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Cigarette Smoke Activates NF κ B and Induces *Muc5b* Expression in Mouse Middle Ear Cells

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Abstract

Objectives—Cigarette smoke exposure is a significant risk factor in the development of otitis media (OM). Nuclear factor kappa B (NF- κ B) is a ubiquitous transcription factor known to mediate cigarette smoke effects on gene regulation in multiple cell types. The *MUC5B* mucin gene contains several putative NF- κ B sites in its promoter and is the predominant mucin expressed in human OM. We hypothesized that in vitro stimulation of a recently developed model system, murine middle ear epithelial cells (MEEC), with cigarette smoke condensate (CSC) activates NF- κ B and subsequently induces *Muc5b* gene expression.

Methods—Luciferase reporter assays, electromobility shift assays (EMSA), and quantitative microplate transcription factor assays (TFA) were performed to evaluate NF- κ B activation with CSC in immortalized murine MEEC (mMEEC). Reverse transcriptase polymerase chain reaction (RT-PCR) assays and quantitative real time RT-PCR were performed to determine whether time course CSC stimulation upregulates *Muc5b* mRNA levels in differentiated mMEEC. Luciferase reporter assays were performed to determine whether CSC activates the *Muc5b* promoter.

Results—Reporter assays, EMSA, and TFA demonstrated three- to five-fold dose-dependent activation of NF- κ B with CSC in mMEEC. CSC stimulation likewise increased *Muc5b* mRNA abundance and induced reporter activity 1.8- to 4.8-fold in plasmids containing –556 and –255 base pairs upstream of the *Muc5b* transcriptional start site in mMEEC.

Conclusions—CSC activates NF- κ B in immortalized MEEC. Furthermore, this activation correlates with CSC-induced *Muc5b* promoter activation and gene expression. Taken together, these results hint that much as in lung cells, the activation of mucins by cigarette smoke is mediated in part by NF- κ B.

Keywords

Muc5b; mouse middle ear epithelial cells; NF-kappaB; cigarette smoke condensate

INTRODUCTION

Otitis Media (OM) is a ubiquitous condition of early childhood accounting for 16 million physician office visits a year¹ at a national cost of 3 to 6 billion dollars.² Exposure to tobacco

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smoke in children has been shown to be a significant risk factor in the development of OM. Links between environmental tobacco smoke (ETS) and OM have been promulgated by various federal agency reports: the Surgeon General,³ the National Research Council,⁴ the U.S. Environmental Protection Agency (EPA),⁵ and the National Cancer Institute (NCI)/California EPA.⁶ Other environmental and genetic risk factors, such as day care and family history of OM, are associated with middle ear disease in childhood.^{7–10} In the presence of smoke exposure, the combined interaction of factors may mean the difference in becoming otitis-prone or not.¹¹ Despite strong casual epidemiologic data, direct mechanisms by which cigarette smoke exposure contributes to middle ear pathology are significantly under-investigated.

Multiple genes increased in inflammatory disease states are regulated by NF- κ B, a ubiquitous deoxyribonucleic acid (DNA)-binding transcription factor.¹² Likewise, NF- κ B is a known mediator of cigarette smoke effects on gene regulation in many cell types.^{13–15} Its activated form has been shown to be increased in bronchial biopsies of smokers.¹⁶ In rabbit middle ear epithelial cells, NF- κ B has been identified as a key intracellular modulator of chronic inflammation.¹⁷ In cancer cells, it is known to directly up-regulate mucin gene expression through promoter interactions.¹⁸

Epithelial cells that encompass mucosal surfaces are increasingly recognized as the first line of defense against infectious or environmentally harmful stimuli, including cigarette smoke. First-line innate immune responses occur at the epithelial mucosal surface.^{19–21} As part of these epithelial defense mechanisms, mucin glycoproteins act as physiologic barriers to contaminants and bacteria by forming mucus, a viscoelastic gel. To date, 18 human mucin (*MUC*) genes have been identified (reviewed by Rose and Voynow²²). These genes encode gene products that are classified as secretory mucins, of which MUC2, MUC5AC, and MUC5B are the best studied, and membrane-tethered mucins, of which MUC1 and MUC4 are presently the best investigated. Within the confines of the middle ear, mucin over-production and the resulting mucous obstruction or stagnation clearly contributes to OM pathology.²³ Although MUC5AC and MUC5B are the primary mucins in respiratory secretions, MUC5B appears to be the predominant mucin involved in human otitis media.^{24–28} However, very little work has evaluated MUC5B mRNA expression in animal and/or cell models of OM. This is likely due to the fact that cellular models of MEEC have only recently been established and that the mouse *Muc5b* gene was only recently cloned.^{29,30} Much like the human *MUC5B* gene, the mouse *Muc5b* promoter contains several putative NF- κ B sites.

