Hydrogen-rich pure water prevents cigarette smoke-induced pulmonary emphysema in SMP30 knockout mice

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[1]**Abbreviations:** COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; Cst, static lung compliance; DI, destructive index; DSB, double-strand break; MLI, mean linear intercept; ɤH2AX, phosphorylated histone H2AX; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; p16INK4A, cyclin-dependent kinase inhibitor 2A; p21CIP1, cyclin-dependent kinase inhibitor 1; ROS, reactive oxygen species; SIPS, stress-induced premature senescence;

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SMP30-KO, senescence marker protein 30 knockout; VC, vitamin C

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is predominantly a cigarette smoke (CS)-triggered disease with features of chronic systemic inflammation. Oxidants derived from CS can induce DNA damage and stress-induced premature cellular senescence in the respiratory system, which play significant roles in COPD. Therefore, antioxidants should provide benefits for the treatment of COPD; however, their therapeutic potential remains limited owing to the complexity of this disease. Recently, molecular hydrogen (H_2) has been reported as a preventive and therapeutic antioxidant. Molecular H_2 can selectively reduce hydroxyl radical accumulation with no known side effects, showing potential applications in managing oxidative stress, inflammation, apoptosis, and lipid metabolism. However, there have been no reports on the efficacy of molecular H_2 in COPD patients. In the present study, we used a mouse model of COPD to investigate whether CS-induced histological damage in the lungs could be attenuated by administration of molecular H_2. We administered H_2-rich pure water to senescence marker protein 30 knockout (SMP30-KO) mice exposed to CS for 8 weeks. Administration of H_2-rich water attenuated the CS-induced lung damage in the SMP30-KO mice and reduced the mean linear intercept and destructive index of the lungs. Moreover, H_2-rich water significantly restored the static lung compliance in the CS-exposed mice compared with that in the CS-exposed H_2-untreated mice. Moreover, treatment with H_2-rich water decreased the levels of oxidative DNA damage markers such as phosphorylated

histone H2AX and 8-hydroxy-2′-deoxyguanosine, and senescence markers such as cyclin-dependent kinase inhibitor 2A, cyclin-dependent kinase inhibitor 1, and β-galactosidase in the CS-exposed mice. These results demonstrated that H_2-rich pure water attenuated CS-induced emphysema in SMP30-KO mice by reducing CS-induced oxidative DNA damage and premature cell senescence in the lungs. Our study suggests that administration of molecular H_2 may be a novel preventive and therapeutic strategy for COPD.

Keywords: Molecular hydrogen, DNA damage, Senescence, Emphysema, Chronic obstructive pulmonary disease, SMP30

Introduction

Chronic obstructive pulmonary disease (COPD) is a severe disease with a relatively high prevalence and mortality rate, and its prevalence has continuously been on the rise owing to an aging society and the cumulative effects of smoking. In 2020, COPD is projected to rank fifth in prevalence and third in cause of death [1]. COPD is characterized by chronic inflammation of peripheral airways and emphysema, represented by enlarged alveolar airspaces and destruction of the lung parenchyma [1,2]. Cells in the respiratory system are constantly exposed to oxidants. Cigarette smoke (CS) is the most important cause of COPD as the major source of oxidants/reactive oxygen species (ROS) in the lungs and throughout the body. Oxidants derived from CS can induce DNA damage [2,3] and stress-induced premature senescence (SIPS) [4–6] in the respiratory system during development of pulmonary emphysema. No COPD treatment has been clinically proven to restore the enlarged alveoli and destroyed lung parenchyma to date. Although antioxidants are expected to provide benefits for patients with COPD, their therapeutic potential remains limited owing to the complexity of this disease.

Molecular hydrogen (H_2) was recently reported as a preventive and therapeutic antioxidant that selectively reacts with the hydroxyl radical ($^{\bullet}OH$), the most cytotoxic ROS generated in mitochondria, and with cytotoxic reduced oxygen radicals [7]. Molecular H_2 has also been proposed for treatment in ischemia–reperfusion injury, neurological diseases, lipid

metabolism diseases, inflammation, and apoptosis, with no known side effects [8,9]. However, there has been no report indicating that molecular H_2 is efficacious for COPD.

We previously reported that senescence marker protein 30 (SMP30), a gluconolactonase involved in vitamin C (VC) biosynthesis that decreases with aging in rats and mice, protected mouse lungs from the oxidative stress associated with aging and smoking [10–14]. SMP30 knockout (SMP30-KO) mice have a shorter lifespan, during which they develop emphysema within 8 weeks of exposure to CS [13,14]. Furthermore, VC treatment successfully prevents CS-induced pulmonary emphysema in this model [15]. Accordingly, we hypothesized that H_2-rich pure water would react with CS-induced oxidants in the lungs to reduce the CS-induced DNA damage and SIPS, resulting in the attenuation of CS-induced pulmonary emphysema in the lungs. To test this hypothesis, we investigated the effects of H_2-rich pure water on lung histopathology and morphology in SMP30-KO mice after 8-week exposure to CS and explored the underlying mechanisms by evaluating the DNA damage and SIPS in the lungs.

Materials and methods

Preparation of H_2-rich pure water

H_2-rich pure water was freshly prepared in cooperation with MiZ Co. Ltd. (Kamakura, Kanagawa, Japan) as previously described [9]. The concentration of H_2 in pure water was

maintained at approximately 7 ppm without opening the bottle. Once the cap was opened, the H_2 concentration significantly decreased after 1 h; therefore, when feeding mice, we changed the glass water bottles twice daily until the end of the experiment.

Mice

Male mice were maintained and bred at the Animal Facility of the Tokyo Metropolitan Institute of Gerontology. SMP30-KO mice were generated from C57BL6 male mice by gene targeting [10]. Mice were maintained in a limited access barrier facility and housed in a humidity (55 ± 10%)- and temperature (24 ± 2 °C)-controlled room under 12-h light/dark cycles. Mice were fed with a VC-deficient chow (CL-2; CLEA Japan, Tokyo, Japan) and had free access to water containing 1.5 g/L VC in 10 μM ethylenediaminetetraacetic acid until the age of 3 months. Afterward, the VC concentration was changed to 0.0375 g/L for 1 month. The mice were then divided into four groups, with 12 animals per group: 1) air-exposed (Air); 2) air-exposed receiving H_2-rich water (Air+H_2); 3) CS-exposed (CS); and 4) CS-exposed receiving H_2-rich water (CS+H_2). Animal experimentation was approved by the Animal Care and Use Committees of the Tokyo Metropolitan Institute of Gerontology and Juntendo University School of Medicine.

Chronic exposure to CS

Mice were exposed to CS for 8 weeks using commercially marketed Peace non-filter cigarettes (29 mg of tar and 2.5 mg of nicotine per cigarette; Japan Tobacco, Tokyo, Japan) and a tobacco smoke inhalation experimental system for small animals (Model SIS-CS; Shibata Scientific Technology, Tokyo, Japan) [14,15]. The experimental settings were: 15 mL stroke volume, six puffs per minute, and 3.5% CS diluted with compressed air. Mice were exposed to either diluted CS or fresh air (control group) for 30 min/day, 5 days/week for 8 weeks.

