Tohoku J. Exp. Med., 2019, **247**, 215-222

Low-Dose Carbon Monoxide Inhibits Rhinovirus Replication in Human Alveolar and Airway Epithelial Cells

Xue Deng,[1] Hiroyasu Yasuda,[2] Takahiko Sasaki[3] and Mutsuo Yamaya[1]

[1]Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[2]Yaotome Ekimae Clinic of Internal Medicine, Sendai, Miyagi, Japan
[3]Department of Internal Medicine, Haranomachi Sasaki Clinic, Sendai, Miyagi, Japan

Carbon monoxide (CO) and nitric oxide (NO) exhibit physiological properties that include the activation of guanylate cyclase. NO inhibits replication of rhinovirus (RV), a major cause of the common cold and exacerbation of bronchial asthma and chronic obstructive pulmonary disease. However, the anti-rhinoviral effects of CO remain unclear. This study investigated whether the exogenous application of low-dose CO could inhibit RV replication in human alveolar and airway epithelial cells. A549 human lung carcinoma cells with alveolar epithelial features and primary cultures of human tracheal epithelial (HTE) cells were pretreated with CO (100 ppm) and infected with a major group RV, type 14 RV (RV14). CO exposure reduced RV14 titers in the supernatants and RV RNA levels in A549 and HTE cells. The treatment with a guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, reversed the inhibitory effects of CO exposure on RV14 replication in A549 cells. Pretreatment of A549 cells with 8-Br-cGMP, a cell-permeable cGMP analog, caused the decrease in RV14 replication, while CO exposure increased cGMP production. CO exposure also increased the expression levels of interferon (IFN)-γ mRNA and protein. In contrast, pretreatment with CO did not increase DNA fragmentation and did not reduce the expression of intercellular adhesion molecule-1, the RV14 receptor, or the number of acidic endosomes, through which RV RNA enters the cytoplasm. These findings suggest that low-dose CO may decrease RV14 replication in alveolar and airway epithelial cells. IFN-γ production, which is induced by CO exposure via guanylate cyclase activation-mediated cGMP production, may be involved in RV14 replication inhibition.

Keywords: airway epithelium; cell biology; infection control; respiratory infections; viral infection
Tohoku J. Exp. Med., 2019 April, **247** (4), 215-222. © 2019 Tohoku University Medical Press

Introduction

Rhinoviruses (RVs) are the major cause of the common cold as well as the most common acute infection illnesses in humans (Turner et al. 1982). RVs are also associated with acute exacerbation of bronchial asthma and chronic obstructive pulmonary diseases (COPD) (Nicholson et al. 1993; Seemungal et al. 2001). Several mechanisms of RV-induced exacerbation of these diseases have been proposed, including virus-induced mucus hypersecretion, airway inflammation, and smooth muscle contraction (Yamaya 2012; Yamaya et al. 2012).

Carbon monoxide (CO) is produced endogenously by heme oxygenase (HO) (Maines 1988) and is present in measurable quantities in the exhaled air of normal subjects (Jarvis et al. 1980). Exhaled CO and arterial blood carboxyhemoglobin concentrations are increased in patients with inflammatory pulmonary diseases, including bronchial asthma, COPD and interstitial lung disease, and upper respiratory tract infections (Zayasu et al. 1997; Yasuda et al. 2005; Hara et al. 2017).

Similar to nitric oxide (NO), CO is a small diatomic gaseous molecule formed when one carbon atom bonds with one O_2 atom. CO exhibits physiological properties mediated in part by activation of guanylate cyclase (Stone and Marletta 1994). CO binds with the heme moiety of purified soluble guanylate cyclase to activate this enzyme and increase cyclic guanosine monophosphate (cGMP) levels (Brüne and Ullrich 1987; Kharitonov et al. 1995). Inhaled CO reduces lung inflammation and decreases airway hyperresponsiveness in mice, and these effects are also mediated through cGMP (Chapman et al. 2001; Ameredes et al. 2003). NO inhibits RV replication and RV-induced cytokine production in human respiratory epithelial cell lines (Sanders et al. 1998). Based on these findings, we hypothesized that CO may inhibit RV replication in human alveolar and airway epithelial cells.

In the present study, we examined the inhibitory effects

Received January 29, 2019; revised and accepted March 25 2019. Published online April 10, 2019; doi: 10.1620/tjem.247.215.
Correspondence: Mutsuo Yamaya, M.D., Ph.D., Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
e-mail: myamaya@med.tohoku.ac.jp

of low-dose CO exposure on RV14 infection in A549 cells, a lung carcinoma cell line with alveolar epithelial features (Lieber et al. 1976), and primary cultures of human tracheal epithelial (HTE) cells. The implications of guanylate cyclase, cGMP and interferon (IFN)-γ were also analyzed to elucidate the mechanism by which CO affects RV14 replication. Additionally, we studied the effects of CO exposure on intercellular adhesion molecule-1 (ICAM-1) expression and on the distribution of acid endosomes, which are associated with RV14 attachment and entry in airway epithelial cells (Greve et al. 1989; Casasnovas and Springer 1994).

Methods

A549 and human tracheal epithelial cell cultures

A549 human lung carcinoma cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in flasks (25 cm^2 surface area; Becton Dickinson, Franklin Lakes, NJ, USA) and then plated at 4×10^5 viable cells/mL in plastic tubes (16 mm in diameter and 125 mm in length; Becton Dickinson) or on coverslips in petri dishes as previously described (Yamaya et al. 2014).