For this study, we hypothesized first that in vitro stimulation of murine MEEC (mMEEC) with the particulate fraction of cigarette smoke (cigarette smoke condensate; CSC) activates NF- κ B, and second, that this activation results in increased *Muc5b* expression in mMEEC.

METHODS

Cell Lines

The mouse middle ear epithelial cell line mMEEC was immortalized by temperature-sensitive simian virus 40 (SV40), allowing for a proliferative phenotype at 33°C and for differentiation at 37°C.³¹ mMEEC were maintained in full growth medium (FGM), which is comprised by Ham's F-12 K culture medium supplemented with 1.5 g/l sodium bicarbonate, 2.0 mmol/L L-glutamine, 10 ng/mL epidermal growth factor (EGF; R&D Systems, Minneapolis, MN), 10 mg/mL insulin/_transferrin_/sodium selenite (Sigma, St. Louis, MO), 2.7 g/l glucose, 500 ng/mL hydrocortisone, 0.1 mmol/L logicessential amino acids and 4% fetal bovine serum. The cells were grown and passaged at 33°C in a 5% carbon dioxide (CO₂)-humidified atmosphere. Prior to experimentation, cells were transferred to a 37°C, 5% CO₂ humidified atmosphere to inactivate the SV-40 virus. Cells were then grown on plastic or on Transwells (Corning Inc.,

Corning, NY) submerged in FGM until they achieved confluence. At that point, they were placed at an air liquid interface (ALI) for 14 days, with FGM being supplied only to the basal side of the cells. These ALI conditions have been reported to further differentiate middle ear cells, with diffuse formation of goblet cells, mimicking in vivo OM metaplastic epithelium.³²

CSC was purchased from Murty Pharmaceuticals (Lexington, KY) where it was prepared using a Phipps-Bird 20-channel smoking machine (Phipps & Bird, Inc., Richmond, VA). The particulate matter from Kentucky standard cigarettes (1R3F; University of Kentucky, Lexington, KY) was collected on Cambridge glass fiber filters (Cambridge Filter Corporation, Gilbert, AZ), and the amount obtained determined by weight increase of the filter. CSC was prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 4% solution (w/v). The average yield of CSC was 26.1 mg/cigarette. The CSC was diluted into DMSO, and aliquots were kept at -80°C . CSC concentrations of 0 to 40 $\mu\text{g/mL}$ total particulate matter (TPM) stimulate cytokine secretion in cultured endothelial cells.³³ Secondhand smoke has been shown to have concentrations estimated to be in the range of this experimental dosage.³⁴

Preparation of Nuclear Extracts

Cells were grown in 75-cm² flasks to 60% to 80% confluence and then stimulated with 10, 20, or 40 $\mu\text{g/mL}$ of CSC in DMSO or plain DMSO for 60 minutes. Cell suspensions were prepared by Trypsin-EDTA treatment, and the cells were washed twice in cold phosphate-buffered saline and pelleted at $500 \times g$. Cytoplasmic and nuclear protein extraction was then performed using a kit from Pierce (Rockford, IL). Briefly, the cell pellets were lysed with cytoplasmic extract reagent I and proteinase inhibitor on ice for 10 minutes. After adding cytoplasmic extract reagent II, the lysates were microfuged for 5 minute at $16,000 \times G$. The supernatant was collected and saved as cytoplasmic extract at -80°C . The nuclear pellet was then resuspended in nuclear extract reagent and proteinase inhibitor, incubated on ice for 40 minutes, and then microfuged at $15,000 \times G$ for 10 minutes. The remaining nuclear extracts were aliquoted and stored at -80°C . The protein concentrations of the extracts were determined in triplicate by using the bicinchoninic acid (BCA) modification of the biuret reaction (Pierce Protein Assay Kit, Pierce, Rockford, IL) scaled for microtiter plate analysis. BSA was used as a standard, and the plates were read at 580 nm on a BioTek 311 microtiter plate reader (BioTek Instruments, Inc., Winooski, VT). Standard curves were generated using computer software. The correlation coefficients for the functions were greater than 0.95 in all experiments.

