cAMP-dependent protein kinase A acts as a negative regulator for nontypeable *Haemophilus influenzae*-induced GM-CSF expression via inhibition of MEK-ERK signaling pathway

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Abstract

Otitis media (OM) is the most common bacterial infection in children, and often leads to conductive hearing loss. The Gram-negative bacterial pathogen, nontypeable *Haemophilus influenzae* (NTHi) is one of the primary causative agents. A classic hallmark of NTHi-induced OM is inflammation in middle ear. While appropriate inflammation is critical for host defense, an overactive response can often be detrimental to the host. Thus, inflammation must be tightly controlled. Here, we show that NTHi up-regulates the expression of granulocyte macrophage-colony stimulating factor (GM-CSF), one of the major proinflammatory mediator, via MEK-ERK signaling pathway in human middle ear epithelial cells. Moreover, cAMP-dependent protein kinase A (PKA) negatively regulates NTHi-induced GM-CSF expression through inhibition of MEK-ERK signaling pathway. Collectively, our studies unveil a new mechanism underlying the tight regulation of GM-CSF expression via a negative cross-talk between cAMP-PKA and MEK-ERK pathways and may shed light on developing new anti-inflammatory strategies.

Introduction

Otitis media (OM) is the most common pediatric infectious disease. Since this disease often leads to conductive hearing loss during the crucial period of speech and language development, children with OM may suffer speech and language disabilities [1,2]. Nontypeable *Haemophilus influenzae* (NTHi) represents one of the most common Gram-negative bacterial pathogen causing OM [3]. Antibiotics are frequently used for the treatment of OM. However, inappropriate antibiotic treatment contributes significantly to the increased multidrug-resistant strains [4,5]. Thus, development of new therapeutic strategies is needed for the treatment of OM based on understanding of its molecular pathogenesis.

An excessive inflammation caused by bacterial infection is a hallmark of OM, characterized by up-regulated proinflammatory mediators [6,7]. Inflammation plays an important role in host defense against bacteria. However, if uncontrolled, excessive inflammation often causes tissue damage and immunopathology such as OM [8,9]. Thus, inflammation must be tightly regulated. Among various proinflammatory mediators, granulocyte macrophage colony-stimulating factor (GM-CSF) plays a critical role in stimulating proliferation and differentiation of granulocytes and macrophages from progenitor cells, and is thus a critical proinflammatory mediator for the host response to infection [10]. The suppression of lipopolysaccharide (LPS)-induced inflammation was observed in GM-CSF-deficient mice [11]. Consistent with this finding, GM-CSF administration in mice led to enhanced up-regulation of proinflammatory cytokines in response to LPS and tumor necrosis factor-α (TNF-α) [12]. Moreover, GM-CSF is identified in middle ear effusion from OM patients [13]. However,

the molecular mechanisms underlying the tight regulation of GM-CSF induction by NTHi remain largely unknown. Understanding the regulatory mechanism of NTHi-induced GM-CSF expression in middle ear may lead to developing new therapeutic strategies for OM.

Cyclic adenosine monophosphate (cAMP) is a second messenger that has been shown to regulate inflammation and immune response [14-18]. Previous studies have shown that cAMP regulates multiple signal pathways via a major downstream effector protein kinase A (PKA) [15]. Increased intracellular cAMP levels in LPS-treated macrophages results in a suppression of proinflammatory cytokines such as TNF-α and IL-1β [19-21]. Thus, activating cAMP signaling may represent an interesting therapeutic strategy to suppress inflammation. Indeed, increasing intracellular cAMP by using phosphodiesterase 4B (PDE4B) inhibitor roflumilast has been recently approved for treating severe chronic obstructive pulmonary disease (COPD) [22]. The role of cAMP-dependent PKA signaling pathway in NTHi-induced GM-CSF expression in middle ear has yet to be investigated.

