

## ORIGINAL RESEARCH

## Nominal carbonic anhydrase activity minimizes airway-surface liquid pH changes during breathing

Ian M. Thornell<sup>1,2,a</sup> , Xiaopeng Li<sup>1,a</sup>, Xiao Xiao Tang<sup>1,2</sup>, Christian M. Brommel<sup>1</sup>, Philip H. Karp<sup>1,2</sup>, Michael J. Welsh<sup>1,2,3</sup> & Joseph Zabner<sup>1</sup>

<sup>1</sup> Department of Internal Medicine, University of Iowa, Iowa City, IA

<sup>2</sup> Howard Hughes Medical Institute, University of Iowa, Iowa City, IA

<sup>3</sup> Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA

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Acid-base, airway epithelium, airway-surface liquid, carbonic anhydrase.

### Correspondence

Joseph Zabner, Department of Internal Medicine, University of Iowa, 6322 PBBB, 169 Newton Rd., Iowa City, IA 52242.  
Tel: 319-356-4419  
Fax: 319-353-6406  
E-mail: Joseph-Zabner@uiowa.edu

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<sup>a</sup>Authors contributed equally to this work.

## Introduction

Respiratory epithelia are covered by a thin ~10  $\mu\text{m}$  layer of liquid termed the airway-surface liquid (ASL) (Widdicombe and Wine 2015). The ASL pH ( $\text{pH}_{\text{ASL}}$ ) is typically a few tenths of a pH unit more acidic than the plasma pH (Jayaraman et al. 2001; Coakley et al. 2003; McShane et al. 2003; Song et al. 2006; Pezzulo et al. 2012; Garland et al. 2013; Abou Alaiwa et al. 2014a).  $\text{pH}_{\text{ASL}}$

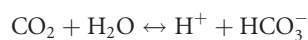
### Abstract

The airway-surface liquid pH ( $\text{pH}_{\text{ASL}}$ ) is slightly acidic relative to the plasma and becomes more acidic in airway diseases, leading to impaired host defense.  $\text{CO}_2$  in the large airways decreases during inspiration (0.04%  $\text{CO}_2$ ) and increases during expiration (5%  $\text{CO}_2$ ). Thus, we hypothesized that  $\text{pH}_{\text{ASL}}$  would fluctuate during the respiratory cycle. We measured  $\text{pH}_{\text{ASL}}$  on cultures of airway epithelia while changing apical  $\text{CO}_2$  concentrations. Changing apical  $\text{CO}_2$  produced only very slow  $\text{pH}_{\text{ASL}}$  changes, occurring in minutes, inconsistent with respiratory phases that occur in a few seconds. We hypothesized that pH changes were slow because airway-surface liquid has little carbonic anhydrase activity. To test this hypothesis, we applied the carbonic anhydrase inhibitor acetazolamide and found minimal effects on  $\text{CO}_2$ -induced  $\text{pH}_{\text{ASL}}$  changes. In contrast, adding carbonic anhydrase significantly increased the rate of change in  $\text{pH}_{\text{ASL}}$ . Using pH-dependent rates obtained from these experiments, we modeled the  $\text{pH}_{\text{ASL}}$  during respiration to further understand how pH changes with physiologic and pathophysiologic respiratory cycles. Modeled  $\text{pH}_{\text{ASL}}$  oscillations were small and affected by the respiration rate, but not the inspiratory:expiratory ratio. Modeled equilibrium  $\text{pH}_{\text{ASL}}$  was affected by the inspiratory:expiratory ratio, but not the respiration rate. The airway epithelium is the only tissue that is exposed to large and rapid  $\text{CO}_2$  fluctuations. We speculate that the airways may have evolved minimal carbonic anhydrase activity to mitigate large changes in the  $\text{pH}_{\text{ASL}}$  during breathing that could potentially affect pH-sensitive components of ASL.

maintenance is vital to respiratory host defense by influencing ASL antimicrobial activity (Pezzulo et al. 2012; Abou Alaiwa et al. 2014b; Shah et al. 2016), mucociliary transport (Clary-Meinesz et al. 1998; Tang et al. 2016), and phagocyte function (Trevani et al. 1999). Abnormalities in these  $\text{pH}_{\text{ASL}}$ -dependent functions likely contribute to several pathological conditions such as cystic fibrosis (Smith and Welsh 1992, 1993; Hug et al. 2003; Quinton 2008; Chen et al. 2010a; Itani et al. 2011), asthma (Hunt

et al. 2000; Kostikas et al. 2002), chronic obstructive pulmonary disease (Kostikas et al. 2002), and acute respiratory distress syndrome (Gessner et al. 2003; Walsh et al. 2006), where  $\text{pH}_{\text{ASL}}$  is acidified relative to nondisease ASL.

The  $\text{pH}_{\text{ASL}}$  is influenced by a number of  $\text{H}^+$ - and  $\text{HCO}_3^-$ -dependent mechanisms (Fischer and Widdicombe 2006).  $\text{HCO}_3^-$  is a powerful buffer because it is in an open-buffering system with  $\text{CO}_2$ , following the elementary equilibrium reaction:



Within the cytoplasm and some extracellular fluid compartments, the  $\text{CO}_2/\text{HCO}_3^-$  buffering system is catalyzed to a nearly diffusion-limited rate by carbonic anhydrase,  $1.6 \times 10^6 \text{ sec}^{-1}$  for carbonic anhydrase isozyme II (Supuran 2008). For most cell types and compartments, the  $\text{CO}_2$  concentration is a nearly constant 5%. However, the  $\text{CO}_2$  concentration in large proximal airways changes during the respiratory cycle (Cochrane et al. 1982). This change is observed in a clinical setting by variations in end-tidal  $\text{CO}_2$ . During inspiration (1–2 sec), the  $\text{CO}_2$  concentration in the large airway lumen approaches that of air (0.04%  $\text{CO}_2$ ), while during expiration (2–4 sec) the  $\text{CO}_2$  concentration approaches 5%.

If carbonic anhydrase is present in the ASL, then fast  $\text{CO}_2$  equilibration will change  $\text{pH}_{\text{ASL}}$  in the large airways during breathing. Here, we tested this prediction using differentiated cultures of porcine airway epithelia. We were surprised to discover that changing the  $\text{CO}_2$  concentration between 0.04% and 5% produced little change in  $\text{pH}_{\text{ASL}}$  over a time course associated with respiration (i.e., a few seconds). We found that nominal carbonic anhydrase activity prevented short-term changes in pH.

## Methods

### Ethical approval

The protocol for isolation of porcine airway epithelia was reviewed and approved by the University of Iowa Animal Care and Use Committee. Newborn piglets were obtained from Exemplar Genetics (Exemplar Genetics, Sioux Center, IA) and were euthanized upon arrival by an overdose of Euthasol via cardiac puncture after sedation with 20 mg/kg Ketamine and 2 mg/kg xylazine.

