AMPK Agonists Ameliorate Sodium and Fluid Transport and Inflammation in Cystic Fibrosis Airway Epithelial Cells

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The metabolic sensor AMP-activated kinase (AMPK) inhibits both the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) Cl^- channel and epithelial Na^+ channel (ENaC), and may inhibit secretion of proinflammatory cytokines in epithelia. Here we have tested in primary polarized CF and non-CF human bronchial epithelial (HBE) cells the effects of AMPK activators, metformin and 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR), on various parameters that contribute to CF lung disease: ENaC-dependent short-circuit currents (I_{sc}), airway surface liquid (ASL) height, and proinflammatory cytokine secretion. AMPK activation after overnight treatment with either metformin (2–5 mM) or AICAR (1 mM) substantially inhibited ENaC-dependent I_{sc} in both CF and non-CF airway cultures. Live-cell confocal images acquired 60 minutes after apical addition of Texas Red–dextran-containing fluid revealed significantly greater ASL heights after AICAR and metformin treatment relative to controls, suggesting that AMPK-dependent ENaC inhibition slows apical fluid reabsorption. Both metformin and AICAR decreased secretion of various proinflammatory cytokines, both with and without prior LPS stimulation. Finally, prolonged exposure to more physiologically relevant concentrations of metformin (0.03–1 mM) inhibited ENaC currents and decreased proinflammatory cytokine levels in CF HBE cells in a dose-dependent manner. These findings suggest that novel therapies to activate AMPK in the CF airway may be beneficial by blunting excessive sodium and ASL absorption and by reducing excessive airway inflammation, which are major contributors to CF lung disease.

Keywords: metformin; cystic fibrosis transmembrane conductance regulator; ENaC; airway surface liquid; inflammation

CLINICAL RELEVANCE

Cystic fibrosis lung disease is characterized by excessive airway surface liquid reabsorption and inflammation, leading to recurrent infections, eventual destruction of lung parenchyma, and respiratory failure. This study, performed using primary human airway epithelial cells, suggests that novel therapies to activate the metabolic sensor AMP-activated kinase (AMPK) in the CF airway may be beneficial both by blunting excessive salt and fluid reabsorption and by reducing excessive airway inflammation.

The serious genetic disease cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) Cl^- channel expressed at the apical membrane in a variety of epithelial tissues, including the lung, where CF lung disease causes considerable morbidity and mortality (1). Defective CFTR function prevents airway epithelium from adequately regulating the airway surface liquid (ASL) volume, which has an adverse effect on mucus clearance from the lung. The CF airway invariably becomes colonized by bacteria such as *Pseudomonas aeruginosa* and develops excessive inflammation, which can cause extensive damage to lung architecture and eventuate in respiratory failure (2). There is growing evidence that a lack of functional CFTR promotes an exaggerated or prolonged inflammatory response to bacterial and other insults, although the underlying pathophysiologic mechanisms are unclear (3–5). Both up-regulation of proinflammatory mediators (e.g., IL-8, IL-6, TNF-α, and GM-CSF) and down-regulation of anti-inflammatory mediators (e.g., IL-10 and inducible nitric oxide synthase) in the CF airway may play an important role in this process (6–8).

Excessive activity of the epithelial sodium channel (ENaC) on the luminal airway surface is a key factor involved in the development of the CF lung phenotype. This defect has been proposed to cause excessive reabsorption of the ASL, reduced airway clearance, and increased susceptibility to chronic inflammation, as demonstrated in a β-ENaC transgenic mouse model (9–11). Therapies to reduce chronic inflammation (steroids, nonsteroidal anti-inflammatory drugs, and statins), combat chronic bacterial colonization (inhaled antibiotics), and enhance airway clearance (hypertonic saline and inhaled recombinant human DNase) have improved outcomes in patients with CF lung disease (12–14). However, therapeutic trials aimed at reducing excessive airway ENaC activity (e.g., through treatment with aerosolized amiloride or its analogs) have proven disappointing, potentially due to poor access of the drug to the channels at the apical membrane, short activity half-life, and/or deleterious effects on airway permeability (14–16). Drugs that can be delivered through the blood that inhibit the channel without directly binding to it at the apical membrane may circumvent these problems and be promising choices for therapy. Furthermore, drugs that target multiple pathogenic factors in CF lung disease and combination therapeutic approaches that attack multiple cellular pathways or targets could have additive or synergistic beneficial effects.

AMP-activated kinase (AMPK) is a ubiquitous Ser/Thr kinase whose activity increases during conditions of metabolic

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and other cellular stresses. AMPK is activated via phosphorylation of the α subunit at Thr-172 in its "activation loop" by upstream AMPKKs (17), which include the LKB1 complex (18), the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) (19), and TGF-β–activated kinase 1 (20, 21). As a cellular metabolic sensor, AMPK acts on a wide variety of substrates and cellular pathways, including the regulation of metabolic pathways (e.g., glycolysis, fatty acid synthesis and oxidation, cellular glucose uptake, and cholesterol synthesis), apoptosis and the cell cycle, nitric oxide production, transcriptional regulation, signaling pathways involved with inflammation, and membrane transport (22–24). Therefore, changes in cellular AMPK activity and function may have broad effects on overall cellular functioning.

Recent work from our laboratory demonstrated that AMPK is more diffusely distributed and has greater activity in primary polarized human bronchial epithelial (HBE) cells derived from patients with CF as compared with HBE cells derived from patients with other non-CF lung diseases (5). Expression of exogenous wild-type CFTR in the CF cells reversed AMPK activation. These CFTR-dependent AMPK activity effects were confirmed in immortalized CF bronchial epithelial (CFBE) cells derived from a ΔF508 homozygote that were stably transfected to overexpress either wild-type or ΔF508 CFTR (5, 25). Moreover, compared with wild-type CFTR–expressing CFBE cells, ΔF508 CFTR–expressing CFBE cells had greater secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-8. Further pharmacologic activation of AMPK in CFBE cells reduced proinflammatory cytokine production, suggesting that AMPK activation in CF airway cells is an adaptive response that reduces inflammation (5). In addition, we and others have previously found that AMPK activators inhibit both CFTR and ENaC channel activity in both the oocyte expression system and in polarized epithelial cells derived from lung and other tissues (24, 26–31). Membrane transport protein regulation by AMPK may provide a sensitive mechanism for the coupling of ion and solute transport to cellular metabolic status (24). Of note, excessive ENaC activity in the CF airway has been implicated in ASL volume depletion and subsequent manifestations of CF lung disease (9–11). Together, these findings led us to hypothesize that AMPK activators may be beneficial in the treatment of CF lung disease.

