Ion transport and regulation of respiratory tract fluid output in dogs

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Chen, Ben T., and Donovan B. Yeates. Ion transport and regulation of respiratory tract fluid output in dogs. J Appl Physiol 90: 821–831, 2001.—To investigate the regulation of respiratory tract fluid output (RTFO), we collected the RTFO in an anesthetized canine model after a series of pharmacological interventions (inhibition of Na<sup>+</sup>-K<sup>+</sup>-ATPase or Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> cotransporter, 250 μl) and physiological challenges (ionic and/or osmotic perturbation in airway luminal water fluxes or by stimulation of the fluxes of ions and associated water fluxes from the submucosa into the airway lumen (14, 15). Specifically, Phillips and colleagues (14) showed that acetylstrophanthidin (a Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup>-ATPase inhibitor), administered to the basolateral side of the tracheal membrane in vitro, decreased the luminal-to-basolateral water flux. We hypothesized that this decrease in fluid absorption was exhibited in vivo as an increase in respiratory tract fluid output (RTFO).

Our laboratory has shown that furosemide (a Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> cotransporter inhibitor), administered to the airway lumen, caused a marked increase in bronchial mucociliary clearance in baboons (26). Because these results were in contradiction with the generally held paradigm that basolateral-to-luminal water flux is coupled with Cl<sup>−</sup> flux, it was not immediately predictable that inhibition of the basolateral Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup>-cotransporter would have resulted in this response. In addition, furosemide has been suggested to have several mechanisms of action that appear to be independent from its action on the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup>-cotransporter (10, 12, 25). To determine whether this response was specific to the inhibition of the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup>-cotransporter, bumetanide, a more specific Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup>-cotransporter inhibitor (40 times more potent than furosemide) was administered to the luminal side of the airway epithelium by aerosol, as well as to the basolateral side by intravenous infusion.

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A hyperosmotic stress and/or cell shrinkage induces upregulation of the Na\(^+-K^+\)-2Cl\(^-\) cotransporter (11). Under the influence of mild airway dehydration, we questioned whether the administration of bumetanide to the airway lumen would enhance or attenuate the observed increase in RTFO. The roles of the epithelial Na\(^+-K^+\)-ATPase to the responses of the mucosa resulting from ionic and osmotic stresses in vivo were unknown. Because fluxes of ions could induce associated water flux across airway epithelium under near-isosmotic condition (2), we questioned whether the inhibition of the Na\(^+-K^+\)-ATPase or the Na\(^+-K^+\)-2Cl\(^-\) cotransporter would increase or decrease the observed responses compared with the unchallenged airways.

We used an anesthetized mechanically ventilated dog model in which the inspired humidity was controlled and the respiratory tract fluid was collected via a catheter having ports that were positioned just caudal to the posterior commissure of the larynx. To determine the response of the RTFO to the inhibition of the Na\(^+-K^+\)-ATPase and Na\(^+-K^+\)-2Cl\(^-\) cotransporter, acetylstrophanthidin and bumetanide were delivered by aerosol to the tracheal lumen. To determine whether airway dehydration would modify these responses, dogs were ventilated with dry air in the presence of these agents. To determine whether decreasing the luminal [Na\(^+\)] and [Cl\(^-\)] under near isosmotic conditions would induce an increase in RTFO, the trachea was challenged with 250 \(\mu\)l of 250 mosmol/kg H\(_2\)O ion-free mannitol solution. The roles of the Na\(^+-K^+\)-ATPase and the Na\(^+-K^+\)-2Cl\(^-\) cotransporter in these responses were evaluated. To determine the extent to which a hyperosmotic challenge of similar volume would further increase the RTFO, the trachea was challenged with 550 mosmol/kg H\(_2\)O ion-free mannitol. To determine any differential responses to luminal and basolateral bumetanide, bumetanide was independently administered by aerosol and by intravenous infusion.

