Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia

Brian Button, Maryse Picher and Richard C. Boucher

Cystic Fibrosis Research and Treatment Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7248, USA

In the lungs, the first line of defence against bacterial infection is the thin layer of airway surface liquid (ASL) lining the airway surface. The superficial airway epithelium exhibits complex regulatory pathways that blend ion transport to adjust ASL volume to maintain proper mucociliary clearance (MCC). We hypothesized that stresses generated by airflow and transmural pressures during breathing govern ASL volume by regulating the rate of epithelial ATP release. Luminal ATP, via interactions with apical membrane P2-purinoceptors, regulates the balance of active ion secretion *versus* absorption to maintain ASL volume at optimal levels for MCC. In this study we tested the hypothesis that cyclic compressive stress (CCS), mimicking normal tidal breathing, regulates ASL volume in airway epithelia. Polarized tracheobronchial epithelial cultures from normal and cystic fibrosis (CF) subjects responded to a range of CCS by increasing the rate of ATP release. In normal airway epithelia, the CCS-induced increase in ASL ATP concentration was sufficient to induce purinoceptor-mediated increases in ASL height and MCC, via inhibition of epithelial Na^+-channel-mediated Na^+ absorption and stimulation of Cl^- secretion through CFTR and the Ca^{2+}-activated chloride channels. In contrast, static, non-oscillatory stress did not stimulate ATP release, ion transport or MCC, emphasizing the importance of rhythmic mechanical stress for airway defence. In CF airway cultures, which exhibit basal ASL depletion, CCS was partially effective, producing less ASL volume secretion than in normal cultures, but a level sufficient to restore MCC. The present data suggest that CCS may (1) regulate ASL volume in the normal lung and (2) improve clearance in the lungs of CF patients, potentially explaining the beneficial role of exercise in lung defence.

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Corresponding author B. Button: 7013 Thurston-Bowles Building, CB#7248, Chapel Hill, NC 27599-7248, USA. Email: bbutton@med.unc.edu

The airway surface liquid (ASL) layer lining the airway surfaces is crucial for mediating mucociliary clearance (MCC) rates and, thus, removal of noxious materials from the lung (Boucher, 2002). The ASL consists of two distinct layers. The upper mucus layer contains mucins secreted from goblet cells and glands and is designed to trap and clear inhaled materials. The underlying periciliary layer (PCL) is a thin ($\sim$7 μm), low-viscosity aqueous layer that acts as a lubricant layer for both cilia beating and movement of the mucus layer over the epithelial surface (Tarran *et al.* 2001). The volume of the ASL is critical for proper lung defence as evidenced by the observation that the airway epithelium, under normal conditions, tightly regulates the height of ASL (Boucher, 2003). For example, when exposed to an 'ASL fluid challenge', normal airway epithelia autoregulate the ASL to a height commensurate with efficient MCC, i.e. $\sim$7 μm, the approximate length of the extended cilium (Tarran *et al.* 2001). These studies demonstrated that ASL autoregulation was associated with reciprocal inhibition of Na^+ transport and activation of Cl^- secretion, the net effect being maintenance of ASL height commensurate with efficient MCC. However, the sensors and response mechanisms that autoregulate volume flow to maintain ASL height at such levels remain unclear.

In cystic fibrosis (CF), mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) are associated with a significant decrease in epithelial Cl^- secretion and excessive Na^+ absorption (Matsui *et al.* 1998*a*). These defects produce unregulated ASL absorption under static culture conditions, resulting in a minimum height of ASL (defined as the height of liquid trapped by folded-over cilia), whereby cilia are incapable of effective beating. Under such conditions, the mucus layer

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collapses onto the airway surface which ultimately forms adhesive plaques. The physical interaction between the mucus layer and the cell surface glycocalyx has been hypothesized to prevent mucus clearance by both cilia activity and coughing (Boucher, 2004).

Despite knowledge of the importance of ASL height in lung health, key aspects of its physiology remain unexplored. For example, little is known about the signals that coordinate the activities of Na^+ absorption and Cl^- secretion to modulate both liquid absorption and secretion to maintain ASL height at physiologically appropriate levels. It has recently been suggested that the signals involved in the regulation of ASL height might, in part, be soluble molecules contained within the ASL (Tarran *et al.* 2005). Emerging evidence suggests that the concentration of ATP and adenosine within the ASL regulates the activity of Na^+ and Cl^- channels via the activation of P1 and P2 purinoceptors located in the apical membrane of airway epithelial cells (Lazarowski *et al.* 2004; Tarran *et al.* 2005). Because the rate of ATP release is a key determinant of ATP and adenosine concentrations on cell surfaces, it is important to identify the physiological stimuli that regulate ATP release by airway epithelia.

The lung is exposed to complex physical forces during breathing and vascular perfusion, which contribute to the regulation of numerous lung functions (Schumacker, 2002). Previous studies have reported that ATP is released from human airway epithelia subjected to physical forces generated by tidal breathing, including mechanical deformation (Kallok *et al.* 1983; Grygorczyk & Hanrahan, 1997; Homolya *et al.* 2000) and liquid shear stress (Guyot & Hanrahan, 2002; Lazarowski *et al.* 2004; Tarran *et al.* 2005). We hypothesized that transmural pressure generated during normal breathing contributes to the maintenance of ASL volume through mechanically induced ATP release and stimulation of purinoceptor-mediated secretion. The lungs also experience increased static pressures during bronchoconstriction (Gunst & Stropp, 1988; Ressler *et al.* 2000) or mucus plugging of the airways. We therefore compared the effects of cyclic and static compressive stress on ASL ATP regulation, ASL height and MCC. Well-differentiated, polarized primary cultures of airway epithelial cells from normal donors and CF patients were utilized in this study and subjected to physiological ranges of static and cyclic compressive stress, then tested for ATP release and metabolism, changes in ASL height, cilia beat frequency (CBF), and rates of mucus transport.

Methods

Cell culture

Human tracheobronchial epithelial cells from normal donors and CF patients were obtained from the University of North Carolina Cystic Fibrosis Tissue Culture Core under the auspices of protocols approved by the UNC Institutional Review Board. Normal epithelial cells were derived from donor lungs and excess tissue of the recipient lung at the time of transplantation, and CF tissues from autopsy- and lung transplant-derived tissues. Cells from the trachea, main stem, and lobar bronchi were isolated by protease digestion (Matsui *et al.* 1998*b*). Isolated cells (10^6 cm^{-2}) were seeded on 12 mm permeable support (0.45 μm pore diameter, Transwell-Clear; Costar) precoated with human placental collagen (Sigma), in Ham's F12-based medium supplemented with 10 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 1 μM hydrocortisone, 30 nM triiodothyronine, 25 ng ml^{-1} epidermal growth factor, and 3.75 μg ml^{-1} endothelial cell growth substance, as previously described (Matsui *et al.* 1998*a*). Cells were maintained under air–liquid conditions, washed every 48–72 h to remove accumulated mucus, and studied as fully differentiated cultures (3–4 week cultures with transepithelial resistances of $\sim$200–400 Ω cm^2). Culture incubations were performed in a well-humidified (> 95%) tissue culture incubator (5% CO_2) at 37°C. In all experiments presented here, excess mucus on the cultures was eliminated by washing the cultures three times with serum-free Ham's F12-based medium 16 h prior to experimentation.

Application of static and cyclic stress to airway cultures

We utilized an in-house-developed system to control the transepithelial pressure exerted on airway cultures. This system, diagrammed in Fig. 1, uses 12 mm × 6 mm conical silicon plugs fitted into the top of the plastic Transwell support to isolate the apical (airway) space in order to apply pressure selectively to this interface (modified from Ressler *et al.* 2000). Two stainless steel ports running through the length of the silicone plug were used to deliver pressure pulses to the culture. Polyethylene (PE) tubing connects one port (input port) to a 36.6 cubic inch pressure reservoir. A microprocessor monitors the chamber pressure via an internal pressure sensor (MPXM2010; Freescale Semiconductor; Freescale, Austin, TX, USA) and controls a microair pump (AA1286; Sensidyne, Clearwater, FL, USA) to maintain constant pressure within the reservoir (at values from atmospheric to 100 cmH_2O). The system obtains the humidified, gassed (5% CO_2) air from the tissue culture incubator in which the complete system is housed. For cultures subjected to CCS, the system's microprocessor controls the operation of a pair of high-speed microsolenoid valves (AWEO128P; The Lee Company, Westbrook, CT, USA) located immediately upstream of the input port and downstream of the output port of the silicon plug. This configuration permits the pressure in the cultures to alternate between chamber pressure (closed state) and atmospheric pressure (open state). This system is multiplexed, capable of producing

identical CCS and SCS waveforms in up to 16 cultures simultaneously (8 CCS and 8 SCS). In cultures subjected to SCS, pressure fluctuations during pump activity were dampened by a latex bladder placed in series with the pressure reservoir to ensure that pressure deviations due to volume leak (associated with CCS) and the subsequent pump activity were minimized. Maximum pressure variations on cultures undergoing SCS were less than ± 0.2 cmH_2O throughout the duration of the pressure exposure (data not shown). This configuration allowed experiments to be performed on paired cultures, exposed to either CCS or SCS, simultaneously.

