Middle Ear Response of \textit{Muc5ac} and \textit{Muc5b} Mucins to Nontypeable \textit{Haemophilus influenzae}

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\textbf{IMPORTANCE} Chronic otitis media with effusion is characterized by middle ear secretion of mucin glycoproteins, predominantly MUC5B; MUC5AC, the other secretory mucin studied frequently, has also been identified in the middle ear. Emerging evidence suggests a dichotomous role for these mucins in innate immune responses. We hypothesized that MUC5AC is an acute responder and MUC5B is expressed at later time points, reflecting a chronic situation.

\textbf{OBJECTIVE} To determine middle ear regulation of MUC5B and MUC5AC following in vitro bacterial and cytokine exposure.

\textbf{DESIGN, SETTING, AND SAMPLES} An in vitro cell-based model of mucin gene regulation was conducted in a basic science laboratory at a tertiary pediatric hospital. The study was conducted from July 1, 2014, to June 30, 2015; data analysis was performed in July 2015.

\textbf{INTERVENTIONS} Nontypeable \textit{H. influenzae} (NTHi) lysates were generated and used to stimulate mouse middle ear epithelial cells (mMEECs) for 2 hours during 3 weeks.

\textbf{MAIN OUTCOMES AND MEASURES} Real-time quantitative polymerase chain reaction, luciferase assays, Western blot assay, and immunofluorescence techniques were performed to determine \textit{Muc5ac} and \textit{Muc5b} expression over time, \textit{Cxcl2} chemokine response, and nuclear factor-\kappaB activation. Luciferase reporter assays were performed to evaluate specific promoter responses after NTHi exposure.

\textbf{RESULTS} Nontypeable \textit{H. influenzae} lysates (200 μg/mL) drove differential mucin gene activation, with \textit{Muc5ac} being induced up to 2.04 fold at 24 hours and 2.79 fold at 96 hours (\(P < .05\)) and \textit{Muc5b} being induced only at more long-term points: 1.61 fold at 96 hours, 1.41 fold at 1 week, and 1.53 fold at 3 weeks (\(P < .05\)). Although NTHi lysates induced robust, early nuclear factor-\kappaB nuclear translocation with nuclear factor-\kappaB-dependent induction of \textit{Cxcl2} expression, the lysates had minimal to no effect on \textit{Muc5ac} and \textit{Muc5b} promoter activity. However, in contrast to NTHi lysates, CXCL2 induced significant transcription of both \textit{Muc5b} and \textit{Muc5ac} as early as 24 hours.

\textbf{CONCLUSIONS AND RELEVANCE} Nontypeable \textit{H. influenzae} lysates activate differential mucin gene activation in mMEECs. Although \textit{Muc5ac} is an early response mucin gene, \textit{Muc5b} appears to react as a chronic response mucin.
titis media is one of the most common conditions of early childhood, accounting for a very high proportion of all pediatric office visits and surgeries annually, at a national health care cost estimated to be greater than $1 billion. Chronic otitis media typically becomes a long-term sequela of acute middle ear infection and is characterized by the persistence of middle ear effusion that is most frequently mucoid. Effusions are predominantly composed of mucin glycoproteins, which are high-molecular-weight O-glycosylated proteins with a mucin (MUC) peptide backbone. More than 20 human (MUC) and mouse (Muc) genes have been identified, with MUC5AC and MUC5B mucins being the predominant polymeric mucin glycoproteins in human airway mucus secretions. Although MUC5B protein has been postulated to be the most abundant mucin in chronic otitis media, MUC5AC has also been identified at the RNA level in middle ear epithelium at baseline and following bacterial exposure. In comparison with MUC5AC, MUC5B regulation in middle ear responses is markedly understudied.

