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Azithromycin inhibits nontypeable *Haemophilus influenzae*-induced MUC5AC expression and secretion via inhibition of activator protein-1 in human airway epithelial cells

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ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi) is one of the most common pathogens in chronic airway infections and exacerbation. The hallmark of chronic respiratory diseases, including cystic fibrosis, diffuse panbronchiolitis and chronic obstructive pulmonary disease, is mucin overproduction. Prolonged macrolide antibiotic therapy at low doses is known to improve clinical outcome in patients with chronic respiratory diseases via anti-inflammatory effects. In this study, we investigated the effects of macrolide therapy on NTHi-induction of the MUC5AC mucin in human airway epithelial cells. A 15-membered macrolide, azithromycin, but not a 14-membered macrolide, clarithromycin, inhibited NTHi-induction of MUC5AC at both the mRNA and protein levels through selective suppression of activation of the transcription factor activator protein-1. Our findings suggest that each macrolide affects MUC5AC production in different ways and that azithromycin is more suitable for the treatment of NTHi-induced respiratory infection.

1. Introduction

The Gram-negative bacterium nontypeable *Haemophilus influenzae* (NTHi) is an important human pathogen that causes otitis media and exacerbates chronic obstructive pulmonary diseases (COPD) (Faden et al., 1996; Foxwell et al., 1998). A hallmark of both otitis media and COPD is mucus overproduction that mainly results from the up-regulation of mucin (Knowles and Boucher, 2002; Lemjabbar and Basbaum, 2002). Mucus secretion plays a role in host protection of mucosal surfaces against pathogens and irritants. However, under diseased conditions such as COPD, diffuse panbronchiolitis and cystic fibrosis, excessive mucus secretion causes airway obstruction and impairment of gas exchange, which makes mucus hyperproduction an important hallmark of pathogenesis. Among the many factors that contribute to mucin hypersecretion in chronic respiratory infection, bacterial infection is one of the most important (Dohrman et al., 1998; Imamura et al., 2004). Therefore, regulatory strategies for the treatment of mucus hyperproduction need to be developed based on an understanding of the molecular pathogenesis of bacterial infections. Recent studies have provided evidence to show that NTHi up-regulates *MUC5AC* mucin gene expression via activation of the p38 mitogen-activated protein kinase (MAPK) (Chen et al., 2004; Wang et al., 2002). Although this information has shown 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.

Macrolide antibiotics are 14-, 15-, and 16-membered ring antimicrobial agents. These antibiotics include erythromycin and clarithromycin that are typical 14-membered macrolides, and azithromycin that is a prototypical 15-membered compound. Macrolide treatment is a well established therapy for respiratory infections. Prolonged, long-term macrolide antibiotic therapy at a low dose has been shown to be effective for the treatment of chronic respiratory diseases such as diffuse panbronchiolitis, cystic fibrosis and COPD (Equi et al., 2002; Kudoh et al., 1998; Suzuki et al., 2001). The beneficial effects of long-term low-dose macrolide therapy are not related to their antimicrobial properties, since the levels of macrolides at low-dose treatments are too low to have sufficient antimicrobial effects. However, long-term therapy of macrolides may increase drug-resistant strains. Here we show that azithromycin and clarithromycin may function as anti-inflammatory agents, and that each macrolide has different effects on mucus production. Our present data might suggest anti-inflammatory mechanisms for the effect of macrolides on modulation of the inflammatory pathways and solve the question as to why one specific macrolide is effective for the treatment of chronic respiratory infection whereas other macrolides are ineffective.

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2. Materials and methods

2.1. Materials

The macrolides clarithromycin (Taishotoyama, Tokyo, Japan) and azithromycin (Pfizer, Groton, CT, USA) were generously donated by the respective companies and were dissolved in dimethyl sulfoxide (DMSO). Dexamethasone was purchased from Sigma (St. Louis, MO). The mouse anti-MUC5AC monoclonal antibody (clone 45M1) was obtained from MONOSAN (Uden, Netherlands). The goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Bio-Rad (Hercules, CA).

2.2. Cell culture

The NCI-H292 (Human airway epithelial) cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. The cells were grown at 37 °C with 5% CO_2 in fully humidified air. For the MUC5AC production studies, cells were exposed to NTHi extracts for RT-PCR or ELISA. Cells were pretreated with azithromycin, clarithromycin or dexamethasone before exposure to NTHi extracts in the inhibition studies. Because the drugs showed dose-dependent response (1, 10, 50 μg/ml), azithromycin and clarithromycin used at the highest concentration (50 μg/ml). But dexamethasone was potent agonist, so dexamethasone used at the lowest concentration (1 μg/ml) (Data not shown). In the case of controls, the cells were incubated with medium alone.

