



Cigarette smoke exposure reveals a novel role for the MEK/ERK1/2 MAPK pathway in regulation of CFTR

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ARTICLE INFO

Article history:

Received 10 June 2014

Received in revised form 19 January 2015

Accepted 6 February 2015

Available online 16 February 2015

Keywords:

CFTR

Cigarette smoke

Airway epithelial cell

MAPK pathway

ABSTRACT

Background: Cystic fibrosis transmembrane conductance regulator plays a key role in maintenance of lung fluid homeostasis. Cigarette smoke decreases CFTR expression in the lung but neither the mechanisms leading to CFTR loss, nor potential ways to prevent its loss have been identified to date.

Methods: The molecular mechanisms leading to down-regulation of CFTR by cigarette smoke were determined using pharmacologic inhibitors and silencing ribonucleic acids (RNAs).

Results: Using human bronchial epithelial cells, here we show that cigarette smoke induces degradation of CFTR that is attenuated by lysosomal inhibitors, but not proteasome inhibitors. Cigarette smoke can activate multiple signaling pathways in airway epithelial cells, including the MEK/Erk1/2 MAPK (MEK: mitogen-activated protein kinase/ERK kinase Erk1/2: extracellular signal-regulated kinase 1/2 MAPK: Mitogen-activated protein kinase) pathway regulating cell survival. Interestingly, pharmacological inhibition of the MEK/Erk1/2 MAPK pathway prevented the loss of plasma membrane CFTR upon cigarette smoke exposure. Similarly, decreased expression of Erk1/2 using silencing RNAs prevented the suppression of CFTR protein by cigarette smoke. Conversely, specific inhibitors of the c-Jun N-terminal kinase (JNK) or p38 MAPK pathways had no effect on CFTR decrease after cigarette smoke exposure. In addition, inhibition of the MEK/Erk1/2 MAPK pathway prevented the reduction of the airway surface liquid observed upon cigarette smoke exposure of primary human airway epithelial cells. Finally, addition of the antioxidant N-acetylcysteine inhibited activation of Erk1/2 by cigarette smoke and precluded the cigarette smoke-induced decrease of CFTR.

Conclusions: These results show that the MEK/Erk1/2 MAPK pathway regulates plasma membrane CFTR in human airway cells.

General significance: The MEK/Erk1/2 MAPK pathway should be considered as a target for strategies to maintain/restore CFTR expression in the lung of smokers.

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1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel that plays a critical role in the lung by regulating airway fluid homeostasis allowing cilia to beat and clear pathogens [1]. Absence of functional CFTR leads to cystic fibrosis a genetic disease associated with impaired mucus clearance, and chronic infection and inflammation [2]. These past few years there has been a lot of interest in the negative regulation of CFTR by pollutants such as cigarette smoke, cadmium, and arsenic [3–6]. We and others have shown that CFTR expression is reduced in the lung of patients who developed chronic obstructive pulmonary disease (COPD) after years of cigarette smoking [7–9]. These findings suggest that suppression of CFTR could contribute to the development of chronic bronchitis seen in COPD which is

characterized by mucus secretion, infection and inflammation similarly to what is observed in the lungs of patients with Cystic Fibrosis.

Suppression of CFTR can occur via degradation by two main pathways: the ubiquitin–proteasome pathway and the lysosomal pathway [10–12]. Plasma membrane CFTR is rapidly endocytosed and undergoes rapid and efficient recycling back to the plasma membrane in human airway epithelial cells, with more than 75% of endocytosed wild-type CFTR recycling back to the plasma membrane [13–15]. The plasma membrane stability of CFTR depends on its biosynthetic processing and post-maturation trafficking, which involves endocytic uptake followed by recycling to the plasma membrane or degradation in the lysosomes [16,17]. The E3 ubiquitin ligase c-Cbl has been shown to facilitate CFTR endocytosis and ubiquitination with subsequent lysosomal degradation [18]. The molecular mechanism by which cigarette smoke alters expression of the CFTR ion channel is still unknown. We have previously shown that using a heterologous expression system, cigarette smoke exposure causes rapid internalization of CFTR. During this internalization, CFTR does not co-localize with

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lysosomes [6] but is instead internalized into an aggresome-like pathway in a calcium-dependent manner [6,19].

Cigarette smoke activates several mitogen-activated protein kinase (MAPK) pathways including the MEK/Erk1/2 MAPK pathway [20]. Activation of this latter MAPK pathway results in cell survival and proliferation [21]. It was recently shown that the MEK/Erk1/2 MAPK pathway can regulate the expression of the epithelial sodium channel ENaC by regulating its interaction with the E3 ubiquitin ligase Nedd4-2 leading to lysosomal degradation of ENaC [22,23]. Whether the MEK/Erk1/2 MAPK pathway also regulates the expression of plasma membrane CFTR is unknown.

Herein, we conducted this study to determine the underlying mechanisms by which cigarette smoke decreases CFTR abundance in human bronchial epithelial cells and determine the role of the MEK/Erk1/2 MAPK pathway in this process. We also evaluated whether the antioxidant N-acetyl-cysteine (NAC) could prevent the cigarette smoke-induced suppression of CFTR.

2. Methods

2.1. Cell culture and reagents

The human bronchial epithelial cell line 16HBE14o- was a generous gift from Dr. Gruenert, an immortalized human bronchial epithelial cell line, and was cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, 10% FBS and penicillin (100 U/ml) and streptomycin (100 µg/ml). The tissue culture plates were coated using human fibronectin (1 mg/ml), collagen I bovine (3 mg/ml), and bovine serum albumin (1 mg/ml). All the cells used in the experiments were between passages 25 and 50, and were grown and maintained at 37 °C in a 5% CO₂ humidified incubator. Primary human bronchial epithelial cells (HBECs) were isolated from excess donor tissue obtained at the time of lung transplantation under a protocol approved by UNC Medical School IRB. Primary HBECs were cultured as previously described and studied when fully differentiated [6,24]. Lactacystin, UO126, UO124, SB203580, and SP600125 were from Calbiochem (La Jolla, CA). PD98059 was purchased from Cell Signaling Technology. The proteasome inhibitor MG132, and the lysosomal inhibitors, leupeptin and chloroquine, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell transfection

16HBE14o- cells were transfected with Erk1 and Erk2 small interfering RNAs (siRNA), cbl siRNA (Ambion), or negative control #1 siRNA (Ambion) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Forty eight hours after transfection, cells were treated with or without 10% cigarette smoke extract (CSE) prepared from Camel cigarettes (R.J. Reynolds). The cells were then lysed in PBS with 1% Triton X-100 containing a cocktail of protease inhibitors (Roche Diagnostics, IN) for protein analysis.