### Isolation, expansion, and culture of porcine airway epithelia

Piglet lungs were excised and proximal large airways, including trachea and main stem bronchi, were dissected

out from the airway tree as described (Li et al. 2016). Next, primary porcine airway epithelia were isolated according to an adapted procedure originally developed for tracheal airway cells. Isolated large airway cells were expanded as recently described (Li et al. 2016) using a method developed to conditionally reprogram airway epithelial cells (Liu et al. 2012; Supryniewicz et al. 2012). Briefly, large airway cells were cultured in F media in the presence of 10  $\mu\text{mol/L}$  Y-27632, a ROCK inhibitor, and low passages of irradiated fibroblast feeder cells NIH-3T3-J2 obtained from Dr. H. Green's laboratory at Harvard University (Rheinwald and Green 1975) and maintained at 37°C with 5%  $\text{CO}_2$ . After two passages of amplification, expanded epithelial cells were separated from feeder cells and seeded onto collagen-coated, semipermeable membranes (Corning #3470) at density of  $10^6 \text{ cells/cm}^2$  and cultured at the air–liquid interface 37°C in a 5%  $\text{CO}_2$  atmosphere as previously described (Zabner et al. 1996) for 2–3 weeks in the absence of feeder cells and ROCK inhibitor. In the first week of seeding at the air–liquid interface, cells were maintained in Small Airway Growth Media (Lonza, Basel, Switzerland) supplemented with 10 ng/mL keratinocyte growth factor for 1 week, after which cells were maintained in USG media. We used the expanded epithelial cells between passage 2 and passage 5 to obtain consistent results.

### Tissue RNA isolation and PCR

Tissue samples from large airway epithelial cells, kidney, intestine, and salivary gland were fixed and stored frozen in RNAlater solution (Invitrogen) followed by thawing and three washes of PBS without divalent cations. Next, the tissues were homogenized in TRIzol reagent solution (Invitrogen) and RNA was extracted using Invitrogen's PureLink RNA Mini Kit according to the manufacturer's protocol. The samples were then processed to cDNA by loading 1000 ng of RNA from each sample using SuperScript IV VILO Master Mix (Invitrogen) according to the manufacturers' protocol. cDNA concentrations of each sample were quantified using NanoDrop 2000 (ThermoFisher), then diluted to 500 ng/ $\mu\text{L}$ . Using Kapa HiFi HotStart ReadyMix PCR Kit a reaction mixture for PCR was made according to the manufacturer protocol using 100 ng of cDNA from each sample with each of the following primer sets for membrane-bound CA isoforms;

CA-IX F: 5'-TCCCACCACAGGAGGAGATT-3' IX R: 5'-ATCCCTGGGAGCCTCAGTAG-3'

CA-XII F: 5'-CCCACTCAACGGATCCAAGT-3' XII R: 5'-CAAACCTGCTGGTTGGCAGTC-3'

Pig Beta Actin F: 5'-CTGCGGCATCCACGAAAC-3' R: 5'-GTGATCTCCTCCTGCATCCTGTC-3'

The thermocycler was programmed with the following settings: Initial denaturation of 3 min at 95°C, followed by 25 cycles of denaturation for 20 sec at 98°C, annealing for 15 sec at 63°C, and extension for 15 sec at 72°C; finishing with a final extension of 1 min at 72°C. PCR product was run using electrophoresis on a 5% agarose gel.

### pH<sub>ASL</sub> measurements

pH<sub>ASL</sub> was measured using the ratiometric fluorescent pH indicator SNARF-1 conjugated to dextran (Molecular Probes, Eugene, Oregon), as previously reported (Pezzulo et al. 2012). SNARF has an estimated response time of ~60 msec (Chen et al. 2010b), which is faster than observed pH changes in this study (10 sec to 5 min). SNARF-1 dextran was applied to the airway-surface liquid as a finely strained powder and 2 h later epithelia were placed in a humidified chamber with 5% CO<sub>2</sub>/21% O<sub>2</sub>/balanced N<sub>2</sub> heated to 37°C and examined by confocal microscopy (Zeiss 510 Meta NLO). pH was calculated as previously described (Pezzulo et al. 2012), but using a nonlinear fit, as pH<sub>ASL</sub> was not completely within the linear range of the dye in this study (e.g., when exposed to apical air). Calibration curves were fitted in GraphPad Prism with a modified acid-base titration equation (Boyarsoy et al. 1988):

$$\text{ratio observed} = a + b \left( \frac{10^{(\text{pH}-\text{pKa})}}{1 + 10^{(\text{pH}-\text{pKa})}} \right)$$

Where variables *a* and *b*, as well as pKa, are obtained from the fit. The *ratio observed* is the recorded 580/640 emission. pH and pKa have their classic definitions. In this study, SNARF-1 dextran had a pKa of 7.44 consistent with 37°C values reported by various groups (Buckler and Vaughan-Jones 1990; Blank et al. 1992; Westerblad et al. 1997; Ch'en et al. 2003). In some experiments, the CO<sub>2</sub> was changed from 5% CO<sub>2</sub> to air (compressed air containing 0.04% CO<sub>2</sub>) every 4 min and the change in pH<sub>ASL</sub> was monitored in real time as described above. To test carbonic anhydrase activity, cells were pretreated with 20 μmol/L acetazolamide (Sigma-Aldrich, St. Louis, MO), a carbonic anhydrase inhibitor, or 1.5 U carbonic anhydrase isozyme II (Sigma-Aldrich, St. Louis, MO), in a final volume of 0.5 μL on the apical surface, then pH<sub>ASL</sub> was monitored as described above.

### Kinetic pH<sub>ASL</sub> analysis

The experimental data (*n* = 5–8 independent experiments performed on cells isolated from *N* = 3–4 pigs) were fitted using the least-squares method in GraphPad Prism with the following equations:

Association phase (removal of CO<sub>2</sub>):

$$\text{pH}_{\text{ASL}} = \begin{cases} t \leq t_0; \text{pH}_{\text{ASL}_0} \\ t > t_0; \text{pH}_{\text{ASL}_0} + (\text{pH}_{\text{ASL}_{\text{max}}} - \text{pH}_{\text{ASL}_0}) \\ \quad \times (1 - e^{-k \times (t-t_0)}) \end{cases}$$

Decay phase (addition of CO<sub>2</sub>):

$$\text{pH}_{\text{ASL}} = \begin{cases} t \leq t_0; \text{pH}_{\text{ASL}_0} \\ t > t_0; \text{pH}_{\text{ASL}_{\text{min}}} + (\text{pH}_{\text{ASL}_0} - \text{pH}_{\text{ASL}_{\text{min}}}) \\ \quad \times e^{-k \times (t-t_0)} \end{cases}$$

Where pH<sub>ASL</sub> is the instantaneous ASL pH, *t* is the instantaneous time, *t*<sub>0</sub> is the time of the removal (association equation) or addition (decay equation) of CO<sub>2</sub>, pH<sub>ASL<sub>0</sub></sub> is the baseline pH<sub>ASL</sub>, pH<sub>ASL<sub>max</sub></sub> is the fitted maximal pH<sub>ASL</sub> (association equation only), pH<sub>ASL<sub>min</sub></sub> is the fitted minimum pH<sub>ASL</sub> (decay equation only), and *k* is the obtained rate constant for the fit. *R*<sup>2</sup> values were >0.98 for all fitted data.

