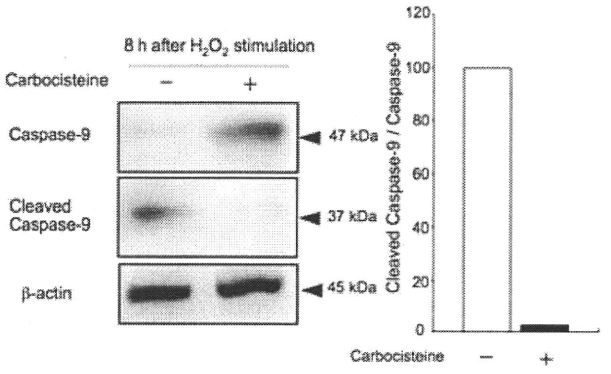

Figure 3 (Left) Western blot analysis of cleaved and non-cleaved caspase-9 and β-actin in human tracheal epithelial cells 8 h after exposure to H_2O_2 (800 μmol/L) in the presence of L-carbocisteine (10 μmol/L) (+) or vehicle (0.1% ddH_2O) (–). Data are representative of five separate experiments. (Right) The intensity of cleaved and non-cleaved caspase-9 in a representative experiment performed 8 h after exposure to H_2O_2 (800 μmol/L) in the presence of L-carbocisteine (10 μmol/L) (+) or vehicle (0.1% ddH_2O) (–). The ratio of cleaved/non-cleaved caspase-9 8 h after exposure to H_2O_2 in the presence of vehicle (0.1% ddH_2O) was set at 100. The intensity of the cleaved caspase-9 band was reduced in the presence of L-carbocisteine.

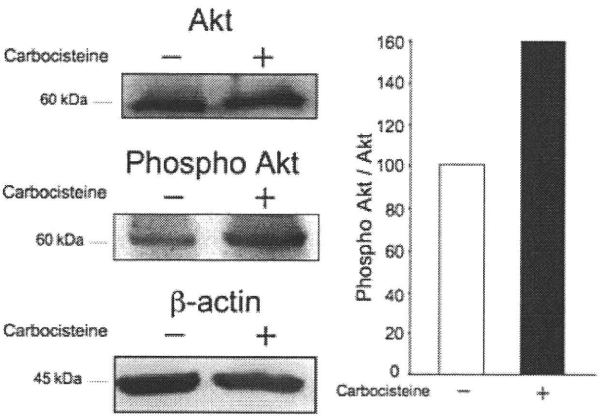

Figure 5 (Left) Western blot analysis of phospho-Akt, Akt and β-actin in cultured human tracheal epithelial cells before exposure to H_2O_2 in the presence of L-carbocisteine (10 μmol/L) (+) or vehicle (0.1% ddH_2O) (–). Data are representative of five separate experiments. (Right) The phospho-Akt/Akt ratio, as measured in a representative western blot experiment using National Institutes of Health Image software, before exposure to H_2O_2, in the presence of L-carbocisteine (10 μmol/L) or vehicle (0.1% ddH_2O) (control). The phospho-Akt/Akt ratio 8 h after exposure to H_2O_2 in the presence of vehicle (0.1% ddH_2O) was set at 100.

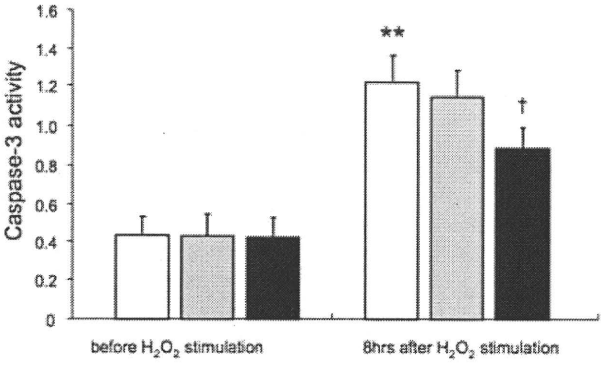

Figure 4 Caspase-3 activity, as measured with a fluorescent detection kit, in cultured human tracheal epithelial cells before and 8 h after exposure to H_2O_2 (800 μmol/L) in the presence of L-carbocisteine (10 μmol/L) (carbocisteine (+)) or vehicle (0.1% ddH_2O, Control) (carbocisteine (–)). Results are mean ± SEM from five samples. Significant differences compared with medium alone and H_2O_2 alone are indicated as $^{**}P < 0.01$ and $^{+}P < 0.05$, respectively. (□) control, (▨) carbocisteine (1 μmol/L), (■) carbocisteine (10 μmol/L).

H_2O_2, cells were pre-incubated with inhibitors of Akt. Pretreatment with L-carbocisteine (10 μmol/L, 72 h) decreased the levels of DNA fragmentation induced by H_2O_2 (800 μmol/L, 24 h) (Fig. 6). Incubation with LY294002 (50 μmol/L, 30 min) or wortmannin (50 nmol/L, 30 min) alone did not change DNA fragmentation levels 24 h after treatment (data not shown). On the other hand, incubation with LY294002 (50 μmol/L, 30 min) or wortmannin (50 nmol/L, 30 min) significantly increased DNA fragmentation 24 h after exposure to H_2O_2 (800 μmol/L) in the cells pretreated with L-carbocisteine (10 μmol/L, 72 h) (Fig. 6).

DISCUSSION

In this study, the proportion of apoptotic human tracheal epithelial cells was concentration- and time-dependently increased after exposure to H_2O_2, and treatment with L-carbocisteine reduced the proportion of apoptotic cells. Exposure to H_2O_2 activated caspase-3 and caspase-9 in the cells, and L-carbocisteine inhibited the activation of these caspases induced by H_2O_2. L-carbocisteine activated Akt phosphorylation, which modulates caspase activation, in human tracheal epithelial cells. Inhibitors of Akt significantly reversed the inhibitory effects of L-carbocisteine on H_2O_2-induced cell apoptosis. These findings suggest that L-carbocisteine may inhibit cell damage induced by H_2O_2 in human airway epithelium through the activation of Akt phosphorylation. In contrast, H_2O_2 did not increase the concentration of LDH in supernatants of epithelial cells, suggesting that H_2O_2 may induce apoptosis of human tracheal epithelial cells, and that L-carbocisteine may inhibit apoptosis of cells induced by H_2O_2.