In the present study, we investigated the role and underlying

molecular mechanism of cAMP- PKA pathway in NTHi-induced GM-CSF expression in human middle ear epithelial cells. We found that NTHi induces GM-CSF expression via MEK-ERK signaling pathway. We further showed that elevated cAMP suppresses NTHi-induced GM-CSF expression through inhibition of ERK activation. Moreover, we demonstrated that cAMP-dependent PKA is involved in negatively regulating NTHi-induced GM-CSF expression. Thus, our study provides the direct evidence for the first time for the negative regulation of GM-CSF induction by NTHi via cAMP-dependent PKA signaling pathway and may help develop new anti-inflammatory therapeutic strategies.

Materials and methods

Reagents and antibodies

PD98059, U0126, forskolin, and H89 were purchased from Enzo Life Sciences. N^6-Phenyl-cAMP (6-Phe-cAMP) was purchased from BioLog. Antibodies for phospho-ERK1/2 (#9101) and total ERK1/2 (#9102) were purchased from Cell Signaling.

Bacterial strains and culture condition

Clinical isolates of NTHi strain 12, 2627, and 9274 were used in this study. NTHi were grown on chocolate agar plate at 37°C in an atmosphere of 5% CO_2 overnight, and inoculated in brain heart infusion (BHI) broth supplemented with 3.5 μg/ml NAD and 10 μg/ml hemoglobin (BD Biosciences). After overnight incubation, bacteria were subcultured into fresh BHI and the log phase NTHi, monitored by measurement of optical density value, was washed and suspended in phosphate-buffered saline (PBS) for *in vitro* cell experiments and for *in vivo* animal experiments.

Cell culture

All media described below were supplemented with 10% (vol/vol) FBS (Sigma-Aldrich). Human middle ear epithelial HMEEC-1 cells were maintained in DMEM (Cellgro) supplemented with BEGM SingleQuots (Lonza). Human lung epithelial A549 cells were maintained in F-12K medium (Gibco). Human cervical epithelial HeLa cells were maintained in MEM medium (Cellgro).

Real-time quantitative RT-PCR analysis

Total RNA was isolated with TRIzol reagent (Life Technologies) by following the manufacturer's instruction. For the reverse transcription reaction, TaqMan reverse transcription reagents (Life Technologies) were used as described previously [23,24]. For quantitative RT-PCR analysis, PCR amplifications were performed by using SYBR Green Universal Master Mix (Life Technologies). In brief, the reactions were performed in triplicate containing 2× Universal Master Mix, 1 μl of template cDNA, 200 nM primers in a final volume of 12.5 μl and they were analyzed in a 96-well optical reaction plate (USA Scientific). Reactions were amplified and quantified by using a StepOnePlus Real-Time PCR System and the manufacturer's corresponding software (StepOnePlus Software v2.3; Life Technologies). The relative quantities of mRNAs were determined by using the comparative Ct method and were normalized by using human cyclophilin for *in vitro* or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for *in vivo* as an endogenous control. The primers for human GM-CSF and cyclophilin were described previously [25]. The primer sequences for mouse GM-CSF and GAPDH are as follows: mouse GM-CSF (Forward 5'-ATGCCTGTCACGTTGAATGAAG-3' and Reverse 5'-GCGGGTCTGCACACATGTTA-3') and mouse GAPDH

(Forward 5'-ACCCAGAAGACTGTGGATGG-3' and Reverse 5'-GGATGCAGGGATGATGTTCT-3'.)

Plasmids and transfections

The expression plasmid of a constitutively active form of MEK (MEK CA) was described previously [26]. All transient transfections were carried out using TransIT-2020 reagent (Mirus) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Cells were stimulated with NTHi for 12 h. Cell culture supernatants were harvested and centrifuged at 15,000 × g for 10 min to remove debris prior to analysis. Human GM-CSF protein was measured with PeproTech Human GM-CSF ELISA Development kit (PeproTech) according to the manufacturer's instructions.

