Airway Surface Liquid Osmolality Measured using Fluorophore-encapsulated Liposomes

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Abstract The airway surface liquid (ASL) is the thin layer of fluid coating the luminal surface of airway epithelial cells at an air interface. Its composition and osmolality are thought to be important in normal airway physiology and in airway diseases such as asthma and cystic fibrosis. The determinants of ASL osmolality include epithelial cell solute and water transport properties, evaporative water loss, and the composition of secreted fluids. We developed a noninvasive approach to measure ASL osmolality using osmotically sensitive 400-nm-diam liposomes composed of phosphatidylcholine/cholesterol/polyethylene glycol-phosphatidylcholine (1:0.3:0.08 molar ratio). Calcein was encapsulated in the liposomes at self-quenching concentrations (30 mM) as a volume-sensitive marker, together with sulfrohodamine 101 (2 mM) as a volume-insensitive reference. Liposome calcein/sulfrohodamine 101 fluorescence ratios responded rapidly (<0.2 s) and stably to changes in solution osmolality. ASL osmolality was determined from calcein/sulfrohodamine 101 fluorescence ratios after addition of microliter quantities of liposome suspensions to the ASL. In bovine airway epithelial cells cultured on porous supports at an air–liquid interface, ASL thickness (by confocal microscopy) was 22 μm and osmolality was 325 ± 12 mOsm. In anesthetized mice in which a transparent window was created in the trachea, ASL thickness was 55 μm and osmolality was 330 ± 36 mOsm. ASL osmolality was not affected by pharmacological inhibition of CFTR in airway cell cultures or by genetic deletion of CFTR in knockout mice. ASL osmolality could be increased substantially to >400 mOsm by exposure of the epithelium to dry air; the data were modeled mathematically using measured rates of osmosis and evaporative water loss. These results establish a ratio imaging method to map osmolality in biological compartments. ASL fluid is approximately isosmolar under normal physiological conditions, but can become hyperosmolar when exposed to dry air, which may induce cough and airway reactivity in some patients.

Key words: water permeability • cystic fibrosis • fluorescence self-quenching • trachea • CFTR

Introduction

The air-bathed surfaces of airways in the nasopharynx, trachea, and distal bronchi represent a unique biological air–fluid interface. The luminal (air-facing) surface of airway epithelia contains a thin (20–75 μm) layer of fluid called the airway surface liquid (ASL).1 The amount of water, salts, osmolytes, and macromolecules in the ASL depends on the transporting properties of the airway epithelia, evaporative water losses, macromolecular secretion, and possibly other factors. The airways in vivo also contain beating cilia, producing convective and surface tension phenomenon, as well as submucosal glands that can secrete fluid and macromolecules onto the airway surface. The ASL could in principle be hyposmolar, isosmolar, or hyperosmolar (compared with blood osmolality) depending on the relative influence of these factors. For example, a hypertoncic ASL might result from evaporative water losses across a relatively water-impermeable barrier, whereas a hypotoncic ASL might result from avid ion absorption or possibly surface tension phenomena (Boucher, 1994, 1999; Quinton, 1994; Widdicombe et al., 1997).

There is conflicting evidence that the ASL is hypertoncic under normal conditions and becomes near iso-tonic in the inherited disease cystic fibrosis (CF; Gilljam et al., 1989; Joris et al., 1993; Smith et al., 1996; Zabner et al., 1998). An increase in ASL salinity in CF has been proposed to inhibit endogenous antimicrobials and promote lung infection (Goldman et al., 1997; Zabner et al., 1998). Changes in ASL osmolality are proposed to play a role in the pathophysiology of asthma and cough, wherein local irritation stimulates neurogenic reflexes (Hahn et al., 1984; Higenbottam, 1984; Daviskas et al., 1996). The composition of the ASL has been difficult to measure because of its small volume. Invasive sampling methods involving filter paper or microcapillary fluid collections have yielded a wide range of ASL salt concentrations (Gilljam et al., 1989; Erjefalt and Persson, 1990; Joris et al., 1993; Smith et al., 1996; Goldman et al., 1997; Knowles et al., 1997; Hull et al., 1998; Zabner et al., 1998; Baconnais et al., 1999; Cowley et al., 2000). In-
Preparation of Liposomes