S-carbocysteine lysine salt monohydrate (S-CMC-Lys) has been reported to act as a scavenger by interfering with the conversion of xanthine dehydrogenase

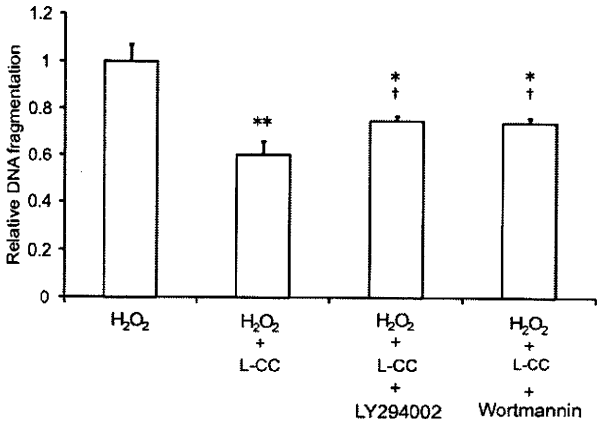

Figure 6 Effects of pre-incubation with LY294002 (50 μmol/L, 30 min) or wortmannin (50 nmol/L, 30 min) on relative DNA fragmentation in primary cultures of human tracheal epithelial cells, 24 h after treatment with H_2O_2 (800 μmol/L) in the presence of L-carbocisteine (L-CC, 10 μmol/L, 3 days) or vehicle (0.1% ddH_2O). Viability of the cells 24 h after stimulation with H_2O_2 (800 μmol/L) in the presence of vehicle was set at 1.0, and all other measurements were normalized to the control values for the purposes of comparison. Results are mean ± SEM from five samples. Significant differences from the values 24 h after treatment with H_2O_2 alone are indicated as $^*P < 0.05$ and $^{**}P < 0.01$. Significant differences from the values after treatment with H_2O_2 in the presence of L-carbocisteine are indicated as $^+P < 0.05$.

to superoxide-producing xanthine oxidase.[26] In contrast to NAC and ambroxol, L-carbocisteine does not have a sulphydryl group that scavenges H_2O_2.[27] However, a recent report demonstrated that L-carbocisteine scavenges reactive oxygen species in cell-free conditions, inhibits the production of reactive oxygen species from rat neutrophils,[28] and has effects similar to those of NAC. These findings suggest that L-carbocisteine has antioxidant effects, as observed in the present study.

The antioxidant effects of mucolytic agents,[1,19] including L-carbocisteine, as observed in the present study, may be associated with reduction of COPD exacerbations.[5,12,18] Furthermore, in patients with bronchial asthma, L-carbocisteine increased the cough threshold, defined as the lowest concentration of capsaicin that induced cough, suggesting that carbocisteine may be a novel therapeutic option for asthmatic patients, especially those with cough variant asthma.[29] However, the clinical benefits of mucolytic agents are still uncertain.[30]

In the present study, in the absence of H_2O_2 exposure, L-carbocisteine increased Akt phosphorylation, which modulates caspase activation.[31] In contrast, phospho-Akt levels decreased after exposure to H_2O_2, and did not differ between cells treated with L-carbocisteine or vehicle. These findings suggest that increased phosphorylation of Akt by pre-incubation with L-carbocisteine may inhibit the activation of caspase-3 and caspase-9 after exposure to H_2O_2. L-carbocisteine may therefore inhibit cell apoptosis induced by H_2O_2 in the human airway epithelium through the activation of Akt phosphorylation.

LY294002 and wortmannin inhibited Akt activation-mediated haeme oxygenase-1 expression, which plays a cytoprotective role against oxidative injury in airway and lung epithelial cells.[32] In the present study, treatment with L-carbocisteine decreased apoptosis of human tracheal epithelial cells induced by H_2O_2 and increased Akt phosphorylation. Treatment of the cells with LY294002 or wortmannin partially but significantly increased cell apoptosis after exposure to H_2O_2, in the presence of L-carbocisteine. These findings suggest that the anti-apoptotic effects of L-carbocisteine may be, at least partially, associated with Akt activation.

The phosphoinositide 3-kinase-Akt pathway has been reported to protect cells from apoptosis.[33] The mechanisms of the anti-apoptotic effects of Akt phosphorylation in airway cells are uncertain. However, in cancer cells, tissue transglutaminase, the most diverse and ubiquitous member of the transglutaminase family of proteins that has been implicated in apoptosis, constitutively activates focal adhesion kinase and the downstream phosphoinositide 3-kinase-Akt survival pathway.[34]

The precise mechanisms by which L-carbocisteine increases Akt phosphorylation are also uncertain. However, NAC, another mucolytic agent, inhibits DNA fragmentation, as well as Akt inactivation induced by the non-steroidal anti-inflammatory drug, diclofenac, in promyelocytic leukaemia cells.[35] Cyclic adenosine monophosphate (cAMP), an inducer of Akt phosphorylation, suppresses Akt inactivation and DNA fragmentation induced by diclofenac.[35] NAC and carbocisteine also increase cAMP levels in hepatic, tracheal and alveolar tissues,[36] suggesting that L-carbocisteine may partly activate Akt phosphorylation by increasing cAMP levels.

NAC may be deacetylated to cysteine, an important precursor of cellular glutathione (GSH) synthesis, thereby stimulating the cellular GSH system,[26] which modulates oxidant-induced cellular damage.[37] Likewise, S-CMC-Lys causes the secretion of GSH by human respiratory cells,[37] suggesting that the antioxidant effect of L-carbocisteine may also be associated with GSH synthesis. Furthermore, reduced production of pro-inflammatory cytokines, including IL-1 and TNF-α,[9,38] may also be associated with the anti-apoptotic effects of L-carbocisteine, in addition to activation of Akt and the GSH system.

In this study, one donor had COPD. However, the effects of H_2O_2 and the antioxidant effects of L-carbocisteine were not different in cells from the COPD patient compared with those from patients without COPD. Although the reasons are uncertain, culturing of the cells may alter epithelial cell function and the influence of COPD may be masked.

Exposure to H_2O_2 did not change the LDH concentrations in culture supernatants, suggesting that H_2O_2 did not induce necrosis of the cells. Oxidative stress-induced cellular responses in B cells may involve necrosis, apoptosis or mitotic arrest, depending on the level of exposure to H_2O_2.[39] H_2O_2 concentrations of

400 and 800 μmol/L appeared to induce apoptosis and not necrosis in tracheal epithelial cells.

