjects sat wearing a nose clip and breathed through a mouthpiece so all their exhaled breath could be directed to the Teflon condenser inside the EcoSc-

reen cooling unit. Collections were made at baseline and the next morning following hypoxic exposure before subjects were removed from the tent. Samples were frozen at −80°C and then batch analyzed with quantification of chloride completed using ion chromatography and sodium measured with inductively coupled plasma mass spectrometry.

Pulmonary arterial pressure

PAP was calculated from the tricuspid regurgitation (TR) velocity as described previously (Yock and Popp, 1984) using the equation $\Delta P = 4V^2$, where *P* is the pressure and *V* (m/s) is the tricuspid regurgitant velocity. The same sonographer performed the echocardiographic measures at baseline and after the 15 hours of hypoxic exposure being performed before the subject was removed from the tent. There were three sonographers who performed these measurements on the subjects, all of them using the following methods for their assessment. Color Doppler was used to locate the TR jet. Data reported are from 16 out of 20 subjects for whom a jet could be visualized and successfully measured. The maximal velocity was determined by careful application of the continuous wave sampler within and parallel to the regurgitation jet.

FIG. 2. DLCO and DLNO in response to normobaric hypoxia. Prehypoxia (white bars) to posthypoxia (black bars) for placebo and amiloride. (A) DLCO; (B) DLNO; (C) Alveolar–capillary membrane conductance (*D_M*); (D) Pulmonary capillary blood volume (*V_c*). Percent change listed for each of the above, placebo versus amiloride, respectively; **p* < 0.05 versus baseline.

Statistical analyses

The SPSS statistical software package (v.22; SPSS, Inc., Chicago, IL) was used for all statistical analyses. Two-factor repeated measure analysis of variance was used to evaluate the main effects of normobaric hypoxia, drug (amiloride vs. placebo), and their interaction on the measures of lung fluid and systemic response to the conditions. Paired samples *t*-tests were performed between percent change from baseline to postexposure to hypoxia metrics (LLS, urine input/output) for the two treatments, with an alpha level of 0.05 used to determine statistical significance. All values presented are mean $\pm$ SD, unless otherwise stated.

Results

Subject characteristics for the 20 subjects who participated in this study are provided in Table 1.

Changes in lung fluid in response to normobaric hypoxia and amiloride

Normobaric hypoxia did not change DLCO, DLNO, or alveolar-capillary membrane conductance (D_M) for both amiloride and placebo conditions (Fig. 2). By contrast, hypoxic exposure caused an increase in pulmonary capillary blood volume (V_C) (hypoxia effect $p < 0.01$); the magnitude of increase in V_C was not different in amiloride versus placebo ($54\% \pm 29\%$ vs. $54\% \pm 52\%$, $p = 0.52$) (Fig. 2). There was a reduction in CT-derived TV in response to hypoxic exposure (hypoxia effect $p \leq 0.01$) that was similar between amiloride and placebo conditions (-49.3 ± 25.7 vs. -46.1 ± 31.2 mL, $p = 0.69$) (Fig. 3). This decrease in TV was not uniform across the lungs, with a minimal reduction (~ 2 mL) in the mid-right lobe, a 10–13 mL reduction in the left lobes and upper right lobe, and a trend for a larger decrease, especially with amiloride, in the lower right lobe (~ 16 mL) (Fig. 4).

FIG. 3. Changes in CT assessed lung TV and calculated EVLW after normobaric hypoxia. Difference from post-hypoxia to prehypoxia for placebo (white bars) and amiloride (black bars) for tissue volume (ΔTV) and EVLW ($= TV - V_C$). Percent change from baseline is listed for EVLW. Absolute change is found in Table 2. EVLW, extravascular lung water; TV, tissue volume.

FIG. 4. CT TV changes stratified by lung lobe after normobaric hypoxia. Difference from posthypoxia to prehypoxia for placebo (white bars) and amiloride (black bars) for TV for lung lobes. LL, lower left; LR, lower right; MR, middle right; TV, tissue volume; UL, upper left; UR, upper right.

There was a similar and significant decrease in EVLW from before to after hypoxic exposure (hypoxia effect $p < 0.01$) with amiloride and placebo ($-8.5\% \pm 3.8\%$ vs. $-7.9\% \pm 5.2\%$, $p = 0.53$) (Fig. 3). CT attenuation distributions showed the same trend for EVLW. Distribution average was shifted to more negative, more leftward skewed, and more kurtotic after hypoxic exposure in both groups suggesting clearance of fluid from the lungs due to the shift toward less attenuation (hypoxia effect $p < 0.05$, Table 3). There was no difference in these changes between amiloride and placebo conditions ($p > 0.05$). Additionally, there was a decrease in PV with hypoxic exposure (hypoxia effect $p < 0.01$) for both conditions, amiloride versus placebo (-9.2 ± 9.7 vs. -11.0 ± 11.0 , $p = 0.52$), suggesting that the decrease in EVLW was not just a shift of fluid from the interstitial to vascular space.

Although there was a decrease in interstitial lung fluid with hypoxia and no effect of amiloride in the gross measures of changes in EVLW or on diffusion capacity or alveolar-capillary membrane conductance, a measure of alveolar fluid, there were still signs of ENaC inhibition. First, utilizing EBC Na^+ to assess changes in alveolar lung fluid suggested a trend for a decrease with placebo, but an increase with amiloride as was expected with amiloride inhibiting ENaC-mediated sodium absorption at the level of the alveolar epithelium. Second, in the 14 subjects fluid input and output were re-

TABLE 2. ABSOLUTE CHANGES

	Placebo		Amiloride	
	Baseline	Post	Baseline	Post
Tissue volume (mL) ^a	834 $\pm$ 137	788 $\pm$ 145 ^a	837 $\pm$ 137	788 $\pm$ 144
Extravascular lung water (mL) ^a	797 $\pm$ 137	736 $\pm$ 143 ^a	802 $\pm$ 137	736 $\pm$ 142 ^a

^a $p < 0.05$ hypoxia effect.