In contrast to primary airway cells, CFBE cells lack significant functional ENaC expression and thus may not be a suitable model system for the study of CF airway pathophysiology. The effects of AMPK activators on salt and fluid transport and the secretion of proinflammatory cytokines in primary polarized HBE cells are unknown. The goals of this study were to examine the effects of AMPK on the secretion of proinflammatory cytokines and salt and fluid transport in primary polarized HBE cells and to assess the potential utility of metformin as a novel therapeutic agent for CF lung disease using this model system. We first investigated the effects of the AMPK activators metformin and AICAR on ENaC and CFTR activity in HBE cells. We next investigated the effects of these drugs on real-time ASL reabsorption rates across polarized HBE cell cultures using live-cell confocal microscopy. We then used multiplex cytokine analysis to determine the effects of AMPK activation on proinflammatory cytokine secretion in both CF and non-CF primary polarized HBE cells. Finally, we tested the effects of lower, more therapeutically relevant concentrations of metformin, an FDA-approved drug for the treatment of diabetes mellitus, given over a 2-week period on ENaC activity and the secretion of proinflammatory cytokines. Our findings suggest that AMPK activation may be a beneficial therapeutic strategy for CF lung disease.

MATERIALS AND METHODS

Primary Human Bronchial Epithelial Cell Culture

HBE cells were cultured from excess pathological tissue following lung transplantation and organ donation under a protocol approved by the University of Pittsburgh Institutional Review Board. Cells were cultured on human placental collagen-coated Costar Transwell filters (Catalog #3470, 0.33 cm^2 0.4-μm pore; Lowell, MA) in 2% Ultroser G medium as previously described and used for experimentation after at least 4 to 6 weeks of culture at an air–liquid interface (32). Non-CF HBE cells were obtained from donors with idiopathic pulmonary fibrosis (five patients), chronic obstructive pulmonary disease (three patients), primary pulmonary hypertension (three patients), or secondary pulmonary hypertension due to congenital heart disease (one patient). Qualitative differences due to disease state were not observed. CF HBE cells were obtained from 13 patients with CF and the following CFTR mutations: ΔF508, W1282X, 1717 G->A, 2789+5 G->A, N1303K, and four unknowns.

Immunoblotting and Quantitation of AMPK Activity

Immunoblotting of polarized HBE cell lysates from individual Transwell filters was performed as described previously (5). Briefly, the intensities of relevant bands were quantitated using a Versa-Doc Imager with Quantity One software (Bio-Rad, Hercules, CA). To measure AMPK activity in the cell lysates, the AMPK-α-pThr-172 band signal in each lane was corrected for local background intensity and normalized to the intensity of β-actin as a loading control.

Measurements of CFTR- and ENaC-Dependent Short-Circuit Current

Short-circuit current (I_{sc}) measurements were performed using modified Ussing chambers designed to accept 0.33-cm^2 Transwells with Ringer's solution in both the apical and basolateral chambers as previously described (5). After the filters were mounted and steady-state I_{sc} was achieved, the ENaC-dependent I_{sc} was calculated as the change in I_{sc} following apical treatment with 10 μM amiloride. After amiloride addition, the CFTR-dependent I_{sc} was defined as the difference between the peak I_{sc} after addition of 10 μM forskolin and 1 mM isobutylmethylxanthine (IBMX) and the final I_{sc} after basolateral treatment with 20 μM bumetanide to block Cl^- secretion across the epithelium (32).

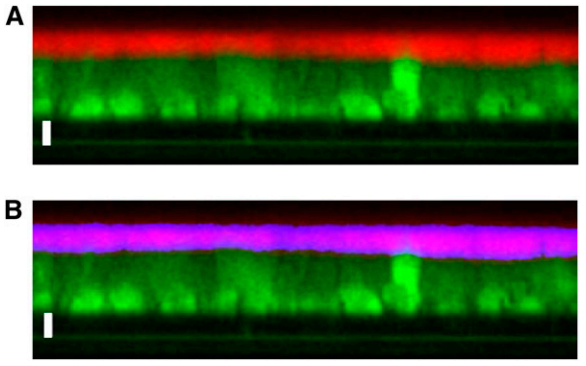

Figure 1. Method for airway surface liquid (ASL) height measurement. A quantity of 2.5 μl PBS containing Texas Red–dextran (10 kD) was applied to the apical surface of differentiated human bronchial epithelial (HBE) cultures and z-scanning confocal images were acquired 30 minutes later (*A*). The labeled ASL was selected using an automated thresholding routine as shown in *blue* (*B*), and the thresholded area was divided by the image width to derive the average ASL height for the entire image (12.33 μm in this example). The cells were loaded with CellTracker Green CMFDA (Molecular Probes) before the experiment to demonstrate the cell shapes and delineate the Transwell filter support. *Bars* = 10 μm.

ASL Height Measurements

To measure the rate of ASL absorption in the presence and absence of AMPK activators, the height of fluorescently labeled fluid on the apical surface of well-differentiated HBE cell cultures was measured serially using confocal microscopy (33). The HBE cells were exposed on both the apical and basolateral sides to 1 mM AICAR, 5 mM metformin, or vehicle control overnight before ASL measurement. Ringer's solution (100 μl) was added to the apical side for this overnight treatment. Subsequently the apical fluid was aspirated with a Pasteur pipette and replaced with 2.5 μl of PBS containing 2 mg/ml Texas Red–dextran (10 kD; Molecular Probes; Eugene, OR). After a 1-minute equilibration period, 50 μl of perfluorocarbon (PFC, FC-77; Sigma, St. Louis, MO) was gently pipetted onto the apical surface to prevent evaporative losses while on the microscope stage. The cultures were imaged on a modified stage of an inverted confocal microscope (Zeiss LSM 510 META; Thornwood, NY) that was fitted with a 37°C serosal bath containing Ringer's solution. Z-scanning was performed with a ×40 oil immersion objective (1.3 NA), with the 543-nm laser at 20% maximal intensity. Three random images were acquired from each filter at 0, 30, 60, 120, 240, and 360 minutes and analyzed in a blinded fashion. The ASL height for each image was then calculated using an automated script in ImageJ software from the NIH (Bethesda, MD). As shown in Figure 1, Gaussian Blur filtering was applied, and the area containing Texas Red–dextran was divided by the image width to yield the average ASL height across the image. Due to irregularities in the HBE cell height, cellular debris, and inconsistent labeling of the ASL, the images were visually inspected to ensure that the automatic measurement represented the true ASL height. If the automatic measurement was not adequate, the ASL height was manually measured in three regions and averaged. In the representative images shown, the light intensity was adjusted to improve the clarity of the image (Figure 1).