2.3. Cell surface biotinylation

The 16HBE14o- cells were rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ to eliminate the proteins present in the media. Cell surface proteins were labeled with 1 mg/ml EZ-Link NHS-SS Biotin (Pierce) for 30 min at 4 °C. Biotinylation was stopped by removing the biotin solution and incubating the cells with PBS containing 1% bovine serum albumin for 10 min at 4 °C to quench any residual NHS-SS biotin. At the end of the experiment, the cells were lysed with PBS-1% Triton X-100 and protease inhibitors (Roche). Biotinylated proteins were incubated with streptavidin beads overnight at 4 °C. After extensive washings, bound proteins were subjected to Western blot analysis. Biotinylated CFTR was detected using a C-CFTR monoclonal antibody (24-1; R&D Systems).

2.4. Immunoblotting

Cells were lysed in PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (Roche). Western blotting was performed as previously described [4]. In brief, 20 µg of the protein was separated with SDS-PAGE in 4–15% polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk in PBS-Tween 20 and immunoblotted with primary antibodies against CFTR antibody (24-1, R&D Systems), phospho-Erk1/2 (Cell Signaling), Erk1/2 (Cell Signaling), or β-actin (Santa Cruz Biotechnology) followed by treatment with appropriate HRP-conjugated secondary antibody (Pierce, Rockford, IL, USA). The signals were detected with enhanced chemiluminescence (Super Signal West Pico; Thermo Scientific) followed by exposure to X-ray films. The protein bands on the X-ray film were scanned, and band density was measured using ImageJ software (NIH).

2.5. ASL height measurements

To measure ASL height, PBS (20 µl) containing 2 mg/ml rhodamine-dextran (10 kDa; Invitrogen, USA) ± 10 µM MEK inhibitor was added to cultures at the start of the experiment for 10 min. To measure ASL secretion, excess fluid was aspirated with a Pasteur pipette to bring ASL height down to ~7 µm, as described by Tarran et al. [25]. Before CS exposure, 10 µM MEK inhibitor was added basolaterally. In all cases, five predetermined points (one central, four 2 mm from the edge of the culture) were XZ scanned using a confocal microscope (Leica SP5; glycerol 63× immersion lens) as described [26]. Cultures were returned to the incubator between time points. For all studies, PFC was added mucosally during imaging to prevent evaporation of the ASL.

2.6. Cigarette smoke extract (CSE) preparation and whole cigarette smoke (CS) exposure

CSE (100%) was prepared as previously described and used to treat 16HBE14o- cells [27]. Primary HBECs were exposed to whole cigarette smoke (CS) after being placed in a specially built smoke exposure chamber that exposes apical but not basolateral surfaces [6,28]. CS was then generated according to the International Organization of Standardization (ISO) standards (35 ml draw over 2 s) using a LC1 smoke engine (Borgwaldt, Richmond, Virginia, USA) and applied to the cultures at a rate of 1 puff every 30 s until the cigarette is smoked (~5 min; ~12 puffs). For ASL experiments cells are treated with CS for about 5 min. This maneuver has previously been shown to drive removal of CFTR from the plasma membrane without inducing gross cellular toxicity [6,28].

2.7. Confocal microscopy

16HBE14o- cells were fixed in ice-cold 100% methanol for 20 min at –20 °C. The slides were then incubated in 1% bovine serum albumin (BSA)/PBS for 30 min, followed by incubation at 37 °C for 1 h with primary antibody against CFTR (24-1; R&D Systems) and the lysosomal marker LAMP-1 (Cell Signaling Technology). After several washings, the slides were incubated at 37 °C for 45 min with appropriate Alexa Fluor® 488- and Alexa Fluor® 594-conjugated secondary antibody. Coverslips were mounted onto slides with Vectashield mounting medium containing DAPI (Vector Laboratories) prior to being imaged on a Leica DMIRE2 inverted confocal microscope using a 63× objective lens.

2.8. Statistical analysis

Data are expressed as mean ± standard error (SE) of at least three independent experiments. The results of the experiments were analyzed by unpaired *t* tests. In all cases, a *p* value of <0.05 was considered as statistically significant.

3. Results

3.1. Effect of lysosomal and proteasome inhibitors on cigarette smoke extract (CSE)-induced decrease of CFTR protein in human airway epithelial cells

Several studies have recently shown that CSE decreases the expression of CFTR in human airway epithelial cells [3,6,27]. Here we used the normal human bronchial epithelial cell line 16HBE14o- that endogenously expresses the ion channel CFTR. The main two pathways leading to CFTR degradation are the proteasomal and lysosomal pathways [5,11,29]. In order to investigate whether the underlying pathway involves either lysosomes or the proteasome, 16HBE14o- cells were treated with CSE in the presence of lysosomal or proteasome inhibitors. As expected, CSE reduced the expression of CFTR (Fig. 1). It has to be noted that only mature CFTR (Band C) is seen on the blots. This result is in agreement with previous report showing that CFTR biogenesis is very efficient (close to 100%) in cells endogenously expressing CFTR such as 16HBE14o- [30]. The lysosomal inhibitors chloroquine (Fig. 1A) and leupeptin (Fig. 1B) both significantly prevented the CSE-induced decrease of CFTR, but they both had no effect on steady state level of CFTR. As previously described [6] the proteasomal inhibitor MG132 did not prevent CFTR diminution after CSE exposure (Fig. 1A). However MG132 alone decreased CFTR expression. We therefore used another proteasomal inhibitor lactacystin (Fig. 1C) which had no effect on steady-state levels of CFTR. Again, this inhibitor could not preclude the loss of CFTR induced by CSE exposure. Taken together our data show that cigarette smoke induces lysosomal degradation of CFTR.