TABLE 3. DIFFERENCES IN EXTRAVASCULAR LUNG WATER USING A HISTOGRAM ANALYSIS OF COMPUTED TOMOGRAPHY ATTENUATION DISTRIBUTIONS

	Placebo		Amiloride	
	Baseline	Post	Baseline	Post
Average (HU)	-889.0 ± 23.4	-894.2 ± 21.1 ^a	-886.3 ± 26.5	-898.3 ± 21.3 ^a
Skew	4.3 ± 0.6	4.6 ± 0.79 ^a	4.3 ± 0.60	4.6 ± 0.69 ^a
Kurtosis	30.9 ± 8.0	35.3 ± 10.1 ^a	31.2 ± 8.9	35.0 ± 10.0 ^a
FWHM (HU)	58.9 ± 12.8	57.8 ± 14.9	61.0 ± 12.1	57.2 ± 13.4

Data are presented as mean ± SD.

^a $p < 0.05$ versus baseline.

FWHM, full-width half-max; HU, Hounsfield units.

corded and although the pairwise comparison was not significant ($p = 0.44$, Table 4), review of the individual responses under each condition shows the variability, and demonstrates that in eight subjects fluid loss was greater with amiloride compared with only four participants, where fluid loss was greater with the placebo than with amiloride, and two participants who showed no real difference between conditions (Fig. 5).

Systemic responses to normobaric hypoxia exposure

The systemic responses to the normobaric hypoxia exposure are presented in Table 4. There was no change in cardiac output with hypoxic exposure (hypoxia effect $p > 0.05$) and no difference between conditions amiloride versus placebo ($p = 0.99$), and the increase in systolic PAP was small (hypoxia effect $p = 0.02$) and not different between amiloride versus placebo visits ($p = 0.41$). Hypoxic exposure caused a significant increase in HR with normobaric hypoxia (hypoxia effect $p < 0.01$) that was not different between conditions ($p = 0.23$). There was a trend for a reduction in NE concen-

tration from pre- to post-hypoxia in the amiloride condition ($p = 0.46$), with no other change in catecholamine concentration being observed. Under both experimental conditions, there was no change in respiratory rate, FVC, FEV₁/FVC, FEF₂₅₋₇₅, and FEF₇₅, suggesting hypoxic exposure had minimal to no effect on lung and airway function (hypoxia and condition effect $p > 0.05$). Hypoxic exposure caused a significant and sustained reduction in SpO₂ (hypoxia effect $p < 0.01$) that was similar between amiloride and placebo conditions ($86\% \pm 3\%$ vs. $85\% \pm 3\%$, $p = 0.29$). Over the course of the hypoxic exposure (~15 hours), SpO₂ decreased below 80% for only 18.3 ± 16.1 and 15.8 ± 15.3 minutes in amiloride and placebo, respectively. No individual presented with signs of HAPE, and subjects demonstrated mild altitude sickness with low modified LLS.

Discussion

In this study, we demonstrated that (1) there was a reduction in lung fluid, specifically interstitial lung fluid, with exposure to normobaric hypoxia and (2) the use of nebulized

TABLE 4. SYSTEMIC RESPONSES TO NORMOBARIC HYPOXIA

	Placebo			Amiloride		
	Baseline	Post	Difference	Baseline	Post	Difference
Cardiac output (L/min)	4.6 ± 1.4	4.2 ± 1.1	-0.3 ± 1.0	4.2 ± 0.9	3.8 ± 1.3	-0.3 ± 1.1
Systolic pulmonary artery pressure (mmHg)	15.9 ± 9.9	17.6 ± 11.5	3.6 ± 5.3	10.6 ± 7.2	16.3 ± 11.5 ^a	6.0 ± 11.0
HR (bpm)	67 ± 9	78 ± 14 ^a	10 ± 9	65 ± 12	79 ± 15 ^a	14 ± 12
SpO ₂ (%)	98 ± 1	92 ± 4 ^a	-11 ± 5	99 ± 1	92 ± 3 ^a	-14 ± 4
EPI (pg/mL)	32.5 ± 25.7	32.7 ± 24.1	0.20 ± 33.0	36.4 ± 50.0	31.0 ± 24.4	-5.4 ± 47.5
NE (pg/mL)	242.2 ± 82.5	207.0 ± 101.4	-35.2 ± 92.6	246.1 ± 133.1	184.9 ± 63.1 ^a	-61.3 ± 114.5
Respiratory rate (breath/min)	15.9 ± 1.6	16.7 ± 1.8	0.8 ± 1.8	15.7 ± 1.8	16.8 ± 1.6	1.1 ± 2.5
FVC (L)	4.9 ± 0.3	4.7 ± 0.3	-0.11 ± 0.05	4.8 ± 0.3	4.8 ± 0.3	-0.08 ± 0.05
FEV ₁ (L)	3.9 ± 0.2	3.9 ± 0.2	-0.02 ± 0.04	3.9 ± 0.2	3.9 ± 0.2	0.03 ± 0.05
FEV ₁ /FVC (%)	81.7 ± 1.4	83.3 ± 1.4	0.6 ± 0.8	82.1 ± 1.4	82.7 ± 1.4	1.6 ± 0.4
FEF ₂₅₋₇₅ (L/s)	3.9 ± 0.2	4.1 ± 0.2	0.2 ± 0.1	3.9 ± 0.2	4.0 ± 0.2	0.02 ± 0.10
FEF ₇₅ (L/s)	1.9 ± 0.1	2.1 ± 0.1	0.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	0.1 ± 0.1
PEF (L/s)	9.0 ± 0.6	8.7 ± 0.6	-0.2 ± 0.2	8.6 ± 0.5	8.6 ± 0.6	0.03 ± 0.2
EBC Na ⁺ (mmol/L)	0.71 ± 0.29	0.58 ± 0.25	-9.7% ± 43.2%	0.61 ± 0.21	0.72 ± 0.51	20.1% ± 92.5%
Fluid input-output (mL)		-136.3 ± 732.4			-272.8 ± 319.6	
Modified Lake Louise Score		1.0 ± 1.2			1.1 ± 1.2	
Percent of time SpO ₂ < 80%		15.8 ± 15.3			18.3 ± 16.1	