Inflammatory Cytokine Expression Analysis

Either apical or basolateral supernatant samples (up to 200-μl aliquots) were used for cytokine expression analysis as indicated in the Figure legends. Samples were analyzed using the human cytokine Lincoplex kit (Linco Research, St. Charles, MO) run on a Bio-Plex suspension array system (Bio-Rad), as previously described (5, 34).

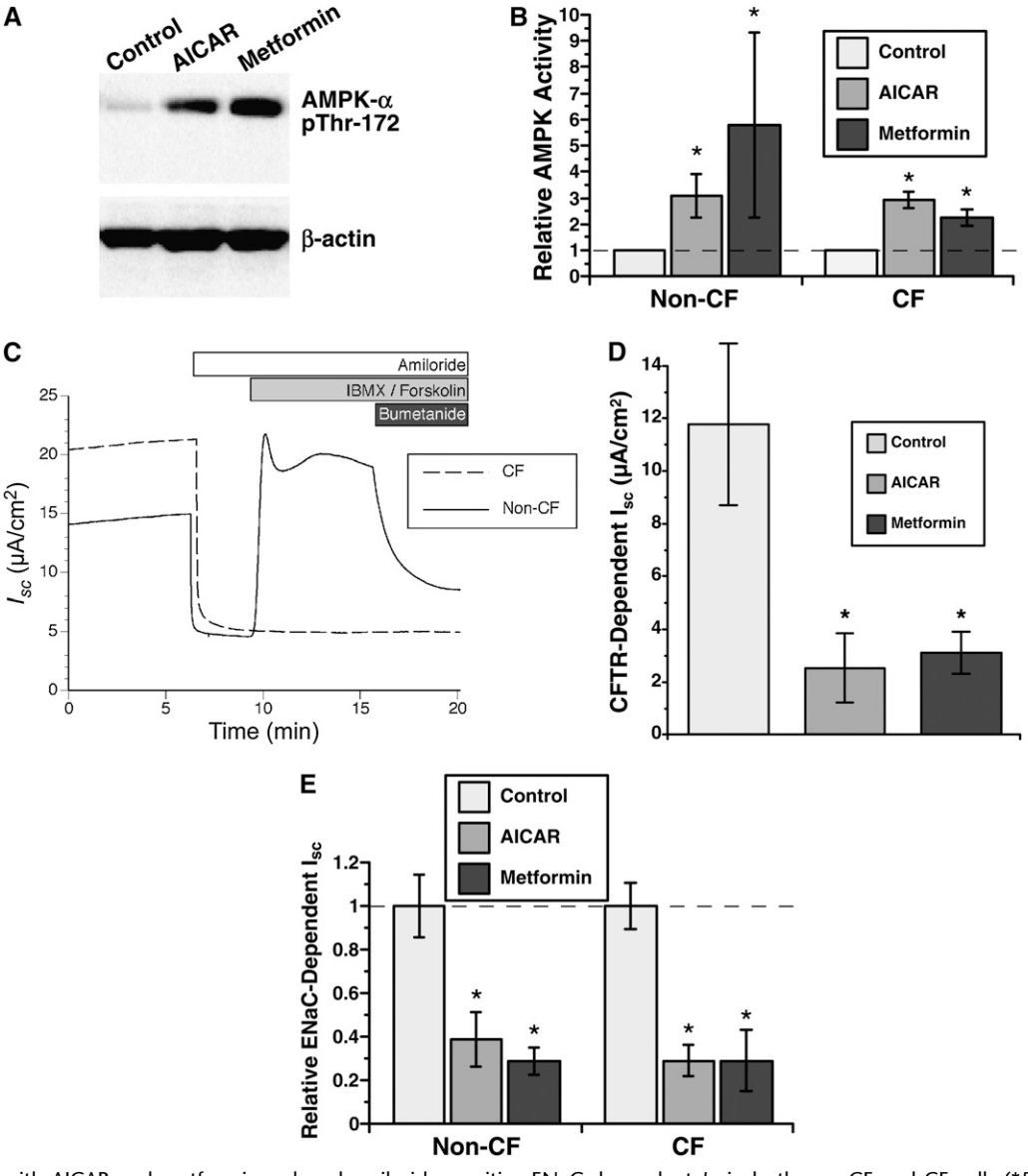

Figure 2. Measurements of cystic fibrosis (CF) transmembrane conductance regulator (CFTR) and ENaC currents in primary airway cells. Differentiated HBE cultures were treated 16 hours before experimentation with either 1 mM AICAR, 5 mM metformin, or vehicle control on both the apical and basolateral sides with the addition of 100 μl of Ringer's solution to the apical side. (*A*) Sample immunoblot for the activated form of AMP-activated kinase (AMPK) (AMPK-α-pThr-172; *upper panel*) and β-actin as a loading control (*lower panel*) taken from lysates of non-CF airway cells. (*B*) AMPK activity of non-CF and CF airway cultures following AMPK activation therapy. Data shown are mean (±SE) AMPK-α-pThr-172 band intensities normalized to that of β-actin in the same lane, as described in Materials and Methods (*$P < 0.05$, unpaired *t* tests, $n = 6$ separate experiments/cell lines for both CF and non-CF cells). (*C*) Representative Ussing chamber short-circuit current (I_{sc}) measurement traces obtained from CF (*dashed line*) and non-CF (*solid line*) airway cells treated with 10 μM amiloride, 1 mM IBMX + 10 μM forskolin, and 20 μM bumetanide at the indicated times. (*D*) Inhibition of CFTR-dependent I_{sc} in non-CF airway cells by both AICAR and metformin as measured by Ussing chamber recordings as described in Materials and Methods (*$P < 0.05$, unpaired *t* tests, $n = 5$ filters for each condition). (*E*) Treatment with AICAR and metformin reduced amiloride-sensitive ENaC-dependent I_{sc} in both non-CF and CF cells (*$P < 0.01$, unpaired *t* tests relative to untreated controls; $n = 16$–18 filters total for each condition obtained from four to five different CF and non-CF cell lines assayed in separate experiments). Results are normalized to the mean ENaC-dependent I_{sc} of the untreated control filters for each cell line within each experiment.

