



## Suplatast tosilate protects the lung against hyperoxic lung injury by scavenging hydroxyl radicals

Kazuhide Fukuhara<sup>a</sup>, Taku Nakashima<sup>a</sup>, Manabu Abe<sup>b</sup>, Takeshi Masuda<sup>a</sup>, Hironobu Hamada<sup>c</sup>, Hiroshi Iwamoto<sup>a</sup>, Kazunori Fujitaka<sup>a</sup>, Nobuoki Kohno<sup>d</sup>, Noboru Hattori<sup>a,\*</sup>

<sup>a</sup> Department of Molecular and Internal Medicine, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, Hiroshima 734-8551, Japan

<sup>b</sup> Department of Chemistry Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan

<sup>c</sup> Department of Physical Analysis and Therapeutic Sciences, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, Hiroshima 734-8551, Japan

<sup>d</sup> Hiroshima Cosmopolitan University, 5-13-18 Ujinanishi, Minami-ku, Hiroshima, Hiroshima 734-0014, Japan

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### ABSTRACT

Prolonged exposure to hyperoxia produces extraordinary amounts of reactive oxygen species (ROS) in the lung and causes hyperoxic lung injury. Although supraphysiological oxygen is routinely administered for the management of respiratory failure, there is no effective strategy to prevent hyperoxic lung injury. In our previous study, we showed that suplatast tosilate, an asthma drug that inhibits T helper 2 (Th2) cytokines, ameliorated bleomycin-induced lung injury and fibrosis through Th2-independent mechanisms. Because bleomycin also generates ROS, we hypothesized that suplatast tosilate might have antioxidant activity and protect the lung against hyperoxic lung injury. To test this hypothesis, mice exposed to hyperoxia were given suplatast tosilate through drinking water. Treatment with suplatast tosilate significantly prolonged mouse survival, reduced the increases in the numbers of inflammatory cells, levels of the pro-inflammatory cytokines/chemokines IL-6 and MCP-1, and protein in bronchoalveolar lavage fluid, and ameliorated lung injury in histological assessment. Suplatast tosilate treatment also significantly inhibited hyperoxia-induced elevations in the levels of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, in bronchoalveolar lavage fluid and 8-isoprostane, a marker of lipid peroxidation, in lung tissue. This finding suggests that suplatast tosilate exerts an antioxidant activity *in vivo*. In addition, we investigated whether suplatast tosilate has a scavenging effect on hydroxyl radical, the most reactive and harmful ROS, using electron paramagnetic resonance spin-trapping. Suplatast tosilate was shown to scavenge hydroxyl radicals in a dose-dependent manner, and its reaction rate constant with hydroxyl radical was calculated as  $2.6 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$ , which is faster than that of several well-established antioxidants, such as ascorbate, glutathione, and cysteine. These results suggest that suplatast tosilate protects the lung against hyperoxic lung injury by decreasing the degree of oxidative stress induced by ROS, particularly by scavenging hydroxyl radicals. Suplatast tosilate might become a potential therapeutic for hyperoxic lung injury.

### 1. Introduction

High concentrations of oxygen are routinely applied for the management of patients with severe respiratory failure, such as acute lung injury and acute respiratory distress syndrome (ARDS). However, prolonged exposure to high concentrations of oxygen can cause hyperoxic lung

injury that mimics the disease condition of ARDS [1–6]. Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, generated under hyperoxic conditions are strongly involved in this lung injury. ROS cause cellular damage by delivering oxidative insults to DNA, proteins, and membranes and by inducing an inflammatory response in the injured lung tissue. Inflammatory cells that

**Abbreviations:** 8-OHdG, 8-hydroxydeoxyguanosine; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; DMPO, 5,5-Dimethylpyrroline-N-oxide; DMS, dimethyl sulfoxide; MPO, myeloperoxidase; Nrf-2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; Th2, T helper 2

\* Corresponding author.

**E-mail addresses:** [respikf127@gmail.com](mailto:respikf127@gmail.com) (K. Fukuhara), [tnaka@hiroshima-u.ac.jp](mailto:tnaka@hiroshima-u.ac.jp) (T. Nakashima), [mabe@hiroshima-u.ac.jp](mailto:mabe@hiroshima-u.ac.jp) (M. Abe), [ta-masuda@hiroshima-u.ac.jp](mailto:ta-masuda@hiroshima-u.ac.jp) (T. Masuda), [hironamada@hiroshima-u.ac.jp](mailto:hironamada@hiroshima-u.ac.jp) (H. Hamada), [hir@hiroshima-u.ac.jp](mailto:hir@hiroshima-u.ac.jp) (H. Iwamoto), [fujikazu@hiroshima-u.ac.jp](mailto:fujikazu@hiroshima-u.ac.jp) (K. Fujitaka), [nokohno@hcu.ac.jp](mailto:nokohno@hcu.ac.jp) (N. Kohno), [nhattori@hiroshima-u.ac.jp](mailto:nhattori@hiroshima-u.ac.jp) (N. Hattori).

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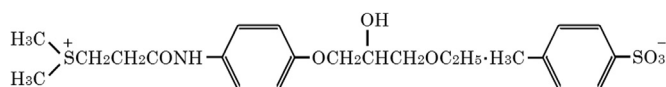


Fig. 1. Chemical structure of suplatast tosilate.

accumulate in the lung also generate ROS, and the lung injury progressively worsens [7–9]. In the management of patients with acute lung injury or ARDS, administration of supraphysiological concentrations of oxygen is required to maintain adequate tissue oxygenation; unfortunately, however, there are no pharmacological therapies to attenuate the resulting hyperoxic lung injury.

Some drugs frequently cause lung injury by generating ROS in the lung; the representative example is bleomycin [10,11]. Because bleomycin is a strong redox-active agent capable of inducing oxidative cleavage of DNA, it is used as an antitumor agent [12]. In mice, intratracheal administration of bleomycin elicits severe inflammation in the lung, followed by pulmonary fibrosis. Interestingly, a previous study from our laboratory reported that suplatast tosilate, an anti-allergic agent only available in Japan, strongly inhibited bleomycin-induced lung injury and fibrosis [13]. Suplatast tosilate (Fig. 1) is widely used as an oral asthma control drug in Japan because it inhibits the production of T helper 2 (Th2) cytokines in animal models of bronchial asthma [14–16]. Although suplatast tosilate has also been prescribed for adult and pediatric patients with allergic rhinitis and atopic dermatitis for more than 20 years in Japan, its long-term toxicity has not been reported.