Data are presented as mean ± SD.

^a $p < 0.05$ versus baseline.

EBC Na⁺, exhaled breath condensate Na⁺; EPI, epinephrine; HR, heart rate; NE, norepinephrine; FEF₇₅, forced expiratory flow at 75% of FVC; Modified Lake Louise Score, out of 30 and averaged over their tent exposure; PEF, peak expiratory flow; Percent of time SpO₂ < 80%, percent of time in tent that Nonin wrist stats dropped below 80%; SpO₂, peripheral oxygen saturation.

FIG. 5. Individual fluid input–output in response to normobaric hypoxia. Each line represents a subject, with the fluid input–output plotted for the amiloride visit and the placebo visit and line connecting the two to show how the responses differed between conditions. Negative fluid loss greater in placebo versus amiloride condition (*dashed lines*); negative fluid loss with amiloride and a positive with placebo or less fluid gain with amiloride (*black lines*). No difference in I/O between conditions (*gray lines*).

amiloride to inhibit ENaC did not affect lung fluid regulation. The results of this study replicate our laboratory's prior findings that exposure to normobaric hypoxia as well as hypobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals (Snyder et al., 2006; Snyder et al., 2008; Taylor et al., 2013), but did not follow our original hypothesis that ENaC inhibition by amiloride would result in greater lung fluid accumulation. The novel findings in this study was the observation that lung fluid regulation was unaffected by ENaC inhibition through inhaled amiloride. The following discussion will highlight the potential mechanisms of lung fluid removal, the importance of ENaC, and the ability of hypoxia to challenge alveolar fluid clearance.

First, what is mediating removal of fluid with exposure to hypoxia?

Consistent with our laboratory's previous findings, we demonstrated a reduction in interstitial lung fluid through CT-derived measures of EVLW with exposure to hypoxia. However, in this current study alveolar fluid clearance rate appeared to be unchanged as there was no change in DLCO or D_M with exposure to hypoxia. One possible explanation for this is that although in both studies subjects were kept in hypoxia until and between all measurements, in the current study the DLCO gas mixture used for post-hypoxia measurements was the same as baseline, where the oxygen concentration was 35%, whereas in the previous study, a special hypoxic DLCO gas mixture was used where the oxygen concentration was 18%. Since the change previously observed was not drastic (+10%), the potential of reoxygenation over the 10 breaths of the nonhypoxic DLCO gas may have limited our ability to measure a change with hypoxia in the current study.

The results of these studies seem to highlight that the observed fluid reduction is predominantly interstitial fluid removal. As such, lymphatic drainage is potentially of greater importance and the primary mediator of the observed reduction in lung fluid. Previous work in sheep and dogs has shown that lymph flow increases 10%–40% with hypoxia (Martin et al., 1986; Levine et al., 1988) and the increases in ventilation experienced with hypoxia also facilitate pumping and production of lymph (Ikomi et al., 1991; Mahe et al., 1991; Pearse et al., 2005; Zawieja, 2009). Additionally, in our previous study we observed an increase in exhaled nitric oxide with normobaric hypoxia exposure (Snyder et al., 2006; Van Iterson et al., 2017).

In the thoracic lymphatic duct of rat, initiation of spontaneous contraction of the phasically nonactive segments results in nitric oxide-mediated relaxation of these segments. This reduction in lymphatic vessel tone improves diastolic filling of the vessels and although contraction rate is reduced, lymphatic contractions are stronger making overall lymphatic pumping more efficient (Gasheva et al., 2006; Gashev, 2008). As such, we hypothesize that the reduction in interstitial lung fluid observed in this study and in previous work in response to normobaric hypoxia is primarily driven by increases in lymphatic fluid clearance mediated by (1) increases in minute ventilation likely elevated due to increases in tidal volume, since we did not observe an increase in respiratory rate and (2) increases in NO mediating relaxation of the lymphatics such that they can more efficiently clear any excess interstitial fluid that is not reabsorbed.

Is impairment of ENaC function really insufficient to cause pulmonary edema in response to hypoxic exposure?

