Novel Cytoplasmic Proteins of Nontypeable *Haemophilus influenzae* Up-regulate Human MUC5AC Mucin Transcription via a Positive p38 Mitogen-activated Protein Kinase Pathway and a Negative Phosphoinositide 3-Kinase-Akt Pathway*

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Nontypeable *Haemophilus influenzae* (NTHi), an important human pathogen that causes chronic otitis media with effusion (COME) in children and exacerbation of chronic obstructive pulmonary disease (COPD) in adults. Mucin overproduction, a hallmark of both diseases, has been shown to directly cause conductive hearing loss in COME and airway obstruction in COPD. The molecular mechanisms underlying mucin overproduction in NTHi infections still remain unclear. Here, we show that NTHi strongly up-regulates MUC5AC mucin transcription only after bacterial cell disruption. Maximal up-regulation is induced by heat-stable bacterial cytoplasmic proteins, whereas NTHi surface membrane proteins induce only moderate MUC5AC transcription. These results demonstrate an important role for cytoplasmic molecules from lysed bacteria in the pathogenesis of NTHi infections, and may well explain why many patients still have persistent symptoms such as middle ear effusion in COME after intensive antibiotic treatment. Furthermore, our results indicate that activation of p38 mitogen-activated protein kinase is required for NTHi-induced MUC5AC transcription, whereas activation of phosphoinositide 3-kinase-Akt pathway leads to down-regulation of NTHi-induced MUC5AC transcription via a negative cross-talk with p38 mitogen-activated protein kinase pathway. These studies may bring new insights into molecular pathogenesis of NTHi infections and lead to novel therapeutic intervention for COME and COPD.

Nontypeable *Haemophilus influenzae* (NTHi), a Gram-negative bacillus, is an important human pathogen in both children and adults (1, 2). In children, it causes chronic otitis media with effusion (COME), one of the most common childhood infections and the leading cause of conductive hearing loss in the United States (3), whereas in adults, it exacerbates chronic obstructive pulmonary disease (COPD), the fourth leading cause of patient deaths in the United States (4, 5). Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections remains a great challenge because of high antigenic variation. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains. Therefore, there is an urgent need for developing novel therapeutic strategies for the treatment of these diseases based on a full understanding of the molecular pathogenesis of NTHi infections.

Although significant progress has been made toward identifying the virulence factors of NTHi, the molecular pathogenesis of NTHi infections is still largely unknown. Interestingly, there is evidence that up-regulation of mucin production induced by bacteria could play an important role. Mucins are high molecular weight glycoproteins that constitute the major component of mucus secretions in the middle ear, trachea, digestive, and reproductive tracts (6). They protect and lubricate the epithelial surface and trap particles, including bacteria and viruses, for mucociliary clearance (7). In COME and COPD, excessive production of mucin occurs, overwhelming the normal mucociliary clearance mechanisms. As mucus levels increase, they contribute significantly to airway obstruction in COPD (8, 9) and conductive hearing loss in COME (10). In addition to the obstructive outcome, mucin has been reported to bind to almost all known bacterial pathogens (11–14). The combination of defective mucociliary clearance and mucin–bacteria interaction could greatly increase the ability of bacteria to persist in a host. To date, 13 mucin genes have been cloned (6, 7, 15–17) and one, MUC5AC, has been shown to be highly expressed in airway and middle ear epithelial cells (18). Furthermore, recent studies have demonstrated that expression level of MUC5AC mRNA in the middle ear is higher in patients with COME than in normal individuals (19). Taken together, these studies strongly suggest that up-regulation of MUC5AC mucin gene plays an important role in the pathogenesis of NTHi infections.

Although little is known about how NTHi up-regulates MUC5AC mucin transcription, previous studies have shown that bacteria can activate transcription of host defense genes via activation of specific signal transduction cascades. Among the commonly known signaling events, the mitogen-activated protein kinase (MAP kinase) pathways are thought to be most important in transmitting extracellular signals from the cell...
surface to the nucleus (20). p38, a major MAP kinase superfamily member, has been shown to be involved in NTHi-induced inflammatory responses (23). In addition to p38 MAP kinase, phosphoinositide 3-kinase (PI 3-kinase) represents another major signaling transducer involved in the regulation of cell proliferation, survival, metabolism, cytoskeleton reorganization, and membrane trafficking (21), as well as bacterial pathogenesis (22). However, the role of both p38 MAP kinase and PI-3 kinase in mucin up-regulation has not yet been explored.