In summary, this study demonstrated that the mucolytic agent, L-carbocisteine, inhibited apoptosis of human tracheal epithelial cells after exposure to H_2O_2, partly through activation of Akt phosphorylation. These effects may be associated with the clinical benefits of mucolytic agents in the treatment of COPD, IPF and cystic fibrosis.

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A Randomized, Single-Blind Study of Lansoprazole for the Prevention of Exacerbations of Chronic Obstructive Pulmonary Disease in Older Patients

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OBJECTIVES: To investigate whether proton pump inhibitor (PPI) therapy reduces the frequency of common colds and exacerbations in patients with chronic obstructive pulmonary disease (COPD).

DESIGN: Twelve-month, randomized, observer-blind, controlled trial.

SETTING: A university hospital and three city hospitals in Miyagi prefecture in Japan.

PARTICIPANTS: One hundred patients with COPD (mean age ± SD 74.9 ± 8.2) participated. They were all ex-smokers and had received conventional therapies for COPD, including smoking cessation and bronchodilators. Patients with gastroesophageal reflux disease or gastroduodenal ulcer were excluded.

INTERVENTION: Patients were randomly assigned to conventional therapies (control group) or conventional therapies plus PPI (lansoprazole 15 mg/d; PPI group) and observed for 12 months.

MEASUREMENTS: Frequency of common colds and COPD exacerbations.

RESULTS: The number of exacerbations per person in a year in the PPI group was significantly lower than that in the control group (0.34 ± 0.72 vs 1.18 ± 1.40; $P < .001$). The adjusted odds ratio with logistic regression for having exacerbation (≥once/year) in the PPI group compared with the control group was 0.23 ($P = .004$). In contrast, there was no significant difference in the numbers of common colds per person per year between the PPI group and the control group (1.22 ± 2.09 vs 2.04 ± 3.07; $P = .12$). PPI therapy significantly reduced the risk of catching frequent common colds (≥3 times/year), the adjusted odds ratio of which was 0.28 ($P = .048$).

CONCLUSION: In this single-blind, nonplacebo-controlled trial, lansoprazole was associated with a significant decrease in COPD exacerbations. More definitive clinical trials are warranted. J Am Geriatr Soc 57:1453–1457, 2009.

Key words: proton pump inhibitor; chronic obstructive pulmonary disease; exacerbation; common cold; lower airway infection

Common colds often predispose patients with chronic obstructive pulmonary disease (COPD) to develop lower airway inflammation,[1] resulting in exacerbations of COPD. Several viruses, such as human rhinoviruses (RVs),[2] respiratory syncytial viruses, and influenza and parainfluenza viruses, are related to exacerbations of COPD.[3,4] Among them, the mechanisms of RV infection are well studied. It was reported that the major type of RV (type 14, RV14) enters the cytoplasm of infected cells after binding to its receptor intercellular adhesion molecule-1 (ICAM-1).[5,6] It has been suggested that destabilization from receptor binding and endosomal acidification mediates the entry of ribonucleic acid of a major rhinovirus, RV14, into the cytoplasm of infected cells.[7]

Proton pump inhibitors (PPIs), such as lansoprazole and omeprazole, are therapeutic agents for gastroduodenal ulcer and gastroesophageal reflux disease (GERD) and have the inhibitory effects on H^+–K^+ ATPase in gastric parietal cells. In addition, they might reduce the production of ICAM-1 in mononuclear cells and gastric mucosa.[8,9]

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Omeprazole increases pH in renal cell vacuoles[10] and inhibits production of proinflammatory cytokines in epithelial cells.[11] These cytokines may be related to pathogenesis of rhinovirus infections and development of COPD exacerbation. Furthermore, it was recently demonstrated that lansoprazole inhibited RV14 infection in cultured human tracheal epithelial cells through the inhibition of ICAM-1 expression and endosomal acidification in the airway epithelial cells.[7] In that report, lansoprazole was also shown to reduce the production of proinflammatory cytokines such as interleukin-1β, interleukin-6, inteleukin-8, and tumor necrosis factor-alpha in the infected epithelial cells.[7] However, the efficacy of PPI therapy for the prevention of the common cold and subsequent exacerbations in patients with COPD has not been investigated. Whether PPI therapy reduced the frequency of common colds and exacerbations of COPD was therefore studied in a prospective, randomized, controlled trial. It was hypothesized that PPI therapy would reduce the frequency of common colds or exacerbations of COPD.

METHODS

Subjects

One hundred six Japanese patients with COPD at a university hospital and three city hospitals in Miyagi prefecture in Japan were enrolled for this study. All patients were ethnically Japanese, were ex-smokers, and fulfilled the criteria of the American Thoracic Society for COPD.[12] All of the participants were treated with bronchodilators, including sustained release theophylline, beta-2 agonists, and inhaled anticholinergic agents. Some of the participants with Stage III and IV COPD received inhaled corticosteroids because of frequent exacerbations of COPD. Patients with obvious bronchial asthma, bronchiectasis, or diffuse panbronchiolitis were excluded from the study. Patients with active gastric or duodenal ulcers and GERD and with symptoms of these diseases were also excluded. All participants received medical examinations for gastroduodenal diseases within 3 years using upper gastrointestinal endoscopy or barium gastric radiography; no abnormalities were found. Furthermore, the presence or absence of GERD symptoms was assessed using the Japanese version of the Carlsson-Dent self-administered questionnaire (QUEST).[13] The subjects with QUEST scores of more than 6 points were defined as positive for GERD symptoms and were excluded from this study.[13] Finally, 103 patients were eligible and were randomly assigned to treatment with a PPI (PPI group) and a control group. The rate and number of common colds and exacerbations in both groups was prospectively compared.

Treatments

A 12-month, randomized, observer-blind, controlled trial was conducted from October 2005 through March 2007. Randomization was performed using a random number table, and the list was held independently of the investigators. Through the randomization, 51 patients were assigned to the PPI group and 52 to the control group. Both groups were treated with conventional therapy for COPD, including bronchodilators and smoking cessation. In addition, patients in the PPI group received lansoprazole (15 mg/d), unless they experienced apparent adverse effects from it. Patients in the control group did not receive lansoprazole. The study was approved by the Tohoku University Ethics Committee.