The risk of serious side effects from suplatast tosilate is extremely low; however, its precise mechanism of action is not fully understood. Nonetheless, we have shown that the inhibitory effect of suplatast tosilate on bleomycin-induced lung injury and fibrosis was not mediated by its action on Th2 cytokines [13]. To the best of our knowledge, there are no new data explaining the anti-allergic action of suplatast tosilate.

Based on these observations, we speculated that suplatast tosilate inhibited bleomycin-induced lung injury and fibrosis via an antioxidant activity that removed the ROS generated by bleomycin. In other words, we hypothesized that suplatast tosilate might have a ROS scavenging activity. To test this hypothesis, we investigated the effects of suplatast tosilate on mice with hyperoxic lung injury, the most prominent animal model for lung injury induced by oxidative stress. In addition, the scavenging effect of suplatast tosilate on hydroxyl radical, a representative ROS, was also analyzed by using electron paramagnetic resonance (EPR; also known as electron spin resonance [ESR]) spin-trapping.

## 2. Materials and methods

### 2.1. Animals

Specific pathogen-free, female C57BL/6 mice weighing 20–22 g were purchased from Charles River Laboratories Japan (Yokohama, Japan). The animals were housed in pathogen-free rooms and maintained on laboratory chow, with free access to food and water. The committee on animal research at the University of Hiroshima approved all procedures.

### 2.2. Oxygen exposure

The mice were exposed to hyperoxia (90 ± 92%) in a sealed 30-l chamber (Allentown, Inc., Allentown, NJ, USA). Food and water were provided ad libitum. Oxygen, provided by an oxygen concentrator (TO-90-5H; Teijin, Tokyo, Japan), was delivered to the chamber to provide eight changes per hour (5 l/min flow rate).

### 2.3. Administration of suplatast tosilate

Suplatast tosilate was synthesized and supplied by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). Suplatast tosilate, dissolved in drinking water at 1 mg/ml, was given to mice ad libitum because we found that oral gavage administration could weaken and even kill mice. From the amount of water drunk, the dose of suplatast tosilate was approximately calculated as 100 mg/kg/day. To prove that suplatast tosilate has a protective effect in hyperoxic lung injury, its administration was started 2 days before the start of hyperoxic exposure. Detailed data regarding the pharmacokinetics of suplatast tosilate after oral administration in rodents were reported in previous studies [29–31].

### 2.4. Bronchoalveolar lavage and cell analysis

The trachea was exposed via a midline incision and cannulated with a tracheal cannula, and the lungs were lavaged three times with 0.5 ml of phosphate-buffered saline. The lavage fluids were pooled and centrifuged at 300g for 10 min at 4 °C. The supernatants were stored at –80 °C before measurement of cytokine and protein concentrations. The cell pellets were resuspended in 1 ml of Dulbecco's modified Eagle's medium, and the total cell numbers were counted using a hemocytometer. Differential cell counts were performed by counting at least 300 cells on a smear prepared using a cytospin (Shandon Inc., Pittsburgh, PA, USA) that was stained with the Diff-Quik stain set (Kokusai Shiyaku, Kobe, Japan).

### 2.5. Histological examination

The lung tissue specimens of the mice were fixed by inflation with a buffered 10% formalin solution. Lung tissue specimens were embedded in paraffin, and the sections were stained with hematoxylin and eosin and thereafter examined by light microscopy.

### 2.6. Protein concentration

The protein concentrations in samples were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

### 2.7. Cytokine measurements

Commercially available ELISA kits for IL-6 and MCP-1 were obtained from R & D Systems (Minneapolis, MN, USA). The concentration of each cytokine in the sample was measured following the manufacturer's protocols.

### 2.8. Total and differential white blood cell counts

About 0.5 ml of blood was drawn from the inferior vena cava and transferred into EDTA tubes. Total and differential white blood cell counts were performed using a TC20™ automated cell counter (Bio-Rad, Hercules, CA, USA).

### 2.9. Quantification of 8-isoprostane

Lung tissue was obtained after 24, 48, and 90 h of hyperoxia. To prevent auto-oxidation, lung tissue was immediately flash frozen in liquid nitrogen at harvest and stored at –80 °C until analysis. Upon thawing, the tissue sample was homogenized in 3 ml of phosphate-buffered saline and centrifuged. The 8-isoprostane content of the supernatants was analyzed using an Oxiselect 8-iso-prostaglandin F2α ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

## 2.10. Quantification of 8-hydroxydeoxyguanosine

As an indicator of oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG) was measured in the bronchoalveolar lavage (BAL) fluid obtained after 24, 48, and 90 h of hyperoxia using the Highly Sensitive ELISA kit for 8-OHdG (NIKKEN SEIL Co., Ltd., Shizuoka, Japan).

## 2.11. Quantification of myeloperoxidase

Myeloperoxidase (MPO) activity was measured in the lung tissue homogenates obtained after 24, 48, and 90 h of hyperoxia using the MPO Mouse ELISA Kit (Abcam, Cambridge, UK), following the manufacturer's instructions.

## 2.12. Assessment of Nrf-2 expression in the lung

Total RNA was isolated from the lungs of mice after 72 h of hyperoxic exposure using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated total RNA was reverse transcribed into cDNA, and real-time quantitative RT-PCR was performed with sequence-specific TaqMan primers and probes for Nrf-2 and the housekeeping gene 18 S rRNA using the ABI Prism 7500 Fast Sequence Detector and SDS analysis software (Applied Biosystems, Foster City, CA, USA). The mRNA level of Nrf-2 was expressed relative to the level of its internal reference, 18 S rRNA.

