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Macrolide antibiotics inhibit respiratory syncytial virus infection in human airway epithelial cells

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ABSTRACT

To examine the effects of macrolide antibiotics on RS virus infection in airways, human tracheal epithelial cells were pre-treated with bafilomycin A_1 and clarithromycin, and infected with RS virus. Viral titers in supernatant fluids and RNA of RS virus, and concentrations of cytokines in supernatant fluids, including interleukin-6 increased with time after infection. Bafilomycin A_1 and clarithromycin reduced viral titers in supernatant fluids of RS virus, RNA of RS virus, the susceptibility to RS virus infection, and concentrations of cytokines induced by virus infection. *N-acetyl-S-geranylgeranyl-L-cysteine*, an inhibitor for a small GTP binding protein of RhoA, isoform A of the Ras-homologus (Rho) family, an active form of which is associated with RS virus infection via binding to its fusion protein (F protein), reduced viral titers in supernatant fluids and RNA of RS virus. Bafilomycin A_1 and clarithromycin inhibited RhoA activation induced by lysophosphatidic acid in the cells. Fasudil, an inhibitor of Rho kinase, also reduced viral titers in supernatant fluids and RNA of RS virus. These findings suggest that macrolide antibiotics may inhibit RS virus infection, partly through the reduced expression of F protein receptor, activated RhoA, and the inhibition of subsequent Rho kinase activation in human airway epithelial cells.

1. Introduction

Respiratory syncytial (RS) virus is one of the important pathogens of common colds (Hayden and Gwaltney, 1988), and is the major cause of viral lower respiratory tract disease in infants and young children (Collins and Crowe, 2006). A relationship was reported between wheezing-associated respiratory illness and RS virus outbreaks in children (Henderson et al., 1979). RS virus infection was also suggested to be an important illness in the elderly and high-risk adults (Falsey et al., 2005; Zambon et al., 2001), and associated with the development of exacerbations of chronic obstructive pulmonary disease (COPD) (Guidry et al., 1991).

RS virus F glycoprotein, the part of the virus that binds to the receptor for RS virus (Collins and Crowe, 2006), can interact with the activated intracellular protein RhoA (Budge and Graham, 2004; Pastey et al., 1999), the isoform A of a small guanosine triphosphatase (GTPase) of the Ras superfamily (Rho, Ras-homologus) (Takai et al., 2001). The F protein promotes fusion of viral and cellular membranes with subsequent transfer of viral genome material into the cell, and promotes syncytial formation of the infected cells (Collins and Crowe, 2006). Pastey et al. (2000) demonstrated the inhibitory effects of a RhoA-derived peptide on syncytium formation induced by RS virus. RhoA signaling is also suggested to relate to cell-to-cell fusion and syncytium formation after RS virus infection (Gower et al., 2005). However, clinically available anti-RS virus agents have not been well studied.

RhoA has various functions including stimulus-evoked cell adhesion and motility, enhancement of contractile response and cytokinesis (Narumiya, 1996; Takai et al., 2001). RhoA functions are modulated by a variety of agents including bafilomycin A_1, one of the macrolide antibiotics and a specific inhibitor of the vascular-ATPase (V-ATPase) (Palokangas et al., 1997), and *N-acetyl-*

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S-geranylgeranyl-L-cysteine (AGGC) (Lu et al., 2004). Macrolide antibiotics bafilomycin A_1, erythromycin, and clarithromycin inhibit infection of rhinovirus (RV) (Jang et al., 2006; Suzuki et al., 2001a, 2002), the major cause of the common cold (Racaniello, 2006), in human airway epithelial cells by the reduction of intercellular adhesion molecule-1 (ICAM-1), the receptor for a major group of RV, and by affecting the acidification of endosomes, where RV RNA enters into the cytoplasm of infected cells. However, the inhibitory effects of macrolides on the infection of RS virus are still uncertain.

Neutrophilic and eosinophilc inflammation in the exacerbations of bronchial asthma and COPD are associated with a variety of mediators including interleukin (IL)-6 and IL-8, the production and secretion of which are stimulated by RS virus in airway epithelial cells as shown previously (Noah and Becker, 1993; Tripp et al., 2005). Bafilomycin A_1, erythromycin and clarithromycin reduce pro-inflammatory cytokines including IL-6 after RV infection in airway epithelial cells (Jang et al., 2006; Suzuki et al., 2001a, 2002). Macrolide antibiotics have clinical benefits in improving the quality of life in refractory asthma patients (Simpson et al., 2008) and reducing COPD exacerbations (Seemungal et al., 2008). However, the inhibitory effects of macrolides on cytokine production after RS virus infection are still uncertain.

We therefore examined the inhibitory effects of bafilomycin A_1 and clarithromycin, macrolide antibiotics, on RS virus infection. We also examined the effects of bafilomycin A_1 and clarithromycin on the production of cytokines, and the RhoA activation to clarify the mechanisms responsible for the inhibition of RS virus infection.

2. Materials and methods

2.1. Media components

Reagents for cell culture media were obtained as follows: Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, phosphate-buffered saline (PBS), and fetal calf serum (FCS) were from GIBCO-BRL Life Technologies, Palo Alto, CA; ultroser G (USG) was from BioSepra, Cergy-Saint-Christophe, France.

2.2. Human tracheal epithelial cell culture

Isolation and culture of the human tracheal surface epithelial cells were performed as described previously (Suzuki et al., 2001a, 2002; Terajima et al., 1997) with some modification (Yamaya et al., 2007). The human tracheal surface epithelial cells were plated at 5×10^5 viable cells/ml in plastic tubes with round bottoms (16 mm diameter and 125 mm length, Becton Dickinson) coated with human placental collagen, because cell attachment in plastic tubes was better than that in glass tubes (data not shown) (Yamaya et al., 2007). Cells were immersed in 1 ml of a mixture of DMEM–Ham's F-12 (DF-12) medium (50/50, vol/vol) containing 2% USG and antibiotics (Suzuki et al., 2001a, 2002; Terajima et al., 1997) in plastic tubes. The tubes were laid and kept stationary in a humid incubator with a slant of ~5°. Because of this position of the plastic tubes, the cells attached and proliferated mainly on the inner surface of the lateral wall of the tubes. The surface area of culture vessels of the plastic tubes covered by the cells became $11.4 \pm 0.2\,cm^2$ ($n = 3$). The opening of the tubes was loosely covered with a screw cap to make air containing carbon dioxide (CO_2) move through the slit. Cells in the tubes were cultured at 37 °C in 5% CO_2–95% air. We confirmed the presence of a dome formation when the cells made confluent cell sheets on days 5–7 of culture using an inverted microscope (MIT-2, Olympus, Tokyo, Japan) (Suzuki et al., 2001a, 2002) as described by Widdicombe et al. (1987).