**MATERIALS AND METHODS**

**System Design**

**Humidity-controlled ventilation system.** A system was designed and constructed to enable positive-pressure ventilation of the dog along with precisely controlled inspired humidity. This system is shown schematically in Fig. 1. Compressed air was divided into two separated streams. One stream was not humidified (~3.0 mg/l, i.e., ~8% at 33°C), and the other stream was humidified using a humidifier (Bird, Palm Springs, CA) to 24–27 mg/l (i.e., 68–76% relative humidity at 33°C). The selection of either dry air or humid air ventilation was achieved by simultaneously opening and closing ball valves in the dry air and humid air conduits. In this way, instantaneous changes from humid to dry air and back to humid air were possible. Two normally open solenoid valves (model 8267C23, Automatic Switch, Florham Park, NJ) were used to vent the humidified or dry air when not being delivered to the dog. To avoid any inadvertent barotrauma, a pressure-relief safety valve ensured that the pressure of the inspired air did not exceed 25 cmH\(_2\)O. The inspiration and expiration of the dog were regulated by a series of normally closed solenoid valves (model 8267C19, Automatic Switch). Inspiratory flow was initiated by a logic valve controller and detected with a pneumotach (Fleisch no. 1) and a pressure transducer (model MP45-14, Validyne). A second pneumotach attached to the endotracheal tube was used to determine the actual ventilatory parameters of the dog. The ventilating system was adjusted to give a measured tidal volume of 160 ml at a respiratory rate of 20 breaths/min in each dog. A mixing chamber (2 liter) in the humidified inspiratory conduit was equipped with a humidity probe (model HM136, Vaisala, Helsinki, Finland) to monitor the humidity of the inspired humidified air. This mixing chamber as well as the intervening tubing were maintained at 30 ± 0.5°C using flexible heating tapes (Omegalux) together with two temperature controllers (model CN8500, Omega). The

![Fig. 1. Schematic of the humidity-controlled ventilation system. Compressed air was conditioned as shown before delivery to the dog. Solid lines, pathway of humid air; dashed lines, pathway of dry air.](http://jap.physiology.org/)
expired tubing was maintained at ~45°C using heating tapes (Omegalux) to prevent condensation. A chamber (0.6 liter) in the expiratory conduit was also maintained at ~45°C using a water bath. A humidity probe (model HM136, Vaisala) inserted into this chamber was used to measure the humidity of the expired air. At 45°C, the relative humidity of the expired air ranged between 60 and 80%, which is the most sensitive and reliable range of the humidity probe. The humidity data from the probes were processed at 0.2 Hz by a humidity processor (model HM136A, Vaisala) and stored in a personal computer.

**Modified endotracheal tube with a suction catheter.** A double-lumen endobronchial tube (32-Fr, Mallinckrodt Medical, St. Louis, MO) was modified to facilitate the quantitative collection of the RTFO while forming an airtight seal to enable the use of positive-pressure ventilation (Fig. 2). The endobronchial tube was shortened, and both proximal and distal cuffs were removed. Two replacement cuffs (Mallinckrodt Medical) were placed adjacent at the tip of the tube. The proximal cuff was used to form an airtight seal in the oropharynx, and the distal cuff was used to secure the position of the endotracheal tube. The distal cuff was confined on the dorsal side of the tube such that mucus being transported to the interarytenoid groove (or posterior commissure) was not interrupted. A catheter (polytetrafluoroethylene, 1.29-mm ID and 1.90-mm OD) was passed inside the lower lumen of the tube. It protruded obliquely through the tube between the cuffs to the tip of the tube where it was secured. The catheter was sealed at the tip, and two holes were drilled on lateral sides of the catheter. The position of the secretion collection ports of the catheter was secured just caudal to the interarytenoid groove to ensure that the ports were kept in close juxtaposition to the epithelium. The other end of the catheter was connected to a preweighed sampling vial (1.5-ml centrifuge tube, Beckman) maintained at 37°C in an incubator. Suction was applied intermittently to the sampling vial such that respiratory tract fluid in the interarytenoid groove was transported via the catheter to the vial.

**Animal Preparation**

Male beagle dogs (Covance), aged 1–2 yr and weighing 12–16 kg, were used. The animals were housed in the Veterans Affairs Chicago Health Care System (West Side, Chicago, IL). The National Research Council’s Guide for the Care and Use of Laboratory Animals was followed throughout this study.