We also used primary cultures of human tracheal epithelial (HTE) cells, which were isolated and cultured using methods previously reported (Yamaya et al. 1992, 2014). HTE cells were cultured in plastic tubes at a density of 5×10^5 viable cells/mL in 1 mL of DMEM–Ham's F-12 medium (DF12 medium) containing 2% Ultroser G (USG) (Yamaya et al. 2014). Tracheas for cell cultures were obtained after death from 5 patients (mean age 73 ± 3 y; 2 females, 3 males) under a protocol approved by the Tohoku University Ethics Committee (2017-1-765 and 2018-1-16). The causes of death included acute myocardial infarction (n = 2), malignant tumors other than lung cancer (n = 1), congestive heart failure (n = 1) and malignant lymphoma (n = 1).

Human embryonic fibroblast cell cultures

Human embryonic fibroblast (HEF) cells (HFL-III cells, Riken Bio Resource Center Cell Bank, Cell No: RCB0523; Japan) were cultured as previously described (Yamaya et al. 2014).

Viral stock

The RV14 (a major group RV) stock was prepared from a patient with a common cold by infecting HEF cells as previously described (Yamaya et al. 2014).

Viral detection and titration

RV14 in the supernatants (cell culture medium) was detected and titrated using HEF cells and endpoint methods by infecting HEF cells in plastic 96-well plates (Becton Dickinson) with 10-fold serial dilutions of virus-containing culture supernatants (Numazaki et al. 1987; Condit 2013; Yamaya et al. 2014). The presence of the typical RV14 cytopathic effects was observed at 7 days. RV14 release rates into the supernatants are expressed as the tissue culture infective dose ($TCID_{50}$)/mL (Yamaya et al. 2014).

RV RNA quantification

To measure RV RNA levels and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels, we performed real-time quantitative polymerase chain reaction with reverse transcription (RT-PCR) using the Taqman technique (Roche Molecular Diagnostic Systems, Mannheim, Germany), as previously described (Yamaya et al. 2014). RV RNA expression level was normalized to the constitutive GAPDH mRNA expression level.

Epithelial cell viral infection and collection of supernatant and RNA

A549 and HTE cells were infected with RV14 [300 μL per tube, 1.0×10^5 $TCID_{50}$ /mL in A549 cells, multiplicity of infection (MOI) of 0.06; 200 μL per tube, 1.0×10^5 $TCID_{50}$ /mL in HTE cells, MOI of 0.04] for 60 min using previously described methods (Yamaya et al. 2014). The cells were cultured at 33°C in 1 mL of DF12 medium containing 2% USG.

The supernatants and RNA were collected or extracted before infection (0 h) and at 24 h and/or 72 h after infection.

Cell treatment with CO and agents

In many *in vivo* experiment reports, animals were exposed to 100-1,000 ppm CO, most commonly 250 ppm of CO, for 1 h per day (Motterlini and Otterbein 2010; Chan et al. 2016), and 250 ppm of CO was considered "low-level of CO" (Ameredes et al. 2003) for *in vivo* experiments. Furthermore, 200 ppm CO was determined to be a ceiling for life and health (Earnest et al. 1997).

Therefore, we reduced the CO concentration to 100 ppm and the exposure time to 10 min to avoid cytotoxicity. A549 cells or HTE cells were pretreated with bubbling air or 100 ppm of CO gas for 10 min per day for 72 h before infection and for an additional 72 h after infection.

For exploring whether cGMP would mimic the effects of CO exposure, A549 cells were pretreated with 8-Br-cGMP (0.2 mM or 2 mM; Sigma-Aldrich, St. Louis, MO, USA) before infection and treated after infection (Koetzler et al. 2009). A549 cells were also pretreated and treated with the guanylate cyclase inhibitor 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 30 μM; Enzo Life Sciences, Farmingdale, NY, USA) to examine the role of guanylate cyclase (Koetzler et al. 2009).

cGMP, IFN-γ and ICAM-1 measurements

To elucidate the mechanism underlying the inhibitory effects of CO exposure, we measured cyclic GMP concentrations in supernatants with a Cyclic GMP assay kit (R&D Systems, Minneapolis, MN, USA). IFN-γ concentrations in whole-cell proteins and IFN-γ mRNA levels in A549 cells were measured with a Quantikine Human IFN-γ Immunoassay kit (R&D Systems) and real-time RT-PCR, respectively.

We also measured the expression levels of ICAM-1, the receptor for RV14 (Greve et al. 1989), using A549 and HTE cells. ICAM-1 mRNA and the concentrations of a soluble form of ICAM-1 (sICAM-1) in the supernatants were analyzed using real-time RT-PCR and a Quantikine Human sICAM-1 immunoassay kit (R&D Systems) as previously described (Yamaya et al. 2014). The expression level of ICAM-1 or IFN-γ mRNA was normalized to the constitutive expression level of GAPDH mRNA.

Measurement of acidic endosomal changes

The distribution and fluorescence intensity of acidic endosomes through which RV RNA enters the cytoplasm (Pérez and Carrasco 1993; Casasnovas and Springer 1994) in A549 cells were measured

with LysoSensor DND-189 dye (Molecular Probes, Eugene, OR, USA) (Yamaya et al. 2014). A549 cells were pretreated with air or 100 ppm CO exposure for 10 min each day for 72 h.

Assessment of DNA fragmentation via cell death detection ELISA

Cell viability was assessed with Cell Death Detection ELISA Plus (Roche Applied Sciences, Mannheim, Germany) (Fortenberry et al. 1999) using supernatants (conditioned medium) from A549 cells. This kit is based on a quantitative sandwich enzyme immunoassay principle and utilizes mouse monoclonal antibodies directed against DNA and histones. This immunoassay allows apoptosis-specific detection and quantification of mononucleosomes and oligonucleosomes, which are released into the cytoplasm of apoptotic cells. Nucleosomes were photometrically detected at 405 nm by measuring peroxidase activity. The final absorbance was obtained by subtracting the observed absorbance of the negative control.