First, at least two types of Na^+ channels have been identified to exist in the alveolar epithelium each with very different regulation, and quite often opposite response to the same stimuli (Eaton et al., 2004; Trac et al., 2017). ENaC is composed of three homologous subunits: α -ENaC, β -ENaC, and γ -ENaC. It is the ratio and combination of these subunits that can produce channels with varying conductances and regulatory properties. When a channel is composed of all three subunits then the channel has high Na^+ selectivity and falls into the highly selective channel (HSC) type. In contrast, nonselective cation channels (NSC) or amiloride-insensitive channels, are composed of at least one α -ENaC subunit and at least one acid-sensing ion channel 1 (ASIC1a) and the channel has low Na^+ selectivity or no selectivity, making it more likely to secrete K^+ rather than absorb Na^+ (Trac et al., 2017). Hypoxia can cause a shift from HSC to NSC as hypoxia reduces HSC or ENaC channels, but increases NSC expression (Jain et al., 2001; Trac et al., 2017), and reduces sodium transport across the airway epithelium (Tomlinson et al., 1999).

In rats it was demonstrated that amiloride caused a greater drop in transepithelial Na^+ flux, measured by NPD, than hypoxia alone. With hypoxia and amiloride, there was no additional reduction in Na^+ current, suggesting that the reduction in Na^+ with hypoxia was amiloride sensitive ENaC mediated (Tomlinson et al., 1999). Furthermore, prior work has demonstrated that total or mean NPD is reduced in HAPE-prone subjects before altitude exposure, suggesting reduced resorption, with only Sartori et al. (2004) showing a significant re-

duction in the amiloride-dependent Na^+ transport (Mairbaurl et al., 2003). Upon ascent to altitude, results continued to be conflicted at times, as Sartori et al. (2004) observed a further decrease in total NPD, specifically only the amiloride-insensitive Na^+ current by $\sim 30\%$, with no change in the amiloride-sensitive Na^+ current, and this was only HAPE-prone subjects.

In contrast, Mairbaurl et al. found that total NPD became more positive due to increased chloride secretion, occurring in response to nasal dryness, and an observed increase in the amiloride-insensitive Na^+ current in both conditions. The amiloride-dependent Na^+ reabsorption decreased in control subjects, whereas remained unchanged in HAPE-prone individuals (Mairbaurl et al., 2003). Additionally, previous cell and tissue work has found that the 40%–50% of the Na^+ and airway fluid clearance occurs through amiloride-insensitive channels (Sakuma et al., 2006; O'Brodovich et al., 2008), with one study in human ATII cells demonstrating that the amiloride insensitive channels made up 70% of the fluid transport (Fang et al., 2006).

Data are conflicting as to which channel Na^+ is moving through to mediate fluid clearance, but recent work by Trac et al. demonstrated that NSC reduction through knocking down either $\alpha\text{-ENaC}$ or ASIC1a reduces alveolar fluid clearance and causes wetter lungs. Furthermore, unlike with ENaC (HSC), where its expression and numbers decrease with hypoxia, NSC increases expression in response to hypoxia and although likely not as effectively they are able to assist in preventing alveolar edema (Trac et al., 2017).

Focusing on the human *in vivo* and *in vitro* work as well as the results of this study suggest that although present, amiloride-sensitive Na^+ transport is not the sole means of alveolar fluid clearance, especially in response to normobaric or hypobaric hypoxia. Measurement of NPD was not performed in this study limiting our ability to directly assess respiratory transepithelial baseline Na^+ transport and the effects amiloride administration had on this in response to the normobaric hypoxia exposure.

Second, one has to also question whether the amiloride dose sufficient for inhibition—how much reached the alveoli and how long was it acting locally on the airway epithelia before being absorbed and circulated systemically. Nebulized amiloride was originally developed for potential use in individuals with cystic fibrosis, where it was hoped it could inhibit the pathological hyperabsorption of Na^+ that occurs through ENaC in these individuals. However, nebulized amiloride showed very poor efficacy in clinical trials and this was attributed to its low potency and short half-life duration on the airway epithelia (Kohler et al., 1986; Knowles et al., 1990; Graham et al., 1993; Pons et al., 2000; Hirsh, 2002).

Understanding these limitations of amiloride, but wanting to inhibit ENaC locally with a FDA-approved nebulized amiloride dose, amiloride was administered three times (5 and 7 hours apart) during the subject's hypoxia exposure. Even with this repeat dosing, drug delivery may have been limited by the aerosol droplet size (larger portion of droplets being outside the respirable range of 1–5 μm), and the lack of a standardized pattern of breathing, which could have reduced alveolar deposition such that the required concentration for effective blockade of 10 $\mu\text{mol/L}$ in the alveoli may not have been reached, and ENaC blockade was then only partial (Noone et al., 1997; Schulz, 1998).

FIG. 6. SpO_2 and change from baseline (ΔSpO_2) during the normobaric hypoxia exposure. The average SpO_2 noted by the CRU nurse every two hours in subjects during their amiloride visit (black squares) and placebo visit (open black circles). Black arrows represent the time when amiloride/placebo was nebulized. The change in SpO_2 from baseline every 2 hours is presented for amiloride (gray squares) and placebo (open gray circles). Standard deviation is not presented on figure to keep figure clear. Amiloride SD: mean ± 4.4 ; range (1.2–5.6). Placebo SD: mean ± 4.4 ; range (3.2–5.9). Amiloride delta SD: mean ± 5.1 ; range (3.9–6.0). Placebo delta SD: mean ± 6.3 ; range (4.9–8.1).