Chemiluminescent Transcription Factor Microplate Assay (TFA)

NF- κB chemiluminescent transcription factor assays (Chemicon, Temecula, CA) were performed per the manufacturer's instructions. Briefly, the nuclear extract samples were thawed and mixed with a double stranded biotinylated oligonucleotide containing the flanked consensus sequence for NF- κB binding (5'-GGGACTTTCC-3'). Cold competition was performed in some groups by including provided unlabeled NF- κB competitor oligonucleotide probes in the binding reaction. TNF- α treated HeLa whole-cell extracts were used as a positive control. Sample nuclear extracts without NF- κB capture probes in the binding reaction served as negative controls. The binding reactions were allowed to sit at room temperature for 1 hour prior to loading them onto a provided 96-well chemiluminescent plate coated with streptavidin. After a 1-hour incubation, the wells were washed three times with $1 \times$ enhanced transcription factor assay buffer and incubated at room temperature with 100 μL of 1:1000 diluted antiNF- κB p65 antibody. After washing, the sample wells were incubated with 1:1000 diluted rabbit IgG-HRP conjugated secondary antibody for 30 minutes. After washing off unbound secondary antibody, chemiluminescent development was performed by adding chemiluminescent detection reagent in chemiluminescent reaction buffer at room temperature for 5 minutes. The absorbance was measured in a microplate luminometer.

Electromobility Shift Assays (EMSA)

A supply of 5' and 3' biotinylated labeled and unlabeled oligonucleotides of the NF- κ B consensus sequence 5'-AGTTGAGGGGACTTTCCCAGG-3' was obtained from Integrated DNA Technologies (Coralville, IA) and annealed to an unlabeled complementary oligonucleotide to generate double-stranded probes for logicradioactive, chemiluminescent EMSA. Nuclear extracts (3 μ g) were equilibrated in binding buffer [10 mmol/L Tris, pH 7.5; 1 mmol/L DTT, 2.5% glycerol, 5 mmol/L MgCl₂, 50 mmol/L KCl, 0.05% NP-40, 0.05 μ g/ μ l poly (dI:dC)-(dI:dC) (Pierce, Rockland, IL) with unlabeled double-stranded oligonucleotide probes (5 pm) in some samples for 15 minutes at room temperature. Double-stranded biotinylated probe (20 fm) was then added to all samples and incubated for an additional 20 minutes at room temperature. Nuclear extracts from 12-O-tetradecanoylphorbol-13-acetate (TPA) + calcium ionophore (CI) stimulated Jurkat cells (Active Motif; Carlsbad, CA) served as a positive control. Samples were loaded onto 6% polyacrylamide gel and run in 0.5 $\times$ TBE at 4°C. The binding reactions were then transferred from the gel to a presoaked nylon membrane (Ambion, Austin, TX) with a semidry electroblotting transfer unit (Owl Scientific, Woburn, MA). The transferred DNA was cross-linked to the membrane using an ultraviolet-light cross linker at 120 mJ/cm² for 60 seconds. The membrane was developed for chemiluminescent detection with a kit from Pierce (Rockland, IL). Briefly, the membrane was first blocked with blocking buffer prior to adding stabilized streptavidin-horseradish peroxidase conjugate. After washing four times, the membrane was equilibrated with substrate equilibration buffer prior to adding luminol enhancer solution in stable peroxide. The membrane was exposed and visualized with a CCD camera equipped Gel Doc 2000 chemiluminescent imaging documentation station (Bio-Rad, Hercules, CA).

Construction of Murine Muc5B Luciferase Reporter Plasmids

The promoter sequence for *Muc5B* was identified on Gen-Bank as accession number AY744445. The following primers tagged with 5' restriction enzyme sequences were obtained from Integrated DNA Technologies (Coralville, IA): -556 F5'-CAGGAGCCCAGAGACTCATC-3'; -359 F5'-ACAAGCCAAG-GTTGTTGTCC-3'; -255 F5'-GGCTTCGAATCCAGAGTAG-3'; and +23 R5'-GCAGGAGGGACTGAGACAAG-3' (XhoI restriction sites on the F primers' 5' end, and BglI restriction site on the R primer 5' end). PCR was performed using these primers and mouse liver genomic DNA extract as template. After purification, resulting products were restricted and ligated to the PGL4 vector (Promega, Madison, WI). After confirmation of the nucleotide sequences, the plasmids were then transformed in competent DH5' cells and amplified with MaxiPrep kits (Promega, Madison, WI).