To calculate the mean pH, the following equation was used:

$$\text{pH}_{\text{mean}} = -\log \left( \frac{10^{-\text{pH}_1} + 10^{-\text{pH}_2}}{2} \right)$$

Where pH<sub>mean</sub> is the mean pH of the proton activities of pH<sub>1</sub> (steady state under 5% CO<sub>2</sub>) and pH<sub>2</sub> (steady state under air).

To determine the rate constants for large airway epithelia exposed to acetazolamide, carbonic anhydrase, or control treatment, the data were fitted using the least-squares method. Consistent with carbonic anhydrase being an enzyme, we observed that equilibrium pH<sub>ASL</sub> values for each experiment were not different. Therefore, we could compare rate constants among these three treatments.

### Simulation of pH<sub>ASL</sub> changes in the respiratory cycle

A program to calculate real-time pH<sub>ASL</sub> changes during the respiratory cycle was coded in MATLAB version R2015a (Mathworks) and is freely available upon request. User-defined input values are the respiration rate, inspiratory:expiratory (I:E) ratio, initial pH<sub>ASL</sub>, and fitted curves obtained using the association and decay equations described above. The program uses pH-dependent rates, which accommodates for pH-dependent unknown variables (e.g., intrinsic buffering capacity) to predict pH<sub>ASL</sub> values in the time domain. Briefly, the program computes the rate of pH change (*dpH/dt*) along the fitted inspiration curve for a user-defined initial pH value and outputs a computed pH with a user-defined time binning (10 points for the smallest respiratory phase in this study). This process is looped for each bin until the end of inspiration is achieved. After completing the inspiration phase,

the program begins the same process on the expiratory curve initiating at the final pH of the inspiration phase. The program then loops between these two phases until a user-defined simulation time is reached. Data were output to a Microsoft Excel file. From these simulations, the mean oscillatory pH ( $\text{pH}_{\text{OSC}}$ ) and the root mean square (RMS) of the oscillatory pH ( $\text{pH}_{\text{RMS}}$ ) were computed. The  $\text{pH}_{\text{RMS}}$  was calculated using the RMS equation for a triangular wave:

$$\text{pH}_{\text{RMS}} = a / \sqrt{3}$$

Where  $a$  is the amplitude of the triangular wave. In some simulations, the steady-state simulation was acidified or alkalinized and the time to recovery to baseline was monitored and fitted with the association (acid addition) or decay equations (alkaline addition) defined above in prism to obtain time constants ( $\tau$ ).

## Statistics

Data are expressed as mean  $\pm$  SEM or regressions fitted by the least-squares method. A  $P$  value less than 0.05 was accepted as significant. Means were compared using ANOVA with Bonferroni or Dunnett correction (for comparing acetazolamide and carbonic anhydrase application to control), and unpaired, or paired t-tests where appropriate. Statistical significance among regressions was computed by comparing the residual sum-of-squares to obtain an F ratio.  $P$  values were obtained in Excel for a given F ratio with corresponding degrees of freedom.

## Results

### Rate of $\text{pH}_{\text{ASL}}$ change in response to $\text{CO}_2$

To assess how changes in  $\text{CO}_2$  affect  $\text{pH}_{\text{ASL}}$ , we studied differentiated cultures of porcine airway epithelium. We varied apical  $\text{CO}_2$  between 5% and 0.04% (air) while maintaining basolateral  $\text{CO}_2$  at 5%. Switching from 5%  $\text{CO}_2$  to air led to a slow  $\text{pH}_{\text{ASL}}$  change that plateaued after  $\sim 4$  min (Fig. 1A). This change was reversible upon reexposure to 5%  $\text{CO}_2$ . These changes occurred with a time constant of approximately 50 sec.

The reaction  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$  is catalyzed by carbonic anhydrase (Kim et al. 2014). Because the observed pH change is slow, we predicted that carbonic anhydrase activity is low within intact ASL. We first assessed mRNA transcripts of carbonic anhydrase isozymes (*Ca*) predicted to be enzymatically active outside of cells, which include the membrane-bound and secreted carbonic anhydrase isozymes. Microarray analysis from Li et al. 2016 (Fig. 1D) revealed that most mRNA transcripts for these isozymes were present at levels lower than *Cftr* mRNA transcripts, which is present in low abundance in airway

epithelia (Trapnell et al. 1991; Chu et al. 1992). *CaXII* mRNA transcripts were present at similar levels to *Cftr*, however, carbonic anhydrase XII protein has low enzymatic activity (Tureci et al. 1998). We also investigated the expression of *CaIX* and *CaXII* in pig trachea by RT-PCR. As shown in Figure 1E *CaIX* and *CaXII* are expressed in intestine and renal cortex tissues, respectively, but not in pig trachea.

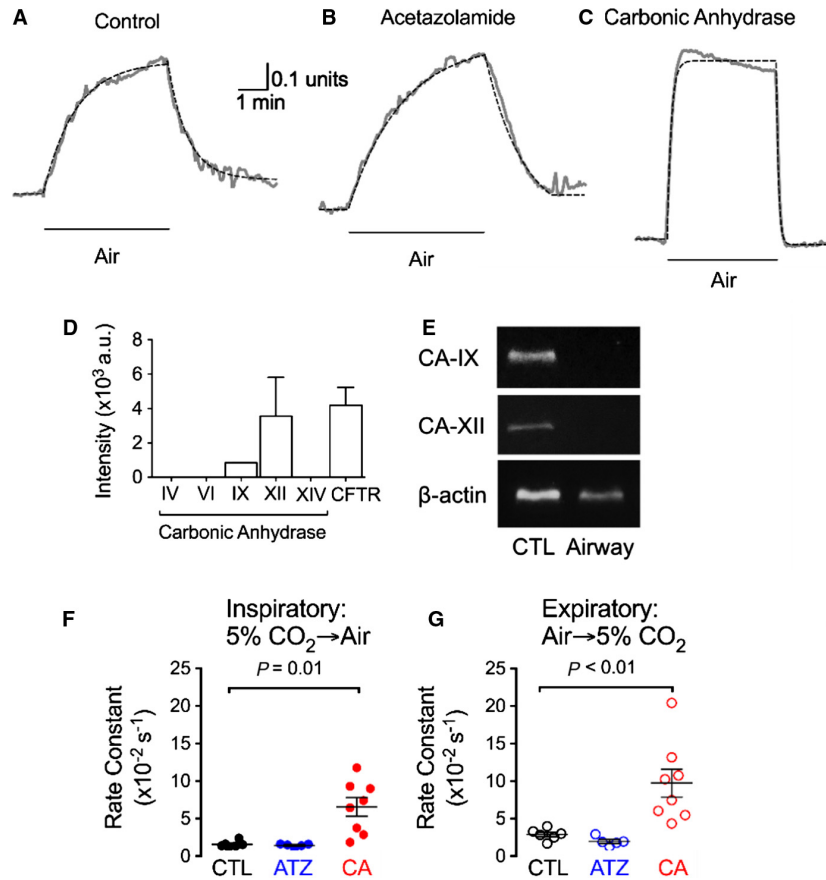
To test the hypothesis that ASL upon differentiated airway cultures has minimal carbonic anhydrase activity, we performed two experiments. We added the carbonic anhydrase inhibitor acetazolamide and found it did not affect  $\text{pH}_{\text{ASL}}$  kinetics induced by apical  $\text{CO}_2$  changes (Fig. 1B). In contrast, adding recombinant carbonic anhydrase to the ASL accelerated the rate of  $\text{pH}_{\text{ASL}}$  change in response to changes in apical  $\text{CO}_2$  (Fig. 1C). Rate constant analysis (Fig. 1F and G) suggests that airway epithelia lack substantial carbonic anhydrase activity resulting in slow changes in  $\text{pH}_{\text{ASL}}$  when  $\text{CO}_2$  concentration changes. These slow changes would minimize the change in  $\text{pH}_{\text{ASL}}$  during breathing.