Statistical analysis

The results are expressed as the means ± SEM. Statistical analyses were performed using two-way repeated measures analysis of variance (ANOVA). Subsequent post hoc analyses were performed using Bonferroni's method. Student's t-tests were performed for comparisons between two groups. Values of $p < 0.05$ were considered significant for all analyses. In the experiments using HTE cell cultures, n refers to the number of donors (tracheae) from whom the cultured epithelial cells were obtained. All analyses were performed using SPSS version 20 (IBM Japan, Tokyo, Japan).

Results

Effects of CO exposure on RV14 replication

RV14 was detected in the supernatants of A549 cells at 24 h, and the viral titers progressively increased between 24 h and 72 h after infection (Fig. 1A). Pretreatment of the cells with CO resulted in significant decreases in the viral titers 24 h and 72 h after infection (Fig. 1A).

Similarly, RV14 was detected in the supernatants of HTE cells at 24 h, and the viral content was progressively

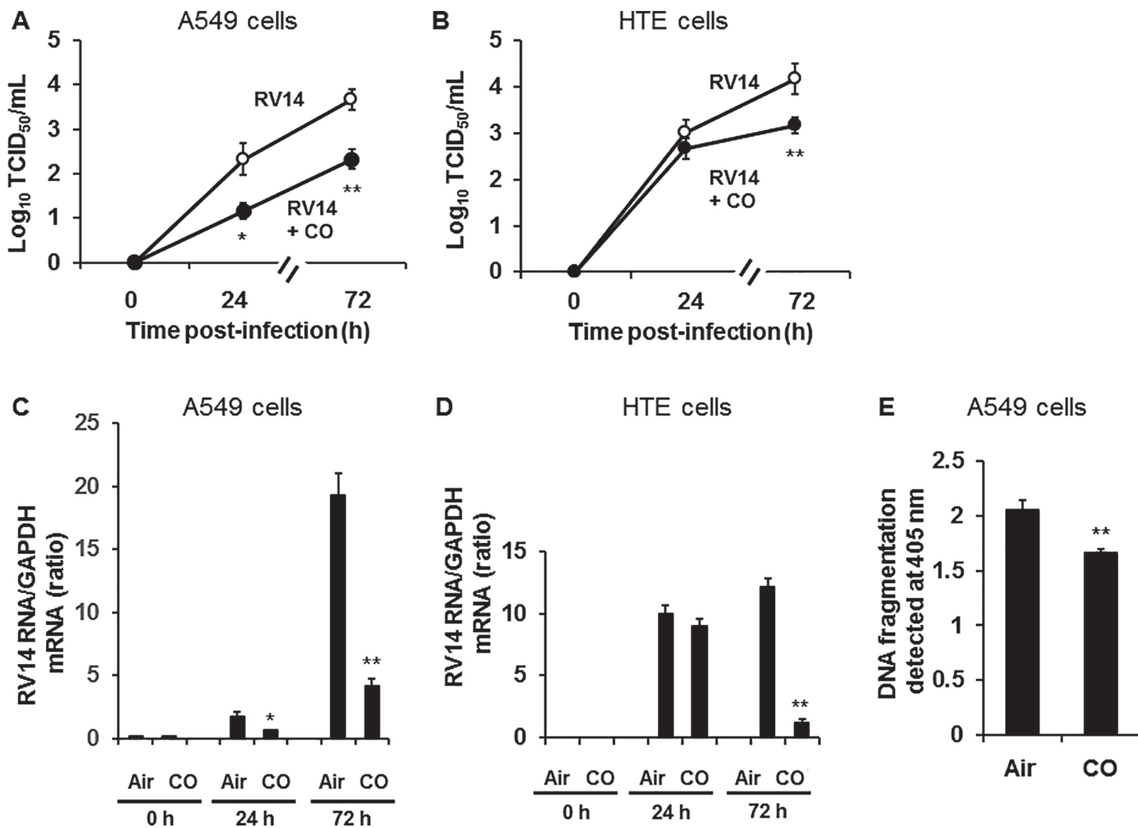

Fig. 1. Effects of CO exposure on RV14 replication and DNA fragmentation.
(A) and (B): Time course of viral release in the supernatants of A549 (A) and HTE (B) cells obtained at different times after RV14 infection. Cells were pretreated by exposing them to air (open circles) or CO gas (closed circles) for 10 min per day for 3 days before RV14 infection and until the experiment ended after infection. RV14 release rates into the supernatants are expressed as the tissue culture infective dose ($TCID_{50}$)/mL.
(C) and (D): RV14 RNA expression in A549 (C) and HTE (D) cells before (0 h) and at 24 h and 72 h after RV14 infection detected by real-time quantitative RT-PCR. RV14 RNA expression was normalized to the constitutive expression of GAPDH mRNA. Cells were pretreated by exposing them to air (Air) or CO gas (CO) before and after RV14 infection.
(E): DNA fragmentation in A549 cells at 24 h after RV14 infection. The potency of DNA fragmentation is expressed as the absorbance value at 405 nm.
(A)-(E): The results are expressed as the means ± SEM of five samples (A549 cells) or five tracheal epithelial cell lines (HTE cells). Significant differences between cells exposed to CO or air are indicated by *$p < 0.05$ and **$p < 0.01$.

increased between 24 h and 72 h after infection (Fig. 1B). Again, treatment of the cells with CO caused the significant decrease in the viral titers at 72 h (Fig. 1B).

The inhibitory effects of CO exposure on RV14 RNA replication in A549 cells were further evidenced by real-time quantitative RT-PCR analysis. RV14 RNA replication in the cells was consistently observed 24 h after infection and was increased between 24 h and 72 h (Fig. 1C). Pretreatment of the cells with CO decreased the RV14 RNA levels at 24 h and at 72 h after infection (Fig. 1C).

Similarly, RV14 RNA replication in HTE cells was consistently detected at 24 h and 72 h after infection (Fig. 1D). Pretreatment of the cells with CO caused the decrease in the RV14 RNA level 72 h after infection (Fig. 1D).