Preparation and morphological evaluation of the lungs

Mice were sacrificed at 8 weeks, and the lungs were subsequently processed as described previously [14,15]. Airspace size was evaluated by determining the mean linear intercept (MLI) [16]. The destructive index (DI) was determined [17] to calculate the destruction of the alveolar wall.

Pulmonary function test

Pulmonary function tests were performed using the FlexiVent system (SCIREQ Scientific Respiratory Equipment, Montreal, Canada) [18]. Static lung compliance (Cst) values were calculated by fitting the Salazar–Knowles equation to the pressure-volume loop. Dynamic lung compliance (Crs) values were calculated using a single-frequency forced

oscillation technique. Tissue elastance and damping were obtained from respiratory system impedance data using a constant-phase model. All parameters were calculated using the FlexiVent software (SCIREQ).

Western blot analysis

The lung tissue was mechanically homogenized with lysis buffer, and homogenates were kept on ice for 45 min. Following centrifugation at 2,500 ×*g* in an Eppendorf tube for 30 min, the supernatant was collected. Transferred membranes were allowed to react with phospho-histone H2AX (Ser139) rabbit monoclonal antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-cyclin-dependent kinase inhibitor 2A (p16INK4A) polyclonal antibody (1:1,000; Proteintech, Chicago, IL, USA) and anti-β-actin monoclonal antibody (1:10,000; Wako, Tokyo, Japan). Western blot signals were acquired using a Fuji LAS-4000 fluorescence imager (Fujifilm Corporation, Tokyo, Japan) and quantified using the Multi Gauge image analysis software (Fujifilm).

Immunohistochemistry

Paraffin-embedded lung sections (4 μm) were incubated with anti-phospho-histone H2AX antibody (S139, 1:5,000; Abcam, Cambridge, UK), anti-β-galactosidase antibody (1:1,000; rabbit IgG fraction, Thermo Fisher Scientific, Waltham, MA, USA),

anti-cyclin-dependent kinase inhibitor 1 (p21WAF1/CIP1) polyclonal antibody (1:3,000; Thermo Fisher Scientific), and anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) polyclonal antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). The ratio of positively immunostained nuclei to the total count of nuclei present in a field at ×400 magnification was determined in 10 different lung areas per mouse.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Analysis of variance was performed using the non-parametric Kruskal–Wallis test. When applicable, the Mann–Whitney *U*-test was used for comparisons between groups. A p-value of < 0.05 was considered significant.

Results

Effects of H_2-rich pure water on histologic and morphometric parameters of the lungs in SMP30-KO mice

Representative histologic images of the lungs from each group are shown in Fig. 1A. Chronic exposure to CS for 8 weeks generated pulmonary emphysema in SMP30-KO mice (Fig. 1A, CS) but not in air-exposed mice (Fig. 1A, Air). Lung tissues from CS+H_2 mice (Fig.

1A, CS+H_2) showed less alveolar destruction than those from CS-exposed mice without H_2 treatment. Compared with the air-exposed group, CS-exposed mice showed significantly greater airspace enlargement, based on MLI values ($p = 0.0233$; Fig. 1B), and alveolar wall destruction, based on DI values ($p = 0.0083$; Fig. 1C); these values were significantly lower in the lungs of mice in the CS+H_2 group ($p = 0.0091$ and $p = 0.0379$, respectively). In contrast, there was no significant difference in lung morphometry between the Air and CS+H_2 groups, which indicates that H_2-rich water attenuates the lung damage induced by CS.

Effects of H_2-rich pure water on lung mechanics in SMP30-KO mice

We performed pulmonary function tests on the lungs from SMP30-KO mice to assess the physiological consequences of chronic exposure to CS. Cst was significantly higher in the CS group than in the other groups. The CS+H_2 group showed marked recovery of lung elasticity, expressed as a decrease in Cst, compared to that in the CS group ($p = 0.0044$; Fig. 2A). Similarly, administration of H_2-rich pure water significantly restored the tissue elastance ($p = 0.0432$; Fig. 2D). In addition, Crs tended to recover in the CS+H_2 group ($p = 0.17$; Fig. 2B), as well as tissue damping ($p = 0.268$; Fig. 2C). These results indicated that H_2-rich pure water could protect pulmonary function during CS exposure in SMP30-KO mice.

Effects of H_2-rich pure water on oxidative DNA damage in the lungs of SMP30-KO mice

To elucidate the mechanisms underlying the preventive effects of H_2-rich pure water on CS-induced pulmonary emphysema, we next evaluated γH2AX protein levels by western blotting as an indicator of DNA damage in the lungs (Fig. 3A, 3B). γH2AX protein levels were significantly higher in the CS group than in the Air group. Moreover, γH2AX expression was significantly lower in the CS+H_2 group than in the CS group ($p = 0.0446$). Detection of γH2AX protein by immunohistochemistry (Fig. 3C, 3D) showed more positively stained cells in alveolar septal areas of the CS group compared with those in the Air ($p = 0.0019$) and CS+H_2 ($p = 0.0124$) groups.

We further evaluated 8-OHdG, a marker of oxidative DNA damage [2], in the lungs by immunohistochemistry (Fig. 3E, 3F). Similar to γH2AX, there were significantly larger numbers of 8-OHdG-positive cells in the CS group compared with the Air ($p < 0.0001$) and CS+H_2 ($p = 0.0159$) groups.

Effects of H_2-rich pure water on stress-induced premature senescence in the lungs of SMP30-KO mice

To confirm that the CS-induced DNA damage causes premature senescence in the lungs, we next evaluated p16 protein levels by western blotting (Fig. 4A, 4B) and p21 levels by immunohistochemistry (Fig. 4C, 4D). Similar to the markers of oxidative DNA damage, p16 and p21 were significantly more strongly expressed in the lungs of the CS group than in those

of the Air ($p = 0.0446$ and $p = 0.0241$, respectively) and CS+H_2 ($p = 0.0239$ and $p = 0.0101$, respectively) groups.

In addition, β-galactosidase protein levels were assessed by immunohistochemistry [6], which revealed a higher number of immunostained cells in alveolar septal areas of the CS group than in the other groups (Fig. 4E). The ratio of β-galactosidase-positive cells was significantly higher in the lungs of the CS group than in those of the control ($p = 0.0471$) and CS+H_2 ($p = 0.0031$) groups (Fig. 4F).

Discussion

The present study demonstrated that administration of H_2-rich pure water attenuated the CS-induced lung damage in SMP30-KO mice, reducing the airspace enlargement and parenchymal destruction. Moreover, the intake of H_2-rich pure water restored the Cst in SMP30-KO mice exposed to CS. The pulmonary restoration was due, at least in part, to the attenuation of CS-induced oxidative DNA damage and premature senescence in the lungs. This is the first study to show the preventive potential of molecular H_2 against CS-induced pulmonary emphysema in a mouse COPD model.

