

Azithromycin, clarithromycin and telithromycin inhibit MUC5AC induction by *Chlamydomphila pneumoniae* in airway epithelial cells

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Running title: Effects of Macrolides on *C. pneumoniae*-induced MUC5AC

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Abstract

Background: Airway mucus hypersecretion is an important problem in chronic respiratory diseases including bronchial asthma. *Chlamydophila pneumoniae* is recently confirmed to be a pathogen in bronchial asthma, but the relationship between *C. pneumoniae* and mucus hypersecretion is uncertain. In this study, we examined whether *C. pneumoniae* induces MUC5AC mucin in airway epithelial cells. We also examined the effects of macrolide and ketolide antibiotics on the *C. pneumoniae*-induced mucus production.

Methods: MUC5AC production in bronchial epithelial cells after stimulation with *C. pneumoniae* was analyzed by ELISA and quantitative RT-PCR. NF κ B and phosphorylated ERK were also analyzed. For inhibition study, cells were pretreated with azithromycin, clarithromycin and telithromycin before stimulation.

Results: *C. pneumoniae* dose-dependently induced MUC5AC production and gene expression. The ERK-NF- κ B pathway was involved in *C. pneumoniae*-induced MUC5AC production. Macrolides and ketolides dose-dependently reduced *C. pneumoniae*-induced MUC5AC production. However, azithromycin was apparently less effective than the other antibiotics. Clarithromycin and telithromycin, but not azithromycin, reduced NF- κ B activation.

Conclusions: Clarithromycin and telithromycin were thought to interfere with the signal pathways between ERK and NF- κ B. These results suggest that airway mucus hypersecretion is one of the mechanisms of *Chlamydophila pneumoniae*-induced bronchial asthma, and that macrolide and ketolide antibiotics represent a novel therapeutic intervention in these patients.

Key words: macrolides, ketolides, asthma, immunomodulatory effects, mucins

1. Introduction

Airway mucus coats the surface of the respiratory tract, acts as a protective barrier against pathogens and foreign irritants and provides the setting for the innate immune system. However, excessive mucus production contributes to restriction of the airway and insufficient gas exchange. This mucus hypersecretion is observed in various chronic airway diseases, such as diffuse panbronchiolitis (DPB), cystic fibrosis, chronic obstructive pulmonary diseases and bronchial asthma. The control of mucus hypersecretion may thus contribute to the treatment of these diseases.

More than 20 mucin genes have been identified in humans, and they are generally classified into membrane-associated and gel-forming mucins [1, 2]. The gel-forming mucins are secreted mucins that provide the viscoelastic properties of the extracellular mucus layer. MUC2, MUC5AC, MUC5B, MUC6 and MUC19 belong to this family, and MUC5AC is strongly expressed in the lung [3]. MUC5AC is up-regulated by various bacterial stimulants.

Bronchial asthma is the most common chronic respiratory disease in the world. It is well known that airway inflammation is a key part of the pathogenesis of bronchial asthma. Airway inflammation is characterized by mucus hypersecretion and immigration of inflammatory cells into the airway lumen. Mucus hypersecretion leads air-flow restriction or obstruction. Pathological findings in the lung of fatal asthma patients have shown increased mucus gland and airway lumen area occupied by mucus [4]. These mucus plugs consist primarily of mucins [5], and MUC5AC and MUC5B are the major components of mucins [6].

Chlamydomydia pneumoniae is one of the causes of pneumonia, and it is probably associated with bronchial asthma. *C. pneumoniae* infection is observed more frequently in stable asthma patients than in healthy controls [7]. It is thought that exacerbation of asthma

can be also triggered by *C. pneumoniae* [8]. Patients with asthma exhibit excess mucin production in the airway; however, it is uncertain whether *C. pneumoniae* affects the production of mucins in airway epithelium.

It is gradually being accepted that macrolide antibiotics not only possess antibacterial activity, but also exert immunomodulatory effects. Macrolides have been shown to improve the survival rate of DPB patients [9]. Clarithromycin reduced the volume of sputum in patients with chronic lower airway diseases [10]. This efficacy is not considered to depend on bactericidal activity, as macrolides have no bactericidal potential against the *Pseudomonas aeruginosa* typically isolated from chronic airway infectious disease patients. The suppression of mucus hypersecretion is seen with erythromycin, clarithromycin and azithromycin *in vivo* and *in vitro* [¹¹⁻¹²]. Telithromycin is a novel ketolide antibiotic that structurally resembles macrolides. It has also been reported that telithromycin has similar immunomodulatory effects as macrolides [13].