### Computational modeling of dynamic $\text{pH}_{\text{ASL}}$ changes in airway epithelia

To further explore how respiratory cycle properties (e.g., respiratory rate and inspiratory:expiratory (I:E) ratio) affect  $\text{pH}_{\text{ASL}}$ , we performed computational modeling using the pH-dependent rates obtained from Figure 1. This approach models the real-time pH changes and allows for integration of the pH-dependent processes (e.g., ASL buffering capacity).

The initial model parameters included a 15 breath per minute (bpm) respiratory rate and an I:E ratio of 1:2, which reflects a typical human respiratory cycle (Cheng et al. 2010). To validate the model, we performed simulations using data beginning at the minimum  $\text{pH}_{\text{ASL}}$  value (i.e., the  $\text{pH}_{\text{ASL}}$  for 5%  $\text{CO}_2$ ) or the maximum  $\text{pH}_{\text{ASL}}$  value (i.e., the  $\text{pH}_{\text{ASL}}$  for air) for each culture. Example simulations for one culture are shown in Figure 2A. For these simulations, we quantified the magnitude of the pH oscillations as root-mean-squared values ( $\text{pH}_{\text{RMS}}$ ) and the mean pH during steady-state oscillations ( $\text{pH}_{\text{OSC}}$ ) (Fig. 2B). For the experiment shown, a  $\text{pH}_{\text{OSC}}$  of 7.36 was reached after  $\sim 4$  min with little oscillation ( $\text{pH}_{\text{RMS}} = 0.002$  pH units). These simulation data suggest that the  $\text{pH}_{\text{ASL}}$  is more alkaline during the respiratory cycle than in the constant presence of 5%  $\text{CO}_2$ , under which most airway culture studies are performed, and the pH does not fluctuate appreciably from breath to breath.

Next, we modeled changes in the respiratory rate (15, 20, and 60 breaths per minute) and the I:E ratios to mimic physiologic or pathologic scenarios (1:2 – physiologic ratio, 1:4 – obstructive airway disease, and 4:1 –



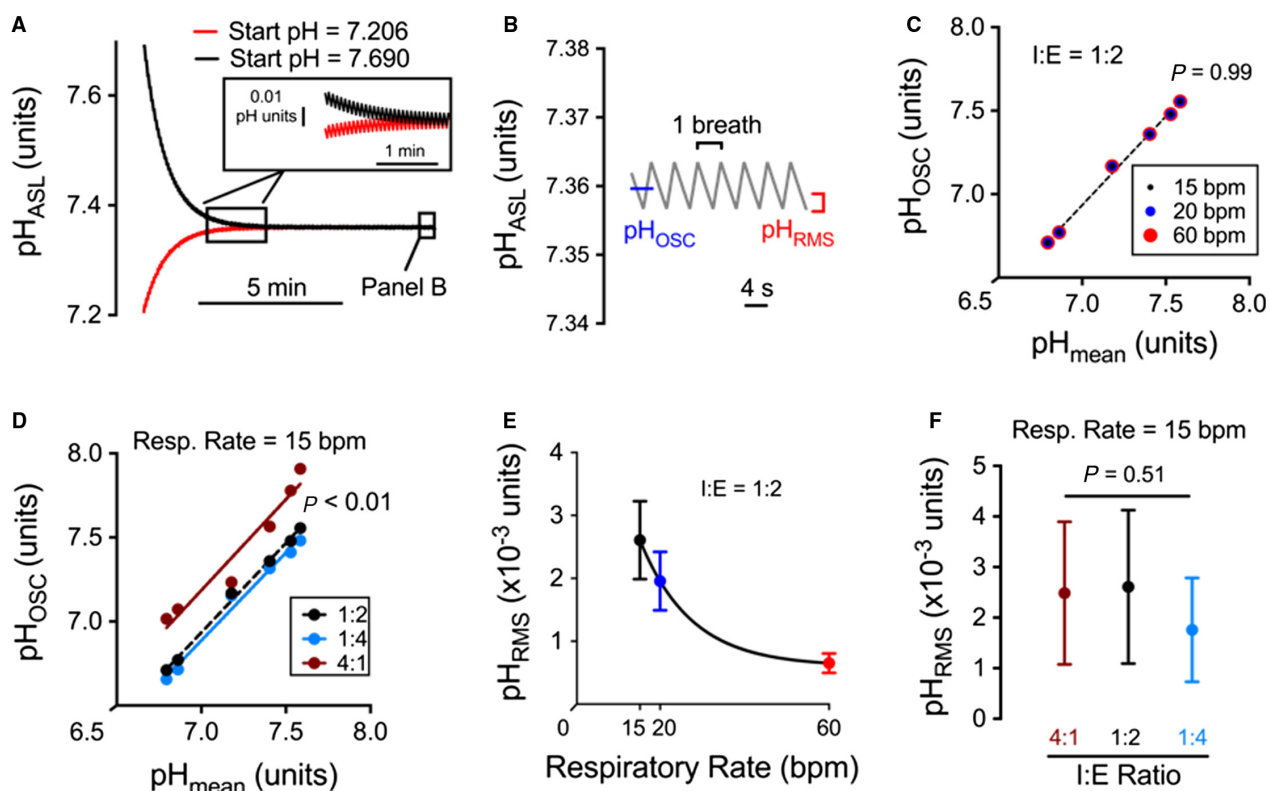
**Figure 1.** Alterations in  $\text{pH}_{\text{ASL}}$  for large airway cultures in response to changes in  $\text{CO}_2$ . A–C, Cells were exposed bilaterally to 5%  $\text{CO}_2$ , followed by an apical shift to air (noted on traces as “Air”). After monitoring  $\text{pH}_{\text{ASL}}$  for 5 min, apical gas was subsequently shifted back to 5%  $\text{CO}_2$ . These maneuvers changed  $\text{pH}_{\text{ASL}}$  from  $\sim 7.2$  to  $\sim 7.7$  in each condition. Each experiment was fitted (black-dotted lines) using the exponential equations described in the Methods. (A) Control conditions. (B) Cells were pretreated with 20  $\mu\text{mol/L}$  acetazolamide, a carbonic anhydrase inhibitor, on the apical surface. (C) Cells were pretreated with 1.5 U carbonic anhydrase on the apical surface. Experiments were performed on matched samples isolated from the same animal. Each trace is a representative example of one experiment. (D) RNA sequencing microarray data for large airway epithelial cells cultured at the air–liquid interface. (E) Pig trachea RT-PCR for CA IX (control: intestine) and CA XII (control: renal cortex). (F and G) Average rate constants for airway epithelia when  $\text{CO}_2$  was shifted from (F) 5%  $\text{CO}_2$  to air or (G) air to 5%  $\text{CO}_2$ . Each dot represents one culture. CTL: control; ATZ: acetazolamide; CA: exogenous carbonic anhydrase.

