

of RV infections and the infection-induced exacerbations of bronchial asthma and COPD. However, Edwards et al. (2006, 2007) demonstrated that a LABA, salmeterol, increased IL-6 production, had no effects on IL-8 production in a bronchial epithelial cell line (BEAS-2B) cells and primary cultures of normal bronchial epithelial cells, and increased CXCL5 expression in primary cells after RV infection. In contrast, in primary cultures of normal bronchial epithelial cells, salmeterol alone reduced the RV-induced production of RANTES (regulated on activation, normal T cells expressed and secreted/CCL5) and interferon- γ -inducible protein 10 (IP-10/CXCL10). Skevaki et al. (2009) demonstrated that the LABA formoterol reduced the release of IL-8 but had no effect on the release of IL-6 from BEAS-2B cells. Thus, the effects of LABAs on the RV infection-induced production of pro-inflammatory cytokines are still controversial.

The major group RVs enter the cytoplasm of infected cells after binding to receptor intercellular adhesion molecule (ICAM)-1 (Greve et al. 1989; Casasnovas and Springer 1994). The entry of the RNA from this group into the cytoplasm of infected cells is suggested to be mediated by destabilization from receptor binding and endosomal acidification (Casasnovas and Springer 1994). Several agents, including glucocorticoids (Suzuki et al. 2000), macrolide antibiotics, bafilomycin (Pérez and Carrasco 1993), and erythromycin (Suzuki et al. 2002), inhibit the replication of the major group RVs through the reduction of ICAM-1 expression or by increasing the endosomal pH. We have demonstrated that the short-acting β_2 agonist procaterol inhibits RV replication in human tracheal epithelial cells (Yamaya et al. 2011). However, inhibitory effects of LABAs on RV replication have not been demonstrated.

The tulobuterol patch is a transdermal patch preparation of the β_2 agonist tulobuterol. The patch is designed to yield sustained β_2 agonistic effects for 24 hours when applied once daily. The tulobuterol patch has been used as a LABA in Japan and has been reported to improve the quality of life in COPD patients (Fukuchi et al. 2005). In the present study, we examined the effects of tulobuterol on the replication of a major group RV, RV14, in primary cultures of human tracheal epithelial cells. We also examined the effects of tulobuterol on the production of ICAM-1 and on the endosomal pH to clarify the mechanisms responsible for the inhibition of RV14 replication.

Materials and Methods

Human tracheal epithelial cell culture

Human tracheal surface epithelial cells were isolated and cultured as described previously (Yamaya et al. 2011). To

enhance RV14 release from the cells and to clarify the inhibitory effects of tulobuterol on viral release, the cells were cultured in rolling tubes (Turner and Couch 2006; Yamaya et al. 2011, 2012). To study the effects of tulobuterol on NF-kappa B (NF- κ B) activation and acidic endosomes before RV infection, the cells were cultured under stationary conditions.

Trachea samples for cell cultures were obtained after death from 41 patients (age, 73 ± 3 years; 15 females, 26 males). No patients had bronchial asthma, but three patients had COPD. The causes of death were malignant tumors other than lung cancer ($n = 23$), acute myocardial infarction ($n = 6$), congestive heart failure ($n = 4$), renal failure ($n = 3$), cerebral bleeding ($n = 2$), dermatomyositis ($n = 2$), and cerebral infarction ($n = 1$). Of the 41 patients, 15 were ex-smokers and 26 had never smoked. This study was approved by the Tohoku University Ethics Committee.

Culturing human embryonic fibroblast cells

Human embryonic fibroblast cells (HFL-III cells, Riken Bio Resource Center Cell Bank, Cell No: RCB0523; Tsukuba, Japan) were cultured as described previously (Yamaya et al. 2011).

Viral stocks

RV14 stocks were prepared from a patient with a common cold by infecting human embryonic fibroblast cells as previously described (Numazaki et al. 1987).

Detection and titration of viruses

RV14 in supernatant fluids (supernatants) was detected and titrated using the endpoint method (Condit 2006) as previously described (Yamaya et al. 2011). TCID₅₀ (tissue culture infective dose) was calculated as previously described (Condit 2006). The rates of change in RV14 concentration in the supernatants are expressed as TCID₅₀ units/ml/24 h (Yamaya et al. 2011).

Quantification of RV RNA

To quantify the RV14 RNA and ribosomal RNA (18S, rRNA) expression in the human tracheal epithelial cells after RV14 infection, two-step real-time quantitative reverse transcription-polymerase chain reaction (RT)-PCR using the Taqman technique (Roche Molecular Diagnostic Systems, Alameda, CA) was performed with TaqMan[®] Gene Expression Master Mix (Applied Biosystems, Bedford, CA) (Yamaya et al. 2011) according to methods previously described by Nolan et al. (2006).

Viral infection of the epithelial cells

Infection of the human tracheal epithelial cells with a stock solution of RV14 (100 μL in each tube, 1.0×10^4 TCID₅₀ units/100 μL , 5.0×10^{-2} TCID₅₀ units/cell) was performed as previously described (Yamaya et al. 2011). The cells were infected with RV14 at 3 days (72 h) after treatment with tulobuterol (0.1 $\mu\text{mol/L}$), except where we describe other concentrations or treatment periods.

Treatment with tulobuterol

To examine the effects of tulobuterol, cultured human tracheal epithelial cells from the same donors were treated with either tulobuterol (0.1 $\mu\text{mol/L}$, supplied from Abbott-Japan Co., Ltd., Tokyo, Japan) or the vehicle (0.001% ethanol) from 3 days (72 h) before RV14 infection until the end of the experiments after RV14 infection (Yamaya et al. 2011), except where we describe other concentrations or treatment periods. This particular dose used was chosen because Ruff et al. (1988) demonstrated smooth muscle relaxation of guinea pig tracheae at 0.1 $\mu\text{mol/L}$ or greater.

To examine the concentration-dependent effects of tulobuterol on RV14 replication and acidic endosomes, the cells were treated with tulobuterol at concentrations ranging from 1 nmol/L to 10 $\mu\text{mol/L}$. Similarly, to examine the time-dependent effects of tulobuterol on RV14 replication and acidic endosomes, the cells were treated with tulobuterol (0.1 $\mu\text{mol/L}$) for time periods ranging from 0 to 3 days (72 h).

To examine the effects of tulobuterol on ICAM-1 mRNA expression in the cells and the concentration of a soluble form of ICAM-1 (sICAM-1) in supernatants, cells were pretreated with tulobuterol (0.1 $\mu\text{mol/L}$) for 3 days before RV14 infection, the supernatants were collected, and RNA was extracted from a sample of the cells just prior to infections.

Collection of supernatants for measurements

The time course of viral release was measured using previously described methods (Yamaya et al. 2011). The supernatants were initially collected at 1 day (24 h) after infection, and then fresh medium with or without tulobuterol was added. Supernatants were also collected at 3 days (72 h) and 5 days (120 h) after infection, and fresh medium with or without tulobuterol was added. Similarly, the supernatants were collected at 7 days (168 h).

Furthermore, to measure RV14 release during the first 24 h, the supernatants were collected at 1 h after RV14

infection, and fresh medium with or without tulobuterol was added. The supernatants were also collected at either 12 h or 24 h after RV14 infection, and fresh medium with or without tulobuterol was added.

Similarly, to examine the effects of tulobuterol on the secretion of IL-1 β , IL-6, and IL-8, supernatants were collected just before infection, and 1 day (24 h), 3 days (72 h), and 5 days (120 h) after RV14 infection.

Effects of tulobuterol on susceptibility to RV infection

The effects of tulobuterol (0.1 $\mu\text{mol/L}$) on the susceptibility to RV14 infection were evaluated as previously described (Yamaya et al. 2011).

Measurement of ICAM-1 expression

The mRNA of ICAM-1 was examined using two-step real-time RT-PCR analysis with the methods described above (*Quantification of rhinovirus RNA*). The concentrations of the soluble form of ICAM-1 (sICAM-1) in supernatants were measured with an enzyme immunoassay (EIA) (Yamaya et al. 2011).

Measurement of changes in acidic endosomes

The distribution and the fluorescence intensity of acidic endosomes in the cells were measured as previously described, using LysoSensor DND-189 dye (Molecular Probes, Eugene, OR) (Gu et al. 1997; Yamaya et al. 2011). The cells on coverslips in Petri dishes were observed with a fluorescence microscope (OLYMPUS IX70; OLYMPUS Co. Ltd., Tokyo, Japan). The excitation wavelength was 443 nm, and the emitted light from the cells was detected through a 505-nm filter. The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®; Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope. The fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and the mean value of fluorescence intensity was expressed as a percentage of the control value compared with the fluorescence intensity of the cells before any treatment.

We studied the effects of a long treatment period with tulobuterol (0.1 $\mu\text{mol/L}$, 72 h) on acidic endosomes because the cells were pretreated with tulobuterol for 3 days before RV14 infection, except when we examined the time- or dose-dependent effects or the effects at other concentrations.

Measurement of cytokine production

We measured IL-1 β , IL-6, and IL-8 levels in the supernatants using specific enzyme-linked immunosorbent assays (ELISAs) as previously described (Yamaya et al. 2011) at all time points.

NF- κ B assay

Nuclear extracts from the cells were prepared using a TransFactor Extraction Kit (BD Bioscience/CLONTECH, Mountain View, CA). The presence of the translocated p50, p65, and c-Rel subunits was assayed using a Trans Factor Family Colorimetric Kit-NF κ B (BD Bioscience/CLONTECH) (Fiorucci et al. 2002; Yamaya et al. 2011).

Statistical analysis

The results are expressed as the mean \pm SE. Statistical analysis was performed using one-way analysis of variance (ANOVA). Subsequent post hoc analysis was performed using Bonferroni's method. For all analyses, values of $P < 0.05$ were considered to be significant. The number of donors (tracheae) from which cultured epithelial cells were used is referred to as n .