Transient Transfection and Luciferase Assays

The pIg κ B Luc reporter construct containing three immunoglobulin G- κ chain NF- κ B binding sites upstream of the luciferase gene has been previously described³⁵ and was generously provided by Frank Ondrey MD, PhD, University of Minnesota, Minneapolis, Minnesota. This NF- κ B reporter plasmid and the *Muc5b* promoter plasmids were transiently transfected into mMEEC cells for luciferase assays as follows. Cell line cultures at 70% confluence were co-transfected with the luciferase plasmids (2 μ g/mL) and a pCMV- β Gal reporter construct (0.4 μ g/mL) (Clontech, Mountainview, CA) in Opti-MEM medium containing 3 μ g/mL of lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 6 hours, the medium was removed, and the cells were placed in FGM. The next day, the cells were treated with CSC at 10, 20, and 40 μ g/mL in DMSO, or plain DMSO for 8 hours. After stimulation, the relative luciferase activity was determined with the dual light reporter gene assay (Tropix, Medford, MA) and a Monolight 2010 plate luminometer (Analytical Luminescence Laboratories, San Diego, CA) according to the manufacturers' instructions. Results for relative luciferase units (RLU) were determined

as a ratio of the luciferase constructs over the pCMV- β Gal reporter to normalize for harvesting efficiency.

RT-PCR and Real Time RT-PCR Analyses of *Muc5b* Gene Expression

For experiments of mucin gene expression, the cells were grown under conditions allowing for further differentiation and goblet cell formation.³² Briefly, mMEEC were grown on transwells submerged in FGM until they achieved confluence. At that point, they were placed at an air liquid interface (ALI) for 14 days, with FGM being supplied only to the basal side of the cells. Ribonucleic acid (RNA) was extracted from these differentiated mMEEC cells exposed to CSC (10, 20, 40 μ g/mL) or vehicle for 2, 8, and 24 hours using TRIzol (Invitrogen, Carlsbad, CA). RNA was precipitated with isopropyl alcohol, washed and dissolved in RNase-free water. Reverse transcription reaction was then performed by using 1 μ g of total RNA from each sample with 10 mmol/L dNTPs, an 50 μ mol/L oligo(dT) and heating this mixture to 65°C for 5 minutes, followed by a 1-minute ice incubation. Then, 200 units of SuperScript III reverse transcriptase enzyme (Invitrogen, Carlsbad, CA), 40 units of RNaseOUT, 0.1 mol/L DTT, and 5 $\times$ first-strand buffer were then added to the mixture and the reaction was incubated at 50°C for 60 minutes. The reaction was then inactivated at 70°C for 15 minutes. At that point, cDNA was used for PCR, using specific pairs of primers as follows: *Muc5b* forward primer, 5'-ACTTGAGGAGGGTTCCAGGT-3'; *Muc5b* reverse primer, 5'-ACAGTGCCAGGGTTTATGC-3' (accession number AJ311906). As an internal control, β -actin was used, and primers for mouse β -actin were obtained from GeneLink (Hawthorne, NY). *Muc5b* and β -actin PCR product sizes are 307 and 349 bp respectively. PCR reactions were carried out in 50- μ L solutions, including 45 μ L of PCR Supermix (Invitrogen, Carlsbad, CA), 2 μ L of cDNA, and 1.5 μ L each of 10 μ mol/L forward and reverse *Muc5b* and β -actin primers. PCR products were separated by electrophoresis on a 2% in 1 $\times$ TAE agarose gel containing 50 ng/mL ethidium bromide and were visualized with a Gel Doc 2000 gel imaging documentation station (Bio-Rad, Hercules, CA).

Real-time RT-PCR was performed on the generated cDNA products in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using the power SYBR green PCR master mix from Applied Biosystems (Branchburg, NJ) with the same primers as described above. β -actin was unchanged by CSC exposure and was used as an internal control for normalizing *Muc5b* mRNA levels in control and experimental samples. Dilution curves confirmed the linear dependence of the threshold cycles on the concentration of template RNA samples. Relative quantification of *Muc5b* mRNA in control and experimental samples was obtained using the standard curve method.