Because mucin overproduction plays an important role in the pathogenesis of COME and COPD, and NTHi is a major pathogen of these diseases, we hypothesize that NTHi up-regulates MUC5AC mucin transcription via activation of specific signal transduction pathways. Here, we show that previously unrecognized cytoplasmic protein components of NTHi up-regulate MUC5AC mucin gene transcription via a positive p38 MAP kinase pathway and a negative PI 3-kinase-Akt signaling pathway. These studies provide new insights into the molecular pathogenesis of NTHi infections and may open up novel targets for therapeutic intervention.

EXPERIMENTAL PROCEDURES

Reagents—SB203580, wortmannin, and LY294002 were purchased from Calbiochem (La Jolla, CA). NTHi lipooligosaccharides (LOS) were a gift from Dr. X. X. Gu (Laboratory of Immunology, NIDCD, National Institutes of Health, Bethesda, MD); Polymyxin B, lipopolysaccharides, and a gift from Dr. X. X. Gu (Laboratory of Immunology, NIDCD, National Institutes of Health, Bethesda, MD). Polymyxin B, lipopolysaccharides, protease inhibitor mixture for bacterial extracts, protease E, and DNase were purchased from Sigma. RNase was obtained from Promega (Madison, WI).

Bacterial Strains and Culture Conditions—NTHi strain 12 and all other NTHi strains used in the study were clinically isolated strains that were kindly provided by Dr. H. Faden (Children's Hospital of Buffalo, State University of New York, Buffalo, NY). The bacterial cells were grown in liquid brain-heart infusion supplemented with NAD and hemin at 37 °C with 5% CO2 as described (23, 24).

Cell Culture—The HeLa (human cervix epithelial) cells were cultured in minimal essential medium. HM3 (human colon epithelial) cells were maintained in Dulbecco's modified Eagle's medium. A549 (human lung epithelial) cells were maintained in F-12 nutrition mixture (Kallestad, modification). All media contained 10% fetal bovine serum (In Vitrogen), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). All cells were cultured in a humidified atmosphere of 5% CO2 and 95% air.

Preparation of NTHi Cytoplasmic Components—The bacterial cells were harvested when they reached middle to late log phase and resuspended in PBS with the same volume (1:10) or one third of the original volume (3:1). The bacterial cell suspension was sonicated on ice three times for 3 min with 5-min intervals between each sonication. Residual cells were removed by centrifugation (10,000 × g, 4 °C for 10 min). Cytoplasmic components were obtained from the supernatant of sonicated bacteria by ultracentrifugation (1,000,000 × g, 4 °C for 1 h), and stored at −80 °C.

RT-PCR Analysis of MUC5AC—Tissue culture dishes (10 cm in diameter) were seeded with 5 × 105 HeLa cells in a 10-ml volume of complete Dulbecco's modified Eagle's medium and incubated for 24 h. The cells were starved in serum-free medium for 18 h and then treated with or without NTHi in duplicate for 5 h. Total RNA was extracted from the lysed cells using an RNeasy mini kit (Qiagen Inc., Valencia, CA) following the manufacturer's instruction and treated with RNase-free DNase I. cDNAs were synthesized with Moloney murine leukemia virus RT (Superscript II, Life Sciences, Gaithersburg, MD) using random hexadeoxynucleotide as primers (Promega). After DNA synthesis, the RT was inactivated by heating the sample at 95 °C for 10 min.

MUC5AC cDNA was amplified with primers 5′-TCC GGC CTC ATC TTC TCC-3′ and 5′-ACT TGG GCA CTG GTC CAT CC-3′. PCR was performed for 15 min at 95 °C, 1 min at 94 °C, 1 min at 57 °C (50 °C for cyclrophilin), and 1 min at 72 °C for each cycle and 7 min at 72 °C after all the cycles. A cycle number that was in the linear range of amplification was selected for PCR analysis: 32 cycles for MUC5AC and 26 for cyclrophilin.