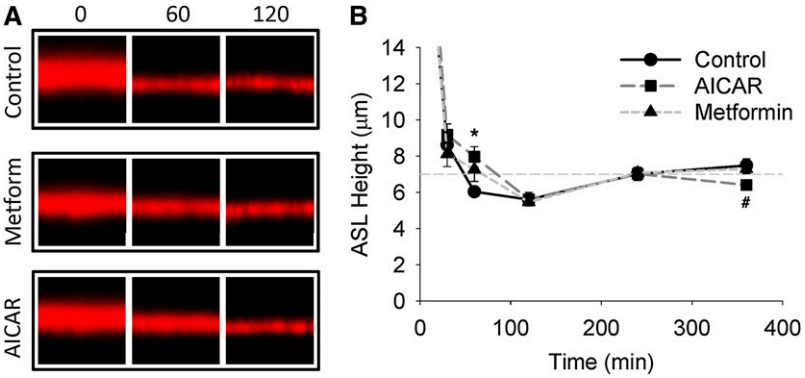

Figure 3. Effect of AMPK activation on the ASL height of non-CF airway cells. A quantity of 2.5 μl of PBS containing 2 mg/ml Texas Red–dextran was applied to the apical surface of differentiated non-CF airway cells that were pretreated overnight with 1 mM AICAR or 5 mM metformin. (*A*) Representative *Z*-scanning confocal microscopy images at 0, 60, and 120 minutes after the addition of the fluorescently labeled PBS. (*B*) Mean ASL height ± SE at various time points up to 360 minutes after apical fluid application (n = 12 filters from three different non-CF tissue donors). *AICAR- and metformin-treated cultures were significantly different from control cultures; #significant difference between AICAR and control cultures (P values are indicated in text).

Metformin Dose–Response and Time Course Studies

Polarized, differentiated CF HBE cells were cultured at an air–liquid interface and the basolateral media were exchanged with fresh media containing 0, 0.03 mM, 0.1 mM, or 1 mM metformin every 2 days. To assure consistent drug delivery, the basolateral media were spiked with metformin on the interim days. Electrophysiologic measurement and basolateral fluid collection for cytokine expression were performed at 0, 2, 4, 7, 10, and 14 days. Transepithelial resistance (R_T) and transepithelial potential difference (V_T) were measured with an epithelial volt-ohmeter using Ag:AgCl electrodes (EVOM; World Precision Instruments, Sarasota, FL). Equivalent short-circuit current (I_{eq}) was calculated by Ohm's law ($I_{eq} = V_T/R_T$), corrected for the Transwell surface area, and normalized to the values obtained on Experiment Day 0. To establish an electrical circuit, 100 μl of media was temporarily applied for 1 hour to the apical surface of the HBE filters. After I_{eq} measurement, the basolateral medium was collected for cytokine analysis and fresh medium applied to the basolateral surface.

Statistics

For the confocal ASL height measurements, all experiments were performed on 12 HBE cell filters cultured from three independent tissue donors. For each individual experiment, the ASL height represents the average of three images/filter. Differences in the ASL height due to AMPK activation were determined by ANOVA with Holm-Sidak *post hoc* analysis at each time interval using Stat-View software (SAS, Cary, NC). All other statistics were performed using unpaired, two-tailed Student's t tests. The P values are indicated in the text, figures and/or legends.

RESULTS

Effects of AMPK Activators on CFTR and ENaC Currents in Primary HBE cells

AMPK has been shown to inhibit CFTR and ENaC activity in polarized epithelial cell lines derived from lung and other tissues (26, 27, 30, 31). To assess the effects of AMPK activators on these ion channels in the more physiologically relevant primary HBE cell model system, differentiated cultures were treated overnight (16 h) with either 1 mM AICAR, 5 mM metformin, or vehicle control in the basolateral medium (Figure 2). Significant AMPK activation with AICAR and metformin treatment occurred in both primary polarized non-CF and CF HBE cell monolayers, as assessed by immunoblotting for the activated form of AMPK (AMPK-α-pThr-172; Figures 2A and 2B). The treatment protocol used and typical responses of both CF and non-CF airway cells to amiloride, IBMX and forskolin, and bumetanide treatment are shown in Figure 2C. Of note, there was little or no cAMP agonist-stimulated I_{sc} in the CF airway cells, consistent with our earlier findings (5). As found in other CFTR-expressing epithelial cells (26, 27), AMPK activation in non-CF cells through overnight AICAR or metformin treatment inhibited CFTR-dependent I_{sc}, which was defined as the difference between the peak I_{sc} measured after IBMX/forskolin treatment and the final I_{sc} after bumetanide treatment (Figure 2D). Very similar effects on CFTR-dependent I_{sc} were also found under these conditions by comparing the I_{sc} measured before and after IBMX/forskolin stimulation (*see* Figure E1 in the online supplement). The AMPK activators AICAR and metformin also dramatically reduced amiloride-sensitive I_{sc} in both non-CF and CF cells to a similar degree (~ 60–70%; Figure 2E). Of note, amiloride-sensitive ENaC currents in untreated CF cells were significantly greater than in non-CF cells in this series of experiments (28.4 ± 7.0 μA/cm^2 versus 11.5 ± 2.7 μA/cm^2; $P < 0.05$, unpaired t test, n = 19–20 filters from six different cell lines for both CF and non-CF). Therefore, AMPK is activated in primary HBE cells by both AICAR and metformin, leading to a reduction in both CFTR-mediated Cl^- secretion and ENaC-mediated Na^+ absorption.