The pathogenesis of COPD is complex, owing to the involvement of recurrent inflammation, oxidative stress (oxidant/antioxidant imbalance), protease/antiprotease imbalance, apoptosis, enhanced senescence of structural cells, and defective repair processes

[4,5,19, 20]. Although CS is clearly the most common risk factor in the development of COPD, only a small proportion of smokers develop the disease, highlighting the presence of specific mechanisms responsible for the susceptibility to CS. CS causes oxidative DNA damage directly or through the generation of ROS, with DNA double-strand breaks (DSBs) being the most severe damage. DSBs can induce apoptosis, cellular senescence, proinflammatory responses, and oncogenesis [2,3,21], and lead to the phosphorylation of histone H2AX at serine 139; thus, phospho-Ser139-H2AX (i.e., γH2AX) is a reliable and sensitive indicator of DSBs [2]. Furthermore, a correlation was demonstrated between DNA oxidation, assessed by the expression of 8-OHdG, and the presence of γH2AX foci; 8-OHdG-positive type II cells contained a higher number of γH2AX foci per cell than those stained negative [3]. 8-OHdG is an established marker of oxidative stress and oxidative DNA damage, and its expression is significantly increased in the peripheral lungs of patients with pulmonary emphysema [22]. The level of 8-OHdG is also increased in the urine of COPD patients and demonstrates a strong correlation with COPD severity, defined by the forced expiratory volume in the first second [23]. The present study demonstrated that molecular H_2 protected the lungs of COPD model mice, at least in part, by reducing the CS-induced DSBs and DNA oxidation, which might be a core mechanism for pulmonary emphysema development. Molecular H_2 may also promote DNA repair and attenuate the susceptibility to CS.

It was recently proposed that CS or other pollutants may accelerate the senescence of lung cells through inducing oxidative stress, thereby accelerating the progression of COPD in some patients. Indeed, molecular markers of senescence are detected in the emphysematous lungs of COPD patients, and *in vitro* exposure of human lung epithelial cells to CS results in enhanced β-galactosidase expression [24]. Moreover, lung fibroblasts from COPD subjects showed increased expression of β-galactosidase and decreased proliferation in culture compared with those from healthy smokers [25,26]. In the current study, we clearly demonstrated that SIPS was enhanced in the lungs of mice with experimental pulmonary emphysema and was attenuated by the administration of H_2-rich water. These findings suggest that the link between DNA damage and SIPS plays a crucial role in the development of pulmonary emphysema and can be modulated by molecular H_2.

The initial finding that cerebral ischemia–reperfusion injury in rats can be improved by inhaling H_2 gas [7] highlighted the biological effects of molecular H_2 as a novel antioxidant, which have been confirmed in a variety of diseases and physiological conditions. Molecular H_2 rapidly diffuses into tissues and cells and is mild enough to not disturb metabolic redox reactions or affect ROS; therefore, there should be minimal adverse effects [7,8]. Molecular H_2 attenuated irradiation-induced lung damage by reducing the oxidative stress [27], and intraperitoneal injection of H_2-rich saline ameliorated airway mucus production and epithelium damage in CS-exposed rats by inhibiting apoptosis [28]. Intraperitoneal injection

of H_2-rich saline attenuated CS-induced lung injury by activating anti-inflammatory and antioxidant pathways and by inhibiting apoptosis [29]. H_2 inhalation significantly reduced inflammatory responses in the lungs and ameliorated lung damage in a COPD-like rat model [30]. In the present study, we demonstrated that H_2-rich water protects against the alveolar destruction induced by exposure to CS by attenuating the oxidative DNA damage and SIPS in the lungs of COPD model mice. Interestingly, chronic clearance of senescent cells improves the established vascular dysfunction associated with aging and chronic hypercholesterolemia [31]. We have also reported that ablation of senescent cells ameliorates aging-related lung dysfunction [18]. To evaluate the therapeutic effect of molecular H_2 on CS-induced pulmonary emphysema, further investigations are needed to determine the potential of molecular H_2 to eliminate DNA-damaged or senescent cells in the lungs after chronic exposure to CS.

This study has several limitations. First, the rapid decrease in H_2 concentration (to approximately 60% within 2 h) may result in an insufficient amount of molecular H_2 in the lungs to affect ROS signaling. Second, we did not fully investigate the effects of molecular H_2 on apoptosis and proinflammatory responses in the lungs, which were described in previous studies [27–30]. Third, each mouse may have received a different amount of molecular H_2, because the mice had free access to H_2-rich pure water during the smoking period. However, no substantial differences in the effects of molecular H_2 were found among

individuals. We believe that supplying molecular H_2 in drinking water is considerably more convenient and safer than other routes of administration for patients with various diseases, including COPD. Thus, molecular H_2 may be a novel and promising treatment for COPD patients. Further experiments and clinical studies are needed for evaluating the therapeutic applications of molecular H_2 in COPD.

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Figure Legends

Fig. 1. Effects of H_2-rich pure water on histologic and morphometric parameters of the lungs in SMP30-KO mice. A. Representative histologic images of lung sections from each group, stained with hematoxylin–eosin. Scale bar = 50 μm. B. MLI data. C. DI values. Values are the mean + SEM (n = 12 for each group). *p < 0.05, **p < 0.01. CS, cigarette smoke; DI, destructive index; MLI, mean linear intercept; SEM, standard error of the mean; SMP30-KO, senescence marker protein 30 knockout.

Fig. 2. Effects of H_2-rich pure water on lung mechanics in SMP30-KO mice. A. Static lung compliance (Cst). B. Dynamic lung compliance (Crs). C. Tissue damping (G). D. Tissue elastance (H). Values are the mean + SEM (n = 5 for each group). *p < 0.05, **p < 0.01. CS, cigarette smoke; SEM, standard error of the mean; SMP30-KO, senescence marker protein 30 knockout.

Fig. 3. Effects of H_2-rich pure water on the expression of ɣH2AX and 8-OHdG in the lungs of SMP30-KO mice. A. Representative western blots of ɣH2AX expression. β-Actin was used as a loading control. Band size is indicated on the right. B. Densitometric analysis of ɣH2AX protein expression, normalized to that of β-actin. C. Representative

immunohistochemistry images of the antibody reaction against ɤH2AX in lung sections from each group. Brown color indicates ɤH2AX-positive alveolar epithelial cells. Scale bar = 50 μm. D. The ratio of ɤH2AX-positive nuclei to the total count of nuclei present in a field at ×400 magnification was determined in 10 different areas of the lung per mouse. E. Representative immunohistochemistry images of the antibody reaction against 8-OHdG in lung sections from each group. Brown color indicates 8-OHdG-positive alveolar epithelial cells. Scale bar = 50 μm. F. The ratio of 8-OHdG-positive nuclei to the total count of nuclei present in a field at ×400 magnification was determined in 10 different areas of the lung per mouse. Values are the mean + SEM (n =12 for each group). *$p < 0.05$, **$p < 0.01$. CS, cigarette smoke; ɤH2AX, phosphorylated histone H2AX; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SEM, standard error of the mean; SMP30-KO, senescence marker protein 30 knockout.