Plasmids, Transfection, and Luciferase Assays—Expression plasmids p38sot(AF) and p38sot(AF) (23, 25) have been described previously. The expression plasmids p110, pS8a (26), Akt KD, and wild-type Akt (27) were kindly provided by D. Stokoe (University of California, San Francisco, CA). The reporter construct MUC5AC contains 3.7-kb 5′-flanking region of the human MUC5AC mucin gene in a luciferase reporter vector pGL3 (18). Transient transfections of cells were performed out in triplicate with Trans IT-LL1 (Panvera, Madison, WI) following the manufacturer's instruction. Forty-two hours after transfection, the cells were treated with NTHi for 4 h and then harvested for luciferase assay. For experiments with inhibitors, HM3 cells stably transfected with MUC5AC-luciferase plasmid were pretreated with inhibitors for 1–2 h, then treated with NTHi for 4 h, and harvested for luciferase assay. Luciferase assays were performed on a Monolight 960 spectrophotometer for Luminescence, San Diego, CA. The NTHi-dependent -fold induction was calculated relative to the luciferase light units obtained in the absence of NTHi treatment. The normalized luciferase activity was thus expressed as relative luciferase activity (-fold induction).

Western Blot Analysis—HeLa and HM3 cells were treated with or without NTHi. Total cell lysates were analyzed by antibodies against phospho-p38 (Thr-180/182), p38, phospho-Akt (Ser-473), and Akt (New England Biolabs, Beverly, MA) as described following the manufacturer's instructions.

RESULTS

NTHi Up-regulates MUC5AC Mucin Gene Transcription—MUC5AC has been identified as a prominent mucin in respiratory secretions (28) and in middle ear effusions of COME (19, 29, 30). To determine the role of NTHi in mucin induction, we first examined MUC5AC mRNA in human epithelial cells treated with NTHi using RT-PCR. As shown in Fig. 1A, MUC5AC mRNA levels significantly increased when the cells were treated with NTHi for 5 h. To investigate whether transcriptional regulation is involved in MUC5AC induction, human epithelial cells including HeLa, HM3, and A549 were transfected with a MUC5AC promoter-luciferase reporter construct and treated with NTHi. The luciferase activity driven by the MUC5AC promoter indeed increased upon exposure to NTHi, suggesting the involvement of transcriptional regulation (Fig. 1B). To further determine whether other clinical isolates of NTHi strains can also up-regulate MUC5AC, we tested a variety of NTHi clinical isolates for MUC5AC-inducing activity. Interestingly, all clinical isolates tested were capable of inducing MUC5AC although their mucin-inducing activity differed quantitatively (Fig. 1C). This result suggests that the mucin-inducing activity of NTHi is well conserved among all 10 strains that were tested. Strain 12, the strain with the most potent MUC5AC-inducing activity, was used for further investigations.

Soluble Cytoplasmic Components of NTHi Play a Major Role in MUC5AC Induction—Having demonstrated that NTHi up-regulates MUC5AC transcription, we next sought to determine the bacterial components responsible for MUC5AC induction. Based on the fact that there has been a dramatic increase of COME cases after antibiotic was introduced as a treatment for otitis media, we postulated that bacterial breakdown components released from lysed bacteria may play an important role in mucin induction. To test our hypothesis, NTHi bacteria were first disrupted by sonication; the mucin-inducing activity of sonicated NTHi was then tested using MUC5AC promoter luciferase assay. As shown in Fig. 2A, NTHi whole bacteria induced modest levels of MUC5AC transcription. However, the mucin-inducing activity was greatly increased when NTHi bacteria were sonicated, indicating that bacterial cell lysis by sonication releases additional potent mucin inducers. To determine which fraction of sonicated NTHi lysates was responsible for the greatly increased activity, the sonicated bacterial lysate was further separated by centrifugation into a pellet, which contains membrane debris as well as residual whole cells, and soluble cytoplasmic fractions (SCF). The SCF fraction was even more potent than sonicated bacterial lysate whereas the activity in the pellet was low, similar to that in nonsonicated whole
bacteria. Because many bacteria are capable of secreting bioactive molecules into the environment, we evaluated the possibility that NTHi produced diffusible mucin inducers. No significant mucin-inducing activity was detected in bacterial culture supernatant (data not shown), suggesting that mucin inducers are not secreted by live intact bacteria.