3.2. Role of MAPK pathways in CSE-induced suppression of CFTR

Cigarette smoke contains over 3000 chemicals including reactive oxygen species (ROS) that can act on various pathways in the cell. Accordingly, CSE can stimulate multiple signaling pathways including mitogen-activated protein kinase (MAPK) pathways. We therefore

investigated whether the main classical MAPK pathways (i.e. p38, JNK, and MEK) contribute to the decrease in the expression of CFTR protein after CSE exposure. As shown in Fig. 2A, inhibition of the MEK/Erk1/2 MAPK pathway using two specific inhibitors, UO126 and PD98059, prevented the loss of CFTR induced by CSE. These results were further confirmed using UO124, the inactive form of UO126 which has no inhibitory property on MEK, and had no protective effect on CFTR after exposure to cigarette smoke (Fig. 2B). UO124 alone had no effect on the expression of CFTR ($p > 0.05$). Although UO126 alone has a trend to increase the expression of CFTR when compared with the control group, this increase failed to reach significance ($p = 0.063$). Conversely, inhibition of the p38 or JNK MAPK pathways had no effect on the suppression of CFTR after exposure of human bronchial epithelial cells 16HBE14o- to CSE (Fig. 2A).

To further confirm the role of Erk1/2 in down-regulation of CFTR by CSE, the expression of Erk1/2 was decreased using silencing RNAs targeting Erk1 and Erk2. As shown in Fig. 3, the expression of Erk1 and Erk2 was decreased by about 50%. No difference in the expression of CFTR was observed between the control and Erk siRNA groups in the absence of CSE treatment. Addition of CSE reduced the expression of CFTR in the control group but reduced expression of Erk1/2 significantly prevented the loss of CFTR protein. To confirm that Erk silencing was sufficient to impair downstream phosphorylation of Erk targets, phosphorylation of Elk was detected. As observed in Fig. S1, addition of CSE increased phosphorylation of Elk which was prevented in cells treated with Erk siRNAs.

3.3. Inhibition of the MEK/Erk1/2 MAPK pathway prevents the loss of CFTR from the plasma membrane of human airway epithelial cells after CSE exposure

CFTR has to be present at the plasma membrane of bronchial epithelial cells to exert its role as a chloride channel and regulate the airway surface hydration [1]. We therefore wanted to determine whether inhibition of

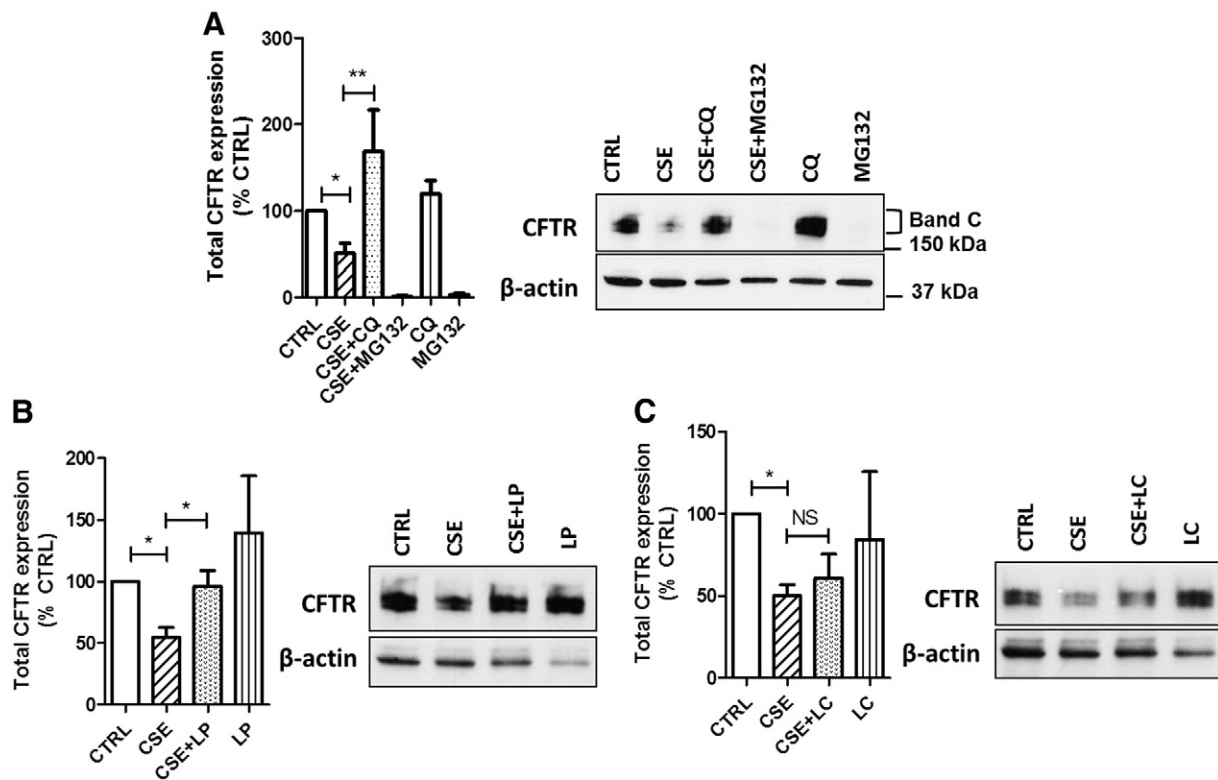


Fig. 1. Effect of the lysosomal inhibitors leupeptin and chloroquine and the proteasomal inhibitor lactacystin on the expression of CFTR after exposure to cigarette smoke extract. 16HBE14o- cells were treated with 10% cigarette smoke extract (CSE) with or without the lysosomal inhibitor leupeptin (LP, 50 μ M) or chloroquine (CQ, 10 μ M), or the proteasome inhibitors lactacystin (LC, 5 μ M) or MG132 for 48 h. CFTR protein was detected by immunoblotting as described in Methods. CTRL, Control. N = 4. *, $p < 0.05$; **, $p < 0.001$; NS, not significant.