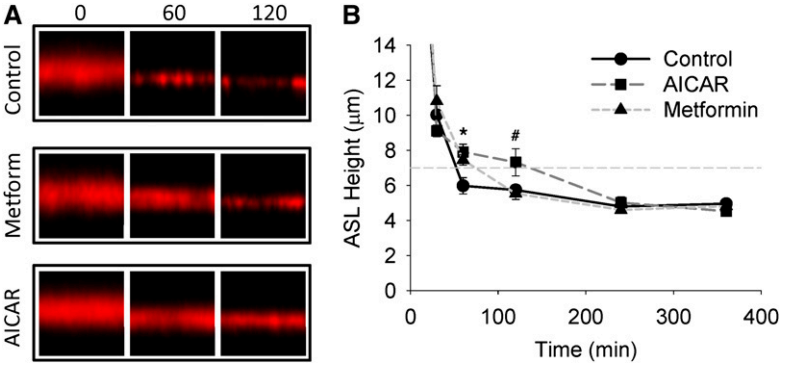

Figure 4. Effect of AMPK activation on the ASL height of CF airway cells. A quantity of 2.5 μl of PBS containing 2 mg/ml Texas Red–dextran was applied to the apical surface of differentiated CF primary airway cells that were pretreated overnight with AICAR or metformin. (*A*) Representative *Z*-scanning confocal microscopy images at 0, 60, and 120 minutes after the addition of the fluorescently labeled PBS. (*B*) Mean ASL height ± SE at various time points up to 360 minutes after apical fluid application (n = 12 filters from three different CF tissue donors). *AICAR- and metformin-treated cultures were significantly different from control cultures; #significant difference between AICAR and control cultures (P values are indicated in text).

Effects of AMPK Activators on ASL Reabsorption Rates in Primary HBE Cells

To determine whether the electrophysiologic alterations caused by AMPK activation are of sufficient magnitude to affect ASL volume and reabsorption rates, the ASL height of HBE cells with and without AMPK activators was assessed at various time points after an apical fluid challenge (cf. Figure 1 and MATERIALS AND METHODS). In the first series of experiments, differentiated non-CF HBE cell cultures were exposed to AICAR and metformin overnight, and the ASL height was then serially measured using confocal microscopy (Figure 3). To label the ASL, 2.5 μl of PBS labeled with 2 mg/ml Texas Red–dextran was applied to the apical surface (Figure 3A). During the initial 0 to 30 minutes after the apical fluid challenge, the ASL height was variable, presumably do to unequal distribution of the dextran and the rapid formation of a fluid meniscus surrounding the Transwell perimeter (33). After this initial redistribution period when no significant differences were observed between control,