Results

Effects of tulobuterol on RV replication in human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to RV14 (5.0×10^{-2} TCID₅₀ units/cell) consistently led to infection. No virus was detected at 1 h after infection, but RV14 was detected in supernatants at 12 h, and the viral content progressively increased between 1 and 12 h after infection (Fig. 1A). Evidence of continuous viral replication was obtained by demonstrating that each of the supernatants collected at either 12 h to 24 h (1 day), 1 day (24 h) to 3 days (72 h), 3 days (72 h) to 5 days (120 h), or 5 days (120 h) to 7 days (168 h) after infection contained significant levels of RV14 (Fig. 1A). The viral titer levels in the supernatants increased significantly with time for the first 3 days (72 h) ($P < 0.05$ by ANOVA). Furthermore, in the tracheal cells from subjects whose cells were infected with RV14, the supernatants collected during 1 (24 h) to 3 days (72 h) after infection contained consistent levels of RV14 (4.52 ± 0.24 log TCID₅₀ units/ml/24 h, $n = 38$).

Treatment of the cells with tulobuterol (0.1 μ mol/L) significantly decreased the viral titers of RV14 in the supernatants from 12 h after infection compared with the titers in the cells treated with vehicle (0.001% ethanol) (Fig. 1A and B).

Furthermore, the selective β_2 -adrenergic receptor antagonist ICI 118551 (1 μ mol/L, Sigma, St. Louis, MO) (Suzuki et al. 2001) reversed the inhibitory effects of tulobuterol (0.1 μ mol/L) on the RV14 titer levels (Fig. 1A and B), whereas ICI 11851 alone did not alter the titer levels (data not shown).

Tulobuterol reduced RV14 release in a concentration-dependent manner (Fig. 1C). Pretreatment of the cells with tulobuterol reduced the viral titers of RV14 in the supernatants at concentrations of 0.1 μ mol/L or greater (Fig. 1C).

The inhibitory effects of tulobuterol on RV14 release were time-dependent. The maximum inhibitory effect was obtained when the cells were pretreated with tulobuterol for 3 days (72 h) (Fig. 1D). Significant inhibitory effects on RV14 release were observed when the cells were treated with tulobuterol (0.1 μ mol/L) for 48 h or longer prior to RV14 infection (Fig. 1D).

The RV14 titer levels in the supernatants of cells collected from 13 ex-smokers over 1 day (24 h) to 3 days (72 h) after infection did not differ from those of the 25 patients who had never smoked (4.58 ± 0.32 log TCID₅₀ units/ml/24 h vs. 4.50 ± 0.21 log TCID₅₀ units/ml/24 h, respectively, $P > 0.02$). Likewise, the RV14 titer levels in the supernatants of the cells from the 35 patients who had COPD did not differ from those of the 35 patients without COPD (data not shown). No virus was detected in the supernatants after infection with ultraviolet (UV)-inactivated RV14 (data not shown).

Treatment with tulobuterol (0.1 μ mol/L) for 3 days (72 h) did not change viability ($99 \pm 1\%$ in tulobuterol vs. $99 \pm 1\%$ in vehicle, $n = 5$, $P > 0.50$), as assessed by trypan blue exclusion. Furthermore, until 7 days (168 h) after initiation of cell culture, the cells made confluent sheets in the tubes in both the culture medium alone and the medium containing tulobuterol (0.1 μ mol/L) at the same time points. The number of cells in the confluent sheets cultured in the medium supplemented with tulobuterol (0.1 μ mol/L) did not differ from that in the medium supplemented with vehicle ($2.1 \pm 0.3 \times 10^6$ of cells/tube in tulobuterol vs. $2.2 \pm 0.3 \times 10^6$ of cells/tube in vehicle, $n = 5$, $P > 0.50$). When lactate dehydrogenase (LDH) concentrations in the supernatants 3 days (72 h) after tulobuterol treatment were measured, the treatment with tulobuterol (0.1 μ mol/L) for 3 days (72 h) did not appear to alter the LDH concentration (29 ± 2 IU/mL/24 h in tulobuterol vs. 30 ± 2 IU/mL/24 h in vehicle, $n = 5$, $P > 0.50$).

Effects of tulobuterol on viral RNA as measured by real-time RT-PCR

Further evidence of the inhibitory effects of tulobuterol on RV14 RNA replication in human tracheal epithelial cells was

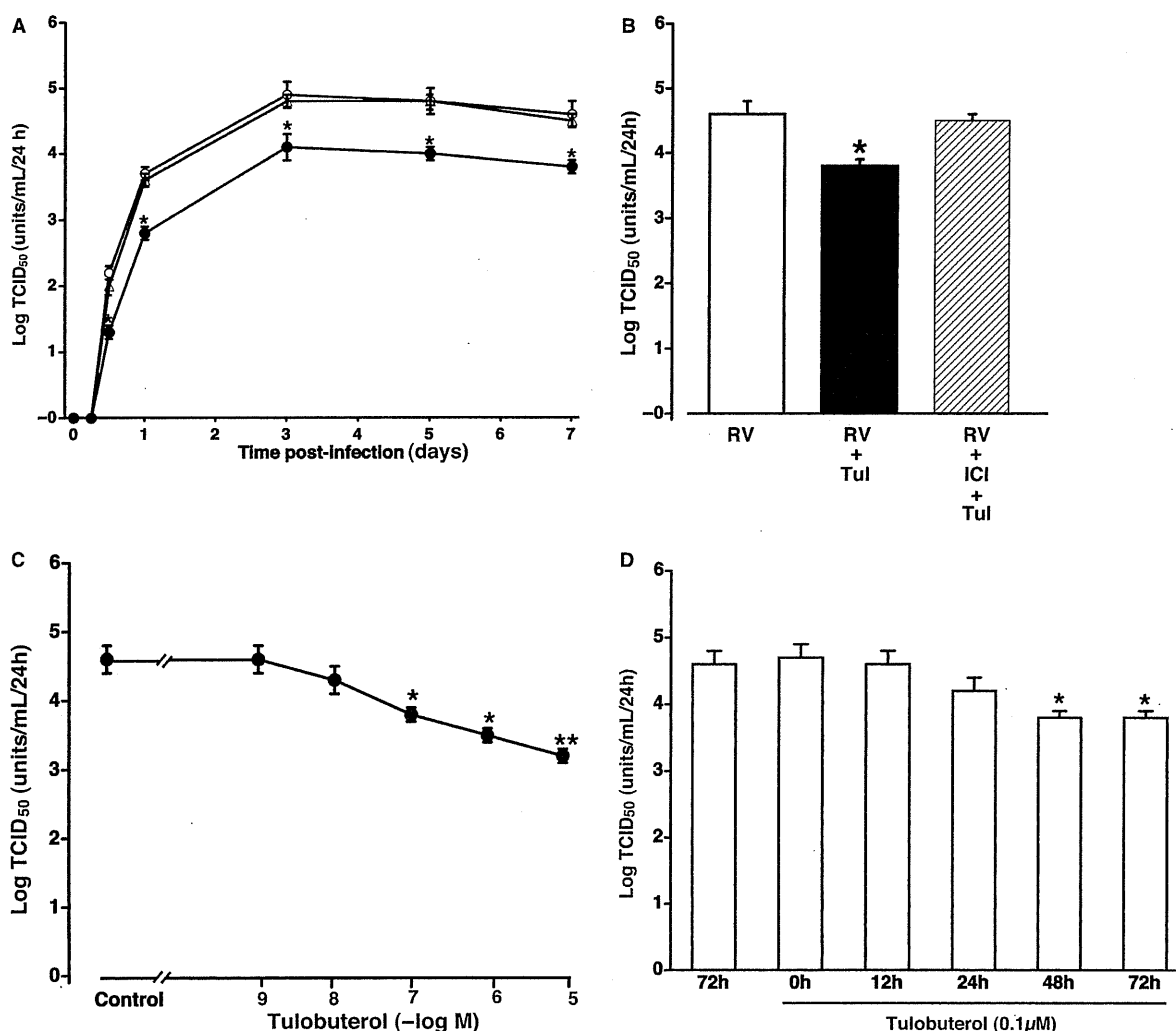


Figure 1. (A) The time course of viral release in the supernatants of human tracheal epithelial cells obtained at different times after exposure to RV14 in the presence of tulobuterol (0.1 $\mu\text{mol/L}$) (closed circles), tulobuterol (0.1 $\mu\text{mol/L}$) plus ICI11851 (1 $\mu\text{mol/L}$) (open triangles), or vehicle (0.001% ethanol) (open circles). The rates of change in RV14 concentration in the supernatants are expressed as TCID₅₀ units/mL/24 h. The results are the mean \pm SE from six different tracheae (two ex-smokers and four nonsmokers). Significant differences from viral infection alone are indicated by * $P < 0.05$. (B) Viral release in the supernatants collected over 1 day (24 h) to 3 days (72 h) after infection in the presence of tulobuterol (0.1 $\mu\text{mol/L}$) (RV + Tul), tulobuterol (0.1 $\mu\text{mol/L}$) plus ICI11851 (1 $\mu\text{mol/L}$) (RV + ICI + Tul), or vehicle (RV). The results are the mean \pm SE from six different tracheae. Significant differences from viral infection alone are indicated by * $P < 0.05$. (C) The concentration-response effects of tulobuterol on the viral release in the supernatants collected over 1–3 days after infection in the cells treated with either tulobuterol or vehicle (Control). The results are the mean \pm SE from six different tracheae. Significant differences from viral infection alone are indicated by * $P < 0.05$ and ** $P < 0.01$. (D) Time course of the effects of tulobuterol (0.1 $\mu\text{mol/L}$) on viral release in the supernatants collected over 1–3 days after infection in the cells treated for times ranging from 0 (0 h) to 3 days (72 h) and the viral release in the cells treated with vehicle for 3 days (72 h). The results are the mean \pm SE from five different tracheae (two ex-smokers and three nonsmokers). Significant differences from before any treatment (time 0) are indicated by * $P < 0.05$.

provided by real-time quantitative RT-PCR analysis. RNA extraction was performed at 1 day (24 h) and 3 days (72 h) after RV14 infection. RV14 RNA in the cells was consistently observed from 1 day (24 h) after infection and increased

between 1 day (24 h) and 3 days (72 h) after infection (Fig. 2). The maximum level of RV14 RNA replication was observed at 3 days (72 h) after infection, whereas RV14 RNA in the cells was not observed before infection.