Because *C. pneumoniae* is now considered to be a pathogen involved in asthma, we hypothesized that *C. pneumoniae* up-regulates MUC5AC in airway epithelial cells. We also investigated the effects of macrolides and ketolides on the MUC5AC production induced by *C. pneumoniae*.

2. Materials and Methods

2.1. Materials

Mouse MUC5AC monoclonal antibody (clone 45M1) was obtained from MONOSAN (Uden, Netherlands). Goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Bio-Rad (Hercules, CA). Rabbit anti-extracellular signal-regulated kinase (ERK) and phosphor-ERK antibodies were obtained from Cell

Signaling Technology (Danvers, MA). Goat anti-rabbit HRP-conjugated secondary antibody was obtained from GE Healthcare (Chalfont St. Giles, United Kingdom). Caffeic acid phenethyl ester (CAPE), PD98059 and U0126 were obtained from Calbiochem (San Diego, CA).

2.2. Preparation of *C. pneumoniae* antigen

The *C. pneumoniae* KK-pn15 strain, which was isolated in our laboratory from a patient with acute pharyngitis, was used as the antigen [14]. The *C. pneumoniae* strain was grown in a HEp-2 cell culture and harvested on day 3 post-inoculation. The elementary bodies (EBs) of the KK-pn15 strain were purified by a method as described previously [15]. At 72 h post-inoculation, infected HEp-2 cells were collected in a sucrose-phosphate-glutamate solution (SPG; sucrose 7.5%, KH_2PO_4 0.052%, $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ 0.1529%, glutamic acid 0.072%) and homogenized with a Teflon homogenizer. After a brief centrifugation at 900 g for 10 min at room temperature to remove cell debris, the supernatant obtained was layered onto a two-layer cushion (bottom layer, 50% wt/vol sucrose solution; top layer, 30% vol/vol Urografin [3,5-diacetamido-2,4,6-triisobenzoic acid; Schering AG, Berlin/Bergkamen, Germany] in 30 mM Tris-HCl buffer [pH 7.3]) and then centrifuged at 8,000 g for 60 min at 4°C with an RPS-25 swing rotor (Hitachi, Tokyo). The precipitate and the turbid bottom layer were suspended together with SPG and then treated with DNase and RNase (final concentration; 20 µg/mL each) for 30 min at 37°C. The suspension was layered onto a continuous Urografin gradient column (40 to 52% vol/vol) and centrifuged at 8,000 g for 60 min at 4°C. Two distinct bands were formed in the gradient column. The presence of a number of EBs was confirmed in the lower band by electron microscopy. After washing with SPG, the EBs were suspended in SPG and stored at -80°C until required.

2.3. *C. pneumoniae* quantification

For DNA extraction, *C. pneumoniae* EBs were suspended in lysis buffer (2 mM Tris-HCl

[pH 8.3], 10 mM KCl, 0.05 mM MgCl₂, 0.5% Triton X-100 and 0.5% Tween20) and 200 µg/ml proteinase K (Sigma, St. Louis, MO). Samples were digested at 55°C for 60 min, followed by 100°C for 10 min, and were then used as DNA extracts. *C. pneumoniae* was quantified by real-time TaqMan PCR targeting the *ompA* gene [16]. Templates were amplified with using a LightCycler system (Roche Diagnostics, Mannheim, Germany).

2.4. Cell culture

NCI-H292 cells were cultured in RPMI-1640 medium with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were grown at 37°C with 5% CO₂ and were subcultured twice weekly. When the cells reached confluence, cells were serum-starved for 24 h and then stimulated with *C. pneumoniae*. For inhibition study, cells were pre-treated with macrolides or signal transduction inhibitors for 30 min before stimulation. As a control, cells were incubated medium alone.

2.5. ELISA

NCI-H292 cells were cultured in 12-well plates and MUC5AC protein was measured by enzyme-linked immunosorbent assay (ELISA) [12]. After stimulation for 24 h, culture medium was kept as the supernatant. Cells were then washed with cold PBS and lysed with lysis buffer (20 mM Tris-HCl [pH 8.0], 133 mM NaCl, 1% NP-40 and 10% glycerol). The preparation was then cleared by centrifugation and the supernatant was saved as the cell lysate. Protein concentrations of the cell lysate were measured. Samples were stored at -80°C until use. Aliquots of supernatant and equal amounts of protein in the cell lysate were incubated at 40°C in a 96-well plate until dry. Plates were blocked with 2% bovine serum albumin for 1 h at room temperature, and incubated with MUC5AC antibody diluted in PBS containing 0.05% Tween 20 for 1 h. Secondary antibody was dispensed into each well for 1 h. Color was developed with 3,3',5,5'-tetramethylbenzidine-peroxydase solution, and the reaction was stopped by adding 2 N H₂SO₄. Absorbance was read at 450 nm.

