Transforming Growth Factor- β 1 and Cigarette Smoke Inhibit the Ability of β ₂-Agonists to Enhance Epithelial Permeability

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Abstract

Chronic bronchitis, caused by cigarette smoke exposure, is characterized by mucus hypersecretion and reduced mucociliary clearance (MCC). Effective MCC depends, in part, on adequate airway surface liquid. Cystic fibrosis transmembrane conductance regulator (CFTR) provides the necessary osmotic gradient for serosal to mucosal fluid transport through its ability to both secrete Cl^- and regulate paracellular permeability, but CFTR activity is attenuated in chronic bronchitis and in smokers. β ₂-adrenergic receptor (β ₂-AR) agonists are widely used for managing chronic obstructive pulmonary disease, and can activate CFTR, stimulate ciliary beat frequency, and increase epithelial permeability, thereby stimulating MCC. Patients with chronic airway diseases and cigarette smokers demonstrate increased transforming growth factor (TGF)- β 1 signaling, which suppresses β ₂-agonist-mediated CFTR activation and epithelial permeability increases. Restoring CFTR function in these diseases can restore the ability of β ₂-agonists to enhance epithelial permeability. Human bronchial epithelial cells, fully redifferentiated at the air-liquid interface, were used for ¹⁴C mannitol flux measurements, Ussing chamber experiments, and quantitative RT-PCR. β ₂-agonists enhance epithelial permeability by activating CFTR via the β ₂-AR/adenylyl cyclase/cAMP/protein kinase A pathway. TGF- β 1 inhibits β ₂-agonist-mediated CFTR activation and epithelial permeability enhancement. Although TGF- β 1 down-regulates both β ₂-AR and CFTR mRNA, functionally it only decreases CFTR activity. Cigarette smoke exposure inhibits

β ₂-agonist-mediated epithelial permeability increases, an effect reversed by blocking TGF- β signaling. β ₂-agonists enhance epithelial permeability via CFTR activation. TGF- β 1 signaling inhibits β ₂-agonist-mediated CFTR activation and subsequent increased epithelial permeability, potentially limiting the ability of β ₂-agonists to facilitate paracellular transport in disease states unless TGF- β 1 signaling is inhibited.

Keywords: cystic fibrosis transmembrane conductance regulator; transforming growth factor- β ; cigarette smoke; epithelial permeability

Clinical Relevance

β ₂-agonists can activate cystic fibrosis transmembrane conductance regulator (CFTR), which leads to an increase in epithelial permeability. Increase in permeability can facilitate serosal to mucosal water transport, and can also facilitate transport of some cationic hydrophilic across the paracellular space (e.g., albuterol). Transforming growth factor (TGF)- β 1, a cytokine up-regulated in chronic airway diseases and cigarette smoke (via TGF- β signaling), suppresses CFTR mRNA and blocks the ability of β ₂-agonists to activate CFTR and enhance epithelial permeability. Therapeutics that block TGF- β signaling can restore CFTR function in chronic airways diseases and in smokers.

Cystic fibrosis (CF) transmembrane conductance regulator (CFTR)-mediated inhibition of Na^+ absorption and activation

of Cl^- secretion provides the osmotic gradient for water to pass through the transcellular/paracellular pathway,

maintaining the appropriate airway surface liquid (ASL) volume and height for efficient mucociliary clearance (MCC) (1, 2).

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Although bronchial epithelial cells express aquaporins, deletion mutants of aquaporins demonstrate regular ASL depth (3, 4). These observations point to a significant contribution of the paracellular space for fluid transport. A number of reports have shown that CFTR controls paracellular permeability of bronchial epithelia (5–7), placing CFTR at a critical juncture in ASL volume regulation. In CF, mutations of the gene encoding CFTR are associated with a significant decrease in epithelial Cl^- secretion, excessive Na^+ absorption (8), and decreased paracellular permeability (5, 6). This effect is mimicked in chronic obstructive pulmonary disease (COPD) and chronic bronchitis (9–12). These diseases resemble mild CF, especially during disease exacerbations, when additional insults, such as inflammation, take a toll on the system. Thus, restoring CFTR function in airway epithelial cells in chronic bronchitis and smokers could help alleviate some of the symptoms of COPD and chronic bronchitis.

β_2 -agonists, used for symptomatic management of airway obstruction, including the one seen in COPD, can stimulate individual components of the MCC apparatus by activating epithelial CFTR and enhancing ciliary beat frequency (CBF). In our earlier study, we demonstrated that β_2 -agonists enhance epithelial permeability (13). In this study, we show that this mechanism is coupled to CFTR activation. Transforming growth factor (TGF)- β 1 and cigarette smoke (CS) interfere with the ability of β_2 -agonists to increase epithelial permeability by down-regulating CFTR mRNA levels. We demonstrate that the CS effect is reversed by blocking TGF- β signaling. These observations can potentially explain the limited β_2 -agonist-mediated epithelial permeability enhancement under inflammatory conditions.