airway pressure release ventilation).  $\text{pH}_{\text{OSC}}$  during steady state was unaffected by the respiratory rate (Fig. 2C) and was directly correlated to the I:E ratio (Fig. 2D).  $\text{pH}_{\text{RMS}}$  was inversely correlated to respiratory rate (Fig. 2E), but unaffected by the I:E ratio (Fig. 2F). In summary, the I:E ratio affects the mean steady-state pH ( $\text{pH}_{\text{OSC}}$ ) and the respiration rate affects the magnitude of pH oscillations ( $\text{pH}_{\text{RMS}}$ ).

Based on our rate analysis, exogenous carbonic anhydrase would increase pH oscillations. To demonstrate this, the simulation was repeated using rates obtained when we added exogenous carbonic anhydrase, vehicle, or acetazolamide (Fig. 3A). The presence of carbonic anhydrase increased ASL  $\text{pH}_{\text{RMS}}$  (Fig. 3B), but not steady-state  $\text{pH}_{\text{OSC}}$  (Fig. 3C) relative to controls. These results were

expected because adding carbonic anhydrase enzymatic activity should alter the pH kinetics, but should not alter the pH equilibrium achieved.

Finally, we modeled  $\text{pH}_{\text{ASL}}$  recovery from simulated acid or alkaline addition to the ASL (Fig. 3D). We found no difference in recovery to an effective 20 nmol/L acid or alkaline addition under control conditions or in the presence of acetazolamide, whereas applying carbonic anhydrase significantly accelerated the time to recovery (Fig. 3E). These data indicate that lack of appreciable airway carbonic anhydrase activity in the ASL would minimize  $\text{pH}_{\text{ASL}}$  changes during respiration and exogenous carbonic anhydrase may help acidic airways achieve a more alkaline pH, at least for a fraction of the respiratory cycle (i.e., flanking peak inspiration).



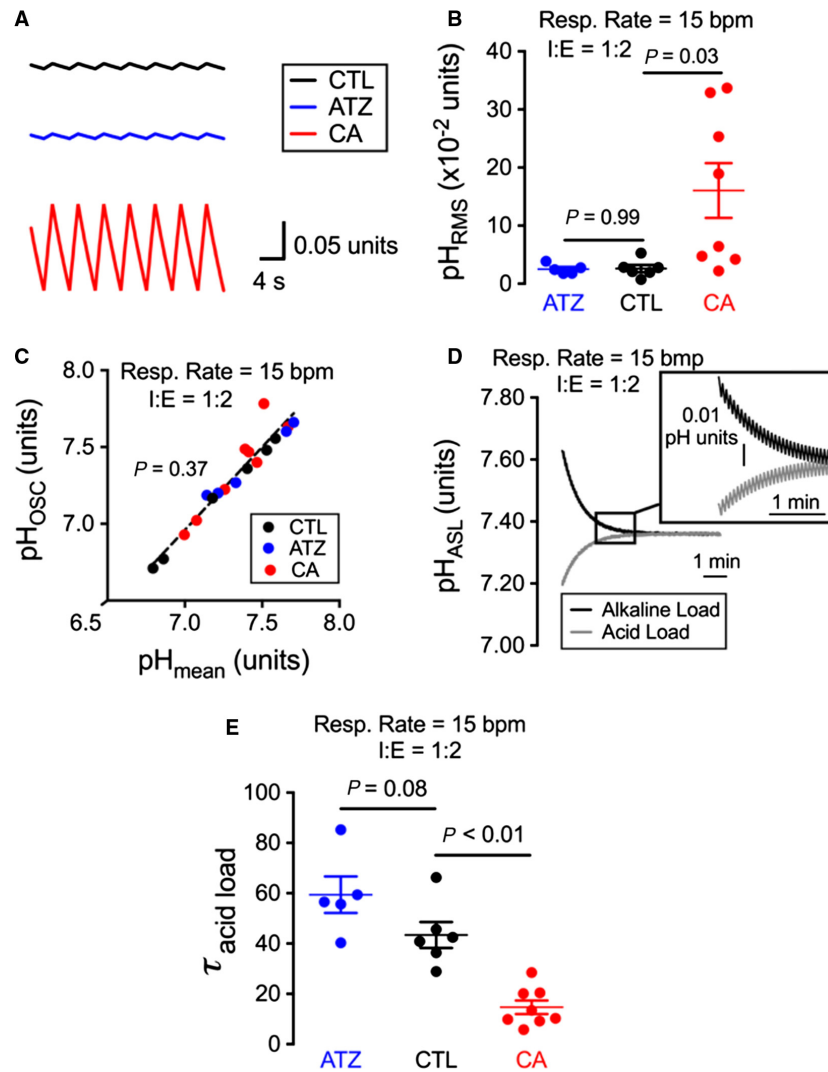
**Figure 2.** Airway  $pH_{ASL}$  is more alkaline during the respiratory cycle than in the constant presence of 5%  $CO_2$ , with minimal breath-to-breath fluctuations. (A) Modeling  $pH_{ASL}$  changes during respiratory cycles with a 15 breath per minute (bpm) respiratory rate and 1:2 I:E ratio. Beginning the simulation at varying pH values does not change steady-state pH achieved. (B) Expansion of the steady-state pH changes from panel A. Parameters that correspond to the mean pH during steady-state oscillation ( $pH_{OSC}$ ) and the bandwidth of the pH oscillation ( $pH_{RMS}$ ) are noted. (C) The I:E ratio was held constant at 1:2, and the respiratory rate was modeled at 15, 20, and 60 bpm. Because  $pH_{OSC}$  will be influenced by the steady-state pH values of a given culture, simulated  $pH_{OSC}$  was plotted as a function of the midpoint  $H^+$  activity between 5%  $CO_2$  and air. There was no change in  $pH_{OSC}$ . (D) The respiratory rate was held constant 15 bpm (typical human breathing cycle), and the I:E ratios were modeled at 1:2 (physiologic ratio), 1:4 (obstructive airway disease), and 4:1 (airway pressure release ventilation). The  $pH_{OSC}$  is directly correlated with relative phase lengths. (E and F)  $pH_{RMS}$  varied inversely with the respiration rate (E) and was not affected by the I:E ratio (F). bpm: breaths per minute. (C and D) Filled circles represent a simulation for rates obtained from different cultures. (E and F) Error bars represent standard error of simulations performed using rates obtained from different cultures.