Statistical Analysis

The statistical difference between experimental and control groups for all experiments was determined by two-tailed Student *t* tests. Significance level was set at *P* < .05.

RESULTS

CSC Increases NF- κ B Activity in mMEEC

NF- κ B is a mediator of cigarette smoke effects in multiple epithelial cell types.^{13–15} To elucidate whether this is also the case in mouse ear epithelium, we performed reporter assays, EMSA, and chemiluminescent NF- κ B TFA as three complementary means to determine whether CSC activates NF- κ B in mMEEC. Reporter gene assays demonstrated statistically significant 2.3, 1.85, and 2.9 increases in activity of the pIg κ B Luc plasmid relative to β -gal in mMEEC with 10, 20, and 40 μ g/mL of CSC, respectively (Fig 1A). EMSA demonstrated increased presence of NF- κ B in nuclear extracts from mMEEC stimulated with 20 μ g/mL CSC for 1 hour (Fig. 1B). This result correlated with quantitative chemiluminescent NF- κ B TFA

results demonstrating that nuclear extracts from mMEEC treated with 20 $\mu\text{g/mL}$ CSC contain a statistically significant increase in NF- κB protein that is capable binding of its consensus oligonucleotide sequence when compared to cells treated with plain DMSO (Fig 1C). Similarly, at the 40 $\mu\text{g/mL}$ dose of CSC there was also increased binding noted, but this did not reach statistical significance ($P < .08$). Taken together, the results shown in Figure 1 qualitatively and quantitatively demonstrate that CSC at 20 $\mu\text{g/mL}$ caused nuclear translocation of activated NF- κB and that CSC at 10, 20, and 40 $\mu\text{g/mL}$ functionally activated transfected NF- κB reporter plasmids in mMEEC.

CSC Increases Expression of Muc5b in a Primary Immortalized Murine Middle Ear Cell Line Grown Under Differentiating Conditions

For these experiments, *Muc5b* expression was evaluated both in mMEEC cells grown on plastic at 37°C and under conditions where they had been reported to differentiate.³² Reverse transcriptase- polymerase chain reaction (RT-PCR) data indicated low levels of *Muc5b* mRNA expression in undifferentiated cells, and CSC did not alter *Muc5b* mRNA levels in mMEEC cells grown submerged on plastic (data not shown). Given the reported lack of goblet cells in MEEC grown on plastic,^{31,32} this finding was expected. To confirm ALI induced differentiation of the mMEEC, in separate experiments periodic acid schiff (PAS) was performed after 14 days of ALI on Transwell membranes (Corning Inc.). We observed PAS staining in goblet cells, whereas no PAS staining was observed in mMEEC cells grown under submerged conditions on plastic (data not shown). RT-PCR data from experiments performed on differentiated cells demonstrated that CSC increased murine *Muc5b* mRNA levels in mMEEC in a dose-dependent fashion at 2 and 8 hours (Fig. 2A). Semi-quantitative densitometry of three separate experiments demonstrated a trend toward CSC-induced increased expression (Fig. 2C). In additional experiments on differentiated mMEEC, quantitative real time PCR results showed statistically significant dose and time course dependent increases in *Muc5b* transcription with CSC (Fig. 2D) with 20 and 40 $\mu\text{g/mL}$ at 2 hours and with 10 and 20 $\mu\text{g/mL}$ at 8 hours. After 8 hours at the higher dose (40 $\mu\text{g/mL}$) and at longer exposure times (24 hours), a decrease in upregulation was noted. This is likely due to increased apoptosis and decreased cell viability that has been previously observed in epithelial cells exposed to these higher CSC doses and times.³⁶

