Therapeutic effect of lecithinized superoxide dismutase on pulmonary emphysema

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Abbreviations: BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; DAPI, 4,6-diamino-2-phenylindole; DPhPMPO, 2-diphenylphosphinoyl-2-methyl-3, 4-dihydro-2H-pyrrole N-oxide; DTPA, diethylenetriamine-N, N, N’, N”, N”-pentaacetic acid; ESR, electron spin resonance; FEV1, forced expiratory volume in first second; FVC, forced vital capacity; H & E, hematoxylin and eosin; IL, interleukin; IPF, idiopathic pulmonary fibrosis; KC, keratinocyte-derived chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMPs, matrix metalloproteinases; NAC, N-acetylcysteine; 8-OHdG, 8-hydroxy-2’-deoxyguanosine; PC, phosphatidylcholine; PC-SOD, lecithinized human Cu/Zn-superoxide dismutase; PPE, porcine pancreatic elastase; QOL, quality of life; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.
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ABSTRACT

No medication exists that clearly improves mortality of chronic obstructive pulmonary disease (COPD). Oxidative molecules, in particular superoxide anions play important roles in the COPD-associated abnormal inflammatory response and pulmonary emphysema, which arises due to an imbalance in proteases and antiproteases and increased apoptosis. Superoxide dismutase (SOD) catalyses the dismutation of a superoxide anions. Lecithinized SOD (PC-SOD) has overcome a number of the clinical limitations of SOD, including low tissue affinity and low stability in plasma. In this study, we examine the effect of PC-SOD on elastase-induced pulmonary emphysema, an animal model of COPD. The severity of the pulmonary inflammatory response and emphysema in mice was assessed by various criteria, such as the number of leucocytes in the bronchoalveolar lavage fluid and the enlargement of airspace. Not only intravenous administration but also inhalation of PC-SOD suppressed elastase-induced pulmonary inflammation, emphysema and dysfunction. Inhalation of PC-SOD suppressed the elastase-induced increase in the pulmonary level of superoxide anions and apoptosis. Inhalation of PC-SOD also suppressed elastase-induced activation of proteases and decreased in the level of antiproteases and expression of pro-inflammatory cytokines and chemokines. We also found that inhalation of PC-SOD suppressed cigarette smoke-induced pulmonary inflammation. The results suggest that PC-SOD protects against pulmonary emphysema by decreasing the pulmonary level of superoxide anions, resulting in inhibition of inflammation and apoptosis and amelioration of the protease/antiprotease imbalance. We propose that inhalation of PC-SOD would be therapeutically beneficial for COPD.
Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death in the world and its prevalence and mortality rates have been increasing (Rabe et al., 2007). COPD is a disease state defined by irreversible and progressive airflow limitation associated with an abnormal inflammatory response. The most important etiologic factor for COPD is cigarette smoking (CS) (Peto et al., 1999; Rabe et al., 2007). Pathologic characteristics of COPD include infiltration of leucocytes, enhanced mucus secretion, dysfunctional airway matrix remodelling and destruction of parenchyma (enlargement of airspace) (Barnes and Stockley, 2005; Owen, 2005; Rabe et al., 2007). Protease/antiprotease imbalance and apoptosis play important roles in this emphysematous lung destruction. Unfortunately, there is no effective drug therapy that is able to significantly and clearly modulate disease progression and mortality (Calverley et al., 2007; Miravitlles and Anzueto, 2009).

It was suggested that oxidative molecules play an important role in the pathogenesis of COPD (Pinamonti et al., 1998; Nadeem et al., 2005; Mak, 2008). In addition to stimulation of the inflammatory response by induction of production of pro-inflammatory cytokines and chemokines, oxidative molecules induce pulmonary cell apoptosis, activate proteases and inactivate antiproteases (Valentin et al., 2005; Rahman and Adcock, 2006; Greenlee et al., 2007).

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified to oxygen and water (Kinnula and Crapo, 2003). Of human SODs, Cu/Zn-SOD accounts for 80% of all SOD activities within the lung (Kinnula and Crapo, 2003). Altered levels of expression and activity of SOD were observed in both COPD patients and animals treated with elastase or CS.
(animal models for COPD) (Kondo et al., 1994; Daga et al., 2003; Valenca et al., 2008) and transgenic mice expressing Cu/Zn-SOD were resistant to elastase- or CS-induced pulmonary emphysema (Foronjy et al., 2006). Furthermore, transgenic mice expressing another type of SOD, extracellular SOD or knockout mice for this protein were resistant or sensitive, respectively, to elastase- or CS-induced pulmonary emphysema through attenuating oxidative fragmentation of ECM (Yao et al., 2010). These results suggest that administration of SOD could be of therapeutic benefit in the treatment of COPD. However, due to its low affinity for tissues and low stability in plasma, there is no report showing that administration of SOD is effective for the treatment of COPD patients or elastase- or CS-induced pulmonary emphysema in animals.