Tulobuterol (0.1 $\mu\text{mol/L}$) decreased the RV14 RNA levels at 1 day (24 h) and at 3 days (72 h) after infection (Fig. 2).

However, ICI 118551 (1 $\mu\text{mol/L}$) reversed the inhibitory effects of tulobuterol (0.1 μM) on RV14 RNA replication, whereas ICI 118551 alone did not alter RNA replication (Fig. 2). The levels of RV14 RNA in the cells treated with ICI 118551 (1 $\mu\text{mol/L}$) alone did not differ from the levels in the cells treated with vehicle (0.001% ethanol) at 1 day (24 h) and 3 days (72 h) after RV14 infection (Fig. 2). In contrast, the level of RV14 RNA in the cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) plus ICI 118551 (1 $\mu\text{mol/L}$) was significantly higher than the level in the cells treated with tulobuterol alone and did not differ from the level in the cells treated with vehicle at 1 day (24 h) and at 3 days (72 h) after RV14 infection (Fig. 2).

Effects of tulobuterol on susceptibility to RV infection

Treatment of the cells with tulobuterol (0.1 $\mu\text{mol/L}$) decreased their susceptibility to RV14 infection. When viral release was measured using supernatants collected 3 days (72 h) after RV14 infection, the minimum dose of

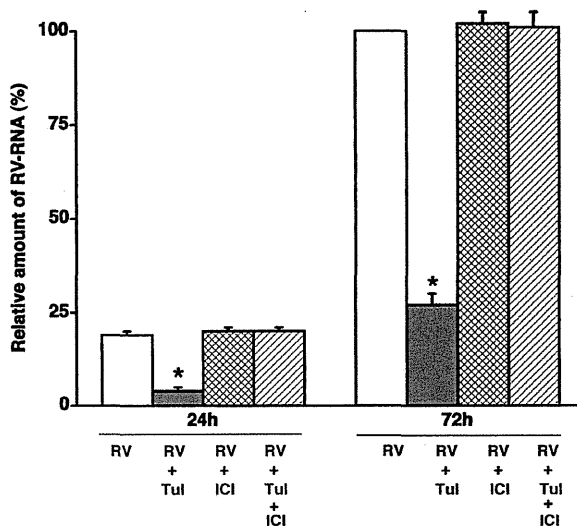


Figure 2. Replication of viral RNA in human tracheal epithelial cells at 1 day or 3 days after infection with RV14 in the presence of tulobuterol (0.1 $\mu\text{mol/L}$) (RV + Tul), ICI 118551 (1 $\mu\text{mol/L}$) (RV + ICI), tulobuterol plus ICI 118551 (RV + Tul + ICI), or vehicle (RV) as detected by real-time quantitative RT-PCR. The results are expressed as the relative amount of RNA expression (%) compared with that of maximal RV14 RNA at day 3 in the cells treated with vehicle and are reported as the mean \pm SE from five samples (two ex-smokers and three nonsmokers). Significant differences from treatment with vehicle (RV) at each time are indicated by * $P < 0.05$.

RV14 necessary to cause infection in the cells treated with tulobuterol (0.1 $\mu\text{mol/L}$, 72 h) (3.3 ± 0.2 log TCID₅₀ units/mL, $n = 5$, $P < 0.05$) was significantly higher than the minimum dose in the cells treated with vehicle (0.001% ethanol) (2.4 ± 0.2 log TCID₅₀ units/mL, $n = 5$).

The selective β_2 -adrenergic receptor antagonist ICI 118551 (1 $\mu\text{mol/L}$) by itself did not alter the minimum dose of RV14 necessary to cause viral release in the supernatants of the cells and did not affect susceptibility (data not shown). In contrast, ICI 118551 reversed the effects of tulobuterol on susceptibility to RV14 infection. The treatment of the cells with tulobuterol (0.1 $\mu\text{mol/L}$) plus ICI 118551 (1 $\mu\text{mol/L}$) decreased the minimum dose of RV14 necessary to cause viral release in the supernatants of the cells (2.5 ± 0.2 log TCID₅₀ units/mL, $n = 5$) compared with the dose in the cells treated with tulobuterol ($P < 0.05$) to the levels in the cells treated with the tulobuterol vehicle (0.001% ethanol).

Effects of tulobuterol on the expression of ICAM-1

Tulobuterol (0.1 $\mu\text{mol/L}$, 72 h) reduced the baseline ICAM-1 mRNA expression in the cells by approximately 35% compared with that of the cells treated with the tulobuterol vehicle (0.001% ethanol) before RV14 infection (Fig. 3A). Furthermore, the concentrations of sICAM-1 in the supernatants of the cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) were significantly lower than those in the cells treated with vehicle before RV14 infection (Fig. 3B).

ICI 118551 (1 $\mu\text{mol/L}$) itself did not change ICAM-1 mRNA expression and sICAM-1 release in the supernatants (data not shown). In contrast, ICI 118551 (1 $\mu\text{mol/L}$) reversed the inhibitory effects of tulobuterol on the ICAM-1 mRNA expression in the cells and sICAM-1 release in the supernatants (Fig. 3A and B). The ICAM-1 mRNA expression level and the concentration of sICAM-1 in the supernatants of the cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) plus ICI 118551 (1 $\mu\text{mol/L}$) were significantly higher than those in the cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) alone and did not differ from the expression level and concentration in the cells treated with vehicle before RV14 infection (Fig. 3A and B).

Effects of tulobuterol on the acidification of endosomes

Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Fig. 4A–C) as described previously (Yamaya et al. 2011). Treatment with vehicle (0.001% ethanol) for 3 days (72 h) did not change the number of acidic endosomes presenting green

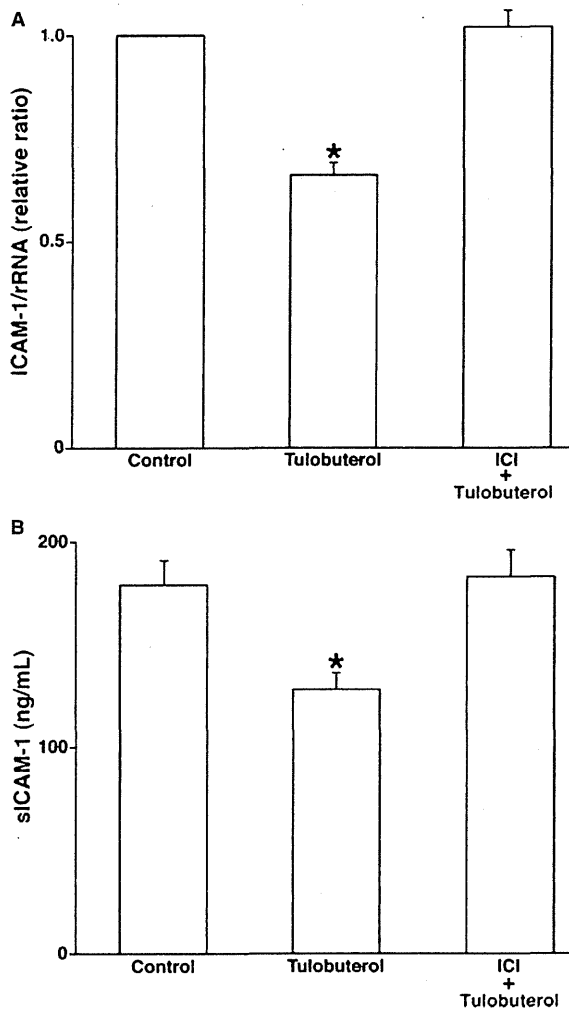


Figure 3. (A) The expression of ICAM-1 mRNA before RV14 infection in human tracheal epithelial cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) (Tulobuterol), tulobuterol plus ICI 118551 (1 $\mu\text{mol/L}$) (ICI + Tul), or the vehicle of tulobuterol (Control) for 3 days. The ICAM-1 mRNA was normalized to the constitutive expression of ribosomal RNA (rRNA). The expression of ICAM-1 mRNA in the cells treated with vehicle (Control) was set to 1.0. The results are the mean \pm SE from five different tracheae (two ex-smokers and three nonsmokers). Significant differences from control values are indicated by * $P < 0.05$. (B) The sICAM-1 concentrations in the supernatants before RV14 infection in human tracheal epithelial cells treated with tulobuterol (0.1 $\mu\text{mol/L}$) (Tul), tulobuterol plus ICI 118,551 (1 $\mu\text{mol/L}$) (ICI + Tul), or vehicle of tulobuterol (Control) for 3 days. The concentrations of sICAM-1 in the supernatants are expressed as ng/mL. The results are the mean \pm SE from five different tracheae. Significant differences from control values are indicated by * $P < 0.05$.

fluorescence in the cells (Fig. 4A and B) or the fluorescence intensity of acidic endosomes (Fig. 4D and E) compared with the intensity in the cells before any treatment. In

contrast, treatment with tulobuterol (0.1 $\mu\text{mol/L}$, 72 h) reduced the number of acidic endosomes with green fluorescence in the cells (Fig. 4C) and the fluorescence intensity of acidic endosomes in the cells (Fig. 4D and E) compared with cells treated with vehicle and before any treatment.

The inhibitory effects of tulobuterol on the fluorescence intensity of acidic endosomes were dose dependent. Significant inhibitory effects were observed at 0.1 $\mu\text{mol/L}$ or greater, and the maximum inhibitory effect was obtained at 10 $\mu\text{mol/L}$ (Fig. 4D). The inhibitory effects of tulobuterol on the fluorescence intensity of acidic endosomes were also time dependent, and significant inhibitory effects were observed when cells were treated with tulobuterol (0.1 $\mu\text{mol/L}$) for 24 h or longer (Fig. 4E). The maximum inhibitory effect was obtained when the cells were treated with tulobuterol for 3 days (72 h) (Fig. 4E).

