Superiority of PC-SOD to other anti-COPD drugs for elastase-induced emphysema and alteration in lung mechanics and respiratory function in mice

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Tanaka K, Sato K-I, Aoshiba K, Azuma A, Mizushima T. Superiority of PC-SOD to other anti-COPD drugs for elastase-induced emphysema and alteration in lung mechanics and respiratory function in mice. *Am J Physiol Lung Cell Mol Physiol* 302: L1250–L1261, 2012. First published April 13, 2012; doi:10.1152/ajplung.00019.2012.—Bronchodilators (such as ipratropium bromide), steroids (such as fluticasone propionate), and newly developed anti-inflammatory drugs (such as roflumilast) are used for patients with chronic obstructive pulmonary disease (COPD). We recently reported that lecithinized superoxide dismutase (PC-SOD) confers a protective effect in mouse models of COPD. We here examined the therapeutic effect of the combined administration of PC-SOD with ipratropium bromide on pulmonary emphysema and compared the effect of PC-SOD to other types of drugs. The severity of emphysema in mice was assessed by various criteria. Lung mechanics (elastance) and respiratory function (ratio of forced expiratory volume in the first 0.05 s to forced vital capacity) were assessed. Administration of PC-SOD by inhalation suppressed elastase-induced pulmonary emphysema, alteration of lung mechanics, and respiratory dysfunction. The concomitant intratracheal administration of ipratropium bromide did not alter the ameliorating effects of PC-SOD. Administration of ipratropium bromide, fluticasone propionate, or roflumilast alone did not suppress the elastase-induced increase in the pulmonary level of superoxide anion, pulmonary inflammatory response, pulmonary emphysema, alteration of lung mechanics, or respiratory dysfunction as effectively as did PC-SOD. PC-SOD, but not the other drugs, showed a therapeutic effect even when the drug was administered after the development of emphysema. PC-SOD also suppressed the cigarette smoke-induced pulmonary inflammatory response and increase in airway resistance. Based on these results, we consider that the inhalation of PC-SOD would be therapeutically beneficial for COPD.

bronchodilator; chronic obstructive pulmonary disease; lecithinized superoxide dismutase

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is a serious global health problem (32). COPD is a disease state defined by irreversible and progressive airflow limitation associated with an abnormal inflammatory responses (32). The most important etiologic factor for COPD is cigarette smoking (CS), and its diagnosis is confirmed by a decrease in the ratio of forced expiratory volume in the first second/forced vital capacity (FEV_1%) (32).

As pulmonary inflammation is believed to play an important role in the progression of COPD (32), anti-inflammatory drugs are necessary for the treatment. Furthermore, to increase the quality of life of patients with COPD, it is essential to improve the symptoms of COPD related to airflow limitations (such as dyspnea). Therefore, bronchodilators (β_2-agonists and anticholinergics) and steroids are presently used for the treatment of COPD (4, 32). However, as there is no effective drug therapy that is able to significantly and clearly modulate both disease progression and mortality (1, 8, 26), new types of medicines, in particular anti-inflammatory drugs that could replace the use of steroids, are required. Roflumilast, an inhibitor of phosphodiesterase-4, is one of a newly developed anti-inflammatory medicines for COPD (9, 13). However, roflumilast did not reduce mortality in patients with COPD (9, 13).

Recent studies suggest that oxidative radicals (such as reactive oxygen species) play an important role in the pathogenesis of COPD (33). Increases in the levels of oxidative radicals have been reported in lung tissues and bronchoalveolar lavage fluid (BALF) from not only patients with COPD and smokers but also from COPD animal models (2, 12, 28, 30). Thus antioxidant molecules have attracted considerable attention as therapeutic candidates for the treatment of COPD.

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently degraded to oxygen and water by catalase or glutathione peroxidase (21). Altered levels of expression and activity of SODs in either lung or red blood cells were observed both in patients with COPD and in animals exposed to CS (10, 15, 22, 42), whereas transgenic mice expressing SOD were reported to be resistant to elastase- or CS-induced pulmonary emphysema (12, 44). However, the low affinity of SOD for tissues and low stability in plasma, with a half-life of a few minutes, are obstacles for its clinical use.