We also examined the cytotoxic effects of CO exposure on A549 cells after RV14 infection. At 24 h after infection, the levels of DNA fragmentation in the culture supernatants of the A549 cells pretreated with CO for 72 h were lower than the levels in supernatants from cells pretreated with air (Fig. 1E).

Effects of guanylate cyclase activation and cGMP on RV14 replication

The binding of CO with guanylate cyclase activates guanylate cyclase and increases cGMP levels (Brüne and Ullrich 1987; Kharitonov et al. 1995). We, therefore, examined the role of guanylate cyclase activation on the inhibitory effects of CO exposure on RV14 replication. Pretreatment of A549 cells with the guanylate cyclase inhibitor ODQ (30 μM) (Koetzler et al. 2009) reversed the decrease in RV14 titers (Fig. 2A) and RV14 RNA replication (Fig. 2B) induced by treatment with CO exposure at

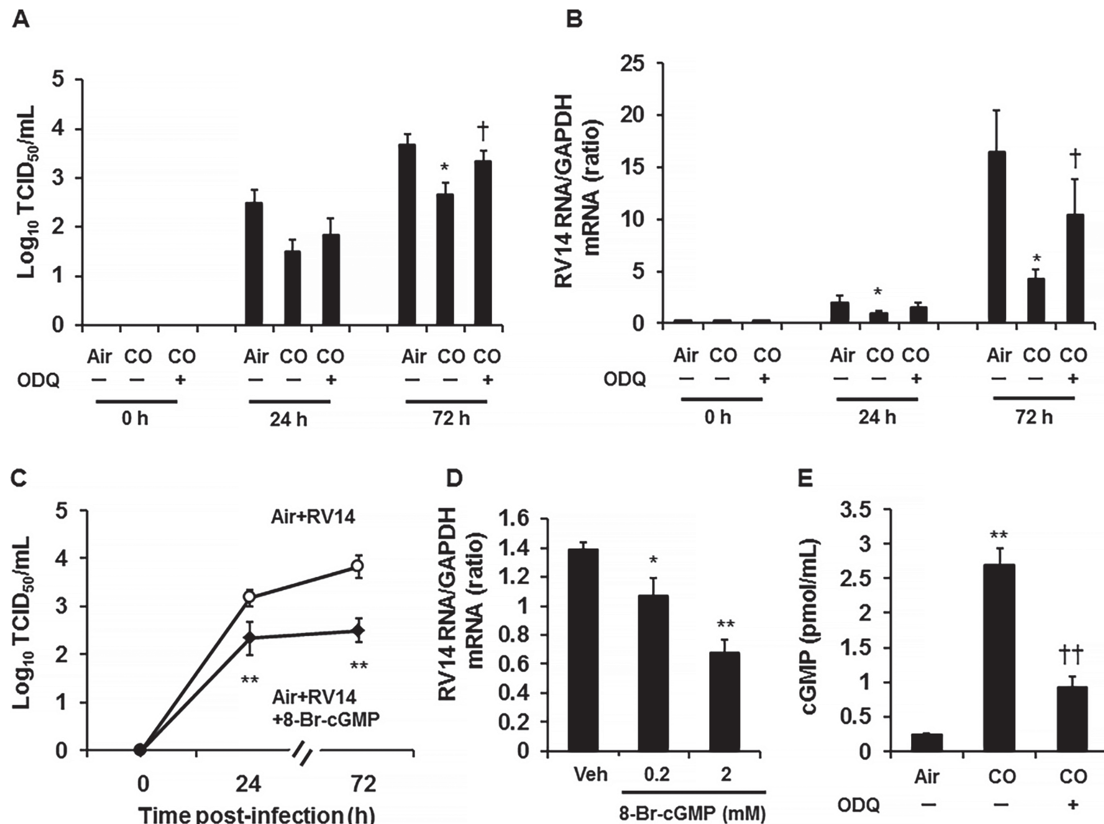

Fig. 2. Effects of ODQ or cGMP on RV14 replication or cGMP production.

(A) and (B): Viral release in the supernatants (A) or the RV14 RNA level (B) before and at 24 h and 72 h after infection in A549 cells pretreated with ODQ (30 μM) or vehicle for 3 days in the presence of air or CO exposure before infection and until the experiment ended after infection. RV14 RNA expression was normalized to the constitutive expression of GAPDH mRNA. The results are expressed as the means ± SEM of five samples (in A549) or five different tracheae. Significant differences after infection from air exposure are indicated by $^*p < 0.05$. Significant differences after infection from CO exposure in the absence of ODQ are indicated by $^{\dagger}p < 0.05$.

(C) and (D): Time course of viral release in the supernatants of A549 cells pretreated with 8-Br-cGMP (2 mM) (closed diamonds) or vehicle (open circles) obtained at different times after infection (C), and RV14 RNA expression in A549 cells pretreated with 8-Br-cGMP (0.2 mM or 2 mM) at 72 h postinfection (D). Cells were pretreated with 8-Br-cGMP for 3 days before infection and until the experiment ended after infection. The results are expressed as the means ± SEM of five samples. Significant differences from RV14 infection in the absence of 8-Br-cGMP (Air+RV14 or veh: vehicle) are indicated by $^*p < 0.05$ and $^{**}p < 0.01$.

(E): cGMP concentrations in the supernatants of A549 cells exposed to air or CO in the absence or presence of ODQ. Significant differences from air exposure or CO exposure in the absence of ODQ are indicated by $^{**}p < 0.01$ or $^{\dagger\dagger}p < 0.01$.

72 h after infection.