Tracheas for cell cultures were obtained after death from 33 patients (age, 74 ± 17 year; 14 females, 19 males) without complications with bronchial asthma or COPD. The causes of death included malignant tumor other than lung cancer ($n = 16$), congestive heart failure ($n = 6$), cerebral bleeding ($n = 3$), acute myocardial infarction ($n = 2$), renal failure ($n = 2$), rupture of an aortic aneurysm ($n = 2$) and cerebral infarction ($n = 2$). Of 33 patients, 9 patients were ex-smokers, and 24 patients had never smoked. This study was approved by the Tohoku University Ethics Committee.

2.3. Culture of human epithelial Hep-2 cells

Human epithelial cell line Hep-2 cells were cultured in Roux type bottles sealed with a rubber plug in minimum essential medium (MEM) containing 5% calf serum supplemented with 5×10^4 U/l penicillin, 50 mg/l streptomycin and 1.7% glucose (Numazaki et al., 1987). The cells were then plated in plastic dishes (96-well plate, Becton Dickinson) or in plastic tubes with round bottoms (Becton Dickinson).

2.4. Viral stocks

Respiratory syncytial (RS) virus was prepared in our laboratory from a patient with a common cold (Numazaki et al., 1987). Serotype of RS virus was identified with the methods as described previously (Peret et al., 1998), and we found that the type of the RS virus used in this study was type A. Stocks of RS virus were generated by infecting Hep-2 cells cultured in plastic tubes in 1 ml of the MEM supplemented with 2% ultra-low IgG FCS, 5×10^4 U/l penicillin, 50 mg/l streptomycin and 1.7% glucose at 33 °C. To obtain the RS virus solution, 7 days after infection with RS virus, Hep-2 cells and culture medium in the tubes were frozen in a short time in ethanol at −80 °C, thawed and sonicated. The virus-containing fluid was frozen in aliquots at −80 °C.

2.5. Detection and titration of viruses

Detection and titration of RS viruses in supernatant fluids were performed with the endpoint methods (Condit, 2006), by infecting replicate confluent Hep-2 cells in plastic 96-well dishes (Becton Dickinson) with serial 10-fold dilutions of virus-containing supernatant fluids. In brief, virus-containing supernatant fluids were 10-fold diluted in MEM supplemented with 2% ultra-low IgG FCS and 1.7% glucose (Numazaki et al., 1987; Terajima et al., 1997), and added into the replicate Hep-2 cells in the wells (200 μl/well) of 96-well dishes. Hep-2 cells in the wells were then cultured at 33 °C in 5% CO_2–95% air for 7 days, and the presence of the big syncytium, which shows typical cytopathic effects (CPE) of RS virus, was examined in all replicate cells as described previously (Condit, 2006; Numazaki et al., 1987). The number of wells which showed CPE of RS virus was counted in each dilution of supernatant fluids. Then, the dilution of virus-containing supernatant fluids which showed CPE in greater than 50% of replicate wells, and the dilution of the fluids which showed CPE in less than 50% of replicate wells were estimated. Based on these data, $TCID_{50}$ (TCID: tissue culture infective dose) was calculated with the methods as previously described (Condit, 2006). Because the human tracheal epithelial cells were cultured in 1 ml of DF-12 medium containing 2% USG in the tubes, viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml (Condit, 2006; Numazaki et al., 1987; Terajima et al., 1997).

2.6. Viral infection of the cells

Infection of RS virus to human tracheal epithelial cells was performed with methods previously described (Ishizuka et al., 2003; Terajima et al., 1997). A stock solution of RS virus (100 μl in each

tube, 1.0×10^4 $TCID_{50}$ units/ml) was added to the human tracheal epithelial cells in the tubes ($(2.0 \pm 0.3) \times 10^6$ of cells/tube, $n = 7$). Then, the multiplicity of infection (moi) was 0.5×10^{-3} $TCID_{50}$ units/cell. We found in preliminary experiments that the viral titers of RS virus stock solution (1.0×10^4 $TCID_{50}$ units/ml), used in this study, measured with the endpoint methods (Condit, 2006) showed similar values compared with those (1.1×10^4 pfu/ml) (pfu: plaque-forming units) measured with the plaque assay method (McKimm-Breschkin, 2004). After a 1-h incubation at 33 °C in 5% CO_2–95% air (Numazaki et al., 1987), the viral solution was removed, and the epithelial cells were rinsed one time with 1 ml of PBS. The cells were then fed with 1 ml of fresh DF-12 medium containing 2% USG supplemented with antibiotics. The opening of the tubes was sealed with rubber plugs and cells were cultured at 33 °C with rolling in an incubator (HDR-6-T, Hirasawa, Tokyo, Japan) as described previously (Ishizuka et al., 2003; Numazaki et al., 1987; Terajima et al., 1997). The supernatant fluids were stored at −80 °C for the determination of viral titers.

2.7. Treatment of the cells with macrolides

In order to examine the effects of macrolides on RS virus infection, the cells were treated with bafilomycin A_1 (10 nM) or clarithromycin (10 μM) (Jang et al., 2006), unless we described other concentrations. Cells were treated with macrolides from 3 days before RS virus infection until the end of the experiments after RS virus infection. A concentration of 10 nM of bafilomycin A_1 was chosen, because we found in preliminary experiments that bafilomycin A_1 at this concentration showed the inhibitory effects on RS viral titers in supernatant fluids with the similar potency, compared with the potency of the inhibitory effects of 10 μM of clarithromycin. A concentration of 10 μM of clarithromycin was chosen, because a concentration of 15 μM of clarithromycin is the maximum serum concentration of macrolides in clinical use (500 mg of oral clarithromycin administration) (Honeybourne et al., 1994).

In order to study the relationship between pre-incubation time and the potency of inhibitory effects, we examined the effects of pre-treatment time on viral titers in supernatant fluids. Cells were pre-treated with macrolides for times ranging from 0 h to 72 h. In preliminary experiments, we found that consistent inhibitory effects were obtained when the cells were pre-treated with macrolides for 72 h. Therefore, cells were pre-treated with macrolides for 72 h (3 days) in this study.

We also studied the relationship between concentration of macrolides and the potency of inhibitory effects.

Macrolides were dissolved in ethanol in this study. However, when we made up the DF-12 medium containing 100 μM of clarithromycin, clarithromycin was dissolved in dimethyl sulfoxide (DMSO, Sigma) (Jang et al., 2006), because of the difficult solubility of it in ethanol.

2.8. Collection of supernatant fluids for viral titer measurements

In order to measure the viral titers in supernatant fluids during 1–3 days after RS virus infection, we used one culture from each trachea after collecting 1 ml of supernatant fluids at 1 day (24 h) after RS virus infection. After collecting supernatant fluids at 1 day after infection, the cells were rinsed with PBS and 1 ml of DF-12 medium containing 2% USG was replaced. Supernatant fluids were also collected at 3 days after infection. Likewise, to measure the viral titers in supernatant fluids during 3–5 days after RS virus infection, after collecting 1 ml of supernatant fluids at 3 days after infection, the cells were rinsed with PBS and 1 ml of the fresh DF-12 medium was replaced. Supernatant fluids (1 ml) were also collected at 5 days after RS virus infection.

