

**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.

*Keywords:* mucus, mucin, chronic obstructive pulmonary disease, immunomodulatory effect, macrolides

## 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.

## **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<sub>2</sub> in fully humidified air. For the MUC5AC production studies, cells were exposed to NTHi extracts for RT-PCR or ELISA. Cells were pre-treated 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<sub>2</sub>. 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-CCACCTCCGAGCCCGTCACTGAG-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 cellular *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'-tetramethylbenzidine-peroxidase solution, and the reaction was stopped by the addition of 2 N H<sub>2</sub>SO<sub>4</sub>. 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 pretreated with or without the macrolides clarithromycin or azithromycin (50 µg/ml), or with dexamethasone (1 µg/ml) for 30 min before the addition of NTHi. Dexamethasone has been reported to inhibit NTHi-induced MUC5AC mucin expression (Komatsu et al., 2008) and was therefore used as a positive control for inhibition. After pretreatment the cells were stimulated with NTHi



for 24 h prior to assay of MUC5AC protein secretion or for 3 h prior to assay of MUC5AC mRNA expression. Azithromycin significantly reduced both MUC5AC mRNA expression (Fig. 2A) and the level of secreted MUC5AC protein (Fig. 2B).

### **3.3. NTHi up-regulates NF- $\kappa$ B and AP-1 activation**

To confirm time-dependent changes in these transcription factors induced by NTHi, we evaluated NTHi-induced activation of NF- $\kappa$ B and AP-1 using a transcription factor assay kit. NCI-H292 cells were incubated with NTHi over a period of one hour during which cell nuclear extracts (10  $\mu$ g of protein) were assayed. NTHi induced the activation of the NF- $\kappa$ B subunits p50 and p65, and the AP-1 subunit c-Jun. In particular, the NF- $\kappa$ B subunits p50 and the AP-1 subunit c-Jun were activated in a time dependent manner (Figs. 3B and 4 respectively).

### **3.4. Azithromycin inhibits NTHi-induced MUC5AC via AP-1 but not via NF- $\kappa$ B**

Since azithromycin decreased the expression of MUC5AC mRNA and protein, we investigated if this effect is mediated by inhibition of NTHi-induced transcription activity. Cells were pretreated with or without macrolides for 30 min and then stimulated with NTHi for one hour following which cell nuclear extracts (10  $\mu$ g of protein) were assayed for NF- $\kappa$ B and AP-1 transcriptional activity. Macrolides did not

inhibit NTHi-induced activation of NF- $\kappa$ B p50/65 (Fig. 5). In contrast, azithromycin, but not clarithromycin, inhibited NTHi-induced activation of the AP-1 subunit c-Jun (Fig. 6).

#### 4. Discussion

In this study, we demonstrated that azithromycin reduced NTHi-induced MUC5AC production by inhibition of AP-1 activation. NF- $\kappa$ B and AP-1 are transcription factors that play a central role in inflammatory immune reactions, and are known to be involved in NTHi-induced mucin expression (Chen et al., 2004). We first confirmed the effect of NTHi on the activity of NF- $\kappa$ B and AP-1 that bind to specific sites upstream of the *MUC5AC* gene. NF- $\kappa$ B is composed of two subunits that, under non-activated conditions reside in the cytoplasm combined with I $\kappa$ B. However, following stimulation by a variety of agents, NF- $\kappa$ B becomes activated and moves to the nucleus. AP-1 is also composed of two variable subunits. The subunits ATF2 and c-Jun are key components of AP-1 and can function as homodimers or heterodimers in the nucleus (Shen et al., 2008). Our results showed that NTHi triggered the activation of NF- $\kappa$ B and AP-1. Inhibition of AP-1 by azithromycin was correlated with a decrease in the mRNA and protein levels of MUC5AC. Thus, AP-1 may be related with regulation of mucus production in the pathogenicity of NTHi. These data suggested the model of NTHi-induced signaling and azithromycin inhibition (Fig. 7). However, the inhibitory effects of azithromycin were incomplete due to the other

transcription factor binding sites that are involved in MUC5AC expression are located downstream of the NF- $\kappa$ B and AP-1 binding sites (Thai et al., 2008). Further experiments are required to determine the precise target of azithromycin.