We examined the effects of 8-Br-cGMP, a cell-permeable cGMP analog (Koetzler et al. 2009), to determine whether cGMP would mimic the effects of the CO exposure. 8-Br-cGMP (2 mM) significantly decreased the viral titers in the supernatants of A549 cells at 24 h and 72 h (Fig. 2C) after infection and dose-dependently decreased the RV14 RNA level 72 h after infection (Fig. 2D).

Effects of guanylate cyclase inhibition on cGMP production increased by CO exposure

We also examined the effects of CO exposure on cGMP production and guanylate cyclase activation. CO exposure markedly increased cGMP levels in the supernatants of A549 cells (Fig. 2E), and pretreatment of the cells with the guanylate cyclase inhibitor ODQ resulted in the decrease of CO exposure-induced cGMP production (Fig. 2E).

Effects of CO exposure on IFN-γ production

Because CO exposure reduced RV14 titers in the supernatants, we examined the effects of CO exposure on the cellular level of IFN-γ that inhibits RV replication (Sentsui et al. 2010). IFN-γ mRNA expression levels were significantly increased at 24 h and 72 h after RV14 infection in A549 cells with air exposure (Fig. 3A), and CO exposure caused the further increase in IFN-γ mRNA expression levels induced by RV14 infection (Fig. 3A).

Similarly, IFN-γ protein levels were increased significantly at 24 h and 72 h after RV14 infection in A549 cells with air exposure, and CO exposure further increased the IFN-γ protein levels increased by RV14 infection at 24 h and 72 h after infection (Fig. 3B).

Effects of CO exposure on ICAM-1 expression

We also examined the effects of CO exposure on the expression of ICAM-1, the receptor for RV14. ICAM-1 mRNA expression levels in A549 and HTE cells were unaltered by the pretreatment with CO before infection (Fig. 4A, B). ICAM-1 mRNA expression levels were increased 24 h after RV14 infection (Fig. 4A, B). CO exposure increased ICAM-1 mRNA expression levels at 24 h after infection compared with those of the cells treated with air (Fig. 4A, B).

The sICAM-1 concentration in the supernatants of the A549 and HTE cells was unaltered by the pretreatment with CO before RV14 infection (Fig. 4C, D). In contrast, sICAM-1 concentrations in the A549 and HTE cells exposed to air and CO increased at 24 h after infection (Fig. 4C, D). The sICAM-1 concentrations at 24 h after infection in cells pretreated with CO tended to be higher than those in the A549 and HTE cells pretreated with air (Fig. 4C, D), but the difference was not significant (Fig. 4C, D).

Effects of CO on acidic endosomes

We also examined the effects of CO exposure on acidic endosomes through which RV RNA enters the cytoplasm. Acidic endosomes in uninfected A549 cells were stained green with LysoSensor DND-189 (Fig. 4E, F). The distribution (Fig. 4E, F) and fluorescence intensity (Fig. 4G) of the acidic endosomes (stained green) in cells exposed to CO did not differ from those in the cells exposed to air.

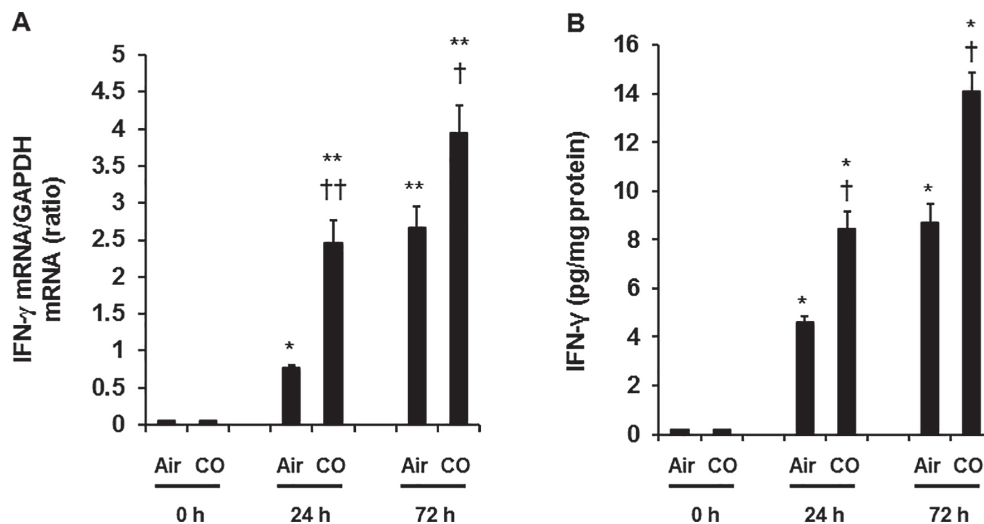

Fig. 3. Effects of CO exposure on IFN-γ production.
IFN-γ mRNA expression (A) and IFN-γ protein production (B) in A549 cells before and at 24 h and 72 h after RV14 infection. Cells were pretreated by exposure to air or CO for 10 min per day for 3 days before RV14 infection and until the experiment ended after infection. IFN-γ mRNA and IFN-γ protein expression levels were normalized to the constitutive expression level of GAPDH mRNA and total protein, respectively. The results are expressed as the means ± SEM of five samples. Significant differences from air exposure before infection are indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. Significant differences from air exposure after infection are indicated by $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$.

