Cigarette Smoke Induces Systemic Defects in Cystic Fibrosis Transmembrane Conductance Regulator Function

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Rationale: Several extrapulmonary disorders have been linked to cigarette smoking. Smoking is reported to cause cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction in the airway, and is also associated with pancreatitis, male infertility, and cachexia, features characteristic of cystic fibrosis and suggestive of an etiological role for CFTR.

Objectives: To study the effect of cigarette smoke on extrapulmonary CFTR function.

Methods: Demographics, spirometry, exercise tolerance, symptom questionnaires, CFTR genetics, and sweat chloride analysis were obtained in smokers with and without chronic obstructive pulmonary disease (COPD). CFTR activity was measured by nasal potential difference in mice and by Ussing chamber electrophysiology *in vitro*. Serum acrolein levels were estimated with mass spectroscopy.

Measurements and Main Results: Healthy smokers (29.45 ± 13.90 mEq), smokers with COPD (31.89 ± 13.9 mEq), and former smokers with COPD (25.07 ± 10.92 mEq) had elevated sweat chloride levels compared with normal control subjects (14.5 ± 7.77 mEq), indicating reduced CFTR activity in a nonrespiratory organ. Intestinal current measurements also demonstrated a 65% decrease in CFTR function in smokers compared with never smokers. CFTR activity was decreased by 68% in normal human bronchial epithelial cells exposed to plasma from smokers, suggesting that one or more circulating agents could confer CFTR dysfunction. Cigarette smoke-exposed mice had decreased CFTR activity in intestinal epithelium (84.3 and 45%, after 5 and 17 wk, respectively). Acrolein, a component of cigarette smoke, was higher in smokers, blocked CFTR by

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Cigarette smoking is associated with multiple systemic disorders and also causes acquired cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction in the respiratory tract.

What This Study Adds to the Field

Cigarette smoking reduces the function of CFTR at multiple sites remote from the respiratory tract, indicating acquired CFTR dysfunction is a systemic phenomenon. Acrolein is an agent detectable in smokers and conferred by cigarette smoking that contributes to this abnormality.

inhibiting channel gating, and was attenuated by antioxidant N-acetylcysteine, a known scavenger of acrolein.

Conclusions: Smoking causes systemic CFTR dysfunction. Acrolein present in cigarette smoke mediates CFTR defects in extrapulmonary tissues in smokers.

Keywords: cystic fibrosis transmembrane conductance regulator; cigarette smoking; chronic obstructive pulmonary disease; acrolein

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Cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial anion channel predominantly expressed in exocrine tissues. Mutations in the gene encoding CFTR are the proximate cause of cystic fibrosis (CF), and its absence causes both airflow obstruction and extrapulmonary manifestations, including pancreatic obstruction, male infertility, chronic constipation, malnutrition, and excess salt loss in the sweat duct (1). CFTR mutations that confer partial function are also associated with exocrine organ disorders, including non-CF bronchiectasis (2), recurrent pancreatitis (3), and congenital bilateral absence of vas deference (4).

A number of extrapulmonary disorders associated with CFTR mutations are also prevalent in individuals who smoke, raising the possibility that CFTR dysfunction may play an important role in these conditions. For example, recurrent idiopathic pancreatitis (5), male infertility (6), cachexia (7), and diabetes mellitus (8) are each independently associated with smoking, and yet the underlying mechanisms remain obscure.

Emerging data indicate that cigarette smoking induces an acquired state of CFTR dysfunction in the respiratory tract even in the absence of CFTR mutations (9, 10) and may contribute to

the pathogenesis of chronic obstructive pulmonary disease (COPD). CFTR dysfunction due to smoke exposure adversely affects mucociliary transport and is also associated with chronic bronchitis (11), a phenotype reminiscent of CF. Although these studies demonstrate the potential role of acquired CFTR dysfunction in lung due to smoking, no studies have evaluated CFTR activity in extrapulmonary organs. Furthermore, animal studies to definitively prove causality have not been conducted or determined the cigarette smoke constituents likely to confer the abnormality. Based on these observations, we hypothesized that cigarette smoke confers CFTR dysfunction in organs remote from the lung and that this could be transmitted by a circulating cigarette smoke constituent.

METHODS

Human Subject Participation

All protocols were approved by the University of Alabama at Birmingham's institutional review board, and all subjects provided written informed consent. Inclusion criteria required age 35 to 80 years and no respiratory illness in the last month that required antibiotics or steroids. A minimum of 10 pack-years tobacco use was required for all patients with COPD, and current smokers were defined as smoking at least 10 cigarettes daily. Former smokers must have been abstinent for 1 year or more, confirmed by measurement of urine cotinine levels less than 10 ng/ml. Spirometry was performed per American Thoracic Society criteria. Patients with COPD must also have had pulmonary obstruction, defined as a post-bronchodilator FEV₁/FVC below the lower limit of normal for age, race, sex, and height based on Hankinson prediction equations. Exclusion criteria include asthma or other lung disease or a change in medications 1 month before enrollment. Modification of the UK National Institute for Health and Clinical Excellence (NICE) criteria (<http://www.nice.org.uk/nicemedia/live/13029/49397/49397.pdf>) was used to determine a clinical diagnosis of COPD.

CFTR genetic testing was also performed, and subjects with CFTR mutations were omitted from further analysis ($n = 5$). DNA testing was performed by the Baylor College of Medicine Genetics Laboratory using a CFTR-related disorders mutation panel. This is an allele-specific genotyping technique for 89 mutations (see Table E2 in the online supplement) and includes testing for mutations common in human populations and reflex testing of the 5T allele.

Sweat Chloride Analysis

Sweat was collected using the Macroduct collection system (Wescor, Logan, UT), as previously reported (12). Chloride concentration was measured at the Center for Sweat Analysis at the University of Colorado and was blinded to disease group.