Microsampling the ASL

At 1 h prior to the experiment, 50 μl of isotonic TES buffered Ringer (TBR; mM: NaCl, 125; KCl, 5.2; CaCl, 1.2; MgCl, 1.2; glucose 10; TES, 10, adjusted to pH 7.4), was applied to the apical surface. Florescent dye, 0.02% Texas Red–dextran (10 kDa; Molecular Probes), was added to the TBR in order to visualize the meniscus with a stereomicroscope (SMZ-2B; Nikon) and avoid ATP release by epithelial damage or mechanical stimulation during microsampling. A mechanical micromanipulator (Narishige; Japan) was used to manoeuvre a fine-tip ($\sim$10 μm) borosilicate microcapillary tube into the

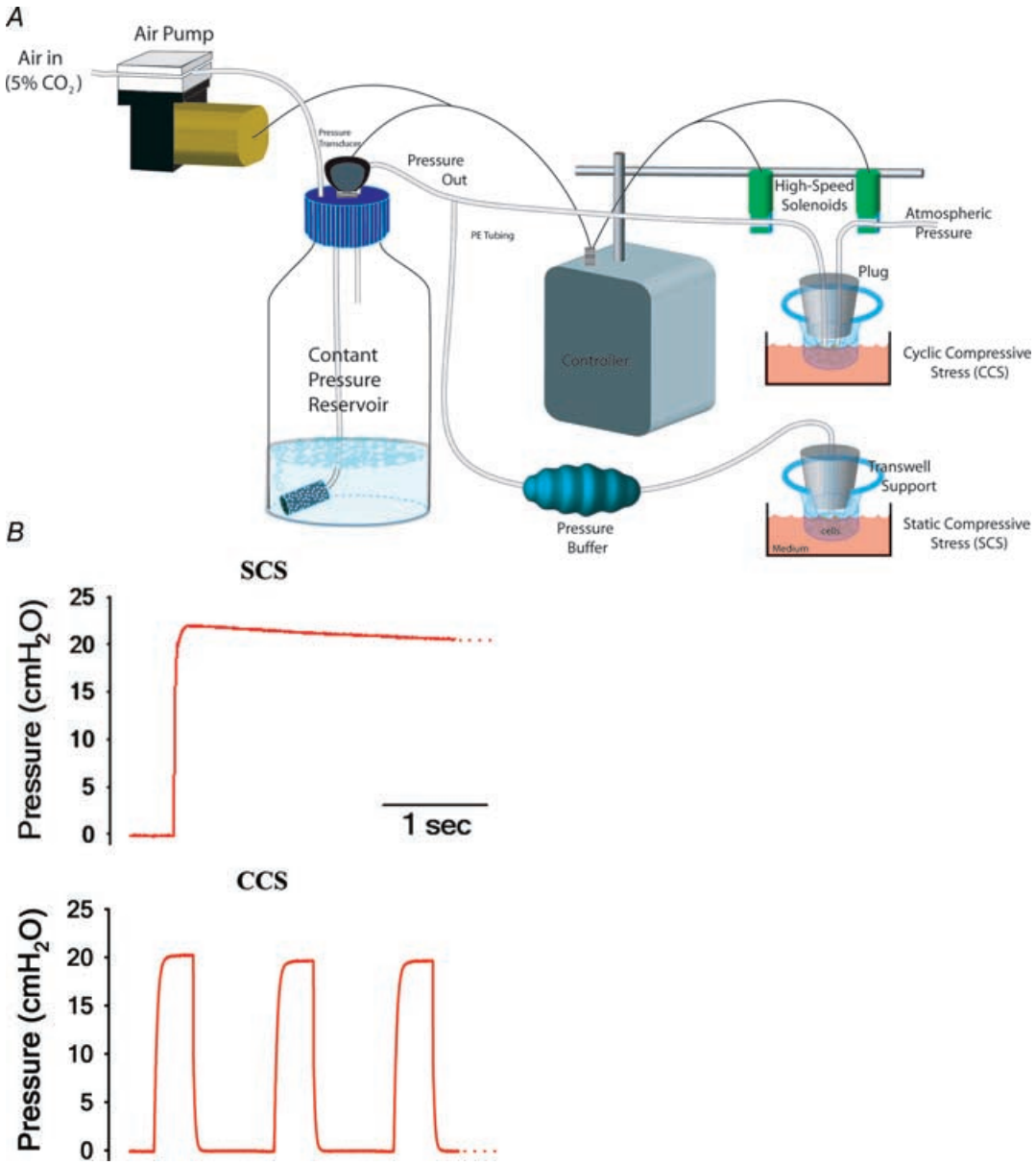

Figure 1. System generating positive static and cyclic compressive stress on human airway cultures
A, a microprocessor controlled the pressure amplitude and timing (in the case of CCS) of transepithelial pressure to the apical surface of the cultured human airway epithelial cells. *B*, sample pressure tracing from a culture exposed to SCS (top) at 20 cmH_2O and CCS (bottom) at 20 cmH_2O for 1 s, every 3 s. See text for details.

meniscus of the ASL. Capillary force was used to gently remove ~10 μl from the apical surface at the indicated time points. The samples were immediately boiled for 2 min to inactivate ectoATPases and stored at −20°C until analysis.

Measurement of ATP by luciferin–luciferase

ATP concentrations in either ASL microsamples or basolateral medium were determined using luciferin–luciferase bioluminescence as previously described (Lazarowski *et al.* 2004). Briefly, a 5 μl sample was added to a test tube and the volume adjusted to 300 μl with HPLC-grade water. One hundred microlitres of the luciferin–luciferase reaction mix (300 μM luciferin, 5 μg ml^{-1} luciferase, 6.25 mM $MgCl_2$, 0.63 mM EDTA, 75 μM dithiothreitol, 1 mg ml^{-1} bovine serum albumin, 25 mM Hepes, pH 7.8) was added to samples with the built-in auto injector of a luminometer (LB953; Berthold Technologies, Wildbad, Germany). Luminescence was detected by the photomultiplier and integrated over 10 s. The recorded arbitrary counts from each sample, counted in duplicate, were compared against an ATP standard curve performed in parallel. The resulting luminescence was shown to be linear between 0.1 and 1000 nM ATP, based on the standard curve. In experiments measuring ATP release rates, cultures were pretreated with a cocktail of ATPase inhibitors (30 μM ebselen and 300 μM $\beta\gamma$Me-ATP) prior to initiation of the experimental procedures (Okada *et al.* 2006). Release rates were calculated from ATP concentrations measured over time as previously described (Okada *et al.* 2006).

Assessment of ectoATPase activity

Airway epithelial cultures exposed to control conditions (atmospheric pressure), CCS or SCS for 1 h were assayed for the rate of cell surface ATP metabolism, as previously described (Picher *et al.* 2004). In brief, the cultures were washed with TBR and preincubated (0.35 ml mucosal/serosal) in Krebs buffer (KRB; mM: 140 Na^+, 120 Cl^-, 5.2 K^+, 25 HCO_3^-, 2.4 HPO_4^-, 1.6 Ca^{2+}, 1.6 Mg^{2+}, 5.2 glucose and 25 Hepes, pH 7.4) for 30 min at 37°C (5% CO_2–95% O_2). The reactions were initiated with 0.01 mM ATP and stopped by transferring 10 μl aliquots to tubes containing 0.3 ml ice-cold water. The samples were boiled for 5 min, filtered and analysed by reversed-phase paired-ion HPLC as previously described (Picher *et al.* 2004).

Assessment of cell viability

Measurements were taken before and during control (atmospheric pressure), CCS, or SCS. Primary cultures of human airway epithelial cells were washed twice with TBR, and then incubated for 30 min with 3 μM calcein-AM (in TBR) at 37°C to stain the cells. The cultures were washed twice with TBR prior to addition of 50 μl ethidium homodimer-1 (1 μM, Live/Dead Kit; Molecular Probes). The cultures were mounted on the stage of an inverted epifluorescence microscopic (Leica) and maintained in 0.5 ml of serum-free Dulbecco's minimum essential medium under resting conditions for 30 min. Cellular calcein (488 excitation/530 nm emission) and ethidium homodimer-1 (560 nm excitation/630 nm emission) fluorescence were recorded in real-time by a cooled CCD camera (OrcaER; Hamamatsu). Images were acquired with a 40× objective using separate filter blocks for visualization of ethidium homodimer-1 and calcein. Images from each condition were counted for stained (red and green) cells, and the number of dead cells per image, minimum of 10 frames per culture, was determined.

Transepithelial resistance measurements

Transepithelial resistance (TER) was assessed using an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL, USA) as previously described (Homolya *et al.* 2000). TER measurements were made in parallel cultures at either given pressure or time points (see results). In these experiments, cultures were removed from the incubator and TER measurements were made immediately following the addition of 100 μl TBR (prewarmed to 37°C) to the apical surface. Following measurement, luminal TBR was carefully aspirated and cultures were returned to the incubator until the next data point. Data represent the comparison of TER of cultures undergoing CCS/SCS relative to the TER of parallel cultures under control (atmospheric pressure) conditions at each data point.

Measurement of ASL height

To visualize the ASL layer, 30 μl TBR containing 0.2% v/v Texas Red–dextran (10 kDa; Molecular Probes) was added to the lumen of freshly washed airway cultures. This volume of TBR results in an initial ASL height of ~20–30 μm, as previously described (Tarran & Boucher, 2002). Images of the Texas Red-labelled ASL were acquired by laser-scanning confocal microscopy (Model 510; Zeiss) using the appropriate filters for Texas Red (540 nm excitation/630 nm emission). To avoid evaporation of the thin ASL layer in low humidity environment, 100 μl of immiscible perfluorocarbon (Fluorinert-77; 3M Corporation) was added to the airway surface following the addition of the labelling dye (Tarran & Boucher, 2002). The height of the ASL was determined by averaging the heights obtained from XZ scans of five predetermined points on the culture. ASL height was measured immediately following the addition of TBR/Texas Red–dextran ($t = 0$) and at various time points after the initiation of continuous CCS, SCS, or control conditions for 24 h.