Mizushima and his co-workers developed PC-SOD, a lecithinized human Cu/Zn-SOD in which four phosphatidylcholine (PC) derivative molecules are covalently bound to each SOD dimer (Igarashi et al., 1992). This modification drastically improves the plasma stability and cellular affinity of SOD (Igarashi et al., 1992; Igarashi et al., 1994; Ishihara et al., 2009). As described in discussion, clinical studies showed that intravenously administered PC-SOD is effective for ulcerative colitis and idiopathic pulmonary fibrosis (IPF) (Broeyer et al., 2008; Suzuki et al., 2008a; Suzuki et al., 2008b) Furthermore, we recently reported that inhalation of PC-SOD is effective against bleomycin-induced pulmonary fibrosis in mice (an animal model for IPF) (Tanaka et al., 2010). We believe that inhalation may be a viable option for administration of PC-SOD, which would improve the quality of life (QOL) of patients treated with this drug. In this study, we have found that inhalation of PC-SOD suppresses elastase-induced pulmonary inflammation, emphysema and dysfunction, through suppression of cell death, activation of proteases, induction of expression of pro-inflammatory cytokines and chemokines and
decrease in the level of α1-antitrypsin (an antiprotease). We propose that inhalation of PC-SOD would be therapeutically beneficial for COPD.
MATERIALS AND METHODS

Chemicals and animals. Paraformaldehyde and porcine pancreatic elastase (PPE) were obtained from Sigma (St. Louis, MO). Novo-Heparin (5000 units) for injection was from Mochida Pharmaceutical Co. (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex Corporation (Kobe, Japan). Terminal deoxynucleotidyl transferase was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 488 goat anti-mouse immunoglobulin G and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). The RNeasy kit was obtained from Qiagen (Valencia, CA), the PrimeScript® 1st strand cDNA Synthesis Kit was from TAKARA Bio (Ohtsu, Japan), and the iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Cytospin® 4 was purchased from Thermo Electron Corporation (Massachusetts, USA), while Mayer’s hematoxylin, 1% eosin alcohol solution and mounting medium for histological examination (malinol) were from MUTO Pure Chemicals (Tokyo, Japan). Unmodified SOD (5190 U/mg) and PC-SOD (3000 U/mg) were from our laboratory stocks (Igarashi et al., 1992). α1-antitrypsin ELISA kit was from Immunology Consultants Laboratory (Newberg, OR). ELISA kits for interleukin (IL)-1β and IL-6 were from Pierce Chemical (Rockford, IL). ELISA kits for tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, and keratinocyte-derived chemokine (KC) were from R&D Systems (Minneapolis, MN). 4,6-diamino-2-phenylindole (DAPI), diethylenetriamine-N, N', N'', N'''-pentaacetic acid (DTPA) and 2-diphenylphosphinoyl-2-methyl-3, 4-dihydro-2H-pyrrole N-oxide (DPhPMPO) were from Dojindo (Kumamoto, Japan). An
antibody against 8-OHdG was from Nikken SEIL (Shizuoka, Japan). Wild-type mice (6-8 weeks old, ICR, male) were used. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Kumamoto University.

**Treatment of mice with PPE, CS and PC-SOD.** Mice maintained under anaesthesia with chloral hydrate (500 mg/kg) were given one intratracheal injection of PPE (50 or 100 µg/mouse) in PBS (30 µl/mouse) by use of a micropipette (p200) to induce pulmonary emphysema. Commercial (non-filtered) cigarettes (Peace®, Japan Tobacco Inc., Tokyo, Japan) that yielded 28 mg tar and 2.3 mg nicotine on a standard smoking regimen were used. For exposure of mice to CS, 15-20 mice were placed in a chamber (volume, 45 L). Mice were exposed to the smoke of 2 cigarettes for 25 min, 3 times/day for 3 days. In the chronic model, mice were exposed to the smoke of 1 cigarette for 35 min, 3 times/day, 5 days/week, for 4 weeks. Each cigarette was puffed 15 times for 5 min.

For intravenous administration of PC-SOD, PC-SOD was dissolved in 5% xylitol and administered via the tail vein. For control mice, 5% xylitol solution was administered. The first administration of PC-SOD was performed just before PPE administration.

For administration of PC-SOD by inhalation, 5-7 mice were placed in a chamber (volume, 45 L). PC-SOD was dissolved in 10 ml of 5% xylitol and an ultrasonic nebulizer (NE-U17 from Omron, Tokyo, Japan) that was connected to the chamber was used to nebulize the entire volume of the PC-SOD solution in 30 min. For control mice, 5% xylitol solution was subjected to nebulization. Mice were kept in the chamber for a
Further 10 min after the 30 min of nebulization. The first inhalation of PC-SOD was performed just before PPE administration.