Nontypeable Haemophilus influenzae (NTHi) is the most common infectious pathogen in acute otitis media. Previous work has demonstrated that middle ear NTHi challenge in mice leads to early overexpression of inflammatory mediators, primarily Cxcl2. Nontypeable H influenzae lysates have been shown to signal through toll-like receptor 2, activating the proinflammatory transcription factor nuclear factor-κB (NF-κB), which leads to early overexpression of MUC5AC in the human airway epithelium. Conversely, we have not noted direct early induction of Muc5b gene expression in vivo and in vitro with NTHi lysate stimulation.

Emerging evidence in knockout and transgenic mice has shown a dichotomous role for Muc5ac and Muc5b mucins in airway immune response. Muc5b is critical for innate immune airway defense since severe airway infections occur in Muc5b-null mice, ultimately resulting in death. The middle ear space of Muc5b-null mice develops fulminant acute bacterial infection with 100% penetrance. However, Muc5ac-null mice do not show susceptibility to airway and middle ear infection but rather a marked reduction in the allergic inflammation that is required for airway hyperreactivity and mucoid occlusion. These findings suggest that chronic Muc5b homeostatic regulation is important in protection from bacteria in the middle ear and that Muc5ac regulates acute and allergic responses. We thus hypothesized a differing Muc5b and Muc5ac response to a time course of NTHi lysates exposure. Specifically, we postulated that NTHi stimulation of murine middle ear epithelial cells would induce early NF-κB activation and induction of Muc5ac expression, with induction of Muc5b expression occurring at more long-term time points.

**Methods**

The study was conducted from July 1, 2014, to June 30, 2015. Institutional review board approval was not requested for this basic science investigation since no human subjects research involving human tissue samples or human cells was performed.

**Bacterial Culture and Preparation of Lysates**

Nontypeable H influenzae, clinical strain 12, was grown on chocolate agar plates before sonication for lysis as previously described. Stock solutions (8 mg/mL) were stored at −20°C.

**Cell Culture**

The mouse middle ear epithelial cells (mMEECs) are immortalized by a temperature-sensitive simian virus 40, allowing for proliferation at 33°C and differentiation at 37°C. The submerged mMEECs were maintained and passaged in full-growth media as previously described. For experiments in submerged cells, full-growth media was replaced with media that lacked fetal bovine serum 4 hours before exposure. Nontypeable H influenzae lysates or mediators were added at 50- to 200-μg/mL concentrations. For Cxcl2 stimulation, we used recombinant mouse Cxcl2 (R&D Systems). The dose was extrapolated from what has been described as the mean interleukin 8 (IL-8) dose present in human middle ear effusions. In vivo and in vitro with NTHi lysate stimulation.

**Real-Time Quantitative Polymerase Chain Reaction**

Expression of messenger RNA (mRNA) (β-actin [NM_007393], Muc5b [NM_028801], Muc5ac [NM_010844], and Cxcl2 [NM_009140]; all GenBank) was evaluated by polymerase chain reaction (PCR) using mouse β-actin primers as an internal control (Gene Link). After treatment, cell lysates were recovered using a lysis buffer (TRIZOL; Life Technologies), and the RNA extraction was performed per the manufacturer’s recommendations. Incubation of DNase I was performed to eliminate genomic DNA contamination (Quanta BioSciences). Reverse transcription of the mRNA was completed (qScript cDNA SuperMix; Quanta BioSciences), and the PCR reactions were performed with a SYBR green method kit for quantification (PerfeCTa SYBR Green SuperMix; Quanta BioSciences). Finally, real-time quantitative PCR analysis was performed using a thermocycler (AB 7900T; Applied Biosystems). The primer sequences used (Integrated DNA Technologies) for Muc5b, Muc5ac, and Cxcl2 have been published. The relative quantification of the gene of interest was performed according to the method described by Pfaffl. Briefly, a comparative quantification method, also called the ΔΔCt method (Ct indicates the threshold cycle, occurring when fluorescence of the sample becomes significantly different from zero), was used to normalize the expression of the gene of interest to the housekeeping gene and to quantitate the expression of the mRNA compared with the control condition. The calculations were made as follows: ΔCt = Ct (gene of interest) − Ct (housekeeping gene), and ΔΔCt = ΔCt (treated con-
dition) – ΔΔct (control condition). The relative expression of the mRNA was then determined using the formula 2^(-ΔΔct).

