ORIGINAL RESEARCH

# The Cystic Fibrosis Transmembrane Conductance Regulator Potentiator Ivacaftor Augments Mucociliary Clearance Abrogating Cystic Fibrosis Transmembrane Conductance Regulator Inhibition by Cigarette Smoke

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## Abstract

Acquired cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction may contribute to chronic obstructive pulmonary disease pathogenesis and is a potential therapeutic target. We sought to determine the acute effects of cigarette smoke on ion transport and the mucociliary transport apparatus, their mechanistic basis, and whether deleterious effects could be reversed with the CFTR potentiator ivacaftor (VX-770). Primary human bronchial epithelial (HBE) cells and human bronchi were exposed to cigarette smoke extract (CSE) and/or ivacaftor. CFTR function and expression were measured in Ussing chambers and by surface biotinylation. CSE-derived acrolein modifications on CFTR were determined by mass spectroscopic analysis of purified protein, and the functional microanatomy of the airway epithelia was measured by 1-μm resolution optical coherence tomography. CSE reduced CFTR-dependent current in HBE cells ($P < 0.05$) and human bronchi ($P < 0.05$) within minutes of exposure. The mechanism involved CSE-induced reduction of CFTR gating, decreasing CFTR open-channel probability by approximately 75% immediately after exposure ($P < 0.05$), whereas surface CFTR expression was partially reduced with chronic exposure, but was stable acutely. CSE treatment of purified CFTR resulted in acrolein modifications on lysine and cysteine residues that likely disrupt CFTR gating. In primary HBE cells, CSE reduced airway surface liquid depth ($P < 0.05$) and ciliary beat frequency ($P < 0.05$) within 60 minutes that was restored by coadministration with ivacaftor ($P < 0.005$). Cigarette smoking transmits acute reductions in CFTR activity, adversely affecting the airway surface. These effects are reversible by a CFTR potentiator *in vitro*, representing a potential therapeutic strategy in patients with chronic obstructive pulmonary disease with chronic bronchitis.

**Keywords:** cystic fibrosis transmembrane conductance regulator potentiator; ivacaftor; cigarette smoke; mucociliary transport; optical coherence tomography

New therapies are needed for the treatment of chronic obstructive pulmonary disease (COPD), which accounts for over $40 billion in annual U.S. healthcare costs (1), and recently surpassed stroke as the third leading cause of death in the United States (2). Smoking is the primary risk factor for the disease, and is responsible for nearly 90% of COPD-related deaths (3). Cigarette smoke may contribute to the pathogenesis of COPD by altering the function of cystic fibrosis (CF) transmembrane conductance regulator (CFTR) (4–8), a protein that functions as an anion channel at the apical surface of airway epithelia.

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## Clinical Commentary

Cigarette smoking can cause acquired cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction, but its impact on the mucociliary transport (MCT) apparatus with and without potentiation of CFTR is poorly characterized. Cigarette smoke extract caused an acute reduction in CFTR channel gating and conferred a severe reduction in MCT *in vitro*. The CFTR potentiator, ivacaftor, reversed these abnormalities, even with chronic administration.

Mutation of the CFTR gene is responsible for CF, an airway disease that shares a similar phenotype with that of patients with COPD that exhibit chronic bronchitis. Indeed, cigarette smoke has been observed to elicit a phenotype characteristic of "acquired CFTR dysfunction," including reduced CFTR activity, enhanced mucus production, and a pronounced impairment of mucociliary transport (MCT) (8). Patients with COPD exhibit reduced CFTR activity, as detected in the upper airways (8), lower airways (9), sweat glands (7, 10), and intestine (7). Furthermore, CFTR dysfunction is independently associated with chronic bronchitis and dyspnea (7–10), and can persist despite smoking cessation (7).

We have previously shown that acquired CFTR dysfunction phenotype can be reversed by the CFTR potentiator, ivacaftor, *in vitro* by activating wild-type CFTR-dependent short-circuit current ($I_{SC}$) after chronic exposure (8). The mechanisms by which CFTR function is reduced by smoking continues to emerge, and its molecular basis is not known. CFTR gating can be acutely blocked by acrolein (7), an important cigarette smoke constituent; this latter mechanism may be amenable to CFTR activator therapy. Other *in vitro* studies in epithelial monolayers showed that, in addition to ion channel dysfunction, cigarette smoke or cigarette smoke extract (CSE) also reduced CFTR mRNA and protein expression (5, 6, 11, 12). Reduction of membrane-bound CFTR levels via mechanisms that do not rely on mRNA expression have also been shown, including CFTR internalization sufficient to impact airway hydration (4). These mechanisms could have important implications as to whether augmentation of CFTR activity by a potentiator, which activates ion channel gating, is a viable approach.

Here, we evaluated the effects of cigarette smoke on CFTR function, focusing on acute-acting pathways that could lend insight into molecular mechanisms, and established that a large negative effect on channel gating of excised patches occurs almost immediately upon exposure that was associated with direct modifications to the protein itself, making CFTR a good candidate for functional rescue by potentiators. Further studies demonstrated that administration of ivacaftor partially restores epithelial function acutely, substantiating its potential as a novel therapy for COPD.

## Materials and Methods

### CFTR Function in Primary Human Airway Epithelial Cells and Tissues

Human sample use was approved by the Institutional Review Boards at University of Alabama Birmingham (no. X080625002) and Massachusetts General Hospital (no. 2008P000178). Primary human bronchial epithelial (HBE) cells were derived from lung explants after written informed consent from subjects with and those without CF by methods described previously (13, 14). Primary human airway cells were grown at an air–liquid interface until terminally differentiated. $I_{SC}$ was measured under voltage clamp conditions using MC8 clamps and P2300 Ussing chambers (Physiologic Instruments, San Diego, CA) (14). Stable plateaus of at least 10 minutes were achieved before addition of each reagent. $I_{SC}$ measurements in human airway explants were conducted after dissection of mucosal layer, and performed as described in cells, with the exception of use of P2307 tissue mounts.