Each dog was fasted overnight but was allowed water ad libitum. It was anesthetized (7 mg/kg) with intravenous propofol (Zeneca Pharmaceuticals, Wilmington, DE) and secured in the supine position. Anesthesia was maintained by a continuous infusion of propofol at the rate of 800–1,000 μg·kg⁻¹·min⁻¹ until dog’s jaw relaxed. The modified double-lumen endotracheal tube with a suction catheter (28-Fr, Mallinckrodt Medical) described in Modified endotracheal tube with a suction catheter was inserted into the trachea under direct laryngoscopic visualization. The collection ports of the catheter were placed just caudal to the interarytenoid groove (or posterior commissure) to collect respiratory tract fluid. After the intubation, intravenous etomidate (Abbott Laboratories, North Chicago, IL) was administered at the rate of 5–10 μg·kg⁻¹·min⁻¹ and the propofol was reduced to 400–500 μg·kg⁻¹·min⁻¹. Etomidate sensitizes the carotid body, resulting an improved blood chemistry. Propofol, a respiratory depressant, was used to suppress the myotonic and clonic effects of etomidate (30). The combination of these two short-acting hypnotics allowed the maintenance of the pH close to 7.3 and the Pco₂ < 45 Torr in each dog. An arterial catheter (20 gauge, 2 in., Becton Dickinson, Sandy, UT) was placed in a femoral artery subcutaneously to monitor blood pressure and to withdraw arterial blood samples. A microspray catheter (Penn-Century, Philadelphia, PA), 40 cm in length and 1 mm in diameter, was inserted via an Opti-Port (Mallinckrodt Medical) through the ventral (inhalation) lumen of the endotracheal tube such that the atomizing nozzle at the end of the catheter protruded ~5 mm past the distal tip of the endotracheal tube (inside the trachea). The Opti-Port was sealed with the catheter in place using a custom-designed sleeve. The endotracheal tube was connected to the ventilation system. A stainless steel syringe (Penn-Century) attached to the microspray catheter was used to pressurize the challenging agent through the catheter into the tracheal lumen. A 15-W heat lamp was used to prevent any condensation on the parts of the endotracheal tube and fittings exposed to room temperature. Water-heated underpads and a blanket were used to maintain the rectal temperature at 38 ± 0.5°C. After the preparation procedure (~40–50 min), the animal was stabilized under mechanical ventilation at the rate of 160 ml/breath and 20 breaths/min using humid air (~72% at 33°C) without an addition of CO₂ for 20 min. Other physiological monitoring included electrocardiogram, hemoglobin oxygen saturation by a pulse oximeter, and rectal temperature (SpaceLabs Medical).

**Protocol**

Each dog underwent three studies: a humid air study, a dry air study, and an intravenous bumetanide study, as shown in Fig. 3, A, B, and C, respectively. The humid air study and the dry air study each consisted of four experiments in each of six beagle dogs. The intravenous bumetanide study consisted of two experiments in each of eight beagle dogs. There was a 7-day period between each experiment for each dog. The sequence of the experiments was randomized.

In the humid air study (Fig. 3A), each dog was ventilated with humid air (~72% at 33°C) at the rate of 20 breaths/min and 160 ml/breath throughout the experiment. During the baseline stabilization period, the RTFO was collected over 1-min periods every 6 min (3 times) to minimize the effect of the stimulation of secretions due to intubation and initial instrumentation. The osmolalities and [Na⁺] and [Cl⁻] in
these initial samples of the intravenous bumetanide study were measured and assumed to be indicative of those in the other two studies in which they were not measured. The RTFOs of these samples are not reported. During the 1-min collection periods, intermittent suction was applied to the collecting catheter to minimize any evaporation or condensation of fluid to or from the collected sample. Immediately after the fifth RTFO collection, the trachea was challenged with a 250-μl aerosol of 250 mosmol/kgH2O ion-free mannitol. The osmolality of the mannitol solution was verified using a vapor pressure osmometer (model 5520, Wescor). The mannitol solution contained 10,000-molecular-weight rhodamine B dextran (1 mg/ml) as a tracer of the percentage of mannitol solution deposited on the airway surface liquid. An arterial blood sample was also taken for analysis of blood gases and pH. The RTFO was collected a total of five times after the mannitol challenge using the same collection protocol above.

In the dry air study (Fig. 3B), the same protocol was followed with the exception that dry air (∼8% at 33°C) was administered for 30 min beginning at the same time as the 0.9% saline administration or pharmacological intervention. Humid air (∼72% at 33°C) ventilation using the same ventilatory parameters was resumed at the same time as each 250 mosmol/kgH2O ion-free mannitol challenge. Using this design, the volume of the collected RTFO was sufficient for analysis and thus the data can be used for comparisons between sets of experiments and studies.