PC-SOD is a lecithinized human Cu/Zn-SOD in which four phosphatidylcholine (PC) derivative molecules are covalently bound to each SOD dimer (19). This modification drastically improves the cellular affinity and plasma stability of SOD without decreasing its enzyme activity (18, 19). We recently reported that administration of PC-SOD by inhalation suppresses elastase- and CS-induced pulmonary inflammatory responses, pulmonary emphysema, and alteration of lung mechanics (39), suggesting that PC-SOD could become new type of anti-inflammatory drug for COPD; in other words, combination application of PC-SOD with a bronchodilator would be therapeutically beneficial for COPD. To propose a clinical protocol for the inhalation of PC-SOD to treat COPD, we examined here the combination application of PC-SOD with ipratropium bromide (an anticholinergic bronchodilator) to treat elastase-induced pulmonary emphysema. We also compared the protective and therapeutic effects of PC-SOD to other

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types of drugs against elastase-induced pulmonary inflammatory responses, emphysema, alteration of lung mechanics, and respiratory dysfunction or CS-induced inflammatory response.

MATERIALS AND METHODS

Chemicals and animals. Porcine pancreatic elastase (PPE), ipratropium bromide, fluticasone propionate, and acetyl-β-methylcholine bromide (methacholine) were obtained from Sigma (St. Louis, MO). Novo-Heparin (5,000 U) for injection was from Mochida Pharmaceutical (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex (Kobe, Japan). Roflumilast was obtained from Sequoia Research Products (Pangbourne, UK). Formalin neutral buffer solution was from WAKO Pure Chemicals (Tokyo, Japan). Cytospin 4 was purchased from Thermo Electron (Boston, MA), whereas Mayer's hematoxylin, 1% eosin alcohol solution, and malinol were from MUTO Pure Chemicals (Tokyo, Japan). PC-SOD (3,000 U/mg) was from our laboratory stocks (19). Diethylenetriamine-*N, 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). ICR mice (4–6 wk old, male) were purchased from Charles River (Yokohama, Japan). 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 and Keio University.

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 U/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 pulmonary level of superoxide anions. The level of superoxide anions was determined by electron spin resonance (ESR) spin trapping with DPhPMPO, as previously described (39). 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 analyses. Lung tissue samples were fixed in 10% formalin neutral buffer solution 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). Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

To determine the mean linear intercept (MLI), 20 lines (500 μm) were drawn randomly on the image of section stained with H & E, and the intersection points with the alveolar walls were counted to determine the MLI. The morphometric analysis at the light microscopic level was conducted by an investigator blinded to the study protocol.

Treatment of mice with PPE, CS, and PC-SOD. Mice maintained under anesthesia with chloral hydrate (500 mg/kg) were given one intratracheal injection of PPE (100 μg/mouse) in PBS (30 μl/mouse) via micropipette (P200) to induce pulmonary emphysema.

Commercial nonfiltered cigarettes (Peace; Japan Tobacco, 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) that was connected to an apparatus producing CS. Mice were exposed to the smoke of two cigarettes for 25 min, three times per day for 3 days.

For administration of PC-SOD by inhalation (60 kU/chamber), 5–8 mice were placed in a chamber (volume, 45 l). PC-SOD (60 kU) was dissolved in 10 ml of 5% xylitol. An ultrasonic nebulizer (NE-U07; Omron, Tokyo, Japan) that was connected to the chamber was used to nebulize the entire volume of the PC-SOD solution (10 ml) over a 30-min period. For control mice, 5% xylitol solution was nebulized over a 30-min period. Mice were kept in the chamber for a further 10 min after completion of the period of nebulization.

The first administration of drugs [PC-SOD (inhalation), ipratropium bromide (intratracheal administration), fluticasone propionate (intratracheal administration), and roflumilast (oral administration)] was performed just before PPE administration or 2 h before the CS treatment to examine the protective effect of each drug. To examine the therapeutic effect (Fig. 7), the first administration of drugs was performed 14 days after the PPE administration.

Measurement of lung mechanics, airway resistance, and $FEV_{0.05}$%. Measurement of lung mechanics and airway resistance was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada), as described previously (34, 39). Mice were anesthetized with chloral hydrate (500 mg/kg), a tracheostomy was performed, and an 8-mm section of metallic tube was inserted into the trachea. Mice were 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.

Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively. All data were analyzed using FlexiVent software (version 5.3) (SCIREQ).

Mice were exposed to nebulized methacholine (1 mg/ml) five times for 20 s, and airway resistance was measured after each methacholine challenge by the snap shot technique. All data were analyzed using FlexiVent software (version 5.3).