We reported previously that lipopolysaccharide (LPS) from Gram-negative bacteria *P. aeruginosa* up-regulates MUC2 mucin transcription (31, 32). Like other Gram-negative bacteria, NTHi also contains LOS, although its LOS differs from LPS in other Gram-negative bacteria in the number of O-side chains (33). NTHi LOS has been shown to induce cytokine expression in epithelial cells (24). Because the NTHi cytoplasmic components may contain LOS, we therefore sought to determine whether LOS was involved in MUC5AC induction. When transfected epithelial cells were treated with LOS, no mucin-induction was detected (Fig. 2B). To corroborate this, the SCF was ultracentrifuged to further spin out bacterial envelope debris and was then pretreated with various concentrations of polymyxin B, which hinders LOS and would neutralize the biological activity of any remaining LOS (24, 33, 34). As shown in Fig. 2C, no significant reduction in NTHi-induced MUC5AC transcription occurred after polymyxin B treatment. Importantly, the potency of the polymyxin B was shown by the fact that it significantly reduced MUC5AC transcription induced by LPS from *Salmonella typhimurium* (Fig. 2D). These data indicate that, unlike NTHi induction of inflammatory cytokines (24) and *P. aeruginosa* induction of MUC2 (31), NTHi induction of MUC5AC does not require LOS.

In addition to LOS, NTHi surface membrane proteins have also been shown to play an important role in the pathogenesis of NTHi infections (4, 23, 35). To determine whether NTHi membrane proteins play an important role in MUC5AC induction, equivalent amounts of envelope proteins and cytoplasmic components were compared for their mucin inducing activity. As shown in Fig. 3A, NTHi cytoplasmic components induced MUC5AC transcription to a much greater degree than envelope proteins. To further verify that the MUC5AC-inducing activity indeed resides in the cytoplasmic fraction rather than being because of an effect of sonication on membrane proteins, the bacteria were disrupted in a French pressure cell, which has been commonly used as an alternative way to completely disrupt bacteria. The cytoplasmic components were separated from the envelope proteins using centrifugation, and their MUC5AC-inducing activity was then assessed. Consistent with the envelope and cytoplasmic components prepared by sonication, NTHi cytoplasmic components prepared using French pressure cell also strongly up-

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** NTHi up-regulates MUC5AC mucin gene transcription. **A**, NTHi up-regulates MUC5AC expression at mRNA level. HeLa (human cervix epithelial) cells were treated with or without NTHi soluble cytoplasmic components in duplicate for 5 h. RT-PCR was then performed to measure the changes in steady-state mRNA levels. Cyclophilin served as a control for the amount of RNA used in each reaction. Similar results were also observed in HM3 (human colon epithelial) cells. Data represent four independent experiments. **B**, NTHi up-regulates MUC5AC transcription in human epithelial cells. A 3.7-kb DNA fragment of the 5′-flanking region of the human MUC5AC mucin gene cloned into a luciferase reporter vector (pMUC5AC3.7luc) was transfected into HeLa, HM3, and A549 (human airway epithelial) cells. Luciferase activity was then assessed in NTHi soluble cytoplasmic components-treated and nontreated cells. Induction by NTHi was detected in all cell lines. **C**, all clinically isolated NTHi strains tested are capable of inducing MUC5AC transcription. HM3 cells stably transfected with pMUC5AC3.7luc were exposed to soluble cytoplasmic components from various NTHi strains as indicated for 4 h. Luciferase activity was then assessed in NTHi-treated and untreated cells. All transfections and luciferase assays were carried out in triplicate. Values represent means ± S.D. (n = 2).
Regulate MUC5AC transcription, whereas the whole bacteria and membrane proteins induced MUC5AC up-regulation to a much lesser extent (Fig. 3B). Therefore, we conclude that the cytoplasmic components of NTHi play a major role in NTHi-induced MUC5AC transcription.

bacteria and various fractions from NTHi as indicated for 4 h. Luciferase activity was then assessed in NTHi-treated and untreated cells. WB, whole intact NTHi bacteria in PBS; SB, sonicated NTHi bacteria in PBS; SCF, soluble cytoplasmic fraction of sonicated bacteria after centrifugation at 10,000 g for 10 min; P, pellet of sonicated bacteria after centrifugation. NTHi LOS does not induce MUC5AC transcription.

HM3 cells stably transfected with pMUC5AC3.7luc were treated with various concentrations of NTHi LOS as indicated for 4 h before being lysed for luciferase assay. C, polymyxin B treatment does not attenuate up-regulation of MUC5AC induced by NTHi soluble cytoplasmic components. NTHi SCF were pretreated with various concentrations of polymyxin B for 10 min before being added to HM3 cells stably transfected with pMUC5AC3.7luc. D, polymyxin B significantly reduced MUC5AC induction by LPS from S. typhimurium. LPS was pretreated with various concentrations of polymyxin B for 10 min at 4 °C and was then added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before being lysed for luciferase assay. All luciferase assays were carried out in triplicate. Values represent means ± S.D. (n = 3).