Materials and Methods

Unless otherwise stated, all materials were obtained from Sigma Chemical Co. (St. Louis, MO). ^{14}C (56.5 mCi/mmol) mannitol was purchased from Perkin Elmer Life Sciences (Boston, MA).

Cell Culture

All experiments were performed with human bronchial epithelial cells,

redifferentiated at the air–liquid interface (ALI). ALI cultures were prepared as previously described (14), except that epinephrine was removed from the cultures 2 days before initiating experiments.

Epithelial Permeability Determination

Epithelial permeability detection studies were performed as described in the online supplement.

Protein Kinase A Activation

Protein kinase (PK) A activation was determined by a fluorescence resonance energy transfer (FRET) approach described previously (15, 16). CBF from the same cells was measured simultaneously. A detailed description is provided in the online supplement.

Electrophysiology Experiments

Ussing chambers were used to determine CFTR activation as described by us previously (17). The input resistance of each filter was measured by application of 1-mV bipolar pulses of 2-second duration. Amiloride (10 μM) was added apically to eliminate epithelial sodium channel influences. After treatment with albuterol or other activators, short-circuit current (I_{SC}) was allowed to stabilize for 30 minutes, and the final resistance of the cultures was determined as an indicator of changes in paracellular permeability.

Smoke Exposure

Normal human bronchial epithelial (NHBE) ALI cultures were exposed to air or CS using a VC10 smoke robot (VITROCELL Systems GmbH, Waldkirch, Germany). Four University of Kentucky research cigarettes were smoked with a puff volume of 35 ml for 2 seconds every 60 seconds (ISO 3,308). The smoke was diluted with 4.5-L/min air and blown over the cell culture filter at a rate of 5 ml/min. Control cells were similarly treated without smoke (air control). This regimen ensures 100% viability of smoked cells with no effect on transepithelial electrical resistance (data not shown) as measured by Epithelial Tissue Volt Ohm Meter (World Precision Instruments, Sarasota, FL). Cells were allowed to recover for 16 hours before initiating experiments.

Statistical Analysis

Unless otherwise stated, data are expressed as mean ($\pm$ SE) from six cultures from at least three lung donors. Statistical

significance was evaluated using unpaired *t* tests for two groups or ANOVA followed by Tukey-Kramer honestly significant difference test for multiple comparisons as appropriate. A *P* value less than 0.05 was considered significant.

Results

β_2 -Adrenergic Agonists Enhance Epithelial Permeability by Signaling through the Adenylyl Cyclase/cAMP/PKA Pathway

To determine whether long-acting β_2 -adrenergic receptor (β_2 -AR) agonists can enhance epithelial permeability, initial epithelial permeability was determined by measuring mannitol flux, as described in MATERIALS AND METHODS. Formoterol, a rapid-onset, long-acting bronchodilator, and salmeterol, a slow-onset, long-acting bronchodilator, were added apically and the cells were incubated for 45 minutes. ^{14}C mannitol was added apically and the apical-to-basolateral mannitol flux was determined. Formoterol and salmeterol both demonstrated an increase in epithelial permeability (Figure 1A). These data confirm that long-acting β_2 -AR agonists are similar to albuterol, which we have previously shown to stimulate permeability (13).

β_2 -ARs signal predominantly through the adenylyl cyclase (AC)/cAMP/PKA pathway. This canonical β_2 -AR signaling pathway involves G α s-mediated activation of a transmembrane AC, followed by cAMP-mediated PKA activation. We first tested if activators of the AC/cAMP/PKA pathway, such as forskolin, can mimic the ability of β_2 -agonists to enhance epithelial permeability. Forskolin (5 μM) increased epithelial permeability, comparable to that of albuterol (Figure 1B). To determine if cAMP signals via PKA or exchange protein activated by cAMP (EPAC), NHBE ALI cultures were treated with analogs that specifically activate either PKA (dibutyryl cAMP; 100 μM) or EPAC (8-CPT-2'-OMe-cAMP; 50 μM). dibutyryl-cAMP, but not 8-CPT-2'-OMe-cAMP, mimicked the effect of forskolin and albuterol, suggesting that cAMP signals via PKA, and not EPAC, to increase epithelial permeability (Figure 1B). The role of transmembrane AC (tmAC) was evaluated by studying albuterol-mediated epithelial permeability increases in the presence or absence of the tmAC