Determination of $FEV_{0.05}$% was performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ) as described previously (34). Mice were tracheotomized and ventilated as described above. The lungs were inflated to 30 cmH₂O over 1 s and held at this pressure. After 0.2 s, the pinch valve (connected to ventilator) was closed, and, after 0.3 s, the shutter valve (connected to negative pressure reservoir) was opened for exposure of the lung to the negative pressure. The negative pressure was held for 1.5 s to ensure complete expiration. $FEV_{0.05}$% was determined using FlexiVent software (version 5.3).

Statistical analysis. All values are expressed as the means ± SE. One-way or 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 $P < 0.05$.

RESULTS

Effect of combination application of PC-SOD with ipratropium bromide on PPE-induced pulmonary emphysema and airway resistance. We considered that the combination application of PC-SOD with a bronchodilator could be useful for the treatment of patients with COPD. On this basis, it is important to examine the effect of a bronchodilator on the pharmacological activity of PC-SOD and vice versa. To begin, we examined the effect of ipratropium bromide on the protective effect of PC-SOD against PPE-induced pulmonary emphysema and alteration of lung mechanics. The extent of PPE-induced pulmonary emphysema was monitored by histopathological analysis and measurement of MLI (an indicator of airspace enlarge-

ment) 14 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 that these phenomena could be suppressed by the daily (from *day 0* to *day 13*) administration of PC-SOD by inhalation (Fig. 1*A*). Furthermore, we found that the simultaneous daily intratracheal administration of ipratropium bromide did not affect this protective effect of PC-SOD in either a positive or a negative manner (Fig. 1*A*). Ipratropium bromide was administered at a dose of 26.7 µg/kg, which is 10 times higher than the clinically used dose. Similar results to those shown in Fig. 1*A* were observed in the presence of 2.67 µg/kg ipratropium bromide (data not shown). The increased MLI by the administration of PPE could be suppressed by treatment of animals with PC-SOD (Fig. 1*B*), a result that was not affected by the

concomitant administration of ipratropium bromide (Fig. 1*B*). We also confirmed that ipratropium bromide did not affect the enzymatic activity of PC-SOD in vitro (data not shown). We previously used ELISA to determine the pulmonary level of PC-SOD after its inhalation. The amount of PC-SOD in the lung tissue was 22.7 ± 2.97 mU/mg tissue after the inhalation of PC-SOD (60 kU/chamber) (38).