## 2.13. EPR spin-trapping analysis of hydroxyl radicals

5,5-Dimethylpyrroline-N-oxide (DMPO) was purchased from LABOTEC Co., Ltd. (Tokyo, Japan), and hydrogen peroxide and ferrous iron (FeSO<sub>4</sub>) were obtained from Wako Pure Chemicals (Osaka, Japan). The capacity of suplatast tosylate to scavenge hydroxyl radicals was examined by EPR spin-trapping using DMPO as a reference trapping agent because it reacts with hydroxyl radicals to form a spin adduct. Hydroxyl radicals were generated by the Fenton reaction started by the addition of hydrogen peroxide (12.4 mM final concentration) to a reaction mixture containing FeSO<sub>4</sub> (0.2 mM final concentration) and DMPO (20 μM final concentration) in the presence of varying concentrations of suplatast tosylate in 66.7 mM phosphate buffer solution (pH 7.4). The EPR signal was measured 1 min after the start of the reaction.

EPR spectra were recorded using an ELEXSYS E500 CW-EPR spectrometer (Bruker BioSpin K.K., Kanagawa, Japan). The measurements were carried out under the following conditions: magnetic field, 347–357 mT; microwave power, 12.0 mW; frequency, 100 kHz; modulation amplitude, 0.1 mT (1.0 Gauss); sweep time, 168 s; time constant, 163.8 ms; received gain, ×66; and temperature, 24 °C. These EPR analyses were performed by the Organic Analysis and Environmental Analysis Team in the Analysis Center of the Panasonic Corporation, Osaka, Japan. The reaction rate of suplatast tosylate with hydroxyl radicals was calculated by using a competition reaction between DMPO and suplatast tosylate with hydroxyl radicals, as previously described [17–19]. The calculation process is shown briefly as follows. Hydroxyl radicals ( $\cdot\text{OH}$ ) react with DMPO or suplatast tosylate (ST) in the reaction system. The reactions produce the two intermediate products (Eqs. (1) and (2)), where  $k_{\text{DMPO}}$  and  $k_{\text{ST}}$  represent the reaction rate constants of DMPO with hydroxyl radicals and suplatast tosylate with hydroxyl radicals, respectively. The rate constant of  $k_{\text{DMPO}}$  has been reported to be  $4.3 \times 10^9 \text{ M}^{-1} \text{ S}^{-1}$  [19,20].



The EPR signal intensity of DMPO-OH (DMPO spin adduct of hydroxyl radicals) was recorded in the absence and presence of suplatast tosylate.

$$d[\text{DMPO} - \text{OH}]/dt = k_{\text{DMPO}}[\text{DMPO}][\cdot\text{OH}] \quad (3)$$

$$d[\text{ST} - \text{OH}]/dt = k_{\text{ST}}[\text{ST}][\cdot\text{OH}] \quad (4)$$

In the competitive reaction, the reduction amounts of DMPO-OH should be equal to the amounts scavenged by suplatast tosylate. Hence, Eq. (5) can be deduced from Eqs. (3) and (4).

$$d[\text{DMPO} - \text{OH}]/dt : d[\text{ST} - \text{OH}]/dt = (1 - X) : X \quad (5)$$

Here, the variable X ( $0 < X < 1$ ) is the inhibition rate of the DMPO-OH signal. From Eqs. (3), (4), and (5), Eq. (6) can be deduced.

$$k_{\text{ST}} = k_{\text{DMPO}} \{X/(1 - X)\} \cdot \{[\text{DMPO}]/[\text{ST}]\} \quad (6)$$

When the concentration of DMPO-OH decreases by 50%, Eq. (6) can be simplified by substituting 0.5 for X.

$$k_{\text{ST}} = k_{\text{DMPO}}[\text{DMPO}]/\text{IC}_{50} \quad (7)$$

The IC<sub>50</sub> represents the concentration of suplatast tosylate that quenches 50% of hydroxyl radicals. Furthermore,  $k_{\text{ST}}$  can be obtained from the slope of the linear regression plot of  $X/(1-X)$  versus the concentration of suplatast tosylate [ST].

## 2.14. Statistical analysis

Statistical analyses were performed using SPSS 17 (SPSS Japan, Tokyo, Japan). Data are presented as the mean ± SEM. Student's *t*-test was used to evaluate statistical differences between the groups. The Kaplan-Meier method was used for survival analysis and comparisons were made using a log-rank test. Differences were considered significant when the *p* value was < 0.05.

## 3. Results

### 3.1. Effect of suplatast tosylate on survival from lethal hyperoxia in mice

To determine whether suplatast tosylate exerts a beneficial effect on survival from lethal hyperoxia in mice, survival was compared between mice administered suplatast tosylate in drinking water (suplatast tosylate-treated group) and mice given only water (control group) during hyperoxic exposure. As shown in Fig. 2A, treatment with suplatast tosylate significantly prolonged survival in mice exposed to hyperoxia. In our mouse hyperoxia model, we found that, after 80 h of hyperoxia, the control group mice became ill, manifested tachypnea, and started dying, although there were no differences in the body weights and food intake between the two groups of mice given water and suplatast tosylate until then. We were thus interested in seeing the effect of suplatast tosylate on survival in mice exposed to hyperoxia for 80 h and then returned to room air. Interestingly, no death occurred in the suplatast tosylate-treated group exposed to hyperoxia for 80 h and returned to room air, whereas a rapid decrease in survival rate was observed in the control group mice immediately after their return to room air (Fig. 2B). Treatment with suplatast tosylate also conferred a prominent survival advantage in the transient hyperoxic exposure model.

### 3.2. Effect of suplatast tosylate on hyperoxic lung injury

To determine whether suplatast tosylate affects the degree of hyperoxic lung injury, the microscopic features of the lungs and the protein concentrations in BAL fluid were compared between the mice of the suplatast tosylate-treated and control groups. As shown in Fig. 3, hyperoxic exposure induced interstitial inflammation and edema in the mouse lungs of both groups that exacerbated over time, but the severity of these conditions was much lower in the suplatast tosylate-treated group.