Fig. 2. Non-LOS molecules released from lysed NTHi by sonication are responsible for the potent MUC5AC-inducing activity. A, effects of various NTHi fractions on MUC5AC induction. HM3 cells stably transfected with pMUC5AC3.7luc were exposed to whole NTHi SCF and envelope proteins. Envelope proteins were separated from the cytoplasmic components by ultracentrifugation of sonicated NTHi. The cytoplasmic and envelope fractions were then added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before luciferase assay. B, a similar potent MUC5AC-inducing activity was also observed in the cytoplasmic components, which were prepared from the disrupted NTHi using French pressure cell, an alternative approach to completely disrupt the bacterial cells. NTHi cells were disrupted using French pressure cell at 1,000 p.s.i. The cytoplasmic components were separated from the envelope components by centrifugation at 10,000 × g at 4 °C for 10 min followed by ultracentrifugation at 1,000,000 × g at 4 °C for 1 h. After centrifugation, the pellet (envelope components) and the cytoplasmic components were added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before being lysed for luciferase assay. Whole Bacteria, NTHi whole bacterial cells; Cyto, cytoplasmic components; EP, envelope proteins. All luciferase assays were carried out in triplicate. Values represent means ± S.D. (n = 3).

Fig. 3. Cytoplasmic components of NTHi play a major role in MUC5AC induction. A, the MUC5AC-inducing activity of the cytoplasmic components of NTHi is much more potent than that of NTHi envelope proteins. Envelope proteins were separated from the cytoplasmic components by ultracentrifugation of sonicated NTHi. The cytoplasmic and envelope fractions were then added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before luciferase assay. B, a similar potent MUC5AC-inducing activity was also observed in the cytoplasmic components, which were prepared from the disrupted NTHi using French pressure cell, an alternative approach to completely disrupt the bacterial cells. NTHi cells were disrupted using French pressure cell at 1,000 p.s.i. The cytoplasmic components were separated from the envelope components by centrifugation at 10,000 × g at 4 °C for 10 min followed by ultracentrifugation at 1,000,000 × g at 4 °C for 1 h. After centrifugation, the pellet (envelope components) and the cytoplasmic components were added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before being lysed for luciferase assay. Whole Bacteria, NTHi whole bacterial cells; Cyto, cytoplasmic components; EP, envelope proteins. All luciferase assays were carried out in triplicate. Values represent means ± S.D. (n = 3).
Proteins Are the Major NTHi Cytoplasmic Components Responsible for MUC5AC Induction—The NTHi cytoplasmic content is a complex mixture containing mainly nucleic acids and proteins. In an effort to better define the mucin inducer, the cytoplasmic fraction was first pretreated with DNase or RNase. Complete digestion of nucleic acids was confirmed by electrophoresis (data not shown). As shown in Fig. 4A, neither DNase nor RNase reduced MUC5AC transcription. NTHi SCF were pretreated with either DNase (94 μg/ml) or RNase (50 μg/ml) or buffer alone overnight, and were then added to HM3 cells stably transfected with pMUC5AC3.7luc for 4 h before being lysed for luciferase assay. B, proteins are the major MUC5AC inducers in NTHi soluble cytoplasmic components. The soluble cytoplasmic components were boiled at 100 °C for 5 min (Heat) or incubated at 37 °C overnight in the presence or absence of protease inhibitor mixture (PI) (1.3 mg/ml). For PE and PBS groups, aliquots of incubation-incubated samples without protease inhibitor were further treated with protease E (PE) (300 μg/ml) or PBS alone as a control for another 2 h at 37 °C before being lysed for luciferase assay. All luciferase assays were carried out in triplicate in HM3 cells stably transfected with pMUC5AC3.7luc. Values represent means ± S.D. (n = 3).
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FIG. 5. Activation of p38 MAP kinase is required for NTHi-induced MUC5AC transcription. A, NTHi SCF induces p38 MAP kinase phosphorylation in HM3 cells. B, SB203580, a specific inhibitor for p38 MAP kinase, attenuates NTHi SCF-induced MUC5AC transcription in a dose-dependent manner. HM3 cells stably transfected with pMUC5AC3.7lu were pretreated with SB203580 for 1 h and were then treated with NTHi SCF for 4 h before being lysed for luciferase assay. C, overexpression of a dominant-negative mutant of either p38α or p38β inhibits NTHi-induced MUC5AC transcription. A dominant-negative mutant of either p38α (p38α DN) or p38β (p38β DN) was transiently co-transfected into HM3 cells with pMUC5AC3.7lu. After 42 h, the transfected cells were treated with or without NTHi SCF for 4 h. The cells were then lysed and assayed for luciferase activity. An empty vector served as a control. All transfections and luciferase assays were carried out in triplicate. Values represent means ± S.D. (n = 3).