2.9. Effects of macrolides on susceptibility to RS virus infection

The effects of macrolides on the susceptibility to RS virus infection were examined as described previously (Suzuki et al., 2001a, 2002; Terajima et al., 1997). The human tracheal epithelial cells were treated with bafilomycin A_1 (10 nM), clarithromycin (10 μM) (Jang et al., 2006) or vehicle of clarithromycin (0.1% ethanol) from 3 days before infection with RS virus until just finishing the RS virus infection. The cells were then exposed to serial 10-fold dilutions of RS virus for 1 h at 33 °C. The presence of RS virus in the supernatant fluids collected for 3–5 days after infection was determined with the methods described above to assess whether infection occurred at each dose of the RS virus used.

2.10. Quantification of RS Virus RNA

To quantify the RS virus RNA and β-actin mRNA expression in the human tracheal epithelial cells after RS virus infection, real-time quantitative RT-PCR using the TaqMan technique was performed as previously described (Heid et al., 1996; Holland et al., 1991; Martell et al., 1999; Suzuki et al., 2002) with some modification (Yamaya et al., 2007). The fragment of RS virus RNA was extracted from the human tracheal epithelial cells before or at either 3 days (72 h) or 5 days (120 h) after infection by RS virus using RNA-Bee (Tel-Test, Inc., Friendswood, TX). cDNA was reverse-transcribed from 10 μg RNA with primers for RS virus using the Omniscript RT Kit (Qiagen K.K., Tokyo, Japan). Then, PCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Briefly, synthesized cDNA (100 ng) was mixed in 40 μl of buffer containing 100 nM forward primer (5′-TGGTGTAGTTGGAGTGCTAGAGAGAGTT-3′), 100 nM reverse primer (5′-TGTCCCTCAGCTTTTTGATATCATC-3′), 250 nM Taqman probe [5′-(FAM) CTAAACAATCAGCATGTGTTGCCATGAGCA (TAMRA)-3′] for RS virus, as previously described (Yamaya et al., 2007). Real-time PCR was performed with StepOne Real-Time PCR System (Applied Biosystems). The standard curve was obtained between the fluorescence emission signals and Cτ by means of 10-fold dilutions of the total RNA, extracted from 1×10^4 $TCID_{50}$ units/ml of RS virus in the supernatant fluids of the Hep-2 cells 7 days after infection with RS virus (1×10^3 $TCID_{50}$ units). Real-time quantitative RT-PCR for β-actin mRNA was also performed using the same PCR products. The standard curve was obtained between the fluorescence emission signals and Cτ by means of 10-fold dilutions of the mRNA extracted from the cells. The expression of β-actin mRNA was used as control, and the expression of RS virus RNA was normalized to the constitutive expression of β-actin mRNA.

2.11. Effects of N-acetyl-S-geranylgeranyl-L-cysteine on RS virus infection

N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) (1 μM) inhibits RhoA activation in pulmonary artery endothelial cells (Lu et al., 2004). In order to examine the effects of AGGC on the RS virus infection, human tracheal epithelial cells were treated with AGGC (1 μM) from 3 days before RS virus infection until the end of the experiments after RS virus infection.

2.12. Measurement of RhoA activation

RhoA activation was assessed by a method described elsewhere (Chikumi et al., 2002; Kadowaki et al., 2004; Yamaguchi et al., 2001) with a modification. A GTP-bound form of RhoA (RhoA-GTP) associated with GST-Rho-binding domain (RBD) was reported to show RhoA activation (Chikumi et al., 2002; Kadowaki et al., 2004; Yamaguchi et al., 2001). Amounts of the RhoA-GTP were

quantified by Western blot analysis in human tracheal epithelial cells. Cells were pre-treated with bafilomycin A_1 (10 nM) or clarithromycin (10 μM) for 3 days. After stimulation with lysophosphatidic acid (LPA) (1 μM, for 5 min) (Mills and Moolenaar, 2003), cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated with glutathione S-transferase (GST) fusion protein including the Rho-binding domain (RBD) of rhotekin. The fusion protein had been bound to glutathione-Sepharose beads (Glutathione Sepharose 4B, Amersham). The incubation was followed by washing with lysis buffer. RhoA-GTP associated with GST-RBD was then released from the beads by the addition of a protein loading buffer (125 mM, Tris/Cl, pH 6.8, 2% glycerol, 4% SDS), and quantified by Western blot analysis using a monoclonal antibody against RhoA (26C4; Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were subsequently incubated with suitable horseradish peroxidase-coupled secondary antibodies. Bands were visualized using an ECL chemiluminescent kit (Amersham Biosciences, Piscataway, NJ) and CCD camera. The bands were quantified by densitometric scanning using ImageJ (http://rsb.info.nih.gov/ij/index.html) (Hegab et al., 2008).

2.13. Effects of Rho kinase inhibitor on RS virus infection

In order to examine the effects of Rho kinase on RS virus infection, human tracheal epithelial cells were pre-treated with fasudil (10 μM, HA1077) (Asahi Kasei Co., Tokyo, Japan), an inhibitor of Rho kinase including RhoA (Uehata et al., 1997; Yin et al., 2007), from 3 days before RS virus infection until the end of the experiments after RS virus infection. RS virus was infected to the cells, and viral titers in supernatant fluids and RS virus RNA were measured. We also examined the effects of Y-27632 (20 μM) (Gower et al., 2005), another Rho kinase inhibitor.

2.14. Measurement of cytokines production

We measured IL-1β, IL-6 and IL-8 of supernatant fluids by specific enzyme-linked immunosorbent assays (ELISAs) as previously described (Suzuki et al., 2001a, 2002; Terajima et al., 1997). In the preliminary experiments, we found that secretion of IL-1β, IL-6 and IL-8 all increased after RS virus infection, and maximum secretion was observed at 3 days after the infection. Therefore, to examine the effects of bafilomycin A_1 and clarithromycin, we measured the secretion of cytokines before and at 3 days after infection with RS virus infection.

2.15. Measurement of changes in pH in the acidic endosomes

The function of RhoA is reported to be regulated by low pH in endosomes (Palokangas et al., 1997). We previously reported that bafilomycin A_1 increases the pH of acidic endosomes in human tracheal epithelial cells (Suzuki et al., 2001a). Therefore, we studied the effects of clarithromycin on the pH of acidic endosomes. The fluorescence intensity of acidic endosomes in the cells was measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes) (Suzuki et al., 2001a, 2002). The effects of clarithromycin on the distribution of acidic endosomes and fluorescence intensity were examined from 100 s before to 300 s after the treatment with clarithromycin (10 μM) or vehicle (ethanol, 0.1%). Furthermore, we studied the effects of long periods of clarithromycin treatment (10 μM, 3 days) on the fluorescence intensity of acidic endosomes. Fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and the mean value of fluorescence intensity is expressed as % of control value compared with the fluorescence intensity of the cells treated with vehicle of clarithromycin (ethanol, 0.1%).