Western blot

Western blots were performed using whole-cell extracts in the lysis buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM $Na_4P_2O_7$, 30 mM NaF, 5 μM $ZnCl_2$, 2 mM Iodoacetic acid, 1% Triton-X, supplemented with 1 mM Na_3VO_4 and PIC), separated on 10% (wt/vol) SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences). The membrane was blocked with 5% (wt/vol) non-fat dry milk in a solution of TBS containing 0.1% Tween 20 (TBS-T). The membrane was then incubated in a 1:1,000 dilution of a primary antibody in 5% (wt/vol) non-fat dry milk-TBS-T. After three washes in TBS-T, the membrane was incubated with 1:5,000 dilution of the corresponding secondary antibody in 5% (wt/vol) non-fat dry milk-TBS-T. Respective proteins were visualized by using Amersham ECL Prime Regent (GE Healthcare Life Sciences).

Mice and animal experiments

C57BL/6J mice (10-12 weeks old) were anesthetized and transtympanically inoculated with $1×10^7$ colony-forming units (c.f.u.) of NTHi, and PBS was inoculated as control. The inoculated mice were then sacrificed 5 h after NTHi inoculation. Dissected mouse middle ears were subjected to total RNA extraction. All animal experiments were carried out in accordance with guidelines of, and were approved by, the Institutional Animal Care and Use Committee (IACUC) at Georgia State University.

Statistical analysis

All experiments were repeated at least three times. Data are shown as mean ± SD. Statistical analysis was analyzed by performing unpaired two-tailed Student's *t*-test. *P<0.05* was considered statistically significant.

Results and discussion

NTHi induces GM-CSF expression in human epithelial cells *in vitro* and mouse middle ear *in vivo*

We have recently reported that NTHi, a major human pathogen of OM and exacerbation of COPD, induces up-regulation of GM-CSF expression in human bronchial epithelial BEAS-2B cells [25,27]. However, the molecular mechanisms underlying the negative regulation of induction of GM-CSF by NTHi remain largely unknown. We first examined whether NTHi induces GM-CSF expression in a number of human epithelial cell lines by performing quantitative PCR (Q-PCR) analysis. NTHi induced GM-CSF expression at mRNA level in human middle ear epithelial HMEEC-1 cells (Figure 1A), human lung
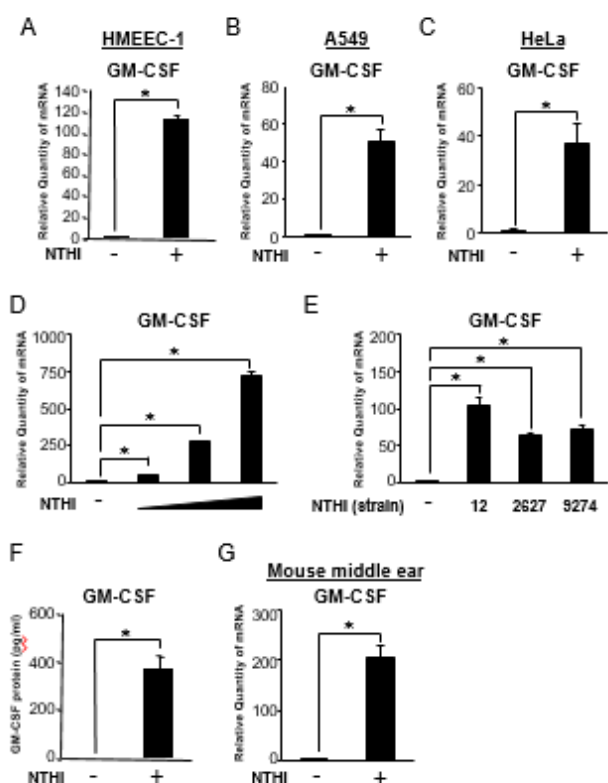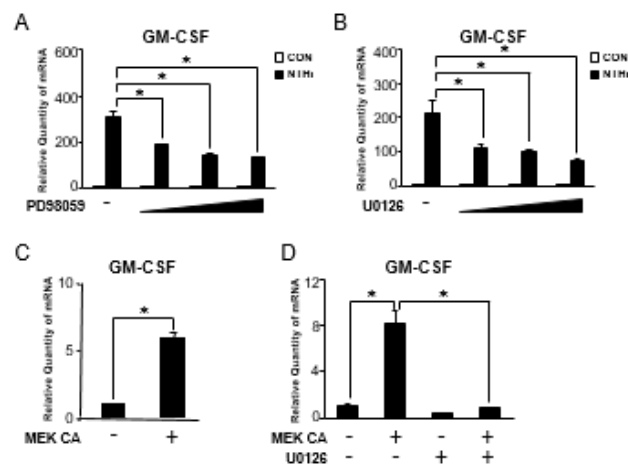
Figure 1. NTHi induces GM-CSF expression in human epithelial cells *in vitro* and mouse middle ear *in vivo*. (A, B and C) HMEEC-1 (A), A549 (B), and HeLa (C) cells were stimulated with NTHi for 5h, and GM-CSF mRNA expression was analyzed by Q-PCR. (D) HMEEC-1 cells were stimulated with increasing doses of NTHi for 5h, and GM-CSF mRNA expression was analyzed by Q-PCR. (E) HMEEC-1 cells were stimulated with various strains of NTHi (12, 2627 and 9274) for 5h, and GM-CSF mRNA expression was analyzed by Q-PCR. (F) HMEEC-1 cells were stimulated with NTHi for 12h, and the protein level of GM-CSF in supernatants was analyzed by enzyme-linked immunosorbent assay. (G) C57BL/6J mice were transtympanically inoculated with NTHi (1×10^7 c.f.u. per ear) for 5h, and GM-CSF mRNA expression was analyzed by Q-PCR. Data are mean ± SD (n = 3); *P < 0.05.