Fig. 4. Effects of H_2-rich pure water on the expression of senescence markers (p16, p21, and β-galactosidase) in the lungs of SMP30-KO mice. A. Representative western blots of p16 expression. β-Actin was used as a loading control. Band size is indicated on the right. B. Densitometric analysis of p16 protein expression, normalized to that of β-actin. C. Representative immunohistochemistry images of the antibody reaction against p21 in lung sections from each group. D. The ratio of p21-positive nuclei to the total count of nuclei

present in a field at ×400 magnification was determined in 10 different areas of the lung per mouse. E. Representative immunohistochemistry images of the antibody reaction against β-galactosidase in lung sections from each group. F. The ratio of β-galactosidase-positive nuclei to the total count of nuclei present in a field at ×400 magnification was determined in 10 different areas of the lung per mouse. Brown color indicates p21- or β-galactosidase-positive alveolar epithelial cells. Scale bar = 50 μm. Values are the mean + SEM (n =12 for each group). *$p < 0.05$, **$p < 0.01$. CS, cigarette smoke; SEM, standard error of the mean; SMP30-KO, senescence marker protein 30 knockout.

Highlights

- Administration of H_2-rich water prevents cigarette smoke-induced emphysema in mice.

- Molecular H_2 reduces oxidative DNA damage and premature senescence in the lungs.

- Supplementation of H_2 can be a novel and promising treatment for COPD patients.

Online Data Supplement

Hydrogen-rich pure water prevents cigarette smoke-induced pulmonary emphysema in SMP30 knockout mice

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Supplementary Methods

Materials

Commercially available primary antibodies were as follows: an anti-cyclin-dependent kinase inhibitor 2A (p16INK4A) polyclonal antibody (Proteintech Group, Chicago, IL, USA), anti-cyclin-dependent kinase inhibitor 1 (p21WAF1/CIP1) polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA), anti-phospho-histone H2AX (Ser139) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-histone H2AX (phospho Ser139) antibody (Abcam, Cambridge, UK), anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA), anti-β-galactosidase rabbit IgG fraction (Thermo Fisher Scientific), anti-β-actin monoclonal antibody (Wako, Tokyo, Japan).

Preparation of hydrogen-rich pure water

H_2-rich pure water was freshly prepared in cooperation with MiZ Co. Ltd. (Kamakura, Kanagawa, Japan) as previously described [1]. Briefly, a pressure-resistant polyethylene terephthalate (PET) bottle (500 mL), with an inserted gas-permeable film, was filled with water. A non-woven fabric containing H_2-generating agent (0.65 g) was

inserted into an acrylic resin tube, then 0.5 mL of water was added, and the tube was tightly closed. The H_2 gas produced was emitted into the water through a check valve attached to the acrylic resin tube. During the reaction, the PET bottle was gradually pressurized to approximately six atmospheres because of the generation of H_2 gas. The reaction was performed for 24 h at 4 °C, and the H_2 gas was dissolved by shaking the bottle for about 30 s. The concentration of H_2 in pure water was maintained at approximately 7 ppm after 24 h without opening the bottle. Once the cap was opened, the H_2 concentration significantly decreased after 1 h; therefore, when feeding mice, we changed the glass water bottles twice daily until the end of the experiment.

Mice

Male mice were maintained and bred at the Animal Facility of the Tokyo Metropolitan Institute of Gerontology. Senescence marker protein 30 knockout (SMP30-KO) mice were generated from C57BL6 male mice by gene targeting [2]. Mice were maintained in a limited access barrier facility and housed in a humidity (55 ± 10%)- and temperature (24 ± 2 °C)-controlled room under 12-h light/dark cycles. Mice were fed with a vitamin C (VC)-deficient chow (CL-2; CLEA Japan, Tokyo, Japan) and had free access to water containing 1.5 g/L VC in 10 μM ethylenediaminetetraacetic

acid until the age of 3 months. Afterward, the VC concentration was changed to 0.0375 g/L for 1 month. The mice were then divided into four groups, with 12 animals per group: 1) air-exposed (Air); 2) air-exposed receiving H_2-rich water (Air+H_2); 3) Cigarette smoke (CS)-exposed (CS); and 4) CS-exposed receiving H_2-rich water (CS+H_2). Animal experimentation was approved by the Animal Care and Use Committees of the Tokyo Metropolitan Institute of Gerontology and Juntendo University School of Medicine.

Chronic exposure to CS

Mice were exposed to CS for 8 weeks using commercially marketed Peace non-filter cigarettes (29 mg of tar and 2.5 mg of nicotine per cigarette; Japan Tobacco, Tokyo, Japan) and a tobacco smoke inhalation experimental system for small animals (Model SIS-CS; Shibata Scientific Technology, Tokyo, Japan) [3–5]. The experimental settings were: 15 mL stroke volume, six puffs per minute, and 3.5% CS diluted with compressed air. Mice were exposed to either diluted CS or fresh air (control group) for 30 min/day, 5 days/week for 8 weeks.

Preparation and morphological evaluation of lungs

Mice were sacrificed at 8 weeks, and the lungs were subsequently processed as described previously [4]. Briefly, mice were weighed and sacrificed by blood collection from the celiac artery and a blood loss from the left atrium with perfusion of phosphate-buffered saline (PBS) through the main pulmonary artery. The lungs were inflated and fixed by intratracheal instillation of 10% buffered formalin (pH 7.4; Wako) at a constant pressure of 25 cm H_2O for 48 h. Frontal plane sections of the lungs were made at the depth of the hilum, followed by further sectioning of each block into two equal-size blocks, also in the frontal plane, and embedded in paraffin. Histological sections were prepared for morphometric evaluation at 4-μm thickness and stained with hematoxylin–eosin. Airspace size was evaluated by determining the mean linear intercept (MLI) [6]. The destructive index (DI) was determined [7] to calculate the destruction of the alveolar wall.

Pulmonary function test

Pulmonary function tests were performed using the FlexiVent system (SCIREQ Scientific Respiratory Equipment, Montreal, Canada) [8]. Mice were euthanized by an intraperitoneal injection of pentobarbital sodium and connected to the FlexiVent system after tracheotomy. Mice were ventilated at a respiratory rate of 150 breaths/min, with a

tidal volume of 10 mL/kg against a positive end-expiratory pressure of 3 cm H_2O. Deep inflation, Snapshot-150, Quickprime-3, and a pressure-volume loop with constantly increasing pressure were consecutively performed six times in each subject. Static lung compliance values were calculated by fitting the Salazar–Knowles equation to the pressure-volume loop. Dynamic lung compliance values were calculated using a single-frequency forced oscillation technique. The pressure, flow, and volume signals obtained from the response to a sinusoidal waveform were fitted to a single compartment model using linear regression. Tissue elastance and damping, which are closely related to tissue resistance and elastance, respectively, were obtained from respiratory system impedance data using a constant-phase model. All parameters were calculated using the FlexiVent software (SCIREQ).