CFTR Ion Transport Assays

CFTR function in primary human bronchial epithelial cells, rectal biopsy samples, murine trachea, and intestines was determined by

short-circuit current measurements in Ussing chambers according to methods described previously (13, 14). CFTR function in murine nasal epithelium was measured by nasal potential difference (NPD) measurements under anesthesia (15).

Murine Studies

A/J mice were exposed in whole-body chambers or nose-only system to room air or diluted mainstream cigarette smoke ($152 \pm 58 \mu\text{g/L}$ of total particulate matter) to the indicated durations of time. Mice were administered acrolein (1 mg/kg) or dimethyl sulfoxide via subcutaneous osmotic pumps (ALZET; Durect Corp, Cupertino, CA) for 4 weeks.

Unitary Conductance Tracings

Single-channel currents were recorded from inside-out patches of HEK 293 cells expressing wild-type CFTR treated with acrolein or dimethyl sulfoxide, and open probability was calculated as reported previously (16).

Estimation of Acrolein

Free circulatory acrolein was measured in serum samples using a mass spectroscopic method described in the online supplement (17). Acrolein modifications were detected in serum proteins by standard Western blots using 1:500 diluted anti-acrolein antibody (Abcam Inc., Cambridge, MA).

Reagents

Acrolein (Acros Organic, Fair Lawn, NJ), forskolin (Calbiochem, San Diego, CA) and CFTR_{inh}-172 (Calbiochem) were obtained as noted; all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Statistics

Descriptive statistics (mean, SD, and SEM) were compared using Student *t* test or analysis of variance, as appropriate. *Post hoc* tests for multiple comparisons after analysis of variance were calculated using Fisher least significant difference. All statistical tests were two-sided and were performed at a 5% significance level (i.e., $\alpha = 0.05$) using GraphPad Prism (La Jolla, CA). Population statistics and regression analyses were performed using SPSS (IBM, Armonk, NY); multiple regression was conducted using a stepwise approach.

Online Supplement

The online supplement includes detailed descriptions of all methods used and additional supporting data.

RESULTS

Systemic CFTR Dysfunction in Cigarette Smokers

To determine whether cigarette smoking is associated with CFTR dysfunction at a site remote from direct exposure, we evaluated

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS UNDERGOING SWEAT CHLORIDE TESTING

	HNS ($n = 33$)	HS ($n = 31$)	CS ($n = 37$)	CFS ($n = 17$)
Age, mean $\pm$ SD, yr	48 $\pm$ 8	50 $\pm$ 7	57 $\pm$ 8*	66 $\pm$ 7*
Female, n (%)	20 (61)	14 (44)	12 (32) [†]	4 (24) [†]
White, n (%)	16 (48)	8 (25)	23 (59)	10 (63)
African American, n (%)	17 (52)	24 (75)	15 (41)	7 (37)
FEV ₁ , mean $\pm$ SD, L	3.09 $\pm$ 0.95	2.87 $\pm$ 0.69	1.99 $\pm$ 0.70*	1.41 $\pm$ 0.66*
FEV ₁ %, mean $\pm$ SD	1.02 $\pm$ 0.10	0.95 $\pm$ 0.12	0.60 $\pm$ 0.15 [†]	0.46 $\pm$ 0.21 [†]
Chronic bronchitis, n (%)	1 (3)	9 (28) [†]	24 (63)*	6 (35)*
Serum sodium, mean $\pm$ SD, mEq/L	139 $\pm$ 2	139 $\pm$ 2	139 $\pm$ 4	140 $\pm$ 3
Serum chloride, mean $\pm$ SD, mEq/L	105 $\pm$ 3	105 $\pm$ 4	104 $\pm$ 3	104 $\pm$ 4
Serum aldosterone, mean $\pm$ SD pg/ml	41.9 $\pm$ 39.1	35.0 $\pm$ 23.5	40.4 $\pm$ 36.7	39.9 $\pm$ 12.3

Definition of abbreviations: CFS = former smoker with COPD; CS = smoker with COPD; HNS = healthy nonsmoker; HS = healthy smoker. *P* values presented are *post hoc* analyses after analysis of variance or Chi-square, as appropriate, compared with healthy nonsmoker group.

* $P < 0.001$.

[†] $P < 0.05$.

Figure 1. Systemic cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction in cigarette smokers and subjects with chronic obstructive pulmonary disease (COPD). (A) Sweat chloride analysis of normal subjects, healthy smokers, smokers with COPD, and former smokers with COPD described in Table 1. The traditional dysfunctional threshold for cystic fibrosis (CF) (60 mEq/L) and CFTR-related disorders (40 mEq/L) in adults of this age group are indicated by dotted lines. * $P < 0.05$, *** $P < 0.0005$, **** $P < 0.00005$. (B) Relationship of CFTR function in cigarette smokers with and without COPD compared with that of patients with CF with various severities of CFTR mutations grouped by clinical phenotype (adapted by permission from Reference 22). The genotype-phenotype curve was generated from individuals in whom CFTR activity was estimated (small circles, labeled in gray) by nasal potential difference testing (x axis) and sweat chloride (y axis). Study subjects (large circles) are plotted based on mean sweat chloride. (C) Relative CFTR activity in healthy smokers, smokers with COPD, and former smokers with COPD in comparison to normal control subjects, as determined by interpolation of the genotype-phenotype curve shown in B. Dotted line represents 100% CFTR activity as determined by Wilschanski and colleagues and was not statistically different from normal control population. (D) The extrapulmonary CFTR assay tracings of intestinal current measurement (ICM) in rectal biopsy samples obtained from a representative smoker and nonsmoker. (E) cAMP-dependent intestinal current (Δ Isc) of rectal biopsy samples derived from smokers and nonsmokers described in Table 2; * $P < 0.05$.