The intravenous bumetanide study (Fig. 3C) was composed of a sham experiment in which 0.9% saline was administered intravenously and an experiment in which bumetanide (0.04 mg/kg) was administered intravenously. The experimental procedures were similar to those contained within the humid air study in which the dogs were challenged with 250 mosmol/kgH2O ion-free mannitol. However, in this study, this osmotic challenge was replaced with a challenge of 550 mosmol/kgH2O ion-free mannitol solution. Analysis of the RTFO

The collected samples of RTFO were weighed. The RTFO as well as the blood samples collected 5 min after the challenge with mannitol (containing fluorescent dextran) were centrifuged at 16,000 rpm at 4°C for 30 min to separate any mucus gel from the RTFO and hematocrit of the blood samples. Only the supernatant of these samples was used. This enabled homogeneous samples to be assayed and facilitated the quantitative retrieval of the samples after measurements. In addition, it minimized any artifact caused by mucus gel sticking to the microelectrodes. The percentage of fluorescent dextran in the supernatant of the RTFO collected 5 min after the mannitol challenge was determined by measuring the fluorescent intensity of the rhodamine B dextran immediately after the experiment. The remaining supernatants of the RTFO and the blood sample were sealed and stored at −70°C. After completion of the studies (<6 mo), the [Na⁺], [Cl⁻], and osmolalities of the supernatant of the RTFO and the blood samples collected 5 min after the mannitol challenge were measured.

Determination of the percentage of fluorescent dextran in the supernatant of the RTFO. The supernatant of the first RTFO collected 5 min after the mannitol challenge was assayed for rhodamine B dextran (1 mg/ml). The supernatant of the RTFO and purified water (background) were added (5 μl) to individual wells on a 96-well (8 × 12) plate (Nunc). Each sample was diluted to 60 μl with purified water (Milli-Qplus, Millipore). The fluorescence due to rhodamine B dextran was measured using Cytofluor II (Biosearch). Excitation and emission filters were set at 530 ± 13 and 620 ± 20 nm, respectively. The percentage of the fluorescent dextran in the supernatant of the RTFO, reflective of the concentration of the deposited mannitol, was calculated from the fluorescent intensity of the supernatant of the RTFO with background subtraction divided by the fluorescent intensity of the initial
mannotol-rhodamine B dextran solution with background subtraction. The partitioning of the dextran between the supernatant and the mucus is unknown. We assumed that the dextran distributed homogeneously between the sol and gel phases of the airway surface liquid.

**Measurement of free [Cl⁻], [Na⁺], and osmolality.** The supernatant of the RTFO and blood samples was stored at −70°C before analysis. The RTFO collected after intubation were obtained using the same six beagle dogs in a separate study using the same preparation procedure (2). All the samples were measured at the same time after the completion of the studies (<6 mo). A Na⁺-selective glass electrode (MI-420, Microelectrodes, Bedford, NH) and a solid-state electrode for Cl⁻ (MI-200, Microelectrodes) were coupled with a double-junction reference electrode (MI-403, Microelectrodes). This reference electrode was composed of an internal glass reference barrel that contained a wire coated with silver chloride equilibrated with a KCl solution (3 M) and an outer reference chamber that was filled with 0.9% saline. In this arrangement, the diffusion of the KCl solution into the microliter sample was minimized. These electrodes were calibrated at room temperature using NaCl solutions of 10, 100, and 400 mM, both before the analysis and immediately after the analysis. The results were fitted to a semilog plot to yield the slope and the intercept: 59.7 ± 0.1 mV/mM and −178.8 ± 0.6 mV for the sodium electrode, −48.7 ± 0.8 mV for the chloride electrode. The osmolality of each sample (8 μl) was measured using a vapor pressure osmometer (model 5520, Wescor) after the free [Cl⁻] and [Na⁺] measurements.

**Statistics**

Results are presented as means ± SE. Statistical significance (P value) of the osmolality, the free-ion concentration, and the dextran percent were calculated using one-way analysis of variance or the t-test. If the t-test failed in normality or variance test, the Mann-Whitney rank-sum test was used. For RTFO data, the P value was calculated using two-way repeated-measures analysis of variance. Bonferroni’s method was used for comparisons. Statistical significance was considered if the P value was <0.05.