The amount of α1-antitrypsin in the plasma and pro-inflammatory mediators in BALF was measured by ELISA according to the manufacturer’s protocol.

**Preparation of BALF and cell count.** BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 units/ml heparin (2 times). About 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents after centrifugation with Cytospin® 4 and the ratios of alveolar macrophages, lymphocytes and neutrophils to total cells were determined.

**Measurement of production of superoxide anions.** The production of superoxide anions was assayed by electron spin resonance (ESR) spin trapping with DPhPMPO, as previously described (Karakawa et al., 2008). Cells collected from BALF were incubated with 0.9% NaCl containing 500 μM DTPA and 10 mM DPhPMPO for 10 min at 37°C. ESR spectra were recorded at room temperature on a JES-TE200 ESR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 min; field modulation width, 0.25 mT; receiver gain, 400; and time count, 0.3 s. Every buffer and solution used in the reaction mixture used for ESR measurement was treated with Chelex 100 resin (Bio-Rad, Hercules, CA) before use to remove metals.
Histological and immunohistochemical analyses and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. Lung tissue samples were fixed for 24 h at a pressure of 25 cmH₂O, and then embedded in paraffin before being cut into 4 µm-thick sections.

For histological examination, sections were stained first with Mayer’s hematoxylin and then with 1% eosin alcohol solution (hematoxylin and eosin (H & E) staining). Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope. Twenty lines (500 µm) were drawn randomly on the image of sections stained with H & E and the intersection points with the alveolar walls were counted to determine the mean linear intercept. The morphometric analysis at the light microscopic level was conducted by a blinded investigator.

For immunohistochemical analysis, sections were treated with 20 µg/ml protease K for antigen activation. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with an antibody against 8-OHdG (1:100 dilution) in the presence of 2.5% BSA and then incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G in the presence of DAPI (5 µg/ml). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

For the TUNEL assay, sections were incubated first with proteinase K (20 µg/ml) for 15 min at 37°C, then with TdTase and biotin 14-ATP for 1 h at 37°C and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 µg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).
Gelatin zymography. The proteolytic activities of MMP-2 and MMP-9 were assessed by SDS-PAGE using zymogram gels containing 0.1% gelatin, as described previously (Namba et al., 2009). The protein concentration was determined by the Bradford method (Bradford, 1976). Following electrophoresis at 4°C (10 µg protein/lane), the gels were washed with 2.5% Triton X-100 for 30 min at room temperature and incubated with zymogram development buffer for 2 days at 37°C. Bands were visualized by staining with Coomassie Brilliant Blue.

Real-time RT-PCR analysis. Real-time RT-PCR was performed as previously described (Namba et al., 2009) with some modifications. Total RNA was extracted from pulmonary tissues using an RNeasy kit according to the manufacturer’s protocol. Samples (2.5 µg RNA) were reverse-transcribed using PrimeScript® 1st strand cDNA Synthesis Kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument; Bio-Rad, Hercules, CA) experiments using iQ SYBR GREEN Supermix, and analyzed with Opticon Monitor Software (Bio-Rad, Hercules, CA). Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, gapdh cDNA was used as an internal standard.

Primers were designed using the Primer3 website. The primers used were (name: forward primer, reverse primer): mf-α 5’-cgctcagccgatttgctatct-3’, 5’-cgagctcgaagttgacttaa-3’; il-1β 5’-gttcccaagcaatatcccaaa-3’, 5’-gtgagtgccagaagttgact-3’; il-6 5’-ctggagtcagagttgact-3’, 5’-cgtcagccgatttgctatct-3’; mip-2α 5’-gttcagaggtgact-3’, 5’-gtgagtgccagaagttgact-3’; mcp-1 5’-cgtcagccgatttgctatct-3’, 5’-gtgagtgccagaagttgact-3’.
Analysis of lung function. Analysis of lung function was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, Canada), as described previously (Kuraki et al., 2002). Mice were anesthetized with chloral hydrate (500 mg/kg), tracheotomised with an 8 mm section of metallic tubing, and mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2-3 cmH₂O. The single-compartment model (snap shot) and the constant-phase model (forced oscillation technique) were applied to analyse lung function. Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively. All data were analysed using FlexiVent software (version 5.3) (SCIREQ, Montreal, Canada).