CFTR activity using the latest assay for sweat chloride successfully implemented in CF clinical trials (12, 18). We enrolled smokers with COPD and nonsmokers with COPD, in addition to healthy nonsmokers and healthy smokers. Six out of 158 screened subjects had insufficient sweat collected for chloride analysis, which was equally dispersed between disease groups. Five subjects were found to be heterozygous for CFTR mutations and were not included in further analysis. Seventeen subjects did not meet spirometry criteria and were thus excluded from further analysis. Demographic variables for those included in the final analysis are shown in Table 1. Smokers with COPD and former smokers with COPD were slightly older than the other patient groups. Healthy nonsmokers had a female sex predilection. Smokers with COPD and former smokers with COPD had a smaller percentage of individuals of African American descent. As expected, spirometry was reduced in subjects with COPD. Sweat chloride was significantly increased in healthy smokers, smokers with COPD, and former smokers with COPD, indicative of reduced CFTR activity in the sweat gland, a site remote from the airway (Figure 1A). Increased sweat chloride was not accounted for by differences in serum chloride, sodium, or aldosterone (Table 1), and sweat chloride in normal control subjects was similar to that in prior publications (see Figure 1B) (19–21). Based on the genotype-phenotype correlation in CF relating CFTR activity measured by NPD and sweat chloride analysis (22) (Figure 1B), the

increase in sweat chloride in COPD subjects is equivalent to a 42% decrement in CFTR function (Figure 1C) and similar to that observed in the airway (11). CFTR dysfunction in the sweat gland was associated with COPD severity (FEV₁), smoking status, chronic bronchitis, bronchitis severity (as measured by Breathlessness,

TABLE 2. UNIVARIATE ANALYSIS OF VARIABLES ASSOCIATED WITH ELEVATED SWEAT CHLORIDE

	Beta	95% CI	P Value
Sex	0.064	−3.86, 8.03	0.489
Age	0.294	0.20, 0.79	0.001
Race	0.103	−2.51, 9.19	0.261
BCSS	0.321	0.92, 3.06	<0.001
Bronchitis	0.231	1.85, 13.96	0.011
FEV ₁	−0.321	−30.82, −9.16	<0.001
MMRC	0.276	1.231, 5.503	0.002
Pack-years	0.309	0.09, 0.33	0.001
Active smoker	0.268	3.02, 14.60	0.003
Ever smoker	0.409	8.74, 20.74	<0.001
COPD	0.348	5.73, 16.82	<0.001
BMI	−0.288	−1.26, −0.303	0.002

Definition of abbreviations: BCSS = Breathlessness, Cough, and Sputum Scale; BMI = body mass index; CI = confidence interval; COPD = chronic obstructive pulmonary disease; MMRC = Modified Medical Research Council scale.

P values in bold are statistically significant.

Figure 2. Systemic cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction caused by whole cigarette smoke *in vivo*. (A, C, and E) Representative tracings for nasal potential difference (A), tracheal short-circuit current (I_{sc}) (C), and intestinal I_{sc} measurements (E) of A/J mice exposed to whole cigarette smoke (four cigarettes, twice daily) or room air control in whole body chambers for 5 weeks before measurements. Mean forskolin (20 μ M) stimulated change in nasal potential difference (B), forskolin (20 μ M) plus IBMX (100 μ M) stimulated change in tracheal I_{sc} (D), and forskolin plus IBMX-dependent change in intestinal I_{sc} (F) of mice shown in A, C, and E. (G) Forskolin plus IBMX-dependent change in intestinal I_{sc} of mice exposed once daily 5 d/wk to whole cigarette smoke for 17 weeks. (H) Intestinal I_{sc} of mice exposed to cigarette smoke through nose-only route for 5 weeks. $n = 8$ –15/condition; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Cough, and Sputum Scale score), dyspnea (as measured by Modified Medical Research Council scale), and body mass index (BMI), among other variables (Table 2); the effect of bronchitis severity and BMI persisted even when smoking and COPD status were included in a multivariate regression model ($\beta = 0.21$ for chronic bronchitis and $\beta = -0.288$ for BMI, respectively; $P < 0.001$, $R^2 = 0.23$) determined by stepwise regression, indicating clinical relevance of sweat chloride abnormality to both respiratory and gastrointestinal systems.

To confirm CFTR dysfunction was present in a nonrespiratory organ, we analyzed CFTR activity by intestinal current measurements from rectal biopsy samples in a cohort of healthy smokers and never smokers undergoing colonoscopy for colorectal cancer screening (Table E1). There were no differences in age, sex, or race. In comparison to nonsmokers, we observed a 60% reduction in cAMP-dependent chloride transport (short-circuit current, I_{sc}) in individuals with a history of smoking, reflecting reduced CFTR activity in these tissues (Figures 1D and 1E). These results established meaningful reductions in CFTR activity at two anatomic sites remote from the respiratory system and unlikely to be directly exposed to substantive amounts of cigarette smoke.