2.6. Real-time quantitative RT-PCR

NCI-H292 cells were cultured in 6-well plates and stimulated for 8 h. Total RNA was extracted 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 were then treated with RNaseH. To quantify the expression of the MUC5AC gene, 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') [17]. 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 was presented as a ratio of hPBGD.

2.7. NF-κB transcription factor assay

Cells cultured in 6-cm dishes were harvested and nuclear extracts from cells were obtained using a nuclear/cytosol fractionation kit (BioVision, Lyon, France) according to the manufacturer's protocol. Activities of p65 were investigated using 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 and capture probe, double-stranded biotinylated oligonucleotides containing the consensus sequence for binding NF-κB, were added to a streptavidin-coated 96-well plate. After incubation, bound p65 was detected using a primary anti-p65 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.

2.8. Western blot analysis

Cells were harvested after treatment, and were washed and homogenized at 4°C in lysis buffer (0.1% sodium dodecyl sulfate [SDS], 1% Igepal CA-630 and 0.5% sodium deoxycholate). Cell lysates (20-50 µg) were resolved by electrophoresis on a 12.5% polyacrylamide gel, and were transferred to a polyvinylidene difluoride membrane. After blocking the membrane in 10% FBS and 0.1% Tween-20 in Tris-buffered saline for 1 h at room temperature, blots were hybridized overnight at 4°C with primary antibodies. After hybridization with secondary antibody, the immunocomplexes were visualized using an ECL Western blotting detection system (GE Healthcare, Chalfont St. Giles, United Kingdom).

2.9. Statistical analysis

All data were expressed as means and standard deviation (SD). Fig. 2C and 2D were analyzed by unpaired *t*-test. In other examinations, one-way analysis of variance was used to determine statistically significant differences between groups. Dunnett's test was used to confirm differences as compared to the control group. Differences were considered to be statistically significance at $p < 0.05$.

3. Results

3.1. Preparation of *C. pneumoniae*

In order to exclude the antibacterial effects of drugs, we used inactivated *C. pneumoniae* EBs. We confirmed that these EBs lost their infectivity because no inclusion body was observed in purified EB-treated HEP-2 cells (data not shown). The number of *C. pneumoniae* used throughout this study was analyzed by real-time PCR and the mean concentration was 6.55 ± 0.50 (SD) \log_{10} copies per 10 µg ($n = 6$). We used *C. pneumoniae* at several protein concentrations, and diluted *C. pneumoniae* correlated well with the quantitative results for the

C. pneumoniae ompA gene (data not shown).

3.2. *C. pneumoniae* induces MUC5AC protein production and gene expression

In order to determine whether *C. pneumoniae* protein induces production of MUC5AC in NCI-H292 cells, we evaluated MUC5AC protein by ELISA and gene expression by quantitative RT-PCR. *C. pneumoniae* dose-dependently increased MUC5AC production (Fig. 1A and 1B) after 24 hours of stimulation. At the mRNA level, *C. pneumoniae* dose-dependently up-regulated MUC5AC gene expression after 8 hours of stimulation with a significant difference at 10 µg/ml (Fig. 1C). For subsequent experiments, we thus used *C. pneumoniae* at a concentration of 10 µg/ml.

3.3. *C. pneumoniae* activates p65 and p50 involved in MUC5AC production

NF-κB is known to be involved in MUC5AC gene expression. To study the involvement of NF-κB, we examined the activity of p65 and p50, major subunits of NF-κB, after *C. pneumoniae*-stimulation. The activity of p65 rose significantly after 20 min, and continued to rise until it peaked at 60 min (Fig. 2A). p50 was also activated after stimulation, with peak activity seen at 40 min (Fig. 2B). Furthermore, in order to determine whether NF-κB is involved in the production of MUC5AC, we evaluated MUC5AC protein using a specific NF-κB inhibitor, CAPE. CAPE (10 µM) decreased the levels of *C. pneumoniae*-induced MUC5AC in the supernatant by 28% (Fig. 2C, $p = 0.050$) and in the cell lysate by 72 % (Fig. 2D, $p = 0.043$).