Next, we sought to identify the downstream target of PI 3-kinase involved in NTHi-induced MUC5AC transcription. Because Akt represents one of the most important signaling molecules downstream of PI 3-kinase (21, 27), it is therefore reasonable to first determine the potential involvement of Akt. We performed Western blot analysis to determine whether NTHi activates Akt. As shown in Fig. 7A (upper panel), phosphorylation of Akt significantly increased after 5 min of treatment with NTHi SCF. The phosphorylation of Akt peaked at 30 min and then declined to the basal level at 5 h after treatment. This finding suggests that, in addition to p38, NTHi SCF also activates Akt. Because we have shown in Fig. 2A that other NTHi fractions are also capable of inducing MUC5AC transcription, we therefore tested these fractions for their ability to activate Akt. Interestingly, most treatments including the whole bacteria induced Akt phosphorylation although their Akt-inducing activity differed quantitatively (Fig. 7A, lower panel). We next sought to determine the involvement of Akt in NTHi-induced MUC5AC transcription. As shown in Fig. 7B, overexpression of a dominant-negative mutant of Akt (Akt KD) enhanced, whereas overexpression of a wild-type Akt reduced, the MUC5AC induction. These results indicate that Akt is also negatively involved in NTHi-induced MUC5AC transcription. Because PI 3-kinase is not the only upstream kinase of Akt, we therefore determined the effect of wortmannin on NTHi-induced Akt phosphorylation to establish the link between the PI 3-kinase and Akt. As shown in Fig. 7C, wortmannin abrogated Akt phosphorylation induced by NTHi cytoplasmic proteins, indicating that Akt indeed acts downstream of PI 3-kinase in response to NTHi.

Having identified p38 MAP kinase as a positive pathway and PI 3-kinase-Akt as a negative pathway involved in NTHi-induced MUC5AC transcription, still unknown is whether or not there is a negative cross-talk between these two signaling pathways. Based on a recent report that inhibition of PI 3-kinase-Akt signaling led to enhanced vascular endothelial growth factor activation of p38 MAP kinase (36), we studied the effect of wortmannin on the phosphorylation state of p38 MAP kinase induced by NTHi. Fig. 7D shows that pretreatment of HM3 cells with wortmannin greatly enhanced phosphorylation of p38 induced by NTHi. To determine whether the activation of PI 3-kinase-Akt pathway may lead to down-regulation of NTHi-induced p38 MAP kinase phosphorylation, an activated, membrane-targeted form of p110 (p110-CαAAAX) was transfected into HM3 cells. As shown in Fig. 7E, NTHi-induced phosphorylation of p38 MAP kinase was attenuated by overexpression of p110-CαAAAX, indicating that activation of PI 3-kinase-Akt indeed leads to down-regulation of p38 MAP kinase phosphorylation induced by NTHi. To further determine whether PI 3-kinase-Akt pathway can bypass the p38 MAP kinase pathway to down-regulate MUC5AC transcription, we first pretreated the cells with SB203580, a specific inhibitor for p38 MAP kinase, and then pretreated the cells with wortmannin, a specific inhibitor for PI 3-kinase, or vice versa, before NTHi was added to the cells. As shown in Fig. 7F, wortmannin no longer enhanced NTHi-induced MUC5AC transcription in the cells that were already pretreated with SB203580, whereas SB203580 was still capable of inhibiting NTHi-induced MUC5AC transcription in the cells that were already pretreated with wortmannin. Taken together, these results demonstrated that activation of PI3-kinase-Akt signaling pathway leads to attenuation of p38 MAP kinase phosphorylation. Thus, it is clear that PI 3-kinase-Akt serves as an inhibitory signaling pathway in NTHi-induced MUC5AC transcription via a negative cross-talk with p38 MAP kinase pathway.