Confirmation of Systemic CFTR Dysfunction in a Murine Model

Based on the association of systemic CFTR dysfunction with smoking, we used an animal model to verify findings in human subjects and provide definitive evidence that nonrespiratory CFTR abnormalities are causally related to cigarette smoking and not epiphenomena associated with other attributes of smokers. Non-CF A/J mice were exposed in whole-body chambers to cigarette smoke twice daily for 5 weeks, and CFTR activity was estimated in the nasal airway by potential difference testing; CFTR function in trachea and intestine were studied using short-circuit current analysis of excised tissues. Cigarette smoke exposure caused a significant decrease in CFTR-mediated ion transport in the nasal airway (Figures 2A and 2B) and trachea (Figures 2C and 2D) in addition to intestinal epithelia (Figures 2E and 2F), the latter representing a nonrespiratory (i.e., systemic) tissue that can be readily tested for CFTR activity. Longer exposures (17 wk once daily) to cigarette smoke further reduced cAMP-dependent I_{sc} in excised ileum (Figure 2G), representing a functional CFTR deficit of 55% when compared with mice exposed to air control and closely resembling the CFTR deficit observed in the rectal biopsy samples of human smokers (Figures 1D and 1E). Intestinal I_{sc} of mice exposed to cigarette smoke through nose-only route for 5 weeks twice daily exhibited a CFTR decrement (Figure 2H) similar to that observed in whole-body smoke chambers (Figures 2E and 2F). The magnitude of CFTR inhibition among tissues compared with the total duration of exposure revealed a relative rank order of nose > trachea > intestine, with a delayed emergence of clinically relevant CFTR blockade in the intestine (Figure 3); this finding is compatible with exposure intensity observed in rodent nose and trachea compared with indirect and less-intense exposure in intestines (23). Taken together, these data provide the first evidence that smoking is causally related to acquired CFTR dysfunction and is responsible for both pulmonary and systemic ion transport abnormalities.

Cigarette Smoke-induced CFTR Dysfunction Can Be Transmitted by Circulatory Agent

The effects of cigarette smoke on CFTR activity at sites remote from direct exposure suggested that CFTR dysfunction could be transmitted by one or more circulating agents. To test this hypothesis, we incubated the plasma of healthy smokers and

Figure 3. Time-course of cigarette smoke effects on pulmonary and systemic cystic fibrosis transmembrane conductance regulator (CFTR) function *in vivo*. Temporal patterns of CFTR dysfunction caused by cigarette smoke are represented by plotting weeks of cigarette smoke exposure versus % decrement in CFTR activity measured in different tissues. Mice were exposed to cigarette smoke twice daily for 2- and 5-week time periods and once daily for 17 weeks. CFTR function was measured in nose (NP, nasal potential difference measurement), trachea, and intestine (Ussing chamber electrophysiology). $n = 8-15$, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

patients with COPD described in Table 1 who also exhibited elevated sweat chloride (i.e., >40 mEq) to the basolateral compartment of primary human bronchial epithelial (HBE) cells derived from healthy donors without CF, a model faithful to CFTR physiology *in vivo* (24). In comparison to HBE cell monolayers exposed to plasma from control subjects with normal sweat chloride (≤ 15 mEq), HBE monolayers exposed to plasma from healthy smokers, smokers with COPD, and former smokers with COPD each exhibited reduced cAMP-dependent anion transport (Figures 4A and 4B); the magnitude of CFTR inhibition was comparable to that present in the sweat gland and intestinal epithelia of mice and humans. Recapitulation of systemic CFTR dysfunction by plasma from smokers who exhibit abnormal CFTR function in the periphery provided strong evidence that one or more circulating agent(s) can cause CFTR dysfunction, warranting further investigation into the possible causative factors.

Acrolein is an important bioactive component of cigarette smoke shown previously to elicit deleterious effects by rapidly reacting with biological nucleophiles, such as threonine, cysteine, histidine, arginine, and lysine residues in proteins, forming toxic adducts via oxidative pathways (25). Acrolein is also produced endogenously by proinflammatory pathways known to be elevated in COPD (26). Modification of CFTR seemed a likely target for circulating acrolein, and progressive decrements in CFTR function due to ongoing acrolein-induced CFTR injury from continued smoking are plausible, depending on the rate of CFTR turnover in tissues. We developed a sensitive method using electrospray ionization–liquid chromatography followed by tandem mass spectrometry to detect and quantify free acrolein in the serum of subjects reported in Table 1. Free acrolein levels were significantly greater in smokers with and without COPD (Figure 4C). Because acrolein is highly reactive, and the recovery of free acrolein from serum is likely to be incomplete, for confirmation we also measured acrolein-modification sites in the serum proteome using Western blot analysis to quantify levels of acrolein-modified proteins in the circulation of smokers. Levels of acrolein-modified serum proteins at a specific molecular weight (20 kD) were consistently elevated in the

Figure 4. Role of circulatory acrolein in mediating cigarette smoke-induced systemic cystic fibrosis transmembrane conductance regulator (CFTR) defects *in vitro*. (A) Representative short-circuit current (I_{sc}) tracing of wild-type human bronchial epithelial (HBE) cells isolated from healthy nonsmokers and studied under voltage clamp conditions after 24-hour exposure of the basolateral compartment to 17 μ L of plasma (diluted in 1 mL media) derived from normal control subjects, healthy smokers, smokers with COPD, and former smokers with COPD. CFTR-dependent anion transport was quantified by sequential addition of amiloride (a, 100 μ M), forskolin (f, 10 μ M), and CFTR_{inh}-172 (i, 10 μ M). (B) Summary of forskolin-stimulated CFTR function in HBE cells exposed to various plasma samples. $n = 6-8$, * $P < 0.05$. (C) Free acrolein concentration in serum obtained from normal control subjects, healthy smokers, smokers with COPD, and former smokers with COPD, as estimated by mass spectrometry based on a relative standard curve. $n = 24, 23, 20$, and 12; * $P < 0.05$. (D) Representative Western blot to detect acrolein-modified proteins; each lane depicts albumin-depleted serum from an individual subject. Ponceau stain is shown as loading control. (E) Ratio of acrolein-modified 20-Kd protein to loading reference. * $P < 0.05$, $n = 12$.

serum of smokers compared with normal control subjects (Figures 4D and 4E), further indicating that the systemic acrolein load is elevated due to smoking.