Western blot analysis

The lung tissue was mechanically homogenized with lysis buffer, and homogenates were kept on ice for 45 min. Following centrifugation at 2,500 × *g* in an Eppendorf tube for 30 min, the supernatant was collected. Protein levels were measured with a BCA kit (Thermo Fisher Scientific). Ten to twenty micrograms of protein were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels. After

transferring the proteins onto Immobilon-P membranes (Merck Millipore Corporation, Darmstadt, Germany), membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 and allowed to react with phospho-histone H2AX (Ser139) rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), p16INK4A polyclonal antibody (1:1,000) and anti-β-actin monoclonal antibody (1:10,000) at 4 °C overnight. Next, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000–1:20,000; GE Healthcare UK, Amersham, UK), followed by the detection with the Clarity western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Western blot signals were acquired using a Fuji LAS-4000 fluorescence imager (Fujifilm Corporation, Tokyo, Japan) and quantified using the Multi Gauge image analysis software (Fujifilm).

Immunohistochemistry

Paraffin-embedded lung sections (4 μm) were deparaffinized in xylene and rehydrated in ethanol. Heat-induced epitope retrieval (10 min at 125 °C) was performed in citrate buffer (pH 6.0; LSI Medience Corporation, Tokyo, Japan). After blocking with 2% bovine serum albumin in PBS, tissue sections were incubated at 4 °C overnight with anti-phospho-histone H2AX (S139) antibody (1:5,000; Abcam), anti-β-galactosidase

antibody (1:1,000), anti-p21 (WAF1, CIP1) polyclonal antibody (1:3,000), and anti-8-OHdG polyclonal antibody (1:500). Next, these tissue sections were incubated with biotin-labeled anti-rabbit IgG antibody (1:300; Agilent Technologies, Santa Clara, CA, USA) and labeled with streptavidin (1:300; Agilent Technologies) for 40 min at 25-28 □. Signals were detected using hydrogen peroxide and 3,3-diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with hematoxylin and dehydrated in xylene. The ratio of positively immunostained nuclei to the total count of nuclei present in a field at ×400 magnification was determined in 10 different lung areas per mouse.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Analysis of variance was performed using the non-parametric Kruskal–Wallis test. When applicable, the Mann–Whitney *U*-test was used for comparisons between groups. A p-value of < 0.05 was considered significant.

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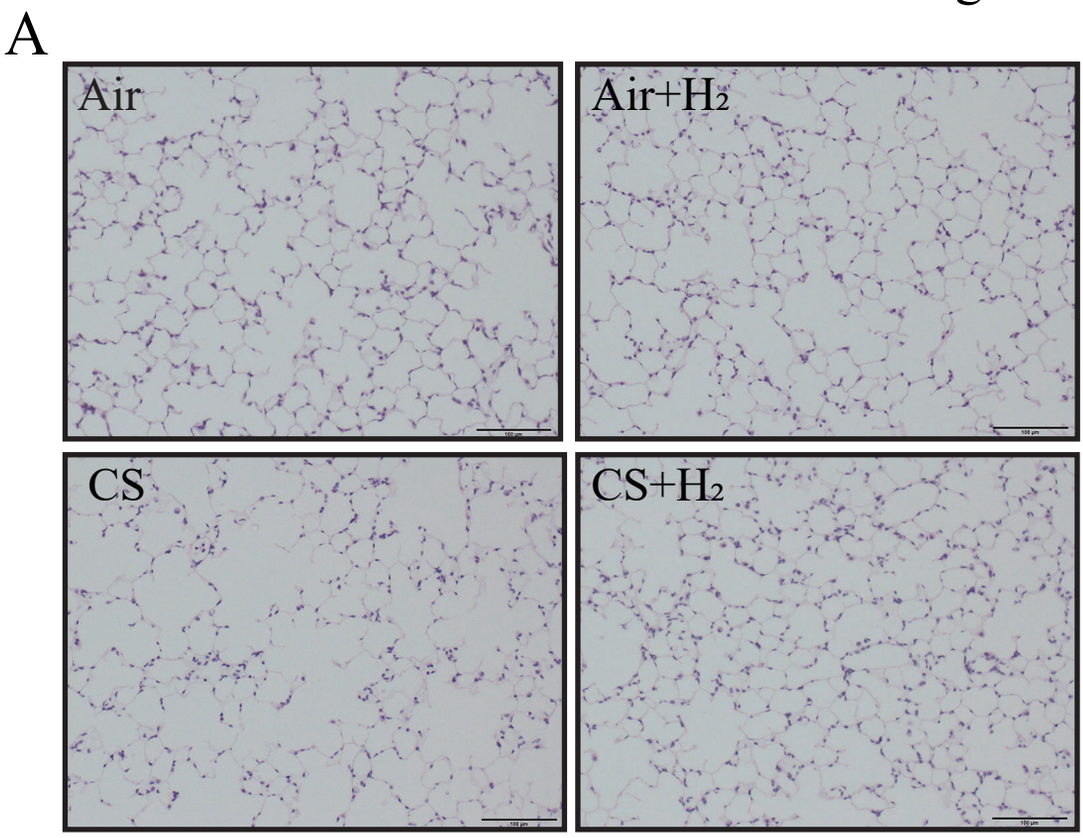
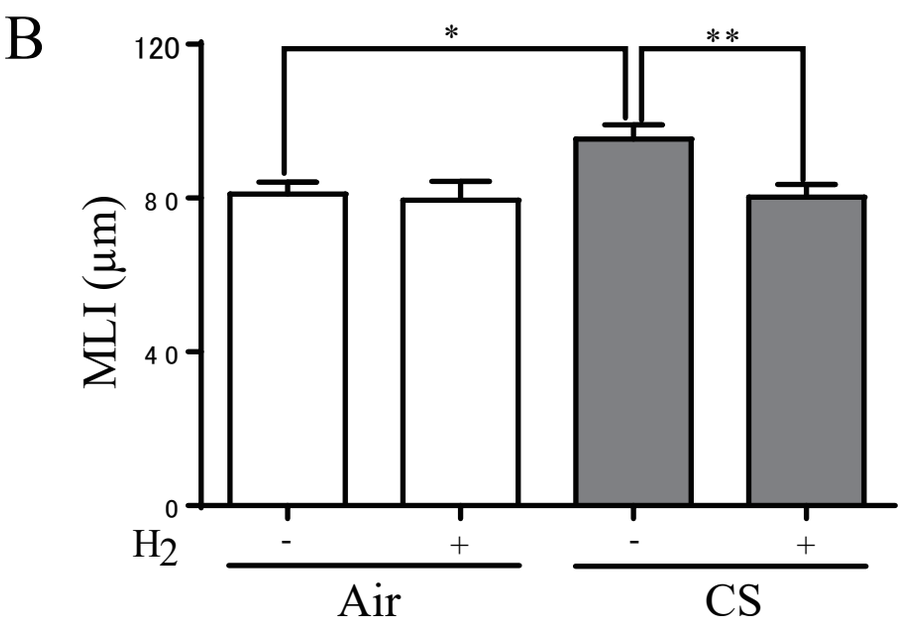
Fig.1 Suzuki.Y et al