Patients with respiratory inflammatory diseases such as diffuse panbronchiolitis, cystic fibrosis and COPD suffer from airway mucus obstruction and airflow limitation due to mucin overproduction. These diseases predispose patients to the development of airway infections because they are damaged the epithelia. Alterations in the chemical character of tracheobronchial mucin, particularly in cystic fibrosis patients, lead to the excessive viscosity, increased elasticity and reduced clearance of mucus. Furthermore, excess mucus may provide additional binding sites for airway pathogens (Chance et al., 1999). NTHi is a bacterium that is a major cause of mucosal respiratory disease and, in particular, it is the most common cause of exacerbation of COPD (Rosell et al., 2005) (Bandi et al., 2001) (King et al., 2008).

In Japan, prolonged macrolide antibiotic treatment at low doses is the therapy of choice for patients with these respiratory diseases in order to improve pulmonary function and clinical outcome. Our experiments have demonstrated an effect of macrolides on mucin overproduction induced by NTHi stimulation of airway epithelial cells. Cigana et al reported IL-8 was regulated by anti-inflammatory effects of azithromycin (Cigana et al., 2006). Azithromycin regulated DNA binding activity of AP-1 c-jun as same as our data. We have shown that azithromycin is more effective than clarithromycin in the prevention of mucin overproduction. These data are supported by clinical reports of diffuse panbronchiolitis patients for which azithromycin therapy was effective (Kobayashi et al., 1995) (Nagata et al., 2001) and by a report

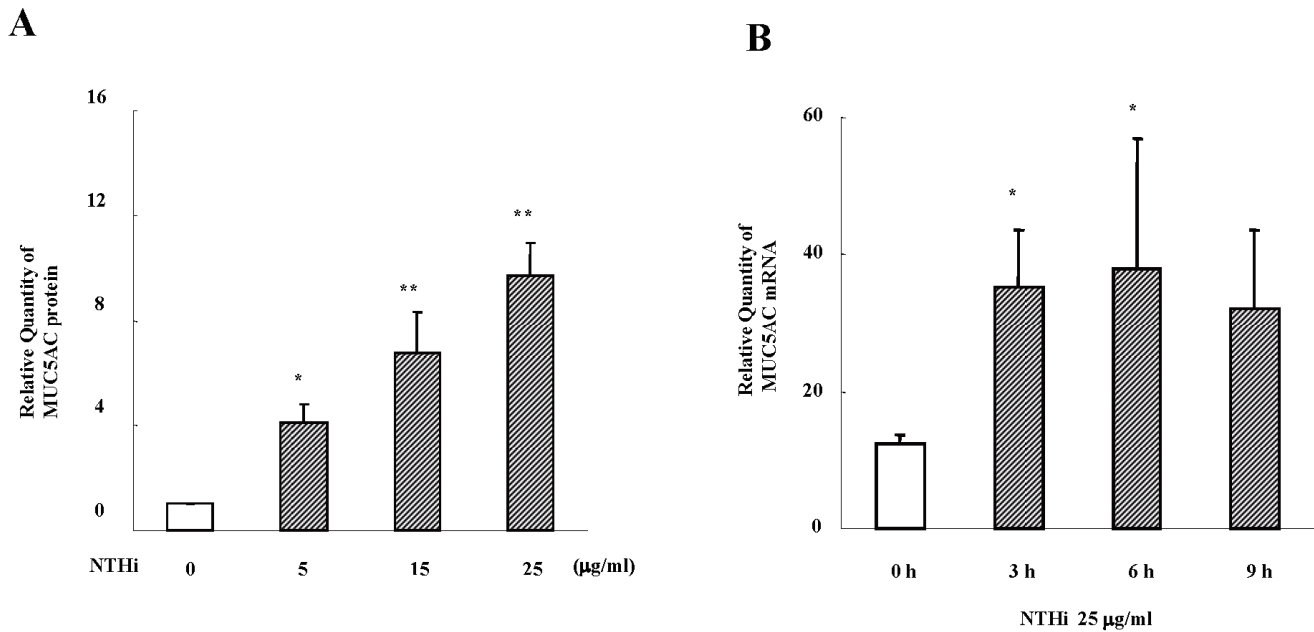
that azithromycin therapy improved the number of respiratory exacerbations and reduced the rate of decline in lung function in patients with cystic fibrosis (Wolter et al., 2002). To eliminate a possibility of the emergence of drug-resistant strain, developments of macrolides without antimicrobial effects were expected.