Diagnosis of Common Colds and COPD Exacerbation

Patients were observed for 12 months, during which time the following 10 symptoms were recorded: sneezing, nasal discharge, nasal congestion, malaise, headache, chills, feverishness, sore throat, hoarseness, and cough. Symptoms were rated for severity on a scale from 0 to 3 and were recorded on daily record cards. Therefore, a daily total of the symptom scores could vary from 0 to 30. A common cold[14] was defined as a total symptom score greater than 5. Patients visited the hospital every 2 weeks, where their doctors evaluated their physical condition. It was also recommended that they visit the hospital for investigator-initiated checks if their total symptom score was greater than 5.

A COPD exacerbation was defined as an acute and sustained worsening of COPD symptoms requiring changes to regular treatment, including antimicrobial therapy and short courses of systemic corticosteroids.[15]

Statistical Analysis

The primary hypothesis was that the occurrence of COPD exacerbation would be lower in the PPI group than in the control group. According to previous studies,[16,17] approximately 60% of patients with COPD would have an exacerbation of COPD per year. In the original power calculations, it was determined that a sample size of 44 participants in each arm would result in 80% power to detect a twofold difference in occurrence of COPD exacerbations between the two groups using a two-tailed alpha level of 0.05.

Two-tailed *t*-tests and chi-square tests were used to compare the demographic and clinical characteristics of the two groups at baseline. Age, smoking history (pack-years), and items of pulmonary function at baseline; the number of common colds per year; and the number of exacerbations per year during the study period are presented as the means ± standard deviations.

Logistic regression was used to examine the adjusted effects on developing a common cold and an exacerbation of COPD of age, sex, influenza vaccination, treatment with inhaled corticosteroids, COPD stage, and use of PPI. Significance was accepted at $P < .05$.

RESULTS

Patient Characteristics

During the study period, one patient in the PPI group and two of the patients in the control group were lost to follow-up. The patient in the PPI group refused to continue the study because of occasional diarrhea with Grade 1 (common terminology criteria for adverse events version 3). One patient in the control group stopped the study because he moved to another prefecture. Another patient in the control group dropped out because he had an acute myocardial infarction 1 month after study enrollment and could not visit the hospital regularly. The remaining 50 patients in

the PPI group and 50 patients in the control group were analyzable.

Age, sex, smoking history, influenza vaccination, use of bronchodilators or inhaled corticosteroids, pulmonary function test, and stage of COPD were not different between the two groups, as shown in Table 1.

Frequencies of Common Colds and Exacerbations in Patients with COPD

The mean number of common colds per person for 12 months was lower, but not significantly so, in the PPI group than in the control group (1.22 ± 2.09 vs 2.04 ± 3.07; $P = .12$, Table 2). During the study period, 27 of 50 patients (54.0%) in the control group and 26 of 50 patients (52.0%) in the PPI group experienced common colds more than once. Fewer patients in the PPI group than in the control group had frequent common colds (≥3 per year), but not statistically significantly so according to a simple chi-square test (6 (12.0%) vs 13 (26.0%); $P = .07$).

Alternatively, the number of COPD exacerbations per person in 12 months was significantly lower in the PPI group than in the control group (0.34 ± 0.72 vs 1.18 ± 1.40; $P = .0003$, Table 2). During the study period, significantly fewer patients in the PPI group than in the control group experienced COPD exacerbations more than once (12 (24%) vs 26 (52%); $P = .004$).

Table 1. Clinical Characteristics of Subjects with Chronic Obstructive Pulmonary Disease (COPD) in Proton Pump Inhibitor (PPI) Group and Control Group

Characteristics	PPI Group (n = 50)	Control Group (n = 50)	*P*-Value
Age, mean ± SD	74.9 ± 8.9	74.8 ± 7.5	.95*
Sex, n (%)			
Male	47 (94.0)	48 (96.0)	>.99†‡
Female	3 (6.0)	2 (4.0)	
Smoking history, pack-years, mean ± SD	48.7 ± 41.0	59.7 ± 33.1	.18*
Influenza vaccination, n (%)	27 (54.0)	18 (36.0)	.07‡
Bronchodilator, n (%)			
Anticholinergic	19 (36.0)	29 (58.0)	.07‡
β2-agonist	23 (46.0)	22 (44.0)	.84‡
Methylxanthine	43 (86.0)	50 (100.0)	.52†
Any type of bronchodilator	50 (100.0)	50 (100.0)	>.99†
Inhaled corticosteroid, n (%)	13 (26.0)	12 (24.0)	.82‡
Pulmonary function, mean ± SD			
Percentage vital capacity	81.5 ± 20.3	80.2 ± 21.0	.77*
FEV1/forced vital capacity	56.3 ± 14.3	56.5 ± 10.8	.93*
FEV1, percentage predicted	65.4 ± 30.5	61.1 ± 23.8	.50*
COPD stage, n			.45‡
I	16 (32.0)	11(22.0)	
II	14 (28.0)	18 (36.0)	
III	17 (34.0)	20 (40.0)	
IV	3 (6.0)	1 (2.0)	

*Data were analyzed using the Student *t*-test.
†Data were analyzed using the Fisher exact test.
‡Data were analyzed using the chi-square test.
SD = standard deviation; FEV1 = forced expiratory volume in 1 second.

Table 2. Frequencies of Common Colds and Exacerbations in Patients with Chronic Obstructive Pulmonary Disease (COPD)

Variables	Proton Pump Inhibitor Group (n = 50)	Control Group (n = 50)	*P*-Value
Common cold			
Total common colds/year, n	61	102	
Number of common colds/year, mean ± SD	1.22 ± 2.09	2.04 ± 3.07	.12*
Patients with ≥1 colds/year, n (%)	26 (52.0)	27 (54.0)	.84†
Patients with ≥3 colds/year, n	6 (12.0)	13 (26.0)	.07†
Exacerbation of COPD			
Total exacerbations/year, n	17	59	
Number of exacerbations/year, mean ± SD	0.34 ± 0.72	1.18 ± 1.40	<.001*
Patients with ≥1 exacerbations/year, n (%)	12 (24.0)	26 (52.0)	.004†

*Data were analyzed using the Student *t*-test.
†Data were analyzed using the chi-square test.
SD = standard deviation.