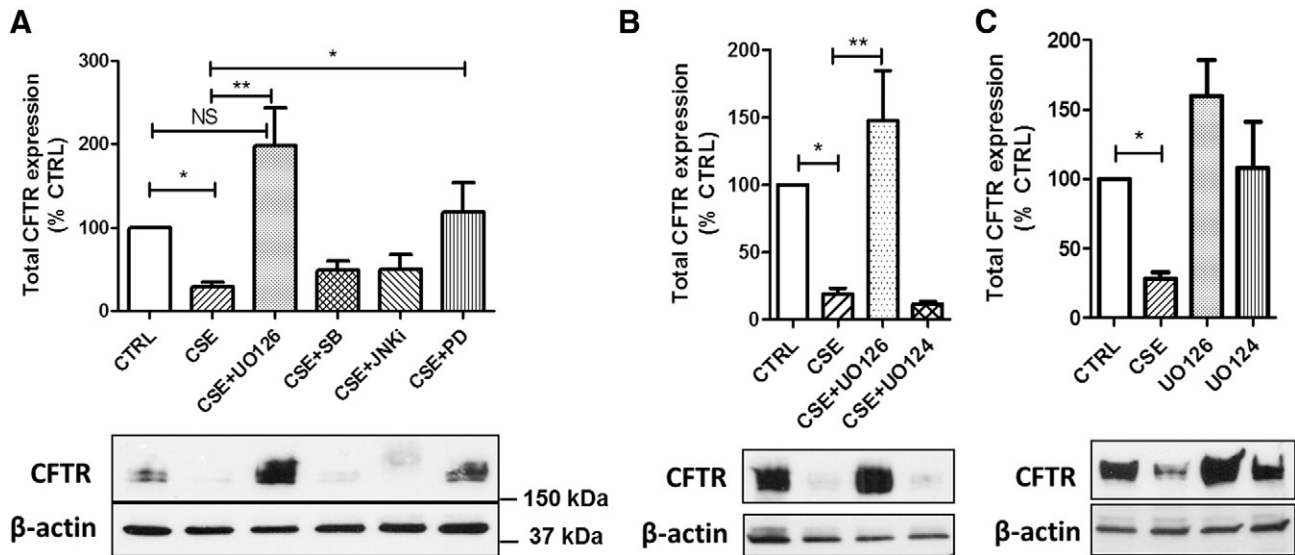


Fig. 2. Role of MAPK inhibitors on CFTR expression after cigarette smoke exposure. 16HBE14o- cells were treated with 10% CSE with or without the MEK/Erk1/2 inhibitors UO126 (10 μ M) or PD98059 (PD, 20 μ M), the p38 inhibitor SB203580 (SB, 20 μ M), the JNK inhibitor SP600125 (JNKi, 20 μ M), or UO124 (10 μ M) for 48 h. CFTR protein was detected by immunoblotting. CTRL, Control. N = 4. *, $p < 0.05$; **, $p < 0.001$; NS, not significant.

the MEK/Erk1/2 MAPK pathway would have any protective effect on plasma membrane CFTR after exposure to CSE. The human bronchial epithelial cells 16HBE14o- were incubated simultaneously with CSE and the MEK/Erk1/2 inhibitor UO126 and CFTR present at the plasma membrane was assessed using cell surface biotinylation. Not only inhibition of the MEK/Erk1/2 MAPK pathway prevented decrease of CFTR protein (Fig. 4A), it also prevented the loss of CFTR from the plasma membrane of airway epithelial cells (Fig. 4B).

3.4. Inhibition of the MEK MAPK pathway prevents cigarette smoke-induced decrease of airway surface liquid (ASL)

CFTR is present at the plasma membrane of bronchial epithelial cells and regulates hydration of the airway surface liquid layer [1]. As seen in cystic fibrosis, absence of functional CFTR results in impaired mucociliary clearance due to reduced ASL. We recently showed that cigarette smoke decreases expression of membrane CFTR in primary human bronchial epithelial cells resulting in impaired ASL [27]. As shown above (Fig. 4B), inhibition of the MEK MAPK pathway prevented

the decrease of plasma membrane CFTR. Accordingly, inhibition of the MEK pathway using the specific inhibitor UO126 prevented the reduction in the height of the ASL observed upon exposure to cigarette smoke (Fig. 4C).

3.5. Role of the E3 ligase c-Cbl on CFTR expression after exposure to cigarette smoke

Ubiquitination of CFTR can lead to proteasomal or lysosomal degradation. The E3 ubiquitin ligase c-Cbl has been shown to be linked to lysosomal degradation of CFTR in airway epithelial cells [18,31]. To determine whether c-Cbl is involved in the CSE-induced degradation of CFTR, we used c-Cbl siRNA to decrease c-Cbl abundance. As shown in Fig. 5, transfection of c-Cbl siRNA reduced c-Cbl expression by ~77%. The CFTR expression was comparable between the control and c-Cbl siRNA groups in the absence of CSE treatment. Conversely, addition of CSE significantly decreased the expression of CFTR in the control group, whereas suppression of c-Cbl expression using siRNA partly prevented the CSE-induced down-regulation of CFTR. These