The alteration of lung mechanics associated with pulmonary emphysema is characterized by a decrease in elastance, which can be monitored by using a computer-controlled small-animal ventilator (23). Total respiratory system elastance (elastance of total lung, including the bronchi, bronchioles, and alveoli) and tissue elastance (elastance of alveoli) were reduced by the PPE treatment (Fig. 1*B*). Treatment with inhaled PC-SOD partially restored these indexes, again in a manner that was not affected by the concomitant administration of ipratropium bromide
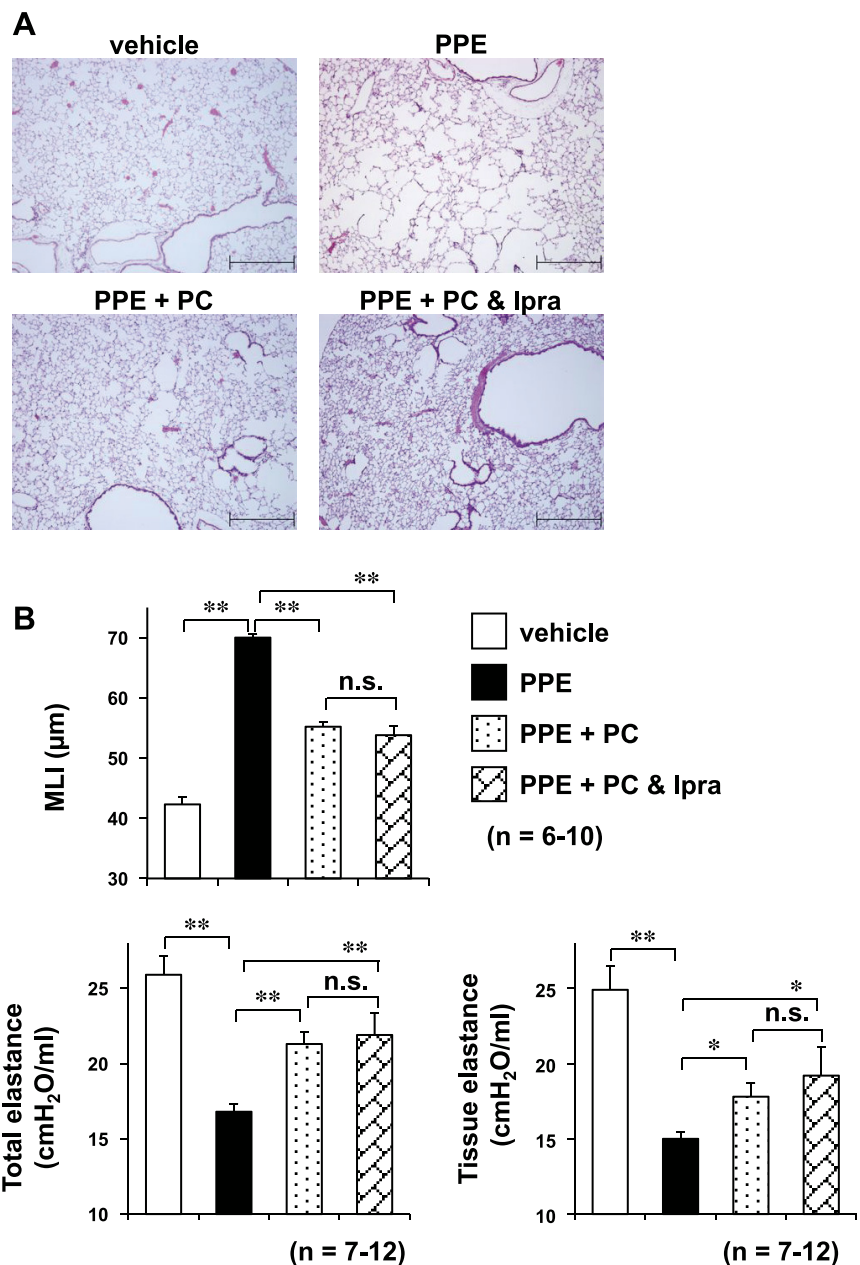

Fig. 1. Effect of ipratropium bromide on the protective effects of lecithinized superoxide dismutase (PC-SOD) against porcine pancreatic elastase (PPE)-induced pulmonary emphysema and alteration of lung mechanics. Mice were treated with or without (vehicle only) PPE (100 µg/mouse) once only on *day 0*. Animals were subsequently treated with PC-SOD (PC; 60 kU/chamber) administered with a nebulizer and/or intratracheal administration of ipratropium bromide (Ipra; 26.7 µg/kg) once daily for 14 days (from *day 0* to *day 13*). Sections of pulmonary tissue were prepared on *day 14* and subjected to histopathological examination (hematoxylin and eosin, H & E staining) (scale bar = 500 µm) (*A*). Airspace size was estimated by determining the MLI as described in MATERIALS AND METHODS (*B*). Total respiratory system elastance (total elastance) and tissue elastance were determined on *day 14* as described in MATERIALS AND METHODS (*B*). Values are means ± SE. *$P < 0.05$; **$P < 0.01$; n.s., not significant.
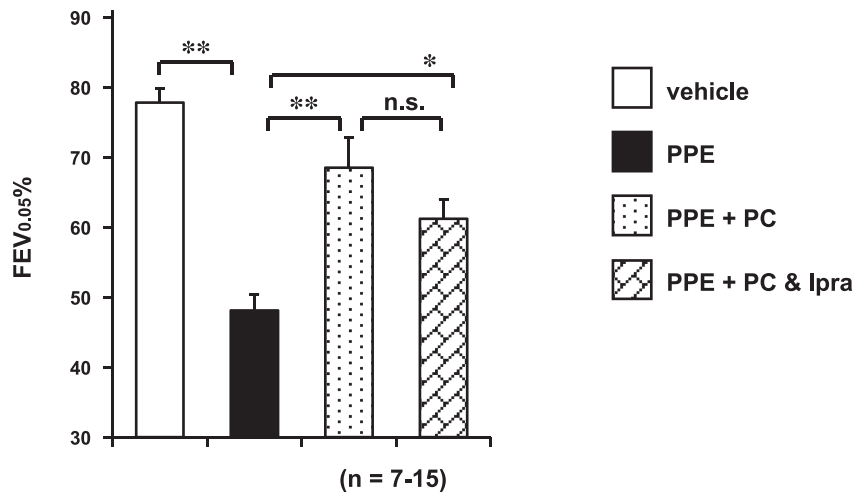
Fig. 2. Effect of ipratropium bromide on the protective effects of PC-SOD against the PPE-induced reduction of forced expiratory volume in the first 0.05 s/forced vital capacity ($FEV_{0.05}\%$). Mice were treated with or without (vehicle only) PPE (100 µg/mouse) once only on *day 0*. Mice were then treated with PC-SOD (PC; 60 kU/chamber) by inhalation and/or intratracheal administration of ipratropium bromide (Ipra; 26.7 µg/kg) for 11 days (from *day 0* to *day 10*). The $FEV_{0.05}\%$ was determined on *day 14* as described in MATERIALS AND METHODS. Values are mean ± SE. *$P < 0.05$; **$P < 0.01$; n.s., not significant.