PC (20 mg), cholesterol, and PEG-PC in the molar ratio 1:0.3:0.08 from chloroform stock solutions were mixed, and the chloroform was evaporated to give a thin lipid film. Multilamellar vesicles were prepared by hydrating the lipid film with 1 ml of buffer containing 1 mM CaCl₂, 0.5 mM MgCl₂, 2.7 mM KCl, 1.47 mM KH₂PO₄, 15 mM Na₂HPO₄, 105 mM NaCl, 2 mM Tris, 30 mM calcium, and 2 mM sulforhodamine 101, pH 7.4. Solution osmolalities were adjusted to 200 or 300 mOsm (with NaCl) as required, and measured on a μ-Osmette osmometer (Precision Instruments Inc.). For preparation of small unformed size liposomes, the vesicle suspension was freeze-thawed four times and passed through 400-nm pore size polycarbonate membrane filters (Avanti Polar Lipids Inc.) using an Avanti mini-extruder. Unencapsulated dye was removed by size exclusion chromatography using a column (Sephadex G-50; Sigma-Aldrich). Suspensions of liposomes were kept at 4°C until use.

Steady-state and Stopped-flow Fluorescence Measurements

Steady-state fluorescence titrations were carried out on a fluorescence plate reader (Fluostar; BMG Labtechnologies Inc.) equipped with temperature regulation and excitation/emission filter sets for calcein (480/530 nm) and sulforhodamine 101 (590/620 nm). Microliter aliquots of stock liposome suspensions were added to solutions containing specified concentrations of NaCl, sucrose, glucose, lactose, or urea. Stopped-flow fluorescence measurements were performed with a Hit-Tech stopped flow apparatus with instrument deadtime <2 ms and 25 kHz data acquisition rate. 0.01 ml of the liposome suspension (0.5 mg lipid/ml) was mixed with an equal volume of buffer to give specified solute gradients. Fluorescence was excited at 480 nm and collected using a >520 cut-on filter. All measurements were done at 37°C.

Cell Culture Experiments

Bovine tracheal cells were cultured on collagen-coated 12-mm-diam snapwell inserts (Costar) with polycarbonate semipermeable membranes at an air-liquid interface at 37°C in a 5% CO₂/95% air atmosphere until fully differentiated as described by Uyekubo et al. (1998). Culture medium was changed every 2–4 d. Cultures...
were generally used 25–30 d after plating, at which time the electrical resistance was >300 Ω cm² and transepithelial potential difference was >30 mV. Cell inserts were mounted (cells facing upward) in a stainless steel perfusion chamber in which the undersurface of the insert was perfused with solutions at specified rates and perfusion pressures. The chamber was maintained at 37°C using a PDMF-2 microincubator (Harvard Apparatus), positioned on the stage of an upright epifluorescence microscope, and enclosed in a 100% humidified air/5% CO₂ tent maintained at 37°C. For depth measurements, the ASL was stained with tetramethylrhodamine-dextran dispersed in a low boiling point perfluorocarbon (boiling point 56°C; Fluorinert FC-72; 3M Co.) as described previously [Jayaraman et al., 2001]. For osmolality measurements, 46 nl of the liposome suspension (5 mg lipid/ml suspended in PBS containing 2 mM CoCl₂) was deposited onto the ASL using a microinjector (Nanoject-II; Drummond Scientific Co.).