Statistical analysis. All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or the Student's t-test for unpaired results was used to evaluate differences between three or more groups or between two groups, respectively. Differences were considered to be significant for values of $P<0.05$. We repeated the experiments at least two times as independent experiments (see figure legends) and selected one representative data to show in figures. The stated number of test sample is not summation of independent plural experiments but is for only one independent experiment.
Results

Effect of PC-SOD on elastase-induced pulmonary emphysema. Pulmonary emphysema was induced in mice given a single (at day 0) intratracheal administration of PPE. The PPE-induced pulmonary inflammatory response can be monitored by determining the number of leucocytes (alveolar macrophages, lymphocytes and neutrophils) in the BALF, 3 days after the administration of PPE (50 µg/mouse). As shown in Fig. 1A, the total number of leucocytes, and individual numbers of alveolar macrophages, lymphocytes and neutrophils were all increased by the PPE treatment. This effect was suppressed by the simultaneous once daily intravenous administration of PC-SOD, suggesting that PC-SOD ameliorates the PPE-induced inflammatory response. However, a higher dose of PC-SOD (30 kU/kg) did not suppress the PPE-induced inflammatory response (Fig. 1A), so in this study PC-SOD exhibited a bell-shaped dose-response profile, similar to that observed previously for intravenous administration of PC-SOD in animal models of other diseases (Ishihara et al., 2009; Tanaka et al., 2010). Intravenous administration of the higher dose (30 kU/kg) of PC-SOD alone (without PPE administration) did not affect the number of leucocytes in the BALF (data not shown).

PPE-induced pulmonary emphysema can be monitored by histopathological analysis and measurement of the mean linear intercept (an indicator of airspace enlargement due to breakdown of the alveolar walls), 3 days after the administration of PPE. Histopathological analysis of pulmonary tissue using H & E staining revealed that PPE administration induced severe pulmonary damage (infiltration of leucocytes and breakdown of the alveolar walls) and these phenomena were suppressed by the intravenous administration of low doses (1.5 and 3 kU/kg), but not of a high dose (30 kU/kg), of PC-SOD (Fig. 1B). The mean linear intercept was increased by the
administration of PPE; this increase was suppressed by intravenous administration of low doses (1.5 and 3 kU/kg) of PC-SOD but was not significantly suppressed at the higher dose (30 kU/kg) (Fig. 1C). Pulmonary tissue damage and the increase in the mean linear intercept 14 days after PPE administration were also suppressed by the intravenous administration of PC-SOD (Fig. 1D and E). We used higher dose of PPE (100 µg/mice) to monitor pulmonary emphysema 14 days after the administration of PPE.

The alteration in lung mechanics associated with pulmonary emphysema is characterised by a decrease in elastance (Kuraki et al., 2002). We thus examined the effect of intravenous administration of PC-SOD on PPE-induced alterations to lung mechanics, using a computer-controlled small-animal ventilator. Total respiratory system elastance (elastance of total lung including bronchi, bronchiole and alveoli) and tissue elastance (elastance of alveoli) were reduced by PPE treatment, and intravenous administration of PC-SOD increased these indexes (Fig. 1F). These results suggest that not only PPE-induced pulmonary emphysema but also PPE-induced pulmonary dysfunction is ameliorated by intravenous administration of PC-SOD.

Effect of inhalation of PC-SOD on elastase-induced pulmonary emphysema.

We recently reported that inhalation of PC-SOD ameliorates bleomycin-induced pulmonary fibrosis (Tanaka et al., 2010). This route of administration does not show a bell-shaped dose-response profile (Tanaka et al., 2010) and may result in higher QOL for patients treated with PC-SOD. Thus, here we examined the effect of inhalation of PC-SOD on PPE-induced pulmonary emphysema. Mice were placed in a chamber connected to an ultrasonic nebulizer, thus exposing them to PC-SOD-containing vapor. We confirmed, by HPLC analysis and measurement of SOD activity, that this treatment
did not affect the structure and activity of the PC-SOD (data not shown). Inhalation of PC-SOD-containing vapor was repeated once daily for 3 or 14 days and the mice were examined for PPE-induced pulmonary disorders. As shown in Fig. 2A, inhaled PC-SOD ameliorated the PPE-induced inflammatory response. This ameliorative effect was observed with not only low doses (30 and 60 kU/chamber) but also a high dose (600 kU/chamber) of PC-SOD, suggesting that the dose-response profile for this administration route is not bell-shaped.

PPE-induced emphysematous lung damage and the increase in the mean linear intercept were also suppressed by inhalation of PC-SOD (Fig. 2B-E), suggesting that inhalation of PC-SOD ameliorates PPE-induced pulmonary emphysema. Again, a bell-shaped dose-response profile was not observed for the ameliorative effect of inhalation of PC-SOD against PPE-induced pulmonary emphysema (Fig. 2B and C). As shown in Table 1, inhalation of unmodified SOD (600 kU/chamber) did not affect the PPE-induced pulmonary inflammatory response and emphysema. This suggests that lecithinization of SOD potentiates its ameliorative effect against PPE-induced lung disorders, as is the case for dextran sulfate sodium-induced colitis and bleomycin-induced pulmonary fibrosis (Ishihara et al., 2009; Tanaka et al., 2010). We also found that inhalation of PC-SOD suppresses PPE-induced decreases in total respiratory system elastance and tissue elastance (Fig. 2F), suggesting that inhalation of PC-SOD ameliorates PPE-induced lung dysfunction. We confirmed that inhalation of PC-SOD alone did not induce pulmonary emphysema and dysfunction (supplemental Fig. 1).