3.4. *C. pneumoniae* phosphorylates ERK

Mitogen-activated protein kinases (MAPKs) are important signals related to MUC5AC production. We focused on ERK and examined the time course of phosphorylation by

Western blotting (Fig. 3A). Phosphorylated ERK band was not seen at the beginning of stimulation, but appeared after 20 min. Phosphorylation of ERK remained at similar levels for 60 min. Moreover, PD98059 (50 μ M) and U0126 (10 μ M), ERK inhibitors, strongly suppressed the production of MUC5AC (Fig. 3B and 3C).

3.5. Macrolide antibiotics suppress *C. pneumoniae*-induced MUC5AC production

We examined the effects of azithromycin, clarithromycin and telithromycin on *C. pneumoniae*-induced MUC5AC production. To eliminate the effects of antibiotic against *C. pneumoniae*, we pretreated cells with macrolide or ketolide antibiotics for 30 min before stimulation. Azithromycin dose-dependently reduced *C. pneumoniae*-induced MUC5AC production (Fig. 4A and 4B). Clarithromycin and telithromycin dose-dependently reduced *C. pneumoniae*-induced MUC5AC production with a significant difference seen at 50 μ g/ml (Fig. 4C-4F). Based on these results, we analyzed the inhibitory effects of macrolide antibiotics at a concentration of 50 μ g/ml.

3.6. Macrolide antibiotics suppress *C. pneumoniae*-induced MUC5AC gene expression

We also examined the inhibitory effects of macrolides at the mRNA level. After pre-treatment with macrolide antibiotics, cells were treated with *C. pneumoniae* for 8 h. Macrolide antibiotics reduced *C. pneumoniae*-induced MUC5AC gene expression to around the same levels as the control, with significant differences seen vs. stimulation alone (Fig. 5).

3.7. Clarithromycin and telithromycin, but not azithromycin suppress *C. pneumoniae*-induced NF- κ B activation.

After pre-treatment with macrolides or ketolides for 30 min, cells were then treated with *C.*

pneumoniae for 60 min. *C. pneumoniae*-induced p65 activation was significantly suppressed by clarithromycin and telithromycin but not azithromycin (Fig. 6A). Activated p50 was suppressed to baseline levels only after treatment with telithromycin (Fig. 6B).

3.8. Macrolide antibiotics do not down-regulate *C. pneumoniae*-induced phospho-ERK

In order to investigate the effects of macrolides and ketolides on MAPK levels, cells pre-treated with macrolides or ketolides were stimulated with *C. pneumoniae* for 20 min. After that, phosphorylation levels of ERK were detected by Western blotting. These drugs did not affect the detection levels when compared to stimulation alone (Fig. 7).

4. Discussion

The present study demonstrates that *C. pneumoniae* protein increases mucus production, and that *C. pneumoniae*-induced mucus production can be suppressed by macrolide and ketolide antibiotics. This indicates a possible therapeutic approach to treat bronchial asthma.

Airway mucus hypersecretion is a pathologic feature seen in obstructive lung diseases. MUC5AC, the major core protein of mucin, is up-regulated by various stimulants, including air pollutants [18], inflammatory mediators [19], cytokines [20] and bacterial components related to chronic airway diseases [12, 21]. In asthma patients, the airway mucus consists primarily of MUC5AC [6].

The relationship between bronchial asthma and pathogenic organisms has recently described in various studies. *Mycoplasma pneumoniae* has been shown to have an association with asthma [22]. *M. pneumoniae* induces MUC5AC production in human lung epithelial cell lines and in airway epithelial cells isolated from asthmatic patients [23]. Rhinovirus infection

related to exacerbation of asthma increases MUC5AC and total mucin concentration in human tracheal epithelial cells [17].

The possible role of *C. pneumoniae* in the pathogenesis of asthma has also been studied. Several studies have reported that *C. pneumoniae* infection is associated with exacerbation of asthma and stable asthma [7, 8]. Surprisingly, in the TELICAST study, about sixty percent of patients showing exacerbation of asthma were found to have *C. pneumoniae* [24]. We demonstrated that *C. pneumoniae* dose-dependently increased MUC5AC production at both the mRNA and protein levels. Considering that airway mucus hypersecretion is one of the pathogenesises of asthma, our study indicates that *C. pneumoniae* may cause exacerbation of asthma by producing airway mucin.