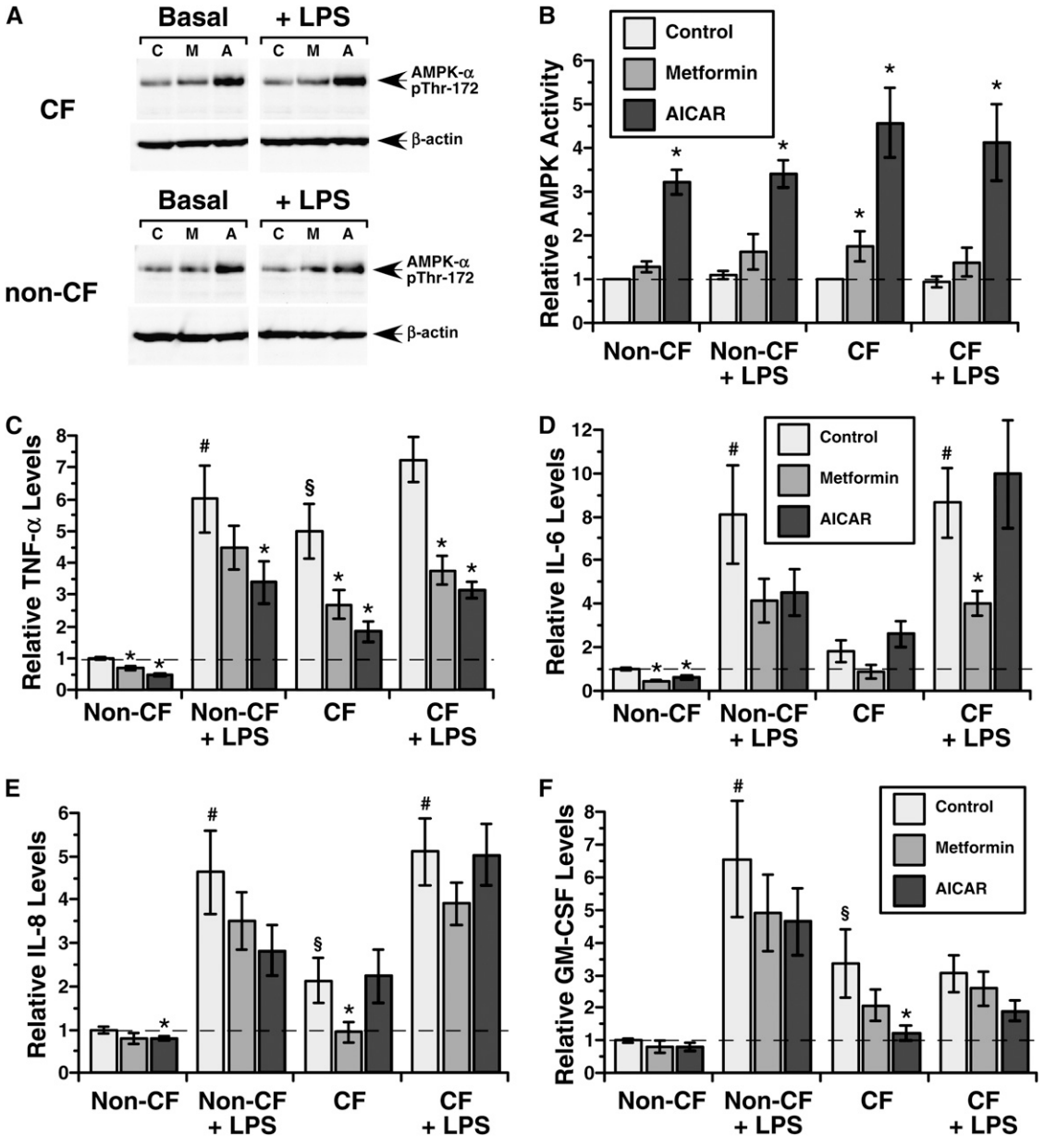

Figure 5. Effects of overnight metformin and AICAR treatment in the presence or absence of LPS on AMPK activity and inflammatory cytokine levels in non-CF and CF airway cells. Differentiated primary airway cultures were treated 16 hours before experimentation with either 2 mM metformin, 1 mM AICAR, or vehicle in the presence or absence of LPS (1 μg/ml). Cytokine concentrations were measured from apical samples collected in 100 μl Ringer's solution, as described in MATERIALS AND METHODS. The cells were then lysed to immunoblot for the activated form of AMPK (AMPK-α-pThr-172) and β-actin. (*A*) Representative immunoblots of CF and non-CF cultures treated with vehicle control (C), metformin (M), or AICAR (A) in the presence or absence of LPS. (*B*) Summary of relative AMPK activities of non-CF and CF cell monolayers treated with AMPK activators ± LPS. Data shown are mean ± SE AMPK activities relative to the untreated condition in the absence of LPS ($^{\#}P < 0.05$, unpaired *t* test relative to control in the absence of LPS). Relative TNF-α (*C*), IL-6 (*D*), IL-8 (*E*), and GM-CSF (*F*) concentrations from non-CF and CF cell cultures treated with AMPK activators ± LPS. Data shown are mean ± SE cytokine concentrations relative to untreated non-CF conditions in the absence of LPS. $^{*}P < 0.05$ relative to control within the same group; $^{\S}P < 0.05$ relative to non-CF control.

AICAR-, or metformin-treated cultures, the ASL height measurements stabilized and became reproducible. At 60 minutes, the ASL height was increased in the AICAR ($P < 0.0001$) and metformin ($P = 0.002$) conditions compared with control non-CF HBE cells, indicating that the rates of ASL absorption were slowed in the AMPK-stimulated cultures (Figure 3B). Subsequently, there was no difference in the measured ASL height between the control and AMPK-stimulated cultures until 6 hours, when the ASL height was lower in the AICAR-treated HBE cells ($P = 0.007$ versus control and $P = 0.023$ versus metformin). Presumably, this decrease in ASL volume reflects a lack of Cl^- secretion due to CFTR inhibition by AICAR (*see* Figure 2 and Ref. 27). These results suggest that the decreases in Na^+ and Cl^- transport caused by AMPK activation are of sufficient magnitude to alter ASL volume and reabsorption rates.

In a second series of experiments, the effects of AMPK activation on ASL volume were assessed in HBE cells cultured from patients with CF (Figure 4). Because HBE cells cultured from CF donors display minimal CFTR-mediated Cl^- secretion, we reasoned that the effects of AMPK activation on ASL volume would primarily reflect decreased Na^+ absorption. As anticipated based on the potent inhibition of ENaC activity by AMPK activation, the ASL height was increased by AICAR at 60 minutes ($P = 0.001$) and 120 minutes ($P = 0.002$) after the apical volume challenge. The ASL height was also increased by metformin at 60 minutes compared with untreated CF HBE cells ($P = 0.009$). In contrast to the wild-type CFTR–expressing cultures, there was no difference in the ASL height at the later time points in CF HBE cells with AMPK activation. These results suggest that AMPK activation slows the rate of ASL absorption in CF HBE cells and as such may augment mucus transport in the CF airway.

Effects of AMPK Activators on the Secretion of Inflammatory Mediators from Primary HBE Cells

In another series of experiments we investigated the effects of AMPK activator treatment on the secretion of various proinflammatory cytokines by HBE cells cultured in the presence or absence of LPS. After overnight treatment with metformin (2 mM) or AICAR (1 mM), AMPK activation was again observed in both non-CF and CF cells (Figures 5A and 5B). Overnight LPS treatment did not have a significant effect on basal AMPK activity or on the ability of AICAR and metformin to induce AMPK activation. Consistent with our data obtained in immortalized CFBE cells (5), primary CF cells had much greater production of TNF-α under baseline conditions than non-CF cells, which was greatly reduced by pharmacologic AMPK activation in both cell types (Figure 5C). Indeed, it appears that an inverse relationship exists between the degree of AMPK activation and TNF-α suppression. As IL-6 (Figure 5D), IL-8 (Figure 5E), and GM-CSF (Figure 5F) are additional important proinflammatory products of the NF-κB and peroxisome proliferator–activated receptor (PPAR) pathways that have been shown to be up-regulated in the CF airway (35), we also investigated the effects of AMPK activators on these mediators. In general, similar significant inhibitory effects or trends were also observed in these inflammatory mediators with AMPK activators.

In summary, these results demonstrate that, although AMPK is already modestly activated in CF airway cells (5), further pharmacologic activation over a relatively short time frame may have beneficial effects by inhibiting excessive ENaC currents, ASL reabsorption, and inflammation, which are major culprits in the pathogenesis of CF lung injury (2). However, the effects and potential utility of AMPK activators at more physiologically relevant doses that may be achieved *in vivo* with these drugs given over longer time periods are not clear based on the preceding studies. We thus performed further studies to determine the specific dose–response relationships and time dependence of the inhibitory effects of metformin on Na^+ absorption and inflammation in CF airway cultures.

Preclinical Studies to Evaluate the Potential Utility of Metformin in the Treatment of CF Lung Disease

In evaluating AMPK activators to pursue for further preclinical studies, we considered drugs that are safe, already FDA-approved for other indications, and available in an oral formulation to be attractive candidates for initial Phase II pilot clinical studies. As AICAR (acadesine) is currently only available in intravenous formulation and has only been tested clinically for brief time periods (hours to days) in the setting of acute myocardial ischemia and reperfusion (36), we decided to pursue additional preclinical studies with metformin, one of the most widely prescribed drugs in the world for the treatment of type 2 diabetes mellitus. Unlike other oral diabetes drugs like sulfonylureas, metformin does not have significant hypoglycemic effects on nondiabetic subjects, and it also does not cause significant weight loss in nonobese subjects with CF (37, 38). These considerations make metformin a potentially attractive choice for use in patients with CF lung disease.