### Transient Transfection and Luciferase Assays
The pIκB luciferase reporter was used to quantify NF-κB activation. The Muc5b promoter plasmid was cloned in our laboratory as previously described. The MUC5AC promoter plasmid contains the reported approximately 1.4-kb sequence of the 5′-flanking sequence of the MUC5AC gene. The Cxcl2 promoter construct for wild-type or mutated for NF-κB binding site have been previously described. The plasmids were transiently transfected into mMEECs for luciferase assays as previously described. After 8 hours, relative luciferase activity was determined with a reporter gene assay (Dual-Light, Applied BioSystems; and Tropix, ThermoFisher Scientific) and a plate luminometer (Mithras Luminometer; Berthold Technologies). Relative luciferase units were determined as a ratio of the luciferase construct counts over the β-galactosidase (βGal) plasmid counts. The βGal plasmid serves as a control for transfection efficiency and total transfected cell numbers. In addition, as mentioned above, serum-free media was used as a vehicle control for NTHi lyse treatments.

### Western Blotting of Mouse p65 Protein
Cells were grown in 6-well plates until subconfluence and then exposed to NTHi lysates for the times indicated. Cells were washed with phosphate-buffered saline and then fractionated using nuclear and cytoplasmic extraction reagents (NE-PER; Pierce Biotechnology) according to the manufacturer’s recommendations. Bicinchoninic acid assay (Thermo Fisher Scientific) was used to assay the total quantity of protein. Western blotting was performed as described previously. The p65 primary antibody (C-20) sc-372 (Santa Cruz Biotechnology) was used at 1:1000 dilution in 2.5% milk solution in phosphate-buffered saline with Tween overnight. The secondary antibody, anti-rabbit, IgG-horseradish peroxidase sc-2030 (Santa Cruz Biotechnology) was used at a 1:10 000 dilution for 1 hour in 2.5% milk solution in phosphate-buffered saline with Tween. Detection was performed with a substrate kit (SuperSignal West Dura Extended Duration Substrate; Pierce Biotechnology).

### Statistical Analysis
The statistical difference between the experimental and control groups was determined by 2-tailed t tests for pairwise comparisons of numeric data and an analysis of variance test followed by the Dunnett test or Wilcoxon rank sum test for multiple group comparisons of numeric data. The significance level was set at P < .05. Data analysis was conducted in July 2015.

### Results

#### NTHi Lysate Effect on Muc5ac and Muc5b Mucin Expression
Real-time PCR data from 3 experiments performed on submerged cells demonstrated that NTHi lysates did not increase early Muc5b expression in mMEECs since there was no significant difference in the level of normalized Muc5b transcripts at 2, 4, 14, 24, and 48 hours with 50 to 200 μg/mL of NTHi lysates compared with the controls (Figure 1A and B). For Muc5ac, NTHi lysate exposure resulted in increased mRNA expression in a dose-dependent fashion at 24 hours (1.6 fold, 1.8 fold, and 2.0 fold with 50, 100, and 200 μg/mL NTHi lysate, respectively) and at 48 hours (1.7 fold, 1.8 fold, and 2.2 fold with 50, 100, and 200 μg/mL NTHi lysate, respectively; but not at earlier time points (Figure 1C and D). Given that upper respiratory epithelial cells are known to activate proinflammatory cytokines on bacterial stimulation via toll-like receptor 2 signaling and induction of NF-κB, we next assayed for Cxcl2 transcripts with NTHi lysate stimulation. As expected, NTHi lysates resulted in an early, dose-dependent, and potent increase in Cxcl2 transcription that began at 2 hours (23.85 fold and 67.7 fold with 100 and 200 μg/mL NTHi lysate, respectively) and persisted up to 48 hours (16.49 fold and 99.23 fold with 100 and 200 μg/mL NTHi lysate, respectively) (all P < .05) (Figure 1E and F). These data indicate that mMEECs are able to mount an early increase in proinflammatory cytokine expression with NTHi stimulation, with a delayed mucin gene response, consisting of Muc5ac, but not Muc5b, expression in the first 24 to 48 hours.