Multivariate Analyses for Risk Factors Related to Frequent Common Colds and COPD Exacerbations

To examine the adjusted effects on developing common colds frequently (≥3 times per year) of potentially confounding factors, logistic regression was performed with age, sex, influenza vaccination, treatment with inhaled corticosteroids, COPD stage, and use of PPI as explanatory variables. Use of PPI was independently and significantly associated with less risk of developing frequent common colds (≥3 times per year), whereas the worst stage of COPD (Stage IV) was independently and significantly associated with greater risk of developing frequent common colds (adjusted odds ratio (AOR) of developing frequent common colds in the PPI group vs the control group = 0.28, 95% confidence interval (CI) = 0.08–0.99, $P = .048$; AOR of developing frequent common colds in patients with Stage IV COPD vs Stage I COPD = 43.2, 95% CI = 2.7–703.2, $P = .008$) (Table 3).

To examine the adjusted effects on developing exacerbations of COPD of potentially confounding factors, logistic regression was performed with age, sex, influenza vaccination, treatment with inhaled corticosteroids, COPD stage, and use of PPI as explanatory variables. Use of PPI was independently and significantly associated with less risk of exacerbations of COPD, whereas aging and worse COPD (Stage III and IV) were independently and significantly associated with greater risk of exacerbation of COPD (AOR of developing exacerbations in the PPI group vs the control group = 0.23, 95% CI = 0.08–0.62, $P = .004$; AOR of developing exacerbation for each year of age = 1.1, 95% CI = 1.0–1.2, $P = .02$; AOR of developing exacerbations in patients with Stage III of COPD vs Stage I = 3.9, 95% CI = 1.1–14.3, $P = .04$; AOR of developing exacerba-

Table 3. Multivariate Analyses of Risk Factors Related to Frequent Common Colds (≥3/Year) and Those Related to Exacerbation of Chronic Obstructive Pulmonary Disease (COPD)

Variable	*P*-Value	Odds Ratio (95% Confidence Interval)
Frequent common cold (≥3/year)		
Use of PPI	.048	0.28 (0.08–0.99)
COPD Stage IV vs I*	.008	43.2 (2.7–703.2)
Exacerbation of COPD		
Age	.02	1.1 (1.0–1.2)†
Use of PPI	.004	0.23 (0.08–0.62)
COPD Stage III vs I*	.04	3.9 (1.1–14.3)
COPD Stage IV vs I*	.006	45.6 (3.0–690.3)

All data were analyzed using the logistic regression method and adjusted for age, sex, influenza vaccination, and treatment with inhaled corticosteroids.
*The group with better outcome.
†Odds ratio that should increase by every 1-year increase in age.
PPI = proton pump inhibitor.

tions in patients with Stage IV of COPD vs stage I = 45.6, 95% CI = 3.0–690.3, $P = .006$) (Table 3).

DISCUSSION

The present study found that treatment with lansoprazole considerably reduced the risk of catching frequent common colds (≥3 per year) in patients with COPD. According to multivariate analysis, the relative risk of catching frequent colds (≥3 per year) in the PPI group compared with the control group was 0.28 (95% CI = 0.08–0.99; $P = .048$). Lansoprazole was found to reduce infection with rhinoviruses and to modulate inflammatory responses in cultured human tracheal epithelial cells after rhinovirus infection.[7] Rhinoviruses are the major cause of the common cold. The major type of rhinoviruses uses ICAM-1 as an infection receptor and enters the cytoplasm by means of endosomal acidification.[6] Infection with rhinoviruses increases the production of several proinflammatory cytokines from infected cells, which are suggested to play an important role in the pathogenesis of rhinovirus infection. Lansoprazole suppressed the expression of ICAM-1 and proinflammatory cytokines such as interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor-alpha and inhibited the acidification of endosomes in human airway epithelial cells.[7] These mechanisms might contribute to the effects of lansoprazole in preventing rhinovirus infection in human tracheal epithelial cells. Furthermore, another PPI, omeprazole, was also reported to inhibit cytokine production of proinflammatory cytokines, ICAM-1 expression, and the acidification of endosomes or vacuoles in various cells.[8,9,11] It is therefore possible that PPI could reduce the frequency of catching colds and the symptoms of colds by suppressing inflammatory cytokines in COPD patients.

The present study also found that treatment with lansoprazole significantly reduced the risk of exacerbations in patients with COPD. According to multivariate analysis, the relative risk of having one or more exacerbations in the PPI group compared with the control group was 0.23 (95% CI = 0.08–0.62; $P = .004$). Viral infection in airways can provoke exacerbations of asthma or COPD. Therefore, the inhibition of viral infection in airway by treatment with PPI could result in reduction in the risks of COPD exacerbation. In addition, the potency of inhibitory effects of PPI therapy on the number of COPD exacerbations per year (by 70% inhibition, $P < .001$) was larger than that on the number of common colds per year (by 40% inhibition, $P = .12$). These findings suggest that the efficiency of PPI treatment in inhibiting exacerbations of COPD seems to be higher than that of inhibiting catching the common cold. Rhinovirus infection causes only patchy damage in airway epithelium,[18] and the cytotoxic effects of an initial rhinovirus infection by itself might be restricted, whereas the airway epithelium infected by viruses produces inflammatory mediators, cytokines, and chemokines.[18] Not only the direct harmful effects of the virus itself, but also immune pathology induced by the immune response could contribute to a syndrome of exacerbation toward respiratory failure.[2,19] PPI could prevent exacerbation in patients with COPD by reducing catching common colds and reducing the production of proinflammatory cytokines in airway epithelial cells after rhinovirus infection.[7]

Acid reflux is a potential trigger of cough[20] and may be a complicating factor in difficult-to-control asthma.[21] It has been reported that patients with COPD are at significantly greater risk of GERD than those without COPD.[22] Another study found that the rate of exacerbations of COPD was higher in patients with GERD symptoms than in those without.[23] Furthermore, GERD symptoms were found to be associated with exacerbation of COPD in a prospective study of Japanese people with COPD.[24] The transient receptor potential vanilloid 1 (TRPV1), an excitatory cation channel, is thought to play an important role in the respiratory symptoms induced by gastroesophageal reflex. TRPV1 is selectively expressed in a subpopulation of nociceptive, primary sensory neurons that promote neurogenic inflammation through neuropeptide release and is activated by a temperature between 42°C and 53°C, low extracellular pH, diverse lipid derivatives, and vanilloid molecules such as capsaicin. In the airways, TRPV1 agonists cause cough, bronchoconstriction, microvascular leakage, hyperreactivity, and hypersecretion. Patients with asthma and COPD are more sensitive to the tussive effects of TRPV1 agonists, and TRPV1 activation may contribute to respiratory symptoms caused by acidic media present in the airways during asthma exacerbation or gastroesophageal reflux–induced asthma or in other conditions.[25] Therefore, it is possible that GERD may be associated with exacerbations of asthma and COPD. To evaluate the effects of PPI on the exacerbations of COPD without GERD, patients with gastroduodenal ulcers and reflex esophagitis in the present study were excluded according to QUEST scores and the results of endoscopic or radiographic analysis of the upper gastrointestinal tract, although 24-hour esophageal pH monitoring was not used because of the requirement of a tertiary care setting and the constrained condition on patients.