We also examined the effects of dibutyryl cyclic-AMP (dibutyryl cAMP, Sigma) on the acidic endosomes to examine the mechanisms of tulobuterol-induced increases in endosomal pH. Treatment with dibutyryl cAMP (100 $\mu\text{mol/L}$) (Gekle et al. 2002) for 72 h reduced the number of acidic endosomes (data not shown) and the fluorescence intensity of acidic endosomes (Fig. 4F). The fluorescence intensity in the cells treated with vehicle did not differ from the intensity observed before any treatment (Fig. 4F).

Effects of tulobuterol on cytokine production

Tulobuterol (0.1 $\mu\text{mol/L}$) reduced the baseline secretion of IL-1 β , IL-6, and IL-8 for 24 h before RV14 infection compared with the levels observed in cells treated with vehicle (0.001% ethanol) (Fig. 5). RV14 infection increased the secretion of IL-1 β , IL-6, and IL-8. Maximum secretion was observed at 1 day (24 h) after RV14 infection for IL-6 and IL-8 and at 3 days (72 h) after infection for IL-1 β . Tulobuterol (0.1 $\mu\text{mol/L}$) also reduced the RV14 infection-induced secretion of IL-1 β , IL-6, and IL-8 compared with the levels observed in the cells treated with vehicle (Fig. 5). UV-inactivated RV14 infection did not affect the secretion of these cytokines.

Treatment of the cells with ICI 118551 (1 $\mu\text{mol/L}$, 72 h) reversed the inhibitory effects of tulobuterol on the baseline and RV14 infection-induced secretion of IL-1 β , IL-6, and IL-8 (Fig. 5), whereas ICI 118551 alone did not affect the secretion of these cytokines (data not shown).

In contrast, ultraviolet-irradiated RV14 did not increase the expression of IL-1 β , IL-6, and IL-8 at any time point after infection (Fig. 5). The secretion of IL-1 β , IL-6, and IL-8 in the supernatants of cells from three ex-smokers

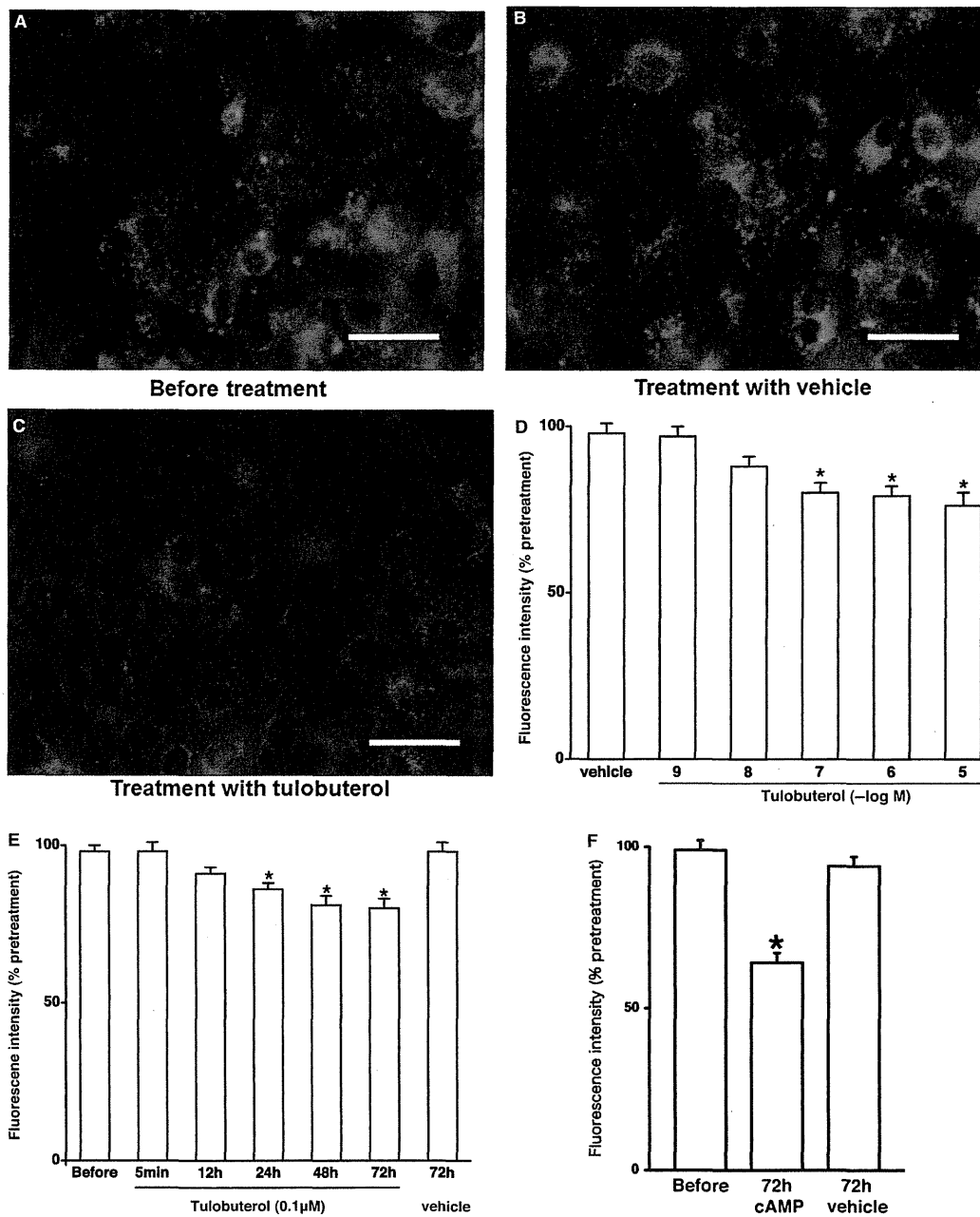


Figure 4. (A–C) Changes in the distribution of acidic endosomes with green fluorescence in human tracheal epithelial cells before (A) and 3 days (72 h) after treatment with tulobuterol (0.1 $\mu\text{mol/L}$) (C) or vehicle (B). The data are representative of five different experiments (two ex-smokers and three nonsmokers). (Bar = 100 μm) (D) The concentration-response effects of tulobuterol on the fluorescence intensity of acidic endosomes 3 days after treatment. The results are the mean \pm SE from five different tracheae. Significant differences from vehicle alone (Vehicle) are indicated by $*P < 0.05$. (E) The time course of the effects of tulobuterol (0.1 $\mu\text{mol/L}$) on the fluorescence intensity of acidic endosomes in the cells treated for times ranging from 0 (Before) to 3 days (72 h) and the fluorescence intensity in the cells treated with vehicle of tulobuterol (vehicle) for 3 days. The results are the mean \pm SE from five different tracheae. Significant differences from before any treatment (Before) are indicated by $*P < 0.05$. (F) The effects of dibutyryl cAMP (100 $\mu\text{mol/L}$) (cAMP) on the fluorescence intensity of acidic endosomes 3 days after treatment. The results are the mean \pm SE from three different tracheae (one ex-smoker and two nonsmokers). Significant differences from before any treatment (Before) are indicated by $*P < 0.05$.

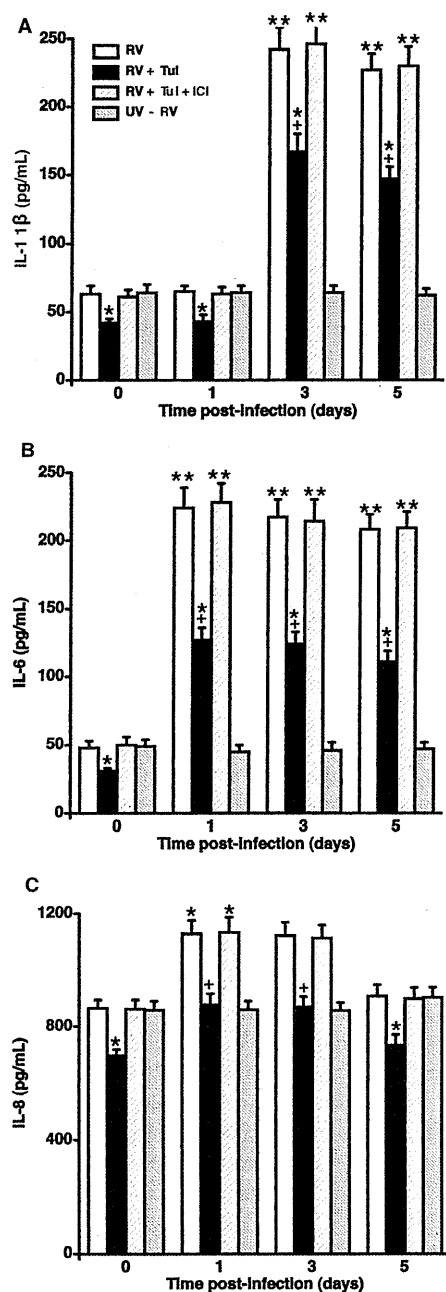


Figure 5. (A–C) Time course changes in the release of cytokines into the supernatants of human tracheal epithelial cells before and after RV14 infection in the presence of tulobuterol (0.1 $\mu\text{mol/L}$) (RV + Tul), tulobuterol plus ICI 118551 (1 $\mu\text{mol/L}$) (RV + Tul + ICI), or vehicle (RV). The results are the mean \pm SE from six different tracheae (three ex-smokers and three nonsmokers). Significant differences from values before RV14 infection (time 0) in the presence of vehicle are indicated by * $P < 0.05$ and ** $P < 0.01$. Significant differences from RV14 infection alone (RV) at each time point after infection are indicated by + $P < 0.05$.

did not differ from those of cells from three patients who had never smoked (data not shown). Similarly, the secretion of IL-1 β , IL-6, and IL-8 in the supernatants of cells from three patients who had COPD did not differ from those from three ex-smokers without COPD complications (data not shown).