**RESULTS**

**Expired Humidity**

When humid air of 24–27 mg/l at 33°C (i.e., 68–76% relative humidity) was delivered to the dog, the humidity of the expired air remained remarkably constant over the 65-min experimental period as shown in Fig. 3, A and C. When dry air (~3 mg/l, 33°C) was delivered to the dog, the expired absolute humidity decreased in an exponential-like manner from 41.5 mg/l to reach 36 mg/l after 30 min (Fig. 3B). A rapid recovery was observed when ventilation with humid air (~72% relative humidity) at 33°C was resumed. The expired absolute humidity reached 41 mg/l by the end of the experiment. Small increases in this expired humidity were observed when an aqueous aerosol was delivered to the trachea, and small decreases were observed when a negative pressure was applied to the suction catheter.

**RTFO**

**Humid air study.** As shown in Fig. 4A, administration of 250 μl of 0.9% saline caused an increase in the RTFO at 5 min compared with the control, with no differences between the 0.9% saline (sham) and the control observed thereafter. In the initial 5 min after the pharmacological interventions, the RTFO was 56.1 ± 16.3 mg after acetylstrophanthidin aerosol (Fig. 4B) and 46.9 ± 9.4 mg after bumetanide aerosol (Fig. 4C) compared with 34 ± 10.5 mg after saline (sham) aerosol (Fig. 4, A, B, or C). At this early time point, these increases were not significant. As noted, the RTFO in the subsequent two collections after the saline (sham) administration (Fig. 4A) returned to the control values (Fig. 4A). The pharmacological responses of acetylstrophanthidin and bumetanide, shown in Fig. 4, B and C, respectively, are now clearly evident. Both agents caused increases in the RTFO at 12 and 18 min, which were about threefold higher than the respective RTFO in the sham experiment (P < 0.05). Twenty-four minutes after the interventions, the increases in RTFO due to acetylstrophanthidin were still apparent (P < 0.05), whereas there was no further indication of any increase in RTFO due to bumetanide.
Compared with the five RTFOs collected immediately after the administration of aerosolized saline in the sham experiment (Fig. 4A), there were increases in the RTFO collected after mannitol challenge at 6, 12, and 18 min (Fig. 4A; \(P < 0.05\)). The interventions of acetylstrophanthidin aerosol or bumetanide aerosol 30 min before these 250 mosmol/kgH\(_2\)O mannitol challenges did not result in any significant differences in RTFO after these mannitol challenges (Fig. 4, B and C, respectively) compared with in the sham study (Fig. 4, B or C).

**Dry air study.** As shown in Fig. 5A, administration of 250 \(\mu\)l of 0.9% saline caused an initial increase in RTFO, compared with the control (Fig. 5A), with no differences between the 0.9% saline and the controls observed thereafter. There was a trend for RTFO in the control experiment to decrease from 5.8 ± 1.5 mg collected at 5 min of dry air ventilation to 2.4 ± 1.0 mg collected at 30 min of dry air ventilation. Compared with administration of saline (sham) aerosol, intervention by acetylstrophanthidin aerosol did not result in a significant increase in RTFO until the 24- and 30-min collections (Fig. 5B). Interestingly, administration of bumetanide aerosol resulted in significant increases in the RTFO at 6, 12, and 18 min, with no increases being discernable thereafter (Fig. 5C). When the dogs were ventilated with dry air, the RTFOs collected after administration of saline aerosol (Fig. 5A vs. Fig. 4A) and acetylstrophanthidin aerosol (Fig. 5B vs. Fig. 4B) were significantly less than when they were ventilated with humid air, as expected, with the exception of the RTFO at 6 and 12 min after intervention of bumetanide aerosol (Fig. 5C vs. 4C). After the resumption of humid air ventilation, it is notable that RTFO in the control experiments recovered from a low of 1.7 ± 0.6 to 5.0 ± 1.5 mg (Fig. 5A). The 250 mosmol/kgH\(_2\)O mannitol challenge caused similar increases in RTFO as the study in which the humid air was delivered throughout the experiments. The prior intervention of either acetylstrophanthidin or bumetanide in combination to dry air ventilation did not result in any significant changes in RTFO, compared with mannitol without any prior intervention (Fig. 5, B and C, respectively).