The timeline of SpO_2 and change from baseline (ΔSpO_2) every 2 hours during the hypoxia exposure shows a trend from a drop in SpO_2 following the amiloride administrations, but this occurs with both placebo and amiloride (Fig. 6). Although complete ENaC blockade was unlikely, the results suggest amiloride was having an inhibitory effect locally as there was a trend for higher EBC Na^+ , a noninvasive assessment of ASL composition, with amiloride compared with placebo. This measurement has its limitations as although the composition of EBC is considered to be a dilute surrogate marker of ASL composition, one cannot be certain what region(s) of the lung the droplets are being formed.

Future work should follow up with NPD measurements to provide an additional measure of changes in ion flux in the airway epithelium in response to hypoxia with and without amiloride. We also have signs that the nebulized amiloride was being absorbed across the epithelia and acting on the kidneys to cause diuresis as there is a trend for a higher net urine output with amiloride. This observed diuretic effect aligns with earlier pharmacokinetic work showing that after aerosol delivery, amiloride plasma concentration peaks by 30 minutes and 50% of amiloride has been excreted by 4–6 hours postadministration (Noone et al., 1997).

Although this study did not show that ENaC was necessary for preventing lung fluid accumulation, it does not discount the role of ENaC in regulating alveolar lung fluid clearance. ENaC has been demonstrated to be necessary for fetal alveolar lung fluid clearance, where knockout of $\alpha\text{-ENaC}$ caused a failure to thrive in mice (Mall et al., 2004). This study and the work of others suggests that the role of amiloride-sensitive ENaC is not primary or solely responsible for maintaining lung fluid homeostasis in response to normobaric or hypobaric hypoxia. ENaC's role in lung fluid

balance is alveolar fluid clearance and in response to normobaric hypoxia we do not observe that this role is challenged, such that it is not needed or necessary to maintain lung fluid balance.

The current study and previous work have demonstrated that exposure to normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals (Snyder et al., 2006; Snyder et al., 2008) meaning that even with amiloride inhibition of ENaC, complete or partial, alveolar fluid clearance is not really challenged as there is not a buildup of interstitial fluid that can potentially move into the alveoli. As such, we conclude that ENaC may not be necessary to maintain gross lung fluid homeostasis in response to normobaric hypoxia in healthy, non-HAPE-susceptible individuals, but instead its role in more fine tuning and alveolar fluid balance and in this exposure there was no alveolar edema to prevent.

Third, was the hypoxic stimulus sufficient to challenge alveolar fluid clearance?

Exaggerated pulmonary hypertension plays an important role in the development of HAPE (Scherrer et al., 1999; Sartori et al., 2000; Sartori et al., 2004). If the hypoxia stimulus is not sufficient, PAP would not be increased due to hypoxic pulmonary vasoconstriction and there would not be a large shift of fluid into the interstitial space (Maggiorini et al., 2001). The estimated capacity of the lymphatics to absorb fluid is between 0.20 and 0.40 mL/kg per hour for each pleural space (Shields, 2009).

Although the conditions (hypoxic tent, CRU environment, level of hypoxia) were the same between this study and our previous study, the degree of hypoxemia experienced by the subjects in the current study was slightly less, with an average SpO₂ around 85% overnight and <20 minutes at a SpO₂ <80% in the current study compared with an average of 82% overnight in the previous study. In the current study, participants demonstrated an increase in HR of <15 bpm, a small increase in PAP, no change in respiratory rate, and no rise in catecholamines, whereas in our previous work we saw an average 14 bpm increase in HR, a doubling of PAP, and an increase in both EPI and NE with 17 hours of normobaric hypoxia exposure (Snyder et al., 2006).

Mazzeo et al. (1994) demonstrated that in response to an acute high-altitude exposure, there is a rapid (within 4 hours) and significant increase in arterial EPI concentrations. Hypoxia directly stimulates the adrenal medulla to release EPI into the circulation, with the increase in EPI concentration directly related to the severity of hypoxia exposure (i.e., the decline in arterial O₂). In calves, Bloom et al. (1977) demonstrated that only in response to intense hypoxia (arterial PO₂ 17.1 ± 2.8 mmHg) did the adrenal medulla secrete physiologically effective amounts of catecholamines. With an average peripheral desaturation >80%, it is unlikely that there was a severe decline in arterial O₂ (80% SpO₂ = PaO₂ ~ 50 mmHg), and as such not a strong enough stimulus for EPI release from the medulla.

Alveolar fluid clearance, where ENaCs plays a role, would only be challenged when net fluid balance is disrupted such that there is more fluid moving from the pulmonary vessels to the interstitial space than can be removed by the lymphatic vessels; as then this excess fluid has the potential to shift into the alveolar space. With no change in catecholamines, no

increase in PAP, and a reduction in lung fluid in the current study, the hypoxia exposure likely did not challenge alveolar fluid clearance such that amiloride-mediated impairment in alveolar transepithelial Na⁺ transport would compromise lung fluid clearance. Additionally, further work is needed to evaluate the role of ENaC in lung fluid, both alveolar and interstitial, to determine if these observations also hold true in HAPE-susceptible individuals.

Conclusion

Acute normobaric hypoxia caused a reduction in lung fluid volume that was unaffected by ENaC inhibition through inhaled amiloride, suggesting amiloride-sensitive ENaC were not necessary to maintain a balance between lung fluid accumulation and lung fluid clearance. We demonstrate a reduction in lung fluid, and as such it is likely that alveolar fluid clearance, where ENaC would be involved, was not significantly challenged. It is possible that amiloride-sensitive ENaC may not be necessary to maintain lung fluid balance in response to hypoxia, but it is more probable that normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals.