Effects on NF- κ B

In cultured human tracheal epithelial cells, tulobuterol (1.0 $\mu\text{mol/L}$, 72 h) produced a small but significant reduction of the amount of p50, p65, and c-Rel of NF- κ B in the nuclear extracts compared with the levels observed in the cells treated with vehicle (Fig. 6A–C) and observed in the cells prior to RV14 infection (data not shown). RV14 infection increased the amount of p50, p65, and c-Rel of NF- κ B in the nuclear extracts in the cells (Fig. 6A–C). Likewise, tulobuterol (1.0 $\mu\text{mol/L}$, 72 h) treatment produced a small but significant reduction in the amount of p50, p65, and c-Rel of NF- κ B induced by RV14 infection (Fig. 6A–C).

Discussion

In this study, we demonstrated that tulobuterol reduced the titers of a major group RV, RV14, in supernatants and also reduced RNA replication of the virus in primary cultures of human tracheal epithelial cells. Pretreatment with tulobuterol reduced the expression of ICAM-1, the receptor for the major group RVs (Greve et al. 1989), and increased the minimum dose of RV14 necessary to cause viral infection. A selective β_2 -adrenergic receptor antagonist, ICI 118551 (Yamaya et al. 2011), reversed the inhibitory effects of tulobuterol on RV14 titer levels, viral RNA replication, and the expression of ICAM-1. Treatment with ICI 118551 also reversed the inhibitory effects of tulobuterol on the susceptibility of the cells to RV14 infection. These findings suggest that the β_2 -adrenoceptor-mediated effects of tulobuterol might inhibit RV14 infection partly through reducing the production of its receptor, as previously reported for the inhibitory effects of agents such as dexamethasone and the short-acting β_2 agonist procaterol (Suzuki et al. 2000; Yamaya et al. 2011).

Tulobuterol reduced the fluorescence intensity of acidic endosomes by 20% and the potency of the inhibitory effects was smaller than that observed with a proton ATPase inhibitor, bafilomycin (Suzuki et al. 2001), and procaterol (Yamaya et al. 2011). The results in the present study suggest that the order of the potency of inhibitory effects on viral replication (bafilomycin > procaterol > tulobuterol) is associated with the potency of the effects on the fluorescence intensity (Suzuki et al. 2001; Yamaya et al. 2011). The exact magnitude, how much the

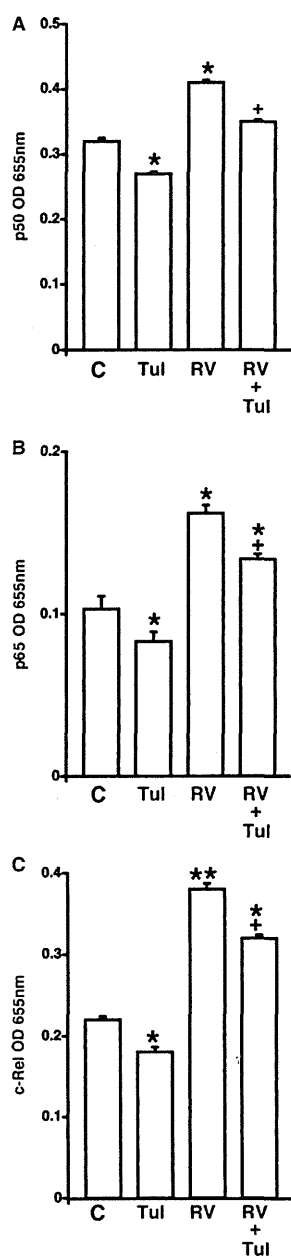


Figure 6. (A–C) Amount of p50 (A), p65 (B), and c-Rel (C) in nuclear extracts in human tracheal epithelial cells treated with tulobuterol (1.0 $\mu\text{mol/L}$) (Tul) or vehicle (C) for 3 days (72 h) before RV14 infection, and the amount in the cells 3 days after infection with RV14 in the presence of tulobuterol (RV + Tul) or in the presence of vehicle (RV). The results are expressed as the OD and represent the mean \pm SE from four different tracheae (two ex-smokers and two nonsmokers). Significant differences from control values (C) before RV14 infection are indicated by * $P < 0.05$ and ** $P < 0.05$. Significant differences from RV infection alone (RV) are indicated by + $P < 0.05$.

inhibitory effects of tulobuterol on the acidic endosomes were associated with reduction of viral replication, could not be determined. However, these findings suggest that the potency of the inhibitory effects of agents on RV replication was associated with the potency of the effects on the acidic endosomes. A lower reduction in the amount of acidic endosomes by tulobuterol might alter rhinoviral replication, and the tulobuterol-mediated reduction of acidic endosomes might also augment the effects on the reduction of RV14 replication in combination with the inhibitory effects on ICAM-1 expression, the receptor for the RV14.

The endosomal pH is regulated by vacuolar H^+ -ATPase (Mellman et al. 1986) and by ion transport across Na^+/H^+ exchangers (Marshansky and Vinay 1996; Nass and Rao 1998). Tulobuterol increases cAMP levels in the cells (Morin et al. 2000), and cAMP increases endosomal pH in kidney epithelial cells through the inhibition of a Na^+/H^+ exchanger (Gekle et al. 2002). We also observed that cAMP increases endosomal pH in human tracheal epithelial cells. These findings suggest that tulobuterol may have an inhibitory effect on Na^+/H^+ exchangers through the increased production of cAMP.

Tulobuterol reduced viral titers to 10% or less and the antiviral activity of tulobuterol is limited. Similarly, the reduction of the release of cytokines by tulobuterol was moderate, and tulobuterol reduced the release of IL-1 β and IL-6 by 40% and 50%, respectively. However, Skevaki et al. (2009) reported that a clinically used inhaled corticosteroid, budesonide, with sufficient antiinflammatory effects, reduces IL-6 in the supernatants of bronchial epithelial cells by approximately 70%. Furthermore, Pan et al. (2006) reported that treatment with budesonide (10^{-8} mol/L) reduces the release of IL-8 by 50% in the supernatants of human bronchial epithelial cells (BEAS-2B) in response to the stimulation with IL-1 β plus pyocyanin, a toxic factor from the bacteria *Pseudomonas aeruginosa*. In the present study, tulobuterol reduced the release of IL-8 in supernatants to baseline levels. These findings suggest that the potency of the inhibitory effects of tulobuterol on IL-1 β , IL-6, and IL-8 release may be sufficient to inhibit airway inflammation. Therefore, tulobuterol may have antiinflammatory effects that are sufficient to drive an inflammatory response.

Neutrophilic and eosinophilic inflammation in the exacerbation of bronchial asthma and COPD by RV infection has also been associated with a variety of mediators including IL-6 and IL-8 (Pizzichini et al. 1998; Seemungal et al. 2000). Tulobuterol inhibits superoxide anion (O_2^-) production by neutrophils and eosinophils (Yasui et al. 2006) and reduces the number of eosinophils in the sputum of asthma patients (Hozawa et al. 2009), although the levels of IL-8 in the induced sputum are not reduced

in COPD patients treated with tulobuterol (Kanehara et al. 2008). Reduced pro-inflammatory cytokine concentrations by tulobuterol during RV14 infection in the present study are consistent with previous findings on the inhibitory effects of procaterol on the plasma levels of cytokines, including IL-1 β , in rats (Izeboud et al. 2004). Salmeterol also inhibits the production of pro-inflammatory cytokines and monokines such as RANTES in bronchial epithelial cells after RV infection (Edwards et al. 2006). Similar to the inhibitory effects of procaterol (Yamaya et al. 2011), tulobuterol may modulate the airway inflammation induced by RV infection.

Intercellular adhesion molecule-1 also plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation observed in patients with bronchial asthma and COPD (Riise et al. 1994; Grunberg and Sterk 1999). The inhibitory effects of tulobuterol on ICAM-1 that we demonstrated in this study are consistent with previous reports that β_2 agonists, such as fenoterol, salmeterol, and procaterol, reduce ICAM-1 expression in airway epithelial cells and fibroblast cells (Oddera et al. 1998; Silvestri et al. 2001; Yoshida et al. 2009; Yamaya et al. 2011) and may also be associated with the inhibitory effects of LABAs, such as formoterol and salmeterol, on the exacerbations of bronchial asthma and COPD (Pauwels et al. 1997; Calverley et al. 2007). However, the antiinflammatory effects of formoterol and salmeterol are still uncertain.

In the present study, tulobuterol reduced the expression of ICAM-1 and pro-inflammatory cytokines. It has been reported that NF- κ B increases the expression of the genes for ICAM-1 and various pro-inflammatory cytokines (Zhu et al. 1996; Papi and Johnston 1999). Tulobuterol administration reduced the levels of p50, p65, and c-Rel of NF- κ B in human tracheal epithelial cells in the present study, and these inhibitory effects on NF- κ B activity are consistent with those of salmeterol and procaterol in lung myofibroblasts (Baouz et al. 2005) and human tracheal epithelial cells (Yamaya et al. 2011). The results for the NF- κ B activation using ELISA-based methods, which were used in the present study, were consistent with those studied with Western blot analysis used to measure the cytosolic amount of I κ B- α (Yamaya et al. 2011, 2012). These findings suggest that tulobuterol might reduce the expression of ICAM-1 partly through the reduction of NF- κ B activation.

Because tulobuterol inhibited NF- κ B activation and reduced cytokine concentration in the supernatants before RV14 infection in the present study, the attenuation of the production of inflammatory cytokines might be due to the inhibition of NF- κ B activation by tulobuterol. We previously reported that RV14 replication stimulates NF- κ B activation (Suzuki et al. 2002). Therefore, the attenuation of RV14 replication observed in the present

study might also be associated with the reduction of cytokine production after RV14 infection.