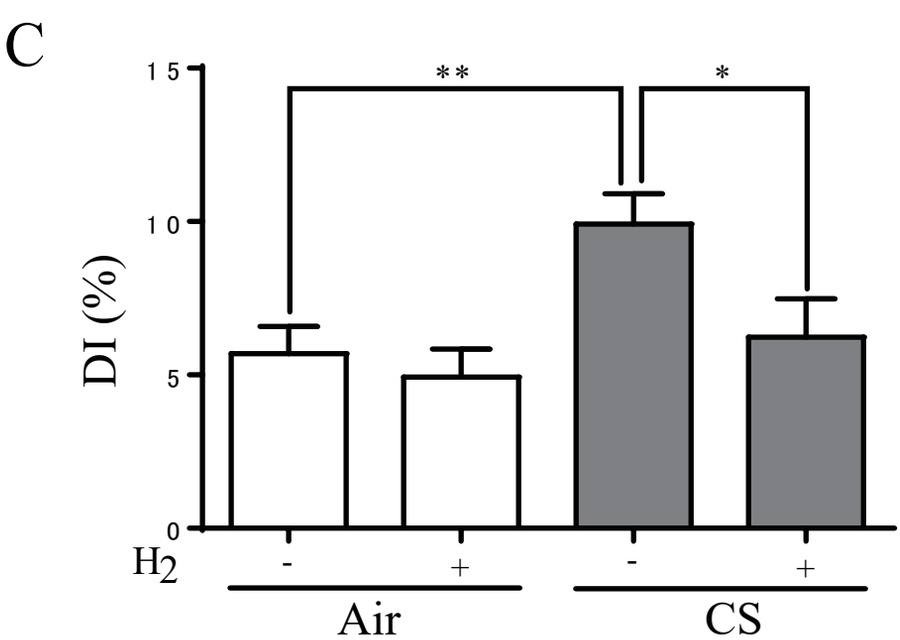


Fig.2 Suzuki.Y et al

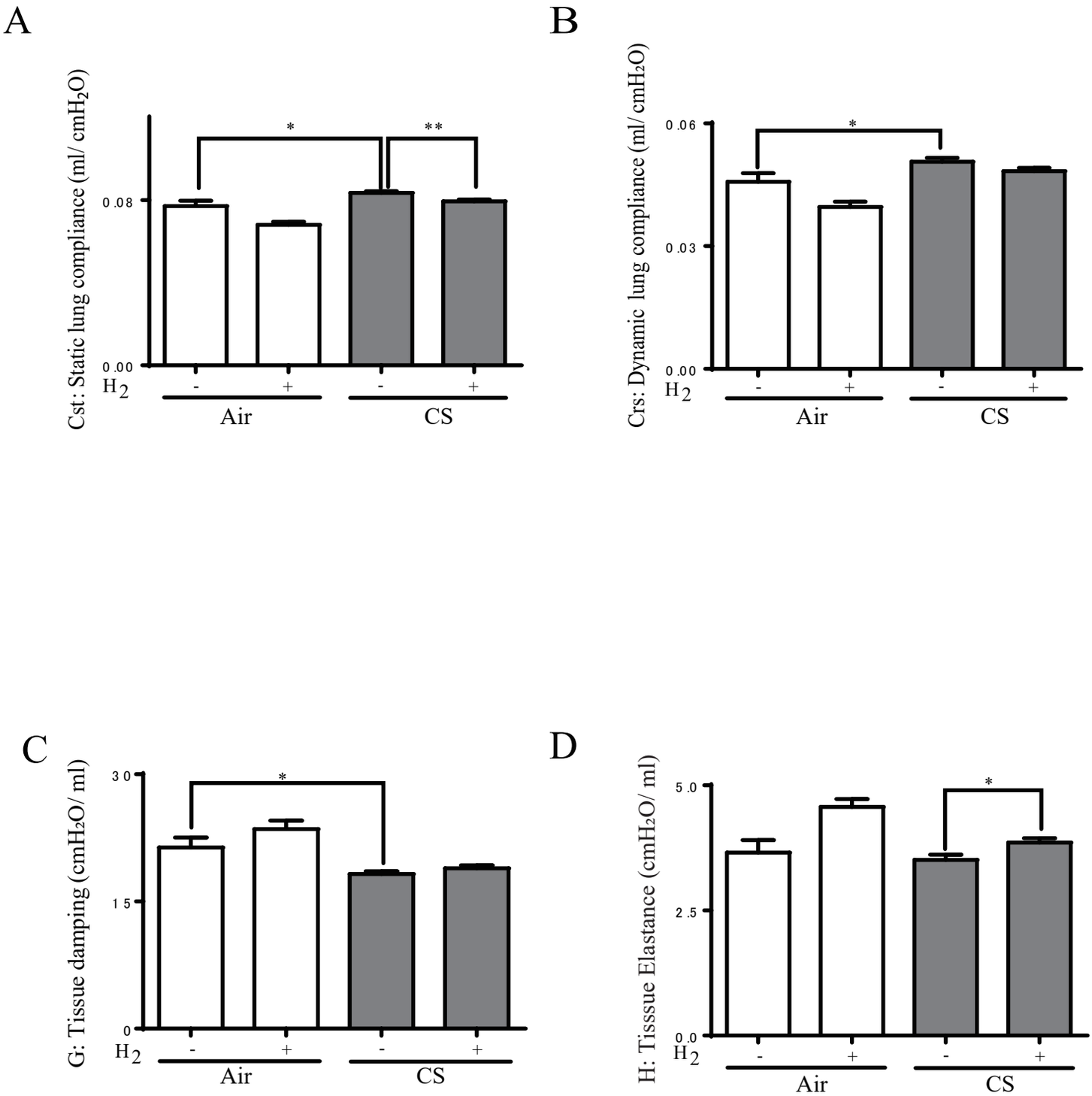
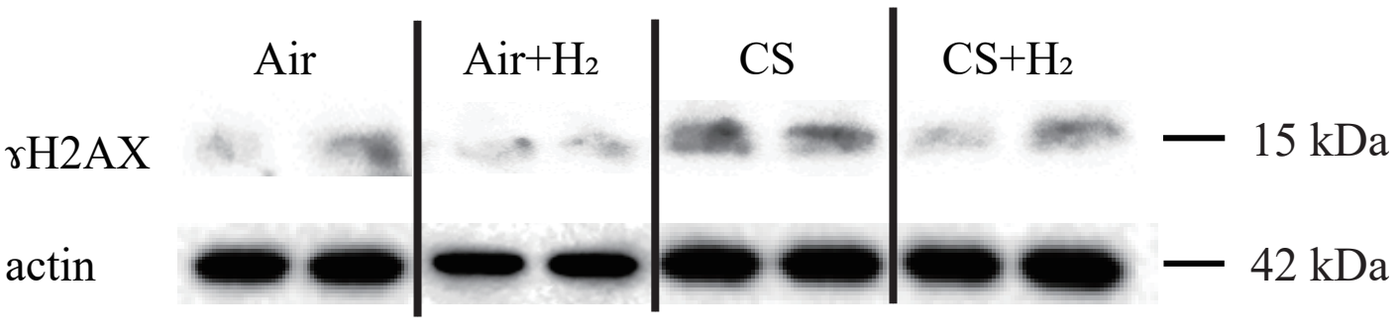