## Discussion

Because the large airway is exposed to oscillations in  $CO_2$  during the respiratory cycle, our goal was to understand how varying  $CO_2$  concentration affects the  $pH_{ASL}$  on differentiated airway epithelia maintained at the air-liquid interface. We found that, after altering  $CO_2$ , the time to plateau was on the order of minutes. In contrast, respiratory cycles occur within seconds and therefore  $pH_{ASL}$  changes are small during breathing. The slow change in  $pH_{ASL}$  was a result of low carbonic anhydrase activity of the ASL. These data are consistent with previous reports that ASL removed from epithelia has minimal carbonic anhydrase activity (Candiano et al. 2007; Kim et al. 2014). Thus, loss of carbonic anhydrase activity in the bulk ASL may represent an evolutionary selection to

minimize dynamic changes in  $pH_{ASL}$  in response to respiration.

At least 16 different carbonic anhydrase isoforms have been described in mammals (Supuran et al. 2008; Imtaiyaz Hassan et al. 2013). The most relevant isoforms to this study are the membrane-bound and secreted forms, including carbonic anhydrase IV, VI, IX, XII, and XIV (Pinard et al. 2015). Carbonic anhydrase IX has been observed in human airways, but only under hypoxic conditions (Polosukhin et al. 2007, 2011). Carbonic anhydrase VI, also known as gustin, is the only isoform that is secreted, and it is expressed highly in the salivary glands (Thatcher et al. 1998). Carbonic anhydrase VI has been found by immunocytochemistry in rat lung (Leinonen et al. 2004) and by mRNA detection in human neuroendocrine bodies (Livermore et al. 2015), but not on surface



**Figure 3.** Modeling changes in pH<sub>ASL</sub> demonstrates that carbonic anhydrase activity appreciably increases predicted pH oscillations during breathing. (A) Modeling pH<sub>ASL</sub> changes under control conditions (CTL) or in response to acetazolamide (ATZ) or carbonic anhydrase treatment (CA). (B and C) Modeling the effect of acetazolamide or carbonic anhydrase on (B) pH<sub>RMS</sub> or (C) pH<sub>OSC</sub>. Carbonic anhydrase promoted a significant increase in pH<sub>RMS</sub>, whereas carbonic anhydrase had no effect on pH<sub>OSC</sub>. (D) Modeling pH<sub>ASL</sub> recovery from a 20 nmol/L acid or alkaline load. (E) Time to recovery ( $\tau$ ) from a 20 nmol/L acid load under control conditions or in the presence of acetazolamide or CA with a respiratory rate of 15 breaths per minute and an I:E ratio of 1:2. CA but not acetazolamide significantly accelerated the time to recovery. CTL, control; ATZ, acetazolamide; CA, exogenous carbonic anhydrase; bpm, breaths per minute. (B, D, E) Filled circles represent a simulation for rates obtained from different cultures.

epithelia of the airways. Carbonic anhydrase XIV was the only membrane-bound isozyme detected at the mRNA level in human infant lung epithelium (Livermore et al. 2015). Recent immunohistochemistry data suggest that carbonic anhydrase XII is expressed in the terminal bud of human respiratory epithelial cells (Lee et al. 2016). Mutations in carbonic anhydrase XII have been implicated in lung disease (Lee et al. 2016), but the mechanism remains unclear. Regardless, we speculate that carbonic anhydrase XII is not likely to regulate pH<sub>ASL</sub> because

carbonic anhydrase XII's subcellular localization suggests lack of direct contact with the ASL (Lee et al. 2016). In addition, carbonic anhydrase XII has low enzymatic activity that is 15% of carbonic anhydrase IV (Tureci et al. 1998). This notion is consistent with our data that the ASL of large airways has minimal carbonic anhydrase activity.

Respiratory rates vary in health and disease. The average respiratory rate in healthy resting humans is approximately 15–20 bpm. During exercise or during respiratory

failure, the respiratory rate can increase to 60 bpm. Our modeling data suggest that these changes would have minimal effect on  $\text{pH}_{\text{ASL}}$ . In patients with obstructive lung disease, such as asthma or chronic obstructive pulmonary disease (COPD), the expiratory phase is increased. Our data suggest that the decrease in  $\text{pH}_{\text{ASL}}$  due to the I:E ratio would be modest ( $\sim 0.1$  pH units). Inverse ratio ventilation is routinely used in intensive care units to ventilate patients that do not respond to conventional mechanical ventilation (Cole et al. 1984). Our model showed that an I:E ratio of 4:1 would significantly increase  $\text{pH}_{\text{ASL}}$ .

Why would the airway evolve to lack carbonic anhydrase in the ASL? Based on the Henderson-Hasselbalch equation, fluctuations in  $\text{CO}_2$  from 5% to 0.04% in the presence of 20 mM  $\text{HCO}_3^-$  and carbonic anhydrase could result in a pH change from 7.4 to 9.5. Thus, we speculate that the large airway evolved to have minimal carbonic anhydrase activity and nonbicarbonate buffering capacity to mitigate extreme alkaline shifts in  $\text{pH}_{\text{ASL}}$ .

Additionally, airway lacking carbonic anhydrase may have been selected for based upon beneficial pH-dependent enzymes, such as  $\beta$ -defensin-3 and LL-37 (Abou Alaiwa et al. 2014b). Simulations with carbonic anhydrase (Fig. 3A) have a pH oscillation magnitude of  $\sim 0.1$  unit, which could affect enzyme function. For example, consider an enzyme whose function is defined by a titration curve and  $\text{pK}_a$  equals that of the midpoint of the ASL pH oscillation. Then an oscillation of 0.1 (midpoint  $\pm 0.05$  units) would undergo a  $\sim 11.5\%$  change in protonation from peak to peak. In contrast, a pH change of 0.01 (midpoint  $\pm 0.005$  units), as seen in the absence of exogenous carbonic anhydrase (Fig. 3A), would change the theoretical enzyme protonation state by only  $\sim 1\%$  from peak to peak.

Our study also has limitations. First, we did not remove mucus from cultured epithelia. However, mucus is present in vivo and it is proposed to have only a minor role in buffering at the physiological  $\text{pH}_{\text{ASL}}$  values (Holma and Hegg 1989; Kim et al. 2014). During inhalation, where  $\text{CO}_2$  is low, pH is  $\sim 7.8$ , and therefore  $\text{HCO}_3^-$  is  $\sim 500 \mu\text{mol/L}$ , high concentrations of mucus may provide additional buffering capacity through its free cysteines ( $\text{pK}_a \sim 8.5$ ). Second, the model does not take into consideration submucosal glands that are present in an intact lung. However, acidic submucosal gland secretions (Widdicombe and Wine 2015) are anticipated to be similar to the perturbations associated with acid loading modeled in Figure 3. Third, prolonged changes in apical  $\text{CO}_2$ , from which our rates were obtained, may have a modest effect on the intracellular pH under the apical membrane. Fourth, additional buffering capacity may be excreted in vivo by the epithelium or the presence of other cell

types (e.g., leukocytes during disease). None of these limitations would affect our conclusion that  $\text{pH}_{\text{ASL}}$  changes were slow in response to  $\text{CO}_2$  due to nominal carbonic anhydrase activity.