The protein concentration in BAL fluid, which reflects protein leakage into the alveolar space, also increased over time after hyperoxic

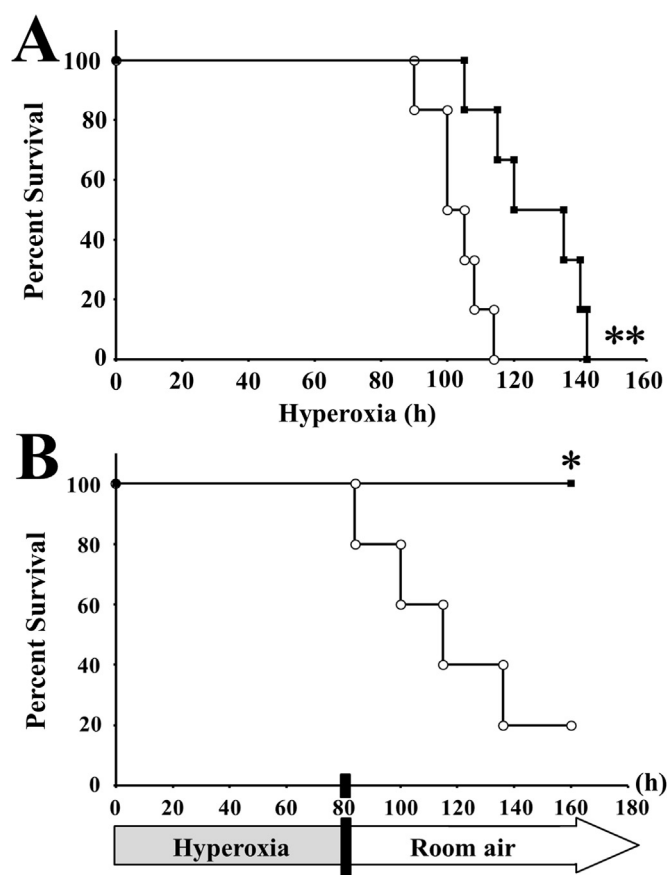


Fig. 2. Survival analysis of mice during and after hyperoxic exposure. (A) Survival was assessed when mice given suplatast tosilate (squares) or water (open circle) were exposed to 90% O<sub>2</sub> continuously until all of the mice had died (n=7 mice/group). (B) Survival was assessed when mice given suplatast tosilate (squares) or water (open circle) were exposed to 90% O<sub>2</sub> for 80 h and returned to the room air condition (n=5 mice/group). Asterisks indicate significant differences determined by Kaplan-Meier analysis. \*p < 0.05, \*\*p < 0.01.

exposure in the mice of both the suplatast tosilate-treated and control groups. However, the protein concentration was significantly lower in the suplatast tosilate-treated group mice than in the control group mice after 72 and 90 h of hyperoxia (Fig. 4A).

### 3.3. Effect of suplatast tosilate on inflammatory indices in BAL fluid

To evaluate the inflammatory response in the lung during hyperoxic exposure, the numbers of inflammatory cells were counted and the levels of the inflammatory cytokines/chemokines IL-6 and MCP-1 were measured in BAL fluid obtained from the mice of the suplatast tosilate-treated and control groups. The recovery rate of BAL fluid typically exceeded 85% and did not differ between the experimental groups. Because lymphocytes and eosinophils were scarcely observed in the BAL fluid obtained from the mice exposed to hyperoxia (data not shown), the macrophage and neutrophil counts are presented. As the hyperoxic lung injury progressed, the numbers of macrophages and neutrophils in BAL fluid increased in the mice of both groups. However, they were significantly lower in suplatast tosilate-treated group mice than in control mice after 72 and 90 h of hyperoxia (Fig. 4B and C). As shown in Fig. 5, the levels of IL-6 and MCP-1 in BAL fluid abruptly increased from 72 to 90 h of hyperoxic exposure in the mice of both groups. Similar to the inflammatory cells, treatment with suplatast tosilate significantly decreased the levels of both IL-6 and MCP-1 in BAL fluid after 72 and 90 h of hyperoxia (Fig. 4D and E). We also measured the Th2 cytokines IL-4 and IL-5 in BAL fluids but their levels were below the detection limits of the ELISA kits at all time points examined in both groups.

### 3.4. Effect of suplatast tosilate on total and differential white blood cell counts during hyperoxic exposure

To evaluate the changes in white blood cell counts during hyperoxic exposure, we counted the numbers of total white cells and neutrophils in peripheral blood at baseline and after 24, 48, 72, and 90 h of