Fig.3-1 Suzuki.Y et al

A



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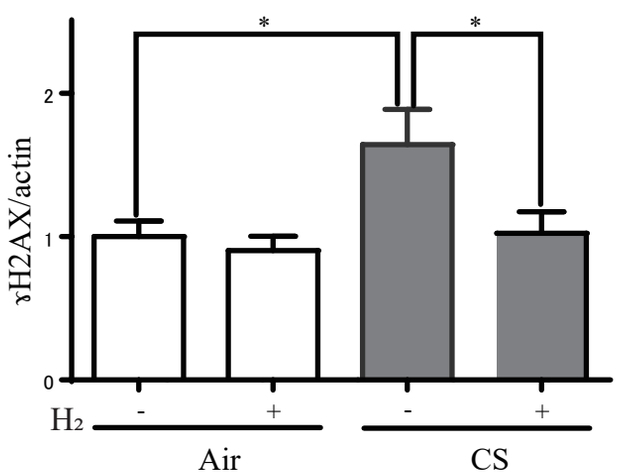

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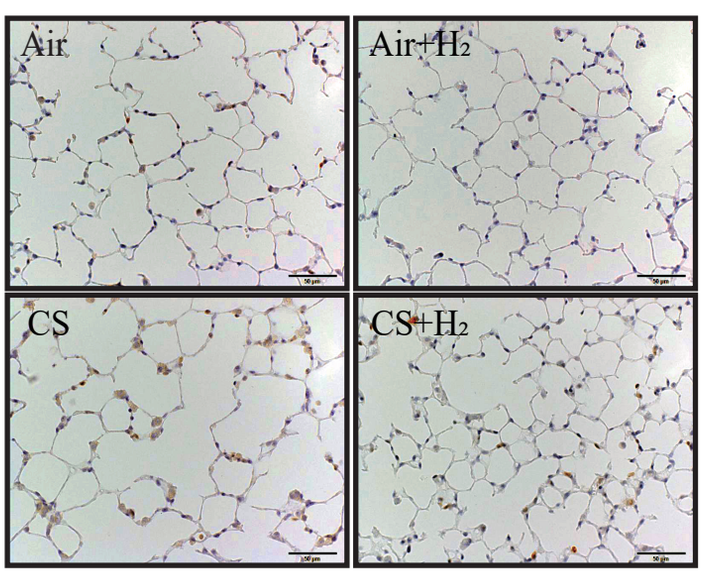

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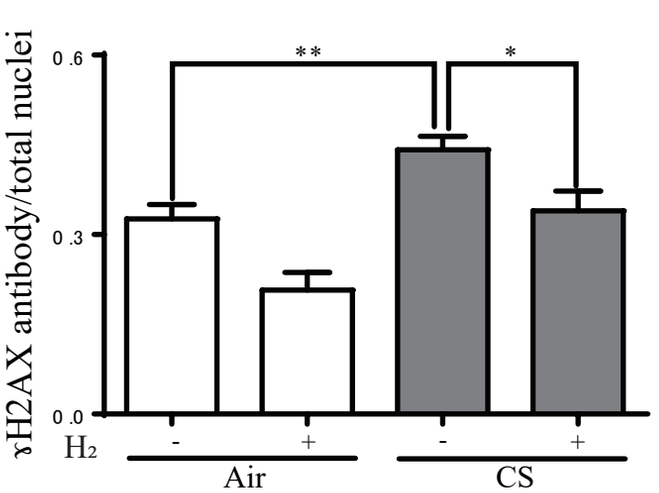

Fig.3-2 Suzuki.Y et al

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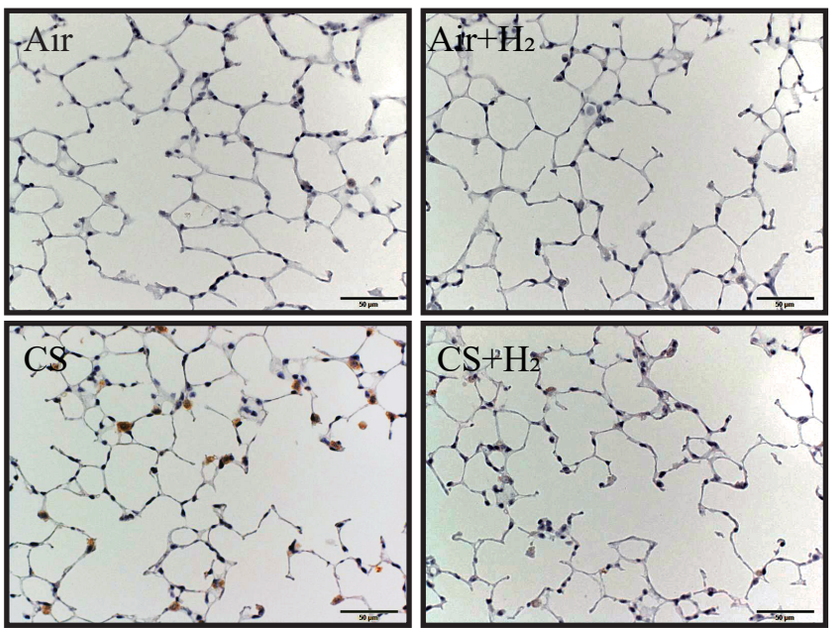

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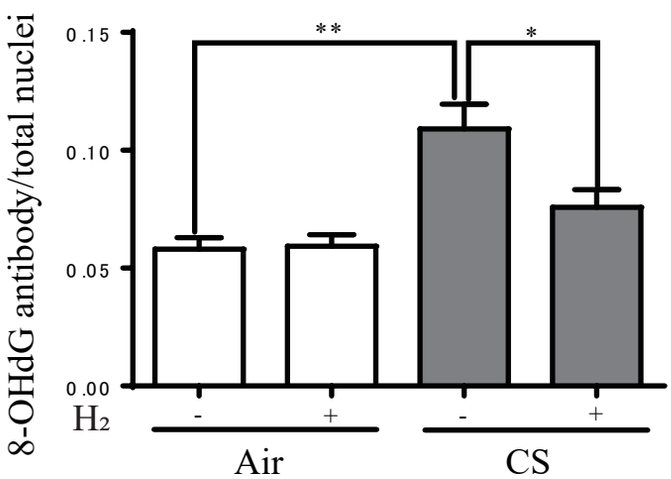