2.16. Statistical analysis

Results are expressed as means ± S.D. Statistical analysis was performed using one-way measures of analysis of variance (ANOVA). Subsequent post hoc analysis was made using Bonferroni's method. For all analyses, values of $P < 0.05$ were assumed to be significant. In the experiments using a culture of human tracheal epithelial cells, n refers to the number of donors (tracheae) from which cultured epithelial cells were used.

3. Results

3.1. Effects of macrolides on RS virus infection of human tracheal epithelial cells

Hep-2 cells did not show any syncytial formation when culture medium 2 h after removing inoculum and washing cells was added to the cells (data not shown), but supernatant fluids 1 day after infection produced syncytial formation on the cells (Fig. 1A). Exposing confluent human tracheal epithelial cell monolayers to RS virus (0.5×10^{-3} $TCID_{50}$ units/cell) consistently led to infection. RS virus was detected in supernatants fluids 1 day after infection, and the viral content progressively increased between 1 day and 5 days after infection (Fig. 1A). RS virus infection of the epithelial cells was constant, and evidence of continuous viral production was obtained by demonstrating that each of the viral titers in supernatant fluids collected during 0–1 day, 1–3 days and 3–5 days after infection contained significant levels of RS virus (Fig. 1A). The viral titers in supernatant fluids increased significantly with time for the 5 days of observation ($P < 0.05$ in each case by ANOVA).

In preliminary experiments, we found that viral titers in supernatant fluids measured using 96-well dishes ($(0.88 \pm 0.11) \times 10^5$ of Hep-2 cells/well in 200 μl of culture medium, $n = 6$) were the same as those using the tubes ($(8.6 \pm 1.1) \times 10^5$ of Hep-2 cells/tube in 1 ml of culture medium, $n = 6$), as described by Numazaki et al. (1987) (data not shown). However, the small amount of RS virus, which did not show the syncytial formation in Hep-2 cells, could not be detected in this system.

Treatment of the cells with bafilomycin A_1 (10 nM) significantly decreased the viral titers in supernatant fluids from 1 day after infection (Fig. 1A). Likewise, treatment of the cells with clarithromycin (10 μM) significantly decreased the viral titers in supernatant fluids from 3 days after infection (Fig. 1A), while the viral titers in supernatant fluids collected during 0–1 day in the cells treated with clarithromycin (10 μM) did not differ from those in the cells treated with vehicle (0.1% ethanol) (Fig. 1A).

Reduction of viral titers in supernatants fluids depended on pre-incubation time (Fig. 1B and C). In the cells pre-treated with bafilomycin A_1 (10 nM), a significant reduction was observed when the cells were pre-treated for 12 h or more, and maximum inhibition was observed when the pre-incubation time was 72 h (Fig. 1B). Likewise, in the cells pre-treated with clarithromycin (10 μM), a significant reduction was observed when the cells were pre-treated for 24 h or more, and maximum inhibition was observed when the pre-incubation time was 72 h (Fig. 1C).

Bafilomycin A_1 reduced viral titers in supernatant fluids concentration-dependently and the maximum effect was obtained at 0.1 μM (Fig. 2A). Clarithromycin also reduced viral titers in supernatant fluids concentration-dependently and the maximum effect was obtained at 100 μM (Fig. 2B). 0.1% of DMSO was contained in the DF-12 medium supplemented with 100 μM of clarithromycin

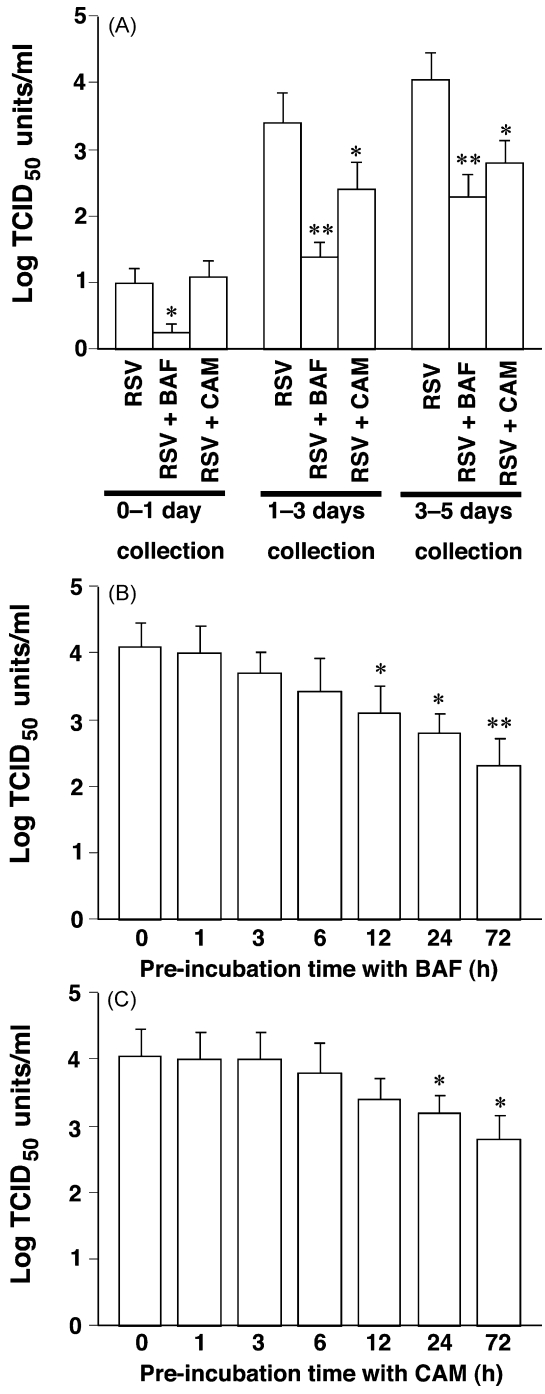

Fig. 1. (A) The time course of RS viral titers in supernatant fluids of human tracheal epithelial cells obtained for indicated days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in the presence of bafilomycin A_1 (10 nM; RSV + BAF), clarithromycin (10 μM; RSV + CAM), or vehicle (0.1% ethanol) (RSV). The viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 5 different tracheae. Significant differences from viral infection alone (RSV) are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$. The small amount of RS virus, which did not show the syncytial formation in Hep-2 cells, could not be detected in this system. (B) and (C) Viral titers in supernatant fluids collected during 3–5 days after infection of the cells pre-treated with bafilomycin A_1 (10 nM, BAF) (B) or clarithromycin (10 μM, CAM) (C) for times ranging from 0 h to 72 h. The viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 5 different tracheae. Significant differences from viral infection alone (time 0) are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

as a solvent. We found that 0.1% of DMSO did not affect viral titers in supernatant fluids (data not shown).