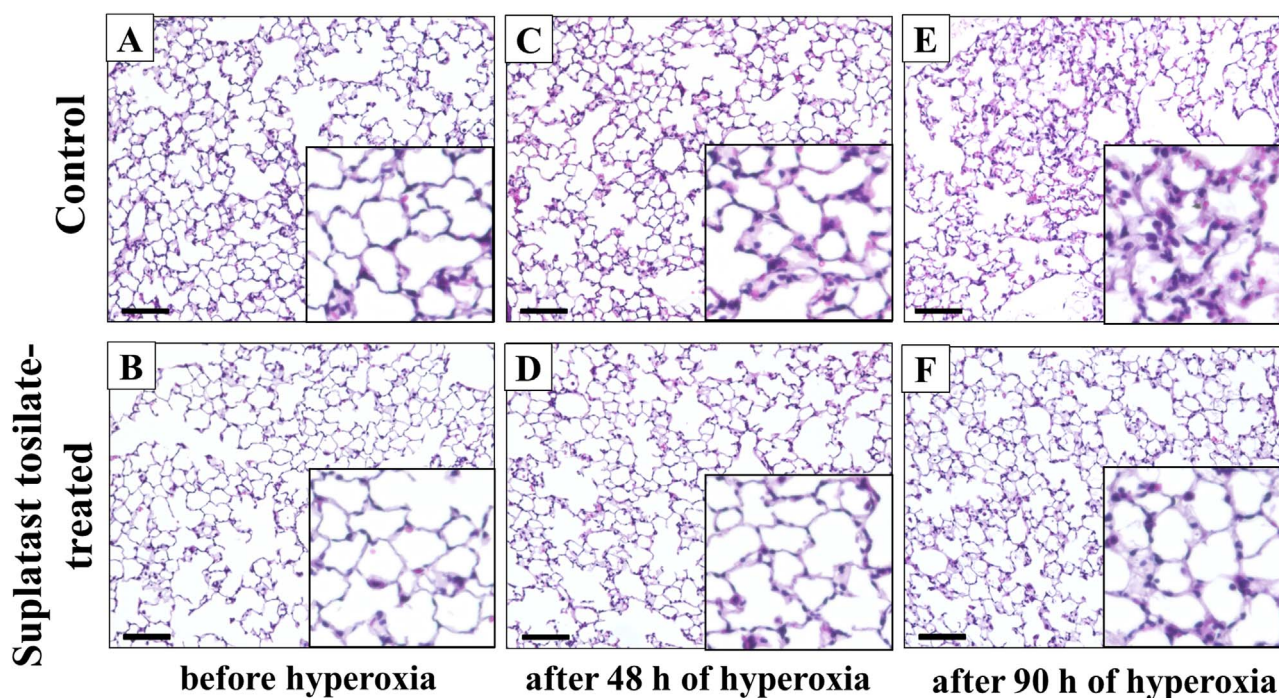


Fig. 3. Lung histology of mice given water or suplatast tosilate during hyperoxic exposure. Formalin-fixed sections of mouse lungs were stained with hematoxylin and eosin. Mice were given water at baseline (A), after 48 h of hyperoxic exposure (C), and after 90 h of hyperoxic exposure (E). Mice were given suplatast tosilate at baseline (B), after 48 h of hyperoxic exposure (D), and after 90 h of hyperoxic exposure (F). Scale bars, 100 μm.

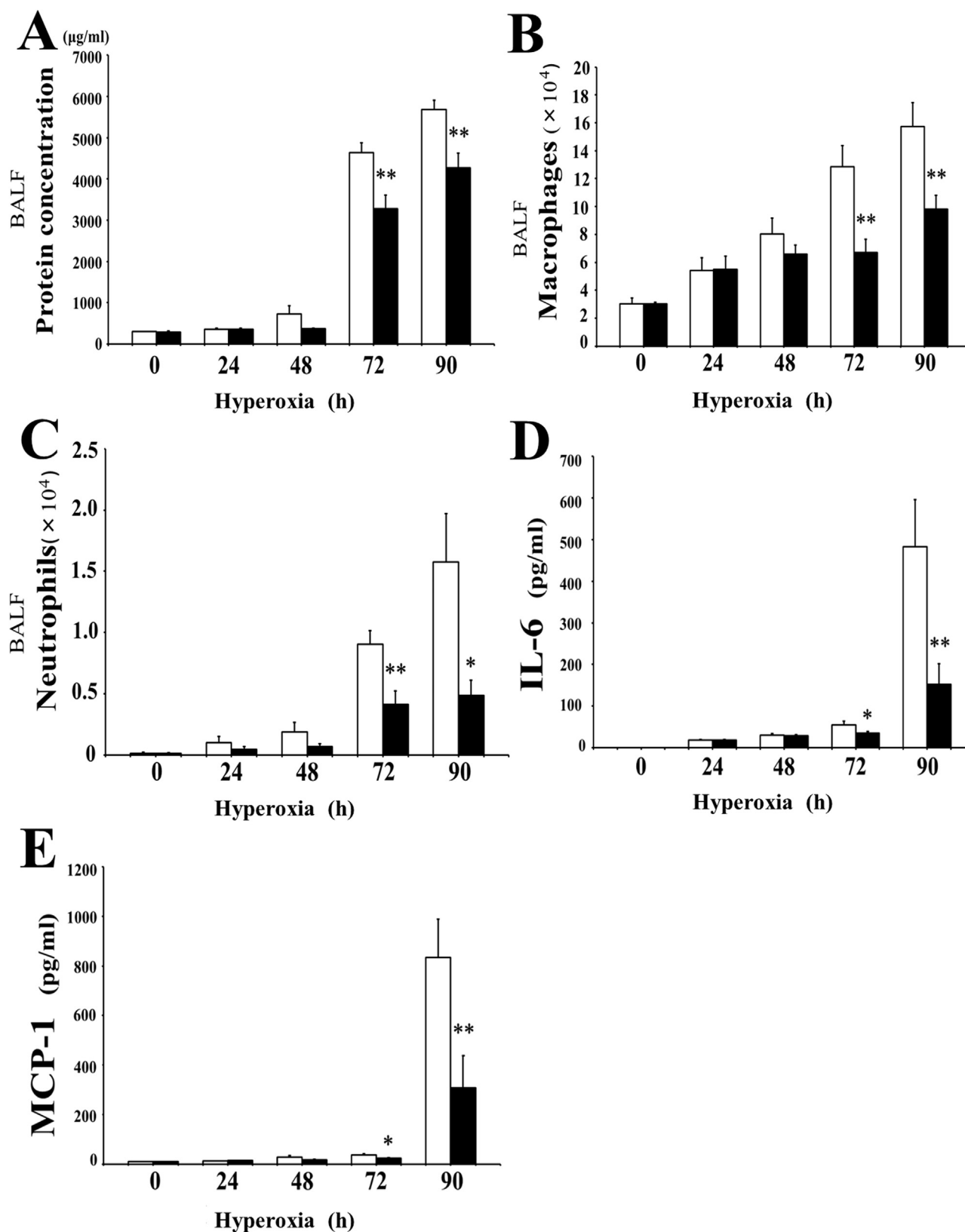


Fig. 4. Total protein concentration (A), numbers of macrophages (B) and neutrophils (C), and concentrations of IL-6 (D) and MCP-1 (E) in bronchoalveolar lavage (BAL) fluid from mice given water or suptalast tosilate during hyperoxic exposure. BAL was performed for mice given water (open bars) or suptalast tosilate (solid bars) at baseline and after 24, 48, 72, and 90 h of hyperoxic exposure. The data are shown as the means  $\pm$  SEM (n=7 mice/group). \*p < 0.05 versus mice given water, \*\*p < 0.01 versus mice given water.

