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Exposure to Cigarette Smoke Condensate Reduces Calcium Activated Chloride Channel Transport in Primary Sinonasal Epithelial Cultures

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Abstract

Background—The cystic fibrosis transmembrane conductance regulator (CFTR) serves as a predominant Cl[−] transport conduit in airway epithelium and is inhibited by cigarette smoke *in vitro* and *in vivo*. Activation of secondary Cl[−] transport pathways through calcium-activated Cl[−] channels (CaCC) has been postulated as a mechanism to bypass defects in CFTR-mediated transport. Because it is not known whether CaCC's are also inhibited by tobacco exposure, the current study was designed to investigate the effect of cigarette smoke condensate (CSC) on CaCC transport.

Study Design—*In vitro* study

Methods—Well-characterized primary murine nasal septal epithelial (MNSE) and human sinonasal epithelial (HSNE) cultures were exposed to CSC in Ussing chambers. We monitored CaCC short-circuit current through stimulation of P2Y purinergic receptors with UTP or ATP and selective inhibition of the CFTR dependent secretory pathway. Characterization of CaCC current was also accomplished in primary airway cells derived from transgenic CFTR^{−/−} (knockout) murine models.

Results—Change in CaCC-mediated current (ΔI_{SC} - representing transepithelial Ca-mediated Cl[−] secretion in $\mu A/cm^2$) was significantly decreased in CSC-exposed wild type MNSE when compared to controls { (32.8 $\pm$ 4.6 vs. 47.5 $\pm$ 2.3; respectively) $p < 0.02$ }. A similar effect was demonstrated in CFTR^{−/−} MNSE cultures (33.4 $\pm$ 2.8 vs. 38.6 $\pm$ 2.0; $p < 0.05$). HSNE cultures also had a significant reduction in I_{SC} (16.1 $\pm$ 0.6 vs. 22.7 $\pm$ 0; $p = 0.008$).

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Conflict of Interest: Dr. Sorscher serves as a consultant for a Birmingham start up company (PNP Therapeutics, Inc) but is not employed by the company and draws no salary from the company. His consulting includes a role as Director/Officer. He acts as a consultant with the approval of UAB and the UAB CIRB. He is compensated for consulting with stock. This company has no relationship to the current manuscript. Dr. Sorscher and Dr. Woodworth are inventors on a patent submitted regarding the possible activity of chloride secretagogues for therapy of sinus disease (Provisional Patent Application Under 35 U.S.C. §111(b) and 37 C.F.R. § 1.53(c) in the United States Patent and Trademark Office). Dr. Woodworth is a consultant for Gyrus ENT, ArthroCare ENT, and is on the GlaxoSmithKline speaker's bureau.

Conclusions—CSC affects multiple pathways fundamental to airway ion transport, including both cAMP and calcium activated Cl^- transport. Inhibition of Cl^- transport may contribute to common diseases of the airways, such as chronic rhinosinusitis and chronic obstructive pulmonary disease.

Keywords

Transepithelial Ion Transport; cigarette smoke condensate; Calcium Activated Chloride Channel; CFTR; Chronic Sinusitis; Chloride Secretion; Murine Nasal Culture; Mucociliary Clearance

Introduction

Sinonasal respiratory epithelium is responsible for the clearance of inhaled pathogens from the nose and paranasal sinuses. The airway surface liquid (ASL) is one component of mucociliary clearance (MCC). ASL is a complex mixture of mucus and periciliary fluid, the composition of which is determined by vectorial transport of ions, such as Cl^- . Cystic fibrosis transmembrane conductance regulator (CFTR) channels contribute the predominant Cl^- transport conduit within respiratory epithelial cells². In the inherited disease cystic fibrosis (CF), there is a mutation in the CFTR protein resulting in impaired exocrine function. In the nose and paranasal sinuses, this effect manifests through dehydration of ASL, and results in chronic stasis of inspissated mucus, bacterial infection, and widespread chronic sinus disease refractory to medical management³.