Acknowledgments

Funding support: NIH HL71478 and HL108962, and CTSA Grant Number UL1 TR000135 from the National Center for Advancing Translational Sciences (NCATS), a component of the National Institutes of Health (NIH).

Author Disclosure Statement

No competing financial interests exist.

References

- Bloom SR, Edwards AV, and Hardy RN. (1977). Adrenal and pancreatic endocrine responses to hypoxia and hypercapnia in the calf. *J Physiol* 269:131–154.
- Boucher RC. (1999). Molecular insights into the physiology of the 'thin film' of airway surface liquid. *J Physiol* 516 (Pt 3): 631–638.
- Chase SC, Wheatley CM, Olson LJ, Beck KC, Wentz RJ, Snyder EM, Taylor BJ, and Johnson BD. (2016). Impact of chronic systolic heart failure on lung structure-function relationships in large airways. *Physiol Rep* 4:pii: e12867.
- Coffman KE, Chase SC, Taylor BJ, and Johnson BD. (2016). The blood transfer conductance for nitric oxide: Infinite vs. finite thetaNO. *Respir Physiol Neurobiol* 241:45–52.
- Dill DB, and Costill DL. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247–248.
- Eaton DC, Chen J, Ramosevac S, Matalon S, and Jain L. (2004). Regulation of Na⁺ channels in lung alveolar type II epithelial cells. *Proc Am Thorac Soc* 1:10–16.
- Fang X, Song Y, Hirsch J, Galletta LJ, Pedemonte N, Zemans RL, Dolganov G, Verkman AS, and Matthay MA. (2006). Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells. *Am J Physiol Lung Cell Mol Physiol* 290:L242–L249.
- Gashev AA. (2008). Lymphatic vessels: Pressure- and flow-dependent regulatory reactions. *Ann N Y Acad Sci* 1131:100–109.
- Gasheva OY, Zawieja DC, and Gashev AA. (2006). Contraction-initiated NO-dependent lymphatic relaxation: A