hyperoxic exposure. As shown in Fig. 5, the numbers of neutrophils in the peripheral blood of mice given water decreased after 48 h of hyperoxic exposure compared with those at baseline and 24 h of

hyperoxic exposure and then drastically increased at 72 and 90 h of hyperoxic exposure; in contrast, those of mice given suptalast tosilate did not change during hyperoxic exposure. This decrease in peripheral

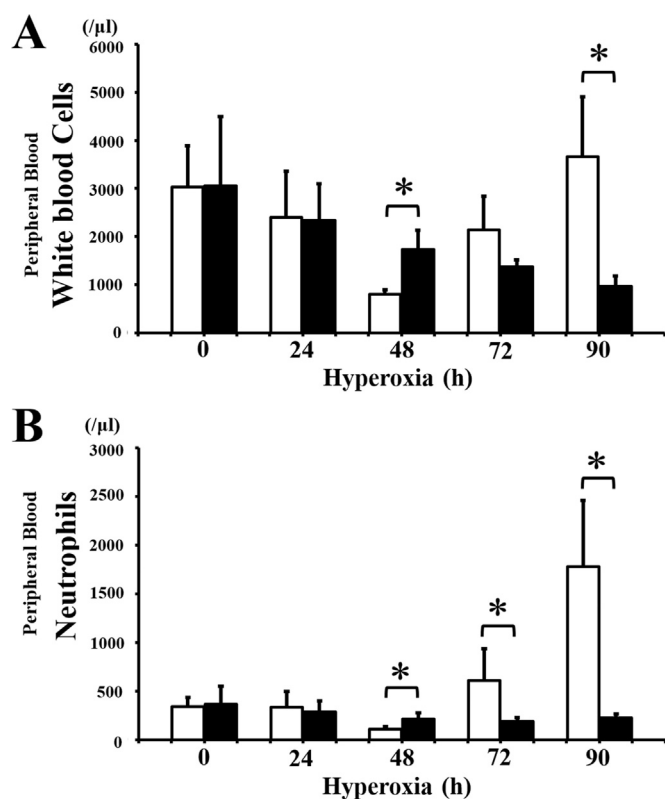


Fig. 5. Numbers of total white cell (A) and neutrophils (B) in peripheral blood from mice given water or suplatast tosilate during hyperoxic exposure. Blood was drawn from mice given water (open bars) or suplatast tosilate (solid bars) at baseline and after 24, 48, 72, and 90 h of hyperoxic exposure. Data are shown as the means  $\pm$  SEM ( $n=7$  mice/group). \* $p < 0.05$  versus mice given water.

blood neutrophils must be caused by pulmonary sequestration of neutrophils occurring at the initiation of harsh inflammation in the lung.

### 3.5. Effect of suplatast tosilate on hyperoxia-induced oxidative stress in the lung

To test the hypothesis that suplatast tosilate exerts an antioxidant effect on hyperoxia-induced oxidative stress in the lung, we measured 8-OHdG, a marker of oxidative DNA damage, in BAL fluid and 8-isoprostane, a marker of lipid peroxidation, in lung tissue after 24, 48, and 90 h of hyperoxia. As shown in Fig. 6A and B, the levels of both 8-OHdG in BAL fluid and 8-isoprostane in lung tissue significantly increased with time, and suplatast tosilate treatment significantly reduced both of these levels at each time point. To evaluate the amount of activated neutrophils infiltrated into the lung, we also measured MPO levels in lung tissues after 24, 48, and 90 h of hyperoxic exposure. As shown in Fig. 6C, the levels of MPO in lung tissues were not different between mice given water and those given suplatast tosilate after 24 and 48 h of hyperoxic exposure. Notably, suplatast tosilate showed an antioxidant effect after 24 and 48 h of hyperoxic exposure when the inflammation levels, particularly neutrophil infiltration, in the lung were not different between mice given water and those given suplatast tosilate. Although we were not able to extract DNA from BAL fluids and thus quantify its amount, we confirmed that there were no significant differences among the amounts of DNA extracted from any of the lung tissue samples (data not shown).

To determine whether the Nrf-2 pathway is involved in the antioxidant effect of suplatast tosilate on hyperoxic lung injury, the expression levels of Nrf-2 in the lungs were compared between mice given water and those given suplatast tosilate after 72 h of hyperoxic

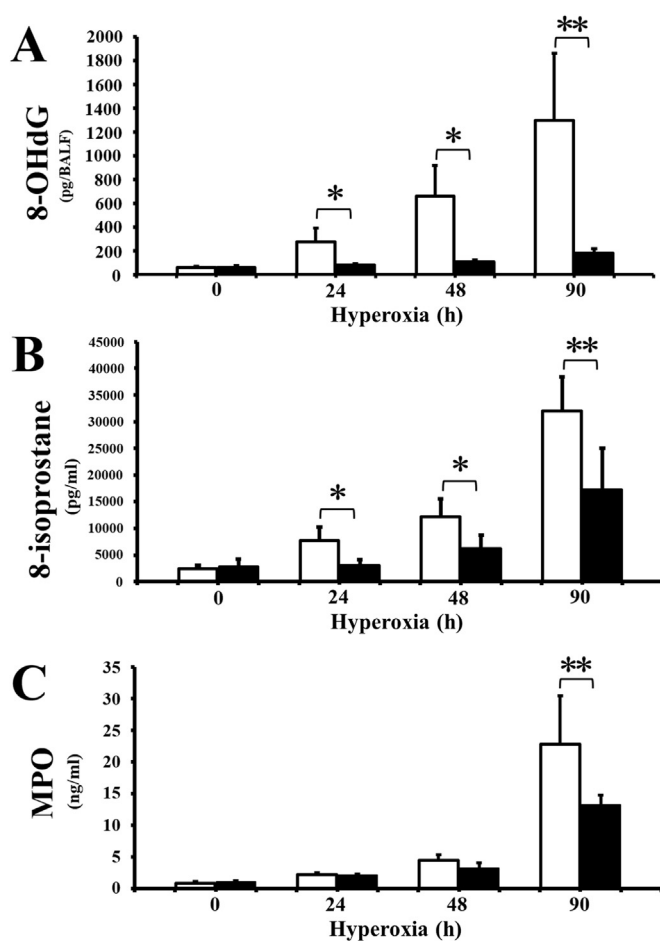


Fig. 6. Oxidative stress assessed by measuring 8-OHdG in BAL fluid (A), 8-isoprostane (B), and MPO (C) in lung tissue. BAL was performed and lung tissues were obtained from mice given water (open bars) or suplatast tosilate (solid bars) at baseline and after 24, 48, and 90 h of hyperoxic exposure. Data are shown as the means  $\pm$  SEM ( $n=7$  mice/group). \* $p < 0.05$ , \*\* $p < 0.01$ .

