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Glucocorticoids inhibit nontypeable *Haemophilus influenzae*-induced MUC5AC mucin expression via MAPK phosphatase-1-dependent inhibition of p38 MAPK

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ABSTRACT

Glucocorticoids are highly effective in the control of many inflammatory and immune diseases. Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, the molecular basis for the inhibitory effect of glucocorticoids on mucin overproduction, a hallmark of chronic respiratory diseases, still remains unclear. Here we show that glucocorticoids markedly inhibit up-regulation of *MUC5AC* induced by NTHi, a major human bacterial pathogen causing chronic obstructive pulmonary disease and otitis media. Inhibition of NTHi-induced *MUC5AC* expression by dexamethasone occurs at the level of p38 MAPK via glucocorticoid receptor. Moreover, glucocorticoids up-regulate MKP-1 expression, which in turn leads to p38 dephosphorylation and the subsequent inhibition of NTHi-induced *MUC5AC* expression. These studies provide new insight into the molecular mechanism underlying glucocorticoid therapy and may lead to novel therapeutic intervention for inhibiting mucin overproduction in patients with NTHi infections.

Mucus overproduction is a hallmark of chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD) and otitis media (OM). Excessive production of mucus, mainly results from up-regulation of mucins, leads to airway mucus obstruction and contributes to morbidity and mortality in these diseases. Mucins, the major component of mucus secretions, are high-molecular weight and heavily glycosylated proteins synthesized by the mucosal epithelial cells lining the middle ear, trachea, and digestive and reproductive tracts. The complexity and diversity of mucin glycosylation determines biological functions of mucins [1,2]. To date, 20 mucin genes have been identified [3,4]. Among these, at least *MUC2*, *MUC5AC*, and *MUC5B* have been shown to play an important role in the pathogenesis of respiratory infectious diseases [3,4]. Under normal conditions, they protect the epithelial cells by binding and trapping the inhaled microbial particles, including bacteria and viruses, for mucociliary clearance, at least in part because of the extraordinary diversity of their carbohydrate side chains [5,6]. However, under diseased conditions such as COPD and OM, the mucociliary clearance mechanism becomes defective. The excessive production of mucin will lead to airway obstruction in COPD and conductive hearing loss in OM [4,6,7].

The Gram-negative bacterium *nontypeable Haemophilus influenzae* (NTHi) is an important human pathogen, which causes OM and exacerbates COPD [8,9]. Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections has been difficult and still remains a great challenge. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, development of alternative therapeutic strategies is urgently needed for the treatment of NTHi infections based on understanding the molecular pathogenesis of NTHi infections. Recent studies have provided evidence that NTHi up-regulates *MUC5AC* mucin expression via activation of p38 mitogen-activated protein kinase (MAPK) [10,11]. However, despite the critical role of mucin in the pathogenesis of NTHi infection, a key issue that has yet to be addressed is how to attenuate mucin overproduction in chronic respiratory diseases, such as COPD and OM.

Glucocorticoids are highly effective in the control of many inflammatory and immune diseases. Their effects are exerted by binding to the intracellular glucocorticoid receptor (GR), which belongs to the family of steroid hormone receptors [12,13]. In addition to their broad use in the treatment of immune and

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inflammatory diseases, adjunctive glucocorticoid therapy has also been used in a variety of bacterial infections, including OM and COPD [14,15]. Because of the importance of glucocorticoids in the treatment of immune and inflammatory diseases as well as bacterial infections, much effort has been made toward demonstrating the effect of glucocorticoids on mucin expression [16–18]. However, the molecular basis for the inhibitory effect of glucocorticoids on mucin overproduction in NTHi infections still remains unclear.

Here we provided the direct evidence that the synthetic glucocorticoid hormone, dexamethasone, inhibits NTHi-induced *MUC5AC* expression *in vitro* and *in vivo*. Inhibition of NTHi-induced *MUC5AC* expression by dexamethasone occurs at the level of p38 MAPK via GR. Moreover, glucocorticoids up-regulate MKP-1 expression, that in turn leads to p38 dephosphorylation and the subsequent inhibition of NTHi-induced *MUC5AC* expression. These studies provide new insight into the molecular mechanism underlying glucocorticoid therapy and may lead to novel therapeutic strategies for inhibiting mucin overproduction in patients with COPD and OM.

Materials and methods

Reagents. Dexamethasone and RU486 were purchased from Sigma (St. Louis, MO). Ro-31-8220, SB203580, PD98059 and SP600125 were purchased from Calbiochem (LaJolla, CA).

Bacterial strains and culture condition. NTHi strain 12, a clinical isolate, was used in this study, and NTHi lysate was prepared as described previously [19]. NTHi lysates (5 μg/ml) were used in all the *in vitro* experiments. We chose to use NTHi lysates because of the following reasons: first, NTHi is known to be highly fragile and undergoes autolysis. Its autolysis can be triggered *in vivo* under various conditions including antibiotic treatment [15,17]. Therefore, using lysate of NTHi represents a common clinical condition *in vivo*, especially after antibiotic treatment.