epithelial A549 cells (Figure 1B), and human cervical epithelial HeLa cells (Figure 1C). Additionally, we determined that NTHi induced GM-CSF expression in a dose-dependent manner (Figure 1D). To further determine the generalizability of our finding, we tested several NTHi strains for GM-CSF mRNA induction [28]. NTHi strain 2627 and 9274 also induced GM-CSF expression at mRNA level in HMEEC-1 cells (Figure 1E). Furthermore, NTHi also induced the expression of GM-CSF at protein level in HMEEC-1 cells (Figure 1F). Consistent with the *in vitro* data, NTHi also enhanced GM-CSF expression at mRNA level in the mouse middle ear (Figure 1G). Together, these data demonstrate that NTHi induces GM-CSF expression at both mRNA and protein levels in human epithelial cells *in vitro* and mouse middle ear *in vivo*.

MEK-ERK signaling is required for NTHi-induced GM-CSF expression in human middle ear epithelial cells

Because we previously demonstrated that the MAP kinase ERK is critical for the regulation of NTHi-induced inflammatory response [26,29], we first examined the effects of PD98059 and U0126, specific ERK inhibitors, on NTHi-induced GM-CSF expression by performing Q-PCR analysis. As shown in Figure 2A and 2B, ERK inhibitors significantly suppressed the enhancement of NTHi-induced GM-CSF
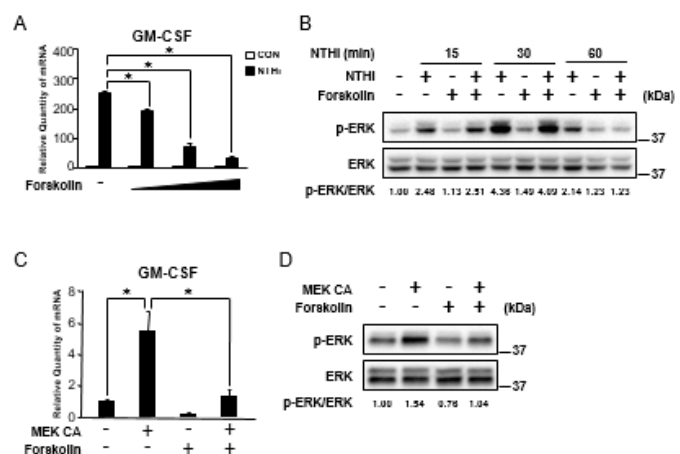