Fig.4-1 Suzuki.Y et al

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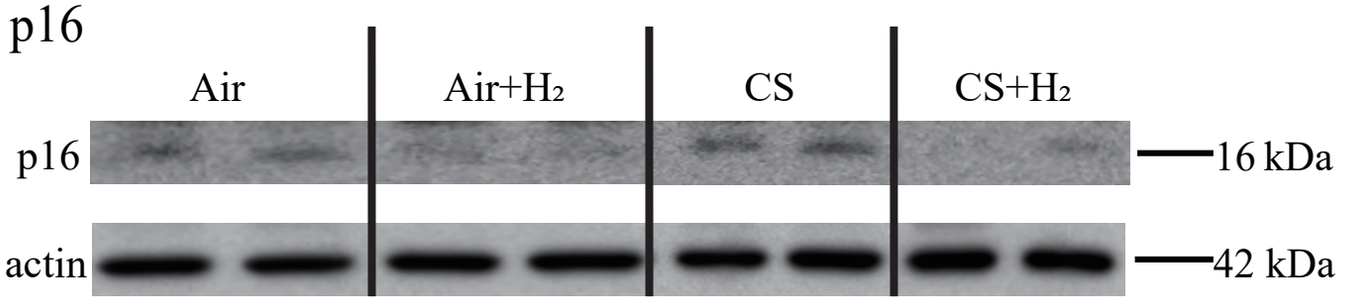

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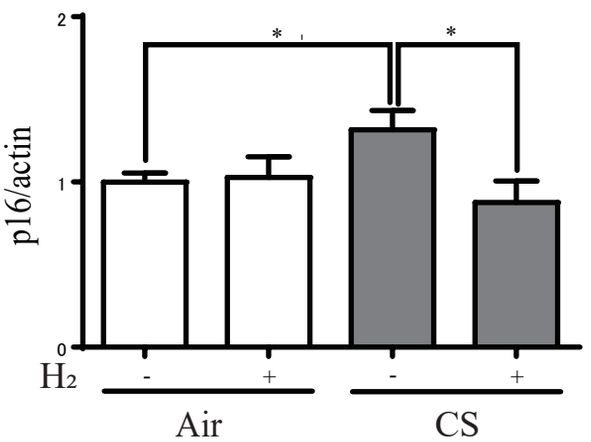

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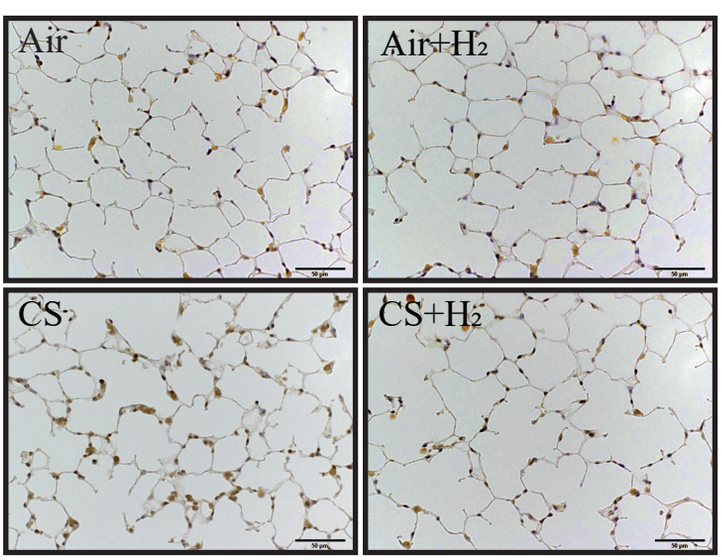

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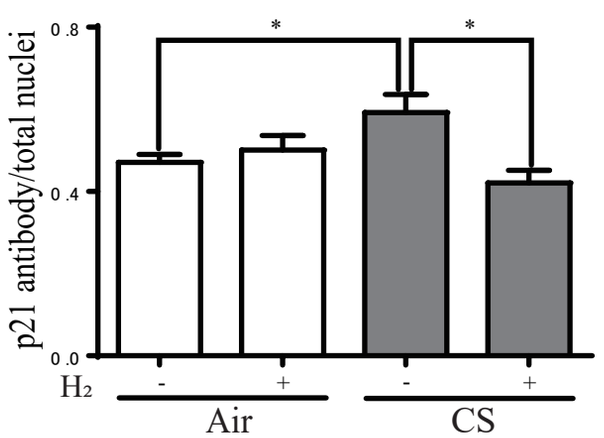
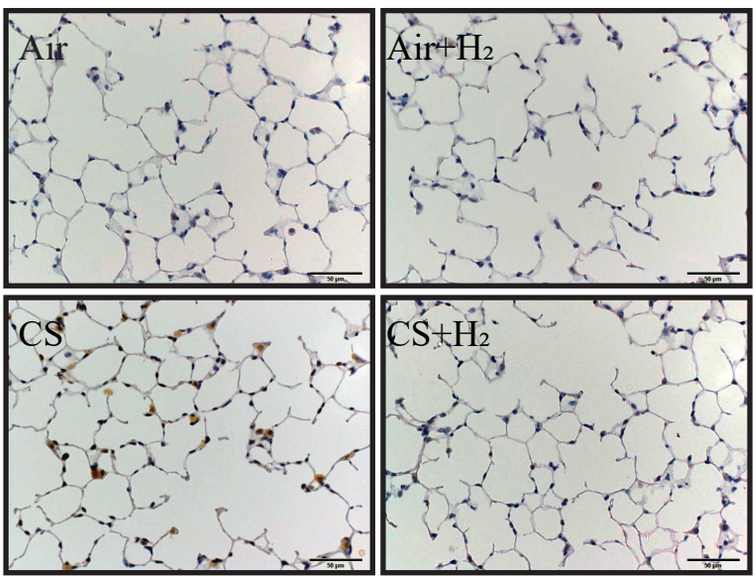
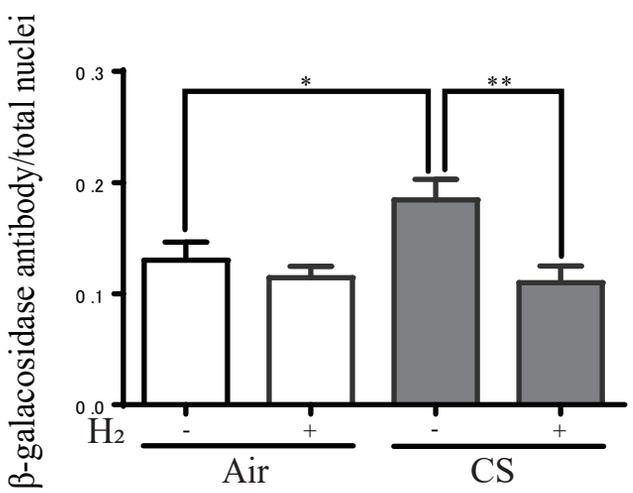


E. β-galacosidase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