Previous studies have shown that various macrolides attenuate MUC5AC production under a variety of conditions *in vitro*, and have analyzed the signaling pathway mediating this attenuation. For example, the MUC5AC production induced by lipopolysaccharide (LPS) derived from *Pseudomonas aeruginosa*, or by human neutrophil peptide-1 (HNP-1), an antimicrobial peptide in neutrophils, was inhibited by azithromycin and clarithromycin through a reduction in the phosphorylation of the MAPkinase ERK1/2 (Ishimoto et al., 2009). In addition, *Chlamydomphila pneumoniae* induced MUC5AC was inhibited by azithromycin, clarithromycin and telithromycin (Morinaga et al., 2009). Other studies have suggested that LPS-induced mucus hypersecretion and NF- $\kappa$ B nuclear translocation was significantly attenuated by roxithromycin, but that p38 and ERK1/2 function was not affected in airway epithelium (Ou et al., 2008). Clarithromycin and azithromycin are known to have different effects on cytokine production and inhibition in murine dendritic cells (Sugiyama et al., 2007). These studies suggest that the signaling pathway by which mucus hypersecretion is modulated varies according to the type of macrolide or stimulant that is used.

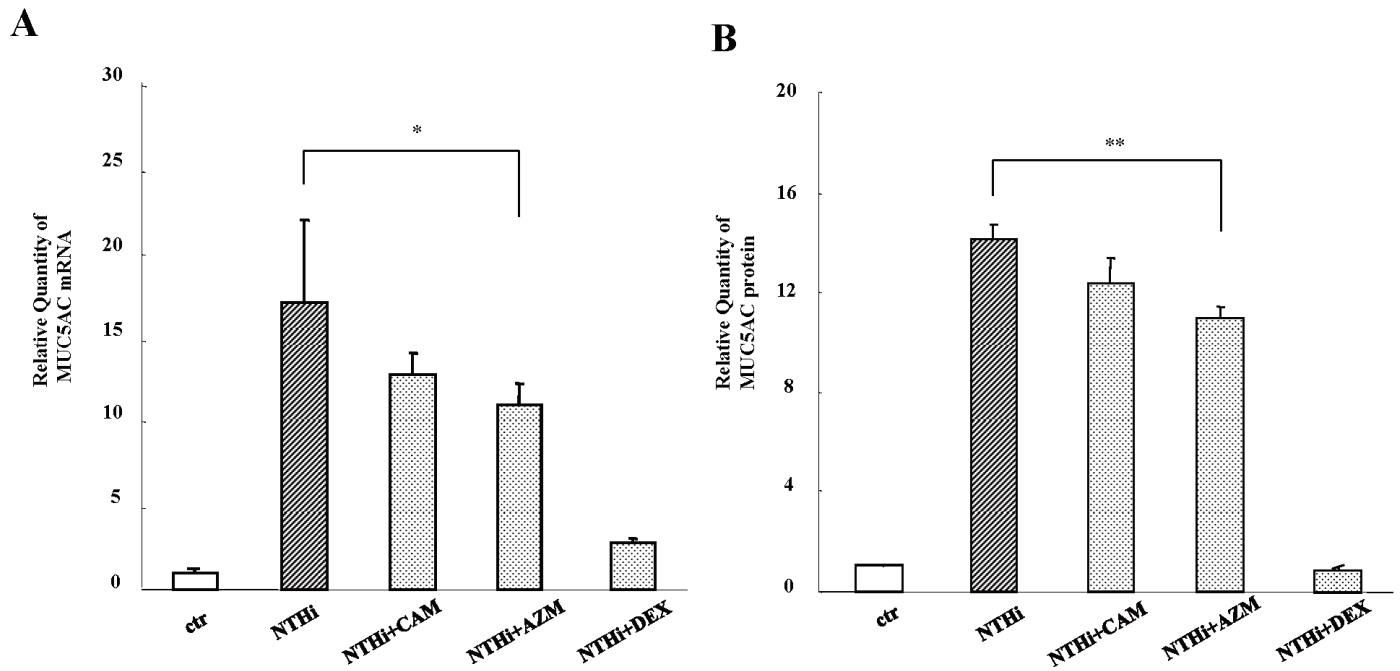
Since different bacteria modulate different signaling pathways in clinical practice, the appropriate macrolide drug for therapy should be chosen based on the type of bacteria that colonize the

lower airway. Our study indicates that azithromycin therapy may be a suitable treatment for respiratory tract infection induced by NTHi.