### CFTR Western Blots and Biotinylation Studies

For Western blotting, cell lysates were normalized for protein content, electrophoresed under SDS-PAGE conditions, and CFTR detected by chemiluminescence using 10B6.2 anti-CFTR antibody (Millipore Corp., Billerica, MA) (15). Data were normalized to tubulin loading control and performed on three occasions for quantification. Surface CFTR was quantified by biotinylation studies, as previously described (8). Briefly, cells were exposed to biotin for 45 minutes, lysed by sonication, and then precipitated with NeutrAvidin beads (Thermo Fisher Scientific, Waltham, MA) followed by CFTR detection, as described previously here. For biochemical detection of acrolein modification, after acute exposure of 16HBE cells, biotin hydrazide was used to label acrolein-modified protein fraction of cell lysates, followed by immunoprecipitation of CFTR and subsequent detection of acrolein by Western blot analysis against streptavadin.

### Macropatch and Single-Channel Recordings

Conventional patch clamp techniques were used to record single-channel currents from inside-out patches from HEK293 cells expressing wild-type CFTR. Pipettes were partially filled with a solution containing 150 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2). Single-channel currents were obtained using an Axopatch 200B patch clamp amplifier (Axon Instruments [AI], Molecular Devices, Sunnyvale, CA) controlled by Clampex software (pClamp 10; Axon Instruments) and digitized (Digidata 1440A interface; AI) at a sampling frequency of 1 kHz. After excision, patches were perfused at pH 7.2 with 150 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes containing 1 mM MgATP and 50 U/ml protein kinase catalytic subunit (Promega, Madison, WI) with or without 1% CSE. Appropriate vehicle (DMSO) controls were used for comparison. Clampfit software (AI) was used to calculate open probability. For display, recordings were filtered at 500 Hz.

### Detection of Acrolein Modifications on Purified CFTR Protein Using Mass Spectroscopy

A stable mammalian cell expression system consisting of HEK293 cells was used to express D-165 human CFTR construct (SUMO*-huCFTR.901FLAG-GFP*). This expression system is capable of yielding sufficient quality and quantity of human CFTR protein suitable for biophysical and structural studies. The methods involved in design and development of this expression system, as well as the biophysical and

functional characterization of CFTR, are described in an earlier report (16).

### The 1-μm Resolution Optical Coherence Tomography Measurements of Functional Microanatomy

The 1-μm resolution optical coherence tomography (μOCT), image acquisition, and processing have been described previously (17). In the current study, cross-sectional images of 0.5 mm × 0.5 mm (x, z) were acquired at a spatial resolution of 2 μm × 2 μm × 1 μm (x, y, z) and a speed of 32–128 frames/s. The methods used to measure airway surface liquid (ASL) depth and ciliary beat frequency from the μOCT data have been described previously (18).

### Reagents

CSE was prepared by bubbling smoke from one 3R4F Research cigarette (University of Kentucky, Lexington, KY) through 1 ml of DMSO at 2 s/10 ml puff, 10 puffs over 3 minutes to make 100% extract (optical density at 320 nm = 0.3 at 100-fold dilution), then applied to cells diluted in media at the percentages shown, an approach similar to the one used extensively in our previous studies *in vitro* (7, 8, 19–21). Ivacaftor was obtained from Selleckchem (Houston, TX). Other agents were purchased commercially from Sigma-Aldrich (St. Louis, MO).

### Statistical Analysis

For quantitative measures, including *in vitro* studies and potential difference testing, descriptive statistics were computed and means compared using Student's *t* test or ANOVA, as appropriate. Statistical tests were two sided and performed at a 5% significance level using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Error bars designate SEM unless indicated otherwise.

## Results

### Cigarette Smoke Acutely Blocks Wild-Type CFTR Activity in Primary Human Airway Cells and in Intact Human Trachea Mucosa

We have previously demonstrated that cigarette smoke exposure, either as CSE (8) or gas whole cigarette smoke (20), inhibits CFTR function when the epithelia are exposed for 24 hours or more; other laboratories have shown similar findings (4, 5, 11). To test whether acute inhibition (i.e., 20 min or less) of CFTR currents also occurred, which would suggest acute-acting mechanisms, primary human airway cells underwent CFTR activation with forskolin (10 μM) in the setting of amiloride (100 μM) to block ENaC channels,