In contrast, Edwards et al. (2007) reported that salmeterol increases IL-6 production and enhances NF- κ B pathway activation following RV infection in a bronchial epithelial cell line (BEAS-2B) and in primary cultures of normal bronchial epithelial cells. Furthermore, another report demonstrated that β_2 agonists do not affect NF- κ B-induced activation of the IL-6 gene in airway smooth muscle cells (Kaur et al. 2008). However, the production of IL-6 after RV infection through the activation of NF- κ B has been reported in A549 cells (Zhu et al. 1996). Fragaki et al. (2006) demonstrated that salmeterol plus corticosteroids reduced IL-6 release in response to *Staphylococcus aureus* in a transformed human tracheal gland cell line partly through the inhibition of NF- κ B. The inhibition of NF- κ B and TNF- α -induced IL-6 production by salmeterol has also been reported in lung myofibroblasts (Baouz et al. 2005). We previously reported that reduced production of IL-6 by the β_2 agonist procaterol is associated with the inhibition of NF- κ B (Yamaya et al. 2011). Thus, these findings suggest that the different effects of β_2 agonists on IL-6 and NF- κ B after RV14 infection or after addition of stimulants may be partly associated with differences in cell type and culture conditions.

In the present study, we observed that tulobuterol reduced the production of ICAM-1 and inflammatory cytokines and that a selective β_2 -adrenergic receptor antagonist ICI 118551 reversed the inhibitory effects of tulobuterol, as reported in a previous study using procaterol (Yamaya et al. 2011). Farmer and Pugin (2000) reported that β -adrenergic agonists increase the cytoplasmic concentration of inhibitory kappa B- α (I κ B- α), by decreasing its degradation. Tulobuterol and procaterol increase intracellular cAMP (Morin et al. 2000; Yamaya et al. 2011), although we did not examine the effects of tulobuterol on the production of cAMP. It has been reported that cAMP-induced signals inhibit NF- κ B activities (Gerlo et al. 2011). These mechanisms may relate to the tulobuterol-induced inhibitory effects on the NF- κ B activity observed in the present study.

Ruff et al. (1988) reported that acetylcholine-induced contraction of the tracheal smooth muscle of guinea pigs was relaxed by tulobuterol and 4-hydroxytulobuterol, a metabolite of tulobuterol (Kubo et al. 1980). They also reported that the potency of 4-hydroxytulobuterol was more than 1000-fold higher than that of tulobuterol (Ruff et al. 1988). The maximum serum concentration of tulobuterol was 2.1–2.4 ng/mL (=10 nmol/L), and the amount of urine excretion of 4-hydroxytulobuterol was approximately half that of tulobuterol (data from Abbott Japan Co. Ltd.), suggesting that the levels of 4-hydroxytulobuterol are similar to those of tulobuterol in serum. We

demonstrated that tulobuterol reduced the RV14 titers in supernatants at concentrations ranging from 0.1 $\mu\text{mol/L}$ to 10 $\mu\text{mol/L}$. These findings suggest that tulobuterol may inhibit RV14 replication at clinically available concentrations, although we could not measure the effects of 4-hydroxytulobuterol.

Because tulobuterol alone did not change the cell viability as assessed by the exclusion of trypan blue and LDH concentrations in supernatants, reduced cytokine release might be partly associated with the inhibition of NF- κ B activation but not with cell injury.

In summary, this is the first report that the β_2 -agonist tulobuterol, which in patch form has been used as a LABA in Japan, reduces RV14 titers in supernatants, reduces RV RNA replication in cultured human tracheal epithelial cells, and decreases the susceptibility of the cells to RV14 infection. These results may occur partly through the reduced expression of ICAM-1, the receptor for the major group RVs, and a reduction in the number of acidic endosomes from which RV14 RNA enters the cytoplasm. Tulobuterol reduced the baseline and RV replication-induced release of IL-1 β , IL-6, and IL-8 in the supernatants. Tulobuterol may inhibit the replication of the major group RVs and modulate the inflammatory responses in the airways after RV replication.

Acknowledgments

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Conflict of Interest

Mutsuo Yamaya is a professor and Hiroshi Kubo is an associate professor in the Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine. This department has received funding from eleven pharmaceutical companies: Abbott Japan, Co., Ltd., Kyorin Pharmaceutical Co. Ltd., Taisho Toyama Pharmaceutical Co., Ltd., GlaxoSmithKline K.K. Co., Ltd., Daiichi Sankyo Co. Ltd., Otsuka Pharmaceutical Co. Ltd., Tanabe Mitsubishi Pharmaceutical Co., Ltd., Teijin Pharma Co., Ltd., Ono Yakuhin Co. Ltd., and AstraZeneca K.K. and Nippon Boehringer-Ingelheim Co., Ltd. Tulobuterol used in the present study was supplied from Abbott-Japan Co., Ltd.

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A study on sleep architecture in patients with chronic respiratory failure under long-term oxygen therapy—Focused on the influence of ventilatory failure (high CO₂) elements on the patient's sleep architecture*

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ABSTRACT

Sleep disturbance related symptoms are common in patients with long-term oxygen therapy (LTOT). Essentially, there were only few previous reports about the sleep architecture in patients with respiratory disease, such as chronic obstructive pulmonary disease (COPD). This study aims to clarify the objective sleep state and the elements that affect sleep architecture in Chronic Respiratory Failure (CRF) patients with focus on clinical cases of chronic hypercapnia. 13 subjects with chronic respiratory failure were enrolled in the study. All the subjects were pre-evaluated by pulmonary function test and Arterial blood gas analysis (ABG) including exercise testing. Polysomnography (PSG) test was performed in each subject with supplemental oxygen. The estimated base line PaCO₂ value that reflects overall PaCO₂ including sleep period was calculated using equation of $PaCO_2[2.4 \times (HCO_3^-) - 22]$ from obtained ABG value just before PSG test. 6 subjects were classified as hypercapnic group (base line PaCO₂ ≥ 45 mmHg) and 7 subjects were non-hypercapnic group (base line PaCO₂ < 45 mmHg). Latency persistent sleep of PSG data was significant higher in patients with hypercapnic than

non-hypercapnic ($p < 0.01$). Periodic Limb Movement was seen in 23.6% of the subjects, however there was no contribution for arousals. Other PSG data include mean SpO₂ were no significant difference. This study suggests that patients with estimated hypercapnia had more disturbed sleep architecture especially significant loss of sleep latency than non-hypercapnic patient with chronic respiratory failure under LTOT. Nocturnal PaCO₂ level or ventilatory function may contribute to sleep disturbance in patients with estimated hypercapnia during LTOT.

Keywords: Chronic Respiratory Failure; Long-Term Oxygen Therapy; Sleep Architecture; Periodic Leg Movement Disorder

1. INTRODUCTION

The goal of Long-Term Oxygen Therapy (LTOT) or Home Oxygen Therapy is to improve respiratory failure in patients with Chronic Respiratory Failure (CRF) and heart failure, which are defined by the partial pressure of arterial oxygen (PaO₂), through maintaining a certain oxygen level at rest and during exertion and sleep [1-3]. Accordingly, application of the therapy and oxygen dosage is usually determined based on the PaO₂ at rest and in accordance with the evaluation of PaO₂ during exertion and sleep [4]. However, patients under LTOT often experience aggravation of the respiratory status of

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which the cause is sometimes not identified.

Many of such patients are hospitalized and discharged repeatedly without being given any novel treatment resulting in the term in which oxygen administration alone cannot maintain the condition and artificial ventilation is required in an intensive care unit.

We noticed that CRF patients under LTOT who experience repeated acute aggravation regardless of hypoxemia resolution with oxygen therapy tend to request a hypnotic prescription and complain of insomnia.

Patients with COPD who need LTOT tend to have insomnia and sleepiness during the daytime in addition to a higher incidence of nightmare compared to healthy subjects [5,6]. Insomnia, early morning awakening and daytime drowsiness are also observed in asthmatic patients not necessarily with respiratory failure [7]. We sometimes have no choice but to prescribe hypnotics for respiratory failure patients who complain of insomnia. Although some studies [7] report that recent hypnotics are relatively safe for use in respiratory failure patients, most hypnotics and sedatives have some muscle relaxant and respiratory depression actions [8-12]. Therefore, hypnotics for sleep improvement should be administered carefully in CRF patients who complain of insomnia and tend to have daytime drowsiness. For safe and rational treatment, it is essential to understand the sleep state in respiratory failure patients without the use of any agent that could influence sleep. And many studies have shown that hypoxemia during sleep causes arousal response and that sleep stages are associated with aggravation and improvement of hypoxemia in respiratory failure patients [13-16].

However, there are few sleep-physiological studies regarding the influence of respiratory disorder on sleep state in chronic respiratory failure patients, especially, the influence of such disorder on the sleep architecture in CRF patients under LTOT. Although one report [17] on the influence of ventilatory failure and hypercapnia on sleep in healthy subjects has been published, the influence on sleep in patients with CRF is yet to be revealed.

We evaluated the sleep architecture in CRF patients under LTOT using polysomnography (PSG) including electroencephalogram, investigated the clinical conditions of the patients focusing on the influence of hypercapnia on sleep architecture, and presented the findings below.

2. PATIENTS AND METHOD

2.1. Subjects

The subjects were 13 male CRF patients of the Respiratory Division in Hachinohe Red Cross Hospital (8 patients with COPD, 2 patients with [diffuse panbronchiolitis]; DPB, 1 patient with [interstitial pneumonitis]; IP,

and 2 patients with [Rheumatoid]; RA lung), all of whom are under LTOT. The oxygen dosage for each patient was determined thorough blood gas analysis at rest, SpO₂ measurement during sleep, and 12 minutes walking test. The controls were 7 male healthy subjects without any findings of sleep disturbance and respiratory failure under PSG, who are outpatients of the Respiratory Division in Hachinohe Red Cross Hospital and matched by gender and age. All the subjects gave written informed consent for using the test results during hospitalization for scientific research. And this study approved by IRB of Hachinohe Red Cross Hospital.

2.2. Polysomnography and Arterial Blood Gas Analysis during Wakefulness

An overnight full-montage polysomnogram was carried out in accordance with the current standards of the American Academy of Sleep Medicine (AASM) [17]. All subjects did not take any xanthine-contained medicine, hypnotics and sedatives in the day of this investigation. The sleep study was started at 8:00 pm and completed at 6:00 am on the following day. The recording montage consisted of continuous recordings of the following physiologic channels: C3-A2 and C4-A1 electro-encephalogram (EEG), right and left electro-oculogram (EOG), a single bipolar electrocardiogram (ECG), chin electromyogram (EMG), oxyhemoglobin saturation by pulse oximetry, chest and abdominal excursion by inductance plethysmography, airflow by an oronasal thermocouple, and body position by a mercury gauge. Scoring of the sleep data were conducted as follows. Initially, an automated computerized analysis of sleep and breathing abnormalities was performed using the Allis Sleepware v. 2.7.43 (Philips Respironics) program. Then, a trained sleep technician and a sleep physician visually assessed and corrected the data in accordance with the current standards of sleep and respiratory scoring.