Acrolein Confers Systemic CFTR Dysfunction

To establish that acrolein inhibits CFTR activity, we exposed non-CF HBE cell monolayers to various concentrations of acrolein to the basolateral compartment, mimicking systemic exposure. Acrolein, but not cotinine, reduced cAMP-dependent I_{sc} in HBE cells (Figure E1), indicating acrolein is a cigarette smoke component that can confer CFTR dysfunction. These findings were dose dependent, as established by evaluation of Calu-3 epithelial cell monolayers (Figures 5A and 5B) after 24-hour exposures. Unitary conductance tracings demonstrated that acrolein had rapid effects on the open-channel probability of CFTR, a measure of its single-channel activity (Figures 5C and 5D) and provides mechanistic explanation for how acrolein mediates the effects of cigarette smoking on CFTR-mediated ion transport. To further validate these findings and to determine the effects of longer duration of low-level acrolein exposure on CFTR expression and function, we treated primary HBE cells daily to 2.5 to 10 ng/mL of acrolein for 7 days. Mass spectroscopic analyses indicated that free acrolein levels applied *in vitro* substantially dissipate within the first hour (Figure E3), indicating its highly reactive nature. As shown in Figures 5G and 5H, long-term exposure to acrolein did not cause

any appreciable change in CFTR protein expression. However, at concentrations of 5 and 10 ng/mL, acrolein significantly reduced CFTR function (Figures 5E and 5F). These data indicate that physiologic exposures of circulatory acrolein in smokers with and without COPD can contribute to CFTR dysfunction, even when levels rapidly dissipate. To further delineate how acrolein contributed to the deleterious effects of circulating cigarette smoke-induced epithelial injury, we used *N*-acetyl cysteine (NAC), a glutathione precursor that scavenges acrolein and that may have clinical usefulness in COPD due to antioxidant effects (27–29). NAC prevented the inhibitory effect of acrolein on cAMP-mediated CFTR activation *in vitro*, demonstrating the potential to abrogate the acrolein-mediated defect (Figure 6A). NAC also blocked inhibition of CFTR conferred by plasma from healthy smokers (Figure 6B), providing evidence that acrolein is a crucial contributor to CFTR dysfunction caused by smoking via a blood-borne and systemically transmitted pathway. *In vivo* studies yielded similar results. A/J mice administered acrolein (1 mg/kg) through continuous infusion by a subcutaneous osmotic pump for 4 weeks increased serum acrolein (12 ng/mL) to levels similar to that observed in smokers with COPD (Figure E2A). Systemic acrolein exposure reduced CFTR function in the nasal epithelium and trachea and was similar in severity to whole cigarette smoke exposure (Figures E2B and E2C). Compared with the vehicle-treated mice, acrolein-exposed mice exhibited decreased CFTR function in intestinal epithelium, and, as observed

in vitro, coadministration of oral NAC (40 μM) completely prevented the suppressive effects of acrolein (Figure 6C).

DISCUSSION

Previous studies have demonstrated that CFTR dysfunction is associated with cigarette smoking and is likely to contribute

to respiratory manifestations of COPD (10, 11, 30, 31). Using a number of complementary studies that include the first evaluation of the effects of cigarette smoke on CFTR biology in a well-controlled animal model, we demonstrate that cigarette smoking is causally related to systemic CFTR dysfunction *in vivo*. Furthermore, *in vitro* and *in vivo* studies establish that

Figure 5. Inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) activity by acrolein. (A) Representative short-circuit current (I_{sc}) tracings of non-CF human bronchial epithelial (HBE) cells exposed to acrolein (2.8 μ g/ml) or vehicle control for 24 hours to the basolateral compartment, then treated with forskolin (10 μ M), and CFTR_{inh}-172 (10 μ M) under voltage clamp conditions in the setting of a chloride secretory gradient and amiloride (100 μ M). (B) Summary of dose-response change in forskolin-stimulated I_{sc} measured in Calu-3 cells exposed to increasing concentrations of acrolein. Acrolein inhibited CFTR with a half-maximal inhibitory concentration of 3.2 μ g/ml. (C) Representative unitary conductance tracings of a membrane patch expressing wild-type CFTR in an inside-out configuration and exposed sequentially to vehicle control (*top*) or acrolein (2.8 μ g/ml, *bottom*) in the presence of protein kinase A (40 U/ml) and MgATP (1 mM). Holding potential was -50 mV. (D) Summary of acrolein effects on open-channel probability of CFTR, as determined by repeated experiments illustrated in C. $^{*}P < 0.05$, $n = 6$. (E) Representative I_{sc} tracing of HBE cells exposed to low-dose acrolein (10 ng/ml) for 7 days with media being changed every 24 hours. Cells were sequentially exposed to amiloride (100 μ M), apical low chloride, forskolin (10 μ M), and CFTR_{inh}-172 (10 μ M). (F) Summary of forskolin-stimulated I_{sc} of cells exposed to acrolein for 7 days. $^{**}P < 0.005$, $^{*}P < 0.05$; $n = 4$. (G) Representative images of Western blot analysis for the expression of CFTR and α -tubulin in HBE cells exposed every day to varying doses of acrolein for 7 days. Western analysis was repeated three separate times, and the summary of densitometric analyses is shown in H. $P =$ not significant, $n = 3$.

circulatory levels of acrolein found in smokers is sufficient to induce the degree of CFTR dysfunction observed in humans and can be pharmacologically blocked.

The CFTR decrements observed in our studies are likely to be clinically significant, as indicated by the association with COPD severity, bronchitis symptoms, and reduced BMI; each of these suggests a role of CFTR in disease expression. Symptoms of bronchitis and dyspnea have been associated with CFTR abnormality detected in the nasal airway (11) and lungs (32). Furthermore, low BMI has been associated with increased mortality in COPD (33) and implicates the role of CFTR deficiency in reducing gastrointestinal tract function in smokers. Genotype-phenotype studies have shown that modest degrees of CFTR abnormality are sufficient to cause disease, particularly when other insults to organ function are also present. For example, studies in individuals who harbor partially functional CFTR mutations demonstrate that CFTR abnormalities greatly increase the risk of recurrent idiopathic pancreatitis (34). Given that at least 15% of patients with idiopathic pancreatitis are heterozygous for CFTR mutations (3, 35), and many causative CFTR mutations confer only modest dysfunction, it seems possible that CFTR dysfunction at the levels we observed is sufficient to increase the risk of both pulmonary and extrapulmonary disease, particularly when compounded by additional insults, such as alcohol, which has also been associated with CFTR dysfunction (36).