Cigarette smoke is a highly toxic substance that greatly affects MCC. Various chemicals in cigarette smoke are known to be toxic in respiratory epithelium⁴⁻⁶. Cigarette smokers have exhibit a higher rate of recurrent sinusitis and chronic rhinosinusitis (CRS) when compared to non-smokers⁷. The effects of cigarette smoke are thought to be secondary to impairment of ciliary function and dehydration of the ASL⁸⁻¹⁰. Patients with chronic bronchitis induced by smoking have histopathological similarities to patients with CF. These similarities include mucus hypersecretion, goblet cell hyperplasia, neutrophilic infiltration of the airway lumen, and bacterial pathogens in the lower airways¹¹. Cigarette smoke extract (CSE) has been shown to decrease epithelial Cl^- secretion in canine trachea and human bronchial epithelial cells^{12,13}. However, CSE lacks the water insoluble components of cigarette smoke. In a prior study by our group, cigarette smoke condensate (CSC), which contains the particulate and water insoluble components of tobacco combustion, was shown to substantially decrease transepithelial transport of Cl^- and ciliary beat frequency¹⁴.

Activation of secondary Cl^- transport pathways through calcium activated chloride channels (CaCC) has been postulated as a potential mechanism to bypass defects in CFTR-mediated Cl^- transport^{15,16}. Although CaCC activity is known to contribute to ASL, MCC, and pathogenesis in diseases, such as CF, no studies to date have evaluated effects of CSC on CaCC. The present study was designed to investigate the effects of CSC on CaCC function in murine nasal septal epithelia (MNSE), human sinonasal epithelia (HSNE), and transgenic CFTR^{-/-} MNSE knockout cultures. The findings indicate that CSC inhibits transepithelial Cl^- secretion mediated by CaCC's, and have important implications regarding both mechanism and recently emerging experimental therapeutics for diseases, such as CF, CRS and COPD.

Materials and Methods

Institutional review board and institutional animal care and use committee approval were obtained prior to the initiation of the study.

Tissue Culture

The culture technique for propagation of MNSE and HSNE at an air-liquid interface has been described in our prior studies^{17–21}. Human sinus epithelium was derived from tissue removed during skull base surgery from individuals without inflammatory disease. Briefly, tissue was harvested and grown on Costar 6.5-mm-diameter permeable filter supports (Corning Life Sciences, Lowell, MA) submerged in culture medium. The media was removed from the surface of the monolayers on day 4 after reaching confluence and the cells fed via the basal chamber. MNSE cultures were genetically identical and derived from the C57 strain and from transgenic CFTR^{-/-} knockout mice. Differentiation and ciliogenesis occurred in all cultures within 10 days to 14 days.

A total of 80 filters were used for completion of these studies. Dose-response relationships and establishment of protocols for the most informative order and application of pharmacologic agents were performed prior to initiation of the study (data not shown). MNSE wild type (wt) and CFTR^{-/-} knockout filters were derived from otherwise genetically identical (congenic) mice. Ussing Chamber analysis was performed only when cell monolayers were fully differentiated with widespread ciliogenesis and transepithelial resistances (R_T) $>500 \Omega \text{ cm}^2$. There was no significant difference between the R_T and I_{SC} of control and CSC-exposed filters when the two groups were compared before the addition of CSC, DMSO, or pharmacologic agents.

Electrophysiology

Solutions and Chemicals—The bath solution contained (mM): 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂PO₄, 1.2 MgCl₂, 10 Glucose. The pH of this solution is 7.3–7.4 when gassed with a mixture of 95% O₂-5% CO₂ at 37°C. Chemicals were obtained from Sigma (St. Louis, MO). Each solution was produced as 1,000× stock and used at 1x in the Ussing Chamber. All experiments were performed with low Cl⁻ (6mM) in the mucosal bath. Pharmacologic preparations were as follows: amiloride (100 μM); INH-172 (10 μM); CSC (200 μg/ml); adenosine deaminase (ADA, 5μg/ml); ATP (200 μM); Niflumic Acid (100 μM), Forskolin (20 μM); UTP (150 μM). Amiloride blocks Na⁺ channels, ensuring that changes observed in short-circuit current (ΔI_{SC}) from various manipulations are attributable to effects on Cl⁻ transport. ATP activates both P2Y receptors, which activate CaCC secretion, and A2B receptors via the breakdown product adenosine, which stimulates CFTR Cl⁻ secretion through cAMP-mediated mechanisms²². To ensure that an observed I_{SC} was due to CaCC, ADA was added to degrade the adenosine byproduct of ATP metabolism. INH-172 is a selective blocker of CFTR-mediated pathways and also inhibits CFTR stimulation, and was used in confirmatory assays to indicate selective I_{SC} activity due to CaCC. UTP activates CaCC I_{SC} via P2Y receptors and was utilized for monitoring CaCC Cl⁻ secretion in wild type and CFTR^{-/-} MNSE cultures. Niflumic acid is a commonly used inhibitor of CaCC I_{SC} , although it also blocks other ion transport pathways, such as CFTR, non-selective cation channels and voltage-gated potassium channels^{23–25}. Forskolin is an activator of cAMP and the CFTR-mediated Cl⁻ secretion pathway and was administered following activation of CaCCs in transgenic CFTR knockout MNSE cultures to confirm absence of CFTR.