Transepithelial potential difference (V_t)

V_t was recorded using a borosilicate glass microelectrode (World Precision Instruments), filled with 3 M KCl, positioned into the ASL by a motorized micromanipulator (MC1000e; SD Instruments) connected to a high-impedance electrometer (World Precision Instruments). A macroelectrode, made from polyethylene tubing containing 3 M KCl–4% agar, was placed in the serosal bath as the reference. To estimate the contribution of active transepithelial ion transport on ASL volume regulation, V_t was measured before (i.e. basal V_t) and after sequential exposure (10 min each) to inhibitors of Cl^- secretion (bumetanide, 10^{-4} M; serosal) and Na^+ absorption (benzamil, 10^{-5} M; serosal), as previously described (Tarran *et al.* 2001). Benzamil was added to the basolateral solution rather than the apical, to avoid disturbing the ASL (Zabner *et al.* 1998; Joo *et al.* 2006). Measurements of V_t were made in normal and CF cultures immediately following the addition of the 30 μl TBR to the apical surface and at 24 h following continuous CCS, SCS, or control conditions to determine the bioelectric properties under conditions identical to the ASL volume measurements (above). As with ASL measurements, perfluorocarbon was added to the luminal surface to avoid evaporation of the thin ASL layer during V_t measurements.

Cilia beat frequency (CBF)

Cultures used were carefully washed with TBR prior to the experiments to eliminate accumulated mucus. After 1 h under control, CCS, or SCS conditions, cultures were immediately placed on an inverted phase contrast microscope (TE 2000; Nikon) with a 20× objective to record cilial movement. High-speed (125 Hz) video images were captured with an 8-bit b/w camera (GS-310 Turbo; Megaplus). The analog signal was digitized via an analog-to-digital converter board (A/D; National Instruments). Specialized software, based on Sisson–Ammons video analysis (Sisson *et al.* 2003), was used to analyse the acquired video images and estimate the average CBF of all motile cilia in each frame.

Measurement of mucociliary transport rates

Fluorescent microspheres (0.02% v/v, 1 μm, yellow–green fluorescence; Molecular Probes) were added to the apical surface of airway epithelial cultures in 30 μl TBR. Measurements of mucociliary transport were made in airway cultures ∼15 min after the addition of the fluorescent microspheres ($t = 0$) and at 24 h following the continuous CCS, SCS, or control, from time-lapse fluorescence images (488 excitation/530 nm emission) acquired during a 3 s exposure with an inverted epifluorescence microscope (Eclipse; Nikon) and a CCD camera (OrcaER; Hamamatsu). The angular velocity of the microspheres was calculated as previously described (Matsui *et al.* 1998*a*).

Statistical analysis

All experiments were performed on airway cultures originating from a minimum of five different patients, in each group. All data are expressed as means ± S.E.M., where n represents the number of different patients utilized. Student's t test for unpaired data was used to assess the difference between two groups. One-way analysis of variance (ANOVA) was performed when more than two groups were compared with a single control, and then differences between individual groups were assessed by a multiple-comparison test (Tukey) when F was < 0.05. In all cases, significance was established at $P < 0.05$.

Results

Application of cyclic and static compressive stress to human airway epithelia

We utilized well-differentiated human airway epithelial cultures from healthy and CF donor lungs to investigate the effects of compressive stress on airway physiology. The cultures were exposed to compressive stress of various magnitudes using a system (Fig. 1*A*) that applies apical to basolateral transepithelial pressure gradients under physiological, thin-film conditions in one of three protocols: (1) static compressive stress (SCS) produced by applying a constant pressure for the duration of the experiment; (2) cyclic compressive stress (CCS) produced by subjecting cultures to a 3 s cycle composed of 2 s at atmospheric pressure (0 cmH_2O transepithelial) and 1 s at a given positive pressure, at a frequency of 20 cycles min^{-1} (CPM) (Fig. 1*B*); or (3) control conditions in which cultures remained at atmospheric pressure for the duration of the experiment. The parameters for CCS where chosen to mimic the mechanical stimulation observed in the airways at a frequency consistent with normal tidal breathing, while the SCS protocol mimics constant stress and pressure, experienced during bronchoconstriction (Gunst & Stropp, 1988; Ressler *et al.* 2000).

Differential effects of static and cyclic stress on ATP release

The relationship between transepithelial pressure and steady-state ASL ATP concentration ([ATP]) was determined for airway cultures subjected to 30 min of either CCS or SCS over a range of transepithelial pressures (from atmospheric pressure to 50 cmH_2O). Cultures subjected to CCS exhibited a curvilinear increase in [ATP] over the pressure range investigated (Fig. 2*A*). The change

in [ATP] was steepest over the range of 0–5 cmH_2O, with an increased rate of change in steady-state [ATP] of 8.3 nM ATP/cmH_2O. At pressures > 5 cmH_2O, the change in [ATP] had declined to 0.3 (nM ATP) cmH_2O^{-1} over the remainder of the pressure range tested. Compared to CCS, the [ATP] was not significantly elevated in cultures subjected to static pressures (SCS) over a similar range of pressures (Fig. 2*A*). [ATP] at 30 min was at steady-state levels, as application of CCS or SCS for 60 min, instead of 30 min, had no significant effect on the [ATP] (data not shown). The measured CCS-induced increase in apical [ATP] was abolished by preincubating the cultures with apyrase, an enzyme that rapidly degrades ATP to AMP (Zimmermann, 2000), confirming that the signal from the luciferin-luciferase assay reflected ATP concentration. Our subsequent experiments were conducted with 20 cmH_2O for both CCS and SCS, unless stated otherwise, because (1) ATP release during CCS was near-maximal and (2) cell viability and epithelial junction integrity were not significantly affected (see next section).

We next investigated whether regulation of [ATP] during compressive stress was polarized, i.e. altered at both the apical and basolateral surfaces in response to compressive stress. Basolateral [ATP] was measured after subjecting airway cultures to 1 h of either control (atmospheric pressure), SCS or CCS. Basolateral [ATP] increased from 4.8 ± 0.4 nM to 9.3 ± 0.2 nM in response to CCS (Fig. 2*B*). As in the case of ASL ATP, application of SCS did not significantly alter steady-state basolateral [ATP] (5.1 ± 0.4 nM) compared to control conditions. The absolute [ATP] between apical and basolateral solutions was quite different, due, in part, to the differences in lavage volume (apical: 50 μl; basolateral: 500 μl). However, the calculated change in ATP mass (compared to control) in the basolateral compartment following CCS was 2.25 pmol, which was similar to that observed in the ASL following CCS (2.43 pmol). Together, these experiments demonstrate that cyclic, but not static, transepithelial pressure induces an increase in [ATP] on both surfaces of human airway epithelia.