To consider the clinical relevance, it is important to examine the effect of the drug on pre-developed lesions in an animal model (Fig. 3). Thus, we examined the effect of
inhalation of PC-SOD on pre-developed pulmonary emphysema. Once-daily inhalation of PC-SOD was started 3 days after the administration of PPE and pulmonary emphysema and function were assessed at day 10. Inhalation of PC-SOD caused suppression of pulmonary emphysema at day 10, suggesting that inhalation of PC-SOD is effective for pre-developed lesions.

The inhalation of PC-SOD also suppressed the PPE-induced alterations in lung mechanics at day 10 (Fig. 3C), suggesting that inhalation of PC-SOD suppresses the PPE-induced lung dysfunction, even when it is administered after the PPE.

Mechanism for the ameliorative effects of PC-SOD on PPE-induced pulmonary emphysema. In order to confirm that inhaled PC-SOD decreases the pulmonary level of superoxide anion, we performed an immunohistochemical analysis to monitor the pulmonary level of 8-OHdG, the damaged nucleotide produced by various ROS, including the superoxide anion (Freeman et al., 2009). As shown in Fig. 4A, the pulmonary level of 8-OHdG was significantly increased by PPE administration and this increase was clearly suppressed by inhalation of PC-SOD, suggesting that production of ROS in the lung was suppressed by inhalation of PC-SOD. We also used ESR analysis to monitor the production of superoxide anion in cells in BALF. The ESR spectrum was consistent with a previously reported DPhPMPO-OOH spectrum (a hyperfine coupling constant of $a^N = 1.24$ mT, $a^{H}_{\beta} = 1.16$ mT, $a^P = 3.95$ mT) (Karakawa et al., 2008). As shown in Fig. 4B and C, the peak of a radical spin adduct of the ESR spectrum corresponding to the amount of superoxide anion (DPhPMPO-OOH adduct) was higher for cells prepared from PPE-administered mice than for cells from control mice. Inhalation of PC-SOD
lowered this peak, suggesting that inhaled PC-SOD suppresses PPE-induced production of superoxide anions in the lung.

As described in the Introduction, pulmonary cell apoptosis plays an important role in the pathogenesis of COPD and PPE-induced pulmonary emphysema. We examined the effect of inhalation of PC-SOD on PPE-induced pulmonary cell death by employing the TUNEL assay. TUNEL-positive cells (indicative of cell death) increased in response to administration of PPE and this increase was suppressed by simultaneous inhalation of PC-SOD (Fig. 4D and E), suggesting that PC-SOD protects pulmonary cells from PPE-induced cell death, and that this effect is involved in the ameliorative effects of inhalation of PC-SOD against PPE-induced pulmonary emphysema.

In order to examine the effect of inhalation of PC-SOD on the PPE-dependent imbalance in proteases and antiproteases, we first examined the activity of MMPs, MMP-2 and MMP-9, using gelatin zymography. The band intensities of MMP-2 and MMP-9, indicative of MMP-2 and MMP-9 activities, were higher for lung tissues prepared from PPE-administered mice than for those from control mice, and this increase was suppressed in mice that had inhaled PC-SOD (Fig. 5A and B). We also examined the serum level of α1-antitrypsin by ELISA and found that the level of α1-antitrypsin was decreased by PPE administration and the level was partially recovered by simultaneous inhalation of PC-SOD (Fig. 5C). These results suggest that inhalation of PC-SOD improves the PPE-dependent protease/antiprotease imbalance and that this effect is involved in the ameliorative effects of inhalation of PC-SOD against PPE-induced pulmonary emphysema.

We also examined the effect of inhalation of PC-SOD on the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokines (MIP-2, MCP-1.
and KC) in lung tissues. The mRNA expression of all of these pro-inflammatory cytokines and chemokines was induced by PPE administration and in most cases this induction was suppressed by inhalation of PC-SOD. We also measured amounts of these pro-inflammatory cytokines and chemokines in BALF by ELISA and confirmed that the data of mRNA expression (supplemental Fig. 2). These results suggest that inhalation of PC-SOD suppresses PPE-induced expression of pro-inflammatory cytokines and chemokines in the lung and that this effect is involved in the ameliorative effects of PC-SOD inhalation on the PPE-induced pulmonary inflammatory response and resulting emphysema.