Radial arterial blood was collected after the subjects were maintained at rest in the supine position for 1 hour with oxygen supplied at the time of admission and the specimens were analyzed using an arterial blood gas analyzer (ABL 700 series, Radiometer, Copenhagen, DK).

2.3. Calculation of Baseline PaCO₂

The ABG obtained from the blood collected at the time of admission is the data during wakefulness. Assuming that bicarbonate ion (HCO₃⁻) concentration derived from the ABG data reflects CO₂ at rest based on the Henderson-Hasselbalch equation and that the concentration includes PaCO₂ during sleep, PaCO₂ at rest including during sleep was estimated using the following prediction Formula (1) [18].

$$\text{PaCO}_2 = 2.4 \times [\text{HCO}_3^-] - 22 \quad (1)$$

Since ventilation is generally suppressed during sleep, ventilatory status during sleep is considered to be an important influential factor affecting the value obtained by the above Formula (1).

2.4. Statistical Analysis

Regarding analysis of the data on the LTOT and Control Groups, the three groups were compared using ANOVA. In regard to analysis of the data on the High CO₂ Group and the Normal CO₂ Group, Cochran-cox test was used.

3. RESULTS

Table 1 shows the demographics and PSG data on each subject group (Table 1). Significant differences

Table 1. Clinical characteristics of control subjects and patients of LTOT (mean ± S.E.).

	Control	LTOT	P-value
n	7	13	
Age (year)	70.4 ± 1.0	72.8 ± 1.6	n.s.
Body-mass-index (kg/m ²)	25.2 ± 1.6	20.8 ± 0.7	<0.01
Total sleep time (min.)	388.1 ± 15.7	288.1 ± 31.8	<0.05
Stage N1 (%)	26.3 ± 1.6	30.9 ± 5.5	<0.05
Stage N2 (%)	55.1 ± 6.8	50.0 ± 5.8	n.s.
Stage N3 (%)	8.3 ± 3.4	0.1 ± 0.1	<0.005
Stage R (%)	10.1 ± 2.3	18.8 ± 2.0	<0.05
Latency persistent sleep (min.)	25.2 ± 4.8	76.9 ± 16.0	<0.05
Sleep Efficiency (%)	72.7 ± 2.8	45.6 ± 3.5	<0.0001
PLMS index (events/h)	2.3 ± 1.4	4.5 ± 1.2	n.s.
Apnea hypopnea index (events/h)	1.9 ± 0.6	14.4 ± 3.9	<0.05
Arousal index (events/h)	11.3 ± 2.3	17.5 ± 2.1	<0.05
SpO ₂ mean (%)	94.0 ± 0.8	94.5 ± 0.7	n.s.
SpO ₂ min. (%)	89.3 ± 0.9	87.8 ± 1.1	n.s.
Desaturation index (events/h)	1.3 ± 0.6	5.7 ± 1.3	<0.05
pH	7.43 ± 0.01	7.41 ± 0.01	n.s.
PCO ₂ (mmHg)	39.0 ± 1.0	45.4 ± 3.2	n.s.
PO ₂ (mmHg)	90.0 ± 2.7	77.4 ± 2.6	<0.01
HCO ₃ ⁻ (mEq/l)	25.2 ± 0.5	28.0 ± 1.3	n.s.
Prediction of PCO ₂ (mmHg)	38.6 ± 1.2	45.2 ± 3.2	n.s.

were found between the LTOT and Control Groups regarding Total Sleep Time (TST), Sleep Efficiency (SE), % stage 1 (N1), % stage 3 + 4 (N3), and % stage REM(R). The values were 288.1 ± 31.8 mins, 45.6% ± 3.5%, 30.9% ± 5.5%, 0.1% ± 0.1%, and 18.8% ± 2.0%, respectively, in the LTOT Group (mean ± S.E.) while they were 388.1 ± 15.7 mins, 72.7% ± 2.8%, 26.3 ± 1.6, 8.3% ± 3.4%, and 10.1% ± 2.3% (mean ± S.E.), respectively, in the Control Group. TST, SE, and % stage 3 + 4 (N3) were significantly lower (*P* < 0.05, *P* < 0.0001, *P* < 0.005) while % stage 1 (N1) and % stage REM (R) were significantly higher (*P* < 0.05, *P* < 0.05) in the LTOT Group. Sleep latency (SL) was 76.9 ± 16.0 mins in the LTOT Group and significantly higher (*P* < 0.05) than that of the Control Group, which was 25.2 ± 4.8 mins (Figures 1 and 2).

Regarding the other PSG parameters, Arousal and Apnea hypopnea indices were significantly higher in the LTOT Group (*P* < 0.05 and *P* < 0.05): 17.5 ± 2.1 times/hour vs. 11.3 ± 2.3 times/hour and 14.4 ± 3.9 times/hour vs. 1.9 ± 0.6 times/hour.

In comparison of the 6 LTOT patients with elevated baseline PCO₂ (PCO₂ ≥ 45) (Hypercapnia: H Group) and 7 patients with normal baseline PCO₂ (PCO₂ < 45) (Normocapnia: N Group) matched by age, hypoxemia (SpO₂ < 90%) was not observed during sleep in both groups and SL was significantly higher (*P* < 0.01) in the H Group: 123.8 ± 18.8 mins in the H Group vs. 36.7 ±

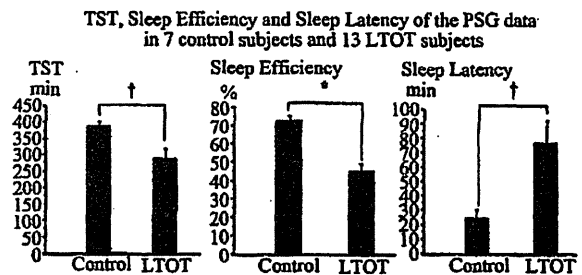


Figure 1. Total sleep time (TST), Sleep Efficiency and Sleep Latency of the PSG data in 7 control subjects and 13 LTOT subjects mean ± SE. †*P* < 0.05, **P* < 0.0001.

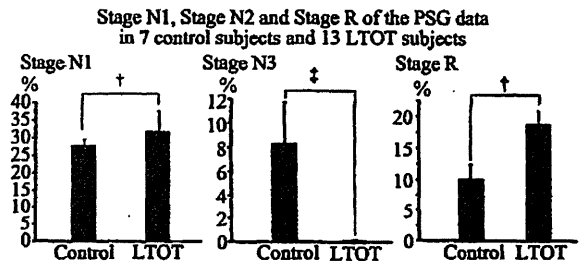


Figure 2. Stage N1, Stage N2 and Stage R of the PSG data in 7 control subjects and 13 LTOT subjects mean ± S.E. †*P* < 0.05, ‡*P* < 0.005.

10.9 mins in the N Group. TST, SE, N1, N2, N3, and R did not differ significantly between the two groups (Figure 3, Table 2).

Although it is noteworthy that Periodic leg movement disorder (PLMD) was observed in 62.5% of the LTOT patients with especially high PLMD complication incidence of 50% in the H Group, but there was no correlation of this finding to sleep interruption (Figure 4).

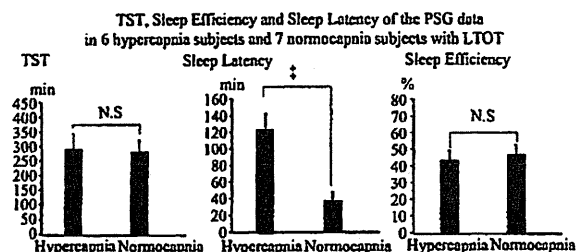


Figure 3. Total sleep time (TST), Sleep Efficiency and Sleep Latency of the PSG data in 6 hypercapnia subjects and 7 normocapnia subjects with LTOT mean ± SE. †P < 0.005.

4. DISCUSSION

CRF in patients is usually diagnosed based on clinical definitions, which are all based on the presence of hypoxemia and categorized depending on whether being accompanied by hypercapnia [19-21]. This means that all CRF patients have hypoxemia and that the study needs to be conducted with the influence of hypoxemia excluded by the means of oxygen administration.

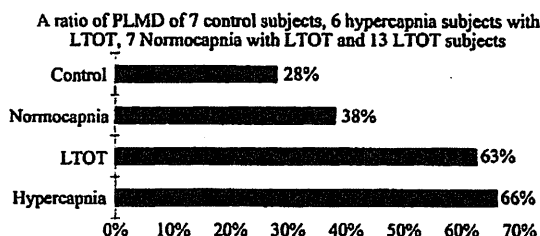


Figure 4. A ratio of PLMD of 7 control subjects, 6 hypercapnia subjects with LTOT, 7 Normocapnia with LTOT and 13 LTOT with LTOT.

Table 2. Clinical characteristics of control subjects and patients of hypercapnia and normocapnia with LTOT (mean ± S.E.).