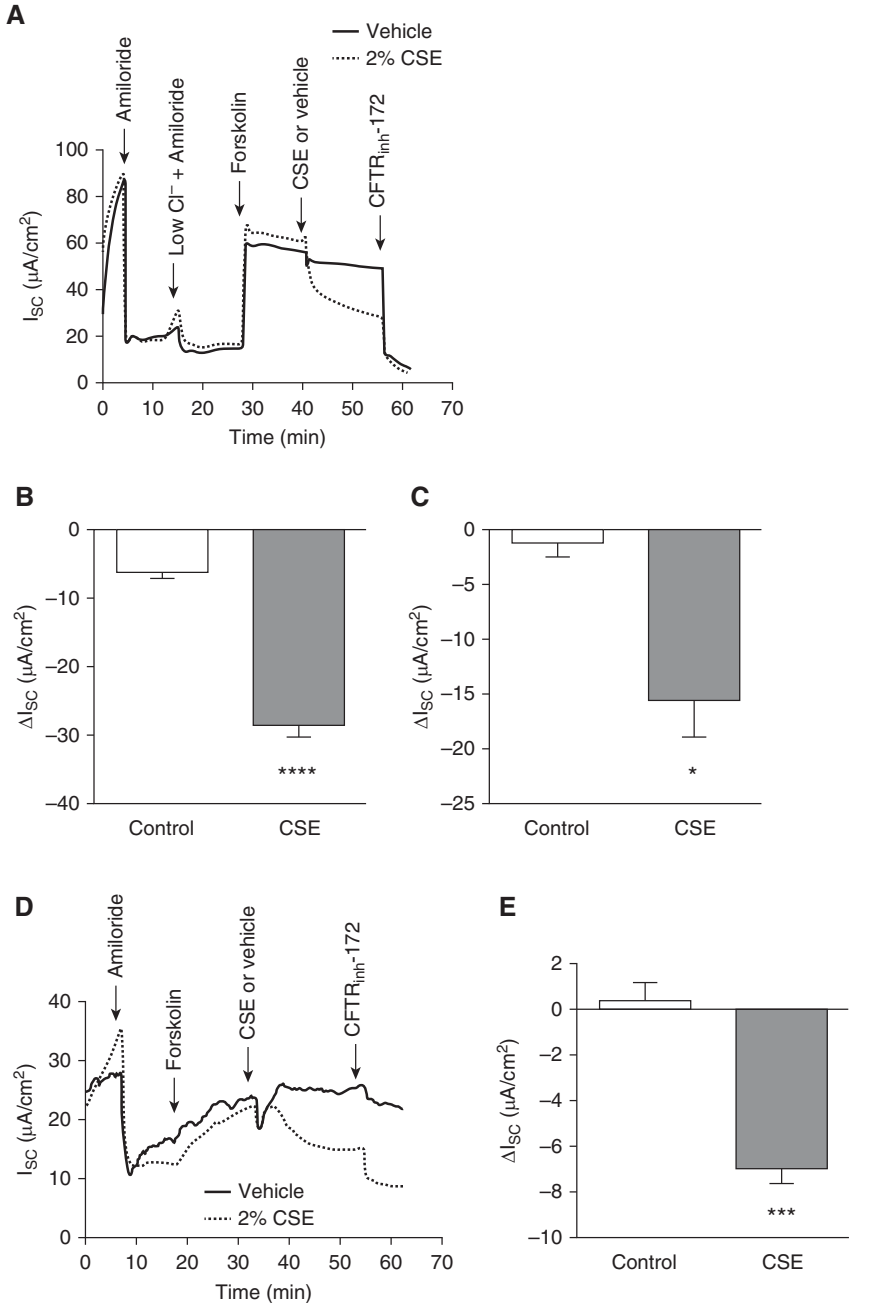


**Figure 1.** Cigarette smoke extract (CSE) acutely reduces cystic fibrosis (CF) transmembrane conductance regulator (CFTR)–dependent short-circuit current ($I_{SC}$). (*A*) Representative $I_{SC}$ tracing of non-CF primary human bronchial epithelial (HBE) monolayers sequentially treated with amiloride (100 μM), chloride-free amiloride (low $Cl^-$), forskolin (20 μM), CSE (2%), or DMSO vehicle control, and $CFTR_{Inh}$-172 (10 μM). (*B*) Summary of forskolin-stimulated $I_{SC}$ shown in *A*. $^{****}P < 0.0001$, $n = 4$/condition. (*C*) Change in $I_{SC}$ when CSE (2%) was added to non-CF HBE cultures before amiloride or forskolin addition. $^{*}P < 0.05$, $n = 3$–4/condition. (*D*) Representative $I_{SC}$ tracing of human tracheal explant from a normal donor, sequentially stimulated with amiloride (100 μM), forskolin (100 nM), CSE (2%), or DMSO vehicle control, followed by $CFTR_{Inh}$-172 (10 μM). (*E*) Summary data of that shown in *D*. $^{***}P < 0.001$, $n = 4$/condition.

followed by addition of CSE (2%) or DMSO vehicle control. As shown in representative $I_{SC}$ tracings, significant (~50%) CFTR inhibition occurred immediately, and was sensitive to $CFTR_{Inh}$-172 (Figure 1A). Summary data showed that CSE inhibited $I_{SC}$ by 28.5 (±1.8) μA/cm$^2$ ($P < 0.0001$), which represented a substantial fraction of forskolin-induced currents (Figure 1B). Complementary studies that examined inhibition of $I_{SC}$ at the outset of the $I_{SC}$ trace, before addition of amiloride or forskolin, also showed an immediate reduction in $I_{SC}$ (Figure 1C) that was sensitive to $CFTR_{Inh}$-172, indicating that even basal levels of CFTR anion transport can be altered by addition of cigarette smoke. As expected, the magnitude of $I_{SC}$ inhibition was less (compare Figures 1B and 1C), because CFTR was not yet fully activated before CSE addition.

We next tested whether this degree of current inhibition was also observed in excised human airway explants to evaluate whether these findings occurred in native tissue. As shown in representative tracings, $I_{SC}$ was again acutely inhibited by addition of CSE after CFTR activation with forskolin (Figure 1D). Summary data indicated a 7.0 (±0.7) μA/cm$^2$ reduction in $I_{SC}$ as compared with a 0.4 (±0.01) μA/cm$^2$ stimulus with forskolin (Figure 1E, abrogating ~50% of CFTR stimulated currents; $P < 0.005$).

To determine the kinetics of reduced CFTR expression under these conditions, we evaluated the effect of CSE on total and cell surface CFTR expression in primary HBE cells at two time points: 20 minutes, representing acute exposure, and 24 hours, representing more chronic exposures studied previously (8). As shown in Figure 2, CSE reduced total CFTR expression and cell surface CFTR expression, but only after chronic exposure; in contrast, 20-minute exposures did not significantly alter either total CFTR expression or cell surface expression, although a trend emerged with the latter, probably reflecting initiation of endocytic recycling (4). In total, these findings indicate that CSE induces an acute blockade of CFTR activity that occurs in very short timeframes in both cells and tissues, and cannot be completely explained by a reduction in cell surface CFTR expression.