3.2. Effects of macrolides on viral RNA by PCR

Further evidence of the inhibitory effects of bafilomycin A_1 and clarithromycin on infection by RS viral RNA replication in human tracheal epithelial cells was provided by real-time quantitative RT-PCR analysis. The RNA extraction was performed before, and at 72 h and 120 h after RS virus infection. RS viral RNA replication in the cells was consistently observed at 72 h and 120 h after infection (Fig. 3), and increased from 72 h to 120 h after infection (Fig. 3). Bafilomycin A_1 (10 nM) and clarithromycin (10 μM) decreased the RS viral RNA at 72 h and 120 h after infection (Fig. 3). RS viral RNA was not detected in the cells before RS virus infection (data not shown).

3.3. Effects of macrolides on susceptibility to RS virus infection

Treatment of the cells with bafilomycin A_1 (10 nM) and clarithromycin (10 μM) decreased the susceptibility of the cells to infection by RS virus. The minimum dose of RS virus necessary to cause infection in the cells treated with bafilomycin A_1 (10 nM, 3 days) (3.2 ± 0.3 log $TCID_{50}$ units/ml, $n = 5$, $P < 0.05$) and clarithromycin (10 μM, 3 days) (3.1 ± 0.3 log $TCID_{50}$ units/ml, $n = 5$,

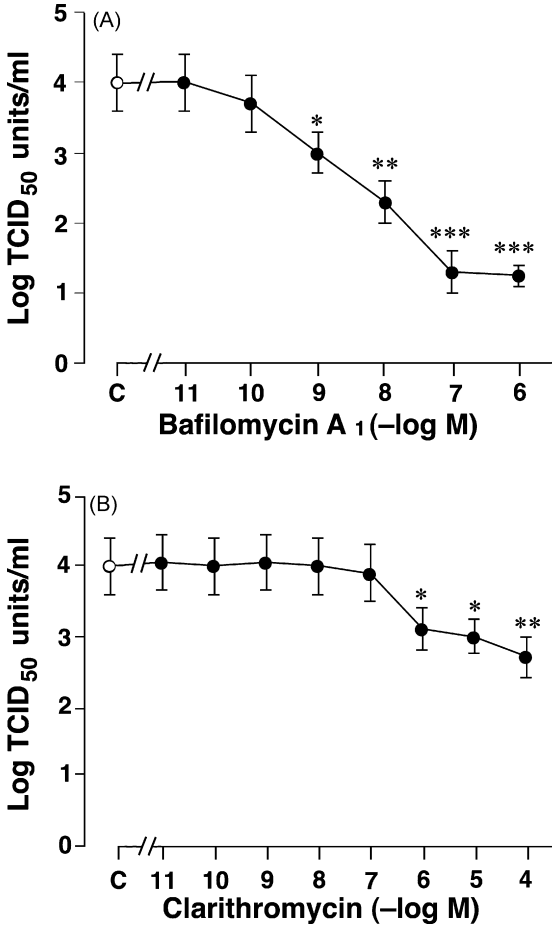

Fig. 2. Concentration–response effects of bafilomycin A_1 (A) and clarithromycin (B) on the viral titers in supernatant fluids collected during 3–5 days after infection. The cells were treated with bafilomycin A_1, clarithromycin or vehicle (control; C, 0.1% ethanol) from 3 days before RS virus infection until the end of the experiments after RS virus infection. The viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 5 different tracheae. Significant differences from vehicle alone (control, C) are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

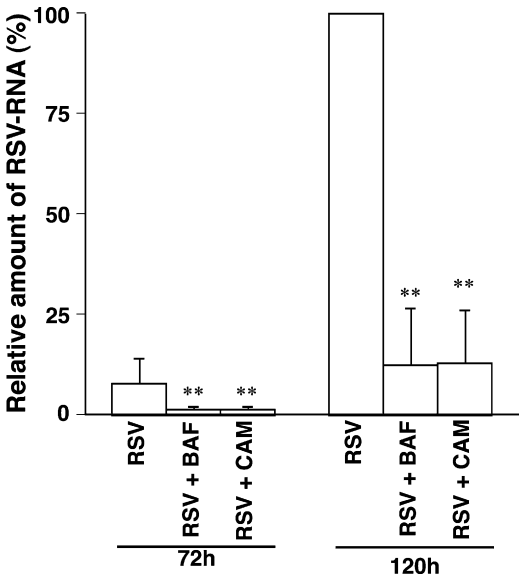

Fig. 3. Replication of RS virus RNA in human tracheal epithelial cells after infections of RS virus in the presence of bafilomycin A_1 (10 nM; RSV + BAF), clarithromycin (10 μM; RSV + CAM) or ethanol (0.1%) as a vehicle (control; RSV) as detected by real-time quantitative RT-PCR. Results are expressed as relative amount of RNA expression (%) compared with those of maximal RS virus RNA at day 5 (120 h) in the cells treated with vehicle, and reported as means ± S.D. from 5 samples. Significant differences from treatment with a vehicle (RSV) at each time are indicated by $^{**}P < 0.01$. Amount of RS virus RNA at day 5 (120 h) in the cells treated with a vehicle was 0.13 ± 0.03 ($n = 5$) compared with the amount of β-actin mRNA.

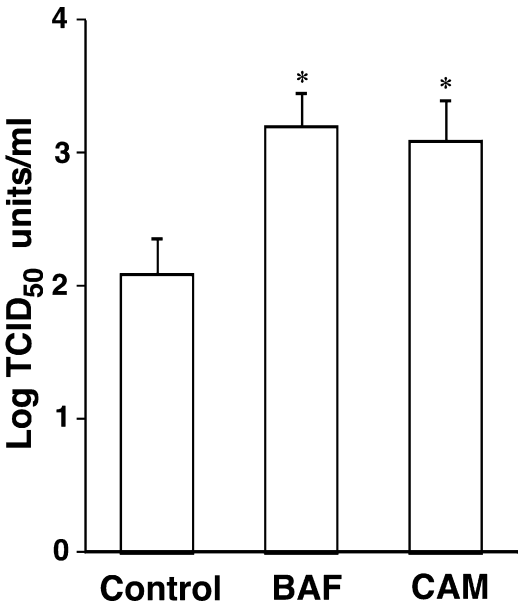

Fig. 4. The minimum dose of RS virus necessary to cause infection in the human tracheal epithelial cells treated with either bafilomycin A_1 (BAF, 10 nM), clarithromycin (CAM, 10 μM, 3 days) or vehicle (control, 0.1% ethanol). The minimum dose of RS virus necessary to cause infection is expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 7 different tracheae. Significant differences from vehicle alone (control) are indicated by $^{*}P < 0.05$.

$P < 0.05$) was significantly higher than that in the cells treated with vehicle (0.1% ethanol) (2.1 ± 0.3 log $TCID_{50}$ units/ml, $n = 5$) (Fig. 4).