## Figure captions



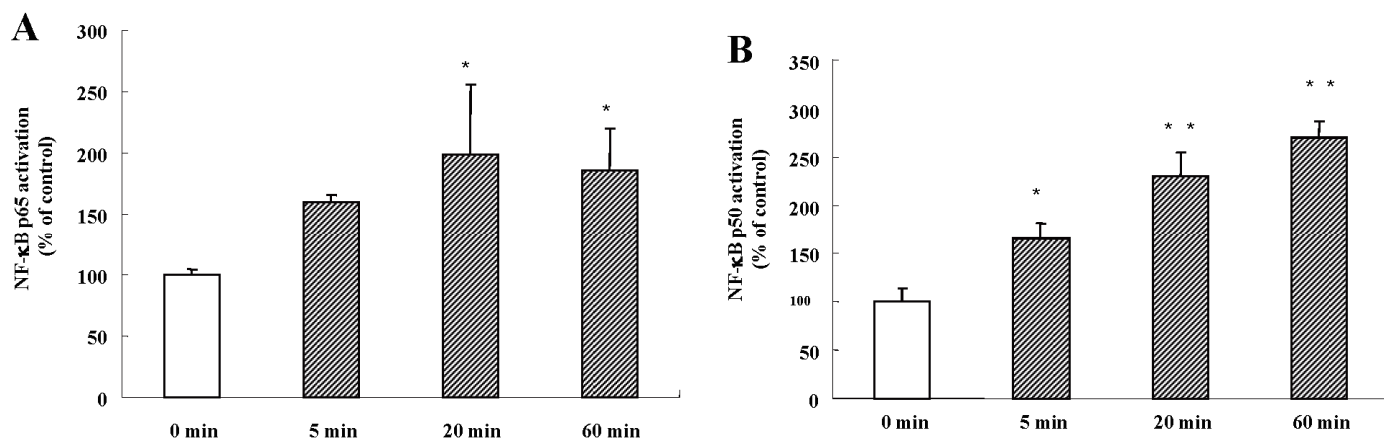
**Fig. 1. Effect of NTHi on cellular MUC5AC expression.** A: NCI-H292 cells were incubated with various concentrations of an NTHi extract for 24 h and the level of the MUC5AC protein secreted into the medium was assayed using an ELISA. NTHi induced the secretion of the MUC5AC protein in a dose-dependent manner. B: NCI-H292 cells were incubated for the indicated times with an NTHi extract (25 µg/ml) following which the mRNA level of MUC5AC was assayed by RT-PCR. NTHi induced MUC5AC mRNA expression at 3 and 6 h. Results are expressed as the mean  $\pm$  S.D. \* $P < 0.05$  \*\* $P < 0.01$



**Fig. 2. Azithromycin inhibits NTHi-induction of MUC5AC secreted protein and mRNA. NCI-H292**

cells were pretreated with or without (control, ctr) the macrolides clarithromycin and azithromycin (50  $\mu\text{g/ml}$ ) or dexamethasone (1  $\mu\text{g/ml}$ ) for 30 min. Subsequently the cells were stimulated by NTHi for 24 h following which cell extracts and the cell supernatant were assayed for MUC5AC mRNA (A) or protein (B) respectively as described in the legend to Fig. 1. The level of the MUC5AC secreted protein induced by NTHi was reduced by azithromycin. Results are expressed as the mean  $\pm$ S.D. \* $P < 0.05$  \*\* $P < 0.01$  vs.