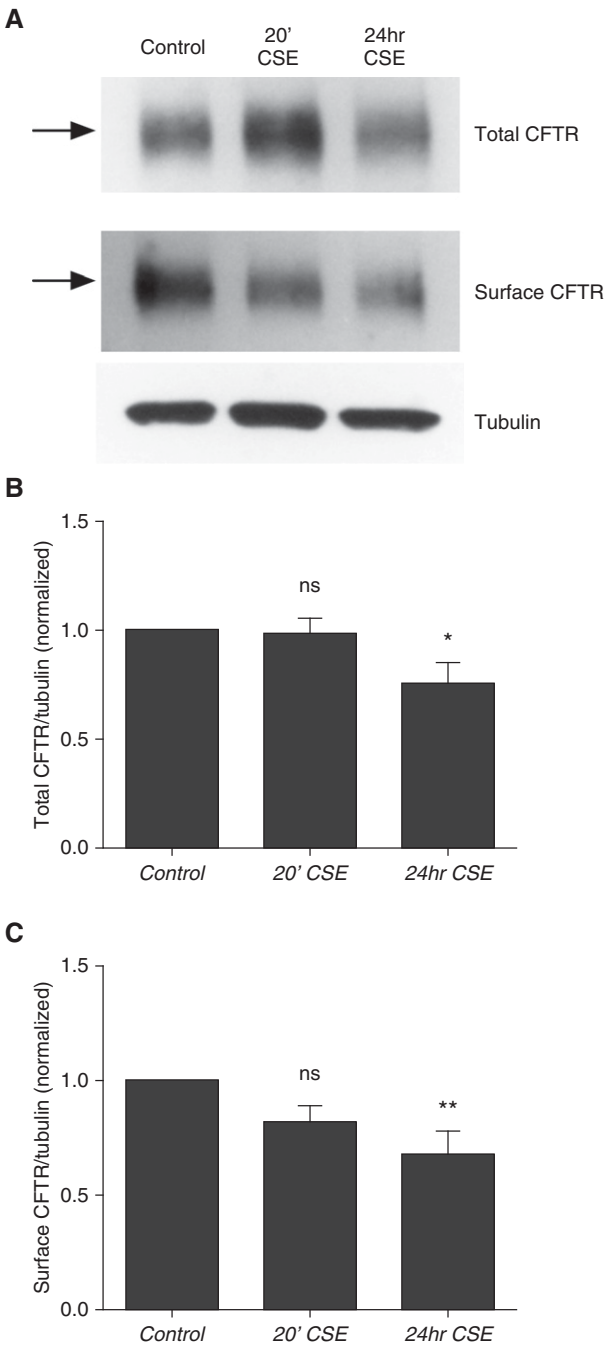


**Figure 2.** CSE reduces surface CFTR expression at 24-hours, but not 20-minute, exposure. (*A*) *Top*: representative Western blot of primary HBE cells exposed to CSE (2%) for 20 minutes or 24 hours to the apical compartment as compared with DMSO vehicle control. CFTR C band indicated by *arrow*. *Middle*: surface CFTR expression quantified by biotinylation followed by Western blot. Surface CFTR indicated by *arrow*. *Bottom*: tubulin loading control. (*B*) Quantitation of total CFTR Western blot, as expressed as CFTR:tubulin ratio in comparison to control. *$P < 0.05$, $n = 4$ blots/condition. (*C*) Surface CFTR:tubulin expression ratio. **$P < 0.01$, $n = 4$ blots/condition. ns, not significant.

### Effects of Cigarette Smoke on CFTR Channel Gating

Acute blockade of CFTR-dependent currents that were not dependent on CFTR expression levels suggested that acute effects on CFTR channel gating may be occurring, as also suggested in a report by Moran and colleagues (22). To confirm, we conducted whole-cell and membrane patch clamp studies on HEK293 cells expressing wild-type CFTR. Upon exposure to CSE (1%), there was an immediate reduction in CFTR

currents (Figures 3A and 3B). Single-channel conductance studies demonstrated a deleterious effect of CSE on CFTR open-channel probability, a fundamental measure of channel gating, which was reduced by 59% (Figures 3C and 3D).

### Cigarette Smoke and Acrolein Covalently Modify CFTR

The acute effects of CSE on CFTR in membrane patches suggested that CFTR itself may be directly impacted by inhalational exposure. Acrolein is one active component of cigarette smoke that we have previously shown affects CFTR gating *in vitro* and channel function *in vivo* (7). Given that acrolein can rapidly modify protein structures (23), and its effects on CFTR activity occurred instantaneously, whereas even 24-hour acrolein exposure did not alter CFTR protein expression (7), we postulated that CSE and acrolein may directly modify CFTR to reduce its ion transport function. To test this, purified recombinant CFTR protein was treated with CSE (1%) acutely and compared with protein samples similarly treated with acrolein (1 μM) and DMSO vehicle. Using nano–liquid chromatography tandem mass spectrometry, acrolein modifications can be detected on protein residues. The unsaturated β-carbon of acrolein readily reacts with cysteine groups to form thioether adducts and the carbonyl carbon irreversibly interacts with the primary amine of lysine residues to form Michael adducts (24–26); in addition, acrolein may also react with the imidazole group of histidine and the guanidino group of arginine. Trypsin-digested CFTR protein reacted with CSE or acrolein yielded eight identical peptides with detectable acrolein modifications. The spectra of representative peptides from purified CFTR contained modified cysteine and lysine residues (Figures 4A and 4B). The amino acid sequence and the change in predicted mass-to-charge ratio (m/z) were consistent with the presence of acrolein adducted via Michael and Schiff base additions to CFTR. Mass spectrometry analysis detected mass changes of 56.02 Da, indicating Michael additions by acrolein on several cysteine and lysine residues (Figures 4B and 4C) and a single mass change of 38.01 Da, suggesting Schiff base addition on Cys524 (Figure 4A).

To confirm acrolein modifications to CFTR biochemically, we labeled protein lysates isolated from acrolein-exposed 16HBE cells with biotin hydrazide, which specifically binds amino acids modified with acrolein, purified with avidin chromatography, and then immunoprecipitated for CFTR, and detected acrolein modifications with streptavidin–horseradish peroxidase. Results showed clear acrolein modifications to isolated CFTR protein (Figure 4D). Moreover, we have previously shown that cigarette smoking causes acrolein modifications in a previous report (7). In aggregate, these findings confirm that CSE and acrolein modify the CFTR protein itself, and does so in a rapid fashion. We postulate that these covalent modifications to CFTR may contribute to altered structure that predisposes CFTR to decreased ion transport, and may also contribute to its instability at the cell surface.