Figure 2. MEK-ERK signaling pathway is required for NTHi-induced GM-CSF expression in human middle ear epithelial cells. (A) HMEEC-1 cells were pretreated with PD98059 (2.5, 5, and 10 μM) for 1 h followed by NTHi stimulation for 5 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (B) HMEEC-1 cells were pretreated with U0126 (1, 5, and 10 μM) for 1 h followed by NTHi stimulation for 5 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (C) HMEEC-1 cells were transfected with a constitutively active form of MEK (MEK CA) for 24 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (D) HMEEC-1 cells transfected with MEK CA for 24 h were treated with U0126 (10 μM) for 2 h, and GM-CSF mRNA expression was analyzed by Q-PCR. Data are mean ± SD (n = 3); *P < 0.05.

expression at mRNA level in a dose-dependent manner, indicating that activation of ERK is required for NTHi induced GM-CSF expression. We next sought to determine if activation of ERK is also sufficient GM-CSF induction by expressing a constitutively active form of MEK (MEK CA), which phosphorylates ERK and results in the activation of ERK. As expected, the expression of GM-CSF was markedly induced by expressing MEK CA in HMEEC-1 cells (Figure 2C). In addition, ERK inhibitor U0126 also markedly suppressed MEK CA-induced GM-CSF expression (Figure 2D). Taken together, these results suggest that MEK-ERK signaling is required for NTHi-induced GM-CSF expression in human middle ear epithelial cells.

Elevated cAMP suppresses NTHi-induced GM-CSF expression via inhibition of ERK in human middle ear epithelial cells

cAMP, a key regulator of inflammatory and immune responses, has long been thought as a promising therapeutic target for inflammation [22,30]. Therefore, we first sought to determine the effect of cAMP in NTHi-induced GM-CSF expression by using forskolin, a potent activator of adenylate cyclase, which leads to an increase in the intracellular cAMP in human middle ear epithelial cells [31]. As shown in Figure 3A, forskolin significantly suppressed NTHi-induced GM-CSF expression in a dose-dependent manner in HMEEC-1 cells. Next, we sought to determine if cAMP elevated by forskolin regulates NTHi-induced ERK phosphorylation by performing western blot analysis. NTHi-induced ERK phosphorylation was markedly suppressed by forskolin in HMEEC-1 cells (Figure 3B). We further confirmed if forskolin can also suppress MEK CA-induced GM-CSF expression and ERK phosphorylation. As shown in Figure 3C and 3D, MEK CA-induced GM-CSF expression and ERK phosphorylation were significantly suppressed by forskolin. Together, these data demonstrate that elevated cAMP suppresses NTHi-induced GM-CSF expression via inhibition of ERK in human middle ear epithelial cells.
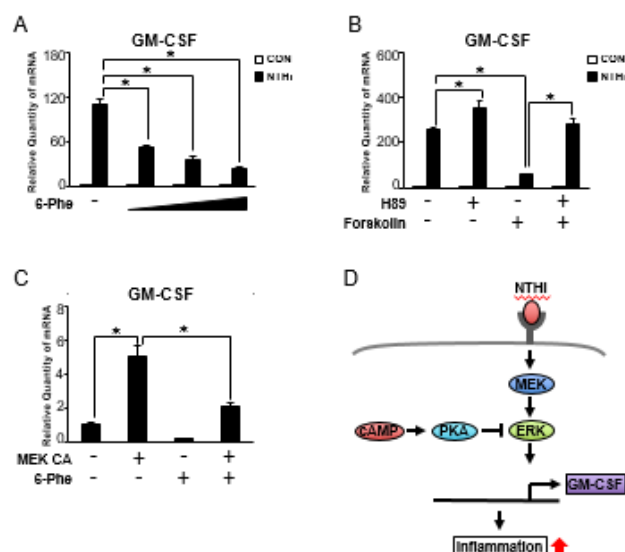
Figure 3. Elevated cAMP suppresses NTHi-induced GM-CSF expression via inhibition of ERK in human middle ear epithelial cells. (A) HMEEC-1 cells were pretreated with forskolin (0.1, 1, and 10 μM) for 1 h followed by NTHi stimulation for 5 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (B) HMEEC-1 cells were pretreated with forskolin (1 μM) for 1 h followed by NTHi stimulation for indicated time, and cell lysates were analyzed by immunoblotting with the indicated antibodies. The fold change of phosphorylated ERK was quantified by densitometry and normalized to total ERK. (C) HMEEC-1 cells transfected with MEK CA for 24 h were treated with forskolin (1 μM) for 2 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (D) HMEEC-1 cells transfected with MEK CA for 24 h were treated with forskolin (1 μM) for 2 h, and cell lysates were analyzed by immunoblotting with the indicated antibodies. The fold change of phosphorylated ERK was quantified by densitometry and normalized to total ERK. Data in A and C are mean ± SD (n = 3); *$P < 0.05$.