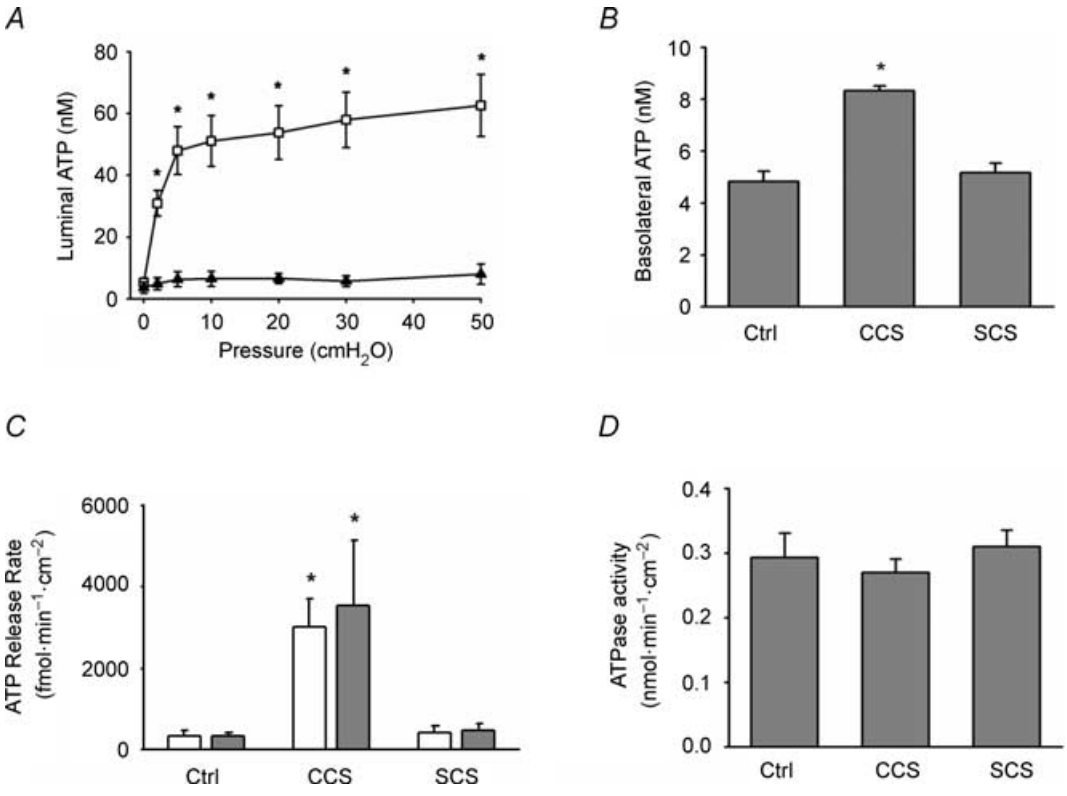

Figure 2. Cyclic, but not static compressive stress increases steady-state ATP level in CF airway cultures
A, relationship between compressive stress pressure amplitude and apical ATP concentration during CCS (□, $n = 9$) and SCS (▲, $n = 7$). Measurements made from individual cultures after 30 min of compressive stress at the indicated pressure. *B*, mean basolateral ATP concentration obtained after 1 h of control conditions ($n = 7$), CCS (20 cmH_2O, 20 CPM, $n = 7$) or SCS (20 cmH_2O, $n = 5$). *C*, ATP release rates in normal (open bars) and CF (filled bars) airway cultures under control (Ctrl) conditions, or after 30 min of CCS (20 cmH_2O, 20 CPM) or SCS (20 cmH_2O), in the presence of a cocktail of ATPase inhibitors (30 μM ebselen and 300 μM $\beta\gamma$Me-ATP) ($n = 4$ in each group). *D*, apical ectonucleotidase activities measured with 10 μM ATP after 1 h exposures to control, CCS (20 cmH_2O, 20 CPM) or SCS (20 cmH_2O) conditions ($n = 5$ in each group). (*Significantly different from control cultures at ambient pressure.)

Extracellular [ATP] reflects the balance between the rates of ATP release and cell surface metabolism (Lazarowski *et al.* 2000). Accordingly, we performed experiments to establish their relative contribution to CCS-induced increase in steady-state [ATP]. First, we measured the rate of ATP release during the initial 30 min of CCS or SCS by a luciferin–luciferase assay in the presence of a cocktail of ectoATPase inhibitors to prevent ATP metabolism (Okada *et al.* 2006). ATP release rates (Fig. 2*C*) in normal cultures undergoing SCS (20 cmH_2O) were not statistically different from control cultures maintained at atmospheric pressure (323.2 ± 145.6 *versus* 423.0 ± 166.8 fmol min^{-1} cm^{-2}, $P < 0.05$). In contrast, CCS (20 cmH_2O, 20 CPM) significantly increased the rate of ATP release to 3008.4 ± 689.8 fmol min^{-1} cm^{-2}. As it has been suggested that CFTR contributes to ATP release (Reisin *et al.* 1994), either directly or via regulation of an ATP release pathway, we next sought to test whether ATP release under basal or compressive stress conditions was altered in CF airway epithelia. As shown in Fig. 2*C*, ATP release rates from CF airway cultures under control, SCS, or CCS conditions was not different from normal cultures (all $P > 0.05$).

Second, to determine whether CCS or SCS altered extracellular ATP metabolism, we measured the capacity of ectoATPase. While the activity of ectoATPases would be expected to increase as a result of increased substrate availability during accelerated ATP release, total ectoATPase capacity (estimated by the rate of metabolism of exogenously applied ATP), was not affected by CCS or SCS (Fig. 2*D*). Collectively, the results in Fig. 2 demonstrate that the elevated ASL [ATP] observed during CCS was the result of increasing the rate of ATP release and not to a decrease in extracellular metabolism.

Cyclic stress-mediated ATP release is a regulated mechanism

Damage to cell integrity can result in the release of intracellular ATP into the thin volume of the ASL. Experiments were therefore designed to determine whether cell viability and integrity were affected by compressive stress. First, we determined whether airway epithelial cells were damaged by compressive stress, using a live/dead assay based on calcein fluorescence and impermeability to ethidium-homodimer of live, non-permeabilized, cells (Lazarowski *et al.* 1997). Figure 3 shows that the number of dead cells was not increased significantly by exposure to 1 h of CCS (20 cmH_2O, 20 CPM) or SCS (20 cmH_2O) compared to control conditions. As positive control for cytolysis, membrane permeabilization by digitonin (50 μM apical) resulted in a substantial increase in ethidium homodimer-1 fluorescence. These results indicate that the compressive stress conditions used in this study for CCS and SCS did not produce significant cytolysis-mediated ATP release.

The impact of compressive stress on epithelial tight junctions was assessed as a second measure of epithelial cell function and barrier integrity. The effect of 1 h CCS on the transepithelial resistance (TER) of airway cultures was measured over a wide range of pressures (from 0 to 100 cmH_2O) (Fig. 4*A*). Only at transepithelial pressures > 60 cmH_2O was TER significantly different from values obtained from paired cultures maintained at atmospheric

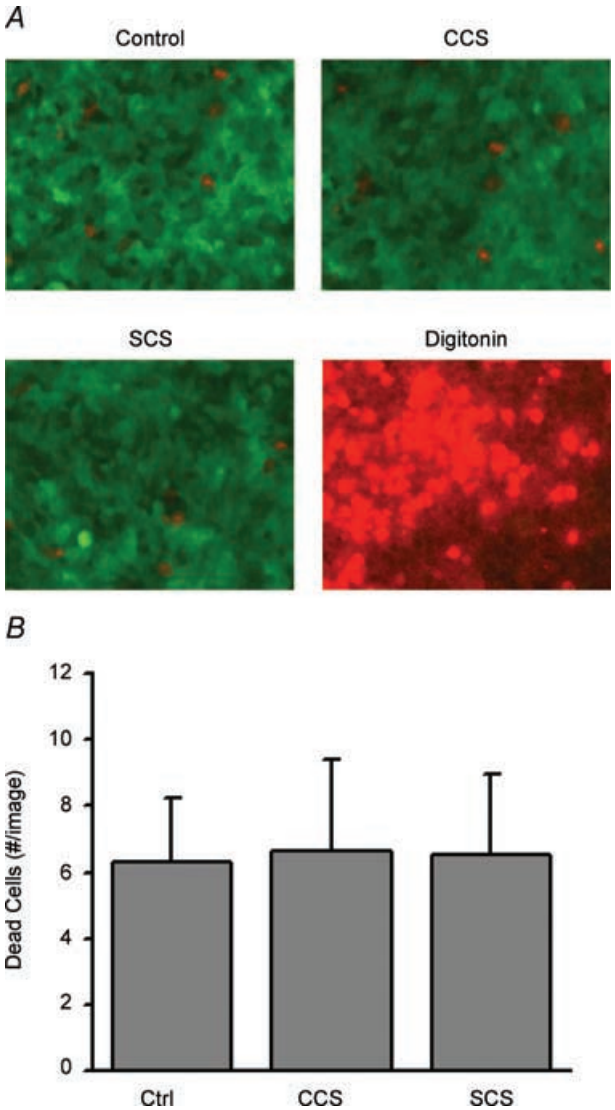

Figure 3. Compressive stress does not affect cell viability
A, representative fluorescence micrographs of CF airway cultures after 1 h under control (ambient pressure), CCS (20 cmH_2O, 20 CPM), or SCS (20 cmH_2O) after staining with calcein-AM (viable cells stain green) and ethidium homodimer-1 (non-viable cells stain red). As a positive control, cell permeabilization with digitonin (50 μM) produced a large change in fluorescence, consistent with cell death. *B*, summary of results for ethidium homodimer-1 labelled cells. ($n = 6$ in each group.)

pressure (0 cmH_2O). Importantly, the pressure chosen for this study, 20 cmH_2O, did not significant affect TER during 24 h CCS or SCS, compared to controls (Fig. 4*B*). Altogether, these experiments indicate that CCS-mediated ATP release was not explained by cell damage or loss of barrier integrity, but rather by regulated mechanisms.

Effects of compressive stress on ASL volume homeostasis

Previous studies have demonstrated that increases in ASL [ATP], by addition of exogenous nucleotides (ATP or UTP) (Knowles *et al.* 1991) or stimulation of ATP release by phasic shear stress (Tarran *et al.* 2005), stimulates net liquid secretion via activation of $P2Y_2$ receptors and adenosine activation of A_{2B} receptors. We therefore assessed the ability of cyclic and static compressive stress to modulate ASL volume homeostasis in human airway cultures. We focused on identifying potential differences in ASL height during oscillatory stress (CCS), which elicited an increase in steady-state [ATP] on the apical epithelial surface *versus* constant stress (SCS), which failed to stimulate ATP release.

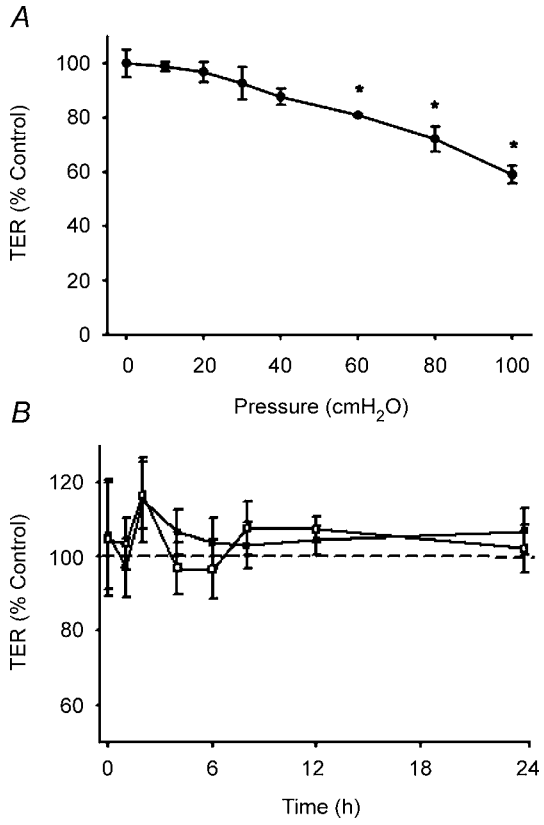

Figure 4. Compressive stress does not affect tight junctional integrity
A, mean transepithelial resistance, as a measure of tight junctional integrity, of CF cultures under CCS for 1 h at various pressure amplitudes ($n = 8$). *B*, time course of transepithelial resistance over 24 h CCS (□, 20 cmH_2O, 20 CPM, $n = 8$) or SCS (●, 20 cmH_2O, $n = 6$), relative to controls at ambient pressure, relative to control cultures at ambient pressure. (*Significantly different from control cultures.)