- self-regulatory mechanism in rat thoracic duct. *J Physiol* 575: 821–832.
- Gille T, Randrianarison-Pellan N, Goolaerts A, Dard N, Uzunhan Y, Ferrary E, Hummler E, Clerici C, and Planes C. (2014). Hypoxia-induced inhibition of epithelial Na⁺ channels in the lung. Role of Nedd4-2 and the ubiquitin-proteasome pathway. *Am J Respir Cell Mol Biol* 50:526–537.
- Graham A, Hasani A, Alton EW, Martin GP, Marriott C, Hodson ME, Clarke SW, and Geddes DM. (1993). No added benefit from nebulized amiloride in patients with cystic fibrosis. *Eur Respir J* 6:1243–1248.
- Hankinson JL, Odencrantz JR, and Fedan KB. (1999). Spirometric reference values from a sample of the general U.S. population. *Am J Respir Crit Care Med* 159:179–187.
- Hirsh AJ. (2002). Altering airway surface liquid volume: Inhalation therapy with amiloride and hyperosmotic agents. *Adv Drug Deliv Rev* 54:1445–1462.
- Hsia CC. (2002). Recruitment of lung diffusing capacity: Update of concept and application. *Chest* 122:1774–1783.
- Hsia CC, Herazo LF, Ramanathan M, and Johnson RL, Jr. (1995). Cardiac output during exercise measured by acetylene rebreathing, thermodilution, and Fick techniques. *J Appl Physiol* 78:1612–1616.
- Hsia CC, and Raskin P. (2005). The diabetic lung: Relevance of alveolar microangiopathy for the use of inhaled insulin. *Am J Med* 118:205–211.
- Hummler E, Barker P, Gatzky J, Beermann F, Verdumo C, Schmidt A, Boucher R, and Rossier BC. (1996). Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet* 12:325–328.
- Ikomi F, Kawai Y, and Ohhashi T. (1991). Beta-1 and beta-2 adrenoceptors mediate smooth muscle relaxation in bovine isolated mesenteric lymphatics. *J Pharmacol Exp Ther* 259: 365–370.
- Jain L, Chen XJ, Ramosevac S, Brown LA, and Eaton DC. (2001). Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. *Am J Physiol Lung Cell Mol Physiol* 280:L646–L658.
- Johnson BD, Beck KC, Proctor DN, Miller J, Dietz NM, and Joyner MJ. (2000). Cardiac output during exercise by the open circuit acetylene washin method: Comparison with direct Fick. *J Appl Physiol* 88:650–658.
- Johnson MW, Taylor BJ, Hulsebus ML, Johnson BD, and Snyder EM. (2012). Hypoxia induced changes in lung fluid balance in humans is associated with beta-2 adrenergic receptor density on lymphocytes. *Respir Physiol Neurobiol* 183:59–65.
- Kerem E, Bistrizter T, Hanukoglu A, Hofmann T, Zhou Z, Bennett W, MacLaughlin E, Barker P, Nash M, Quittell L, et al. (1999). Pulmonary epithelial sodium-channel dysfunction and excess airway liquid in pseudohypoaldosteronism. *N Engl J Med* 341:156–162.
- Knowles MR, Church NL, Waltner WE, Yankaskas JR, Gilligan P, King M, Edwards LJ, Helms RW, and Boucher RC. (1990). A pilot study of aerosolized amiloride for the treatment of lung disease in cystic fibrosis. *N Engl J Med* 322:1189–1194.
- Kohler D, App E, Schmitz-Schumann M, Wurtemberger G, and Matthys H. (1986). Inhalation of amiloride improves the mucociliary and the cough clearance in patients with cystic fibrosis. *Eur J Respir Dis Suppl* 146:319–326.
- Levine BD, Kubo K, Kobayashi T, Fukushima M, Shibamoto T, and Ueda G. (1988). Role of barometric pressure in pulmonary fluid balance and oxygen transport. *J Appl Physiol* 64: 419–428.
- Liu Y, Menold E, Dullenkopf A, Reissnecker S, Lormes W, Lehmann M, and Steinacker JM. (1997). Validation of the acetylene rebreathing method for measurement of cardiac output at rest and during high-intensity exercise. *Clin Physiol* 17:171–182.
- Maggiorini M, Melot C, Pierre S, Pfeiffer F, Greve I, Sartori C, Lepori M, Hauser M, Scherrer U, and Naeije R. (2001). High-altitude pulmonary edema is initially caused by an increase in capillary pressure. *Circulation* 103:2078–2083.
- Mahe L, Chapelain B, Gargouil YM, and Neliat G. (1991). Characterization of beta-adrenoceptor subtypes and indications for two cell populations in isolated bovine mesenteric lymphatic vessels. *Eur J Pharmacol* 199:19–25.
- Mairbaurl H, Weymann J, Mohrlein A, Swenson ER, Maggiorini M, Gibbs JS, and Bartsch P. (2003). Nasal epithelium potential difference at high altitude (4,559 m): Evidence for secretion. *Am J Respir Crit Care Med* 167:862–867.
- Mall M, Grubb BR, Harkema JR, O'Neal WK, and Boucher RC. (2004). Increased airway epithelial Na⁺ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 10: 487–493.
- Martin DJ, Baconnier P, Benchetrit G, Royer F, and Grimbert FA. (1986). Effect of acute hypoxia on lung fluid balance in the prerecruited dog lung. *Bull Eur Physiopathol Respir* 22: 335–340.
- Matthay MA, Folkesson HG, and Clerici C. (2002). Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev* 82:569–600.
- Matthay MA, Folkesson HG, and Verkman AS. (1996). Salt and water transport across alveolar and distal airway epithelia in the adult lung. *Am J Physiol* 270:L487–L503.
- Mazzeo RS, Wolfel EE, Butterfield GE, and Reeves JT. (1994). Sympathetic response during 21 days at high altitude (4,300 m) as determined by urinary and arterial catecholamines. *Metabolism* 43:1226–1232.
- Mentz WM, Brown JB, Friedman M, Stutts MJ, Gatzky JT, and Boucher RC. (1986). Deposition, clearance, and effects of aerosolized amiloride in sheep airways. *Am Rev Respir Dis* 134:938–943.
- Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, et al. (2005). Standardisation of spirometry. *Eur Respir J* 26:319–338.
- Motley HL, Cournaud A, et al. (1947). The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. *Am J Physiol* 150:315–320.
- Noone PG, Regnis JA, Liu X, Brouwer KL, Robinson M, Edwards L, and Knowles MR. (1997). Airway deposition and clearance and systemic pharmacokinetics of amiloride following aerosolization with an ultrasonic nebulizer to normal airways. *Chest* 112:1283–1290.
- O'Brodovich H, Yang P, Gandhi S, and Otulakowski G. (2008). Amiloride-insensitive Na⁺ and fluid absorption in the mammalian distal lung. *Am J Physiol Lung Cell Mol Physiol* 294: L401–L408.
- Olson LJ, Snyder EM, Beck KC, and Johnson BD. (2006). Reduced rate of alveolar-capillary recruitment and fall of pulmonary diffusing capacity during exercise in patients with heart failure. *J Card Fail* 12:299–306.
- Pearse DB, Searcy RM, Mitzner W, Permutt S, and Sylvester JT. (2005). Effects of tidal volume and respiratory frequency on lung lymph flow. *J Appl Physiol* 99:556–563.
- Pons G, Marchand MC, d'Athis P, Sauvage E, Foucard C, Chaumet-Riffaud P, Sautegau A, Navarro J, and Lenoir G. (2000). French multicenter randomized double-blind placebo-