CSC was purchased from Murty Pharmaceuticals (Lexington, KY) and is comprised of the particulate matter of cigarette smoke, including thousands of toxic chemicals (e.g. nicotine, nitrosamines, heavy metals, cadmium, phenol, anthracyclic hydrocarbons, and chemical carcinogens) dissolved in dimethyl sulfoxide (DMSO). In order to ensure changes in short-circuit current measurements were not secondary to DMSO, all control experiments were performed with the corresponding amount of this solvent. The optimum concentration of CSC for Ussing chamber analysis was determined in a previous study to be 200 μg/ml¹⁴.

Short circuit (I_{SC}) measurements—Transwell inserts (Costar, Corning Life Sciences) were mounted in a modified, vertical Ussing Chamber to investigate pharmacologic manipulation of ion transport. Monolayers were continuously short-circuited after fluid resistance compensation using automatic VCC 600 voltage clamps (Physiologic Instruments, San Diego, CA). Transwell filters were mounted in bath solution warmed to 37°C, and the solution continuously gas-lifted with a 95% O₂/5% CO₂ mixture. The I_{SC} was monitored at 1 measurement per second. By convention, a positive deflection in I_{SC} was defined as the net movement of anion in the serosal to mucosal direction.

Statistical Analysis

Statistical analysis was performed using two-tailed paired *t*-test, unpaired *t*-test, and analysis of variance, as appropriate.

Results

Initial stimulation with CaCC-stimulated currents (ΔI_{SC}) in MNSE cultures were significantly decreased when exposed to CSC ($32.83 \pm 4.6 \mu\text{A}/\text{cm}^2$) in comparison to DMSO controls ($47.53 \pm 2.25 \mu\text{A}/\text{cm}^2$; $p = 0.02$). (Figure 1&2) To isolate apical CaCC-mediated current, CFTR^{-/-} knockout mice were utilized. CFTR^{-/-} knockout cultures also had significant inhibition of ΔI_{SC} when compared to controls ($33.4 \pm 2.78 \mu\text{A}/\text{cm}^2$ vs. $38.56 \pm 1.96 \mu\text{A}/\text{cm}^2$, respectively; $p = 0.03$) confirming inhibition of CaCC Cl⁻ current. (Figure 1&3) The absence of response following the combination of forskolin and purinergic (P2Y) stimulation confirmed a lack of CFTR-mediated pathways in the transgenic CFTR knockout cultures. In trials with HNSE cultures, there was also a significant reduction in ΔI_{SC} with CSC exposure (CSC, $16.06 \pm 0.6 \mu\text{A}/\text{cm}^2$ vs. control, $22.7 \pm 0 \mu\text{A}/\text{cm}^2$; $p = 0.008$). (Figure 1)

Discussion

The studies presented here examined the effects of CSC on CaCC-mediated Cl⁻ secretion. Prior reports have demonstrated tobacco smoke byproduct inhibition of CFTR-mediated Cl⁻ secretion in models of lower airway cell epithelium. In a recent study by our group, inhibition of CFTR-mediated Cl⁻ transport by CSC was demonstrated in MNSE and HSNE cultures¹⁴. While much is known regarding CFTR structure and function, the Cl⁻ channel TMEM16A was only recently identified as the major candidate channel responsive to calcium-dependent activation²⁶. Because secondary Cl⁻ transport pathways through CaCC's represent a unique target for therapy of CF and other diseases of MCC, TMEM16A could represent a potential therapeutic avenue for treatment in the setting of cigarette smoke related mucociliary dysfunction^{15,16}. To our knowledge, this is the first study to examine the effects of CSC specifically on CaCC-mediated transepithelial Cl⁻ secretion.