2.3. Bacterial culture and extract

The NTHi strain was grown on chocolate agar at 37 °C with 5% CO_2. NTHi crude extracts were made using a previously reported method (Wang et al., 2002). In brief, NTHi were harvested from a plate of chocolate agar after overnight incubation and incubated in 30 ml of brain–heart infusion (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with NAD (3.5 μg/ml). After overnight incubation, NTHi were centrifuged at 10,000×*g*, for 10 min at 4 °C. The supernatant was then discarded and the resulting NTHi-pellet was suspended in 10 ml PBS and sonicated. Residual cells were removed by centrifugation (10,000×*g* for 10 min at 4 °C) and filtered using a 0.2 μm filter (Millipore Co., Billerica, USA). The lysate was stored at −80 °C.

2.4. RT-PCR

Total RNA was extracted from NCI-H292 cells cultured in a 60 mm dish using QuickGene-Mini80 and QuickGene RNA cultured cell kits (FUJIFILM Co., Tokyo, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and was then treated with RNaseH. To quantify the expression of the *MUC5AC* gene, PCR primers and Taqman probes were designed and used as reported previously (Forward primer, 5′-CAGCCACGTCCCCTTCAATA-3′; Reverse primer, 5′-ACCGCATTTGGGCATCC-3′; Taqman probe, 5′-6-FAM-CCACCTCC-GAGCCCGTCACTGAG-TAMRA-3′) (Inoue et al., 2006). MUC5AC was amplified for 40 cycles (15 s at 95 °C, and 30 s at 60 °C) using a LightCycler system. To normalize MUC5AC expression, human porphobilinogen deaminase (hPBGD) was also measured using an hPBGD primer set (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Data are presented as a ratio of hPBGD.

2.5. ELISA

The MUC5AC protein level was measured using an enzyme-linked immunosorbent assay (ELISA) according to a procedure described previously (Imamura et al., 2004). After NTHi stimulation for 24 h, the culture medium was collected as the cell supernatant. This supernatant was then incubated at 40 °C in a 96-well plate until dry. The plates were blocked with 2% bovine serum albumin for 1 h at room temperature and were then incubated with the anti-MUC5AC antibody diluted in PBS containing 0.05% Tween 20 for 1 h. Horseradish peroxidase (HRP)-conjugated anti-goat IgG was then dispensed into each well. After 1 h, the plates were washed three times with PBS. Color was developed using a 3, 3′, 5, 5′-tetramethyl-benzidine-peroxidase solution, and the reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was read at 450 nm.

2.6. Transcription factor assay

Cells cultured in 60 mm dishes were harvested and nuclear extracts were obtained using a nuclear/cytosol fractionation kit (BioVision, Lyon, France) according to the manufacturer's protocol. DNA binding activity of NF-κB was measured with an NF-κB transcription factor assay kit (Upstate, Temecula, CA) according to the manufacturer's instructions. Briefly, 10 μg of samples from untreated or treated cells, as well as a capture probe consisting of double-stranded biotinylated oligonucleotides containing the consensus sequence for the binding of NF-κB, were added to a streptavidin-coated 96-well plate. After incubation, NF-κB p50 or p65 subunits that were bound were detected using a primary anti-p50 or anti-p65 antibody. The plate was then incubated with the secondary antibody, a chromogenic substrate was added to the cells, and the absorbance of each sample was read using a microplate reader. DNA binding activity of the transcription factor Activator protein-1 (AP-1) was determined using the ELISA-based TransAM AP-1 kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. Briefly, samples (10 μg of protein) from untreated or treated cells were added to the oligonucleotide-coated 96-well plate. After incubation, bound c-Jun was detected using a primary anti-c-Jun antibody. The plate was incubated with secondary antibody, a chromogenic substrate was added to the cells, and the absorbance of each sample was read using a microplate reader.

3. Results

3.1. NTHi up-regulates MUC5AC mucin mRNA expression and protein secretion

To determine the effect of NTHi concentration on MUC5AC protein expression, NCI-H292 cells were incubated with various concentrations of NTHi for 24 h and the level of the MUC5AC protein secreted into the culture supernatant was evaluated using an ELISA. The level of secreted MUC5AC increased with NTHi addition in a dose-dependent manner (Fig. 1A). Based on this result, a concentration of 25 μg/ml of NTHi was chosen for further studies. We next evaluated the effect of NTHi on the induction of MUC5AC mRNA expression over a period of 9 h following NTHi addition (Fig. 1B) using RT-PCR. The expression of MUC5AC mRNA was increased by NTHi as early as 3 h after NTHi addition, which was the earliest time point tested. MUC5AC mRNA expression was maximally induced after 6 h of NTHi stimulation.

3.2. Azithromycin inhibits NTHi-induced MUC5AC mRNA expression and protein secretion

To determine the effect of macrolides on NTHi-induced MUC5AC mRNA expression and protein secretion, NCI-H292 cells were