(Fig. 1B). Administration of ipratropium bromide alone did not affect PPE-induced pulmonary emphysema and alteration of lung mechanics (see Fig. 4, *A* and *B*). The protective effect of inhaled PC-SOD against the PPE-induced alterations seen in Fig. 1 is consistent with that reported previously (39).

As the diagnosis of COPD is confirmed by a decrease in $FEV_1\%$, it is important to evaluate the manner in which drugs proposed for the treatment of COPD affect respiratory function related to $FEV_1\%$ in animal models. Given the recent report of a protocol to measure $FEV_{0.1}\%$ in the mouse (34), we applied basically the same technique to monitor PPE-induced respiratory dysfunction. To begin, we periodically monitored FEV in PPE-administered and control mice and found that the $FEV_{0.05}\%$ clearly decreased in PPE-treated mice (data not shown). As shown in Fig. 2, this decrease was significantly suppressed in mice treated with PC-SOD. The concomitant administration of ipratropium bromide slightly decreased the $FEV_{0.05}\%$ compared with PC-SOD treatment alone, but the difference was not statistically significant. Note that, to avoid a temporary increase in $FEV_{0.05}\%$ due to the bronchodilator effects of ipratropium bromide, the administration of the latter was discontinued on *day 10*, and the assay was performed on *day 14*.

We subsequently examined the effect of PC-SOD on the bronchodilator activity of ipratropium bromide. As shown in Fig. 3, the dose-dependent increase in airway resistance (bronchoconstriction) induced by inhaled methacholine was completely suppressed by preadministration of ipratropium bromide, confirming its bronchodilator activity. On the other hand, inhaled PC-SOD did not affect the airway resistance in either the presence or absence of ipratropium bromide (Fig. 3), suggesting that PC-SOD neither has bronchodilator activity nor affects the bronchodilator activity of ipratropium bromide.

Comparison of protective and therapeutic effects of various drugs against PPE-induced pulmonary emphysema. We then examined the effect of different types of drugs used clinically in the treatment of COPD (fluticasone propionate and roflumilast, as well as ipratropium bromide) on PPE-induced pulmonary emphysema, alteration of lung mechanics, and respiratory dysfunction. Dosages that were considered standard (16.7 µg/kg fluticasone propionate, 1 mg/kg roflumilast, and 2.67 µg/kg ipratropium bromide) and elevated (167 µg/kg fluticasone propionate, 5 mg/kg roflumilast, and 26.7 µg/kg ipratro-

pium bromide) were used (see discussion). As shown in Fig. 4, *A* and *B*, neither the intratracheal administration of fluticasone propionate nor ipratropium bromide suppressed the PPE-induced pulmonary damage or the increase in MLI. Amelioration of the PPE-induced pulmonary damage and emphysema was observed with the oral administration of roflumilast (Fig. 4, *A* and *B*); however, the extent of amelioration was less apparent than that seen with treatment with inhaled PC-SOD (Fig. 1, *A* and *B*). We also examined the effect of these drugs on PPE-induced alterations in lung mechanics. As shown in Fig. 4B, the restoration of total respiratory system elastance and tissue elastance was observed only with the higher dose of roflumilast, and the extent of restoration was lower than that seen with PC-SOD (Fig. 1B). Furthermore, none of these drugs affected PPE-induced respiratory dysfunction (decrease in $FEV_{0.05}\%$) (Fig. 4C). The results in Fig. 4 thus suggest that treatment with PC-SOD offers a greater protective effect than other types of
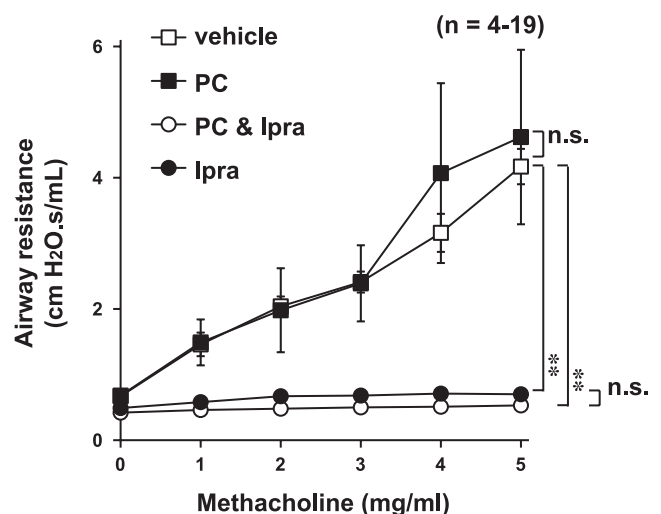