In normal cultures under control conditions, the 30 μl liquid challenge (added at $t = 0$) was absorbed over a period of ~12 h, followed by maintenance of the ASL height at levels corresponding to extended cilia (~7 μm) (Fig. 5*A* and *B*). This biphasic nature of ASL volume regulation, i.e. rapid removal of liquid from airway surfaces followed by a steady-state volume, suggested that active ion transport systems shifted from an absorptive to a balanced phenotype. Subjecting cultures to continuous CCS for 24 h did not alter the overall pattern of ASL volume regulation, i.e. early liquid absorption followed by a steady-state ASL volume. However, the initial rate of liquid absorption was significantly reduced by CCS compared to control conditions (Fig. 5*B*). In addition, steady-state ASL height was increased during CCS to 13.2 ± 1.7 μm, compared to 7.9 ± 0.9 μm under control conditions. In contrast, application of SCS for 24 h did not significantly alter either the initial rate of absorption or steady-state ASL height (7.5 ± 1.7 μm) compared to controls.

Reflecting the ion transport defects of CF airway epithelia, the CF cultures under control conditions (Fig. 5*A* and *B*) absorbed the added liquid more rapidly than normal cultures during the initial phase and removed all of the available liquid from airway surfaces before a 7 μm plateau phase was achieved. The steady-state ASL height in CF cultures at 24 h was 3.9 ± 0.8 μm, a level that reflects the minimal height of the cilia collapsed onto the surface of the epithelium. However, in CF cultures subjected to CCS for 24 h (Fig. 5*C*), the initial rate of liquid absorption was significantly reduced when compared to control conditions. More importantly, the steady-state ASL height was increased 3-fold by CCS to 11.9 ± 3.4 μm, compared to control CF cultures. In CF cultures subjected to SCS for 24 h, neither the initial rate of absorption of the exogenously applied solution nor the steady-state ASL height (3.2 ± 0.4 μm) was significantly altered. Collectively, these data suggest that rhythmic stimulation is required to maintain ASL to heights that are compatible with proper mucociliary clearance.

Experiments were designed to identify the mechanisms mediating the CCS-mediated increase in ASL height of CF epithelia. First, we tested whether the activation of purinoceptors, due to the stimulation of ATP release during CCS, but not SCS, was responsible for the restoration of normal ASL height by CCS but not SCS. We therefore repeated steady-state ASL height measurements on the apical surface of CF airway cultures 24 h after initiation of CCS or SCS in the presence of apyrase, an enzyme that efficiently cleaves released ATP. In marked contrast to CCS alone, CCS–apyrase-treated CF cultures (Fig. 5*D*, CCS + APY) exhibited accelerated volume absorption and ASL depletion similar to CF cultures under static or SCS conditions. These data

strongly implicate ATP as the principal mediator of increased ASL volume in CF cultures during CCS.

To investigate the putative role of ATP-induced Cl^- secretion in CCS-mediated ASL volume regulation in CF, additional cultures were pretreated with a blocker of Cl^- secretion, bumetanide (10^{-4} M, serosal), then subjected to CCS for 24 h. Bumetanide significantly inhibited the CCS-mediated restoration of normal ASL height (Fig. 5*D*, CCS + BUM). These data support a critical role for the ATP-mediated stimulation of Cl^- secretion in the adjustment of ASL height during CCS that was not observed during SCS.

Bioelectric properties of airway epithelia during compressive stress

The biphasic nature of ASL volume regulation, i.e. rapid removal of liquid from airway surfaces, followed by a steady-state volume, suggests that active ion transport systems are adjusted from an absorptive to a balanced absorptive/secretory phenotype. The modulation of steady-state ASL volume by CCS suggests that CCS-mediated modulation of ion transport mechanisms must occur. To identify the ion transport processes involved in the rebalancing of ASL height during CCS, transepithelial potential difference (V_t) measurements under thin-film conditions were performed before and after sequential exposure to inhibitors of Cl^- secretion (bumetanide, 10^{-5} M) and Na^+ absorption (benzamil, 10^{-5} M) immediately following the addition of 'excess' liquid (TBR, 30 μl) and at steady state (24 h later), under conditions identical to the ASL volume measurements. It should be noted that while the drug-sensitive voltages are not completely independent measurements, as the application of one blocker will change the driving force of the other ion (Willumsen *et al.* 1989; Willumsen & Boucher, 1991), the experiments here were designed to minimize this effect, and to evaluate the

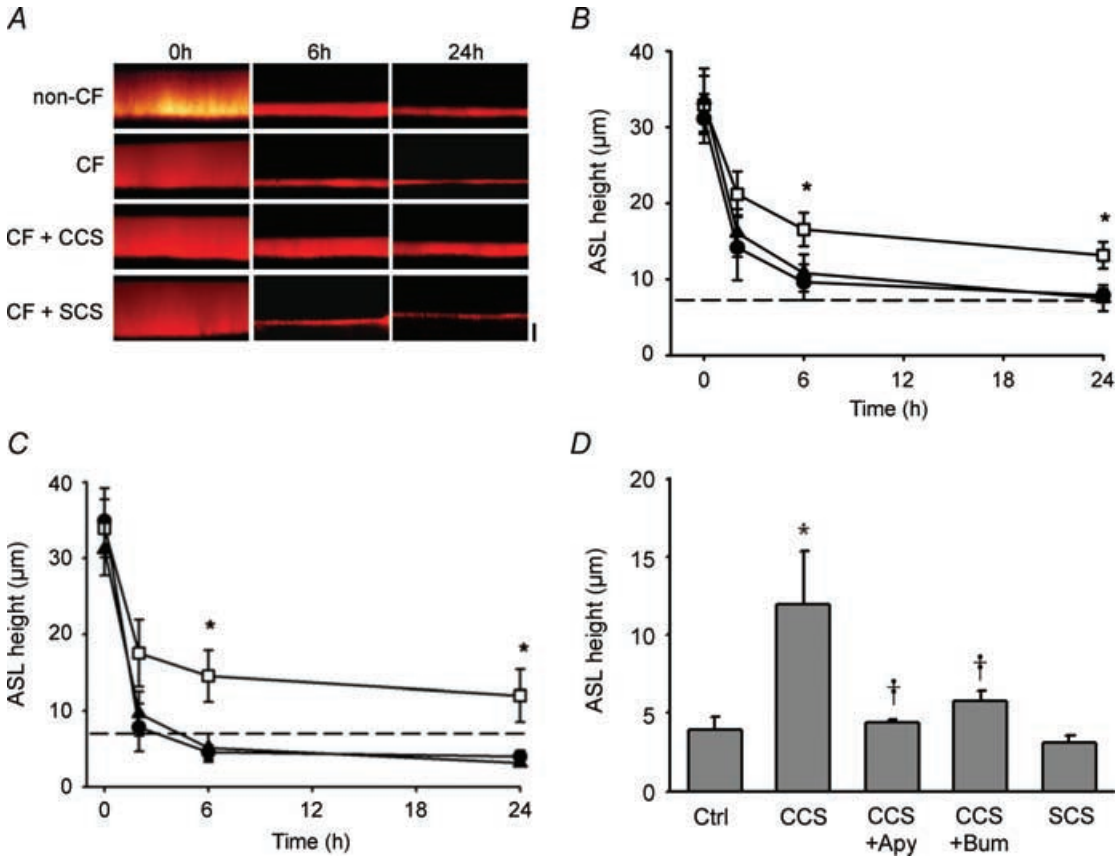

Figure 5. CCS, but not SCS induces net liquid secretion in normal and CF airway epithelial cultures
A, XZ confocal images of ASL at 0, 6 and 48 h after mucosal addition of 30 μl TBR containing Texas red–dextran to normal and CF airway cultures under control conditions and CF cultures undergoing CCS (20 cmH_2O, 20 CPM) or SCS (20 cmH_2O). Scal bar = 10 μm. *B* and *C*, time course of changes in ASL height on normal (*B*) and CF (*C*) cultures under control (●), CCS (□), or SCS (▲) conditions. ($n = 6$ in each condition.) Dotted line denotes ASL height of normal airway cultures in steady-state conditions. *D*, mean ASL height of CF cultures (at 24 h) under control, SCS, or CCS conditions in the absence and presence of apyrase (Apy; 5 U ml^{-1}, $n = 9$) or bumetanide (10^{-5} M, $n = 6$). (*Significantly different than control cultures. †Significantly different from CCS alone.)

relative contribution of Na^+ and Cl^- in each experimental condition. Hence, bumetanide was added first, as it has negligible effects on both the intracellular sodium activity (a_{Na}) and apical membrane potential (i.e. driving force for sodium).