NF- κ B has been shown to play an important role in mucin production in numerous studies. In this study, *C. pneumoniae* activated both p65 and p50. Although CAPE decreased *C. pneumoniae*-induced MUC5AC production, it did not suppress it completely. These findings suggest that NF- κ B is partly associated with the MUC5AC production in the airway epithelial cells after *C. pneumoniae* contact. NF- κ B is not the only transcription factor that up-regulates MUC5AC gene expression. The AP-1 transcription factor binding site and NF- κ B binding site are located in the upstream of the MUC5AC gene [25]. AP-1 may be also involved in MUC5AC gene expression after *C. pneumoniae*-stimulation. Furthermore, *C. pneumoniae* up-regulated ERK phosphorylation and *C. pneumoniae*-induced MUC5AC production was inhibited by PD98059 and U0126 pre-treatment. These findings suggest that the ERK pathway may be associated with *C. pneumoniae*-induced mucin production. Other MAPKs, such as p38 and JNK, were not involved in *C. pneumoniae*-induced MUC5AC production (data not shown).

In this study, *C. pneumoniae*-induced MUC5AC production was suppressed by macrolides and ketolides. Azithromycin suppressed *C. pneumoniae*-induced MUC5AC at mRNA level

after 8 h stimulation as well as the other drugs but appeared to be less effective at protein level after 24 h stimulation than the others. This finding of azithromycin may reflect shortness of its effect. With regard to NF- κ B levels, although telithromycin markedly suppressed p65 and p50 activity, clarithromycin suppressed only p65 activity, despite showing similar effects as telithromycin with regard to MUC5AC mRNA and protein production. This indicates that p65 may play a more important role in MUC5AC production than p50. Azithromycin also decreased p65 activation when compared to positive controls, but there was no statistically significant difference in the results for protein levels. Differences in effectiveness among macrolides have also been observed in cytokine production *in vivo* [26]. The suppressive effects vary depending on their actions against upstream signal transduction or transcriptional factors.

As mentioned above, each drug attenuated NF- κ B activation, but did not inhibit the phospho-ERK after *C. pneumoniae* stimulation. This indicates that macrolides and ketolides inhibit pathways between ERK and NF- κ B to interfere with *C. pneumoniae*-induced MUC5AC expression.

Macrolides and telithromycin have recently attracted attention in the treatment of bronchial asthma. Additional therapy with clarithromycin in the treatment of severe bronchial asthma patients significantly improved quality-of-life scores with reducing airway concentrations of IL-8 and neutrophil numbers [27]. In patients with exacerbation of asthma, co-treatment with telithromycin improved symptom scores and lung functions [24]. The reasons for the clinical benefits of these drugs among asthma patients are not well understood. Although macrolides and ketolides have antibacterial activity against *C. pneumoniae*, we consider that the effects of drugs in our data are results of immunomodulatory effects because noninfectious *C. pneumoniae* EBs were used. Our results suggest that macrolides and ketolides attenuate excessive mucus production, which then alleviates asthma symptoms. Considering the role of

C. pneumoniae in exacerbation of asthma, our results support such clinical benefits in these patients.

In this study, we used these antibiotics at a concentration of 50 µg/ml. The mean steady-state concentrations of these antibiotics in alveolar macrophages after oral administration are reported to reach over 300 µg/ml [28, 29], while those in the epithelial lining fluid reached over 3 µg/ml [28-30]. Therefore, the concentration of antibiotics used in this study may be attainable in clinical cases.

In conclusion, *C. pneumoniae* up-regulates MUC5AC production in airway epithelial cells and macrolides and ketolide suppress this MUC5AC production by interfering with signal transduction. Because mucus hypersecretion is thought to be one of the mechanisms of *C. pneumoniae*-induced exacerbation of bronchial asthma, macrolide and ketolide antibiotics may represent a novel therapeutic intervention in the treatment of bronchial asthma.

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Figure legends

Fig. 1. Production and gene expression of MUC5AC protein after *C. pneumoniae* stimulation. Cells were stimulated with 0.1, 1.0 or 10 $\mu\text{g/ml}$ *C. pneumoniae*. As a control, cells were treated with culture medium alone. (A, B) After 24 hours of stimulation, protein levels were measured by ELISA and are given in terms of % above control (n = 4). *C. pneumoniae* dose-dependently increased MUC5AC production in the supernatant (A) and in the cell lysate (B). Data are expressed as the means \pm S.D. (C) After 8 hours of stimulation, gene expression levels were measured by quantitative RT-PCR and were normalized against hPBGD (n = 3). *C. pneumoniae* dose-dependently increased MUC5AC gene expression, with a significant difference seen at 10 $\mu\text{g/ml}$. * indicates $p < 0.05$ and † indicates $p < 0.01$ vs. controls, respectively.