Figure 1. β_2 -agonists enhance epithelial permeability. (A) Long-acting β_2 -agonists enhance epithelial permeability. The ability of the β_2 -agonists formoterol (10 μ M) and salmeterol (10 μ M) to enhance epithelial permeability was determined as described in MATERIALS AND METHODS. Both agonists enhance epithelial permeability. (B–D) Short-acting β_2 -agonists increase epithelial permeability via the adenylyl cyclase (AC)/cAMP/protein kinase (PK) A pathway. (B) Normal human bronchial epithelial (NHBE) cells exposed to AC/cAMP/PKA pathway activators forskolin (5 μ M), 8-CPT-2'-OMe-cAMP (50 μ M; exchange protein activated by cAMP [EPAC]-specific analog) and dibutyryl (db) cAMP (100 μ M) or vehicle controls were compared with albuterol (10 μ M). Forskolin and dbcAMP (PKA-activating analog) demonstrate permeability increases comparable to that observed with albuterol, whereas 8-CPT-2'-OMe-cAMP did not, suggesting that cAMP signals via PKA to increase epithelial permeability. (C) The role of the classical AC/cAMP/PKA pathway was further confirmed using the adenylyl cyclase inhibitor, MDL-12,330A (25 μ M). MDL-12,330A inhibits the ability of albuterol to increase epithelial permeability. (D) PKA inhibitor, KT-5720 (10 μ M), inhibits the ability of albuterol to increase epithelial permeability, confirming the role of AC/cAMP/PKA pathway. Each $n = 3$ lungs (6 filters); * $P < 0.05$.

inhibitor, MDL-12,330A (Figure 1C). MDL-12,330A (25 μ M) inhibited albuterol-mediated increases in epithelial permeability. These data demonstrate that activation of tmAC constitutes a key step in β_2 -agonist-mediated epithelial permeability increases.

PKA activation and cAMP, independent of PKA activation, can have diverse effects that directly or indirectly impact the permeability of tight junctions. cAMP can directly alter tight junction permeability by activating E3 ubiquitin ligase (18), which is responsible for ubiquitination and degradation of tight junction proteins (18, 19). To determine if cAMP directly affects tight junction permeability or mediates its effects via PKA, the ability of β_2 -agonists to enhance epithelial permeability was studied in the

presence or absence of KT-5720, a PKA inhibitor. Cells were preincubated with KT-5720 (10 μ M) or vehicle. KT-5720 blocked the ability of albuterol to enhance epithelial permeability (Figure 1D), suggesting that β_2 -agonists enhance epithelial permeability via cAMP-dependent PKA activation.

We further confirmed albuterol-mediated activation of PKA in NHBE ALI cultures by a FRET-based approach (15, 16). Cells were perfused apically with buffer containing albuterol (10 μ M) followed by forskolin (20 μ M). Figure 2A shows representative traces of changes in the FRET ratio (PKA activation) and CBF increases in response to albuterol and forskolin. Perfusion with albuterol activated PKA (Figure 2B), indicated by an increase in the FRET ratio. Forskolin also caused an increase in FRET ratio. In both cases, there

was a simultaneous increase in CBF (Figures 2B and 2C).

β_2 -Agonist-Mediated Increase in Epithelial Permeability Is a Function of CFTR Activation

The activation of CFTR by the AC/cAMP/PKA pathway by β_2 -agonists has been well established by us and many others (20, 21). CFTR also interacts with β_2 -AR via PDZ interactions (22). Although the main role of CFTR is believed to be the regulation of Cl^- and possibly HCO_3^- secretion, a number of reports have shown that CFTR is also involved in regulating paracellular permeability and, hence, paracellular fluid transport (5–7). To determine if CFTR activation by β_2 -AR is involved in increasing epithelial permeability, β_2 -agonist-mediated enhancement of epithelial permeability was studied in the presence or absence of two specific CFTR inhibitors, CFTR_{inh}172 (20 μ M) (23) and GlyH101 (5 μ M). Both CFTR_{inh}172 and GlyH101 significantly decreased albuterol's ability to enhance epithelial permeability (Figure 3A), suggesting that β_2 -AR-mediated activation of CFTR is needed for increased epithelial permeability. Although there was a general trend that GlyH101 was more effective at suppressing permeability enhancements by β_2 -agonists than CFTR_{inh}172, the difference was not statistically significant. To confirm the role of CFTR, epithelial permeability was studied in Δ F508 homozygous and Δ F508/G551D heterozygous CF HBE cells. Albuterol increased epithelial permeability in Δ F508 CF HBE cells, but not in Δ F508/G551D heterozygous CF HBE cells (Figure 3B), similar to the data obtained with CFTR_{inh}172. This may suggest that homozygous Δ F508 cells have some measurable CFTR activity after epinephrine starvation at the apical surface, which was confirmed in Ussing chamber experiments (data not shown). Preincubating Δ F508 homozygous cells with the CFTR corrector (24, 25), VX-809 (500 nM), and the potentiator, VX-770 (1 μ M), did not cause a further increase in epithelial permeability by albuterol. For G551D/ Δ F508 heterozygous cells, preincubation with VX-770 (1 μ M) led to a significant increase in epithelial permeability when stimulated with albuterol, and reached levels comparable to the ones seen in NHBE cells (Figure 3B). To determine why the correction of G551D/ Δ F508 resulted in