Cell culture. A549 (human lung epithelial), HMEEC-1 (human middle ear epithelial), HM3 (human colon epithelial) and HeLa (human cervix epithelial) were maintained as described previously [11,19]. All media contained 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Plasmids, transfection and luciferase assay. The constitutively active forms of MKK3(E) and MKK6(E) and report constructs of *MUC5AC* were described previously [11,19]. The expression plasmids of the antisense and wild-type MKP-1 were kindly provided by Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Dr. C. Desbois-Mouthon (INSERM U-402, Faculté de Médecine Saint-Antoine, Paris, France), respectively [20,21]. Transient transfections of cells were carried out in triplicate with TransIT-LT1 reagent (Mirus, Medison, WI) following manufacturer's instruction. For experiments with inhibitors, the transfected cells were pretreated with or without chemical inhibitors including RU486 (1 μM), Ro-31-8220 (1 μM) and SB203580 (1 μM) for 1 h prior to NTHi treatment.

Real-time quantitative PCR (Q-PCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions as described previously [10,11,19], and the reverse transcription reaction was performed using TaqMan reverse transcription reagents (Applied Biosystems). PCR amplification was performed by using TaqMan Universal Master Mix (Applied Biosystems). Reactions were amplified and quantified by using as ABI 7700 sequence detector and the manufacturer's corresponding software (Applied Biosystems). Relative quantity of mRNAs were obtained by using the comparative threshold cycle (C_t) Method.

Western blot analysis. Western blots were performed as described and following the manufacturer's instructions [19]. Briefly, western blots were performed using whole cell extracts, separated on 8–10% SDS–PAGE gels and transferred to polyvinylidine difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with a solution of TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated in a 1:1000 dilution of a primary antibody. After another three washes in TBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody. The membrane was reacted with chemiluminescence reagent ECL (Amersham Biosciences) to visualize to blots. Antibodies against phospho-p38 (Thr180/Tyr182) and p38 were purchased from Cell Signaling Technology (Beverly, MA).

Animal experiments. BALB/c mice were purchased from Charles River Lab. and 7–8 weeks old male BALB/c mice were used in this study. Animals were intratracheally inoculated with 50 μl of NTHi corresponding to 1×10^7 CFU of live NTHi. Mice were sacrificed by intraperitoneal injection of sodium pentobarbital (100 mg/kg in 100 μl of saline) 3 h after NTHi inoculation. Total RNA was isolated from the lung tissues using TRIzol® Reagent following manufacturers instruction. Reverse transcription and Q-PCR analysis of mouse *Muc5ac* were conducted as described above. For inhibition study, mice were pretreated with 1 mg/kg of dexamethasone intraperitoneally 1 h before NTHi inoculation. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Rochester

Statistical analysis. Statistical analysis was performed with Student *t-test*. *P* values of less than 0.05 were considered statistically significant.

Results and discussion

Glucocorticoids inhibit NTHi-induced MUC5AC expression in vitro and in vivo

NTHi, an important human respiratory pathogen, up-regulates *MUC5AC* mucin, a primary innate mucosal defense response in mammalian airways [10,11,19]. To evaluate the effect of glucocorticoids on *MUC5AC* mucin expression, we first confirmed whether NTHi up-regulates *MUC5AC* expression in human epithelial cells. NTHi-induced MUC5AC transcription in a does-dependent manner. Moderate up-regulation of MUC5AC was observed with 5 μg/ml of NTHi lysate corresponding to 1×10^7 CFU of live NTHi, which is the concentration used *in vivo* NTHi-induced pneumonia model (data not shown) [22]. NTHi indeed up-regulated *MUC5AC* expression at mRNA level in a time-dependent manner not only in HeLa cells (Fig. 1A), but also in human lung epithelial A549 and middle ear epithelial HMEEC-1 cells (Fig. 1B), as assessed by Q-PCR analysis. Recently, it was reported that the synthetic glucocorticoid hormone, dexamethasone, represses *MUC5AC* gene expression in lung epithelial cells [16,17]. We thus assessed the effect of dexamethasone on NTHi-induced *MUC5AC* expression at transcriptional level by using *MUC5AC* promoter-driven luciferase reporter construct in human epithelial cells. As shown in Fig. 1C and D, dexamethasone markedly inhibited NTHi-induced *MUC5AC* expression at transcriptional level in a dose-dependent manner. To further confirm the inhibitory effect of dexamethasone on NTHi-induced *MUC5AC* expression *in vivo*, we next determined whether dexamethasone also inhibited NTHi-induced *MUC5AC* expression in the lungs of the mice. Consistent with our *in vitro* finding, dexamethasone also attenuated NTHi-induced *Muc5ac* mRNA expression in the lungs of mice (Fig. 1E). Together, these data demonstrate that glucocorticoids inhibit NTHi-induced *MUC5AC* expression *in vitro* and *in vivo*.