	Control	Hypercapnia	Normocapnia	Control vs Hypercapnia	Control vs Normocapnia	Hypercapnia vs Normocapnia
n	7	6	7			
Age (year)	70.4 ± 1.0	71.0 ± 3.0	74.3 ± 1.5	n.s.	n.s.	n.s.
Body-mass-index (kg/m ²)	25.2 ± 1.6	20.3 ± 1.0	21.2 ± 0.9	<0.05	<0.05	n.s.
Total sleep time (min.)	388.1 ± 15.7	292.1 ± 55.8	284.7 ± 39.1	n.s.	<0.05	n.s.
Stage N1 (%)	26.3 ± 1.6	35.5 ± 10.9	27.0 ± 4.6	<0.05	<0.005	n.s.
Stage N2 (%)	55.1 ± 6.8	47.7 ± 11.3	52.0 ± 5.8	n.s.	n.s.	n.s.
Stage N3 (%)	8.3 ± 3.4	0.2 ± 0.1	0.1 ± 0.1	<0.05	<0.05	n.s.
Stage R (%)	10.1 ± 2.3	16.4 ± 3.5	20.9 ± 2.2	n.s.	<0.01	n.s.
Latency persistent sleep (min.)	25.2 ± 4.8	123.8 ± 18.8	36.7 ± 10.9	<0.0005	n.s.	<0.005
Sleep Efficiency (%)	72.7 ± 2.8	43.9 ± 4.0	47.0 ± 5.8	<0.0001	<0.005	n.s.
PLMS index (events/h)	2.3 ± 1.4	6.5 ± 2.2	2.8 ± 0.7	n.s.	n.s.	n.s.
Apnea hypopnea index (events/h)	1.9 ± 0.6	13.7 ± 8.1	15.0 ± 3.0	n.s.	<0.001	n.s.
Arousal index (events/h)	11.3 ± 2.3	17.4 ± 2.6	17.5 ± 3.4	n.s.	<0.001	n.s.
SpO ₂ mean (%)	94.0 ± 0.8	95.7 ± 1.2	93.6 ± 0.8	n.s.	n.s.	n.s.
SpO ₂ min. (%)	89.3 ± 0.9	88.7 ± 1.7	87.0 ± 1.6	n.s.	n.s.	n.s.
Desaturation index (events/h)	1.3 ± 0.6	4.2 ± 1.4	7.0 ± 2.2	n.s.	<0.05	n.s.
pH	7.43 ± 0.01	7.38 ± 0.02	7.43 ± 0.01	<0.05	n.s.	<0.05
PCO ₂ (mmHg)	39.0 ± 1.0	54.4 ± 4.6	37.8 ± 1.5	<0.005	n.s.	<0.005
PO ₂ (mmHg)	90.0 ± 2.7	77.7 ± 3.6	77.2 ± 4.1	<0.05	<0.05	n.s.
HCO ₃ ⁻ (mEq/l)	25.2 ± 0.5	32.0 ± 1.6	24.5 ± 0.6	<0.005	n.s.	<0.001
Prediction of PCO ₂ (mmHg)	38.6 ± 1.2	54.9 ± 3.9	36.8 ± 1.4	<0.005	n.s.	<0.001

Accordingly, the primary endpoint of this study was to investigate the influence of chronic hypercapnia in patients with hypoxemia resolved by means of LTOT.

In order to compare the influence of respiratory failure in hypoxemia and ventilatory failure patients under LTOT, baseline PaCO₂ values that reflect the ventilatory state during sleep were derived from the bicarbonate ion (HCO₃⁻) values using the prediction formula [18].

This is based on the fact that PaCO₂ becomes higher due to the physiological suppression of ventilation during sleep. In addition, PaCO₂ values at wakefulness do not reflect the values during sleep. Thus, we derive the baseline PaCO₂ during sleep from HCO₃⁻ that reflects longer-term PaCO₂ and needs prolonged period to change.

Assuming that the predicted PaCO₂ at rest reflects PaCO₂ during sleep, the LTOT patients were categorized by the level of PaCO₂ during sleep and PSG data for each group were compared.

Regarding nocturnal sleep architecture in CRF patients under LTOT, it was characterized by shorter TST, larger % stage N1, smaller % stage N3, and longer sleep latency, namely, lower sleep efficiency compared to healthy subjects even with the maintenance of oxygen level during sleep by means of oxygen administration.

These characteristics are evidences of so-called sleep disturbance of which the pattern was different from the pattern attributable to certain events and accompanied by arousal responses such as observed in obstructive sleep apnea hypopnea syndrome (OSAHS). Rather, it was similar to have sleep architecture in sleep disturbance observed in generalized anxiety disorder. Although some studies report that high PaCO₂ is responsible for arousals [22], a causal relationship between high PaCO₂ and arousals was not found in LTOT patients. Future studies may reveal such causal-relationship through long-term follow up of sleep variation in respiratory failure patients. This study probably failed to prove the association of high PaCO₂ with arousals due to a lack of sleep tests in matched patient groups before the patients contracted respiratory failure. When hypercapnia and normocapnia patients in the LTOT Group were compared, sleep latency was shorter in hypercapnia patients. The influence of PaCO₂ was considered to be one of the causes.

In patients classified into the LTOT Group, PLMD complication incidence was around 50% with no relation to PaCO₂ accumulation.

Regarding insomnia related to respiratory diseases, there are many reports on bronchial asthma [7,18]. COPD patients present various symptoms depending on the disease types and severity. The most frequent symptom in patients at Stage 2 or higher is "exertional dyspnea" while severe breathing difficulty that interrupts sleep at night is rare unless accompanied by bronchial

asthmatic attack [23] or accidental pneumothorax.

Pulmonary emphysema at the stationary phase rarely causes insomnia because it is rarely accompanied by symptoms of cough and sputum. Significant hypoxemia during REM sleep associated with decrease in PaO₂ partial pressure during wakefulness is reported regarding sleep in COPD patients [24].

However, there are no reports on sleep state in LTOT patients. We found in this study that sleep in LTOT patients is characterized by shorter TST, larger Stage N1, longer sleep latency and lower sleep efficiency compared to controls. These characteristics are similar to the sleep pattern in patients with generalized anxiety disorder.

Regarding the subjects in this study, in whom normal oxygen saturation is maintained during sleep by means of oxygen administration, possible causes of sleep disturbance are biological and physical stressors such as respiratory muscle fatigue and factors related to anxiety disorders due to slow aggravation of the ventilatory state.

Guilleminault [25] *et al.* first reported the incidence of PLMD in sleep disorder and revealed that PLMD was observed in 11.3% of chronic insomnia patients. Coleman [26] *et al.* reported that PLMD was observed in 3.5% of hypersomnia patients and this incidence was raised to 18% in such patients aged 65 or over. Since the incidence of PLMD is high in the patients with cerebrovascular disorder and myelopathy, damage to the inhibitory system between the central nerve and ventral horn cell that functions during sleep may be associated with decrease in the threshold of the disease onset [27]. Variance in sympathetic nerve activity in the pons and reticular formation seems to be associated with periodic onset of PLMD since the interval of muscle contraction matches the fluctuation of breathing, arterial and cerebrospinal fluid pressure, which is suppressed by sympathetic nerve system during sleep [28]. No healthy subjects in this study were diagnosed as having PLMD based on the PSG data, probably due to the small number of the cohort. On the other hand, PLMD was found in 62.5% of the LTOT patients although the number of the cohort is so small. Studies with a larger number of cohorts may be required in the future.

Respiratory disease, especially COPD, has been considered to be a cause of insomnia since the incidence of insomnia is higher in patients with this disease compared to healthy controls [29]. Many studies in the past investigated the sleep state before commencement of LTOT. In addition to this, we classified patients depending on whether they had hypercapnia and compared the differences in sleep state among the groups. We supposed that frequent measurements of nocturnal oxygen concentration during sleep could affect sleep testing and insertion of the Swan-Ganz catheter for testing presents high risk

to the LTOT patients. Therefore, assuming that the predicted PaCO₂ derived from the value of HCO₃⁻, which is obtained from the ABG analysis of the blood collected at the time of admission while the patient is awake, reflects the PaCO₂ during sleep, we estimated the PaCO₂ level during sleep using the prediction formula for PaCO₂ at rest including during sleep (PaCO₂ = 2.4 × [HCO₃⁻] - 22) [18].

Using this data, the subjects were classified into High PaCO₂ and Normal PaCO₂ Groups and compared. The results showed prolonged sleep latency in the High PaCO₂ Group, for which intensified insomniac symptoms as in the previously mentioned COPD cases seemed to be a possible cause since the patients in this study all had severe respiratory failure.

Some reports suggest increase in Stage 1 observed in the group with OSAHS and hypercapnia as the cause of sleepiness. Continued arousal state due to decreased cerebral blood flow during sleep is also a possibility. Chemical, neurogenic, and myogenic controls function to modulate cerebral blood flow [30-32] and carbon dioxide is considered to affect the cerebral blood flow most [30]. A study by Wollf [33] et al. and subsequent studies [34,35] confirm that increase in partial pressure of arterial CO₂ dilates the cerebral blood vessel while the decrease contracts the vessel.

However, the mechanism concerning PaCO₂ increase is yet to be confirmed since there are two opinions at the moment: relaxation of the smooth muscle in the cerebral blood vessel due to a chemical mechanism [36,37] and cerebral blood flow increase via neuroreflex [38]. Regarding the influence of PaCO₂ on LTOT patients, it is conjectured that decrease in ventilation increases PaCO₂ partial pressure, which dilates the cerebral blood vessel and increases cerebral blood flow resulting in the volume of cerebral blood flow equal to or larger than the flow during wakefulness. However, the possibility of prolonged sleep latency and decrease in TST was suggested. It may be necessary to take actual PaCO₂ during sleep using the non-invasive method of measuring carbon dioxide concentration, classify the patients by the values, and make comparisons.

Prolonged sleep latency, shortened sleep time and decreased deep sleep were found in CRF patients with hypercapnia even with exclusion of the influence of hypoxia, which suggested the possible influence of CO₂.

This study did not measure PaCO₂ directly during sleep. Because there is a possibility that the collection of ABG sample during sleep can affect sleep architecture. This is regarded as major limitations of this study. It will be also examined sleep architecture after the intervention with assisted ventilation.

In conclusions, maintaining a certain oxygen level during sleep may not be sufficient to achieve adequate

sleep in CRF patients with hypercapnia under LTOT.

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Abbreviations

Long-term oxygen therapy (LTOT);
 Chronic obstructive pulmonary disease (COPD);
 Chronic Respiratory Failure (CRF);

Arterial blood gas analysis (ABG);
 Polysomnography (PSG);
 Total Sleep Time (TST);
 Periodic leg movement disorder (PLMD).