**Fluorescence Microscopy**

Fluorescence was measured using a Nipkow wheel-type confocal microscope (Leitz with Technical Instruments confocal/coaxial module) with photomultiplier detector, cooled CCD camera (Photometrics), and custom filter sets (Chroma) for tetramethylrhodamine, calcine, and sulforhodamine 101. Fluorescence was detected using a Nikon 50× extralong working distance air objective (numerical aperture 0.55, working distance 8.5 mm). For osmolality measurements, calcine and sulforhodamine 101 fluorescence were recorded at specified times after application of the liposome suspension. Background fluorescence was under 1% of measured fluorescence in all measurements. For ASL thickness measurements, the microscope fine focus was driven by a microstepper motor to record the axial fluorescence profile as the focal plane moved through the ASL volume. ASL thickness was determined to better than 2 μm accuracy using a reconstruction technique as described previously (Jayaraman et al., 2001).

**Measurements in Mouse Trachea In Vivo**

Mice (25–35 g body weight, wild-type and Cambridge CF null mice; CFRDP Transgenic Core Facility) were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) 10 min after pretreatment with atropine (1 mg/kg intraperitoneal). As described previously [Jayaraman et al., 2001], atropine was used to avoid induction of gland secretions by the surgical procedure that was seen in 10–25% of untreated mice. A midline incision was made in the neck to expose the trachea for measurement of ASL and transepithelial potential difference. The ASL was stained by instillation of 0.5–1 μl of the liposome suspension using a microcatheter inserted into a feeding needle introduced via the mouth. The mouse was positioned on the microscope stage for fluorescence measurements as described above. In some experiments, mice breathed dry air through a cannula inserted via a tracheostomy. After instillation of the liposome suspension through the cannula, fluorescence was measured 2–3 and 9–12 mm distal to the tip of the cannula. After completion of the measurements, mice were killed by an overdose of pentobarbital (150 mg/kg). Animal protocols were approved by the UCSF Committee on Animal Research.

**R E S U L T S**

Fig. 1 (top) depicts the strategy for measurement of osmolality by ratio imaging microscopy. Liposomes of 400-nm diameter composed of PC, PEG-PC, and cholesterol were generated by extrusion in the presence of calcine and sulforhodamine 101. After chromatographic separation from external fluorophore, the dye-encapsulated liposomes functioned as a ratioable osmotic sensor. Increasing external osmolality resulted in osmotic shrinkage and increased dye concentration. The green fluorescence of calcine decreases because of self-quenching, whereas the red fluorescence of sulforhodamine 101 does not change. As shown in Fig. 1 (bottom), the green-to-red fluorescence ratio (F_green/F_red) decreased with increasing external osmolality. Liposome size, lipid composition, fluorescent dyes, and dye concentrations were optimized to give stable osmolality-sensing liposomes that interacted minimally with cell membranes and ASL constituents (see discussion). The dependences of F_green/F_red on external osmolality for different [calcine] are shown; 30 mM calcine was used for subsequent measurements.

The osmolality-sensing liposomes were further characterized. Size analysis by quasi-elastic light scattering indicated a unimodal size distribution with liposome diameter 382 ± 4 nm. Liposome osmotic water permeability was measured by stopped-flow fluorescence quenching. Fig. 2 A shows the time course of calcine...
fluorescence in response to a sudden change in solution osmolality to different values (200–600 mOsm) as indicated. Osmotic equilibration was >95% complete in under 1 s, with a computed osmotic water permeability coefficient ($P_f$) of 0.006 cm/s. To determine whether liposome volume remained stable after osmotically induced volume changes, the time course of calcein fluorescence was measured in response to increasing solution osmolality with different solutes, including the impermeable solute sucrose, and the biologically important solutes NaCl, lactate, glucose, and urea. Fig. 2 B shows stable liposome volume for at least 1 h in response to gradients of NaCl, lactate, and glucose, as shown by the identical $F_{green}/F_{red}$ versus osmolality relationships. As expected, urea equilibrated rapidly within a few seconds, as measured directly by stopped-flow light scattering (Fig. 2 B, inset). Fortunately, except in the kidney or in renal failure, biological urea concentrations are generally <7 mM (10 mg/dl). Dye leakage from liposomes was measured from the time course of calcein fluorescence in the absence of an osmotic challenge. There was <1% increase in fluorescence over 1 h (not shown), with no significant decrease in fluorescence after the addition of 5 mM cobalt (as CoCl$_2$), a membrane-impermeable static quencher of calcein fluorescence (Wallach and Steck, 1963). Together with measurements done over longer times, it was concluded that both dyes leaked out of liposomes by <5% in 5 h.