In normal cultures under control (atmospheric pressure) conditions, benzamil-sensitive V_t dominated the pattern of bioelectrics immediately after the addition of TBR ($t = 0$, Fig. 6*A*), consistent with active liquid absorption (Fig. 5*A*). As the excess volume was absorbed, reaching the steady-state ASL height (24 h), bumetanide-sensitive V_t remained constant (Fig. 6*A*), while benzamil-sensitive V_t decreased significantly (from 6.3 ± 0.4 to 4.5 ± 0.2 mV; $P = 0.004$). This data suggests that the pattern of ion transport shifted from an absorptive to a more 'balanced' phenotype to maintain ASL volume. In normal cultures undergoing continuous CCS, a greater bumetanide-sensitive V_t at 24 h was observed (4.5 ± 0.6 *versus* 8.3 ± 0.8 mV), representing a shift to a more secretory phenotype that was consistent with the elevated steady-state ASL height (Fig. 5*B*). However, application of SCS for 24 h had no effect on the magnitude of bumetanide-sensitive V_t (5.2 ± 1.0 mV), mirroring the lack of effect of SCS on ASL height.

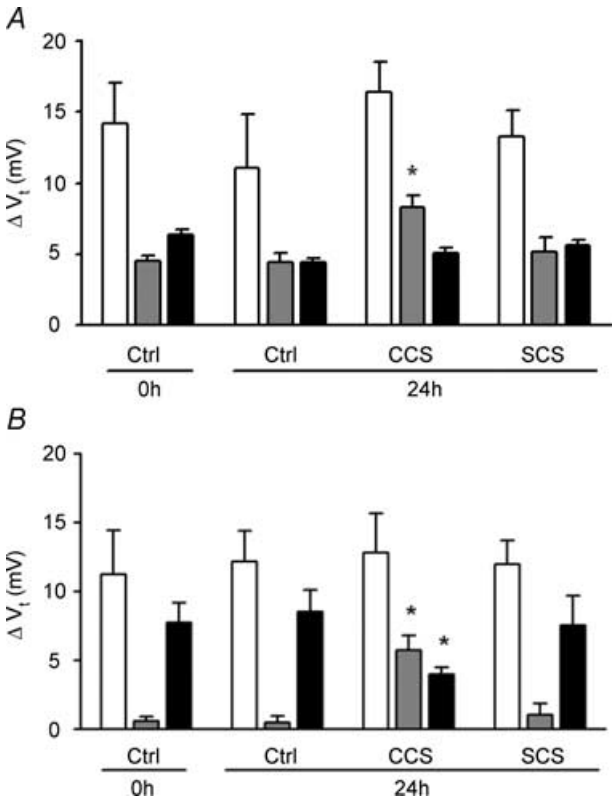

Figure 6. CCS alters the pattern of Na^+ and Cl^- bioelectrics in normal and CF airway cultures
Bar graphs depicting total potential difference (V_t) (open bars) and changes in V_t in response to sequential application of bumetanide (10^{-4} M, basolateral; grey bars) and benzamil (10^{-5} M, apical; black bars) in normal (*A*, $n = 5$) and CF (*B*, $n = 6$) cultures under control, CCS (20 cmH_2O, 20 CPM) or SCS (20 cmH_2O) at 0 and 24 h after mucosal addition of 30 μl TBR. (*Significantly different from control cultures (Ctrl) at 24 h.)

While the basal V_t in cultures of CF tracheobronchial epithelia at atmospheric pressure conditions were not significantly different from normal cultures, their pattern of bioelectrics in the presence of ion transport blockers was significantly different from those of normal airway epithelia (Fig. 6*B*). There was (1) a virtual absence of bumetanide-sensitive V_t in CF cultures relative to the normal cultures at $t = 0$ ($P < 0.0001$); and (2) at 24 h, the magnitude of benzamil-sensitive V_t in CF cultures was not reduced compared to $t = 0$, despite the fact that all available liquid had been absorbed from the surface of the epithelium. Applying continuous CCS to CF cultures dramatically altered the pattern of Na^+ and Cl^- bioelectrics. After 24 h, CCS exhibited a significantly increased bumetanide-sensitive V_t (0.4 ± 0.4 *versus* 5.7 ± 1.1 mV) and a reduced benzamil-sensitive V_t (8.5 ± 1.6 *versus* 4.0 ± 0.5 mV) compared to $t = 0$ (control) cultures. Similar to normal culture data, CF cultures subjected to continuous SCS failed to exhibit significant changes in either benzamil- or bumetanide-sensitive V_t. Together, these data suggest that CCS, but not SCS, adjusts the ASL height of CF airway epithelia through alterations in both Cl^- secretion and Na^+ absorption across the apical membrane.

Effect of compressive stress on cilia beating and mucus transport

Cilia beat frequency in human airway epithelia is regulated by changes in intracellular Ca^{2+} concentrations (Zhang & Sanderson, 2003). While a variety of agonists induce Ca^{2+} mobilization, ATP-mediated purinoceptor activation is amongst the most efficient signalling pathways. We therefore investigated whether CCS-mediated ATP release regulates cilia beating activity and mucus transport rates. Measurements of cilia movement in normal cultures by high-speed phase-contrast video microscopy indicated that 1 h of continuous CCS significantly increased CBF from 7.4 ± 0.6 to 10.8 ± 0.2 bps (Fig. 7*A*). However, cultures undergoing 1 h of continuous SCS did not exhibit significant changes in CBF rate (6.9 ± 0.6 bps). The relationship between CCS and CBF was tested with apyrase (5 U ml^{-1}) added to the ASL prior to CCS. Enzymatic removal of ASL ATP completely inhibited CCS-induced increase in CBF.

Cilia beat frequency and ASL height are key factors determining the rate of clearance of mucus from the surface of ciliated airway epithelia. We therefore assessed the impact of compressive stress on mucociliary transport (MCT) rates by recording the movement of fluorescent microspheres (1 μm diameter) (Matsui *et al.* 1998*b*)

added to the apical surface of CF cultures (in 30 μl TBR). MCT was measured ~15 min after the addition of the fluorescent microspheres ($t = 0$) and at various time points over the subsequent 3 h of under continuous CCS, SCS, or control conditions. During this time, ASL volume was not a limiting factor (i.e. ASL was not depleted). In well-differentiated CF cultures subjected to 1 h continuous CCS, the angular velocity of fluorescent microspheres doubled (from 32.4 ± 1.1 to 65.4 ± 8.3 μm s^{-1}), compared to control conditions (Fig. 7*B*). In contrast, 1 h continuous SCS did not alter the rate of MCT (30.5 ± 1.2 μm s^{-1}) on CF airway epithelia.

As a consequence of proper balancing of ASL volume, MCT persists for several days in normal cultures after the addition of TBR containing fluorescent beads (Matsui *et al.* 1998*a*). However, MCT is abolished in CF cultures 4–6 h after the addition of fluorescent beads as a result of ASL depletion. Based on the finding that 24 h continuous CCS maintained the ASL layer of CF cultures to a height that should enable ciliary beat, we tested the long-term impact of compressive stress on MCT in CF airway cultures. Figure 8*A* and *B* shows that, contrary to control conditions (MCT = 4.5 ± 0.4 μm s^{-1}), the CF cultures chronically subjected to CCS sustained MCT for 48 h after the addition of 30 μl TBR containing fluorescent beads at levels (47.6 ± 2.7 μm s^{-1}), similar to rates measured in normal cultures. In cultures subjected to continuous SCS, the rate of MCT of CF cultures 48 h after volume addition (3.6 ± 1.7 μm s^{-1}) was not statistically different from control CF cultures. Together, these studies suggest

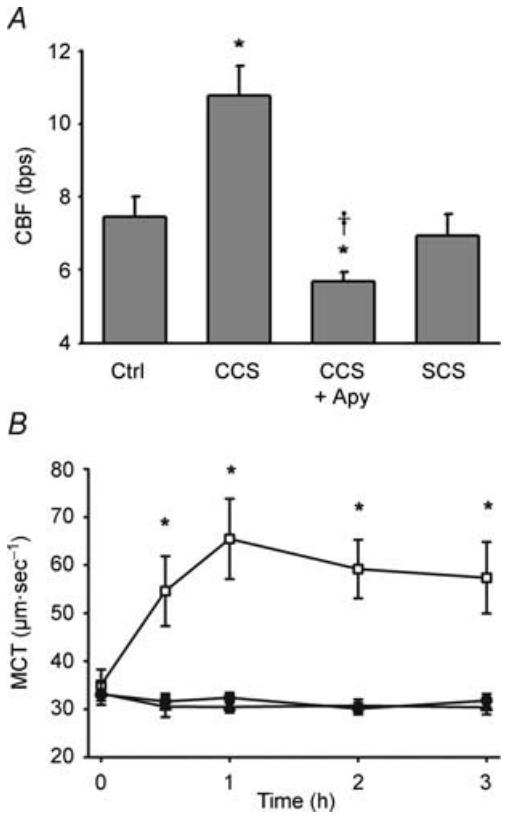

Figure 7. CCS, but not SCS, increases cilia beat frequency (CBF) and mucociliary transport (MCT) rates
A, summary of CBF measurements from CF cultures after 1 h control (Ctrl, $n = 10$), SCS (20 cmH_2O, $n = 7$) or CCS (20 cmH_2O, 20 CPM, $n = 10$) alone or in the presence of apyrase (CCS + APY, $n = 5$). *B*, rates of MCT after the addition of 30 μl TBR to CF cultures under control (●), CCS (□, 20 cmH_2O, 20 CPM), or SCS (▲, 20 cmH_2O) ($n = 7$ in each group). (*Significantly different from control cultures. †Significantly different from CCS alone.)