Figure 2. β_2 -agonist signaling in airway epithelial cells leads to PKA activation and ciliary beat frequency (CBF) increase. (A) A representative trace of PKA activation and CBF increase as determined by the fluorescence resonance energy transfer (FRET) approach described by us previously (16). NHBE air-liquid interface (ALI) cultures infected with the FRET pair regulatory subunit II-CFP (RII-CFP) and catalytic subunit-YFP (CAT-YFP) were perfused apically with buffer, albuterol (10 μ M), and then forskolin (5 μ M), in that order. (B) Perfusion with albuterol leads to PKA activation as demonstrated by an increase in the FRET ratio. (C) PKA activation corresponds to an increase in CBF. All $n = 3$ lungs (5 filters); * $P < 0.05$.

improved permeability response than Δ F508 alone, we measured the change in I_{sc} in corrected Δ F508 cells (treated with VX-809 and VX-770) versus corrected G551D/ Δ F508 cells (VX-770). A change in I_{sc} of 3.0 μ A/cm² was observed for corrected Δ F508 cells versus 6.8 μ A/cm² observed with corrected G551D/ Δ F508 cells (data not shown). Thus, heterozygous cells showed larger responses to CFTR rescue therapy compared with Δ F508 homozygous cells.

Given these data, we wanted to test if there is any correlation between CFTR activity and epithelial permeability. Albuterol-mediated CFTR activation in NHBE ALI cultures, grown on Snapwell filters (Corning Inc., Corning, NY), was modulated by increasing doses of GlyH101 (1, 2.5, 5, 10, and 15 μ M). In parallel, age- and lung-matched cultures grown on 12-mm Transwell filters (Corning Inc.) were treated identically, and permeability was measured. A linear relationship ($r^2 = 0.75$) between albuterol-mediated CFTR activation and epithelial permeability increases was found (Figure 3C).

Weiser and colleagues (7) demonstrated that CFTR exerts its effect on paracellular permeability via myosin light chain kinase (MLCK), demonstrating that the MLCK-specific inhibitor, ML-7, blocked the ability of cAMP to increase epithelial permeability in bronchial epithelial cell lines. Thus, β_2 -agonist-mediated enhancement of epithelial permeability was studied in the presence or absence of ML-7 (10 μ M). ML-7 did not inhibit albuterol-mediated increases in epithelial permeability (data not shown), suggesting that the CFTR-mediated increase in permeability was not dependent on MLCK phosphorylation in primary NHBE cells.

TGF- β 1 Inhibits the Ability of β_2 -Agonists to Enhance CFTR Activity and Overall Transepithelial Permeability

TGF- β 1 has been shown to interfere with β_2 -AR signaling (26). We therefore tested the potential consequence of TGF- β 1 exposure on albuterol-mediated CFTR

activation. NHBE ALI cultures were pretreated with TGF- β 1 (10 ng/ml) or vehicle for 16 hours. Then, albuterol-mediated increases in epithelial permeability were determined. TGF- β 1 pretreatment inhibited albuterol-mediated increases in epithelial permeability (Figure 4A). Pretreatment with TGF- β 1 (10 ng/ml for 16 h) did not alter the initial permeability of the epithelium (see Figure E1 in the online supplement). We then determined if TGF- β 1 interferes with β_2 -agonist-mediated CFTR activation. NHBE ALI cultures grown on Snapwell filters were treated with TGF- β 1 (10 ng/ml) or vehicle for 16 hours and mounted in Ussing chambers. Albuterol (10 μ M) was added apically in the presence of amiloride, and the increase in I_{sc} was determined. TGF- β 1 pretreatment led to a significant inhibition of β_2 -agonist-mediated CFTR activation (Figure 4B).

To determine the mechanism by which TGF- β 1 inhibits β_2 -agonist-mediated CFTR activation, NHBE cells were pretreated

with TGF- β 1 for 16 hours, total RNA was isolated, and levels of β_2 -AR and CFTR mRNA were determined. Pretreatment of NHBE ALI cultures with TGF- β 1 led to β_2 -AR and CFTR mRNA down-regulation (Figure 4C), confirming previous observations (27, 28).