Fig. 2. Activation of NF- κ B after stimulation with 10 $\mu\text{g/ml}$ *C. pneumoniae* for the indicated time periods (n = 3). (a and b) *C. pneumoniae*-induced p65 activation was observed after 20 min and gradually increased within 60 min (A). Activated p50 was observed after 20 min and peaked at 40 min (B). Results are expressed as means \pm SD. * indicates $p < 0.05$ and † indicates $p < 0.01$ versus results at 0 min. (C and D) Cells were pretreated with specific a NF- κ B inhibitor, CAPE, for 30 min. CAPE at 10 μM suppressed MUC5AC production in the supernatant (C, $p = 0.050$ vs. stimulation alone) and in the cell lysate (D, $p = 0.043$ vs. stimulation alone).

Fig. 3. Phosphorylation of ERK after *C. pneumoniae* stimulation. (A) Cells were treated with 10 µg/ml *C. pneumoniae* for the indicated time periods. ERK and phospho-ERK were detected by Western blotting. Phospho-ERK was not seen initially, but appeared after 20 min. Data is representative of three separate experiments. (B and C) The effects of PD98059 and U0126, ERK inhibitors, on MUC5AC production were analyzed at the protein level by ELISA. PD98059 (50 µM) and U0126 (10 µM) markedly reduced MUC5AC production both in the supernatant (B) and in the cell lysate (C). † indicates $p < 0.01$

Fig. 4. Effects of macrolide and ketolide antibiotics on MUC5AC production induced by *C. pneumoniae*. Cells were pretreated with drugs for 30 min and were then stimulated with 10 µg/ml *C. pneumoniae* for 24 h (n = 4). MUC5AC protein was analyzed by ELISA both in the supernatant (A, C and E) and in the cell lysate (B, D and F). Azithromycin (A and B), clarithromycin (C and D) and telithromycin (E and F) dose-dependently suppressed *C. pneumoniae*-induced MUC5AC production. At 50 µg/ml, all of the drugs except azithromycin significantly decreased MUC5AC production when compared to stimulation alone in the cell lysate ($p = 0.050$). * indicates $p < 0.05$ and † indicates $p < 0.01$.

Fig. 5. Effects of macrolide and ketolide antibiotics on MUC5AC gene expression induced by *C. pneumoniae*. Cells were pretreated with each drug at 50 µg/ml for 30 min and were then stimulated by 10 µg/ml of *C. pneumoniae* for 8 h (n = 5). Each drug significantly suppressed *C. pneumoniae*-induced MUC5AC gene expression. † indicates $p < 0.01$ versus stimulation alone.

Fig. 6. Effects of macrolide and ketolide antibiotics on NF- κ B. Cells were pretreated with each drug at 50 μ g/ml for 30 min, and were then stimulated by *C. pneumoniae* for 60 min (n = 4). All drugs suppressed p65 activation (A), but only telithromycin suppressed p50 activation (B). † indicates $p < 0.01$ vs. stimulation alone.

Fig. 7. Effects of macrolide and ketolide antibiotics on ERK phosphorylation. Cells were stimulated with *C. pneumoniae* for 20 min after pretreatment with each drug. Equal amounts of protein were analyzed. Drugs did not affect the detection levels of phospho-ERK when compared to stimulation alone. Data are representative of three separate experiments.