A dose–response and time-course experiment was performed to examine the effects of metformin on ENaC currents and proinflammatory mediators in polarized, well-differentiated CF primary airway cells. The cultures were treated with 0, 0.03, 0.1, and 1 mM metformin, and the equivalent I_{sc} (I_{eq}) and inflammatory profiles were examined at Days 0, 2, 4, 7, 10, and 14. These concentrations were chosen because 0.03 mM is in the range of what may be reached with a typical metformin dosing regimen of 1,000 mg twice daily in an adult patient (38). Both a time- and dose-dependent inhibition of I_{eq} by metformin was observed (Figure 6). Because the majority of the basal I_{eq} in CF HBE cells is amiloride-sensitive, the decreased I_{eq} values observed after metformin treatment most likely correlate with ENaC inhibition. As shown in the online supplement (Figure

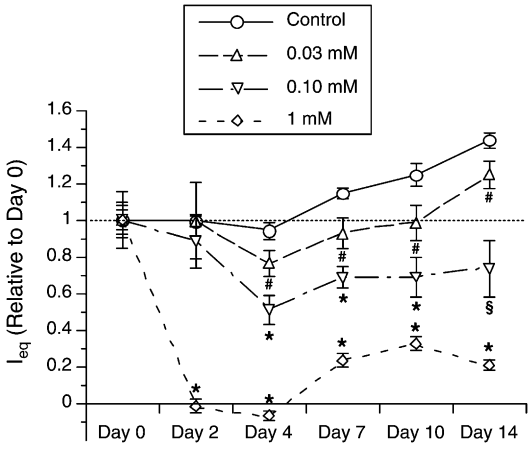

Figure 6. Dose–response and time dependence of the metformin effect on I_{eq} across differentiated CF airway cell cultures. The I_{eq} across polarized CF airway cultures was serially measured after the addition of 0, 0.03, 0.1, and 1 mM metformin. Data shown are mean $I_{eq} \pm$ SE normalized to the values obtained on Day 0; $n = 3$–4 cultures. $^*P < 0.01$, $^{\S}P < 0.05$, and $^{\#}0.05 < P < 0.10$ versus control conditions at the respective time point.

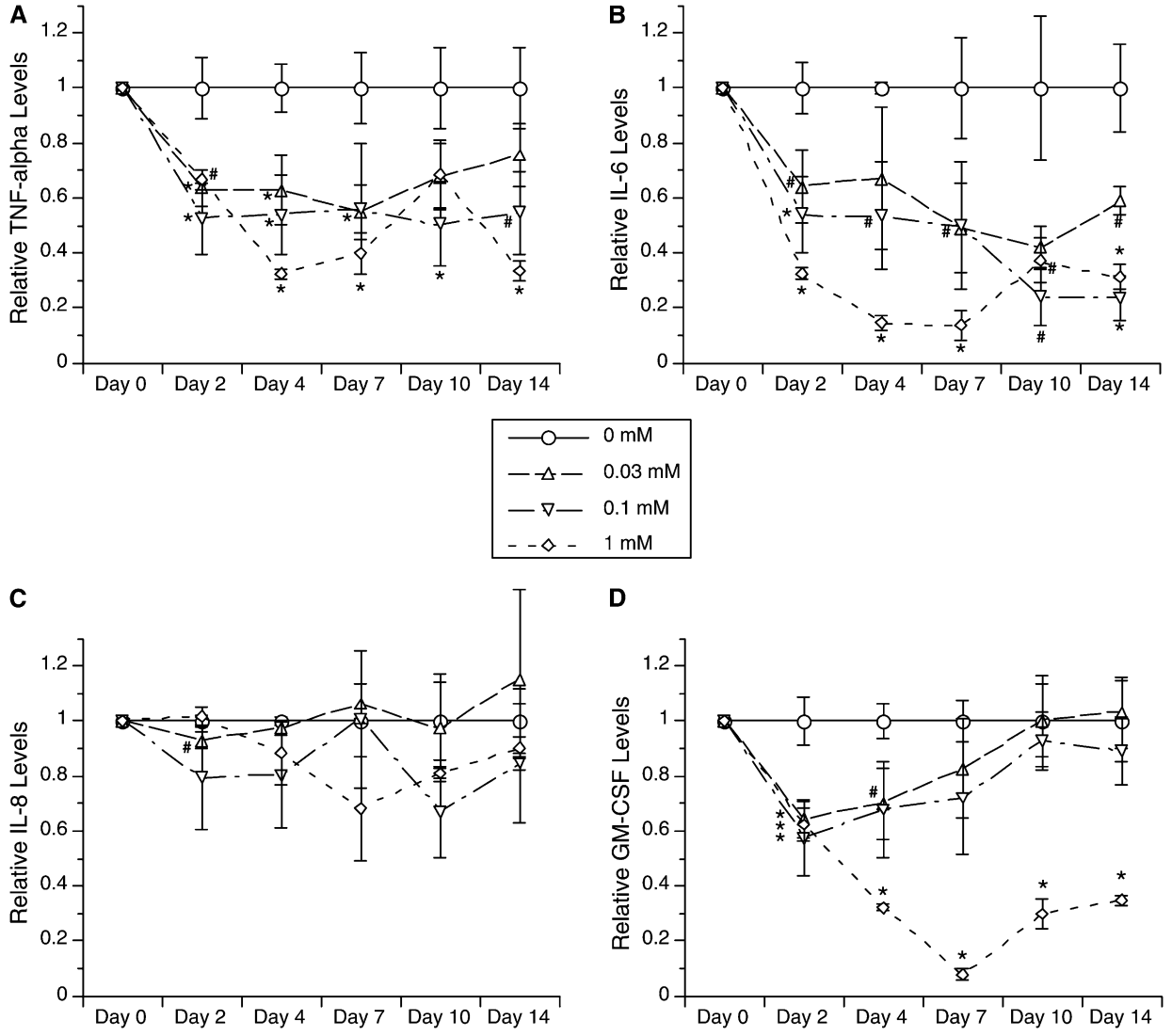

Figure 7. Dose–response and time dependence of the metformin effect on secretion of cytokines from differentiated CF airway cell cultures. After the addition of 0, 0.03, 0.1, and 1 mM metformin, serial 48-hour samples of the basolateral media were collected and subsequently used for multiplex cytokine analysis. Data shown are mean (± SE) TNF-α (*A*), IL-6 (*B*), IL-8 (*C*), and GM-CSF (*D*) concentrations normalized to untreated control conditions at the respective time point; $n = 3$–4 cultures. $^{*}P < 0.05$, $^{\#}0.05 < P < 0.10$.