#### Long-term NTHi Lysate Effect on Muc5b and Muc5ac Expression in mMEECs Grown Under Differentiating Conditions
To evaluate the long-term effect of NTHi on middle ear epithelium, mMEECs were grown at air-liquid interface under conditions in which they have been reported to differentiate, with every-other-day 2-hour exposures to NTHi vs control. Although there was also no significant difference in the level of Muc5b RNA at 48 hours with NTHi compared with the control, under these conditions, a statistically significant 1.79-fold induction (P < .001) of gene expression was noted at 96 hours with NTHi lysate exposure. The induction was sustained over time with 1.41-, 1.19-, and 1.53-fold inductions at 1, 2, and 3 weeks, respectively (P < .001) (Figure 2A). Comparatively, Muc5ac expression increased from a mean fold change of 1.97 at 48 hours to maximal fold change induction of 2.79 at 96 hours (P < .001) before returning to baseline from 1 to 3 weeks without any significant difference relative to control (Figure 2B). These data demonstrate that, compared with Muc5ac, Muc5b responses to bacterial insult and injury occur at a less robust but more chronic and sustained level.

#### NTHi Lysate Effect on NF-κB, Muc5b, or Muc5ac Promoter Activity
To elucidate whether NTHi lysates activate NF-κB in submerged murine middle ear cell lines, we performed reporter assays, Western blot assays, and immunostaining for the NF-κB p65 subunit as complementary methods to test for NF-κB activation. Reporter gene assays demonstrated 2.1-fold, 3.2-fold, and 4.3-fold increases in pIκB luciferase plasmid normalized with βGal in mMEECs with 50, 100, and 200 μg/mL of NTHi lysates (all P < .05) (Figure 3A). This promoter response was confirmed by the Western blot assay analysis
Submerged mouse middle ear epithelial cells were grown to 80% confluence, deprived of serum for 6 hours, and treated for 2, 4, 14, and 24 hours or 24 and 48 hours with 50 to 200 μg/mL of NTHi lysates before real-time quantitative polymerase chain reaction was performed as indicated, with β-actin serving as the gene of reference. A and B. No statistically significant changes for Muc5b expression were noted at any of the time points or doses used. C and D. A dose-dependent increase in Muc5a expression was noted at 24 and 48 hours, but not at earlier time points, with NTHi lysate stimulation. E and F. A potent and robust dose-dependent increase of Cxcl2 expression was noted at all time points. The log_{10} scale was used to standardize across measured genes. The bars represent ΔΔCt-fold changes with errors. 

\[ P < .001 \] comparing 50, 100, and 200 μg/mL of NTHi lysates with no NTHi lysates, determined using 2-tailed t tests.
demonstrating early nuclear translocation of p65 in mMEECs exposed to 200 μg/mL of NTHi lysates at 30 minutes and 1 hour (Figure 3B).

Because of the observed direct NF-κB response to NTHi in mMEECs, we assayed for activation of the Muc5b and Muc5ac promoters following NTHi stimulation since these promoters contain NF-κB response elements.49,50 In line with the real-time PCR data, no activation of the Muc5b or Muc5ac promoter was seen with NTHi stimulation after overnight incubation of mMEECs with NTHi lysates (Figure 4). Therefore,
although NTHi is clearly able to drive NF-κB activity in mMEECs, much like in human cells, this NF-κB response does not result in direct upregulation of Muc5b and Muc5ac mucin gene promoters.