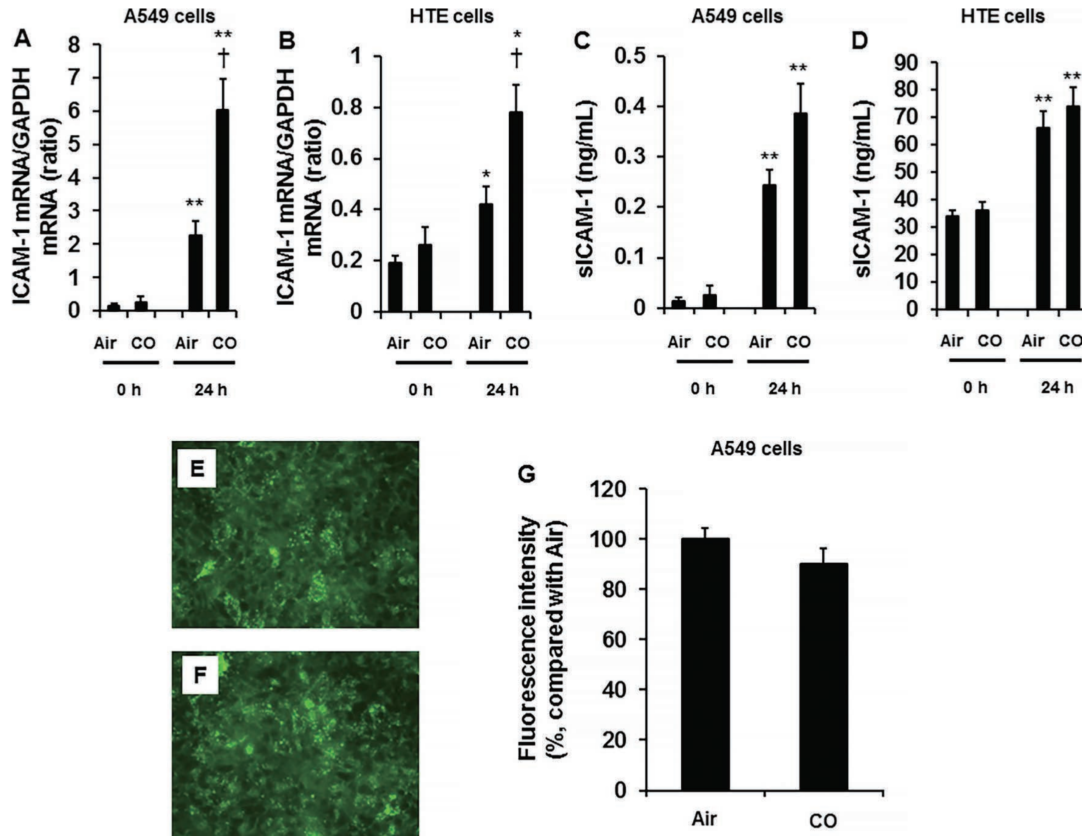

Fig. 4. Effects of CO exposure on ICAM-1 expression and acidic endosomes.
(A)-(D): ICAM-1 mRNA expression (A and B) and sICAM-1 concentrations (C and D) in the supernatants of A549 (A and C) or HTE cells (B and D) before (0 h) and 24 h after RV14 infection. Cells were pretreated by exposure to air or CO for 10 min per day for 3 days before infection and until the experiment ended after infection. The expression of ICAM-1 mRNA was normalized to the constitutive expression of GAPDH mRNA. The results are expressed as the means ± SEM of five samples (in A549 cells) (A and C) or five tracheal epithelial cell lines (in HTE cells) (B and D). Significant differences from air exposure before infection are indicated by *$p < 0.05$ or **$p < 0.01$. Significant differences from air exposure 24 h after infection are indicated by $^{\dagger}p < 0.05$.
(E) and (F): Distribution of acidic endosomes exhibiting green fluorescence in A549 cells at 72 h after pretreatment with exposure to air (E) or CO (F) for 10 min per day for 3 days (magnification × 200).
(G): The fluorescence intensity of acidic endosome in A549 cells at 72 h after pretreatment with exposure to air or CO for 10 min per day for 3 days. The fluorescence intensity was measured in 100 cells, and the mean value of the fluorescence intensity in the air-exposed cells was set to 100%. The results are expressed as the means ± SEM of four samples.

Discussion

In the present study, we demonstrated that exposure of A549 and HTE cells to CO significantly decreased RV14 titers in the supernatants and viral RNA replication in the cells. Pretreatment of A549 cells with the guanylate cyclase inhibitor ODQ reversed the inhibitory effects of the CO exposure on RV14 titers and RV14 RNA replication, and pretreatment of the cells with 8-Br-cGMP decreased the RV14 titers and RV14 RNA replication, although the concentration of 8-Br-cGMP (0.2 and 2 mM) (Koetzler et al. 2009) used in the present study was much higher than that observed in the culture supernatants (Fig. 2D, E). CO exposure increased cGMP production in A549 cells, and the guanylate cyclase inhibitor ODQ inhibited the cGMP production induced by CO exposure. CO exposure also increased IFN-γ production in infected and uninfected A549 cells. CO modulates the guanylate cyclase-GMP signaling pathway (Verma et al. 1993; Hawkins et al. 1994), and 8-Br-cGMP induces IFN-γ production in type 1 T-cells (Niedbala et al. 2002). These findings suggest that CO exposure may decrease RV14 replication in alveolar and airway epithelial cells by increasing IFN-γ, which inhibits RV replication (Sentsui et al. 2010), via cGMP production induced by guanylate cyclase activation.

In the present study, cells were exposed to 100 ppm CO for 10 min per day for 72 h before infection. In many *in vivo* experiment reports, animals were exposed to 100-1,000 ppm CO, most commonly to 250 ppm CO, for 1 h per day (Motterlini and Otterbein 2010; Chan et al. 2016), and 250 ppm CO was considered “low-level of CO” (Ameredes et al. 2003) for *in vivo* experiments. Furthermore, 200 ppm CO was determined to be a ceiling for life and health (Earnest et al. 1997). Because we were unable to find any

in vitro reports describing a suitable CO concentration, we reduced the CO concentration to 100 ppm and exposure time to 10 min to avoid cytotoxicity.