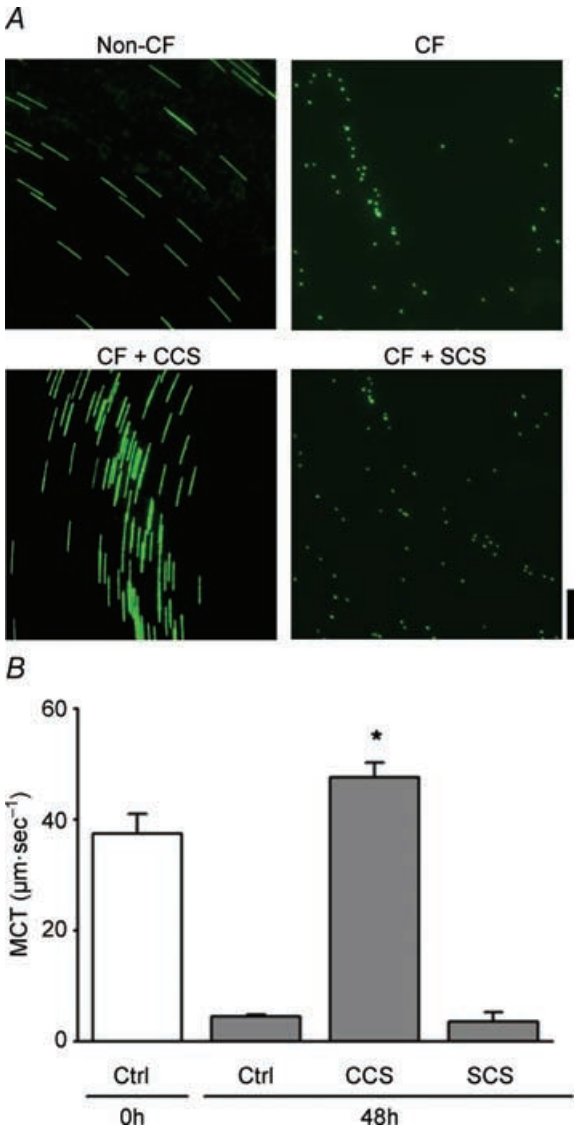

Figure 8. Long-term preservation of MCT on cultured CF airway epithelium undergoing CCS
A, representative 2 s exposures of fluorescent microspheres on CF airway cultures 48 h after the addition of 30 μl TBR under control and CCS (20 cmH_2O). A similar exposure from a normal culture, at 48 h, is shown for comparison. Scale bar = 100 μm. *B*, summary of MCT rates before (control; open bars) and after 48 h (filled bars) of control, SCS (20 cmH_2O), or CCS (20 cmH_2O, 20 CPM). $n = 5$–7 in each group. (*Significantly different from control cultures (Ctrl) at 48 h.)

that rhythmic, and not static, compressive stress can restore normal ASL height, cilia beating and mucus transport to CF airway epithelia.

Discussion

Human airway epithelia are low-resistance (Boucher, 1994), water-permeable (Verkman *et al.* 2000) barriers that are unable to sustain osmotic gradients. The capacity to both absorb Na^+ and secrete Cl^- provides the epithelium with the means to fine tune the amount of liquid in the ASL to levels that mediate efficient mucus clearance (Boucher, 2003). A central question is how airway epithelia sense the needs for ASL on airway surface and produce ASL volumes optimal for lung defence.

One requirement for lung defence is tidal breathing, raising the possibility of a link between tidal breathing, ASL volume, and the rates of mucociliary clearance. As a mechanism to link these functions, we hypothesized that (1) the mass of nucleotides and nucleosides on airway surfaces is regulated by the rate of ATP release triggered by airflow/transmural pressure-induced mechanical stress, and (2) the mass of nucleotides and nucleosides in the ASL determines ASL volume/height via the activation of purinoceptors on the apical membrane.

Despite evidence that nucleotides are important ASL volume regulators, information describing the regulation of the release of nucleotides by airway cells is sparse. Mechanical stress applied to a variety of cell types appears to be a common mechanism for ATP release. Indeed, the process of normal tidal breathing exposes airway cells to complex mechanical forces. Movement of air across the surface of the airway imparts a shear stress on the surface epithelium, peeking at 0.5 dyne cm^{-2} during tidal breathing (Tarran *et al.* 2005). Trans-airway pressure gradients also occur during respiration. For example, during passive expiration during normal dial breathing, transmural pressure of $\sim$8.5 cmH_2O are routinely generated (Levitzky, 1991). During forced expiration, more dispersed transmural pressure gradients are generated within the airways that can approach 20 cmH_2O in the proximal airways.

In addition to oscillatory forces, the airway cells can be subjected to continuous, non-phasic, transmural pressures. For example, during bronchoconstriction, constriction of smooth muscles in the airway wall causes overlying airway epithelium to fold into crevasses, producing continuous, non-oscillatory, positive trans-epithelial pressure (Gunst & Stropp, 1988; Ressler *et al.* 2000). The magnitude of the resulting positive pressure exerted on airway epithelia has been previously estimated (Ressler *et al.* 2000) and can exceed 30 cmH_2O.

Mechanical stresses on airways are even more extreme during cough (Leith, 1985). In the initial, 'compression phase' of a cough, the combination of closure of the glottis with the rapid onset expiratory muscle activity produces very rapid changes (600–1600 $cmH_2O\,s^{-1}$) in subglottic transpulmonary pressures, reaching sustained pressures as high as 200 cmH_2O. During the expiration phase of coughing, this pressure abruptly falls, producing peak air flow rates of greater than 500 l min^{-1} (Leith, 1985). The level of surface shear stress under these conditions can reach as high as 1700 dynes cm^{-2} (Basser *et al.* 1989).

In the present study, we used a novel, computer-controlled device to impart oscillatory positive trans-epithelial pressures, at a frequency consistent with normal tidal breathing, to well-differentiated human airway epithelial cultures. While this system does not recapitulate the substantial elongation/stretching of the epithelium manifested *in vivo* during tidal breathing, the cell compression utilized in this system did expose cells to transepithelial pressure gradients typical of tidal breathing *in vivo*. Using this model system of phasic airway compression, we established the relationship between the magnitudes of cyclic compressive stress (CCS) and steady-state ASL ATP concentrations (Fig. 2*A*). The relationship between ASL [ATP] and transepithelial pressure with CCS was steepest in the physiological range of normal, non-forced, breathing (i.e. 0–5 cmH_2O). At pressures of greater than 5 cmH_2O the magnitude of change in ATP release relaxed. Using a cocktail of ectonucleotidase inhibitors to inhibit endogenous ectoATPase activities (Fig. 2*B*) and independent measurements of endogenous ecto-ATPase activities (Fig. 2*C*), our results demonstrated that the change in apical [ATP] during CCS was not due to a reduction in cell surface metabolism but rather to the stimulation of ATP release.

We also provided evidence that CCS raises bilaterally [ATP] in liquids bathing primary cultures of human airway epithelial cells (Fig. 2*A* and *B*). This finding is in agreement with previous studies describing the stimulation of both apical and basolateral release of ATP, and subsequent activation of P2Y purinoceptors, by physical deformation of cultured airway epithelia (Homolya *et al.* 2000). However, this result differs from experiments subjecting airway cells to apical surface perfusion-generated shear stress (Tarran *et al.* 2005) in which apical [ATP] was selectively raised. Collectively, these data suggest that mechanical strains imparted by perfusion-induced apical shear stress are confined to the apical cellular domains, whereas compressive stress is transmitted to both apical and basolateral domains (Tschumperlin *et al.* 2004).

An interesting observation was that static compressive stress (SCS), in the form of non-oscillatory transepithelial pressure, at the same magnitude used during CCS, did not stimulate significant changes in steady-state ATP concentrations (Fig. 2*A*). Since CCS increased steady-state ASL [ATP] by increasing the rate ATP release, the absence of a change in ASL [ATP] during SCS suggests that (1)

ATP release is triggered by the rate of change in pressure and/or (2) the mechanisms involved in sensing the stress or ATP release rapidly adapt. Similarly, perfusion-generated shear stress was shown to induce ATP release from cultured human airway epithelia in response to phasic, but not continuous, shear stress (Tarran *et al.* 2005).

The action of ATP released during CCS can be mediated by a large group of cell surface ATP receptors, i.e. purinoceptors, including members of the P2X and P2Y families (Leipziger, 2003). While P2X receptors are ligand-gated ion channels that are activated by ATP and are cation selective, the P2Y receptors are G-protein-coupled receptors that predominantly couple to Gq, activating phospholipase C (Schwiebert & Zsembery, 2003). Studies have demonstrated that airway epithelia are likely to express a number of isoforms of both the metabotropic P2Y (Communi *et al.* 1999; Cressman *et al.* 1999) and inotropic P2X (Korngreen *et al.* 1998; Taylor *et al.* 1999; Zsembery *et al.* 2003) receptors. However, elucidation of specific physiological roles for P2Y and P2X purinoceptors in response to airway ATP has been significantly hindered by the general absence of high-affinity antagonists that exhibit suitable subtype selectivity. Several lines of evidence suggest that $P2Y_2$ is the predominate purinoceptor involved in ATP-mediated ASL homeostasis. Fist, it has been shown on human airway epithelial cells that UTP and ATP equally regulate changes in intracellular Ca^{2+} mobilization (Mason *et al.* 1991; Homolya *et al.* 1999) and ion transport (Jiang *et al.* 1993; Benali *et al.* 1994; Hwang *et al.* 1996) suggesting a common purinoceptor (Brown *et al.* 1991). Of the purinoceptors present in human airway epithelial cells, only the $P2Y_2$ receptor exhibits such pharmacology (Muller, 2002). Second, studies using $P2Y_2$ receptor-deficient mice demonstrate that intracellular Ca^{2+} mobilization during mechanical stress is mediated mainly by $P2Y_2$ receptors (Homolya *et al.* 2000). Finally, ATP-mediated inhibition of Na^+ transport requires hydrolysis of phosphatidylinositol-bisphosphates (PIP_2) (Yue *et al.* 2002) via $P2Y_2$ receptor activation (Kunzelmann *et al.* 2005). While P2Y receptors represent the predominate purinoceptors involved in eliciting changes in ion transport and fluid regulation, numerous studies support the role of both P2Y (Morse *et al.* 2001) and P2X (Korngreen *et al.* 1998; Ma *et al.* 1999) purinoceptors in the regulation of cilia beating, and hence, the rate of mucus clearance.