NTHi Effects on the Cxcl2 Promoter

We next explored whether the noted Cxcl2 induction with NTHi lysates (Figure 1C) was the result of NF-κB activity at the Cxcl2 promoter. For these experiments, luciferase assays were performed with promoter constructs containing 531 base pairs and deletions (−184 and −124 base pairs) of the Cxcl2 promoter upstream of a luciferase gene. To determine specific NF-κB effects, mutant plasmids containing a mutated, putative, NF-κB response proximal element were also used (Figure 5A). The results indicated that 200 μg/mL of NTHi lysates resulted in 1.43-fold ($P = .001$), 1.32-fold ($P = .049$), and 1.26-fold ($P = .03$) activation of the −531, −187, and −124 lengths of the Cxcl2 promoter constructs containing the intact NF-κB response site. However, this activation was completely abrogated (ie, below constitutive levels) in each of the promoter constructs when the NF-κB response site was mutated (Figure 5B-D).

CXCL2 Effects on Muc5b and Muc5ac Expression

As an initial strategy to determine whether CXCL2 can function as a cytokine that drives mucin gene expression in mMEECs, we explored whether stimulation of these cells with CXCL2 results in activation of Muc5b and Muc5ac expression. For Muc5b mRNA levels, we noted a 1.35-fold increase at 24 hours ($P = .009$) and a 2.0-fold increase at 48 hours ($P = .002$) with 300 pg/mL of CXCL2 stimulation. For Muc5ac mRNA levels, we noted a 1.54-fold increase at 24 hours ($P = .005$) and a 2.1-fold increase at 48 hours ($P = .01$) with 300 pg/mL of CXCL2 stimulation. Furthermore, overnight CXCL2 stimulation resulted in a 1.77-fold statistically significant activation of the Muc5b promoter ($P = .03$), but not the Muc5ac promoter ($P = .86$). These data suggest that proinflammatory cytokines, such as CXCL2, may act as indirect mediators of NTHi mucin gene response effects in mouse middle ear mucosa (eFigure in the Supplement).

Discussion

Mucins are large, heavily O-glycosylated proteins that play a key role in the innate immune system but can also contribute to airway obstruction when oversecreted. However, to our knowledge, the exact role of different mucin subtypes in the promotion of otitis media progression has not been previously investigated.

Although most mucin subtypes can be identified in middle ear epithelium at the RNA level, MUC5B has been identified as the predominant mucin glycoprotein secreted into the middle earspace of patients with chronic mucoid middle ear effusion. A recent study of Muc5b-null mice demonstrated the critical role of this mucin in airway defense including the middle ear. These knockout mice showed a severe and fulminant propensity to airway and middle ear infection, leading to early death. An overproduction of Muc5ac was demonstrated by histologic techniques in the Muc5b-null mice (probably to compensate for the lack of Muc5ac), but the overproduction failed to protect the airways from infection. However, Muc5ac-knockout mice do not show any difference compared with wild-type mice in terms of airway or middle ear infection susceptibility. As such, it is clear that there must be a dichotomous role involving the function of MUC5B and MUC5AC glycoproteins in the airway and ear. In humans with asthma, as MUC5AC expression increases, MUC5B expression in the lung either remains stable or decreases markedly.
however, to our knowledge, this type of time-dependent association between the 2 secretory mucins has never been studied in the middle ear.

Our data are in line with the hypothesis that MUC5AC is secreted earlier upon infectious and inflammatory stimuli and in a more robust fashion compared with MUC5B, which responds in a comparatively mutated but more sustained fashion. One can perhaps extrapolate that, compared with the acute responder (MUC5AC), MUC5B is more critical for long-term baseline homeostasis than is MUC5AC, as has been postulated. 42, 43