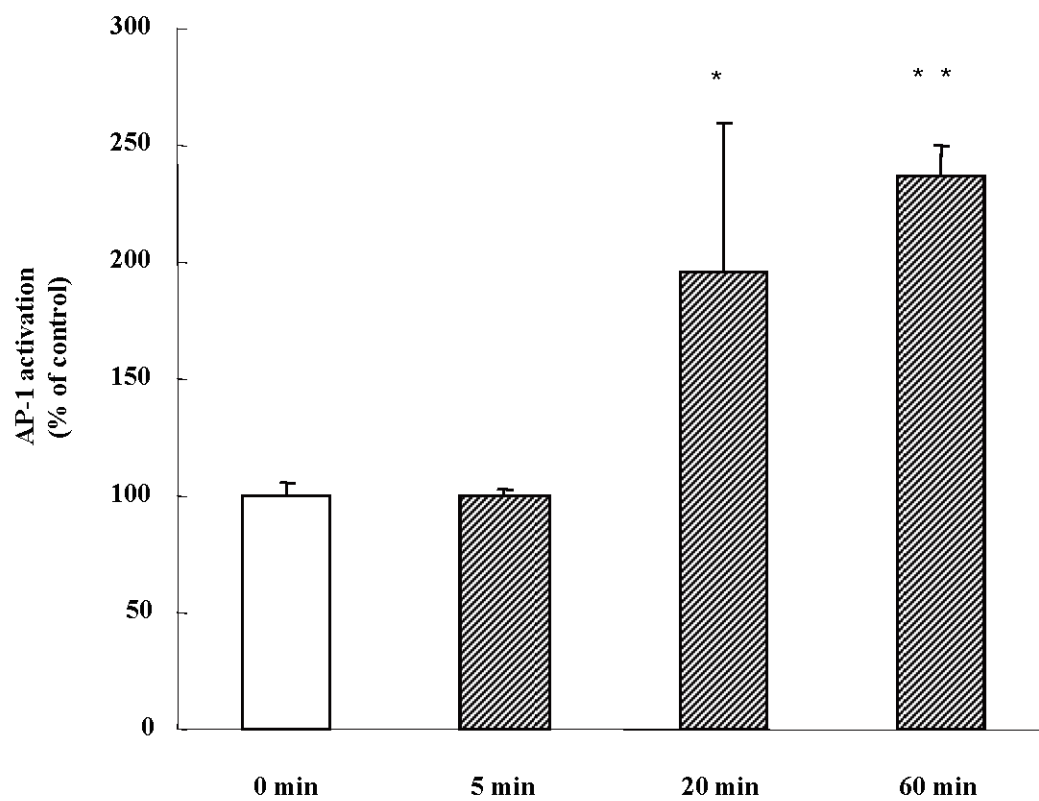
NTHi treated. AZM: azithromycin, CAM: clarithromycin, DEX: dexamethasone



**Fig. 3. NTHi induces activation of both of the NF-κB subunits p50/p65 in a time dependent manner.**

NCI-H292 cells were treated with NTHi for the indicated times following which the activity of the NF- κB subunits p65 (A) and p50 (B) were assayed using a commercial transcription factor assay kit. Results are expressed as the mean  $\pm$ S.D. \*P<0.05, \*\*P<0.01