Because TGF- β decreased mRNA levels of both β_2 -AR and CFTR, we tried to determine which suppression by TGF- β 1 is more important for the decreased β_2 -agonist-mediated permeability change seen. To do this, we looked at CFTR activation independently of β_2 -AR receptor-mediated signaling. Fully differentiated NHBE ALI cultures were pretreated with TGF- β 1 for 16 hours, cells were mounted in Ussing chambers and stimulated with forskolin (5 μ M). At the end of the experiment, CFTR_{inh}172 was added to confirm that the change in *I*_{sc} was CFTR specific. TGF- β 1 pretreatment significantly inhibited forskolin-mediated epithelial permeability increases (Figure 4D). TGF- β 1 pretreatment significantly decreased forskolin-mediated CFTR activation (Figure 4E). Next, we looked at the effect of TGF- β 1 on the ability of β_2 -agonists to activate the AC/cAMP/PKA pathway using the FRET approach described above with concurrent CBF measurements. NHBE ALI cultures were treated with 10 ng/ml TGF- β 1 or vehicle for 16 hours. Cells were perfused apically with buffer, albuterol, and forskolin (in that order). PKA activation was defined as the change in FRET ratio from baseline to the albuterol or forskolin peak. Figure 5A shows representative traces from TGF- β 1-treated and untreated cells for changes in FRET ratio and CBF. There was no statistically significant difference in albuterol-mediated stimulation of PKA and CBF in the absence or presence of TGF- β 1 pretreatment (Figures 5B and 5C). TGF- β 1 pretreatment also did not alter forskolin-mediated PKA or CBF activation (Figures 5D and 5E). Thus, TGF- β 1 pretreatment did not have a significant impact on signaling by β_2 -agonists as assessed by PKA activation and CBF stimulation, despite decreases in overall β_2 -AR mRNA levels. These data confirm that TGF- β 1-mediated decrease in β_2 -AR mRNA does not affect β_2 -AR signaling, but that the decrease in CFTR mRNA and function is critical for TGF- β 1 to inhibit β_2 -agonist-mediated epithelial permeability increases.

Figure 3. β_2 -agonists increase epithelial permeability by activating cystic fibrosis (CF) transmembrane conductance regulator (CFTR). (A) CFTR inhibition also inhibits β_2 -agonist-mediated increases in epithelial permeability. Two chemically distinct CFTR inhibitors, CFTR_{inh}172 (20 μ M) and GlyH101 (10 μ M), inhibit the ability of albuterol to increase epithelial permeability, suggesting that CFTR activation enhances epithelial permeability. All $n = 5$ lungs (10 filters). (B) Effect of the CFTR potentiator, VX-770, and corrector, VX-809. Albuterol increases epithelial permeability in Δ F508 homozygous CF HBE cells. Preincubation with VX-809 and VX-770 does not lead to a statistically significant increase in epithelial permeability by albuterol. Albuterol does not demonstrate epithelial

Figure 4. Transforming growth factor (TGF)- β 1 inhibits β_2 -agonist-mediated CFTR activation and increases in epithelial permeability. (A) TGF- β 1 inhibits albuterol-mediated increases in epithelial permeability as measured by mannitol flux. NHBE ALI culture cells exposed to TGF- β 1 (10 ng/ml for 16 h) failed to demonstrate an increase in epithelial permeability when treated with albuterol (10 μ M). $n = 6$ lungs (6 filters). (B) TGF- β 1 attenuates β_2 -agonist-mediated CFTR activation in NHBE cells. NHBE ALI cultures grown on Snapwells demonstrate an attenuated albuterol-induced CFTR specific I_{sc} increase in TGF- β 1-treated cells. $n = 6$ lungs (6 filters). I_{sc} for vehicle-treated cells was 29.28 ± 3.56 μ A/cm 2 and for TGF- β 1-treated cells was 7.76 ± 1.81 μ A/cm 2 . (C) TGF- β 1 treatment significantly decreases CFTR mRNA levels with a modest, but significant, decrease in β_2 -adrenergic receptor (β_2 -AR) mRNA. $n = 6$ lungs (6 filters). (D) NHBE ALI cultures grown on 12-mm Transwell inserts were pretreated with TGF- β 1 (10 ng/ml), and the forskolin-mediated increase in epithelial permeability was determined. Inhibition of CFTR function by TGF- β 1 translates to an inability of forskolin to increase epithelial permeability. $n = 3$ lungs (6 filters). (E) NHBE ALI cultures grown on Snapwells were treated with recombinant TGF- β 1 (10 ng/ml) or vehicle for 16 hours. Cells were mounted in Ussing chambers and Cl^- efflux was stimulated with forskolin (5 μ M). TGF- β 1 signaling inhibits forskolin-mediated CFTR activation. $n = 3$ lungs (3 filters). I_{sc} for vehicle-treated cells was 8.4 ± 0.85 μ A/cm 2 and for TGF- β 1-treated cells was 1.6 ± 0.39 μ A/cm 2 . * $P < 0.05$.