There are several limitations in the present study. The study design was not placebo controlled, and the scale of the study was not large. Furthermore, 24-hour esophageal pH monitoring was not used to diagnose GERD, which had

a higher sensitivity for acid regurgitation. The possibility that subjects with occult acid reflux could have been enrolled is not completely denied.

In conclusion, adding low-dose, long-term lansoprazole to the conventional therapies would have beneficial effects on reduction of the frequency of catching common colds and prevention of exacerbations in patients with COPD without any severe adverse effects. Further investigation of PPI therapy in patients with COPD with a large scale regarding safety and efficacy should be done.

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Author Contributions: Takahiko Sasaki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Takahiko Sasaki, Katsutoshi Nakayama, and Mutsuo Yamaya contributed to study supervision, reviewed the data, wrote the report, and recruited patients. Hiroyasu Yasuda, Motoki Yoshida, Takaaki Asanuma, Takashi Ohrui, and Hiroyuki Arai were clinical investigators. Hiroyasu Yasuda, Motoki Yoshida, and Takashi Ohrui were pulmonologists who diagnosed COPD and exacerbations of COPD. Jun Araya, Kazuyoshi Kuwano, and Hiroyuki Arai contributed to study supervision and study design.

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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 C_T 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 C_T 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

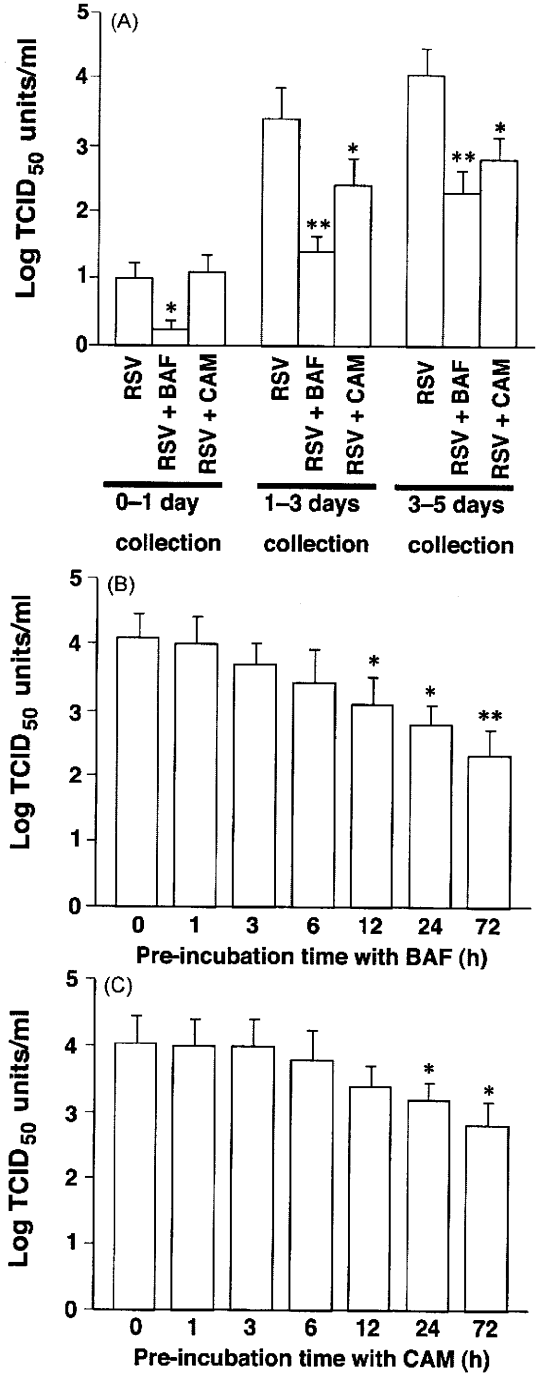

Fig. 1. (A) The time course of RS viral titers in supernatant fluids of human tracheal epithelial cells obtained for indicated days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in the presence of bafilomycin A_1 (10 nM; RSV+BAF), clarithromycin (10 μM; RSV+CAM), or vehicle (0.1% ethanol) (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, $n = 5$,

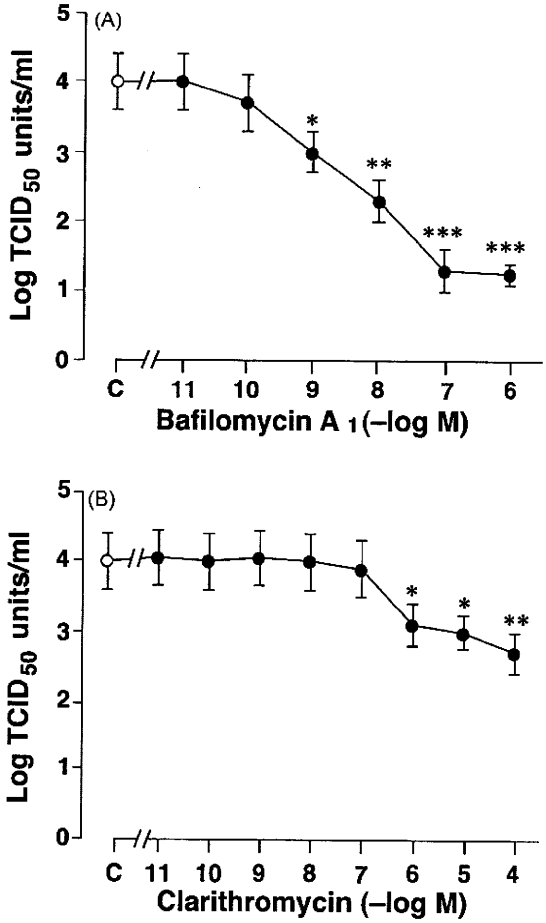

Fig. 2. Concentration–response effects of bafilomycin A_1 (A) and clarithromycin (B) on the viral titers in supernatant fluids collected during 3–5 days after infection. The cells were treated with bafilomycin A_1, clarithromycin or vehicle (control; C, 0.1% ethanol) from 3 days before RS virus infection 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 $^{***}P < 0.001$.