**Intravenous bumetanide study.** The increase in RTFO after aerosolized bumetanide (Figs. 4C and 5C) was not observed after 0.04 mg/kg of intravenous bumetanide (Fig. 6). Compared with the intravenous saline experiment [sham; in which the RTFOs were 8.3 ± 1.5, 8.8 ± 2.1, and 7.1 ± 1.7 mg (Fig. 6)], the RTFOs in the intravenous bumetanide experiment were 7.5 ± 1.4, 4.0 ± 0.8, and 3.7 ± 0.5 mg (Fig. 6), with the RTFO at 12 and 18 min reaching statistical significance. The increase in RTFO due to the 550 mosmol/kgH\(_2\)O mannitol challenge (Fig. 6) was understandably larger than for the 250 mosmol/kgH\(_2\)O challenge (Fig. 4A; \(P < 0.05\)). As with the 250 mosmol/kgH\(_2\)O challenge, the response decreased over the subsequent 24 min. There appeared to be a nonsignificant trend for intravenous bumetanide to cause a slight decrease in the response to the 550 mosmol/kgH\(_2\)O mannitol challenge in each of the five samples (Fig. 6).
Osmotic stress. As shown in Fig. 7, the osmolality of 282 ± 7 mosmol/kgH₂O in the RTFO collected during the stabilization period immediately after intubation before the intravenous bumetanide study was similar to the 298 ± 2 mosmol/kgH₂O of the blood samples. The increases in osmolality due to dry air ventilation can be appreciated by comparing the osmolality in the sham study. The 30-min dry air ventilation caused an increase in the osmolality in the RTFO from 314 ± 6 mosmol/kgH₂O 5 min after either the 250 mosmol/kgH₂O mannitol challenge or, in the case of the iv experiments, 5 min after the 550 mosmol/kgH₂O mannitol challenge (P = 0.03). In the humid air and dry air studies, there were no significant differences in osmolality of the RTFO collected 5 min after the challenge to increase from 146 ± 9 and 97 ± 13 mM (Fig. 8A) to 173 ± 23 and 108 ± 15 mM (Fig. 8B), respectively, albeit these did not reach statistical significance. The [Cl⁻] and [Na⁺] in the RTFO collected after intubation in the intravenous bumetanide study were 161 ± 9 and 101 ± 5 mM, respectively (Fig. 8).

Fluorescent dextran percent. The percentage of fluorescent dextran in the RTFO collected 5 min after mannitol challenge is shown in Fig. 9. The percentages of the fluorescent dextran in the collected RTFO samples did not vary significantly between any of the experiments in the humid air study (P = 0.84), in the dry air study (P = 0.81), or in the intravenous bumetanide study (P > 0.9). These results indicate that the
deposition pattern and mass of the aerosolized mannitol challenges were reproducible.

DISCUSSION

We demonstrated that, in vivo, the inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase was exhibited as an increase in RTFO. Phillips and colleagues (14) showed that, during homeostatic conditions, the basolateral-to-luminal water flux was smaller than the luminal-to-basolateral water flux, the latter being associated with transepithelial Na\(^{+}\) transport and the activity of the Na\(^{+}\)-K\(^{+}\)-ATPase. Thus the increase in RTFO together with an increase in ciliary beat frequency (26) were likely the underlying mechanisms resulting in an acetylstrophanthidin-induced increase in bronchial mucociliary clearance observed by Winters and Yeates (26).

The dose of bumetanide delivered in these experiments (63 μg) is roughly equivalent to 2.5 mg of furosemide, which is four times less than the dose (10 mg) deposited by Winters and Yeates (26). Because the microspray catheter delivers a 20-μm-diameter aerosol predominantly to the trachea during two consecutive inspiratory maneuvers (<10 s), and Winters and Yeates delivered a 5.8- to 6.9-μm-diameter aerosol to the tracheobronchial airways over a 6-min period (26), it is likely that the pharmacological doses to the trachea were similar. On the assumption that furosemide and bumetanide have similar mechanisms of action when administered to the airway lumen, the data presented herein (Fig. 4C and Fig. 5C) indicate that the increase in the mucus transport rate per ciliary beat in baboons, which was attributed to a furosemide-induced increase in airway fluid (26), was, indeed, the case. On this basis, bumetanide delivered by aerosol to the airways would cause a predictable increase in bronchial mucociliary clearance (27), similar to that observed after aerosol delivery of furosemide (26).