CS Attenuates β_2 -Agonist-Mediated CFTR Activation and Permeability Increases via TGF- β Signaling

CS exposure has been shown to decrease surface CFTR levels and activity (11, 29, 30). NHBE ALI cultures were exposed to the smoke regimen outlined in MATERIALS AND

METHODS. Cells were allowed to recover for 16 hours. ALI medium was replaced with transport buffer, and initial mannitol flux was determined using ^{14}C -mannitol and vehicle or albuterol (10 μ M). Albuterol enhanced epithelial cell permeability in air-exposed cells, whereas no increase in

mannitol permeability was observed in cells exposed to CS (Figure 6A). CS exposure did not affect the initial permeability of cultures after 16 hours using the smoke regimen outlined in MATERIALS AND METHODS (Figure E1B). These data suggest that CS inhibits the ability of β_2 -agonists to increase epithelial cell permeability.

CS is known to induce TGF- β 1 signaling (26, 31). Addition of a TGF- β receptor (TGFBR)-2 neutralizing antibody to the apical and basolateral side of cultures before smoke exposure prevented the effects of CS described previously here, and restored the ability of albuterol to increase epithelial permeability compared with cells treated with goat IgG alone (Figure 6A).

Next, we sought to determine if CS alters mRNA levels of either TGF- β 1, CFTR, or β_2 -AR. NHBE ALI cultures were exposed to the smoke regimen outlined in MATERIALS AND METHODS. Cells were allowed to recover for 16 hours. Total RNA was isolated and levels of TGF- β 1, CFTR, and β_2 -AR were determined. CS decreased CFTR mRNA levels, while not affecting β_2 -AR or TGF- β mRNA levels (Figure 6B). Preincubation with an anti-TGFBR2 antibody partially rescued CS-induced CFTR mRNA down-regulation. We then determined if CS also inhibits CFTR activity in Ussing chamber experiments, and whether this inhibition could be reversed by a TGFBR2-neutralizing antibody. CS exposure indeed inhibited CFTR activity (Figure 6C), and there was a modest, but significant, restoration of CFTR function with the TGFBR2-neutralizing antibody.

Discussion

β_2 -AR agonists are important for symptomatic management of airway obstruction, including that seen in COPD. In our earlier work, we demonstrated that albuterol, a short-acting β_2 -agonist bronchodilator, enhances epithelial permeability. Given the ability of β_2 -agonists to also activate CFTR (11, 32) and

Figure 3. (Continued). permeability increases in G551D/ Δ F508 heterozygous CF HBE cells. Preincubation with VX-770 restores epithelial permeability increases comparable to those observed in NHBE ALI cultures. $n = 8$ filters (1 lung for heterozygous; 2 lungs for homozygous). (C) CFTR activity parallels the epithelial permeability increase. NHBE ALI cultures on Snapwells were mounted in Ussing chambers and were pretreated with increasing concentrations of GlyH101 (1, 2.5, 5.0, 10, and 15 μ M). Albuterol (10 μ M) was added apically and Cl^- efflux determined. In parallel, age- and lung-matched cultures, grown on 12-mm Transwell inserts, were treated identically, and the change in epithelial permeability was determined as mentioned in MATERIALS AND METHODS. A scatter plot of change in short-circuit current (ΔI_{sc}) versus change in epithelial permeability (ΔP_{app} , normalized) demonstrates a reasonably linear relationship between CFTR activity and change in epithelial permeability ($r^2 = 0.75$), suggesting that CFTR activation could be important for epithelial permeability increases. $n = 5$ lungs (5 filters); * $P < 0.05$.