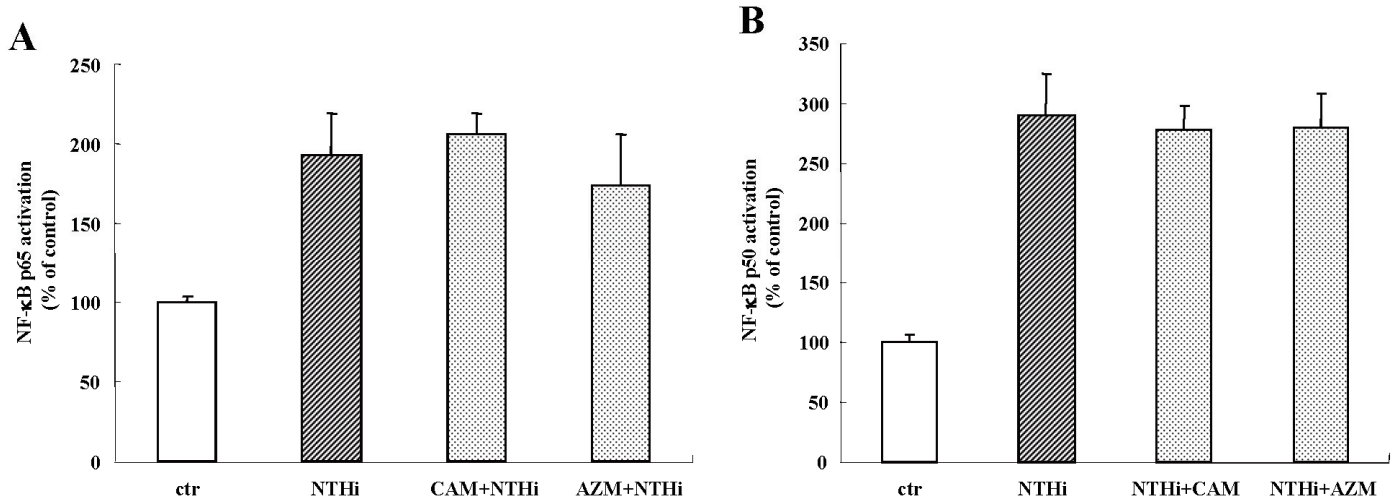




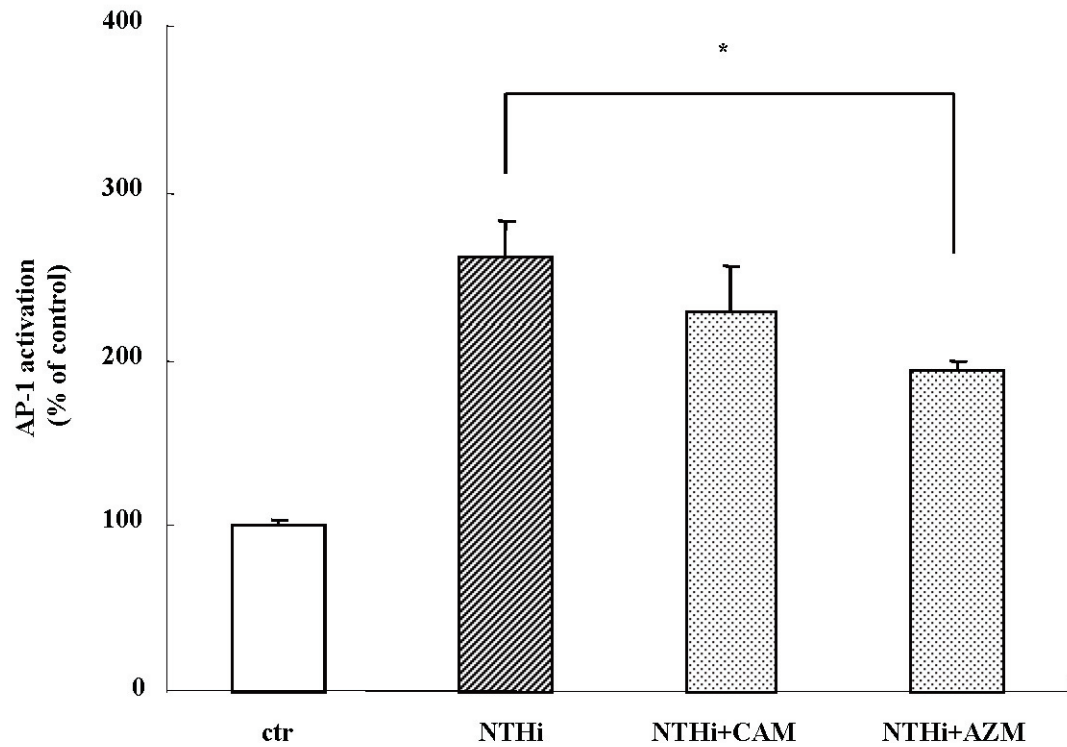
**Fig. 4. NTHi induces activation of the AP-1 subunit of c-Jun in a time dependent manner. NCI-H292**

cells were treated with NTHi for the indicated times following which AP-1 activity was assayed using a