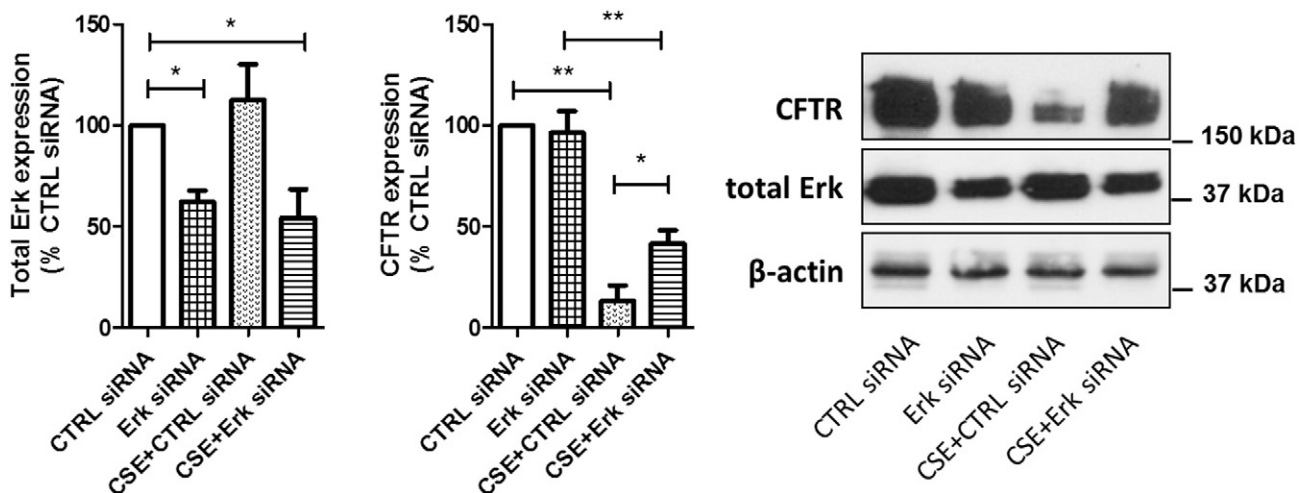


Fig. 3. Decreased expression of Erk1 and 2 prevents the CSE-induced suppression of CFTR. 16HBE14o- cells were incubated with Erk1 and 2 siRNAs or control siRNA. Forty eight hours later 16HBE14o- cells were incubated with 10% CSE for 24 h. CFTR and Erk 1 and 2 proteins were detected by immunoblotting. β -actin was detected to confirm equal loading between samples. CTRL, Control. N = 4. *, $p < 0.05$; **, $p < 0.001$.

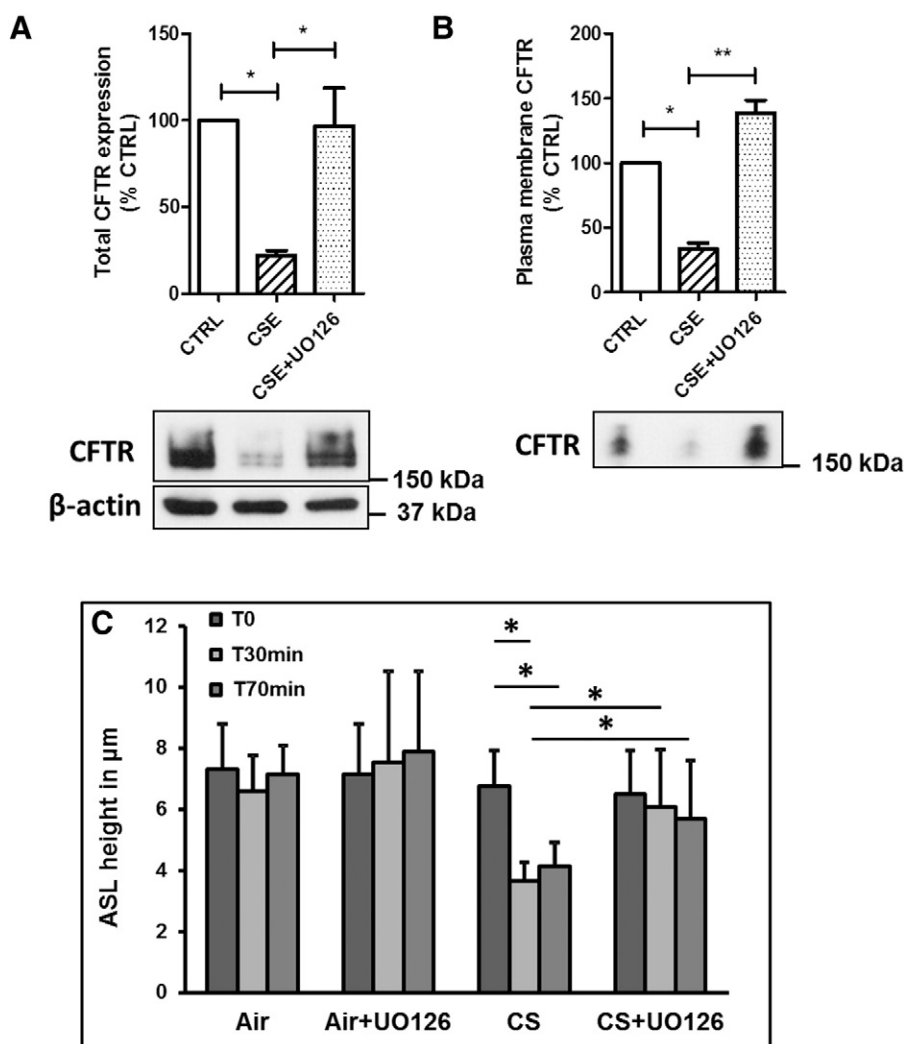


Fig. 4. Inhibition of the MEK/Erk1/2 MAPK pathway prevents loss of plasma membrane CFTR after cigarette smoke exposure. (A) and (B) 16HBE14o- cells were treated with 10% Camel cigarette smoke extract (CSE) with or without UO126 (10 μ M) for 48 h. CFTR expression (total (A) or plasma membrane (B)) was detected as described in Methods. CTRL, Control. N = 4. (C) Primary human bronchial epithelial cells were pretreated with 10 μ M UO126 and then exposed to air or cigarette smoke (CS) as described in Methods section. ASL was measured at the indicated time. N = 6 from two normal donors. *, $p < 0.05$; **, $p < 0.001$.