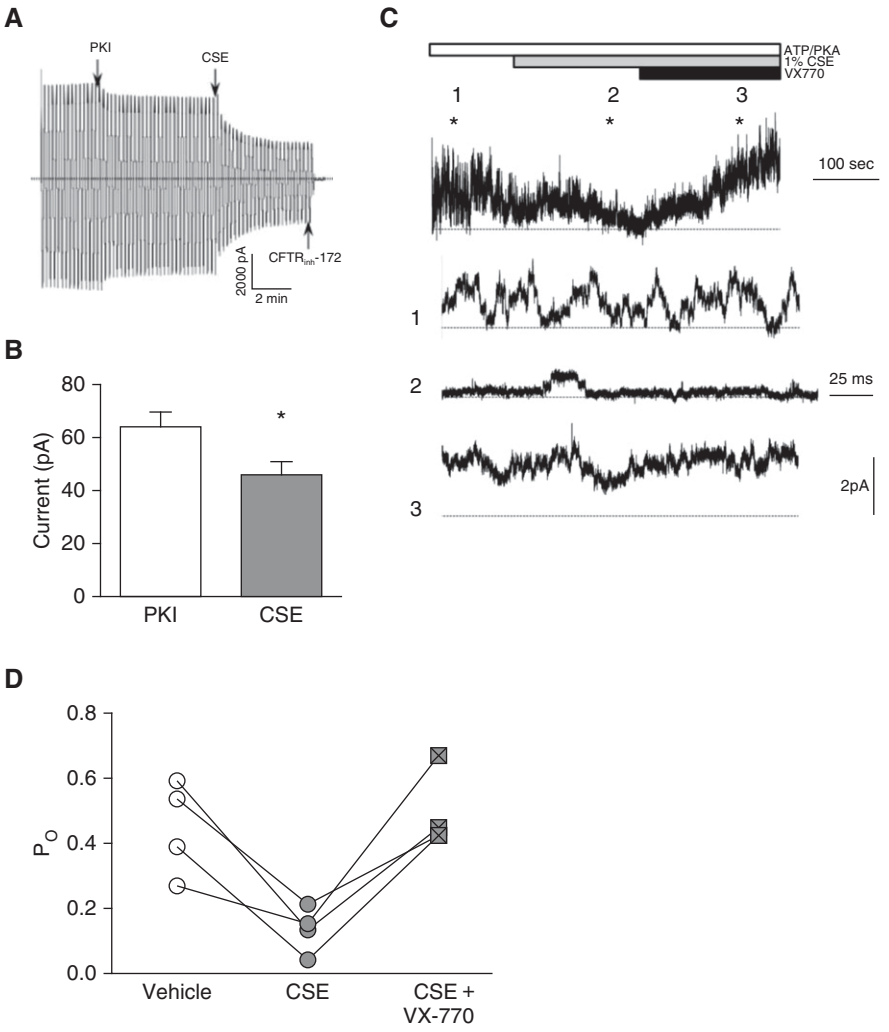


**Figure 3.** CSE acutely reduces CFTR-dependent channel opening. (*A*) Representative recording from macropatch of HEK293 cells expressing wild-type CFTR then exposed to low-dose protein kinase A (PKA) inhibitor (PKI; 40 U/ml) to ascertain phosphorylation status, then CSE (1%). $CFTR_{Inh}$-172 (10 μM) was added to confirm CFTR dependence. Holding potential was −50 mV. (*B*) Summary data of that shown in *A*. *$P < 0.05$, $n = 4$–6/condition. (*C*) Unitary conductance tracing of inside-out patch derived from wild-type CFTR expressing HEK293 cells showing significant and immediate inhibition of CFTR channel opening upon addition of CSE (1%). Holding potential was −50 mV. Experiment performed in the presence of 1 mM MgATP plus 40 U/ml of the catalytic subunit of PKA. *$P < 0.05$, $n = 4$. *Dotted lines* represent zero current level. (*D*) Changes in the probability of opening ($P_o$) for CFTR channels observed in *C* are summarized following addition of vehicle, CSE (1%) and ivacaftor (VX-770).

### The Effects of Acute and Chronic Cigarette Smoke on the Functional Microanatomy of Primary HBE Cells

Given the deleterious effects of cigarette smoke on CFTR-dependent anion transport, we next examined the impact of