exposure. As shown in Fig. 7, no difference was found between them, suggesting that the protective effect of suplatast tosilate on hyperoxic lung injury is not strongly mediated by the Nrf-2 pathway.

### 3.6. Hydroxyl radical-scavenging activity of suplatast tosilate evaluated by EPR spin-trapping analysis

To test the hypothesis that suplatast tosilate exerts a scavenging activity on ROS, the capacity of suplatast tosilate to scavenge hydroxyl radical, a representative ROS, was examined using the EPR spin-trapping method with DMPO as a reference trapping agent. Fig. 8A(a) shows the EPR spectrum of the DMPO-hydroxyl radical spin adduct without suplatast tosilate in the reaction mixture. When suplatast tosilate was added to the reaction, the EPR signal intensity decreased in proportion to the increase in the suplatast tosilate concentration [Fig. 8A(b)–(d)]. From the slope of the linear plot (Fig. 8B and C), the half-maximal inhibitory concentration was calculated as 3.4  $\mu\text{mol/ml}$  and the reaction rate constant of suplatast tosilate with hydroxyl radical was calculated as  $2.6 \times 10^{11} \text{ M}^{-1} \text{ S}^{-1}$ , which corresponded to the diffusion limit.

## 4. Discussion

In the present study, we showed that, in a mouse model of hyperoxic lung injury, treatment with suplatast tosilate ameliorated lung inflammation, improved the survival rate, and prolonged survival.

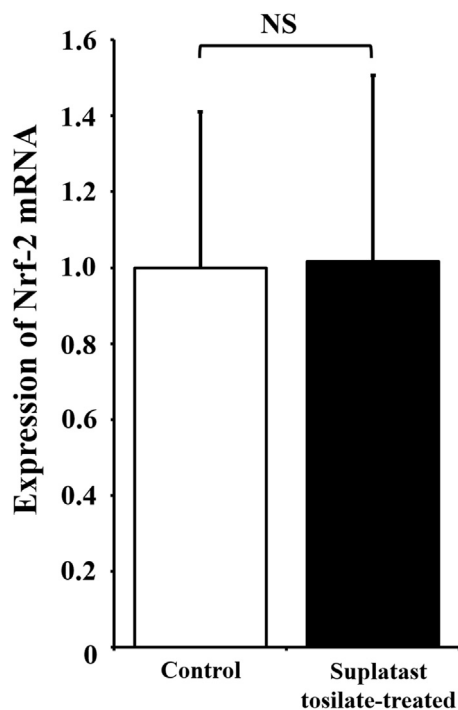
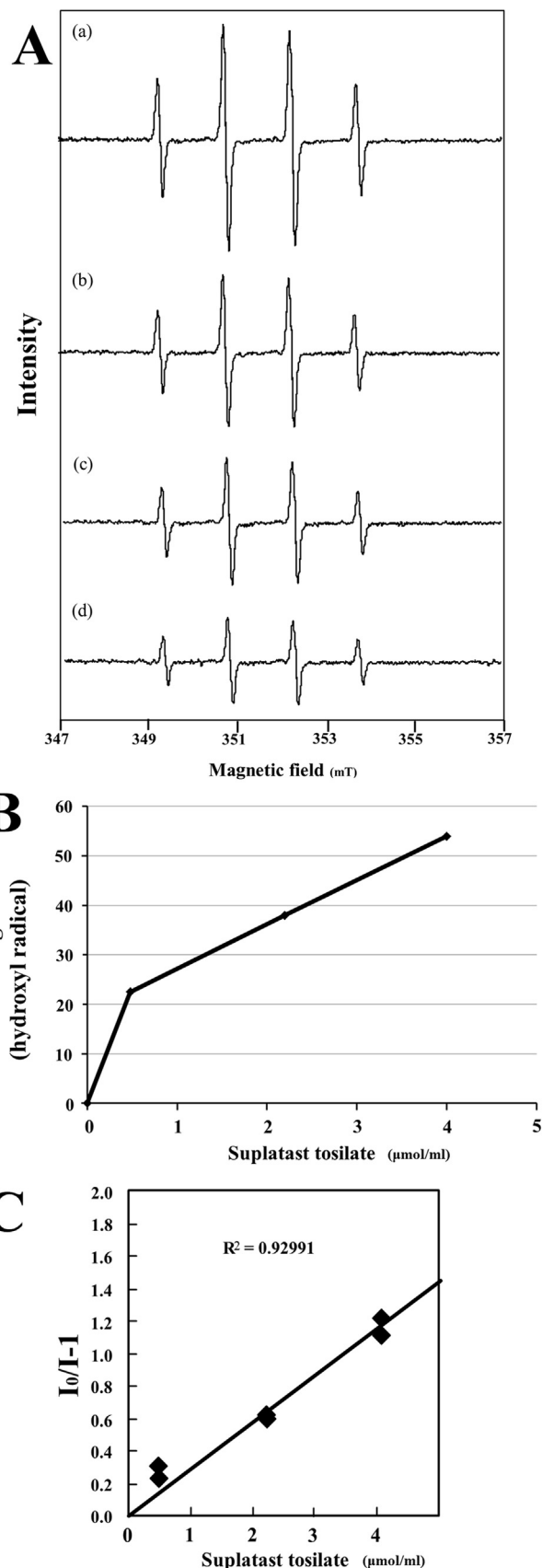


Fig. 7. Assessment of Nrf-2 mRNA expression levels in mouse lungs after 72 h of hyperoxic exposure. Expression level of Nrf-2 in lungs relative to the internal reference 18S rRNA was assessed in mice given water (open bar) or suplatast tosilate (solid bar) after 72 h of hyperoxic exposure. Data are shown as the means  $\pm$  SEM (n=5 mice/group). NS: not significant.