3.4. Effects of N-acetyl-S-geranylgeranyl-L-cysteine on RS virus infection

N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) (1 μM), an inhibitor of RhoA (Lu et al., 2004), reduced the viral titers of RS virus in supernatant fluids, when the cells were treated with AGGC from 3 days before RS virus infection until the end of the experiments after RS virus infection (Fig. 5A). Likewise, AGGC (1 μM) decreased the RS viral RNA in the cells at 120 h after infection (Fig. 5B).

3.5. Effect of macrolides on RhoA activation

A GTP-bound form of RhoA (GTP-bound RhoA, RhoA-GTP) associated with GST-RBD was reported to show RhoA activation (Chikumi et al., 2002; Kadowaki et al., 2004; Yamaguchi et al., 2001). Amounts of the RhoA-GTP were quantified by Western blot analysis in human tracheal epithelial cells. The band of RhoA-GTP was faintly observed in the baseline conditions, and the density of the RhoA-GTP was increased by stimulation with LPA (1 μM) (data not shown). The relative ratio of the band density of RhoA-GTP compared with that of total RhoA in the cells stimulated with LPA (1 μM) was 0.85 ± 0.07 ($n = 5$). Bafilomycin A_1 (10 nM) and clarithromycin (10 μM) reduced the density of the bands of RhoA-GTP caused by the stimulation with LPA (1 μM) (Fig. 6 and Table 1).

3.6. Effects of Rho kinase inhibitor on RS virus infection

Fasudil (10 μM, HA1077) (Asahi Kasei Co., Tokyo, Japan), an inhibitor of Rho kinase including RhoA (Uehata et al., 1997; Yin et al., 2007), reduced the viral titers in supernatant fluids collected during 3–5 days after infection (2.9 ± 0.4 *vs* 3.9 ± 0.5 log $TCID_{50}$ units/ml, $n = 5$, $P < 0.05$), when the cells were treated with fasudil from 3 days before RS virus infection until the end of the experiments after RS virus infection (Fig. 7A). Fasudil (10 μM) also reduced the RS viral RNA at 5 days (120 h) after infection (24 ± 3%, $n = 5$, $P < 0.01$) (Fig. 7B). In contrast, Y-27632 (20 μM) (Gower et al., 2005), another Rho kinase inhibitor, did not reduce viral titers in supernatant fluids (3.9 ± 0.4 log $TCID_{50}$ units/ml in Y-27632 *vs* 4.1 ± 0.5 log $TCID_{50}$

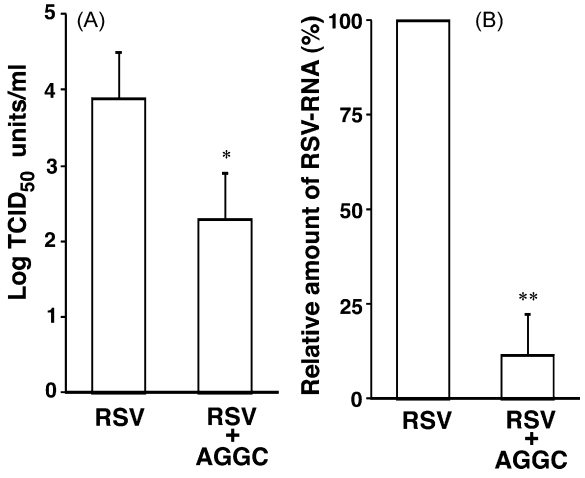

Fig. 5. (A) RS viral titers in supernatant fluids collected during 3–5 days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in human tracheal epithelial cells, treated with either *N-acetyl-S-geranylgeranyl-L-cysteine* (AGGC) (1 μM; RSV + AGGC) or vehicle (0.1% ethanol; RSV) from 3 days before RS virus infection until the end of the experiments after RS virus infection. The viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 5 different tracheae. Significant differences from viral infection alone (RSV) are indicated by $^{*}P < 0.05$. (B) Replication of RS virus RNA in human tracheal epithelial cells 5 days after infections of RS virus in the presence of AGGC (1 μM; RSV + AGGC) or a vehicle (0.1% ethanol; RSV) as detected by real-time quantitative RT-PCR. Results are expressed as relative amounts of RNA expression (%) compared with those of RS virus RNA at day 5 (120 h) in the cells treated with vehicle (RSV), and reported as means ± S.D. from 5 samples. Significant differences from treatment with a vehicle (RSV) are indicated by $^{**}P < 0.01$. Amount of RS virus RNA at day 5 (120 h) in the cells treated with a vehicle (RSV) was 0.13 ± 0.03 ($n = 5$) compared with the amount of β-actin mRNA.

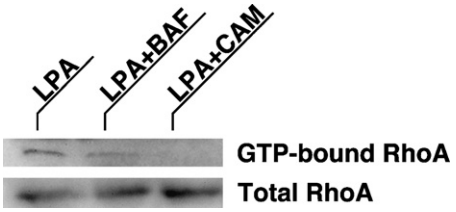

Fig. 6. Inhibitory effects of pre-incubation with bafilomycin A_1 (LPA + BAF, 10 nM, 72 h) and clarithromycin (LPA + CAM, 10 μM, 72 h) on the activation of RhoA (GTP-bound RhoA, RhoA-GTP) by lysophosphatidic acid (LPA) (1 μM, for 5 min) in human tracheal epithelial cells. Data are representative of five different experiments.

units/ml in RS virus alone, $n = 5$, $P > 0.20$) and RS virus RNA in the cells at 5 days (120 h) after infection (101 ± 1%, $n = 5$, $P > 0.20$).

3.7. Effect of macrolides on cytokines production

To examine the effects of bafilomycin A_1 and clarithromycin on cytokines production after RS virus infection, the human tracheal epithelial cells were treated with bafilomycin A_1 (10 nM), clarithromycin (10 μM) or vehicle (0.1% ethanol) from 3 days before RS virus infection until the collection of the supernatant fluids after RS virus infection. The secretion of IL-1β, IL-6 and IL-8 all increased after RS virus infection (Fig. 8), and maximum secretion was observed at 3 days after the infection (data at 1 day and 5 days not shown). Treatment with bafilomycin A_1 (10 nM) and clarithromycin (10 μM) reduced the concentrations of IL-1β, IL-6 and IL-8 3 days after RS virus infection as well as baseline concentrations of these cytokines before RS virus infection (Fig. 8). In contrast, ultraviolet-irradiated RS virus did not increase IL-1β, IL-6 and IL-8 (Fig. 8).

Table 1
Inhibitory effects of macrolides on RhoA activity.

Condition	Density ratio of RhoA-GTP/total RhoA ($n = 5$, mean ± S.D.)	P value
LPA	0.85 ± 0.07	–
LPA + BAF	0.43 ± 0.07	$P < 0.05$
LPA + CAM	0.16 ± 0.09	$P < 0.05$

LPA: lysophosphatidic acid (1 μM), BAF: bafilomycin A1 (10 nM), CAM: clarithromycin (10 μM). Results are expressed as relative ratio of the band density of RhoA-GTP compared with that of total RhoA. Significant differences from treatment with LPA alone are indicated by $P < 0.05$.