**Effect of PC-SOD on CS-induced inflammatory response.** PPE-induced pulmonary emphysema is a convenient and reproducible model of COPD and thus this model has been used frequently for evaluation of drugs for COPD. However, it is believed that the CS-induced pulmonary emphysema model is more relevant as an animal model of COPD, because it induces the disease using the same stimulus rather than just replicating one of the mechanisms of the disease. Thus, we examined the effect of PC-SOD on CS-induced pulmonary emphysema. Mice were assessed for a pulmonary inflammatory response at 3 days, following exposure to CS. We found that this treatment induced an inflammatory response, as was the case for treatment with elastase (Fig. 6A). As shown in Fig. 6A, intravenously administered PC-SOD ameliorated the CS-induced increase in the total number of leucocytes, and individual numbers of alveolar macrophages, lymphocytes and neutrophils in the BALF, suggesting that intravenous administration of PC-SOD ameliorates CS-induced pulmonary inflammation. As shown
in Fig. 6B, inhalation of PC-SOD also ameliorated the CS-induced inflammatory response.

We also examined the effect of PC-SOD on CS-induced pulmonary emphysema and dysfunction. Exposure of mice to CS for 4 weeks caused emphysematous lung damage and the increase in the mean linear intercept and this emphysema was suppressed by simultaneous inhalation of PC-SOD (supplemental Fig. 3A and B). We also found that exposure of mice to CS for 4 weeks caused decreases in total respiratory system elastance and tissue elastance and this decrease was suppressed by simultaneous inhalation of PC-SOD (supplemental Fig. 3C). These results suggest that inhalation of PC-SOD is effective for the treatment of CS-related pulmonary inflammation, emphysema and lung dysfunction, including COPD.
Discussion

In this study, we used PC-SOD, a derivative of SOD with higher stability in plasma and a higher affinity for tissue, which shows greater therapeutic effects than SOD in animal models of various inflammatory diseases, such as IPF, colitis, focal cerebral ischemic injury and spinal cord injury-induced motor dysfunction (Hori et al., 1997; Tamagawa et al., 2000; Ishihara et al., 2009; Tanaka et al., 2010). We have clearly shown that PC-SOD ameliorates pulmonary emphysema. This result indicates the therapeutic potential of SOD against COPD-related pulmonary emphysema and is consistent with previous results that show transgenic mice expressing SOD bear a phenotype of resistance to pulmonary emphysema (Foronjy et al., 2006; Petrache et al., 2008). In a phase I clinical study, intravenously administered PC-SOD (40-160 mg) had a terminal half-life of more than 24 h, with good safety and tolerability (Broeyer et al., 2008; Suzuki et al., 2008a). Recently published results of a phase II clinical study have shown that intravenously administered PC-SOD (40 or 80 mg) significantly improves the symptoms of ulcerative colitis patients, which involves ROS (Suzuki et al., 2008b). A phase II clinical study has shown that intravenously administered PC-SOD (40 or 80 mg) is therapeutically effective against IPF as judged by monitoring the serum level of marker proteins (lactate dehydrogenase and surfactant protein-A). Since the safety and efficacy of PC-SOD were shown in not only the animal model but also in clinical studies, the application of PC-SOD for COPD is realistic.

Here we have shown that not only intravenous administration but also inhalation of PC-SOD ameliorates pulmonary emphysema. We believe that inhalation is a clinically more valuable route of administration than the intravenous route for the following two reasons. The first is that PC-SOD administered by inhalation does not have a bell-shaped
dose-response profile. Bell-shaped dose-response curves are of clinical concern as they may reflect the presence of side effects. The lack of a bell-shaped dose-response profile upon inhalation has also been observed for bleomycin-induced pulmonary fibrosis (Tanaka et al., 2010). Since the efficacy of intravenous administration of higher doses of PC-SOD on bleomycin-induced pulmonary fibrosis was restored by simultaneous administration of catalase, which converts hydrogen peroxide to water and oxygen, the ineffectiveness of high doses of PC-SOD is likely to be caused by accumulation of hydrogen peroxide (Tanaka et al., 2010). However, the reason why inhalation of PC-SOD does not show the bell-shaped dose-response profile remains unknown. The second reason is that patients treated with PC-SOD administered by inhalation would have a higher QOL than those treated intravenously. Although a phase II clinical study has shown that intravenously administered PC-SOD (40 or 80 mg) is effective for both ulcerative colitis (Suzuki et al., 2008b) and IPF, the main obstacle against proceeding into the next stage of clinical study is the poor QOL for patients undergoing the current clinical protocol of PC-SOD administration (daily intravenous infusion for 4 weeks). Furthermore, in a phase II clinical study for IPF, the plasma levels of markers (lactate dehydrogenase and surfactant protein-A) but not forced vital capacity (FVC) were modified by intravenous administration of PC-SOD, suggesting that a longer period of treatment with PC-SOD is required to improve FVC in IPF patients. However, daily intravenous infusion for a longer period is not practical. Therefore, we propose that inhalation of PC-SOD for a longer period may be effective not only for IPF but also for COPD and would maintain the QOL of patients. The therapeutic potential of inhalation of PC-SOD for the treatment of COPD is also supported by observations made in this study: inhalation of PC-SOD ameliorated not only PPE-induced pathological alterations
but also PPE-induced functional changes, and inhalation of PC-SOD was effective even for pre-developed pulmonary emphysema (stimulation of spontaneous restoration from pulmonary emphysema and suppression of progression of pulmonary dysfunction).