While the mechanism of ATP release under both basal and stimulated conditions remains elusive, it has been suggested that ATP release involves the activity of one or more ion channels of the 'ATP binding cassette' family (reviewed by Schwiebert, 1999). However, whether one member of this family, the cystic fibrosis transmembrane conductance regulator (CFTR), contributes to ATP release remains controversial. It has been suggested that CFTR mediates ATP release directly (Reisin *et al.* 1994; Schwiebert *et al.* 1995; Pasyk & Foskett, 1997) or facilitates its release by regulating a separate ATP release pathway (Sugita *et al.* 1998; Braunstein *et al.* 2001). However, other studies have failed to find a link between CFTR and ATP release (Reddy *et al.* 1996; Grygorczyk & Hanrahan, 1997; Watt *et al.* 1998). Our studies, using primary human airway epithelial cultures demonstrate that ATP release rates under basal conditions (control) as well as stimulated (CCS) were identical between normal and CF cultures lacking CFTR. These findings are consistent with a prior study utilizing another type of mechanical stress (hypotonic shock), which demonstrated no difference in ATP release rates between normal and CF airway epithelium (Okada *et al.* 2006). Together, these data argue against the involvement of CFTR in human airway epithelial ATP release under either basal or stress-stimulated conditions.

An important issue with respect to mechanical release of cellular ATP in any study is whether ATP release is regulated or occurs as a consequence of cell damage. The procedures used in this study were designed to avoid any significant contribution of epithelial damage to ATP release. Nevertheless, the lysis of a very small number of cells (approximately 10 in a 1 cm^2 culture), containing millimolar ATP (Lazarowski *et al.* 2004), would release sufficient ATP into the small volume of ASL in our cultures to account for the initial increase in [ATP] measured during CCS. However, once ATP is released on epithelial surfaces, it is rapidly metabolized by a complex ecto-enzyme system that dephosphorylates and transphosphorylates purine and pyrimidine molecules. The half-life of ATP in this environment, as measured by the degradation of exogenously added [γ-^{32}P]ATP, is on the order of seconds (Lazarowski *et al.* 2004). Therefore, the continuous lysis of many cells over time (estimated at 60 cells per culture per minute) would have to occur to account for the steady-state ATP accumulation observed during CCS (Fig. 2*A*). Our cytolysis assay used in this work has the sensitivity to detect the $> 1\%$ incidence of damaged cells required for sustained (> 1 h) increases in [ATP]. The fact that we did not detect evidence for CCS-induced increased cell lysis, strongly argues against such a mechanism for ATP release during CCS.

The physiological importance of CCS-induced ATP release was examined by measuring the purinoceptor-mediated epithelial responses that support mucus clearance. First, we investigated the regulation of ASL height on human airway cultures under control conditions. Under control conditions, the recovery of steady-state ASL height following the addition of a small excess volume to the apical surface of normal cultures displayed a biphasic pattern. Our results investigating the pattern of bumetanide- and benzamil-sensitive V_t suggest that the initial rapid decline in liquid height was dominated by Na^+ absorption, followed by a

plateau, steady-state, phase reflecting more balanced Na^+ absorption *versus* Cl^- secretion. A key finding in this work is that continuous CCS rebalanced ASL volume at higher plateaus in normal human epithelial cultures. Measurement of ASL volume homeostasis revealed that the initial absorption rate of the added liquid was reduced by CCS and was followed by a plateau level that was significantly higher than controls at atmospheric pressure (Fig. 5*B*). The importance of this finding is that there is no unique level of ASL height; rather, in response to increased CCS-induced ATP release and increased ASL [ATP], ASL height/volume also increased. We speculate that this capacity allows for normal airway epithelium to respond to airway stresses with an appropriate volume of ASL. It is worth noting, however, that these experiments were performed in the absence of mucus. *In vivo*, we speculate ASL in excess of the $\sim$7 μm PCL is transferred to the overlaying mucus layer, resulting in the increased hydration state of the mucus and accelerated clearance.

Airway epithelial cultures from CF patients failed to maintain normal ASL height under static conditions. Liquid absorption was (1) increased and (2) failed to slow as ASL height approached 7 μm (Fig. 5*C*). A defect in Na^+ transport regulation of ASL volume was revealed in measures of the magnitude of benzamil-sensitive V_t, which were not reduced at $t = 24$ compared to $t = 0$, as observed with normals. The absence of a bumetanide-sensitive V_t during conditions of volume depletion identified a role for Cl^- secretion in ASL volume homeostasis. Importantly, both defects reflect absent CFTR function.

Continuous CCS restored ASL height on CF airway epithelia to levels sufficient for effective mucus transport. The pattern of bioelectrics of CF cultures under continuous CCS were consistent with CCS-mediated ATP release and activation of purinoceptors, leading to an inhibition of Na^+ absorption and stimulation of CaCC-mediated Cl^- secretion (Fig. 6*B*) (Lazarowski *et al.* 2004). The net result was the conversion of the CF epithelium from unregulated Na^+ absorption to an epithelium that modulated Na^+ absorption and exhibited Cl^- secretion. The role of the ATP signalling pathway in this conversion was supported by the observation that enzymatic removal of ATP using apyrase prevented the CSS-mediated restoration of ASL volume in the CF cultures.

Mucociliary clearance depends on the coordinated activity of cilia beating within the low-viscosity PCL layer. Various chemical mediators stimulate ciliary beat frequency (CBF) via intracellular signals including cAMP, cGMP, nitric oxide, and Ca^{2+} mobilization (Satir & Sleigh, 1990; Salathe *et al.* 2000), and extracellular ATP is amongst the most potent Ca^{2+}-dependent stimulators of cilia activity (Evans & Sanderson, 1999; Lansley & Sanderson, 1999; Zhang & Sanderson, 2003). Cilia beating is also stimulated during mechanical stimulation (Lansley & Sanderson, 1999), a finding consistent with our observation of CCS-mediated stimulation in ciliary activity (Fig. 7*A*). The ligand mediating CCS-induced increases in CBF could be ATP itself or the metabolic product adenosine, which has been shown to increase CBF on human airway epithelia through A_{2b} receptors (Morse *et al.* 2001) by a cAMP-independent signalling pathway (Zhang & Sanderson, 2003). However, we found that apyrase, which degrades ATP and is predicted to increase ASL adenosine concentration, completely inhibited the CCS-induced stimulation of cilia beating activity. These results suggest that ATP-mediated purinoceptor activation represents the predominant mechanism stimulating CBF on CCS-stimulated airway epithelia.

The human airway epithelial cultures utilized in this study display rotational displacement of mucus, as evidenced by the coordinated movement of fluorescent microspheres in the ASL air–liquid interface (Matsui *et al.* 1998*a*). While normal cultures maintain this transport for many days, CF airway cultures cease transport of the microspheres 6–8 h after excess liquid is added due to hyperabsorption of the ASL layer (Fig. 8*A* and *B*). A key finding in the present study was that mucus transport activity could be maintained in CF airway cultures for several days under continuous CCS conditions (Fig. 8*A* and *B*). Collectively, these experiments demonstrate that CCS restores MCC on CF airway epithelia by a mechanism involving increased ATP release and purinoceptor-mediated rebalancing of ion fluxes across the apical membrane, increased ASL height, and increased cilia beating activity and mucus transport.

In summary, our data demonstrate that oscillatory compressive stress, mimicking stress observed in the lung during tidal breathing, increases the rate of ATP release onto the surface of the airways. The consequence of an elevated ASL ATP concentration is to shift the pattern of ion transport from absorption to secretion, via activation of apical purinoceptors, producing liquid secretion and acceleration of MCC. In this way, the normal lung can respond to stress-inducing stimuli on the intrapulmonary airways by stimulating secretion and accelerating clearance, thus promoting lung health. Our data also demonstrated that application of constant, non-oscillatory, stresses of identical magnitude did not stimulate airway epithelial ATP release. Therefore, during asthma-associated bronchoconstriction, the constant airway stresses generated would not be expected to result in the stimulation of either ASL volume or MCC. Finally, dependence of rhythmic mechanical stresses on ATP release rates may account for the preservation of MCC in young CF patients prior to the onset of chronic airway obstruction by mucus plugs. The beneficial effects of physical and deep-breathing exercise in CF patients (Dodd & Prasad, 2005) may reflect the effects of increased physical forces on the airways that stimulates ATP release, and hence, airway hydration and mucus clearance.

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