Figure 5. TGF- β 1 signaling does not inhibit β ₂-agonist-mediated PKA activation. PKA activation and CBF increases by albuterol were determined in TGF- β 1-treated and untreated cells by a FRET analysis approach that we have used previously (16, 17). Human immunodeficiency virus-pseudotyped lentiviruses encoding fluorescently tagged PKA subunits (RII-CFP and CAT-YFP) under transcriptional control of the FoxJ1 promoter (expression solely in ciliated cells) were used to infect undifferentiated NHBE cells, which were then allowed to differentiate at the ALI. β ₂-AR is known to associate with PKA type II (51, 52). NHBE ALI cultures expressing these PKA subunits were treated with recombinant TGF- β 1 (10 ng/ml) or vehicle for 16 hours. ALI cultures were placed in a customized chamber and mounted at room temperature onto the stage of a Nikon E600Fn microscope (Nikon Instruments Inc., Melville, NY). Cells were perfused apically with buffer containing albuterol or forskolin. The Metafluor FRET module was used to measure CFP/YFP ratios. CBF was simultaneously monitored in these cells. (A) A representative trace for CBF and FRET ratio from vehicle-treated and TGF- β 1-treated NHBE cells expressing the RII-CFP and CAT-YFP subunits of PKA. (B and C) TGF- β 1 does not inhibit albuterol-mediated PKA activation or CBF increases, suggesting that down-regulation of β ₂-AR mRNA does not translate to a functional inhibition. (D and E) TGF- β 1 does not inhibit forskolin-mediated PKA activation or CBF increases, suggesting that the AC/cAMP/PKA pathway is intact. Baseline CBF was between 4.5 and 7.5 Hz (mean, 5.5 ± 0.4 Hz) for untreated cells and 5–8.5 Hz (mean, 6.7 ± 0.45 Hz) in TGF- β 1-treated cells. All $n = 3$ lungs (5 filters).

increase CBF (33), they can play an important role in boosting MCC apart from their established roles as bronchodilators.

The binding of β ₂-agonists to their cognate receptors at the airway epithelium results in activation of the canonical AC/cAMP/PKA pathway. At the epithelium, this signaling results in PKA-mediated CFTR activation, increased epithelial permeability (13), and enhanced CBF. Thus, β ₂-agonists have the potential to stimulate all major components of the MCC apparatus. Here, we focused on intracellular signaling and paracellular permeability. We first examined the mechanism by which β ₂-agonists enhance epithelial permeability, followed by the effect of detrimental factors, such as CS, and enhanced TGF- β signaling. Using stimulators and inhibitors of components

of the AC/cAMP/PKA pathway, we could attribute β ₂-agonist-mediated epithelial permeability increases to the classical AC/cAMP/PKA pathway. Although it is widely accepted that agonist binding to β ₂-adrenergic receptor leads to PKA activation, we also confirmed this in our primary bronchial epithelial cell culture model using a FRET approach reported by us previously (34), looking at PKA activation and CBF increases of single cells in real time. Because β ₂-AR activation activates CFTR, and because some reports implicate CFTR in epithelial permeability regulation (35–37), we studied whether β ₂-agonists acted through CFTR to increase epithelial permeability. Our observations point to a role of CFTR: the CFTR-specific inhibitors, CFTR_{inh}172 and GlyH101, were successful in blocking β ₂-agonist-mediated

epithelial permeability increases, albeit to a different degree. We also observed a linear correlation between CFTR activity and increase in epithelial permeability. Given these data, we would have expected that cells from patients with CF would not respond with an increase in paracellular permeability in response to β ₂-agonists. This was the case in G551D/ Δ F508 heterozygous cells, but not in cells homozygous for Δ F508, as shown here and in our previous data (13). However, we do not believe that this observation is contradictory to the general hypothesis that CFTR is involved in regulating paracellular permeability. Our data are in line with observations of Hanrahan's group, in which CFTR potentiation by genistein decreased transepithelial electrical resistance in CF HBE cells at 37°C (6). Δ F508 molecules

Figure 6. Cigarette smoke (CS) inhibits the ability of albuterol to increase epithelial permeability in a TGF- β 1-dependent manner. (A) Albuterol enhances epithelial permeability in control (air-exposed) cells, while having no effect on cells exposed to CS. Anti-TGFR2 neutralizing antibody (added 3 h before exposure apically and basolaterally; 25 μ g/ml) restores the ability of albuterol to increase permeability in smoke-exposed cells when compared with goat IgG used as control. $n = 4$ lungs (8 filters). (B) NHBE ALI cultures were exposed to the smoke regimen described in MATERIALS AND METHODS. Total RNA was collected and mRNA levels of CFTR, β 2-AR, and TGF- β 1 were determined. CS exposure down-regulates CFTR mRNA, but does not affect TGF- β 1 and β 2-AR mRNA levels. Preincubation with anti-TGFR2 antibody suppresses the inhibitory effect of CS on CFTR mRNA. $n = 3$ lungs (4 filters). *Significantly different from air-exposed cells ($P < 0.05$). **Significantly different from smoke-exposed and smoke plus goat IgG exposed cells. (C) NHBE ALI cultures grown on Snapwells were exposed to CS. Separately, cells were treated with anti-TGFR2 antibody. Cells were mounted in Ussing chambers and Cl^- efflux in response to albuterol addition was determined. Anti-TGFR2 neutralizing antibody causes a modest, but statistically significant, restoration of CFTR function in smoke-exposed cells. Isc values are: air, $12 \pm 2.9 \mu\text{A}/\text{cm}^2$; smoke, $5.3 \pm 0.75 \mu\text{A}/\text{cm}^2$; smoke + anti-TGFR2, $7.33 \pm 1.41 \mu\text{A}/\text{cm}^2$; $n = 6$ lungs (6 filters). *Significantly different from air-exposed cells ($P < 0.05$). **Significantly different from smoke-exposed cells ($P < 0.05$).