PKA negatively regulates NTHi-induced GM-CSF expression via inhibition of MEK-ERK signaling pathway in human middle ear epithelial cells

Having shown that cAMP is involved in NTHi-induced GM-CSF expression in HMEEC-1 cells, we next investigated the role of the cAMP effector PKA [15]. As shown in Figure 4A, a selective PKA activator (6-Phe-cAMP) significantly suppressed NTHi-induced GM-CSF expression in a dose-dependent manner. This data indicates that cAMP-dependent activation of PKA is involved in the negative regulation of NTHi-induced GM-CSF expression. We further evaluated the involvement of PKA in NTHi-induced GM-CSF expression in HMEEC-1 cells treated with a selective PKA inhibitor (H89) and forskolin. As shown in Figure 4B, inhibition of NTHi-induced GM-CSF expression by forskolin was markedly reversed by H89. Next, we determined whether MEK CA-induced GM-CSF expression is regulated by PKA. As shown in Figure 4C, MEK CA-induced GM-CSF expression was significantly suppressed by PKA activator 6-Phe-cAMP. Taken together, PKA negatively regulates NTHi-induced GM-CSF expression via inhibition of MEK-ERK signaling pathway in human middle ear epithelial cells (Figure 4D).

In summary, our study demonstrates for the first time that cAMP-dependent PKA pathway acts as a negative regulator of MEK/ERK-mediated GM-CSF induction by NTHi. This finding is of particular translational interest as the MEK-ERK signaling pathway has long been thought as a promising therapeutic target. Understanding the intrinsic link between cAMP and ERK activation provides new therapeutic potentials to diseases in which ERK signaling is activated. It is important to note, however, that due to the critical role that the MEK-ERK signaling pathway plays on apoptosis and cell growth, extended manipulation of this pathway could lead to unwanted side effects. Therefore, treatments involving the manipulation of the MEK-ERK

Figure 4. PKA negatively regulates NTHi-induced GM-CSF expression via inhibition of MEK-ERK signaling pathway in human middle ear epithelial cells. (A) HMEEC-1 cells were pretreated with 6-Phe-cAMP (6-Phe, 0.1, 0.3, and 0.5 mM) for 1 h followed by NTHi stimulation for 5 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (B) HMEEC-1 cells were pretreated with H89 (20 μM) and forskolin (1 μM) for 1 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (C) HMEEC-1 cells transfected with MEK CA for 24 h were treated with 6-Phe-cAMP (0.5 mM) for 2 h, and GM-CSF mRNA expression was analyzed by Q-PCR. (D) A schematic diagram illustrating that cAMP-dependent PKA negatively regulates NTHi-induced GM-CSF expression via inhibition of MEK-ERK signaling pathway. Data are mean ± SD (n = 3); *$P < 0.05$.

signaling pathway should be limited to short term usage. Future studies may provide a unique understanding of the nuances of NTHi-induced inflammation and provide direction in developing novel therapeutic strategies. Not only will this study provide critical understanding in controlling the immune response to a bacterial infection, but also its implications could be applied to other infectious and inflammatory diseases.

Conflicts of interest

The authors declare no conflict of interest.

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