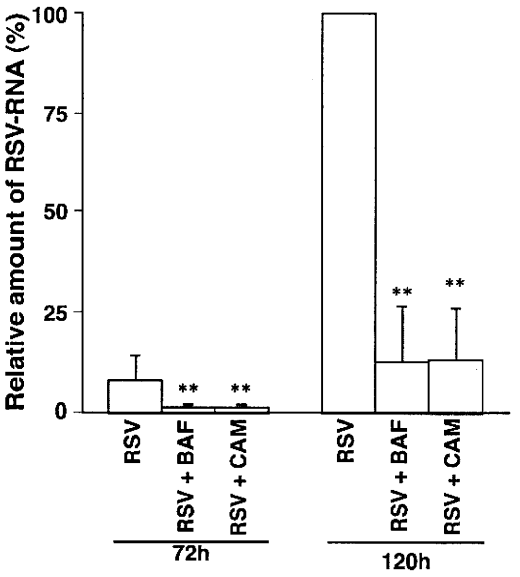

Fig. 3. Replication of RS virus RNA in human tracheal epithelial cells after infections of RS virus in the presence of bafilomycin A_1 (10 nM; RSV+BAF), clarithromycin (10 μM; RSV+CAM) or ethanol (0.1%) as a vehicle (control; RSV) as detected by real-time quantitative RT-PCR. Results are expressed as 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.

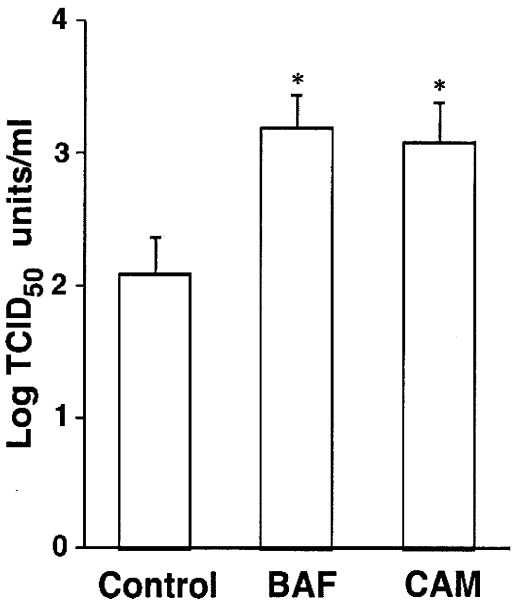

Fig. 4. The minimum dose of RS virus necessary to cause infection in the human tracheal epithelial cells treated with either bafilomycin A_1 (BAF, 10 nM), clarithromycin (CAM, 10 μM, 3 days) or vehicle (control, 0.1% ethanol). The minimum dose of RS virus necessary to cause infection is expressed as $TCID_{50}$ 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 $TCID_{50}$

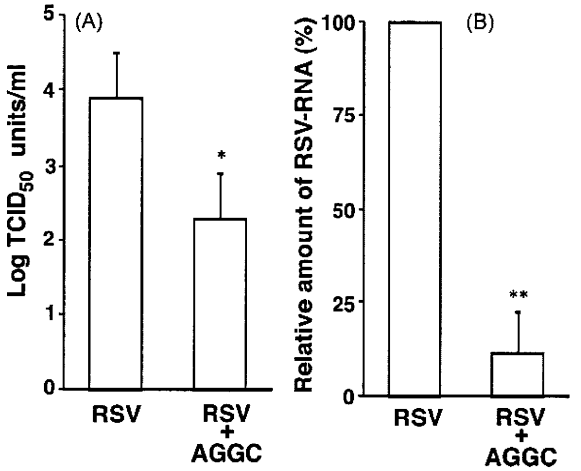

Fig. 5. (A) RS viral titers in supernatant fluids collected during 3–5 days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in human tracheal epithelial cells, treated with either *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC) (1 μM; RSV+AGGC) or vehicle (0.1% ethanol; RSV) from 3 days 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.

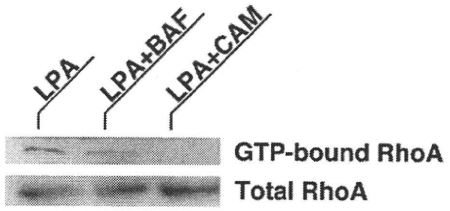

Fig. 6. Inhibitory effects of pre-incubation with bafilomycin A_1 (LPA + BAF, 10 nM, 72 h) and clarithromycin (LPA + CAM, 10 μM, 72 h) on the activation of RhoA (GTP-bound RhoA, RhoA-GTP) by lysophosphatidic acid (LPA) (1 μM, for 5 min) in human tracheal epithelial cells. Data are representative of five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

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 $P < 0.05$.

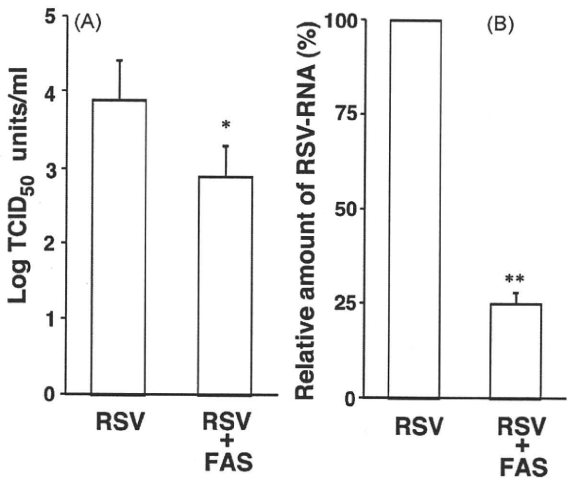

Fig. 7. (A) RS viral titers in supernatant fluids collected during 3–5 days after exposure to 10^{-3} $TCID_{50}$ units/cell of RS virus in human tracheal epithelial cells treated with either fasudil (10 μM; RSV + FAS) or vehicle (double distilled water; RSV) from 3 days before RS virus infection until 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 $^{**}P < 0.01$.