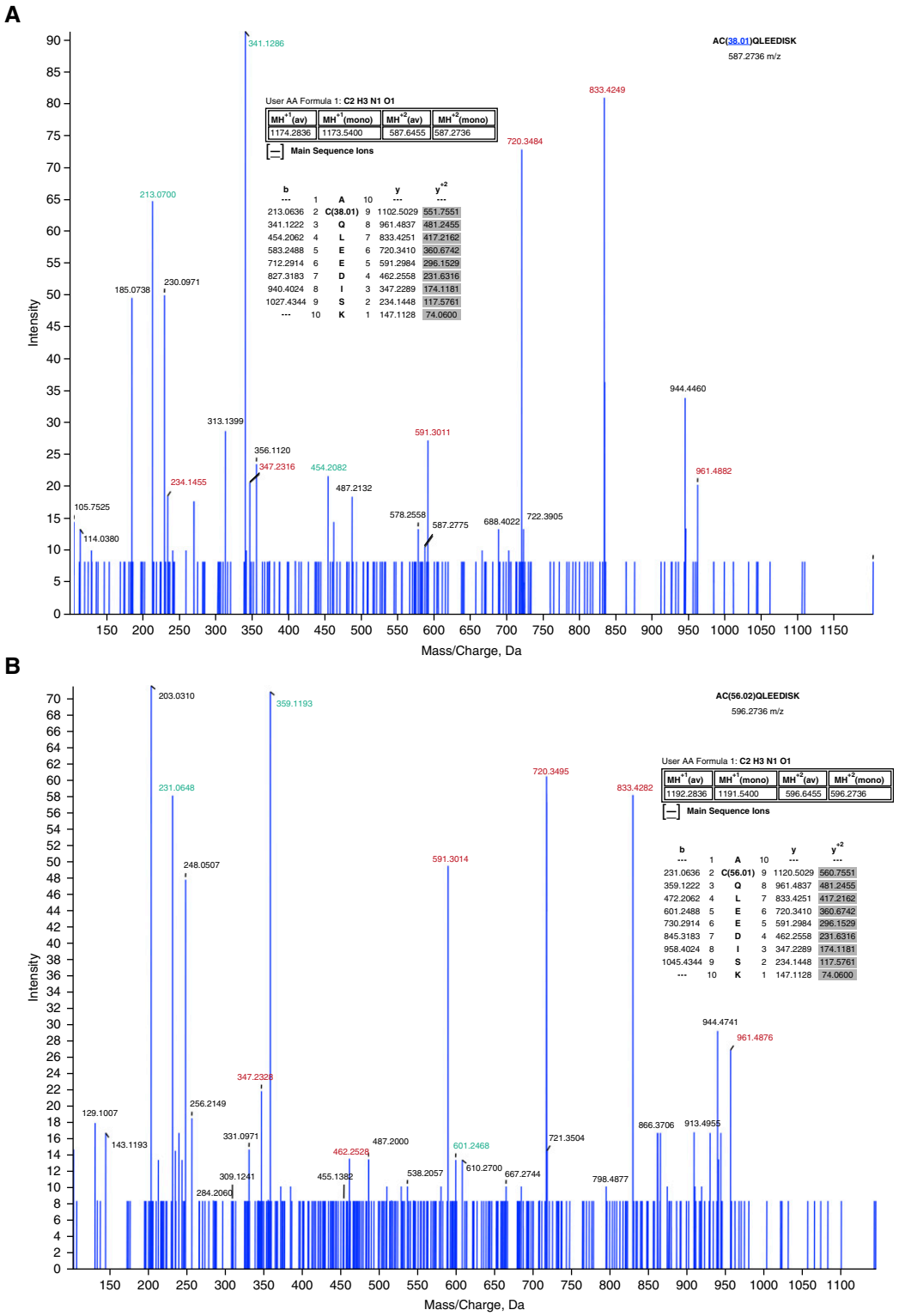


**Figure 4.** Covalent modification of CFTR by cigarette smoke and acrolein. Purified recombinant CFTR protein was treated with 1 μM acrolein or 1% CSE for 30 minutes at 37°C, resolved on SDS-PAGE gel, trypsin digested, and analyzed by nano–liquid chromatography tandem mass spectrometry. Spectra are shown for acrolein modifications of cysteine by Schiff's base addition (+38 addition [*A*]) and for a Michael addition on a lysine (+56 addition [*B*]) that resulted from treatment of CFTR with both acrolein and CSE addition, but did not occur after treatment with vehicle control. The masses of the theoretical fragment ions representing both kinds of acrolein modifications are listed in a *table inset* within the spectra. (*C*) Summary of specific CFTR amino acids modified by acrolein and CSE (highlighted in *red* with their molecular weight within the native CFTR sequence) within eight CFTR peptide

C

| Position | Sequence | Modification |
|---|---|---|
| Cys 524 | AC(38.01)QLEEDISK | Schiff Base |
| Cys 524 | AC(56.02)QLEEDISK | Michael Addition |
| Cys 647 | LM(ox)GC(56.02)DSFDQFSAER | Michael Addition |
| Cys 1395 | QAFADC(38.01)TVILC(Carbamidomethyl)HER | Schiff Base |
| Lys 464 | GQLLAVAGSTGAGK(56.02) | Michael Addition |
| Lys 1334 | SVIEQFPGK(56.02) | Michael Addition |
| Lys 1177 | FIDM(ox)PTEGKPTK(56.02) | Michael Addition |
| Lys 532 | AC(Carbamidomethyl)QLEEDISK(56.02) | Michael Addition |

D

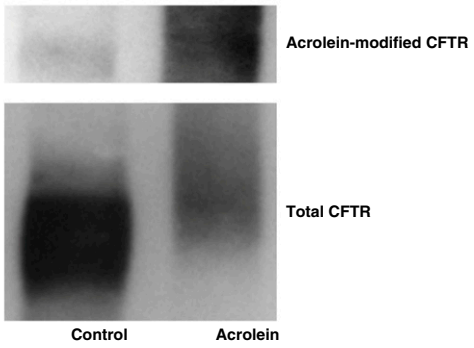


**Figure 4.** (Continued). fragments detected by matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis. (*D*) 16HBE cells acutely treated with acrolein (5 ppm, 10 min) as compared with cells exposed to vehicle control were analyzed for changes in total protein (*bottom*) and for fraction of CFTR proteins that were modified by acrolein using biotin hydrazide directed toward acrolein-modified CFTR residues (*top*). *Blots* are representative of three separate studies.

acute smoke exposure on the airway surface, which is severely affected in COPD (27). For this task, we used μOCT imaging, because it provides an integrated measure of multiple aspects of the functional microanatomy of the airway surface without additions of contrast dye or microparticles (18, 28), and used mucosal CSE addition, as expected to occur *in vivo*. CSE had a deleterious effect on ASL depth (Figures 5A and 5B), and ciliary beating (Figures 5A and 5C) that was present as early as 20 minutes, indicative of the importance and impact of acute-acting pathways on the MCT apparatus. These results demonstrated that reduced CFTR-mediated anion secretion, together with excessive mucus expression (8, 29), rapidly and severely inhibits the mucociliary clearance (MCC) apparatus of airway monolayers.