In addition to bumetanide being a Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter inhibitor, bumetanide has also been shown to inhibit apical Cl\(^{-}\) channels. Specifically, bumetanide inhibited Cl\(^{-}\) absorption through the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels in the apical membrane of sweat ducts (21). This is consistent with the studies of Ropke et al. (22), who showed in rabbit nasal epithelia that bumetanide administered to luminal surface inhibited short-circuit- and amiloride-induced basolateral to luminal Cl\(^{-}\) efflux. This alternate mechanism of action of bumetanide and furosemide has a structural basis. The structure of these cotransporter inhibitors is similar to that of the inhibitors of chloride channels (7). When epithelia were maintained under open circuit conditions, the luminal-to-basolateral Cl\(^{-}\) fluxes were larger than the basolateral-to-luminal Cl\(^{-}\) fluxes, resulting in a net Cl\(^{-}\) absorption (3, 16). Phillips and Yeates (15) showed that when the cAMP-dependent Cl\(^{-}\) channels

Fig. 8. Comparisons of unbound Na\(^{+}\) and Cl\(^{-}\) concentrations of the respiratory tract fluid output collected 5 min after the 250 mosmol/kgH\(_2\)O mannitol challenge in the humid air study (A) and the dry air study (B). Ion concentrations in the respiratory tract fluid output collected during the stabilization period in the iv bumetanide study were used as a control value in A and B. Values are means ± SE for 6 beagle dogs.

Fig. 9. Comparison of the percentage of intensity of fluorescent dextran in the respiratory tract fluid output collected 5 min after mannitol challenge compared with that in the initially administered mannitol solution in the humid air study in which 6 dogs were ventilated with humid air before the 250 mosmol/kgH\(_2\)O mannitol challenge (A), in the dry air study in which 6 dogs were ventilated with dry air for 30 min before the 250 mosmol/kgH\(_2\)O mannitol challenge (B), and in the iv bumetanide study in which 8 dogs were ventilated with humid air before the 550 mosmol/kgH\(_2\)O mannitol challenge (C). Values are means ± SE.
were inhibited with diphenylamine-2-carboxylate under open-circuit conditions, there was a 38% attenuation of the potential difference and the luminal-to-basolateral water flux was reduced more than the basolateral-to-luminal water flux, resulting in a basolateral-to-luminal net water transport (15). These quiescent, open-circuit conditions are different from, and should not be confused with, the acetylcholine-induced Cl−-dependent liquid secretion reported by Trout et al. (23). Uyekubo and colleagues (24) showed, that under open-circuit conditions, forskolin, an activator of CFTR, increased net fluid absorption across cultured bovine tracheal epithelia by ~2.5-fold and that 5-nitro-2-(3-phenylpropylamino)benzoate, a CFTR blocker, markedly inhibited net fluid absorption on cultured human and bovine airway epithelia (24). In addition, Zabner and colleagues (31) have shown that the presence of apical CFTR channels is necessary for maximal water absorption. Thus bumetanide administered to the airway lumen likely inhibited apical Cl− channels and increased airway hydration by impairing luminal-to-basolateral Cl− and Na+ flux, as well as their associated water fluxes.

Alternate explanations for our observations and those of others can be conceived if there were an Na+-K+-2Cl− cotransporter on the apical membrane. However, there are no immunohistochemical studies showing the presence of the Na+-K+-2Cl− cotransporter on the apical surface of the airway epithelium.

In these studies, we observed a decrease in RTFO after 0.04 mg/kg intravenous of bumetanide, whereas Winters and Yeates (26) reported an increase in RTFO after a roughly equivalent dose of 2 mg/kg intravenous of furosemide, at least as far as inhibition of the Na+-K+-2Cl− cotransporter is concerned. Phillips and Yeates (15) showed that basolateral administration of furosemide to ovine tracheal epithelia under quiescent conditions appeared to inhibit luminal to basolateral water fluxes more that basolateral-to-luminal water fluxes. This is consistent with the increase in RTFO and tracheobronchial mucociliary clearance reported by Winters and Yeates (26). The decrease in RTFO by intravenous bumetanide reported here indicates that furosemide and bumetanide have different pharmacological profiles in terms of their effects on transepithelial water fluxes when administered intravenously. We have no other explanation at this time.