CSC Increases the Activity of the Murine Muc5b Promoter in mMEEC

To determine whether the observed increases in *Muc5b* mRNA levels with CSC were due to increased transcriptional regulation, studies were performed to determine whether CSC increased promoter activity of constructs of differing lengths of the *Muc5b* 5' flanking region (Fig. 3A). Luciferase reporter gene assays (Fig. 3B, 3C) demonstrated statistically significant relative Luciferase unit (RLU) 1.8, 2.2, and 1.2 fold activation of the -556 bp *Muc5b* promoter construct in MEEC with 10, 20, and 40 $\mu\text{g/mL}$ respectively ($P < .001$). Similarly, results show 3.7, 4.8, and 1.3 fold activation of the -229 bp *Muc5b* promoter in MEEC with 10, 20, and 40 $\mu\text{g/mL}$ of CSC respectively ($P < .001$). The results suggest that the observed increase in *Muc5b* mRNA expression induced by CSC is at least partially regulated at the transcriptional level. As shown in Figure 3A, MatInspector analysis (Genomatix MatInspector, Munich, Germany, www.genomatix.de; v. 3.0, 0.80/Opt. -0.03) shows the presence of two AP-1 and two NF κB cis-sites in the *Muc5b* -556 construct and one AP-1 and one NF κB site in the -255 construct.

DISCUSSION

Tobacco smoke exposure has been shown to be a significant epidemiologic risk factor for the development of otitis media^{3,4,6,11,37} but mechanisms whereby cigarette smoke products contribute to the pathogenesis of otitis media have not been previously studied. NF- κB has been demonstrated to be a major intracellular mediator of cigarette smoke effects, and studies

have shown that cigarette smoke products activate NF- κ B in macrophages,¹³ human lung epithelial cells,¹⁴ and squamous cell lines.¹⁵ In studies using lung epithelial cells, CSC-mediated effects have been noted to be dose and time dependent, resulting in the activation of numerous genes with NF κ B cis-sites in their 5'-upstream flanking sequences.^{15,36,38} Increased expression levels of NF- κ B have been shown in bronchial biopsies of smokers.¹⁶ Given these reports, and the fact that NF- κ B has also been purported to be a major mediator of the chronic inflammatory response seen in OM,¹⁷ we first sought to investigate whether CSC would be able to activate NF- κ B in mMEEC.

Our data shown in Figure 1 qualitatively and quantitatively demonstrate that CSC causes nuclear translocation of NF- κ B and functional activation of NF- κ B in mMEEC. Luciferase reporter results show that CSC induces activation of consensus NF- κ B reporter constructs transiently transfected into mMEEC (Fig. 1A). EMSA results (Fig. 1B) demonstrate increased binding of an NF- κ B binding complex in 20 μ g/mL CSC stimulated mMEEC nuclear extracts. Chemiluminescent transcription factor assay results (Fig 1C) further confirm that CSC is able to activate nuclear translocation of NF- κ B, where it is able to bind to its consensus oligonucleotide sequence.

In the lung cell biology field, studies have shown that cigarette smoke mediated NF- κ B activation increases transcription of the secreted mucin gene *MUC5AC* in respiratory epithelial cells through a signal transduction pathway involving the epidermal growth factor receptor (EGFR).³⁹⁻⁴¹ Because mucin overproduction is also a key pathologic feature of OM, the second aim of this study was to determine whether mucin gene induction would occur in the middle ear cell models exposed to cigarette smoke products. As opposed to the lower airways, where MUC5AC is the predominant mucin in secretions, studies in human OM have shown that MUC5B is the predominant mucin in chronic middle ear disease.^{24-27,28} Therefore, we analyzed whether CSC activated *Muc5b* transcription in a murine-cultured cell model of OM, mMEEC.

Our results demonstrate both qualitatively and quantitatively that CSC upregulates *Muc5b* mRNA levels in differentiated murine middle ear cell line mMEEC at 2 and 8 hours (Fig. 2A, C, and D). Notably, these findings are consistent with the EMSA and TFA results shown in Figure 1, which demonstrate maximal consensus sequence binding of an NF- κ B binding complex at 20 μ g/mL of CSC, the same dose at which maximum *Muc5b* mRNA induction is noted. At the higher CSC dose (40 μ g/mL) and at longer exposure times (24 hours) a decrease in *Muc5b* mRNA upregulation was noted even to below basal levels. This is likely due to the increased apoptosis and decreased cell viability previously observed in epithelial cells exposed to these higher CSC doses and times.³⁶ Considering that the *Muc5b* promoter contains several putative NF- κ B binding sites, we also sought to investigate whether CSC could activate *Muc5b* promoter reporter plasmids. The -556 bp *Muc5b* promoter plasmid includes two possible NF- κ B sites while the -229 bp *Muc5b* promoter plasmid has only one NF- κ B cis-site (Fig 3A). Our luciferase reporter data (Fig 3B and C) show that CSC induces similar functional activation of both *Muc5b* promoter deletion constructs transfected into mMEEC. One might have expected the induction to have been greater in the longer deletion construct with one more NF- κ B site, but it is possible that the longer construct contains a negative regulator binding element. Also, it is possible that other transcription factors such as Sp1 and AP1 contribute to promoter activity in both plasmids. EMSA and chromatin immunoprecipitation (ChIP) assays are currently ongoing in our lab to determine whether NF- κ B actually binds these specific cognate sites in the *Muc5b* promoter, and whether other transcription factors also play a pivotal role in mMEEC after CSC exposure. Finally, site specific mutagenesis directed luciferase constructs are being generated to determine if these sites are indeed responsible for the CSC induced increased *Muc5b* promoter activity.