### Ivacaftor Reverses Acquired CFTR Abnormalities and Augments Airway Surface Hydration and Ciliary Beating *In Vitro*

Although CFTR potentiators were originally developed to restore activity to mutant CFTR localized to the cell surface, some CFTR potentiators, including the clinically approved potentiator, ivacaftor, also augment wild-type CFTR function in human airway epithelia (13, 30) (as opposed to F508 del CFTR that does not reside at the cell surface and exhibits little response to ivacaftor [31]). In excised patches after inhibition of CFTR by CSE, ivacaftor significantly augmented CFTR open channel probability (Figures 3C and 3D). These results indicate that CSE modified CFTR, even in excised patch where the influence of other proteins are minimal, are responsive to CFTR potentiators, and thus confirmed the relevance of this mechanism for the rescue of CFTR function.

To test whether potentiated CFTR channel gating could also improve epithelial function, we again turned to μOCT imaging of primary HBE monolayers, conducted in cells simultaneously exposed to CSE and ivacaftor and monitored acutely. Ivacaftor robustly increased ASL depth in CSE-exposed cells within 60 minutes (Figures 5A and 5B), which was accompanied by a trend toward improved ciliary beating at 20 minutes, but not 60 minutes (Figures 5A and 5C). Ivacaftor treatment without CSE exposure also enhanced ASL depth, indicative of the responsive nature of the MCC apparatus to augmented anion secretion, even in healthy non-CF monolayers (Figures 5A and 5B). In total, these data strongly indicate that the CFTR potentiator, ivacaftor, partially abrogates the downstream manifestations of acquired CFTR dysfunction induced by CSE on airway epithelia, ultimately resulting in increased airway surface hydration and ciliary beating necessary for efficient MCT. Collectively, these data demonstrate that CFTR potentiation represents an attractive therapeutic target to address mucus stasis in COPD, as demonstrated by models predictive of *in vivo* results in CF trials.

## Discussion

A number of mechanisms are known to contribute to acquired CFTR dysfunction; however, the mechanistic basis, relative importance, and impact on functional phenotype are still emerging. This article adds definitive details to this pathway, and in particular the importance and physiological significance of acute-acting mechanisms. In addition to biochemical changes in CFTR expression that occur on a chronic time frame, our results point out the importance of acute inhibition of CFTR activity through altered channel gating by CSE, and that its underlying molecular mechanism may be due to covalent modifications of the CFTR protein itself. Acute inhibition of $I_{SC}$ was immediate and robust, even in native epithelia, supporting previous observations of altered CFTR gating observed in CFTR-transduced *Xenopus* oocytes (22). Reversal by ivacaftor in the same studies lends credence to this observation, and rule out loss of channels as the primary cause (in addition to the nature of excised patch studies, where protein recycling machinery is absent). We further identified that purified CFTR undergoes chemical modification upon CSE exposure by Michael addition, modifying CFTR fragments with the acrolein moieties at key positions within the CFTR molecule. This provides key mechanistic information on the molecular level for how CFTR function is modified