Fig. 1

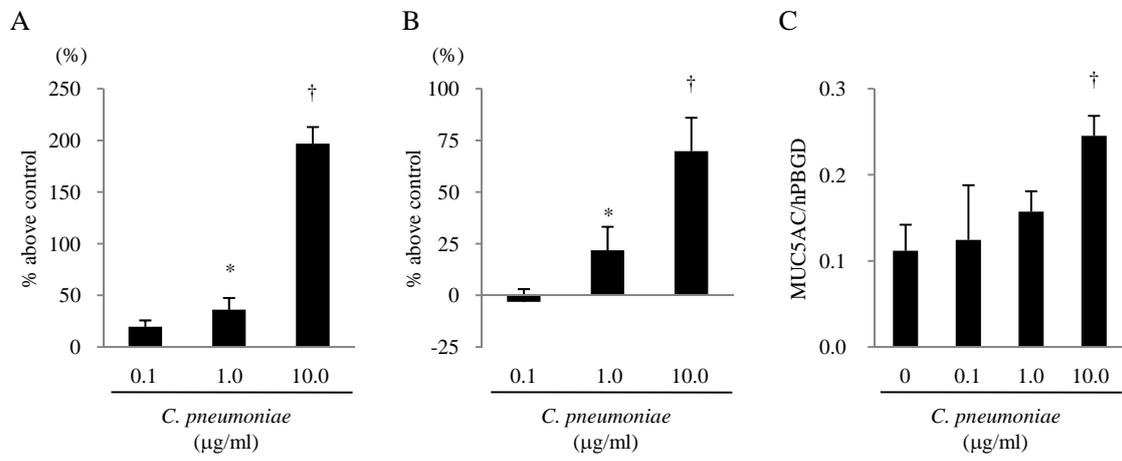


Fig. 2

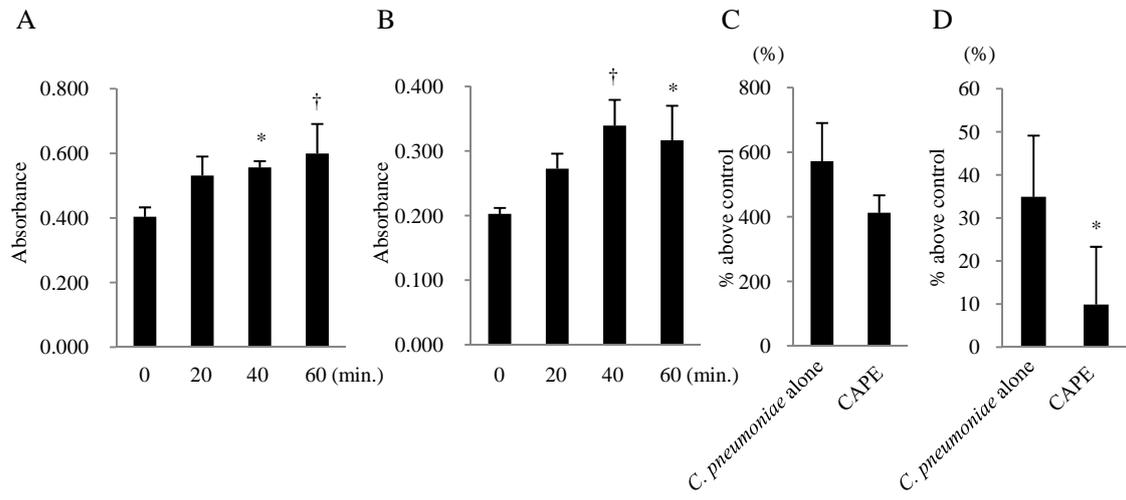


Fig. 3

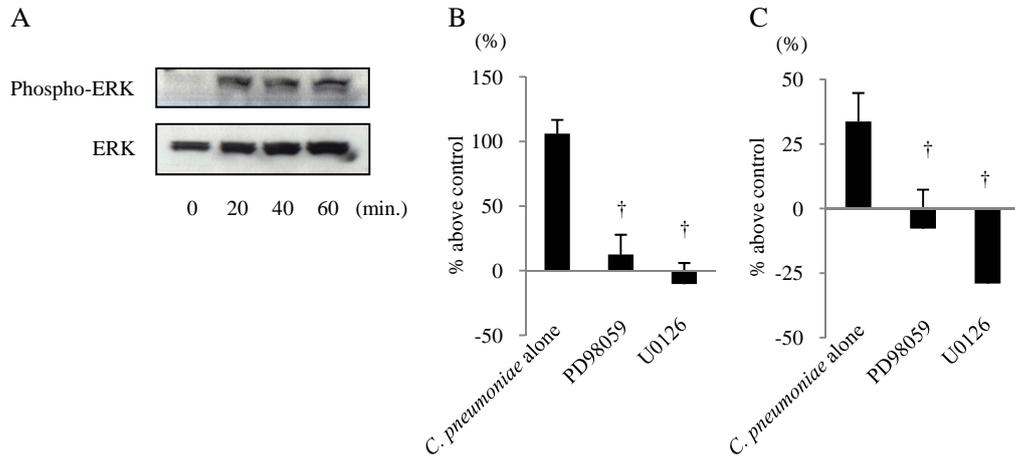