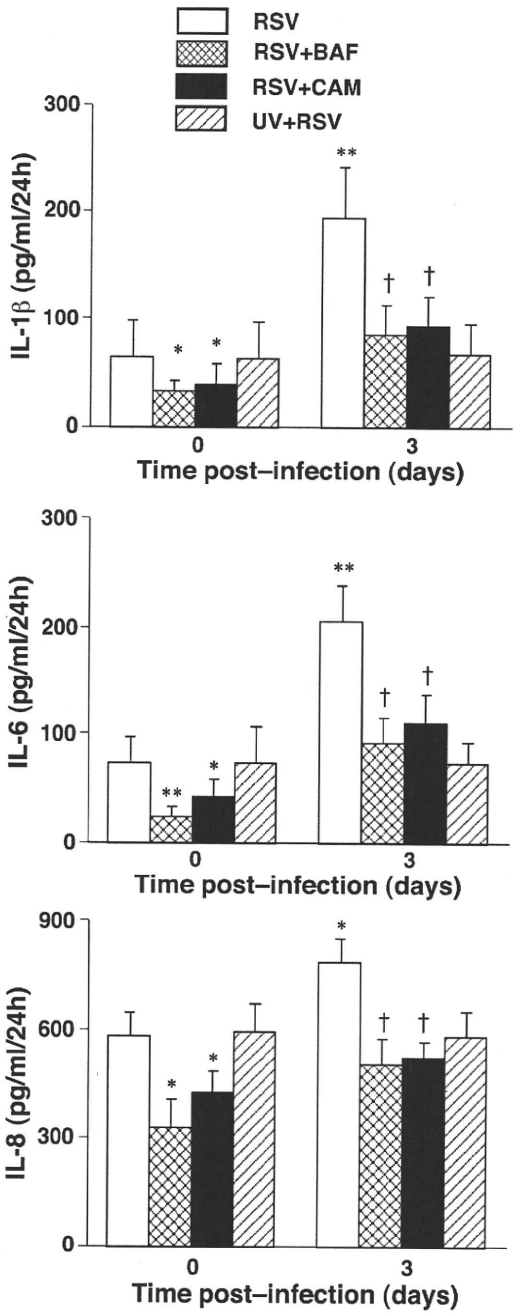

Fig. 8. Release of cytokines (IL-1β, IL-6 and IL-8) in supernatant fluids of human tracheal epithelial cells before (time 0; for 24 h before infection) and 3 days after RS virus infection (during 1–3 days after infection) in the presence of either bafilomycin A_1 (10 nM; RSV + BAF), clarithromycin (10 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 $^{\dagger}P < 0.05$.

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).

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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Short communication

L-Carbocisteine reduces neutrophil elastase-induced mucin production

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ABSTRACT

Human neutrophil elastase (HNE) exists in high concentrations in airway secretions and produces mucus hypersecretion in patients with chronic obstructive pulmonary disease (COPD). L-Carbocisteine improves the quality of life and reduces exacerbation in COPD patients. However the precise mechanism is uncertain. We examined the effects of L-carbocisteine on HNE-induced mucus hypersecretion and on the production of reactive oxygen species (ROS) which is associated with mucin production induced by HNE. NCI-H292, a human lung mucoepidermoid carcinoma cell line, was treated with or without HNE and L-carbocisteine. MUC5AC mRNA expression and ROS production in the cells, and MUC5AC protein concentration in supernatants were measured. HNE increased MUC5AC mRNA expression and MUC5AC protein concentration in supernatants in the cells. L-Carbocisteine reduces HNE-induced mRNA expression and protein secretion of MUC5AC. L-Carbocisteine also reduced ROS production in the cells induced by HNE. Reduction of HNE-induced mucus secretion by L-carbocisteine in the pulmonary epithelial cells may partly relate to the reduction of ROS.

1. Introduction

Mucus hypersecretion relates to dyspnea and sputum which are symptoms in patients with chronic obstructive pulmonary disease (COPD) (Macnee, 2007), cystic fibrosis and bronchial asthma. MUC5AC mucin is a major component of airway mucus in the respiratory tract, and is closely linked to goblet cell hyperplasia and mucus hypersecretion (Zheng et al., 2007). Neutrophil influx into the airways has been reported in patients with COPD (Macnee, 2007), and human neutrophil elastase (HNE) induces MUC5AC mucin secretion and goblet cell hyperplasia in cultured airway epithelial cells (Zheng et al., 2007). On the other hand, a mucolytic agent L-carbocisteine inhibits infection of rhinovirus, the major cause of common colds, and acts as a selective scavenger of oxidants (Yasuda et al., 2006b; Nogawa et al., 2009). Mucolytic agents including L-carbocisteine reduce the frequency of COPD exacerbation and common colds, and improved quality of life (Yasuda et al., 2006a). Because purulent sputum increases in COPD exacerbations, these findings suggest the reduced sputum production by the treatment with L-carbocisteine might relate to a clinical benefit in COPD. However, the inhibitory effects of L-carbocisteine have not been studied on HNE-induced mucin production. In this study, we examined the effects of L-carbocisteine on MUC5AC production induced by HNE in NCI-H292, a human lung mucoepidermoid carcinoma cell line. We also examined the effects of L-carbocisteine on ROS production which stimulates MUC5AC production in airway epithelial cells (Shao and Nadel, 2005).

2. Materials and methods

2.1. Cell culture and study protocol

NCI-H292 (ATCC, Rockville, MD), a human lung mucoepidermoid carcinoma cell line, was cultured in RPMI 1640 medium supplemented with 10% FBS (Moregate Biotech, Bulimba, QLD, Australia), and 1% Antibiotic-Antimycotic® (Gibco, Grand Island, NY) in a humidified 5% CO_2 atmosphere at 37 °C.

H292 cells in 6-well culture plates were pre-incubated with L-carbocisteine (1–100 μM) or control vehicle (PBS), and then co-incubated with HNE (100 nM, 30 min) (Human Sputum Elastase, Elastin Products, Co., Owensville, MO) or the equivalent volume of control vehicle (50:50, glycerol: 0.02 M sodium acetate, pH 5.0) for 30 min (Shao and Nadel, 2005). The concentrations of L-carbocisteine (1–100 μM) were chosen because the maximum concentrations of L-carbocisteine in the serum become more than 10 μM after oral ingestion of 1500 mg of L-carbocisteine (De Schutter et al., 1988). Serum-free medium was replaced and then cells were cultured for 8 h. For MUC5AC mRNA quantification, total RNA extraction was performed 8 h after stimulation with HNE. For MUC5AC protein content measurement, culture supernatants were

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