In fact, treatment with CO exposure did not increase the levels of DNA fragmentation, and did not reduce ICAM-1 expression or the number of acidic endosomes in A549 cells. Although we did not measure the pH of the culture medium, the color of the culture medium, which contained phenol red, of the cells exposed to CO was similar to that of the cells exposed to air during the cell culture in an incubator. These findings suggest that 100 ppm CO exposure had no apparent cytotoxicity and that the reduced RV14 replication observed in the cells exposed to CO might not be caused by cytotoxic effects of CO exposure in the present study.

CO is toxic and lethal to living things in high doses (> 1,200 ppm) (Chance et al. 1970; Earnest et al. 1997), and CO is an industrial pollutant, resulting in chronic hypoxia at high levels (Schwela 2000). However, the discovery of the physiological actions of NO introduced the idea that a small and ubiquitous diatomic gas molecule other than O_2 may be associated with many cellular processes (Palmer et al. 1987); thus, novel physiological actions of CO, including those on airway inflammation and hyperresponsiveness (Chapman et al. 2001; Ameredes et al. 2003), were investigated.

Unlike NO, which interacts indiscriminately with several intracellular targets, CO reacts exclusively with transition metals. Because of its inherent chemical reactivity, CO is relatively stable compared with NO (Boczkowski et al. 2006). Endogenous CO production occurs via constitutive HO (HO-2) and inducible HO (HO-1) activity, which are also the enzymes that catabolize heme (Maines 1988; Motterlini and Otterbein 2010).

CO has been studied as a cytoprotective and homeostatic molecule with important physiological and pathophysiological signaling capabilities in the lung (Chapman et al. 2001), airways (Ameredes et al. 2003), platelets (Brüne and Ullrich 1987) and muscle (Chan et al. 2016). CO was reported to relieve pulmonary arterial hypertension in sheep experiencing hypoxia (Nachar et al. 2001). Furthermore, CO inhalation has been suggested for clinical use due to its anti-inflammatory effects against ventilator-induced lung injury (Dolinay et al. 2004). Thus, the therapeutic potential of CO for clinical applications is being discussed (Rosas et al. 2018).

We previously reported that various agents, including macrolides, inhaled corticosteroids, β_2 agonist and mucolytic agents, inhibited RV14 replication in HTE cells (Yamaya 2012; Yamaya et al. 2012; 2014) by reducing expression of ICAM-1, the RV14 receptor (Greve et al. 1989), and the number of acidic endosomes, through which RV RNA enters the cytoplasm (Casasnovas and Springer 1994; Pérez and Carrasco 1993). However, in the present study, CO exposure did not decrease ICAM-1 expression levels in A549 and HTE cells or the number of acidic endosomes in A549 cells, suggesting that CO exposure inhibits RV14 replication via mechanisms other than reducing RV14 attachment and entry via acidic endosomes.

In summary, CO exposure inhibited RV14 replication in human alveolar and airway epithelial cells. The inhibitory effect of CO on RV14 replication may occur partially via a guanylate cyclase activation pathway to induce cGMP and subsequently produce IFN-γ. Our data suggest that applying low-dose CO may be a potential therapy for RV infection and infection-induced exacerbation of bronchial asthma and COPD.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (No. 22790744) of the Japanese government. We also thank FUKUDA DENSHI Co. Ltd. and Taiyou Touyou Sanso Co. Ltd. for supplying the CO gas cylinder. We thank the staff at the Biomedical Research Unit and the Department of Pathology at Tohoku University Hospital for providing technical support.

Conflict of Interest

The authors declare no conflict of interest.

References

Ameredes, B.T., Otterbein, L.E., Kohut, L.K., Gligonic, A.L., Calhoun, W.J. & Choi, A.M. (2003) Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **285**, L1270-1276.

Boczkowski, J., Poderoso, J.J. & Motterlini, R. (2006) CO-metal interaction: vital signaling from a lethal gas. *Trends Biochem. Sci.*, **31**, 614-621.

Brüne, B. & Ullrich, V. (1987) Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.*, **32**, 497-504.

Casasnovas, J.M. & Springer, T.A. (1994) Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. *J. Virol.*, **68**, 5882-5889.

Chan, M.C., Ziegler, O., Liu, L., Rowe, G.C., Das, S., Otterbein, L.E. & Arany, Z. (2016) Heme oxygenase and carbon monoxide protect from muscle dystrophy. *Skelet. Muscle*, **6**, 41.

Chance, B., Erecinska, M. & Wagner, M. (1970) Mitochondrial responses to carbon monoxide toxicity. *Ann. NY Acad. Sci.*, **174**, 193-204.

Chapman, J.T., Otterbein, L.E., Elias, J.A. & Choi, A.M. (2001) Carbon monoxide attenuates aeroallergen-induced inflammation in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **281**, L209-216.

Condit, R.C. (2013) Principles of virology. In *Fields Virology,* sixth ed., edited by Knipe, D.M. & Howley, P.M., Lippincott Williams & Wilkins, Philadelphia, PA, pp. 21-51.

Dolinay, T., Szilasi, M., Liu, M. & Choi, A.M. (2004) Inhaled carbon monoxide confers antiinflammatory effects against ventilator-induced lung injury. *Am. J. Respir. Crit. Care Med.*, **170**, 613-620.

Earnest, G.S., Mickelsen, R.L., McCammon, J.B. & O'Brien, D.M. (1997) Carbon monoxide poisonings from small, gasoline-powered, internal combustion engines: just what is a "well-ventilated area"? *Am. Ind. Hyg. Assoc. J.*, **58**, 787-791.

Fortenberry, J.D., Owens, M.L. & Brown, L.A. (1999) S-nitrosoglutathione enhances neutrophil DNA fragmentation and cell death. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **276**, L435-

442.

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E. & McClelland, A. (1989) The major human rhinovirus receptor is ICAM-1. *Cell*, **56**, 839-847.

Hara, Y., Shinkai, M., Kanoh, S., Fujikura, Y., Rubin, B.K., Kawana, A. & Kaneko, T. (2017) Arterial carboxyhemoglobin measurement is useful for evaluating pulmonary inflammation in subjects with interstitial lung disease. *Intern. Med.*, **56**, 621-626.