escape endoplasmic reticulum-associated degradation. The extent of endoplasmic reticulum-associated degradation escape is different in different tissues. LeSimple and colleagues (6) and Carvalho-Oliveira and colleagues (38) observed between 10 and 50% of Δ F508 molecules in CF HBE cells at the apical surface. This may be sufficient in our cell cultures to regulate paracellular permeability. Consistent with this hypothesis, correction of Δ F508/G551D heterozygous CF HBE cells with Vx-770 demonstrated increased CFTR activity (Isc = $6.8 \mu\text{A}/\text{cm}^2$) and a better response in epithelial permeability changes by albuterol when compared with Δ F508 cells corrected with Vx-809 and Vx-770 (Isc = $3.0 \mu\text{A}/\text{cm}^2$).

The exact mechanism by which CFTR modulates epithelial permeability remains unclear. Weiser and colleagues (7) demonstrated that CFTR modulates phosphorylation of MLCK, and changes in paracellular permeability can be blocked by ML-7. However, ML-7 had no effect on β 2-agonist-mediated increases of epithelial permeability in our studies. This discrepancy could be explained by the model system used, namely, that of primary bronchial epithelial cells versus cell lines. The secretagogue used to stimulate CFTR may be important as well. It is widely known that PKA-dependent phosphorylation of MLCK can regulate tight junction permeability (39, 40). It is

possible that the authors were able to inhibit this aspect of cAMP-mediated epithelial permeability increase using ML-7. In our model, cAMP synthesis is a function of β 2-AR activation that ensures that cAMP remains compartmentalized, precluding it from having other pleiotropic effects on tight junction permeability.

Another possibility is that increases in epithelial permeability are due to CFTR molecules activated in subcellular/intracellular locations (41). CFTR is involved in a range of intracellular functions like endosomal trafficking, lysosomal acidification, and also protein maturation. Alternatively, activation could lead to redistribution of CFTR molecules, resulting in an epithelial permeability increase. At least one report mentions that CFTR redistributes close to tight junctions on activation (42).

We examined the effect of CS and TGF- β 1, two factors commonly encountered in patients suffering from chronic bronchitis, on the ability of β 2-agonists to enhance epithelial permeability. Both CS and recombinant TGF- β 1 were able to inhibit β 2-agonist-mediated CFTR activation and consequently increase in epithelial permeability (confirming that epithelial permeability is a function of CFTR activation). Although CS itself has been shown to increase epithelial permeability, these changes are transient, and return to

normal within 12 hours of exposure (43). We measured the inhibitory effect of CS on albuterol-mediated permeability changes at 16 hours after smoke exposure, when values had returned to normal (Figure 6). Thus, the inhibitory effect is not related to the well known, but only transient, CS-induced baseline permeability changes. Confirming previous reports in different cell types (27, 44), TGF- β 1 decreased both β 2-AR and CFTR mRNA. However, by looking at β 2-AR and CFTR function independently of each other, we were able to confirm that CFTR was functionally inhibited. It is possible that, apart from decreasing CFTR biogenesis, TGF- β 1 signaling interferes with CFTR function or localization directly. CS decreases CFTR mRNA and consequently decreases the ability of β 2-agonists to enhance epithelial permeability. A neutralizing antibody to TGFR2 rescued the effect of CS on CFTR mRNA decreases and on β 2-agonist-mediated epithelial permeability increases, but had only a modest effect on restoring Cl^- efflux, suggesting that CS induces TGF- β signaling. CS can affect apical CFTR function directly (29), or indirectly by altering plasma membrane fluidity (45, 46). TGF- β 1 decreases CFTR mRNA levels, and could affect intracellular CFTR function as well. If this is the case, the neutralizing antibody could restore the increase in epithelial permeability by restoring

CFTR intracellular expression more than apical expression, and would therefore have only a modest effect on apical Cl^- efflux.

Finally, we have reported that albuterol transits the epithelium paracellularly by increasing paracellular permeability, and

thus its own transport. Inhibition of the signaling pathway involved in increasing paracellular permeability can directly impact transepithelial flux of albuterol and consequently its onset of action. Therapeutic regimens that block TGF- β 1 signaling might be able to restore the ability of

β_2 -agonists to enhance MCC in smokers and other patients with chronic airway diseases, in whom TGF- β signaling is up-regulated. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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