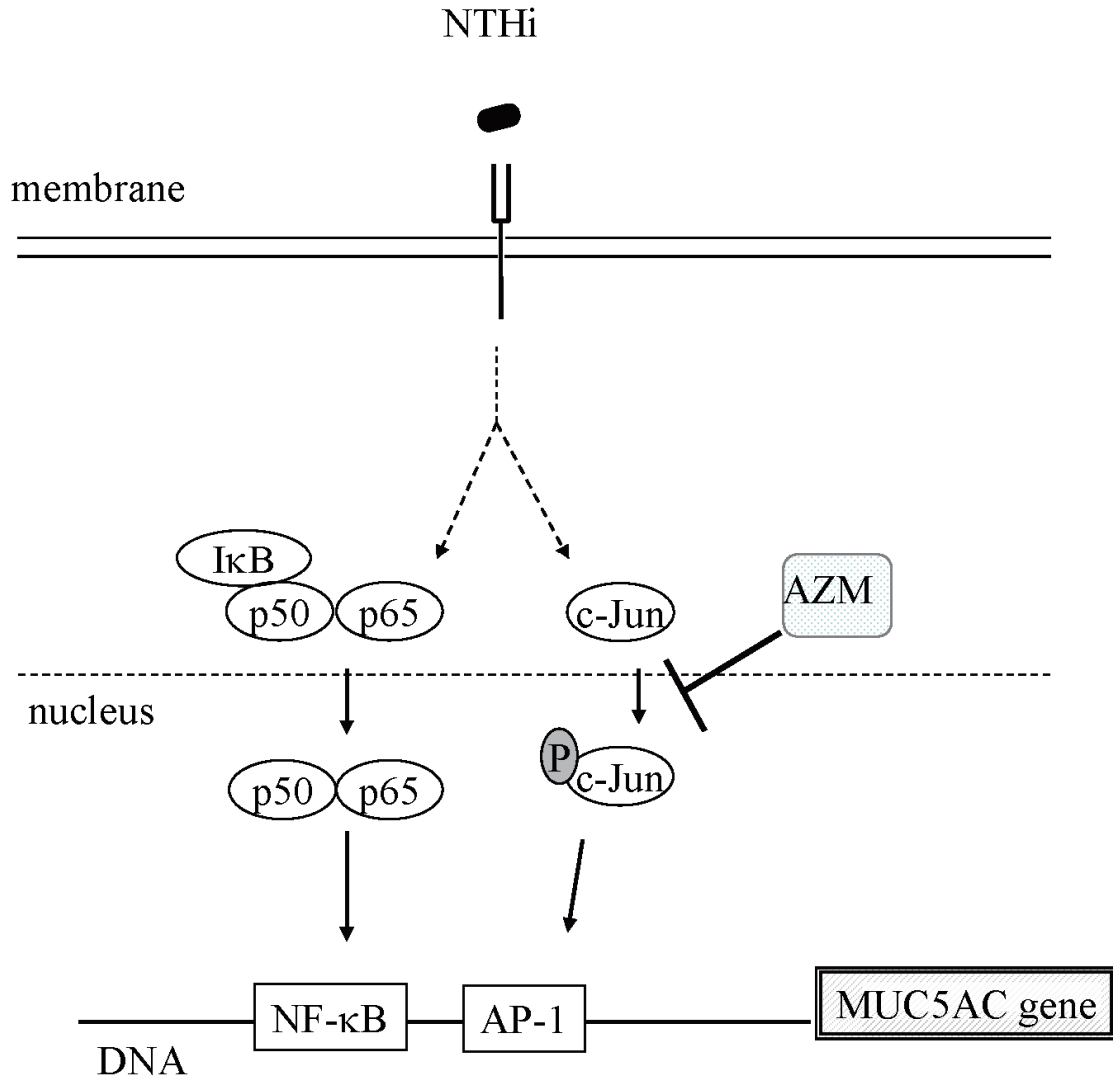
commercial transcription factor assay kit. Results are expressed as the mean  $\pm$ S.D. \*P<0.05, \*\*P<0.01



**Fig. 5. Macrolides do not inhibit NTHi-induced activation of NF-κB p50/p65.** NCI-H292 cells were pretreated with or without the macrolides clarithromycin and azithromycin for 30 min. Subsequently the cells were stimulated with NTHi for one hour. Nuclear extracts of harvested cells were assayed for the transcriptional activity of NF-κB p65 (A) and p50 (B) using a commercial kit. AZM: azithromycin, CAM: clarithromycin



**Fig. 6. Azithromycin inhibits NTHi-induced AP-1 activation.** NCI-H292 cells were pretreated with and without the macrolides clarithromycin and azithromycin for 30 min. Subsequently the cells were stimulated with NTHi for one hour. Nuclear extracts of harvested cells were assayed for AP-1 transcriptional activity using a commercial kit. NTHi-induced AP-1 activation was inhibited by azithromycin. Results are expressed as the mean  $\pm$ S.D. \* $P < 0.01$  AZM: azithromycin, CAM: clarithromycin



**Fig. 7. The signal pathway of NTHi-induction of MUC5AC expression and its modulation by azithromycin.** As indicated, NTHi induces MUC5AC expression via activation of the p65/p50 subunits of NF-κB, and of c-Jun, a subunit of AP-1. Azithromycin inhibits NTHi-induced MUC5AC expression via suppression of activation of c-Jun. AZM: azithromycin

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