Sweat chloride testing is a traditional test used in the evaluation of adults with late-onset bronchiectasis. Sweat chloride values obtained in our cohort were in the intermediate range, below

the traditional diagnostic threshold of CF (e.g., 60 mEq/L), but approximate that seen in individuals with CFTR-related disorders (e.g., 40 mEq/L) (22). Because smoking may affect sweat chloride levels, smoking status may need to be considered as a covariate in individuals being evaluated for atypical cases of CF, such as adults who present for evaluation later in life; a similar adjustment may be needed for NPD (9, 11).

Based on the inhibition of CFTR conferred by plasma, elevated circulatory levels of acrolein, and recapitulation of acquired CFTR dysfunction by subcutaneous acrolein *in vivo* that approximated clinically relevant serum concentrations, our studies demonstrate that acrolein is one crucial contributor to acquired and systemically transmitted CFTR dysfunction. Aside from direct inhalation via cigarette smoke, acrolein can also be produced through endogenous pathways associated with inflammation (26). Our findings are also supported by studies demonstrating the acute effects of acrolein on CFTR function, albeit at concentrations unlikely to be observed in systemic circulation for sustained periods (37). Acrolein protein modification occurs in the epithelia of patients with COPD and could partially explain persistent decrements in CFTR activity observed due to smoking, because acrolein-protein adducts persist despite prolonged smoking cessation and would be unlikely to be detected in former smokers with COPD (38). Although we did not detect elevated free acrolein in former smokers, these sources of chronic low-level acrolein production, in addition to the highly reactive nature of acrolein that limits its detection, may explain why sweat chloride remains elevated in former smokers with COPD.

Given that the effect of acrolein and other cigarette smoke constituents alter the open-channel probability of CFTR, reversal of this

Figure 6. Neutralization of acrolein-induced cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction. (A) Mean forskolin-dependent change in I_{sc} in HBE cells exposed to acrolein (2.8 μ g/ml), acrolein plus *N*-acetyl cysteine (NAC, 300 μ M), an acrolein scavenger, or vehicle control for 24 hours to the basolateral compartment. $^{**}P < 0.005$, $^{*}P < 0.05$, $n = 8$, performed twice independently. (B) CFTR-dependent forskolin-stimulated I_{sc} was measured after basolateral exposure of plasma (see Figure 4) from normal control subjects, healthy smokers, and the same healthy smokers coincubated with NAC for 24 hours. $^{*}P < 0.05$, $n = 8$, performed twice independently. (C) Similarly, administration of acrolein (1 mg/kg) in mice via osmotic pumps for 4 weeks rendered decreased CFTR function in intestinal epithelium, and such a decrease was effectively overcome by oral administration of NAC (40 μ M) in drinking water. $n = 16$.

pathway could be an important therapeutic approach to address the protean manifestations conferred by CFTR dysfunction. Based on the effective neutralization of CFTR dysfunction induced by acrolein and smoker plasma by NAC, our results demonstrate that the inhibition of acrolein-mediated CFTR dysfunction could represent a viable therapeutic approach to limit the deleterious effects of cigarette smoking. The beneficial effects of NAC observed on lung function and exacerbations in patients with COPD have largely been attributed to its antioxidant and mucolytic properties, and the significance of acrolein neutralization in these patients has not been studied (29, 39). Although prior attempts to use NAC as a treatment for COPD have demonstrated modest but inconsistent benefit, the doses used were not sufficient to achieve the serum or tissue levels necessary to counter acrolein-mediated events (28, 40).

Although we have successfully identified that acrolein strongly inhibits CFTR, our findings do not rule out that other agents present in cigarette smoke might also contribute to acquired CFTR dysfunction, including that transmitted by the systemic route. Oxidative stress (41), cadmium (42), hypoxia (43), and the unfolded protein response (44) have each been associated with CFTR dysfunction and are potential contributors to the acquired phenotype, particularly in the lung, where exposures to these stimuli would be expected to be particularly prominent.

Extrapulmonary manifestations are increasingly recognized to contribute to the symptom burden of lung diseases related to cigarette smoking, including COPD (45). We establish that CFTR abnormalities occur in organs remote from direct exposure, including the sweat gland and distal rectum of humans and the intestinal epithelia of mice. These findings are potentially significant, as the results could have implications for end-organ physiology outside the respiratory tract. However, extensive testing of end-organ pathology and function is required to better understand the implications of acquired CFTR dysfunction. A number of conditions more prevalent in smokers are directly related to CFTR function, including chronic pancreatitis (5), male infertility (6), and cachexia (7). Given that CFTR is known to have an important role in the gastrointestinal, pancreatic, and reproductive systems, and that genetic CFTR mutations also cause pancreatitis (3, 35), malnutrition (46), and male infertility (47), these data support the need for better studies to evaluate whether cigarette smoking is a contributor to these nonrespiratory conditions through acquired CFTR dysfunction. Considering the protean manifestations of smoking, it would be naive to expect that CFTR abnormalities play a causative role in all systemic manifestations of smoking or COPD. Although systemic inflammation has been postulated as a significant contributor to common nonpulmonary manifestations of COPD (48), including coronary artery disease (49), COPD myopathy (50), and cognitive dysfunction (51), CFTR dysfunction is unlikely to contribute to these disorders, as the protein has not been mechanistically implicated in their pathology.