We have proposed that there is ion-associated water transport across airway epithelium that is distinct from, but coexisting with osmotically driven water transport (2). When the dogs were ventilated with dry air, the decrease in expired humidity (Fig. 3) and the increases in osmolality (Fig. 7) and [Na+] and [Cl−] concentrations (Fig. 8) in the RTFO were consistent with observations by other investigators (13, 19). We propose that the osmotically driven water flux, in part, is counterbalanced by an increase in ion-associated water flux due to the increase in ion concentrations in the RTFO during dry air ventilation, consistent with the observations of Price (18). The osmotic pressure difference across the epithelium causes a considerable driving force for water transport, with 40 mosmol/kgH2O being equivalent to a hydrostatic pressure of 1,034 cmH2O (1). Freed and Davis (5) demonstrated that, despite an extraordinary severe dry air challenges to the peripheral airways, the osmolality of the airway surface fluid was maintained between 450 and 500 mosmol/kgH2O with only small decreases in the volumes of airway surface fluid. As the volume of airway surface liquid continues to decrease, the osmotic gradient due to impermeant osmolytes in the airway surface liquid contributes more to the osmotically driven water transport. These osmolytes possibly induce water transport into the airway lumen so as to maintain a minimal volume of the surface barrier and prevent damage of the epithelium. Also, the balance of these two distinct water transport processes across airway epithelium may provide a mechanism that contributes to the onset of the rapid recovery in airway hydration after the termination of dry air ventilation (4).

The inhibition of an increase in mucociliary clearance after isocapnic ventilation with dry air in the presence of furosemide observed by Daviskas et al. (4) is not inconsistent with our findings. In our experiments, the administration of bumetanide to airway lumen induced a transient increase in RTFO for 18 min (Figs. 4C and 5C). In their study design, mucociliary clearance was measured only after the inhalation of furosemide for 7 min, the deposition of radioactive aerosol (2 min), and the subsequent clearing of the oropharynx and esophagus for ~10 min followed by dry air hypreventilation for 6–8 min (i.e., a total of ~25 min). Thus it is not surprising that the transient increase in RTFO after furosemide aerosol was not observed in those experiments because the inhibitory effect due to the action of furosemide on the cotransporter was likely dominant after the diffusion of furosemide across the epithelium. These data and our data, showing that intravenous bumetanide also reduced RTFO, are consistent with the preliminary data of Wong and Yeates (28), who showed that furosemide, delivered intravenously, inhibited the induced increase in bronchial mucociliary clearance after hypreventilation with dry air.

It is notable that the 250 mosmol/kgH2O ion-free mannitol challenge caused a prolonged increase in RTFO compared with the administration of aerosolized saline (Fig. 4A). Considering that the airway contains ~100–250 μl of fluid, it is reasonable to assume that 250 μl of the ion-free mannitol dilute the ion contents of the airway secretion to ~50% of their initial concentration (Fig. 9A). Thus these observations could result from an obligatory reduction of [Na+] and [Cl−] in the RTFO by the 250 mosmol/kgH2O ion-free mannitol challenge. A reduction in [Cl−] in the airway lumen increases the electrochemical gradient for a Cl− efflux across the apical membrane (20). Assuming that [Cl−] in the airway lumen is in dynamic equilibrium with the intracellular [Cl−] (9), then decreasing [Cl−] in the airway lumen will decrease intracellular [Cl−]. This decrease in intracellular [Cl−] upregulates the Na+-
K⁺-Cl⁻ cotransporter (8) and associated water transport (32). As shown in Fig. 8, the ion concentrations of the RTFO after the ion-free mannitol challenges (250 μl) were only marginally lower than those measured in the RTFO collected at the beginning of the experiment, indicating that Na⁺ and Cl⁻ were transported into the airway lumen in conjunction with an increase in basolateral-to-luminal water flux (2). These increases in RTFO were relatively independent from any remaining action of the pharmacological agents. When the trachea was challenged with the 550 mosmol/kgH2O mannitol (Fig. 6), the considerably larger increase in RTFO was likely due to the combined effects of Na⁺ and Cl⁻ depletion together with an increase in the osmotic gradient across the epithelium (2).

The tracheobronchial airways respond to changes in [Na⁺] and [Cl⁻], osmolality, and volume of the airway lining fluid so as to return these parameters to their homeostatic values. From the present studies, as well as those of Price et al. (18) and Jiang et al. (9), we can deduce that the defense of airway ion concentrations has precedence over the maintenance of airway surface fluid volume regulation. Thus manipulation of airway surface ion concentrations by adding ion-free isosmotic solutions with impermeant osmolytes can potentially be used as an effective mechanism to increase airway hydration and facilitate the clearance of secretions in patients with inspissated mucus possibly without the potential side effects of toxicity-induced changes in airway caliber. These data clearly confirm our previous prediction that delivery of cardiac glycosides by aerosol to the airways increases the removal of secretions by increasing the volume of airway surface fluid removed from the tracheobronchial airways. The delivery of an isosmotic solution of a low-dose cardiac glycoside or an impermeant osmolyte could have potential therapeutic benefits.

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