Administration of suplatast tosilate also significantly decreased the amounts of 8-OHdG in BAL fluid and 8-isoprostane in lung tissue. In addition, EPR spin-trapping analysis revealed that suplatast tosilate can scavenge hydroxyl radicals. These results suggest that suplatast tosilate protects against hyperoxic lung injury by decreasing the degree of oxidative stress induced by ROS, particularly through its ability to scavenge hydroxyl radicals.

The most interesting result of this study is that treatment of mice exposed to hyperoxia with suplatast tosilate significantly reduced the amounts of both 8-OHdG in BAL fluid and 8-isoprostane in lung tissue compared with untreated mice exposed to hyperoxia. 8-OHdG is a hydroxyl radical-damaged guanine nucleotide of nuclear and mitochondrial DNA that serves as a sensitive biomarker of *in vivo* oxidative DNA damage [21]. Because alveolar epithelial cells represent a primary target of hyperoxic lung injury [22], most 8-OHdG in BAL fluid should be derived from oxidatively damaged alveolar epithelial cells. The ability of suplatast tosilate to reduce the 8-OHdG level in BAL fluid suggests that suplatast tosilate protected alveolar epithelial cells against oxidative stress induced by hyperoxia. In turn, 8-isoprostane is a specific product of lipid peroxidation formed in cell membranes by ROS peroxidation of arachidonic acid that serves as a sensitive biomarker of oxidative stress in tissue [23]. The ability of suplatast tosilate to reduce the 8-isoprostane level in lung tissue suggests that suplatast tosilate protected the lung against a broad range of oxidative stresses induced by hyperoxia. These results indicate that orally administered suplatast tosilate exerts a strong antioxidant activity *in vivo*. Because the calculated dose of suplatast tosilate used in the present study was higher than the dose for human use, further experiments with a lower dose of suplatast tosilate should be conducted.

Another notable finding of this study, obtained using EPR spin-trapping analysis, is that suplatast tosilate was able to scavenge hydroxyl radicals *in vitro*. The reaction rate constant of suplatast tosilate with hydroxyl radicals was calculated as  $2.6 \times 10^{11} \text{ M}^{-1} \text{ S}^{-1}$ , which is faster than that of several well-established antioxidants, such as



**Fig. 8.** (A) Effect of suplatost tosilate on hydroxyl radicals generated by the Fenton reaction. Electron paramagnetic resonance (EPR) spectra of DMPO-OH spin adducts in the absence of suplatost tosilate (a) and in the presence of 0.48  $\mu\text{mol/ml}$  suplatost tosilate (b), 2.2  $\mu\text{mol/ml}$  suplatost tosilate (c), and 4.0  $\mu\text{mol/ml}$  suplatost tosilate (d). (B) Scavenging activity of suplatost tosilate on hydroxyl radicals. Suplatost tosilate eliminated hydroxyl radicals in a dose-dependent manner with a half-maximal inhibitory concentration of 3.4  $\mu\text{mol/ml}$ . (C) Reciprocal plot of  $I_0/I-1$  value versus suplatost tosilate concentration.  $I_0$  and  $I$  are the EPR peak area in the absence and presence of various concentrations of suplatost tosilate.

ascorbate, glutathione, and cysteine [24–26]. Because the hydroxyl radical is the most reactive and harmful ROS and is considered responsible for the development of hyperoxic lung injury [7,27,28], we believe that this scavenging effect of suplatost tosilate on hydroxyl radicals was also exerted *in vivo* and thus protected the lung from hyperoxic injury. In addition, we can suggest that another mechanism is involved in the *in vivo* antioxidant activity of suplatost tosilate. Suplatost tosilate absorbed by the digestive system is readily metabolized into dimethyl sulfide (DMS) in various tissues [29–31]. Because a previous study showed that DMS can exert a strong antioxidant activity [32,33], DMS metabolized from suplatost tosilate *in vivo* might play an unignorable role in protecting the lung against the oxidative stress induced by hyperoxia. In addition, we are expecting that another metabolite of suplatost tosilate, N-(4-(3-ethoxy-2-hydroxypropoxy)phenyl)acrylamide, would also have strong antioxidant activities. However, further study is necessary to elucidate this point.

In the present study, the protective role of suplatost tosilate against hyperoxic lung injury was also shown by its ability to inhibit the increases in the number of neutrophils and the levels of the pro-inflammatory cytokines/chemokines IL-6 and MCP-1 in BAL fluid from hyperoxia-exposed mice. Hyperoxic lung injury is initiated by ROS-induced damage of alveolar epithelial cells [7,8,34,35] and is followed by the prominent infiltration of inflammatory cells into lung tissue [36]. These inflammatory cells, particularly neutrophils and macrophages, generate ROS and cause a vicious cycle of oxidatively induced lung injury. Therefore, the inhibitory effect of suplatost tosilate on both the numbers of neutrophils and levels of IL-6 and MCP-1 in BAL fluid suggests that suplatost tosilate breaks this vicious cycle in hyperoxic lung injury.

In conclusion, suplatost tosilate protected the lung against oxidative stress induced by hyperoxic exposure and prolonged the survival of mice exposed to hyperoxia. This protective effect appeared to be mediated by the ability of suplatost tosilate to scavenge hydroxyl radicals; however, we also speculate that DMS metabolized from suplatost tosilate *in vivo* might be involved in this protection. Because suplatost tosilate has been widely used as an asthma control drug in Japan, the results of this study suggest that suplatost tosilate could also be potentially used for the treatment of high oxidative stress-related diseases of the lung such as hyperoxic lung injury and pulmonary fibrosis.

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## Conflicts of interest

The authors disclose no potential conflicts of interest.

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