Drugs for COPD should suppress both the inflammatory response and emphysematous lung destruction. As ROS, especially superoxide anions, are suggested to induce both an inflammatory response and emphysematous lung destruction (Mak, 2008), PC-SOD was predicted to suppress both of these events. In fact, we showed that inhalation of PC-SOD suppresses a PPE-induced increase in leucocytes in BALF and the expression of pro-inflammatory cytokines and chemokines. We also showed that inhalation of PC-SOD suppresses PPE-induced emphysematous lung destruction. Both apoptosis and protease/antiprotease imbalance seem to be involved in emphysematous lung destruction associated with COPD (Demedts et al., 2006; Rabe et al., 2007; Petrache et al., 2008). We have shown that inhalation of PC-SOD suppresses PPE-induced pulmonary cell death and protease/antiprotease imbalance (activation of MMPs and decrease in the level of $\alpha_1$-antitrypsin). We recently reported that PC-SOD protects cultured lung epithelial cells from menadione (a superoxide anion-releasing drug)-induced cell death (Tanaka et al., 2010). It has also been reported that oxidative molecules activate MMPs and suppress the expression of $\alpha_1$-antitrypsin (Desrochers and Weiss, 1988; Greenlee et al., 2007; Mak, 2008; Wan et al., 2008). Thus, it seems that a PC-SOD-dependent decrease in the level of superoxide anions is responsible for the inhibitory effect of PC-SOD on PPE-induced pulmonary cell death and the protease/antiprotease imbalance.

One of the current standard clinical protocols for treatment of COPD patients is administration of a long-acting $\beta_2$-agonist or anticholinergic along with corticosteroid
inhalation. This combination regime reduces the annual rate of exacerbation and improves health status and spirometric values, although it does not improve the mortality rate with statistical significance (Calverley et al., 2007). β₂-agonists and anticholinergics are effective in improving the airflow limitation associated with COPD (Rabe et al., 2007). On the other hand, some reports have suggested that treatment with corticosteroids does not clearly modulate the inflammatory response in COPD patients or in a CS-induced pulmonary emphysema animal model (Rabe et al., 2007; Fox and Fitzgerald, 2009). Based on these previous observations and those in this study that inhalation of PC-SOD is effective against the CS-induced inflammatory response, we consider that a combination regime of administration of a long-acting β₂-agonist (or anticholinergics) along with inhalation of PC-SOD, instead of corticosteroids, may be therapeutically beneficial for COPD patients.
Authorship Contributions.

Participated in research design: Ken-ichiro Tanaka, Tohru Mizushima.

Conducted experiments: Ken-ichiro Tanaka, Yuta Tanaka, Yuri Miyazaki.

Contributed new reagents or analytic tools: Takushi Namba, Keizo Sato, Kazutetsu Aoshiba.

Performed data analysis: Ken-ichiro Tanaka, Keizo Sato.

Wrote or contributed to the writing of the manuscript: Ken-ichiro Tanaka, Kazutetsu Aoshiba, Arata Azuma, Tohru Mizushima.
References


Footnotes.

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Figure Legends

Figure 1. Effect of intravenous administration of PC-SOD on PPE-induced pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 or 100 µg/mouse) once at day 0 were intravenously administered the indicated doses of PC-SOD (1.5, 3 or 30 kU/kg) once daily for 3 days (from day 0 to day 2) (A-C) or 14 days (from day 0 to day 13) (D-F). The total cell number, and numbers of alveolar macrophages, lymphocytes and neutrophils were determined at day 3 as described in the Materials and Methods (A). Sections of pulmonary tissue were prepared at days 3 or 14 and subjected to histopathological examination (H & E staining) (B, D). Airspace size was estimated by determining the MLI as described in the Materials and Methods (C, E). At day 14, total respiratory system elastance and tissue elastance were determined as described in the Materials and Methods (F). Values are mean ± S.E.M. *P<0.05; **P<0.01. Data are representative of two independent experiments.

Figure 2. Effect of inhalation of PC-SOD on PPE-induced pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 or 100 µg/mouse) once at day 0 inhaled the indicated doses of PC-SOD (30, 60 or 600 kU/chamber) once daily for 3 days (from day 0 to day 2) (A-C) or 14 days (from day 0 to day 13) (D-F). Inflammatory response (A), airspace size (B-E) and lung mechanics (F) were assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *P<0.05; **P<0.01. Data are representative of three independent experiments.
Figure 3. Effect of PC-SOD on pre-developed pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 µg/mouse) once at day 0 inhaled the indicated doses of PC-SOD (kU/chamber) once daily from day 3 to day 9. Airspace size (A and B) and lung mechanics (C) were assessed at day 10 as described in the legend of Fig. 1. Values are mean ± S.E.M. *P<0.05; **P<0.01. Data are representative of two independent experiments.