E2), the metformin-dependent inhibition of I_{eq} at 0.03 and 0.1 mM occurred largely via an increase in the transepithelial resistance (R_T) (or a decrease in the transepithelial conductance [$= 1/R_t$]), rather than an effect on the transepithelial voltage (V_T). At the highest concentration (1 mM), both R_T and V_T were affected. In addition, metformin treatment generally inhibited the basolateral secretion of the various pro-inflammatory mediators previously studied (Figure 7). The lowest concentration of metformin (0.03 mM) caused significant or marginally significant reductions in both TNF-α (Figure 7A) and IL-6 (Figure 7B) starting within 2 days of treatment; this persisted throughout the 14-day study period. Higher metformin concentrations generally caused greater inhibition at most time points. The dose-dependent effects of metformin on IL-8 (Figure 7C) were generally marginal in these experiments, possibly because the IL-8 concentration was above the upper limit of the assay for a number of the samples. GM-CSF levels (Figure 7D) were significantly reduced by all metformin concentrations at 2 days, but then there was recovery back toward untreated control levels by the later time points for the cells treated with 0.03 and 0.1 mM metformin. In summary, these results demonstrate that lower, more pharmacologically relevant concentrations of metformin have an inhibitory effect on both ENaC and proinflammatory mediators in polarized primary CF airway cells.

DISCUSSION

Because AMPK has previously been shown to modulate transepithelial ion transport and inflammation, we tested the hypothesis that therapies to activate AMPK in the CF airway would have a beneficial effect in primary human airway epithelia. We found that treatment with AICAR or metformin, which activate AMPK via different mechanisms, caused a dose-dependent decrease in sodium absorption and inflammatory cytokine secretion from primary CF airway cells. Furthermore, AMPK activation decreased the ASL absorption rate after apical fluid administration. As excessive salt and fluid absorption and exuberant inflammation are characteristic pathological features of CF lung disease, these results provide hope that

therapeutic strategies to augment AMPK activity will have a beneficial effect in the CF airway.

The ability of AMPK-activating drugs to inhibit both ENaC and CFTR activity are consistent with previous results from our and other laboratories. Because these drugs inhibit chloride secretion in addition to sodium absorption, ASL height measurements were performed to clarify whether AMPK activation would have the net result of increasing or decreasing the ASL volume. After the apical fluid challenge, when HBE cells assume a predominantly absorptive function (10), both AICAR and metformin augmented the ASL height, suggesting that under these conditions the predominant electrophysiologic effect of AMPK activation was that of ENaC inhibition. Subsequently, when the apical fluid bolus had been absorbed, there was a trend toward a lower ASL height after AMPK activation in non-CF airway cell cultures. Presumably at this later time point, when HBE cells assume a predominantly secretory function (10), the decreased ASL volume by AMPK activation reflects CFTR inhibition. Given the paucity of functional CFTR in the CF airway, it is unlikely that CFTR inhibition by AMPK activators will cause a significant reduction in chloride secretion *in vivo*.

AMPK activation has long been known to inhibit HMG-CoA reductase (39), the rate-limiting enzyme in sterol biosynthesis and the production of isoprenoids, which are increased in the CF airway and may contribute to excessive inflammation there (40, 41). Moreover, pharmacologic activation of AMPK has been reported to attenuate LPS-induced expression of proinflammatory cytokines through down-regulation of IκK activity and inhibition of NF-κB (42). These findings led us to hypothesize that therapies to activate AMPK in the CF airway may be beneficial in reducing the expression of proinflammatory mediators that contribute to excessive CF airway inflammation (5). AMPK activity was generally inversely related to the elaboration of TNF-α, IL-6, IL-8, and GM-CSF by primary differentiated non-CF airway cells. While the secretion of these cytokines was increased in airway cells cultured from CF donors and after LPS exposure, AMPK activation continued to mitigate inflammatory cytokine secretion under these conditions. Furthermore, the time-course and dose–response experiments with metformin strengthen the notion that AMPK activation decreases inflammation in CF airway epithelia at concentrations similar to those achieved with oral dosing of the drug *in vivo*. Overall, these results indicate that metformin may have beneficial effects on inflammation in patients with CF lung disease.

In addition to the potential beneficial effects of metformin on airway epithelium, metformin may have additional benefits in the treatment of patients with CF. The incidence of CF-related diabetes mellitus (CFRD) has been increasing with the increased lifespan of individuals with CF, currently occurring in approximately 30% of adult patients with CF as per the CF patient registry. While CFRD is traditionally believed to be caused by insulinopenia due to pancreatic β cell fibrosis, recent studies have suggested that impaired insulin sensitivity contributes to CFRD and that insulin resistance frequently develops during infections (37, 43). Small observational studies of patients with CFRD have demonstrated that metformin is not associated with adverse events such as hypoglycemia or lactic acidosis (37). Furthermore, the efficacy of metformin was comparable to that of insulin, in regard to HbA_{1C} reduction and weight gain. Therefore, the available data suggest that metformin therapy is not only safe in patients with CF, but it may potentially improve CFRD, mitigate airway inflammation, and augment ASL volume and mucociliary clearance.

AMPK activation has a relatively modest effect on ASL hydration and inflammation at metformin concentrations that are likely achieved after typical dosing regimens. However, it is possible that small changes in airway physiology have significant effects on mucus clearance and inflammation. Therapies such as azithromycin slow the rate of lung function decline and lower the risk of exacerbation in patients with CF, yet have relatively modest effects on cultured cells (44). Furthermore, our studies do not account for the anti-inflammatory effect of AMPK activation on leukocytes (45, 46), which may further mitigate airway inflammation *in vivo*.

AMPK activation in primary CF airway epithelial cultures caused a dose-dependent reduction in Na^+ absorption, ASL absorption, and inflammatory cytokine production. Previous studies conducted in primary human airway cultures have proven to adequately predict *in vivo* nasal potential difference and/or clinical response. For example, the *in vitro* response of cultured HBE cells to amiloride, UTP, chlorzoxazone, denufosol, and 17 β-estradiol have all subsequently been confirmed in the human respiratory tract *in vivo* (9, 47–50). Thus, the beneficial effects of AMPK activation on primary CF cultures provide ample rationale for further studies to determine its clinical utility in the treatment of CF airways disease. In summary, these preclinical experiments using metformin and AICAR on airway epithelia provide promise that AMPK activation represents a novel therapeutic strategy to both augment airway surface hydration and mitigate inflammation.

Conflict of Interest Statement: K.R.H. holds stock in Bristol-Myers Squibb (up to $1,000), and has received sponsored research grants from the National Institutes of Health ($100,000 or more), Cystic Fibrosis Foundation ($100,000 or more), and American society of Nerphrology ($50,000–$100,000). None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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