In the present experiments, CaCC-mediated Cl⁻ transport was inhibited in both murine and human culture models of upper airway epithelium. Through pharmacologic manipulation of CFTR-mediated current and Na⁺ channel blockade, we focused on apical CaCC ΔI_{SC} and examined the effects of CSC in these *in vitro* culture models. Transgenic CFTR^{-/-} MNSE cultures provided an excellent validation of CFTR-independence of the pathways investigated here. There is some evidence to suggest that CaCC's play a more prominent role in murine (versus human) airway epithelium, based on earlier findings that CF transgenic mice do not develop significant sinopulmonary disease²⁷. Thus, confirmation of these effects in a human culture model was required. Notably, studies of HSNE cultures also exhibited significantly decreased CaCC-mediated I_{SC} when exposed to CSC, corroborating this effect across species.

The mechanisms by which CSC inhibits transepithelial CaCC-mediated Cl⁻ secretion are currently unknown. Our previous studies have demonstrated inhibition of CFTR-mediated Cl⁻ transport, which could be secondary to a decrease in cAMP-mediated cell signaling in response to forskolin¹⁴. Others have shown decreased expression of CFTR mRNA, protein, and function in response to cigarette smoke extract *in vitro*¹². As CaCC's have a distinct mechanism of action through intracellular Ca²⁺ second messenger signaling, this pathway may represent a distinct target of CSC and will be a focus of future investigations. Alternatively, a general stimulation of the cellular Cl⁻ secretory gradient (e.g. via effects on basolateral transport mechanisms) may also contribute to the findings shown here. The present experiments therefore provide a foundation for molecular analysis (RT-PCR, protein expression) of CSC effects on other epithelial transport mechanisms in the future²⁸.

Conclusion

The present investigation expanded on a prior study by our group demonstrating inhibition of transepithelial Cl⁻ current by CSC. We found that CSC specifically confers inhibition of CaCC-mediated Cl⁻ current in MNSE cultures, confirmed our findings in transgenic CFTR^{-/-} airway epithelial monolayers, and also validated the role of CaCC's in a human culture model of sinonasal epithelium. The findings from this study lend support to the hypothesis that sinonasal mucus is inefficiently cleared in response to cigarette smoke secondary to a decrease in Cl⁻ secretion, which causes a reduction in the periciliary fluid layer volume. The results point to a more profound effect of cigarette smoke on MCC than previously appreciated. The Cl⁻ secretory pathways inhibited by CSC include CFTR, CaCC, and possibly other ion transport mechanisms in the airways. Pharmacologic stimulation of Cl⁻ transport represents a potential therapeutic target for individuals with CRS and other diseases of mucociliary dysfunction that are contributed by frequent tobacco smoke exposures.

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Figure 1.

The change in CaCC-stimulated current (ΔI_{SC}) in wild type MNSE, CFTR knockout MNSE, and HSNE cultures was significantly decreased when exposed to CSC in comparison to DMSO controls.

Wild Type MNSE

Figure 2.

Representative I_{sc} tracing of wild type MNSE cultures. Wild type MNSE cells grown on transwell permeable supports were mounted in modified Ussing chambers under short-circuit conditions and sequentially exposed to amiloride, INH-172, CSC or DMSO control, ADA, ATP, and niflumic acid. Note no changes in Cl^- dependent I_{sc} (after amiloride) until the application of ATP. Either DMSO control (upper tracing) or 200 μ g/ml of CSC was added to the apical surface at the start of the experiment. Decreased ATP-stimulated CaCC I_{sc} was observed in the upper tracing (arrows). Niflumic acid (NA) inhibition was considered unreliable due to non-specificity and variable results.

CFTR^{-/-} Knockout MNSE Cultures

Figure 3.

Representative I_{sc} tracing of CFTR^{-/-} MNSE cultures. Cultures were sequentially exposed to amiloride, CSC or DMSO control, UTP, niflumic acid, and forskolin. Decreased ATP-stimulated CaCC I_{sc} was observed in the upper tracing (arrows). The addition of forskolin confirms lack of CFTR in these transgenic mice.