- controlled trial on nebulized amiloride in cystic fibrosis patients. The Amiloride-AFLM Collaborative Study Group. *Pediatr Pulmonol* 30:25–31.
- Roughton FJ, and Forster RE. (1957). Relative importance of diffusion and chemical reaction rates in determining rate of exchange of gases in the human lung, with special reference to true diffusing capacity of pulmonary membrane and volume of blood in the lung capillaries. *J Appl Physiol* 11:290–302.
- Sakuma T, Gu X, Wang Z, Maeda S, Sugita M, Sagawa M, Osanai K, Toga H, Ware LB, Folkesson G, et al. (2006). Stimulation of alveolar epithelial fluid clearance in human lungs by exogenous epinephrine. *Crit Care Med* 34:676–681.
- Sartori C, Allemann Y, Duplain H, Lepori M, Egli M, Lipp E, Hutter D, Turini P, Hugli O, Cook S, et al. (2002). Salmeterol for the prevention of high-altitude pulmonary edema. *N Engl J Med* 346:1631–1636.
- Sartori C, Allemann Y, Trueb L, Lepori M, Maggiorini M, Nicod P, and Scherrer U. (2000). Exaggerated pulmonary hypertension is not sufficient to trigger high-altitude pulmonary oedema in humans. *Schweiz Med Wochenschr* 130:385–389.
- Sartori C, Duplain H, Lepori M, Egli M, Maggiorini M, Nicod P, and Scherrer U. (2004). High altitude impairs nasal trans-epithelial sodium transport in HAPE-prone subjects. *Eur Respir J* 23:916–920.
- Savourey G, Guinet A, Besnard Y, Garcia N, Hanniquet AM, and Bittel J. (1995). Evaluation of the Lake Louise acute mountain sickness scoring system in a hypobaric chamber. *Aviat Space Environ Med* 66:963–967.
- Scherrer U, Sartori C, Lepori M, Allemann Y, Duplain H, Trueb L, and Nicod P. (1999). High-altitude pulmonary edema: From exaggerated pulmonary hypertension to a defect in transepithelial sodium transport. *Adv Exp Med Biol* 474:93–107.
- Schulz H. (1998). Mechanisms and factors affecting intrapulmonary particle deposition: Implications for efficient inhalation therapies. *Pharm Sci Technol Today* 1:336–344.
- Scillia P, Delcroix M, Lejeune P, Melot C, Struyven J, Naeije R, and Gevenois PA. (1999). Hydrostatic pulmonary edema: Evaluation with thin-section CT in dogs. *Radiology* 211:161–168.
- Shaker SB, Dirksen A, Laursen LC, Skovgaard LT, and Holstein-Rathlou NH. (2004). Volume adjustment of lung density by computed tomography scans in patients with emphysema. *Acta Radiol* 45:417–423.
- Shields TW. (2009). *General Thoracic Surgery*. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, PA.
- Snyder EM, Beck KC, Hulsebus ML, Breen JF, Hoffman EA, and Johnson BD. (2006). Short-term hypoxic exposure at rest and during exercise reduces lung water in healthy humans. *J Appl Physiol* 101:1623–1632.
- Snyder EM, Johnson BD, and Beck KC. (2005). An open-circuit method for determining lung diffusing capacity during exercise: Comparison to rebreathe. *J Appl Physiol* 99:1985–1991.
- Snyder EM, Olson TP, Johnson BD, and Frantz RP. (2008). Influence of sildenafil on lung diffusion during exposure to acute hypoxia at rest and during exercise in healthy humans. *Eur J Appl Physiol* 103:421–430.
- Swenson ER. (2013). Hypoxic pulmonary vasoconstriction. *High Alt Med Biol* 14:101–110.
- Tamhane RM, Johnson RL, Jr., and Hsia CC. (2001). Pulmonary membrane diffusing capacity and capillary blood volume measured during exercise from nitric oxide uptake. *Chest* 120:1850–1856.
- Taylor B, Summerfield DT, Issa AN, Kasak AJ, and Johnson BD. (2013). Lung fluid regulation at high altitude. *FASEB J* 27:1207.12.
- Tomlinson LA, Carpenter TC, Baker EH, Bridges JB, and Weil JV. (1999). Hypoxia reduces airway epithelial sodium transport in rats. *Am J Physiol Lung Cell Mol Physiol* 277:L881–L886.
- Trac PT, Thai TL, Linck V, Zou L, Greenlee M, Yue Q, Al-Khalili O, Alli AA, Eaton AF, and Eaton DC. (2017). Alveolar nonselective channels are ASIC1a/alpha-ENaC channels and contribute to AFC. *Am J Physiol Lung Cell Mol Physiol* 312:L797–L811.
- Van Iterson EH, Snyder EM, and Johnson BD. (2017). The influence of 17 hours of normobaric hypoxia on parallel adjustments in exhaled nitric oxide and airway function in lowland healthy adults. *High Alt Med Biol* 18:1–10.
- Vivona ML, Matthay M, Chabaud MB, Friedlander G, and Clerici C. (2001). Hypoxia reduces alveolar epithelial sodium and fluid transport in rats: Reversal by beta-adrenergic agonist treatment. *Am J Respir Cell Mol Biol* 25:554–561.
- Wheatley CM, Baker SE, Morgan MA, Martinez MG, Morgan WJ, Wong EC, Karpen SR, and Snyder EM. (2015). Effects of exercise intensity compared to albuterol in individuals with cystic fibrosis. *Respir Med* 109:463–474.
- Wheatley CM, Baldi JC, Cassuto NA, Foxx-Lupo WT, and Snyder EM. (2011a). Glycemic control influences lung membrane diffusion and oxygen saturation in exercise-trained subjects with type 1 diabetes: Alveolar-capillary membrane conductance in type 1 diabetes. *Eur J Appl Physiol* 111:567–578.
- Wheatley CM, Foxx-Lupo WT, Cassuto NA, Wong EC, Daines CL, Morgan WJ, and Snyder EM. (2011b). Impaired lung diffusing capacity for nitric oxide and alveolar-capillary membrane conductance results in oxygen desaturation during exercise in patients with cystic fibrosis. *J Cyst Fibros* 10:45–53.
- Wheatley CM, Morgan WJ, Cassuto NA, Foxx-Lupo WT, Daines CL, Morgan MA, Phan H, and Snyder EM. (2013). Exhaled breath condensate detects baseline reductions in chloride and increases in response to albuterol in cystic fibrosis patients. *Clin Med Insights Circ Respir Pulm Med* 7:79–90.
- Yock PG, and Popp RL. (1984). Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. *Circulation* 70:657–662.
- Zawieja DC. (2009). Contractile physiology of lymphatics. *Lymphat Res Biol* 7:87–96.

Address correspondence to:
 Courtney M. Wheatley, PhD
 Division of Cardiovascular Diseases
 Mayo Clinic Scottsdale
 13400 East Shea Boulevard
 Scottsdale, AZ 85259

E-mail: wheatley.courtney@mayo.edu