In summary,  $\text{pH}_{\text{ASL}}$  oscillations in the respiratory cycle are small, avoiding extreme pH values that may be caustic to airway epithelia. However, given that  $\text{pH}_{\text{ASL}}$  is more acidic in many airway diseases (e.g., in CF due to decreased  $\text{HCO}_3^-$  secretions) and contributes to host defense defects, delivering or increasing ASL carbonic anhydrase may be useful to increase  $\text{pH}_{\text{ASL}}$ . An increase in carbonic anhydrase activity would increase the pH oscillation magnitude, achieving more alkaline pH values for a fraction of the respiratory cycle.

## Conflict of Interest

The University of Iowa has licensed CF pigs to Exemplar Genetics, and MJW receives royalties from the license. No conflicts of interest, financial, or otherwise are declared by the other authors.

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## References

- Abou Alaiwa, M. H., A. M. Beer, A. A. Pezzulo, J. L. Launspach, R. A. Horan, D. A. Stoltz, et al. 2014a. Neonates with cystic fibrosis have a reduced nasal liquid pH; a small pilot study. *J. Cyst. Fibros.* 13:373–377.
- Abou Alaiwa, M. H., L. R. Reznikov, N. D. Gansemer, K. A. Sheets, A. R. Horswill, D. A. Stoltz, et al. 2014b. pH modulates the activity and synergism of the airway surface liquid antimicrobials beta-defensin-3 and LL-37. *Proc. Natl. Acad. Sci. U.S.A.* 111:18703–18708.
- Blank, P. S., H. S. Silverman, O. Y. Chung, B. A. Hogue, M. D. Stern, R. G. Hansford, et al. 1992. Cytosolic pH measurements in single cardiac myocytes using carboxy-seminaphthorhodafluor-1. *Am. J. Physiol.* 263:H276–H284.
- Boyarsky, G., M. B. Ganz, R. B. Sterzel, and W. F. Boron. 1988. pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of  $\text{HCO}_3^-$ . *Am. J. Physiol.* 255:C844–C856.
- Buckler, K. J., and R. D. Vaughan-Jones. 1990. Application of a new pH-sensitive fluoroprobe (carboxy-SNARF-1) for intracellular pH measurement in small, isolated cells. *Pflugers Arch.* 417:234–239.



- Candiano, G., M. Bruschi, N. Pedemonte, L. Musante, R. Ravazzolo, S. Liberatori, et al. 2007. Proteomic analysis of the airway surface liquid: modulation by proinflammatory cytokines. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292: L185–L198.
- Ch'en, F. F., E. Dilworth, P. Swietach, R. S. Goddard, and R. D. Vaughan-Jones. 2003. Temperature dependence of  $\text{Na}^+$ - $\text{H}^+$  exchange,  $\text{Na}^+$ - $\text{HCO}_3^-$  co-transport, intracellular buffering and intracellular pH in guinea-pig ventricular myocytes. *J. Physiol.* 552:715–726.
- Chen, J.-H., D. A. Stoltz, P. H. Karp, S. E. Ernst, A. A. Pezzulo, T. O. Moninger, et al. 2010a. Loss of anion transport without increased sodium absorption characterize newborn porcine cystic fibrosis airway epithelia. *Cell* 143:911–923.
- Chen, Y. C., A. Ostafin, and H. Mizukami. 2010b. Synthesis and characterization of pH sensitive carboxySNARF-1 nanoreactors. *Nanotechnology* 21:215503.
- Cheng, L., O. Ivanova, H. H. Fan, and M. C. Khoo. 2010. An integrative model of respiratory and cardiovascular control in sleep-disordered breathing. *Respir. Physiol. Neurobiol.* 174:4–28.
- Chu, C. S., B. C. Trapnell, S. M. Curristin, G. R. Cutting, and R. G. Crystal. 1992. Extensive posttranscriptional deletion of the coding sequences for part of nucleotide-binding fold 1 in respiratory epithelial mRNA transcripts of the cystic fibrosis transmembrane conductance regulator gene is not associated with the clinical manifestations of cystic fibrosis. *J. Clin. Invest.* 90:785–790.
- Clary-Meinesz, C., J. Mouroux, J. Cosson, P. Huitorel, and B. Blaive. 1998. Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *Eur. Respir. J.* 11:330–333.
- Coakley, R. D., B. R. Grubb, A. M. Paradiso, J. T. Gatzky, L. G. Johnson, S. M. Kreda, et al. 2003. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 100:16083–16088.
- Cochrane, G. M., C. G. Newstead, R. V. Nowell, P. Openshaw, and C. B. Wolff. 1982. The rate of rise of alveolar carbon dioxide pressure during expiration in man. *J. Physiol.* 333:17–27.
- Cole, A. G., S. F. Weller, and M. K. Sykes. 1984. Inverse ratio ventilation compared with PEEP in adult respiratory failure. *Intensive Care Med.* 10:227–232.
- Fischer, H., and J. H. Widdicombe. 2006. Mechanisms of acid and base secretion by the airway epithelium. *J. Membr. Biol.* 211:139–150.
- Garland, A. L., W. G. Walton, R. D. Coakley, C. D. Tan, R. C. Gilmore, C. A. Hobbs, et al. 2013. Molecular basis for pH-dependent mucosal dehydration in cystic fibrosis airways. *Proc. Natl. Acad. Sci. U.S.A.* 110:15973–15978.
- Gessner, C., S. Hammerschmidt, H. Kuhn, H. J. Seyfarth, U. Sack, L. Engelmann, et al. 2003. Exhaled breath condensate acidification in acute lung injury. *Respir. Med.* 97:1188–1194.
- Holma, B., and P. O. Hegg. 1989. pH- and protein-dependent buffer capacity and viscosity of respiratory mucus. Their interrelationships and influence on health. *Sci. Total Environ.* 84:71–82.
- Hug, M. J., T. Tamada, and R. J. Bridges. 2003. CFTR and bicarbonate secretion by epithelial cells. *News Physiol. Sci.* 18:38–42.
- Hunt, J. F., K. Fang, R. Malik, A. Snyder, N. Malhotra, T. A. Platts-Mills, et al. 2000. Endogenous airway acidification. Implications for asthma pathophysiology. *Am. J. Respir. Crit. Care Med.* 161:694–699.
- Imtaiyaz Hassan, M., B. Shajee, A. Waheed, F. Ahmad, and W. S. Sly. 2013. Structure, function and applications of carbonic anhydrase isozymes. *Bioorg. Med. Chem.* 21:1570–1582.
- Itani, O. A., J. H. Chen, P. H. Karp, S. Ernst, S. Keshavjee, K. Parekh, et al. 2011. Human cystic fibrosis airway epithelia have reduced  $\text{Cl}^-$  conductance but not increased  $\text{Na}^+$  conductance. *Proc. Natl. Acad. Sci. U.S.A.* 108:10260–10265.
- Jayaraman, S., Y. Song, L. Vetrivel, L. Shankar, and A. S. Verkman. 2001. Noninvasive in vivo fluorescence measurement of airway-surface liquid depth, salt concentration, and pH. *J. Clin. Invest.* 107:317–324.
- Kim, D., J. Liao, and J. W. Hanrahan. 2014. The buffer capacity of airway epithelial secretions. *Front. Physiol.* 5:188.
- Kostikas, K., G. Papatheodorou, K. Ganas, K. Psathakis, P. Panagou, and S. Loukides. 2002. pH in expired breath condensate of patients with inflammatory airway diseases. *Am. J. Respir. Crit. Care Med.* 165:1364–1370.
- Lee, M., B. Vecchio-Pagan, N. Sharma, A. Waheed, X. Li, K. S. Raraigh, et al. 2016. Loss of carbonic anhydrase XII function in individuals with elevated sweat chloride concentration and pulmonary airway disease. *Hum. Mol. Genet.* 25:1923–1933.
- Leinonen, J. S., K. A. Saari, J. M. Seppanen, H. M. Myllyla, and H. J. Rajaniemi. 2004. Immunohistochemical demonstration of carbonic anhydrase isoenzyme VI (CA VI) expression in rat lower airways and lung. *J. Histochem. Cytochem.* 52:1107–1112.
- Li, X., X. X. Tang, L. G. Vargas Buonfiglio, A. P. Comellas, I. M. Thornell, S. Ramachandran, et al. 2016. Electrolyte transport properties in distal small airways from cystic fibrosis pigs with implications for host defense. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 310:L670–L679.
- Liu, X., V. Ory, S. Chapman, H. Yuan, C. Albanese, B. Kallakury, et al. 2012. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *Am. J. Pathol.* 180:599–607.
- Livermore, S., Y. Zhou, J. Pan, H. Yeger, C. A. Nurse, and E. Cutz. 2015. Pulmonary neuroepithelial bodies are polymodal airway sensors: evidence for  $\text{CO}_2/\text{H}^+$  sensing. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 308:L807–L815.