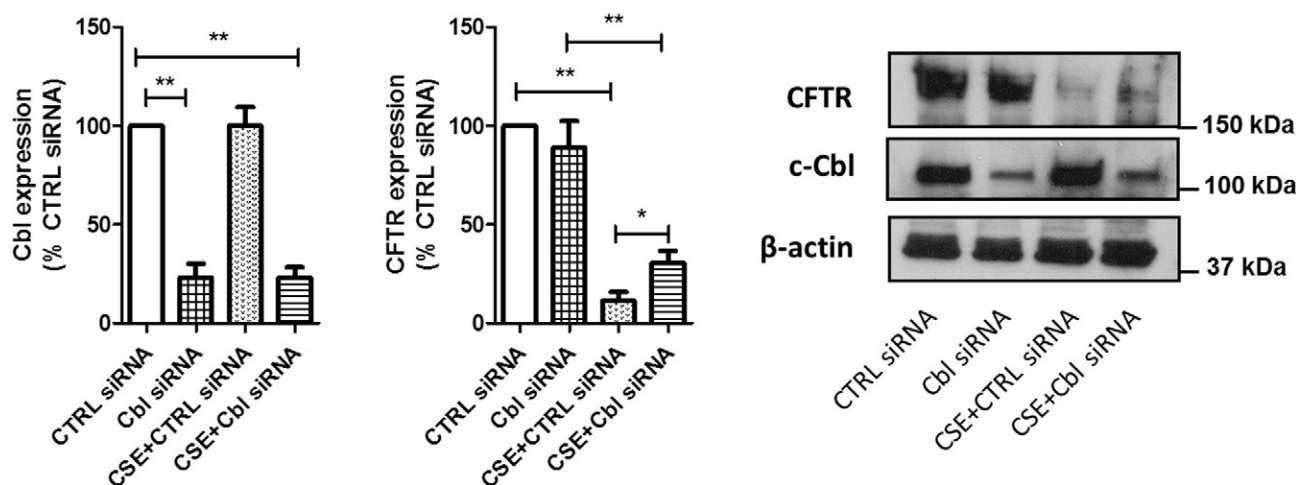


Fig. 5. Effect of the E3 ligase c-Cbl on CFTR expression after exposure to CSE. 16HBE14o- cells were transfected with c-Cbl or control siRNA for 48 h, followed by treatment with 10% Camel cigarette smoke extract (CSE) for 24 h. CFTR and c-Cbl were detected by immunoblotting. β-actin was detected to confirm equal loading between samples. CTRL, Control. N = 4. *, $p < 0.05$; **, $p < 0.001$.

data indicate that the E3 ligase c-Cbl contributes to suppression of CFTR upon cigarette smoke exposure.

3.6. Inhibition of the MEK/Erk1/2 pathway prevents lysosomal degradation of CFTR

Lysosome-associated membrane protein 1 (LAMP-1) is a marker of the lysosomes and was used to determine the intracellular localization of CFTR. As shown in Fig. 6 and S2, using confocal microscopy the CFTR signal was reduced after exposure to CSE as expected. No co-localization of CFTR with LAMP-1 was observed in 16HBE14o- cells in those conditions and could be due to CFTR degradation preventing its detection with the antibody used. However, inhibition of lysosomal degradation using chloroquine (CQ) allowed detection of CFTR in the lysosomes when the cells were exposed to CSE. The co-localization of CFTR with LAMP-1 was reduced in the presence of the MEK/Erk1/2 inhibitor UO126. These results suggest that CSE induces lysosomal degradation of CFTR in human bronchial epithelial cells via activation of the MEK/Erk1/2 MAPK pathway.

3.7. The antioxidant N-acetylcysteine (NAC) prevents the CSE-induced loss of CFTR

Cigarette smoke contains many chemical compounds as well as reactive oxygen species (ROS) that can trigger activation of signaling pathways such as MEK/Erk1/2 [20]. N-acetylcysteine (NAC) is an antioxidant that can inhibit ROS directly via the redox potential of its thiol or indirectly by increasing intracellular glutathione levels. We therefore investigated whether NAC could prevent the loss of CFTR after exposure to CSE. Cells were treated with NAC and CSE simultaneously and CFTR expression was analyzed 24 h later. As observed in Fig. 7A, addition of 10 mM NAC prevented the loss of CFTR due to CSE exposure. NAC alone had no effect on CFTR expression (Fig. 7A). Since the data presented above show that CSE alters the expression of the CFTR protein via activation of the MEK/Erk1/2 MAPK pathway, we investigated whether NAC prevented the loss of CFTR after CSE exposure by blocking activation of Erk1/2 (phosphorylation). CSE induced activation of Erk1/2 as shown by detection of phosphorylated Erk1/2 (Fig. 7B) which was inhibited in the presence of 10 mM NAC. A lower concentration of 2 mM NAC had little effect on CSE-induced decrease of CFTR or phosphorylation of Erk1/2 (Fig. 7A and C).

4. Discussion

In this study we investigated the molecular mechanism by which cigarette smoke suppresses expression of CFTR in human bronchial epithelial cells. Our results revealed that cigarette smoke negatively regulates CFTR via activation of the MEK/Erk1/2 MAPK pathway. We found that cigarette smoke leads to internalization of the CFTR ion channel, and inhibition of the MEK/Erk1/2 MAPK pathway prevented the cigarette smoke-induced loss of CFTR as well as decreased of airway surface liquid (ASL). In addition we show that the antioxidant NAC prevented the loss of CFTR by inhibiting Erk1/2 phosphorylation.