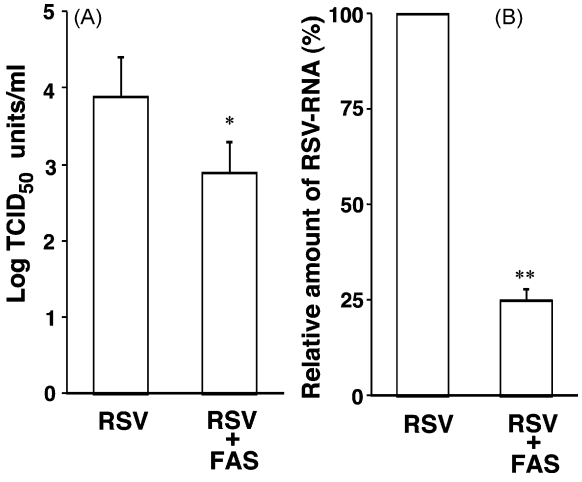

Fig. 7. (A) RS viral titers in supernatant fluids collected during 3–5 days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in human tracheal epithelial cells treated with either fasudil (10 μM; RSV + FAS) or vehicle (double distilled water; RSV) from 3 days before RS virus infection until the end of the experiments after RS virus infection. The viral titers in supernatant fluids are expressed as $TCID_{50}$ units/ml. Results are means ± S.D. from 5 different tracheae. Significant differences from viral infection alone (RSV) are indicated by $^{*}P < 0.05$. (B) Replication of RS virus RNA in human tracheal epithelial cells 5 days after infections of RS virus in the presence of fasudil (10 μM; RSV + FAS) or vehicle (double distilled water; RSV) as detected by real-time quantitative RT-PCR. Results are expressed as relative amounts of RNA expression (%) compared with those of RS virus RNA at day 5 (120 h) in the cells treated with vehicle (RSV), and reported as means ± S.D. from 5 samples. Significant differences from treatment with a vehicle (RSV) are indicated by $^{**}P < 0.01$.

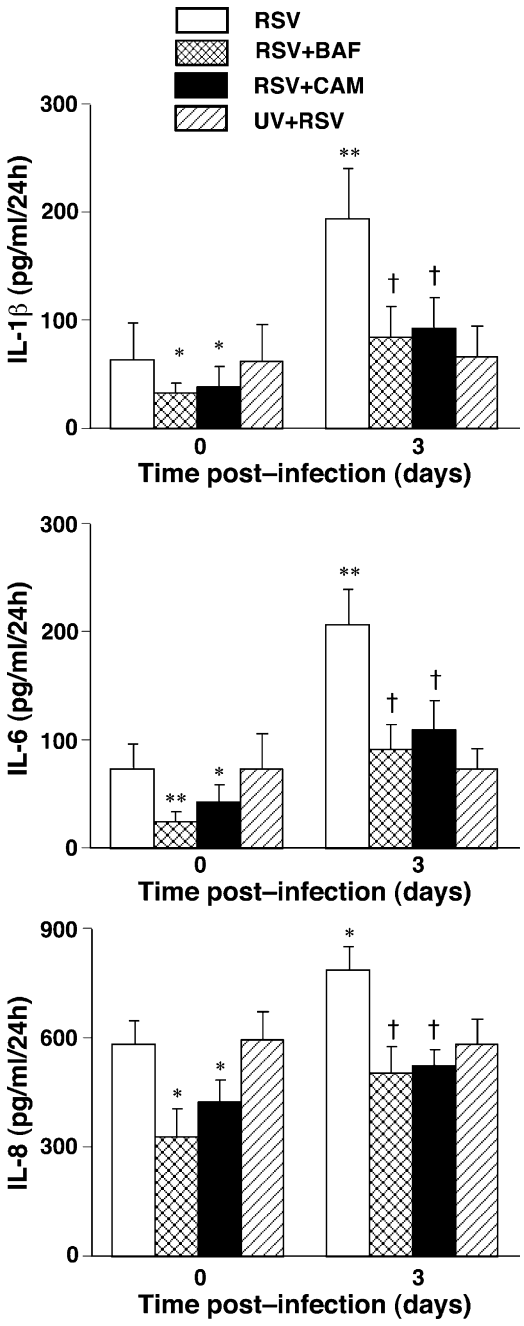

Fig. 8. Release of cytokines (IL-1β, IL-6 and IL-8) in supernatant fluids of human tracheal epithelial cells before (time 0; for 24 h before infection) and 3 days after RS virus infection (during 1–3 days after infection) in the presence of either bafilomycin A_1 (10 nM; RSV + BAF), clarithromycin (10 μM; RSV + CAM) or vehicle (0.1% ethanol; RSV), or after UV-inactivated RS virus (UV-RSV) infection. The rates of change in cytokine concentration in the supernatant fluids are expressed as pg/ml/24 h. Results are means ± S.D. from 5 different tracheae. Significant differences from values before RS virus infection (time 0) in the presence of vehicle (0.1% ethanol; RSV) are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$. Significant differences from values of RS virus (RSV) plus vehicle 3 days after RS virus infection are indicated by $^{\dagger}P < 0.05$.

3.8. Effect of clarithromycin on pH in the acidic endosomes

The effect of clarithromycin on the changes in the distribution and the fluorescence intensity of acidic endosomes were examined from 100 s before until 300 s after the treatment with clarithromycin (10 μM) or vehicle (ethanol, 0.1%). Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189, and green fluorescence from acidic endosomes was observed in a granular pattern in the cytoplasm (data not shown). Clarithromycin (10 μM) decreased the number and the fluorescence intensity of acidic endosomes with green fluorescence in the cells with time. The fluorescence intensity from acidic endosomes in the epithelial cells treated with clarithromycin (10 μM) for 300 s was significantly reduced by 32 ± 6% ($n = 5$, $P < 0.05$) compared with that in the cells treated with vehicle (ethanol, 0.1%). The fluorescence intensity from acidic endosomes in the epithelial cells treated with clarithromycin (10 μM) for 3 days (72 h) was also reduced by 89 ± 2% ($n = 5$, $P < 0.01$).

4. Discussion

In the present study, we have shown that viral titers in supernatant fluids and RNA of RS virus in the human tracheal epithelial cells increased with time, and bafilomycin A_1, one of the macrolide antibiotics and a specific inhibitor of the vascular-ATPase (V-ATPase) (Palokangas et al., 1997) and a widely used macloride antibiotic clarithromycin reduced viral titers of RS virus in supernatant fluids concentration-dependently, RNA of RS virus replication, and the susceptibility to RS virus infection. *N-acetyl-S-geranylgeranyl-L-cysteine* (AGGC), an inhibitor for a small GTP binding protein of RhoA, reduced viral titers in supernatant fluids and RNA replication of RS virus. Bafilomycin A_1 and clarithromycin inhibited activation of RhoA induced by lysophosphatidic acid (LPA) in the cells. Because activated RhoA interacts with the RS virus F protein, these findings suggest that bafilomycin A_1 and clarithromycin may inhibit RS virus infection, partly through the reduction of activated RhoA in the cells. Furthermore, bafilomycin A_1 and clarithromycin reduced concentrations of cytokines, including IL-1β IL-6, and IL-8 in supernatant fluids. Macrolide antibiotics may also modulate airway inflammation induced by RS virus infection.