- McShane, D., J. C. Davies, M. G. Davies, A. Bush, D. M. Geddes, and E. W. Alton. 2003. Airway surface pH in subjects with cystic fibrosis. *Eur. Respir. J.* 21:37–42.
- Pezzulo, A. A., X. X. Tang, M. J. Hoegger, M. H. Alaiwa, S. Ramachandran, T. O. Moninger, et al. 2012. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 487:109–113.
- Pinard, M. A., B. Mahon, and R. McKenna. 2015. Probing the surface of human carbonic anhydrase for clues towards the design of isoform specific inhibitors. *Biomed. Res. Int.* 2015:453543.
- Polosukhin, V. V., W. E. Lawson, A. P. Milstone, S. M. Egunova, A. G. Kulipanov, S. G. Tchuvakin, et al. 2007. Association of progressive structural changes in the bronchial epithelium with subepithelial fibrous remodeling: a potential role for hypoxia. *Virchows Arch.* 451:793–803.
- Polosukhin, V. V., J. M. Cates, W. E. Lawson, A. P. Milstone, A. G. Matafonov, P. P. Massion, et al. 2011. Hypoxia-inducible factor-1 signalling promotes goblet cell hyperplasia in airway epithelium. *J. Pathol.* 224:203–211.
- Quinton, P. M. 2008. Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis. *Lancet* 372:415–417.
- Rheinwald, J. G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–343.
- Shah, V. S., D. K. Meyerholz, X. X. Tang, L. Reznikov, M. Abou Alaiwa, S. E. Ernst, et al. 2016. Airway acidification initiates host defense abnormalities in cystic fibrosis mice. *Science* 351:503–507.
- Smith, J. J., and M. J. Welsh. 1992. cAMP stimulates bicarbonate secretion across normal, but not cystic fibrosis airway epithelia. *J. Clin. Invest.* 89:1148–1153.
- Smith, J. J., and M. J. Welsh. 1993. Fluid and electrolyte transport by cultured human airway epithelia. *J. Clin. Invest.* 91:1590–1597.
- Song, Y., D. Salinas, D. W. Nielson, and A. S. Verkman. 2006. Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. *Am. J. Physiol. Cell Physiol.* 290:C741–C749.
- Supryniewicz, F. A., G. Upadhyay, E. Krawczyk, S. C. Kramer, J. D. Hebert, X. Liu, et al. 2012. Conditionally reprogrammed cells represent a stem-like state of adult epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 109:20035–20040.
- Supuran, C. T. 2008. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discov.* 7:168–181.
- Supuran, C. T., A. Di Fiore, and G. De Simone. 2008. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. *Expert Opin. Emerg. Drugs* 13:383–392.
- Tang, X. X., L. S. Ostedgaard, M. J. Hoegger, T. O. Moninger, P. H. Karp, J. D. McMenimen, et al. 2016. Acidic pH increases airway surface liquid viscosity in cystic fibrosis. *J. Clin. Invest.* 126:879–891.
- Thatcher, B. J., A. E. Doherty, E. Orvisky, B. M. Martin, and R. I. Henkin. 1998. Gustin from human parotid saliva is carbonic anhydrase VI. *Biochem. Biophys. Res. Comm.* 250:635–641.
- Trapnell, B. C., C. S. Chu, P. K. Paakko, T. C. Banks, K. Yoshimura, V. J. Ferrans, et al. 1991. Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* 88:6565–6569.
- Trevani, A. S., G. Andonegui, M. Giordano, D. H. Lopez, R. Gamberale, F. Minucci, et al. 1999. Extracellular acidification induces human neutrophil activation. *J. Immunol.* 162:4849–4857.
- Tureci, O., U. Sahin, E. Vollmar, S. Siemer, E. Gottert, G. Seitz, et al. 1998. Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. *Proc. Natl. Acad. Sci. U.S.A.* 95:7608–7613.
- Walsh, B. K., D. J. Mackey, T. Pajewski, Y. Yu, B. M. Gaston, and J. F. Hunt. 2006. Exhaled-breath condensate pH can be safely and continuously monitored in mechanically ventilated patients. *Respir. Care* 51:1125–1131.
- Westerblad, H., J. D. Bruton, and J. Lannergren. 1997. The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. *J. Physiol.* 500(Pt 1):193–204.
- Widdicombe, J. H., and J. J. Wine. 2015. Airway Gland Structure and Function. *Physiol. Rev.* 95:1241–1319.
- Zabner, J., S. C. Wadsworth, A. E. Smith, and M. J. Welsh. 1996. Adenovirus-mediated generation of cAMP-stimulated Cl<sup>-</sup> transport in cystic fibrosis airway epithelia in vitro: effect of promoter and administration method. *Gene Ther.* 3:458–465.