In summary, the findings presented here indicate that cigarette smoking causes CFTR dysfunction in nonpulmonary tissues that can be transmitted by circulating agents such as acrolein and support investigations into the contribution of CFTR dysfunction to extrapulmonary end-organ manifestations of smoking.

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References

- Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. *N Engl J Med* 2005;352:1992–2001.
- Bienvenu T, Sermet-Gaudelus I, Burgel PR, Hubert D, Crestani B, Bassinet L, Dusser D, Fajac I. Cystic fibrosis transmembrane conductance regulator channel dysfunction in non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med* 2010;181:1078–1084.
- Sharer N, Schwarz M, Malone G, Howarth A, Painter J, Super M, Braganza J. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. *N Engl J Med* 1998;339:645–652.
- Chillon M, Casals T, Mercier B, Bassas L, Lissens W, Silber S, Romey MC, Ruiz-Romero J, Verlingue C, Claustres M, et al. Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. *N Engl J Med* 1995;332:1475–1480.
- Cote GA, Yadav D, Slivka A, Hawes RH, Anderson MA, Burton FR, Brand RE, Banks PA, Lewis MD, Disario JA, et al. Alcohol and smoking as risk factors in an epidemiology study of patients with chronic pancreatitis. *Clin Gastroenterol Hepatol* (In press)
- Gaur DS, Talekar M, Pathak VP. Effect of cigarette smoking on semen quality of infertile men. *Singapore Med J* 2007;48:119–123.
- Balasubramanian VP, Varkey B. Chronic obstructive pulmonary disease: effects beyond the lungs. *Curr Opin Pulm Med* 2006;12:106–112.
- Will JC, Galuska DA, Ford ES, Mokdad A, Calle EE. Cigarette smoking and diabetes mellitus: evidence of a positive association from a large prospective cohort study. *Int J Epidemiol* 2001;30:540–546.
- Cantin AM, Hanrahan JW, Bilodeau G, Ellis L, Dupuis A, Liao J, Zielinski J, Durie P. Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. *Am J Respir Crit Care Med* 2006;173:1139–1144.
- Kreindler JL, Jackson AD, Kemp PA, Bridges RJ, Danahay H. Inhibition of chloride secretion in human bronchial epithelial cells by cigarette smoke extract. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L894–L902.
- Sloane PA, Shastry S, Wilhelm A, Courville C, Tang LP, Backer K, Levin E, Raju SV, Li Y, Mazur M, et al. A pharmacologic approach to acquired cystic fibrosis transmembrane conductance regulator dysfunction in smoking related lung disease. *PLoS ONE* 2012;7:e39809.
- Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, Sagel SD, Hornick DB, Konstan MW, Donaldson SH, et al. Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *N Engl J Med* 2010;363:1991–2003.
- Rowe SM, Sloane P, Tang LP, Backer K, Mazur M, Buckley-Lanier J, Nudelman I, Belakhov V, Bebek Z, Schwiebert E, et al. Suppression of CFTR premature termination codons and rescue of CFTR protein and function by the synthetic aminoglycoside NB54. *J Mol Med (Berl)* 2011;89:1149–1161.
- Hug MJ, Derichs N, Bronsveld I, Clancy JP. Measurement of ion transport function in rectal biopsies. *Methods Mol Biol* 2011;741:87–107.
- Du M, Keeling KM, Fan L, Liu X, Kovacs T, Sorscher E, Bedwell DM. Clinical doses of amikacin provide more effective suppression of the human CFTR-G542X stop mutation than gentamicin in a transgenic CF mouse model. *J Mol Med (Berl)* 2006;84:573–582.
- Hickman-Davis JM, McNicholas-Bevensee C, Davis IC, Ma HP, Davis GC, Bosworth CA, Matalon S. Reactive species mediate inhibition of alveolar type II sodium transport during mycoplasma infection. *Am J Respir Crit Care Med* 2006;173:334–344.
- Andreoli R, Manini P, Corradi M, Mutti A, Niessen WM. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2003;17:637–645.
- Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Drevinek P, Griese M, McKone EF, Wainwright CE, Konstan MW, et al. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* 2011;365:1663–1672.
- Sebro R, Levy H, Schneck K, Dimmock D, Raby BA, Cannon CL, Broeckel U, Risch NJ. Cystic fibrosis mutations for p.F508del compound heterozygotes predict sweat chloride levels and pancreatic sufficiency. *Clin Genet* 2011;82:546–551.