Measurements of ASL osmolality were done in cultures of bovine airway epithelial cells grown at an air–liquid interface. Fig. 3 A shows the experimental setup along with images of the ASL recorded using calcein (green) and sulforhodamine 101 (red) filter sets, shown with pseudocolored ratio image with osmolality scale. (B) $F_{green}/F_{red}$ measured by fluorescence microscopy for liposomes prepared in 200- or 300-mOsm solutions and suspended in solutions of different osmolalities (open circles, SEM, $n = 5$). The same liposomes were added to the ASL (closed squares) of cell cultures ($n = 7$–9 cultures). (C) Determination of ASL depth by z-scanning confocal microscopy as described in MATERIALS AND METHODS. Representative z-scans for tetramethylrhodamine-stained ASL before (left) and after (middle) addition of the liposome suspension, and after exposure to dry air for 10 min (right). See text for averaged ASL depths.

**Figure 3.** ASL osmolality in monolayer cultures of airway epithelial cells. (A, left) Schematic of apparatus for measurement of liposome fluorescence in ASL. Snapwell inserts containing cell monolayers grown at an air–liquid interface were mounted in a perfusion chamber enclosed in a 100% humidity, 5% CO$_2$ tent. (A, right) Images of liposome-stained ASL recorded using calcein (green) and sulforhodamine 101 (red) filter sets, shown with pseudocolored ratio image with osmolality scale. (B) $F_{green}/F_{red}$ measured by fluorescence microscopy for liposomes prepared in 200- or 300-mOsm solutions and suspended in solutions of different osmolalities (open circles, SEM, $n = 5$). The same liposomes were added to the ASL (closed squares) of cell cultures ($n = 7$–9 cultures). (C) Determination of ASL depth by z-scanning confocal microscopy as described in MATERIALS AND METHODS. Representative z-scans for tetramethylrhodamine-stained ASL before (left) and after (middle) addition of the liposome suspension, and after exposure to dry air for 10 min (right). See text for averaged ASL depths.
Exposure to dry air in which ASL thickness decreased to 8 μm because of evaporative water loss.

Various maneuvers were carried out to change ASL osmolality. Fig. 4 A shows control experiments in which small volumes of hypotonic or hypertonic saline were added to the ASL. As expected, calcein fluorescence and apparent ASL osmolality changed promptly. Fig. 4 B summarizes ASL osmolalities measured under control conditions, in the presence of the transport inhibitors glibenclamide and NPPB (to inhibit CFTR) and amiloride (to inhibit ENaC) and after exposure to dry air. ASL osmolality was not significantly changed by the transport inhibitors, but was increased considerably by rapid evaporative water loss resulting from exposure to dry air.

Because of the important role of osmosis in maintaining ASL osmolality when evaporative water loss occurs, we measured osmotic water permeability across airway cell layers from the time course of ASL volume after an osmotic challenge. 200 μl of hypotonic saline (150 mOsm) or hypertonic saline (600 mOsm) containing the volume marker blue dextran (0.1 g/ml) was added to apical surface of the cell monolayer. 10 μl aliquots of luminal fluid were removed at specified times for determination of volume marker concentration. Fig. 4 C shows the time course of fluid secretion after adding hypertonic saline to ASL. The computed transepithelial osmotic water permeability coefficient (Pf), assuming a smooth epithelial cell surface, was (5.5 ± 1.2) × 10⁻²³ cm/s (n = 4 cultures) for induced fluid secretion and (6.2 ± 2.0) × 10⁻³ cm/s for induced fluid absorption (hypotonic saline added to ASL). These values are lower than that of 0.022 cm/s reported in cultures of human airway cells (Matsui et al., 2000).