Hep-2 cells did not show any syncytial formation when culture medium 2 h after removing inoculum and washing cells was added to the cells. In contrast, supernatant fluids 1 day after infection produced syncytial formation on the cells, showing that supernatants fluids 1 day after infection contained significant amounts of RS virus. These findings suggest that day 0–1 virus was the production of new virions.

RhoA, isoform A of the Ras-homologus (Rho) family (Takai et al., 2001), has various functions including stimulus-evoked cell adhesion and motility, enhancement of contractile response and cytokinesis (Narumiya, 1996). Furthermore, the activated form of RhoA moves to the cell membrane and is implicated in the RS virus infection (Collins and Crowe, 2006; Pastey et al., 1999). Reduction of activated RhoA (GTP-bound RhoA) by macrolides, observed in this study, is consistent with a previous report in which AGGC reduces activated RhoA in endothelial monolayer (Lu et al., 2004). The association between inhibition of RhoA activity and inhibition of RS virus infection observed in this study is also consistent with previous studies demonstrating the inhibitory effects of a RhoA-derived peptide on syncytium formation induced by RS virus (Pastey et al., 2000) and the inhibition of RS virus infection in Hep-2 cells by tiotropium, a cholinergic antagonist (Iesato et al., 2008). Bafilomycin A_1 and clarithromycin might inhibit RS virus infection partly through the reduction of activated RhoA, the receptor for RS virus F protein.

As Pastey et al. (1999) demonstrated, RhoA amino acids 67–110 bind to RS virus F protein amino acids 146–155, and facilitates virus-induced syncytium formation. They also reported that pretreatment of RS virus with the RhoA peptides 77–95 block RS virus replication (Pastey et al., 2000). These observations initially suggested that RhoA-derived peptides might inhibit RS virus replication by disrupting an *in vivo* interaction between RS virus F protein and RhoA. However, Budge and Graham (2004) reported that the antiviral activity of RhoA-derived peptides is not due to competitive inhibition of RS virus F protein–RhoA interaction, but is rather a function of the peptides' intrinsic biophysical properties. On the other hand, the region of the peptide 77–95, most critical for inhibition of RS virus, is not exposed on the cell surface, and optimal antiviral activity of RhoA-derived peptide requires oxidation of an internal cysteine residue (Budge et al., 2003). The site of RhoA inactivation by bafilomycin A_1 and clarithromycin needs to be studied.

RhoA activation is also associated with various roles on the entry and exocytosis of viruses other than RS virus (Clement et al., 2006; Loomis et al., 2006; Veettil et al., 2006). RhoA inhibition by macrolides, as shown in this study, might also inhibit these processes in RS virus infection, although we did not examine them.

Bafilomycin A_1 inhibits small GTPase including RhoA in various cells by increasing the pH (Palokangas et al., 1997). The mechanisms for RhoA inactivation by clarithrmycin are uncertain. However, we demonstrated that clarithromycin reduced fluorescence intensity from acidic endosomes in human tracheal epithelial cells. These inhibitory effects of clarithromycin are consistent with other macrolides including bafilomycin A_1 and erythromycin as we previously reported (Suzuki et al., 2001a, 2002). Clarithromycin might act on human tracheal epithelial cells and inactivate RhoA, at least partly, through the increased pH in acidic endosomes.

The Rho kinase inhibitor Y-27632 alters the pattern of RS virus F protein localization in infected cells (Gower et al., 2001), but does not reduce RS viral titers in supernatant fluids of Hep-2 cells (Gower et al., 2005), as we also observed in the present study in human tracheal epithelial cells. In contrast, we demonstrated that another Rho kinase inhibitor, fasudil, reduces RS virus replication and release into supernatant fluids. These findings suggest that downstream pathways of RhoA such as Rho kinase may relate to RS virus replication, although the precise role of RhoA and its downstream signals in the RS virus infection is still uncertain (Budge and Graham, 2004). The functions of fasudil other than Rho kinase inhibition, including protein production and calcium movement in the cells (Moore et al., 2004; Shibuya et al., 1988), may differ from those of Y-27632.

Heparan sulfate, chondroitin sulfate and ICAM-1 also act as receptors for RS virus (Collins and Crowe, 2006), and Iesato et al. (2008) reported that tiotropium reduces RS virus replication partly due to the inhibition of ICAM-1 expression as well as inhibition of RhoA activity in Hep-2 cells. Reduced expression of ICAM-1 by bafilomycin A_1 (Suzuki et al., 2001a) and clarithromycin (Jang et al., 2006) might also relate to clarithromycin mediated-inhibition of RS virus infection, observed in the present study, although we did not examined ICAM-1 expression in this study.

The serum or plasma concentrations of clarithromycin in clinical use are reported to be between 3 μM and 15 μM (Honeybourne et al., 1994; Rodvold, 1999). On the other hand, in the epithelial lining fluids of the respiratory tract, clarithromycin concentrations are higher than those in the serum (Rodvold, 1999). Therefore, epithelial cells in human trachea may be exposed to clarithromycin at concentrations of 10 μM or more.

Macrolide antibiotics reduce the frequency of COPD (Seemungal et al., 2008; Suzuki et al., 2001b; Yamaya et al., 2008). In addition to various mechanisms proposed previously including rhinovirus infection (Suzuki et al., 2002), inhibition of RS virus infection, observed in this study, may be also associated with reduced frequency of COPD exacerbations.

In the present study, RS virus infection increased the production of IL-1β, IL-6 and IL-8. Bafilomycin A_1 and clarithromycin reduced RS virus infection-induced production of IL-1β, IL-6 and IL-8 as well as baseline production of these cytokines before RS virus infection. Because bafilomycin A_1 and clarithromycin reduced viral titers in supernatant fluids, the inhibiting effects of macrolides on RS virus infection and on cytokine production themselves might be associated with the reduced production of these pro-inflammatory cytokines in the cells treated with bafilomycin A_1 and clarithromycin after RS virus infection.

In summary, this is the first report that macrolide antibiotics including clarithromycin, a widely used macloride antibiotic, and bafilomycin A_1 inhibit infection by RS virus and decrease the susceptibility of cultured human tracheal epithelial cells to RS virus infection, partly through the reduced expression of activated RhoA, a RS virus F protein receptor. Marolide antibiotics also reduced baseline and RS virus infection-induced release of pro-inflammatory cytokines in supernatant fluids including IL-1β, IL-6 and IL-8. Macrolide antibiotics may modulate the airway inflammation after RS virus infection in COPD and bronchial asthma.

Conflict of interest statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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