Dr. Welsh's laboratory was the first to report that cigarette smoke inhibits chloride ion transport across tracheal epithelium [32]. Several reports have shown that cigarette smoke inhibits the expression and function of the ion channels CFTR and ENaC [6,8,33,34] and recently, a cigarette smoke-induced increase in intracellular calcium has been implicated in CFTR internalization [19]. However, key questions remain regarding the molecular pathway leading to CFTR deregulation. In this study, we used normal bronchial epithelial cell line 16HBE14o- which endogenously expresses CFTR since many studies used cells derived from cancer or heterologous systems with cells overexpressing CFTR. Consistent with a previous study where Bafilomycin A1 prevented CFTR inhibition, here we show that the lysosomal inhibitors, chloroquine and leupeptin, prevented the loss of CFTR, whereas inhibition of the proteasome had no effect. However, CFTR could not be detected in the lysosomes using immunohistochemistry in the presence of CSE and could be due to CFTR degradation preventing its detection. Absence of co-localization of CFTR and LAMP-1 was previously reported [6] even though lysosomes have been shown to contribute to CFTR degradation [10]. Conversely, addition of the lysosomal inhibitor chloroquine, which prevents acidification of the lysosomes, allowed co-localization of CFTR with the lysosomal marker LAMP-1 (see Fig. 6). Bafilomycin A1 inhibits smoke-induced calcium release and also prevents CFTR diminution [19]. Thus, sorting out effects caused by altered lysosomal calcium release versus inhibition of lysosomal degradation is hard to differentiate and additional studies will be required. Interestingly, the heavy metal arsenic has been shown to trigger lysosomal degradation of the CFTR ion channel in airway epithelial cells [18,31]. Taken together, these results suggest that targeting the lysosomes would prevent CFTR degradation upon exposure to pollutants.

Lysosomal degradation of membrane proteins is generally associated with monoubiquitination. C-Cbl is an E3 ligase previously reported to

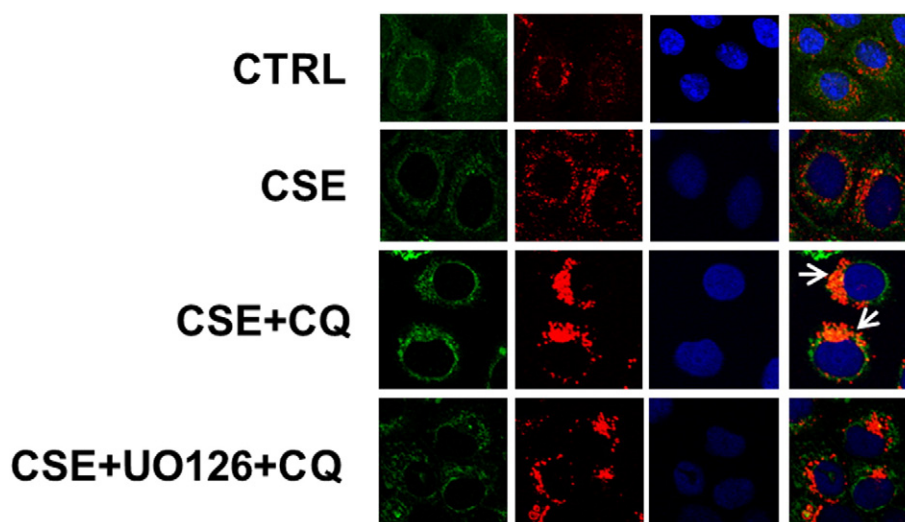


Fig. 6. Effect of CSE on intracellular localization of CFTR. Representative confocal microscopic images of CFTR (Alexa488, green color) and LAMP-1 (Alexa594, red color)-stained cells. 16HBE14o- cells were treated with 10% CSE with or without the lysosomal inhibitor chloroquine (CQ, 20 μ M/ml) and the MEK/Erk1/2 MAPK inhibitor UO126 (10 μ M) for 24 h. Representative images showing the co-localization of CFTR and LAMP-1 are shown. CTRL: Control. Original magnification 630 \times .