The osmotic water permeability of the cell layer sets limits on the theoretical magnitude of the difference between ASL and perfusate osmolalities. As depicted in Fig. 5 (top), water is lost from the ASL by evaporation (Je) and replaced from the tracheal wall by osmosis (Jv). ASL osmolality (FASL) is then calculated from the equation: J_e = J_v = P_f v_w S(FASL - Fb), where v_w is the partial molar volume of water (18 cm³/mol), S is surface area, and F_b is perfusate osmolality.
Evaporative water loss (Je) during exposure to dry air was measured gravimetrically to be \(1.1 \times 10^{-5}\) microliters water loss per centimeter squared area, and independent of solution salt content (0–500 mM NaCl). Fig. 5 (bottom) shows the predicted dependence of ASL osmolality on Pf and relative humidity, showing the expected increase in ASL osmolality with decreasing Pf. Using the measured Pf of \(5.5 \times 10^{-3}\) cm/s and dry air (0% humidity), the predicted ASL osmolality is 403 mOsm, in good agreement with the measured value of 419 mOsm from Fig. 4 B. This simple model provides a quantitative description of the quasi-steady-state increase in ASL osmolality after exposure to dry air, but is probably inaccurate for long exposure times because of ASL solute transport driven by the increased solute concentration.

Cultured airway cell monolayers represent an imperfect model of the intact airway epithelium because they are not exposed to time-varying air flows with changing moisture, oxygen, and carbon dioxide content. In addition, the intact airways have a considerably more complex structure than uniform monolayers of cultured cells. Therefore, we measured ASL osmolality in the mouse trachea in vivo. Microliter aliquots of liposome suspension were instilled into the lower trachea using a small catheter introduced through a feeding needle. The liposomes rapidly dispersed throughout the trachea. Fig. 6 A shows the fluorescently stained exposed trachea as seen using calcein (green) and SR (red) filters. Fig. 6 B shows \(F_{\text{green}}/F_{\text{red}}\) values for liposomes in solutions of known osmolalities (open circles) and in the trachea (closed squares). ASL osmolality was 330 ± 36 mOsm \((n = 5)\) in wild-type mice, not significantly different from 321 ± 27 mOsm in cystic fibrosis (CFTR-null) mice. ASL osmolalities measured using liposomes prepared in 200 and 300 mOsm buffers were not significantly different. Additional studies were done on wild-type mice in which a rectangular window was created in the anterior tracheal wall to visualize the posterior tracheal mucosa through a transparent plastic window as described previously (Jayaraman et al., 2001). ASL osmolalities measured through the window did not differ significantly from those measured without the window, and ASL depth (range 50–68 \(\mu\)m) was not affected by instillation of the liposome suspension (Fig. 6 C). Further studies using intravenously administered FITC-dextran (10 kD) showed that the tracheal wall did not become leaky to macromolecules under the conditions of our experiments.

Further studies were done on wild-type mice to determine whether the ASL could become hyperosmolar when exposed to dry air. Mice were allowed to breath dry air (0% humidity) through a tracheal cannula for 10 min before measurement of ASL osmolality. This maneuver mimics tracheostomy breathing, and is probably a good model for the ASL in the nasopharynx under normal ventilation conditions. We found a significant increase in ASL osmolality to 438 ± 11 mOsm at
2–3 mm distal to the tip of the cannula, decreasing to 340 ± 10 mOsm at 9–12 mm distal to the tip of the cannula in mice ventilated with dry air.