DISCUSSION

NTHi has now become well established as an important human pathogen in both children and adults. In children, it causes COME, one of the most common childhood infections and the leading cause of conductive hearing loss in children (1–4). In adults, it causes lower respiratory tract infections in the setting of COPD, the fourth leading cause of patient death in the United States (5). Mucin overproduction, a hallmark of both diseases, has been shown to directly cause conductive hearing loss in COME and airway obstruction in COPD. The
molecular mechanisms by which mucin is up-regulated in NTHi infections still remain poorly understood. In the present study, we performed experiments to determine the involvement of NTHi in up-regulation of MUC5AC mucin gene transcription in human epithelial cells. Here, we show that NTHi cytoplasmic proteins up-regulate MUC5AC transcription via a positive p38 MAP kinase signaling pathway and a negative PI 3-kinase-Akt signaling pathway (Fig. 8).

A major finding in this study is the experimental evidence for the involvement of bacterial cytoplasmic proteins in MUC5AC induction. This result, although rather unexpected, may well explain why many patients still have persistent symptoms such as middle ear effusion in COME even after intensive treatment with antibiotics (37). One of the major characteristics of NTHi is its tendency to autolyze. Its autolysis can be triggered in vitro when the bacteria culture is old, and in vivo under various conditions including antibiotic treatment. Clinical microbiology studies have shown that most effusions from the patients with COME were negative on bacteria culture, whereas bacterial DNA could be detected by PCR in 80% of effusions, often in the absence of viable bacteria on culture (30). In addition, DeMaria et al. (38) reported that endotoxin was present in 67% of middle ear effusions that were negative as determined by culture for any bacterium. Despite some potential underestimation of the prevalence of viable bacteria by conventional culture, these results clearly indicate that bacterial breakdown products or components released from lysed bacteria persist in the middle ear even after bacteria die and thus may act as long lasting stimuli of mucin production and inflammatory responses (37). Taken together, our present study and the previous findings suggest that the cytoplasmic proteins released from the lysed NTHi bacteria after treatment with antibiotics may contribute substantially to the pathogenesis of otitis media by directly up-regulating MUC5AC mucin transcription.

Another unexpected finding in this study is the negative effect of NTHi LOS on MUC5AC transcription. We previously showed that LPS from other Gram-negative bacteria such as Pseudomonas aeruginosa and S. typhimurium up-regulates MUC2 and MUC5AC transcription (31, 39). Additionally, induction of proinflammatory cytokines by NTHi LOS has also been reported (24). Based on these studies, we initially expected to observe a stimulating effect of LOS on MUC5AC. The negative effect shown in Fig. 2 (B and C) is unexpected, because it was in sharp contrast to the up-regulation of mucin by LPS from S. typhimurium and P. aeruginosa. In comparison with LPS, LOS lacks an O-specific polysaccharide (33). Therefore it seems logical that the lack of O-specific polysaccharide may...
account for the negative effect on MUC5AC induction. However, this notion is not supported by the fact that LPS molecules purified from a polysaccharide-deficient strain and a wild-type strain of \textit{P. aeruginosa} were equipotent in induction of MUC2 (31), suggesting that lipid A and the sugar core region are sufficient for mucin induction. In view of the structure of other regions, LOS also appears to differ from LPS in lipid A (33). An antigenic analysis of NTHi lipid A by Apicella \textit{et al.} (40) showed that a monoclonal antibody specific for the lipid A portion of NTHi LOS recognized the lipid A determinant on most NTHi strains but did not recognize the lipid A of 39 stains from 14 non-\textit{H. influenzae} species. Thus, differences in the lipid A region between NTHi LOS and other bacterial LPS may be responsible for the difference in mucin induction. Although no direct up-regulation on MUC5AC by NTHi LOS was shown in \textit{vitro}, our data do not preclude the possibility that LOS may indirectly up-regulate MUC5AC \textit{in vivo} by inducing cytokines such as TNF-\alpha, which has been shown to up-regulate mucin (15).

In the present study, we provided evidence for the first time that activation of p38 MAP kinase is required for up-regulation of MUC5AC by NTHi cytoplasmic protein(s). In addition, we showed that PI 3-kinase-Akt signaling pathway is also activated by NTHi, which, however, leads to down-regulation of MUC5AC.
pathogenesis of NTHi-induced infections and lead to novel therapeutic intervention for COME and COPD.

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