20. Farrell PM, Kosciak RE. Sweat chloride concentrations in infants homozygous or heterozygous for F508 cystic fibrosis. *Pediatrics* 1996;97:524–528.
21. Liu L, Gardecki JA, Nadkarni SK, Toussaint JD, Yagi Y, Bouma BE, Tearney GJ. Imaging the subcellular structure of human coronary atherosclerosis using micro-optical coherence tomography. *Nat Med* 2011;17:1010–1014.
22. Wilschanski M, Dupuis A, Ellis L, Jarvi K, Zielenski J, Tullis E, Martin S, Corey M, Tsui LC, Durie P. Mutations in the cystic fibrosis transmembrane regulator gene and in vivo transepithelial potentials. *Am J Respir Crit Care Med* 2006;174:787–794.
23. Schlesinger RB. Comparative deposition of inhaled aerosols in experimental animals and humans: a review. *J Toxicol Environ Health* 1985;15:197–214.
24. Dvorak A, Tilley AE, Shaykhiev R, Wang R, Crystal RG. Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome? *Am J Respir Cell Mol Biol* 2011;44:465–473.
25. Jia L, Liu Z, Sun L, Miller SS, Ames BN, Cotman CW, Liu J. Acrolein, a toxicant in cigarette smoke, causes oxidative damage and mitochondrial dysfunction in RPE cells: protection by (R)-alpha-lipoic acid. *Invest Ophthalmol Vis Sci* 2007;48:339–348.
26. Stevens JF, Maier CS. Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res* 2008;52:7–25.
27. Zuin R, Palamidese A, Negrin R, Catozzo L, Scarda A, Balbinot M. High-dose N-acetylcysteine in patients with exacerbations of chronic obstructive pulmonary disease. *Clin Drug Investig* 2005;25:401–408.
28. Decramer M, Rutten-van Molken M, Dekhuijzen PN, Troosters T, van Herwaarden C, Pellegrino R, van Schayck CP, Olivieri D, Del Donno M, De Backer W, et al. Effects of N-acetylcysteine on outcomes in chronic obstructive pulmonary disease (Bronchitis Randomized on NAC Cost-Utility Study, BRONCUS): a randomised placebo-controlled trial. *Lancet* 2005;365:1552–1560.
29. Tse HN, Raiteri L, Wong KY, Yee KS, Ng LY, Wai KY, Loo CK, Chan MH. High-dose N-acetylcysteine in stable COPD: the 1-year, double-blind, randomized, placebo-controlled HIACE study. *Chest* 2013;144:106–118.
30. Cantin AM. Cellular response to cigarette smoke and oxidants: adapting to survive. *Proc Am Thorac Soc* 2010;7:368–375.
31. Clunes LA, Davies CM, Coakley RD, Aleksandrov AA, Henderson AG, Zeman KL, Worthington EN, Gentzsch M, Kreda SM, Cholon D, et al. Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration. *FASEB J* 2012;26:533–545.
32. Dransfield MT, Wilhelm AM, Flanagan B, Tidwell SL, Raju SV, Gaggar A, Steele C, Tang LP, Liu B, Rowe SM. Acquired CFTR dysfunction in the lower airways in COPD. *Chest* 2013;144:498–506.
33. Celli BR, Cote CG, Marin JM, Casanova C, Montes de Oca M, Mendez RA, Pinto Plata V, Cabral HJ. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med* 2004;350:1005–1012.
34. Ooi CY, Dorfman R, Cipolli M, Gonska T, Castellani C, Keenan K, Freedman SD, Zielenski J, Berthiaume Y, Corey M, et al. Type of CFTR mutation determines risk of pancreatitis in patients with cystic fibrosis. *Gastroenterology* 2011;140:153–161.
35. Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. *N Engl J Med* 1998;339:653–658.
36. Raju SV, Wang G. Suppression of adenosine-activated chloride transport by ethanol in airway epithelia. *PLoS ONE* 2012;7:e32112.
37. Alexander NS, Blount A, Zhang S, Skinner D, Hicks SB, Chestnut M, Kebbel FA, Sorscher EJ, Woodworth BA. Cystic fibrosis transmembrane conductance regulator modulation by the tobacco smoke toxin acrolein. *Laryngoscope* 2012;122:1193–1197.
38. Kitaguchi Y, Taraseviciene-Stewart L, Hanaoka M, Natarajan R, Kraskauskas D, Voelkel NF. Acrolein induces endoplasmic reticulum stress and causes airspace enlargement. *PLoS ONE* 2012;7:e38038.
39. Anderson MP, Rich DP, Gregory RJ, Smith AE, Welsh MJ. Generation of cAMP-activated chloride currents by expression of CFTR. *Science* 1991;251:679–682.
40. Tanel A, Averill-Bates DA. Inhibition of acrolein-induced apoptosis by the antioxidant N-acetylcysteine. *J Pharmacol Exp Ther* 2007;321:73–83.
41. Cantin AM, Bilodeau G, Ouellet C, Liao J, Hanrahan JW. Oxidant stress suppresses CFTR expression. *Am J Physiol Cell Physiol* 2006;290:C262–C270.
42. Rennolds J, Butler S, Maloney K, Boyaka PN, Davis IC, Knoell DL, Parinandi NL, Cormet-Boyaka E. Cadmium regulates the expression of the CFTR chloride channel in human airway epithelial cells. *Toxicol Sci* 2010;116:349–358.
43. Guimbellot JS, Fortenberry JA, Siegal GP, Moore B, Wen H, Venglarik C, Chen YF, Oparil S, Sorscher EJ, Hong JS. Role of oxygen availability in CFTR expression and function. *Am J Respir Cell Mol Biol* 2008;39:514–521.
44. Bartoszewski R, Rab A, Fu L, Bartoszevska S, Collawn J, Bebek Z. CFTR expression regulation by the unfolded protein response. *Methods Enzymol* 2011;491:3–24.
45. Chatila WM, Thomashow BM, Minai OA, Criner GJ, Make BJ. Comorbidities in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2008;5:549–555.
46. Pencharz PB, Durie PR. Pathogenesis of malnutrition in cystic fibrosis, and its treatment. *Clin Nutr* 2000;19:387–394.
47. Dodge JA. Male fertility in cystic fibrosis. *Lancet* 1995;346:587–588.
48. Nussbaumer-Ochsner Y, Rabe KF. Systemic manifestations of COPD. *Chest* 2011;139:165–173.
49. Sin DD, Man SF. Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. *Circulation* 2003;107:1514–1519.
50. Casadevall C, Coronell C, Ramirez-Sarmiento AL, Martinez-Llorens J, Barreiro E, Orozco-Levi M, Gea J. Upregulation of pro-inflammatory cytokines in the intercostal muscles of COPD patients. *Eur Respir J* 2007;30:701–707.
51. Borson S, Scanlan J, Friedman S, Zuh E, Fields J, Aylward E, Mahurin R, Richards T, Anzai Y, Yukawa M, et al. Modeling the impact of COPD on the brain. *Int J Chron Obstruct Pulmon Dis* 2008;3:429–434.