**DISCUSSION**

The fluorescent liposome sensor developed here provided a ratioable signal to map osmolality in macroscopic or microscopic biological compartments. The liposome sensor was applied to important issues in airway physiology—the absolute osmolality of the ASL, and the hypotheses that ASL osmolality is altered in cystic fibrosis and can be increased by exposure to dry air. The methodologies for fluorescent staining of the ASL in cell culture models and intact mouse trachea were recently developed and validated in measurements of ASL [Na⁺], [Cl⁻], and pH (Jayaraman et al., 2001). The principal conclusions of this study are that, under normal conditions, ASL osmolality is approximately isosmolar with external osmolality; that pharmacological inhibition or genetic knockout of CFTR in cystic fibrosis does not result in altered osmolality; and that exposure to dry air can substantially increase ASL osmolality. These results extend previous findings that ASL [Na⁺] and [Cl⁻] are each >100 mM, that [HCO₃⁻] is ~8 mM, and that these concentrations do not differ significantly in CF. The remaining unmeasured cation to account for the osmotic gap is probably K⁺, and the remaining unmeasured anions probably include proteins and small organic anions. Our results indicate that substantial quantities of unmeasured osmolytes are not present in the ASL, and that surface tension phenomena do not contribute significantly to osmotic balance.

The design of the liposomes presented a challenge given the requirements of bright ratioable fluorescence, excellent dye encapsulation, minimal interactions with ASL components, rapid osmotic equilibration, and slow solute equilibration. The inclusion of PEG-labeled PC at 8 mol percent conferred a polar exterior, resulting in decreased incorporation of lipidic constituents from the ASL as well as decreased solute and dye permeabilities. The 400-nm-diam liposome size was optimal to minimize fusion to the cell surface, prolong solute equilibration time (compared with smaller liposomes), and encapsulate sufficient fluorophore to give bright signals in the ASL. Calcein was chosen at the volume-sensitive fluorophore because of its excellent self-quenching properties with minimal spectral shift, pH-insensitivity, and sustained incorporation in liposomes. In addition, the availability of a potent quencher of calcein fluorescence (Co²⁺; Wallach and Steck, 1963) permitted detection and quenching of small amounts of calcein that may leak from liposomes. Other volume-sensing strategies (chlorocarboxyfluorescein self-quenching, and various fluorophore-quencher pairs) were found to be inferior. Sulforhodamine 101 was chosen as the volume-insensitive reference fluorophore because of its bright red fluorescence, lack of self-quenching, pH independence, and sustained incorporation in liposomes. Sulforhodamine 101 was used previously as a reference dye in measurements of Golgi pH using a liposome fusion method (Seksek et al., 1995). Finally, dye concentrations and osmolality of the liposome preparation were optimized to maximize the sensitivity of the calcein/sulforhodamine 101 fluorescence ratio to solution osmolality.

The findings here of an approximately isosmolar ASL, taken together with water permeability measurements and the computations in Fig. 5, suggest that ASL osmolality differs little from serum osmolality under normal physiological conditions. Manoeuvres to increase or decrease ASL osmolality by nebulization of hypertonic or hypotonic solutions are predicted to produce only transient changes in ASL osmolality. However, although the osmotic water permeability of the airway epithelium is fairly high, evaporative water loss by tracheostomy or rapid dry air breathing can substantially increase ASL osmolality. The potential clinical relevance of this observation with respect to cough and reactive airway disease will require further investigation.

The lack of effect of CFTR inhibitors in the cell culture studies, the comparable ASL osmolarities in wild type and CF mice, and the fairly high water permeability of the tracheal epithelium suggest that ASL osmolality is not an important factor in the pathogenesis of CF. These results are in agreement with our recent findings of similar salt concentrations ([Na⁺] and [Cl⁻]) using the same cell culture and mouse systems. However, it is recognized that cell cultures are imperfect models of the airways, and CF mice are imperfect models of human CF because the mice develop little pulmonary disease. Definitive noninvasive measurements in human airways may be required to definitively establish the role of ASL fluid composition in the pathogenesis of CF. Notwithstanding these caveats, investigation of alternative CF pathogenesis mechanisms are warranted, such as abnormalities in gland secretory function.

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