Inflammation associated with acute bacterial infection is also postulated to contribute to chronic otitis media. 44, 45 Studies 46, 47 have shown that NTHI induction of the proinflammatory transcription factor NF-κB leads to potent induction of middle ear mucosal hyperplasia in vivo as well as to up-regulation of proinflammatory cytokines, such as IL-1β and Cxcl2. In the present study, we identified marked NF-κB-dependent induction of Cxcl2 in mMEECs, validating previous work by Lim et al 19 performed in human middle ear epithelial cells. A functional murine homologue of IL-8, Cxcl2 has been postulated 48 to act as a critical mediator of bacterial-induced inflammation in the airways, leading to mucin hypersecretion. The Cxcl2 receptor is critical for mucin overproduction in the lower airway epithelium of mice following viral challenge. Transgenic mice that overexpress IL-1β increase production of Cxcl2 and also exhibit mucoid metaplasia, potentially through Cxcl2-mediated release. 49 With consideration of this background information, it is noteworthy that Cxcl2, but not NTHI lysates, was able to activate Muc5b expression at early time points.

The Cxcl2 cytokine directed only moderate induction of the Muc5b promoter and no induction of the Muc5ac promoter. Given that both of the mucin transcripts were induced transcriptionally, regulation of MUC5AC is an important mechanism by which Cxcl2, it is possible that the mechanism for Cxcl2-mediated increase in mucin transcripts is via stabilization of mRNA. The IL-8–induced posttranscriptional, but not transcriptional, regulation of MUC5AC is an important mechanism by which Cxcl2, which again was completely abrogated (below baseline levels) when the proximal NF-κB site was mutated. D, There was statistically significant induction of the 124-bp–length Cxcl2 promoter with 200 μg/mL of NTHi, which again was completely abrogated (below baseline levels) when the proximal NF-κB site was mutated. A, Plasmid maps showing the Cxcl2 promoter sequence of 531, 187, and 124 base pairs (bp) inserted upstream of the start codon into a PGGL3 vector (Promega). For each plasmid length, a plasmid with a mutated proximal nuclear factor–κB (mNF-κB) site at 57 bp upstream of the start codon was also used. Analysis of variance was conducted using the Dunnett test. B, There was statistically significant induction of the 531-bp–length Cxcl2 promoter with 100 and 200 μg/mL of NTHi, which was completely abrogated (below baseline levels) when the proximal NF-κB site was mutated. C, There was statistically significant induction of the 187-bp–length Cxcl2 promoter with 200 μg/mL of NTHi, which again was completely abrogated (below baseline levels) when the proximal NF-κB site was mutated. D, There was statistically significant induction of the 124-bp–length Cxcl2 promoter with 200 μg/mL of NTHi, which again was completely abrogated (below baseline levels) when the proximal NF-κB site was mutated.
nism for IL-8–driven increases in MUC5AC mRNA levels in lung epithelial cells.\(^3\)

Taken together, our findings in this study lead us to speculate that it is not NTHi lysates that lead directly to chronic mucin overexpression in the middle ear epithelium. Rather, it is likely that secondary, indirect effects of repeated bacterial stimulation, such as cytokine hypersecretion, immune cell recruitment, and middle ear mucosal metaplasia, create the conditions allowing mucin upregulation and overproduction as postulated by others.\(^4\) A limitation of this work is that the immortalized cell line used does not secrete much mucin protein. As such, mucin-specific effects are limited to analyzing RNA levels, which may not always translate to protein levels in secretions. Future and ongoing work in our laboratory aim to investigate intermediary molecular mechanisms by which NTHi affects mucin gene expression, but early data, such as those reported herein, are highly suggestive that cytokines, such as CXCL2, play a critical role.

### Conclusions

Our data have demonstrated that NTHi lysates activate a differential response in mucin gene activation, with Muc5ac being induced at 24 to 96 hours and Muc5b being induced at more long-term time points (96 hours to 3 weeks) and only in differentiated cells. Furthermore, much like in human middle ear cells, NTHi drives a potent, early, proinflammatory epithelial cell response mediated through NF-κB activation. This early inflammatory response is likely critical in driving subsequent, more chronic, middle ear mucin gene upregulation.
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Original Investigation Research


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