Fig. 4

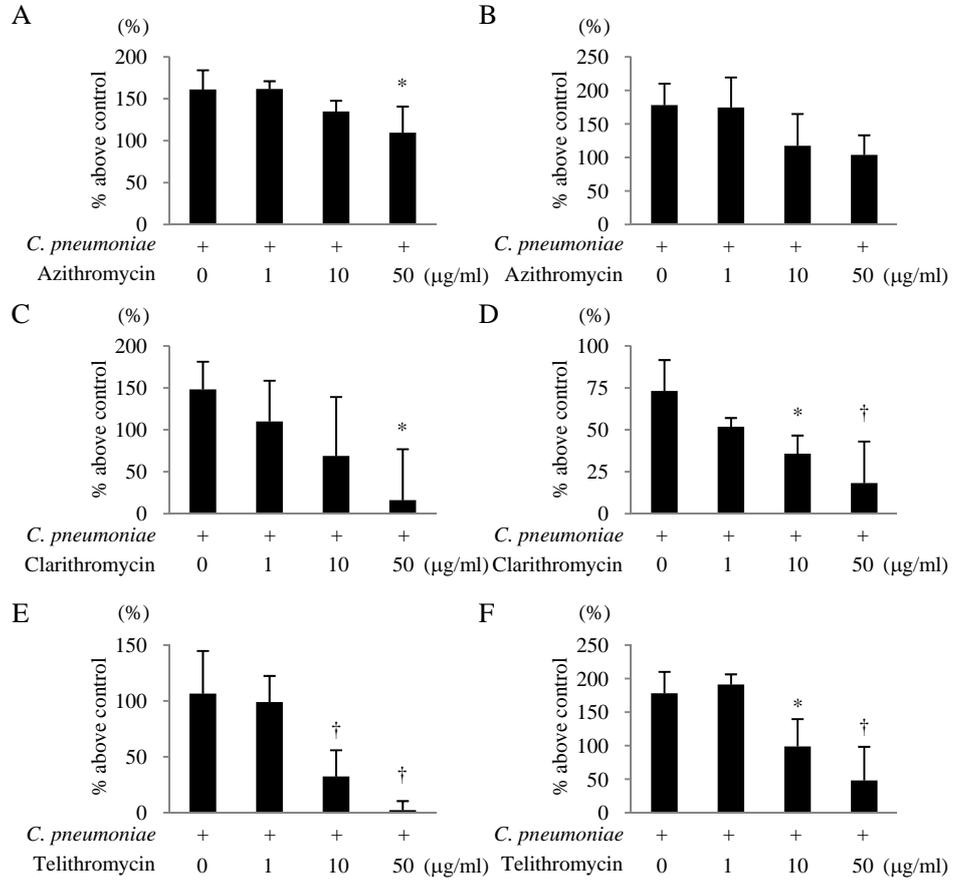


Fig. 5

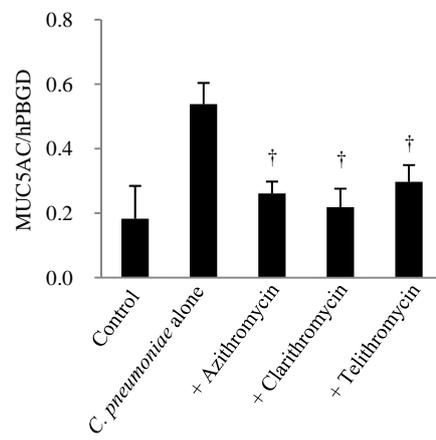


Fig. 6

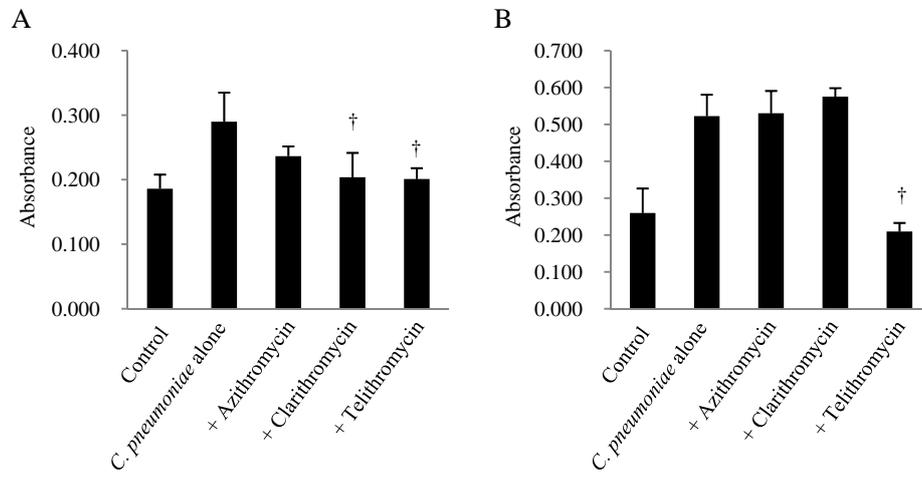


Fig. 7