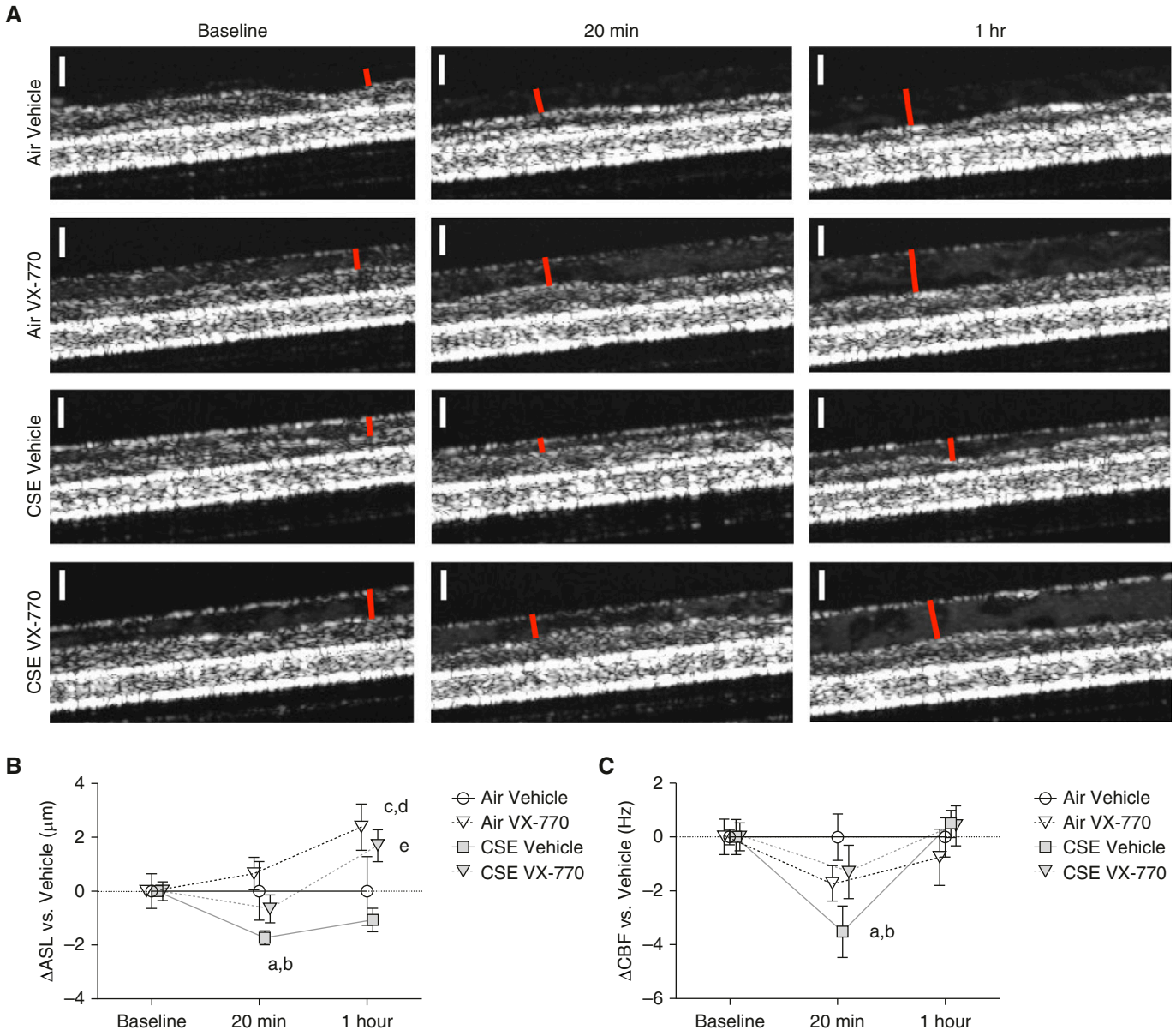


**Figure 5.** The 1-μm resolution optical coherence tomography (μOCT) reveals significant changes in airway surface liquid (ASL) depth and ciliary beat frequency (CBF) after acute CSE exposure and/or ivacaftor (VX-770) treatment of HBE cells expressing wild-type CFTR. (*A*) Representative μOCT images of each experimental group at baseline, 20 minutes, and 1 hour posttreatment indicating the surface microanatomy (*red lines*, ASL; *scale bars*, 10 μm). (*B*) After 20 minutes of exposure, CSE-treated cells exhibit diminished ASL when compared with baseline ($^{a}P < 0.05$) and to air control cells at the same time point ($^{b}P < 0.05$); 1 hour of ivacaftor exposure markedly augmented ASL depth, as compared with that at baseline ($^{c}P < 0.05$) and to DMSO control at 1 hour ($^{d}P < 0.05$). Similarly, at 1 hour, cells treated with both CSE and ivacaftor display significantly higher ASL levels than those exposed only to CSE ($^{e}P < 0.005$). (*C*) CSE exposure significantly reduced CBF at 20 minutes when compared with baseline ($^{a}P < 0.0005$) and air control at the same time point ($^{b}P < 0.005$). $n = 4$–6 and repeated twice using cells collected from two separate donors.

by smoking. It also is completely consistent with our prior observations that acrolein itself, a prominent component of cigarette smoke, confers abnormal gating and does so rapidly (7). CFTR has been shown to be susceptible to posttranslational modifications that alter its functional properties (16); hence, this is another example of the importance of posttranslational reactions modifying (or regulating) CFTR function.

The findings demonstrating the clear contribution of acute-acting pathways to acquired CFTR dysfunction do not discount the importance of chronic mechanisms that also clearly contribute. Reduced CFTR mRNA expression has been reported in both human airway cells exposed to cigarette smoke *in vitro* and nasal samples procured from human smokers (8, 11). Reduced CFTR mRNA transcripts have also been demonstrated in the lungs of patients with COPD at the time of explantation (32), although a decrease in CFTR mRNA expression was not observed in a recent study that relied on endobronchial sampling of airway basal cells (33). Protein

levels are also affected by cigarette smoke, which induces CFTR internalization through a $Ca^{2+}$-dependent pathway (4). This is consistent with reduced levels of surface CFTR expression that have been reported by our group (8); whether this is induced by Michael reactions that impact CFTR residues could be addressed in future studies.

Previous studies have demonstrated that cigarette smoking can contribute to acquired CFTR dysfunction, potentially contributing to the pathogenesis of airway disease, such as COPD, and in particular those with chronic bronchitis (4–8). However, most of these studies have evaluated the effects of chronic cigarette smoke exposure on ion transport or ASL depth at a single, chronic time point, and have not captured the kinetics of molecular mechanisms altered by smoke exposure. The functional anatomic imaging performed here clearly demonstrates the deleterious effects of cigarette smoke on ASL depth that occur within 60 minutes, providing strong evidence that cigarette smoke–induced acquired CFTR dysfunction contributes to the pathogenesis of COPD. Overall, the results support a number of epidemiologic studies that link chronic bronchitis symptoms and severity to the degree of CFTR abnormality (7–10).

Although evidence is unequivocal that smoking cessation is absolutely critical to limit disease progression, chronic bronchitis symptoms can often persist for a large number of patients with COPD who successfully quit smoking, and still others never successfully quit smoking despite the desire to do so; likewise, CFTR dysfunction also can persist despite smoking cessation, as shown by our group in two separate cohorts (7, 10). There are still large unmet therapeutic needs to be addressed, even in those individuals that have stopped smoking. Thus, reduced CFTR function in COPD-related chronic bronchitis represents an important therapeutic opportunity worthy of further exploration. Reversal of CFTR gating abnormalities induced by cigarette smoke, acrolein, hypoxia, or other factors is particularly attractive, as augmenting channel opening is the principal mechanism by which CFTR potentiators, such as ivacaftor, confers its benefit (13, 30). This does not discount the potential for other mechanisms, such as those that augment expression or address epigenetic regulation, which may also be of interest, especially when CFTR function is affected by other mechanisms, such as loss of cell differentiation and proteolytic degradation of the CFTR protein (34). Stimulation of a depleted number of residual channels, such as that observed in patients with R117H/5T CF who exhibit reduced CFTR activity due to a reduced number of channels, also may play a role (35). The recently published pilot evaluation of ivacaftor in patients with COPD confirms the feasibility of using CFTR modulators in COPD. Although this pilot trial was statistically underpowered and likely of too short in duration to detect definitive changes in lung function, there were improvements in CFTR activity and respiratory symptoms, especially in those patients whose CFTR activity was diminished at baseline (36).

Another important finding revealed by functional anatomic imaging is that the addition of ivacaftor, even when administered to healthy monolayers, increased ASL depth. This is consistent with clinical data showing that MCC in healthy individuals can be pharmacologically stimulated over basal rates (37), and is also compatible with the notion that CFTR is submaximally activated under healthy conditions and susceptible to stimulation when required, such as after the exposure to noxious stimuli or respiratory pathogens (38). This suggests the potential for CFTR potentiators or activators to offer a protective function even in those without genetic or acquired CFTR dysfunction, a concept worthy of prospective study. ■

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