Figure 4. Effect of PC-SOD on the PPE-induced increase in the level of 8-OHdG, production of superoxide anions and pulmonary cell death. Mice treated with PPE inhaled PC-SOD (kU/chamber) for 3 days (from day 0 to day 2) (A, D, E) or 1 day (day 0) (B and C) as described in the legend of Fig. 2. Sections of pulmonary tissue were prepared at day 3 (A, D, E). Sections were subjected to immunohistochemical analysis with an antibody against 8-OHdG or DAPI staining (A). Cells in BALF were collected at day 1, incubated with a spin trap agent (DPhPMPO) and subjected to radical adduct ESR spectrum analysis to determine the amount of superoxide anion present (B). The intensity of the ESR signal of the superoxide anion adduct (DPhPMPO–OOH adduct shown by the bar in (B)) was determined. Sections were subjected to TUNEL assay or DAPI staining (D). The number of TUNEL-positive cells was counted (E). Values are mean ± S.E.M. *P<0.05; **P<0.01. Data are representative of two independent experiments.

Figure 5. Effect of PC-SOD on PPE-induced MMP activation, decrease in the serum level of α1-antitrypsin and alteration in gene expression. Mice treated with PPE inhaled PC-SOD (kU/chamber) for 3 days (from day 0 to day 2) (A, B) or for 1 day (day 0) (C, D) as described in the legend of Fig. 2. Pulmonary tissues were
obtained at day 3 and MMP-2 and MMP-9 activities were measured as described in the Materials and Methods (A). The intensity of the bands corresponding to latent form of MMP-2 and MMP-9 was determined and expressed relative to the control samples (vehicle) (B). The serum level of α1-antitrypsin was determined by ELISA at day 1 (C). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the gapdh gene are expressed relative to the control samples (vehicle) (D). Values are mean ± S.E.M. *P<0.05; ** P<0.01. Data are representative of two independent experiments.

Figure 6. Effect of PC-SOD on CS-induced inflammatory response. Mice were exposed to CS for 3 days as described in the Materials and Methods. Mice were also intravenously administered PC-SOD (kU/kg) (A) or inhaled with PC-SOD (kU/chamber) (B) as described in the legends of Figs. 1 and 2. Inflammatory response was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *P<0.05; **P<0.01. Data are representative of two independent experiments.
Table 1.

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<table>
<thead>
<tr>
<th></th>
<th>PPE (50) (n = 8)</th>
<th>+ U-SOD (600 kU/chamber) (n = 4)</th>
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<tr>
<td>Total cells (x 10⁵ cells)</td>
<td>4.9 ± 0.33</td>
<td>4.9 ± 0.35</td>
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<tr>
<td>Alveolar macrophages (x 10⁵ cells)</td>
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<td>4.7 ± 0.33</td>
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<td>Lymphocytes (x 10⁴ cells)</td>
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<td>Neutrophils (x 10⁴ cells)</td>
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<td>1.3 ± 0.19</td>
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<tr>
<td>Mean linear intercept (µm)</td>
<td>58.2 ± 1.30</td>
<td>57.7 ± 0.37</td>
</tr>
</tbody>
</table>

Table Legend.

**Table 1.**  Effect of inhalation of unmodified SOD on PPE-induced pulmonary emphysema. Mice were treated with a single dose of PPE (50 µg/mouse) at day 0 and inhaled unmodified SOD (U-SOD, 600 kU/chamber) once daily for 3 days (from day 0 to day 2). Inflammatory response and the mean linear intercept were assessed as described in the legend of Fig. 1. Values are mean ± S.E.M.
Fig. 2

A

**Total cells**

- Vehicle (n = 8)
- PPE (50) (n = 13)
- + PC (30) (n = 4)
- + PC (60) (n = 6)
- + PC (600) (n = 4)

**Alveolar macrophages**

- Vehicle (n = 8)
- PPE (50) (n = 13)
- + PC (30) (n = 4)
- + PC (60) (n = 6)
- + PC (600) (n = 4)

**Lymphocytes**

- Vehicle (n = 8)
- PPE (50) (n = 13)
- + PC (30) (n = 4)
- + PC (60) (n = 6)
- + PC (600) (n = 4)

**Neutrophils**

- Vehicle (n = 8)
- PPE (50) (n = 13)
- + PC (30) (n = 4)
- + PC (60) (n = 6)
- + PC (600) (n = 4)

B

**vehicle**

- PPE (50)
- + PC (30)
- + PC (60)
- + PC (600)

C

**Mean linear intercept (µm)**

- Vehicle (n = 7)
- PPE (50) (n = 8)
- + PC (30) (n = 4)
- + PC (60) (n = 6)
- + PC (600) (n = 5)