Notably, the higher dose of CSC (40 μ g/mL) induced activity of the *Muc5b* promoter constructs in contradistinction to the RT-PCR results showing a decrease in *Muc5b* mRNA levels. There are two possible explanations for this. First, luciferase assays measure whether the exogenously transfected *Muc5b* promoter is upregulated by transcription factors activated by CSC independent of whether there is a potential for diminished cell viability (because of internal normalization with β Gal co-transfection) which could account for the decrease in actual gene transcripts measured with RT-PCR. Second, the luciferase assays were performed on mMEEC grown on plastic, while RT-PCR measures of gene transcription were performed on differentiated cells grown on air liquid interface (ALI). It is possible that higher doses of CSC are more toxic to the differentiated cells. We are currently performing measures of cell viability and apoptosis in mMEEC exposed to CSC under both growth conditions.

In conclusion, dissolved cigarette smoke particulate matter activates NF- κ B in immortalized MEEC. Furthermore, this activation correlates with CSC-induced *Muc5b* promoter activation and gene expression in mMEEC. Taken together, these results indicate that much as in lung cells, the activation of mucin gene expression by cigarette smoke is mediated in part by NF- κ B.

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Fig. 1.

Cigarette smoke condensate (CSC) increases nuclear factor kappa B (NF- κ B) activity in murine middle ear epithelial cells (mMEEC). (A) Luciferase assay results with the pIgkBLuc reporter construct⁴² activity normalized to β -gal activity demonstrate significant increases in functional NF- κ B activity with CSC (*all $P < .05$). (B) Electromobility shift assays (EMSA) demonstrate increased binding of an NF- κ B consensus oligonucleotide binding complex with nuclear extracts from mMEEC treated with 20 μ g/mL CSC. (C) Chemiluminescent transcription factor assays quantitatively demonstrate statistically significant increases in nuclear NF- κ B with 20 μ g/mL CSC (* $P = .03$).

Fig. 2.

Cigarette smoke condensate (CSC) upregulates *Muc5b* gene expression in differentiated murine middle ear epithelial cells (mMEEC). (A) Reverse transcriptase polymerase chain reaction (RT-PCR) shows increased expression of *Muc5b* mRNA with increasing doses of CSC at 2 and 8 hours. (B) Murine organ tissue controls for PCR primers demonstrate an amplicon of 309 bp, signifying expression of *Muc5b* mRNA in stomach but not in spleen, as expected. (C) Semiquantitative densitometry from three separate RT-PCR experiments. (D) Quantitative real-time PCR demonstrates statistically significant increases in *Muc5b* mRNA at 2 and 8 hours (* $P = .01$, ** $P < .001$).

Fig. 3.

Luciferase reporter activity in murine middle ear epithelial cells (mMEEC) after stimulation with CSC. (A) Putative nuclear factor kappa (NFκB) and AP1 transcription factor sites are identified in the 1.7-kb murine *Muc5b* promoter. Two plasmids containing different lengths of the *Muc5b* promoter as indicated by dashed lines were cloned into PGL4 Luc (as described in the Methods) vectors and co-transfected along with a β-gal reporter plasmid into mMEEC. (B) Results show significant activation of the -556 bp *Muc5b* promoter construct relative to β-gal with CSC stimulation (*P < .001). (C) Similarly, results show significant activation of the -229 bp *Muc5b* promoter construct relative to β-gal with CSC (*P < .001).