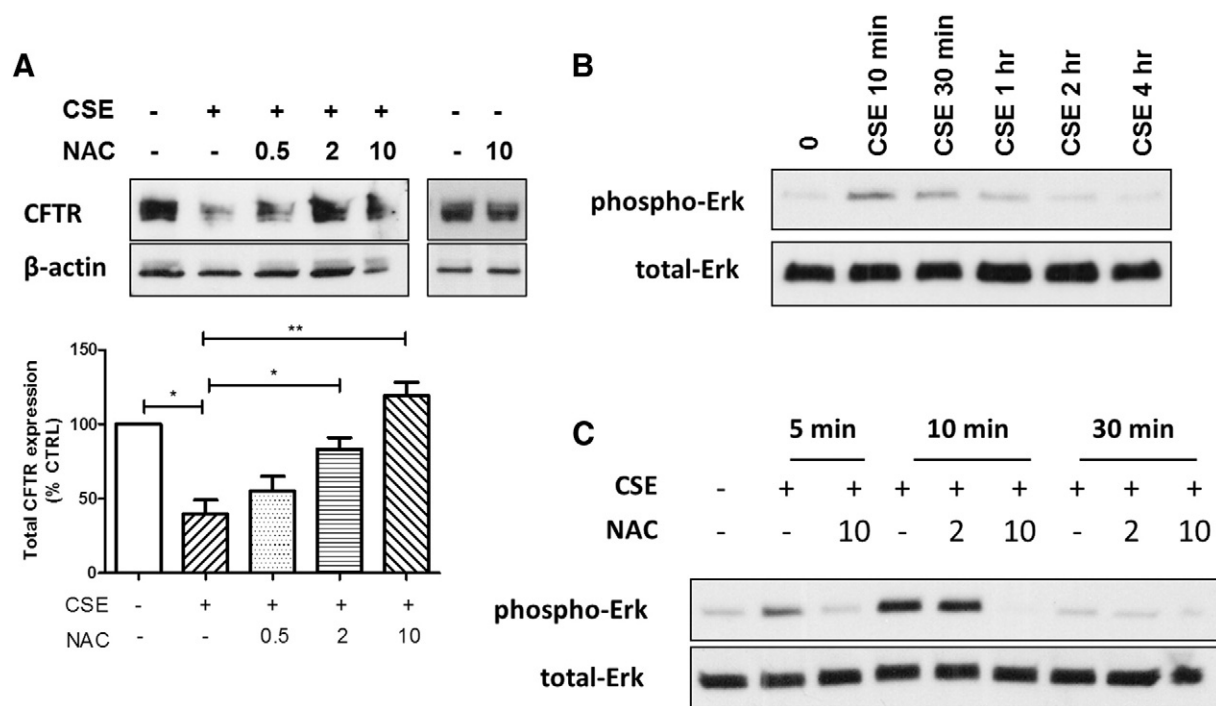


Fig. 7. The antioxidant N-Acetylcysteine (NAC) prevents suppression of CFTR upon cigarette smoke exposure. (A) 16HBE140- cells were treated with 10% CSE with or without 0.5, 2, or 10 mM NAC. CFTR protein was detected by immunoblotting. $N = 4$. *, $p < 0.05$; **, $p < 0.001$. (B) 16HBE140- cells were treated with 10% CSE for the indicated times. PhosphoErk1/2 and total Erk1/2 were detected by immunoblotting. $N = 4$. (C) 16HBE140- cells were treated with 10% CSE with or without NAC (2 or 10 mM) for the indicated time. Phospho-Erk1/2 was detected by immunoblotting. Blots are representative of at least three experiments.

facilitate the lysosomal degradation of CD5, gp130, as well as CFTR [18,31, 35,36]. However, decreasing the expression of c-Cbl using silencing RNA prevented the CSE-induced suppression of CFTR suggesting that c-Cbl contributes to regulation of CFTR in human bronchial epithelial cells.

The epithelial sodium channel ENaC which interacts with CFTR in bronchial epithelial cells [37,38] is degraded by the lysosomes after activation of the MEK/Erk1/2 MAPK pathway by interacting with the E3 ligase Nedd4-2 [39–41]. Here we show that pharmacological inhibition of MEK using UO126 or PD98059, or genetic inhibition of Erk1/2 using siRNAs prevented the cigarette smoke-induced suppression of CFTR. Most importantly, inhibition of the MEK/Erk1/2 MAPK pathway prevented the loss of CFTR from the plasma membrane of the human bronchial epithelial cells 16HBE as well as prevented the cigarette smoke-induced decreased of ASL. This is an important finding since CFTR needs to be present at the apical membrane of airway epithelial cells to play its role as a chloride channel to maintain fluid homeostasis in the lung. Activation of the Erk1/2 pathway by the pollutant cadmium was reported to increase CFTR activity in kidney cells [42]. In this latter study, CFTR activity was measured 5 min after exposure to cadmium. It is therefore possible that activation of Erk1/2 has distinct effects depending of the type of cells studied (renal versus airway epithelial cells) and/or the time after MEK/Erk1/2 activation (short-term versus long-term). Since both CFTR and ENaC are down-regulated following activation of the MEK/Erk1/2 MAPK pathway, it might be an unrecognized pathway to regulate plasma membrane ion channels.

About 20% of smokers develop chronic obstructive pulmonary disease (COPD) but over 90–95% of patients with COPD were smokers (<http://www.goldcopd.com>). Some reports indicate that as many as 50% of smokers develop COPD if an advanced age is reached [43]. Cigarette smoke contains over 3000 chemicals as well as reactive oxygen species that can lead to activation of several signaling pathways including the MEK/Erk1/2 MAPK pathway [20]. For example our team has recently showed that cadmium, a toxic heavy metal present in cigarette smoke, induces secretion of the pro-inflammatory cytokine IL-8 via an Erk1/2-dependent pathway when added to human bronchial

epithelial cells [44]. Interestingly, sustained activation of Erk1/2 has been found in mice and rats exposed to cigarette smoke [45,46]. Most importantly, analysis of phospho-Erk1/2 revealed that patients with COPD (emphysema) have elevated Erk activation when compared to healthy control subjects [20,45]. Based on our results we predict that sustained activation of Erk in the lung will contribute to suppression of CFTR expression.

NAC has been used in patients with COPD with mixed success [47, 48]. Recently it was shown that higher doses might be required to obtain beneficial effects [49]. Several studies reported that long-term high dose of NAC treatment may reduce the risk of exacerbations and improve lung function (FEV_1) [50,51]. The doses used in our study are within the range of doses used in clinical practice (4–10 mM daily) [50]. We observed that 2 mM NAC had very little inhibitory effect on activation of Erk1/2 MAPK pathway, whereas 10 mM prevented activation of Erk1/2 and consequently loss of CFTR protein (see Fig. 7A and C). It is important to note that the cells were not pre-treated with NAC so the protective effect of NAC is not due to increased levels of glutathione but rather by acting directly as an antioxidant. In addition, the inhibition of Erk1/2 activation was seen only after 5–10 min (see Fig. 7). Interestingly, Varelogianni et al. reported that NAC increases chloride efflux via activation of the CFTR chloride channel in human bronchial epithelial cells expressing the CFTR mutant deltaF508 [52]. This latter mutation is the most common mutation leading to cystic fibrosis (CF). Since CF cells have higher Erk1/2 activation when compared to control non-CF cells [53] it is possible that NAC could inhibit the MEK/Erk1/2 pathway in CF cells resulting in rescue of deltaF508-CFTR. Accordingly, a recent study identified kinase inhibitors, including inhibitors of the Ras/Raf/MEK/Erk1/2 pathway as potent correctors of deltaF508-CFTR.

5. General significance

Due to the role of CFTR in the bronchial epithelium and its potential role in chronic bronchitis seen in COPD patients, our data suggest that

NAC would benefit COPD patients with chronic bronchitis by inhibiting activation of the MEK/Erk1/2 pathway resulting in stabilization of plasma membrane CFTR. Members of the MEK/ERK pathway may serve as novel biomarkers of tobacco smoke exposure.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2015.02.004>.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

ECB was funded by the NIH/NHLBI (grant HL109969). RT was funded by the National Institutes of Health/Food and Drug Administration (FDA) [grant numbers HL108927 and HL120100]. Research reported in this publication was in part supported by the National Institutes of Health and the FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Food and Drug Administration.

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