Hawkins, R.D., Zhuo, M. & Arancio, O. (1994) Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. *J. Neurobiol.*, **25**, 652-665.

Jarvis, M.J., Russell, M.A. & Saloojee, Y. (1980) Expired air carbon monoxide: a simple breath test of tobacco smoke intake. *Br. Med. J.*, **281**, 484-485.

Kharitonov, V.G., Sharma, V.S., Pilz, R.B., Magde, D. & Koesling, D. (1995) Basis of guanylate cyclase activation by carbon monoxide. *Proc. Natl. Acad. Sci. USA*, **92**, 2568-2571.

Koetzler, R., Zaheer, R.S., Wiehler, S., Holden, N.S., Giembycz, M.A. & Proud, D. (2009) Nitric oxide inhibits human rhinovirus-induced transcriptional activation of CXCL10 in airway epithelial cells. *J. Allergy Clin. Immunol.*, **123**, 201-208. e9.

Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W. & Todaro, G. (1976) A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer*, **17**, 62-70.

Maines, M.D. (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.*, **2**, 2557-2568.

Motterlini, R. & Otterbein, L.E. (2010) The therapeutic potential of carbon monoxide. *Nat. Rev. Drug Discov.*, **9**, 728-743.

Nachar, R.A., Pastene, C.M., Herrera, E.A., Riquelme, R.A., Sanhueza, E.M., Troncoso, S. & Llanos, A.J. (2001) Low-dose inhaled carbon monoxide reduces pulmonary vascular resistance during acute hypoxemia in adult sheep. *High Alt. Med. Biol.*, **2**, 377-385.

Nicholson, K.G., Kent, J. & Ireland, D.C. (1993) Respiratory viruses and exacerbations of asthma in adults. *BMJ*, **307**, 982-986.

Niedbala, W., Wei, X.Q., Campbell, C., Thomson, D., Komai-Koma, M. & Liew, F.Y. (2002) Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. *Proc. Natl. Acad. Sci. USA*, **99**, 16186-16191.

Numazaki, Y., Oshima, T., Ohmi, A., Tanaka, A., Oizumi, Y., Komatsu, S., Takagi, T., Karahashi, M. & Ishida, N. (1987) A microplate method for isolation of viruses from infants and children with acute respiratory infections. *Microbiol. Immunol.*, **31**, 1085-1095.

Palmer, R.M., Ferrige, A.G. & Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524-526.

Pérez, L. & Carrasco, L. (1993) Entry of poliovirus into cells does not require a low-pH step. *J. Virol.*, **67**, 4543-4548.

Rosas, I.O., Goldberg, H.J., Collard, H.R., El-Chemaly, S., Flaherty, K., Hunninghake, G.M., Lasky, J.A., Lederer, D.J., Machado, R., Martinez, F.J., Maurer, R., Teller, D., Noth, I., Peters, E., Raghu, G., et al. (2018) A phase II clinical trial of low-dose inhaled carbon monoxide in idiopathic pulmonary fibrosis. *Chest*, **153**, 94-104.

Sanders, S.P., Siekierski, E.S., Porter, J.D., Richards, S.M. & Proud, D. (1998) Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J. Virol.*, **72**, 934-942.

Schwela, D. (2000) Air pollution and health in urban areas. *Rev. Environ. Health*, **15**, 13-42.

Seemungal, T., Harper-Owen, R., Bhowmik, A., Moric, I., Sanderson, G., Message, S., Maccallum, P., Meade, T.W., Jeffries, D.J., Johnston, S.L. & Wedzicha, J.A. (2001) Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **164**, 1618-1623.

Sentsui, H., Wu, D., Murakami, K., Kondo, T. & Matsumura, T. (2010) Antiviral effect of recombinant equine interferon-γ on several equine viruses. *Vet. Immunol. Immunopathol.*, **135**, 93-99.

Stone, J.R. & Marletta, M.A. (1994) Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry*, **33**, 5636-5640.

Turner, R.B., Hendley, J.O. & Gwaltney, J.M. Jr. (1982) Shedding of infected ciliated epithelial cells in rhinovirus colds. *J. Infect. Dis.*, **145**, 849-853.

Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. & Snyder, S.H. (1993) Carbon monoxide: a putative neural messenger. *Science*, **259**, 381-384.

Yamaya, M. (2012) Virus infection-induced bronchial asthma exacerbation. *Pulm. Med.*, **2012**, 834826.

Yamaya, M., Azuma, A., Takizawa, H., Kadota, J., Tamaoki, J. & Kudoh, S. (2012) Macrolide effects on the prevention of COPD exacerbations. *Eur. Respir. J.*, **40**, 485-494.

Yamaya, M., Finkbeiner, W.E., Chun, S.Y. & Widdicombe, J.H. (1992) Differentiated structure and function of cultures from human tracheal epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **262**, L713-724.

Yamaya, M., Nishimura, H., Nadine, L., Kubo, H. & Nagatomi, R. (2014) Formoterol and budesonide inhibit rhinovirus infection and cytokine production in primary cultures of human tracheal epithelial cells. *Respir. Investig.*, **52**, 251-260.

Yasuda, H., Yamaya, M., Nakayama, K., Ebihara, S., Sasaki, T., Okinaga, S., Inoue, D., Asada, M., Nemoto, M. & Sasaki, H. (2005) Increased arterial carboxyhemoglobin concentrations in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **171**, 1246-1251.

Zayasu, K., Sekizawa, K., Okinaga, S., Yamaya, M., Ohrui, T. & Sasaki, H. (1997) Increased carbon monoxide in exhaled air of asthmatic patients. *Am. J. Respir. Crit. Care Med.*, **156**, 1140-1143.