Fig. 3. Effect of PC-SOD on the ipratropium bromide-dependent decrease in airway resistance. Mice were treated with PC-SOD (PC; 60 kU/chamber) by inhalation and/or intratracheal administration of ipratropium bromide (Ipra; 26.7 µg/kg). After 1 h, mice were exposed to nebulized methacholine 5 times, and airway resistance was determined after each methacholine challenge as described in the MATERIALS AND METHODS. Values are means ± SE. **$P < 0.01$; n.s., not significant.
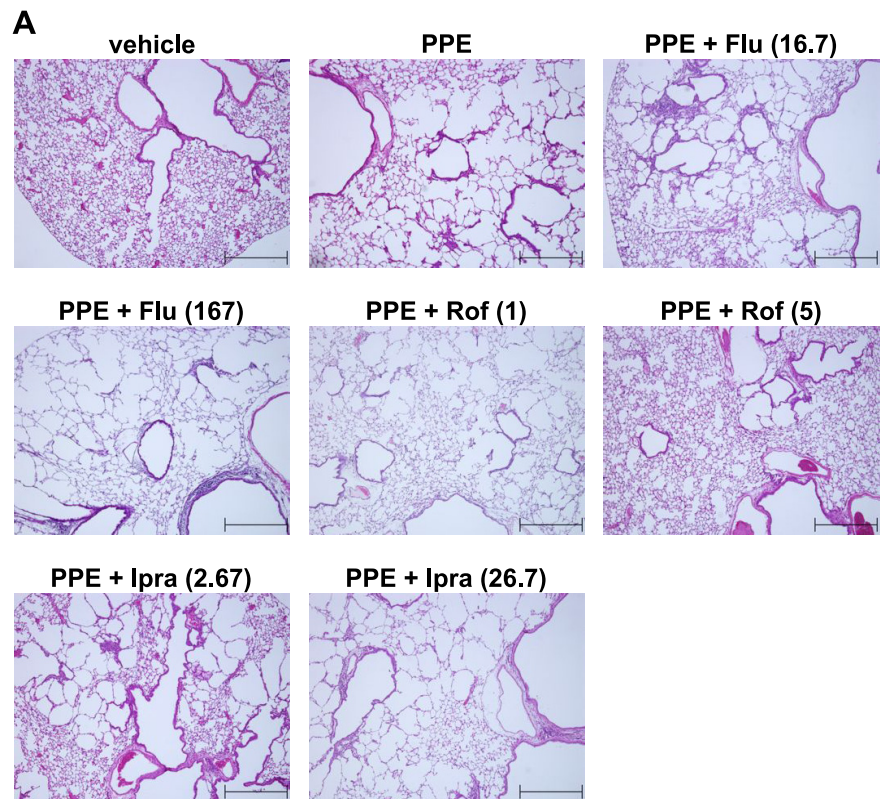
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Fig. 4. Effect of different drugs on PPE-induced pulmonary emphysema. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. Fluticasone propionate (Flu; μg/kg) and ipratropium bromide (Ipra; μg/kg) or roflumilast (Rof; mg/kg) were administered intratracheally or orally, respectively, once daily for 14 days (from *day 0* to *day 13*). Histopathological examination (scale bar = 500 μm) (*A*), determination of the mean linear intercept (MLI) (*B*), measurement of total respiratory system elastance (total elastance) and tissue elastance (*B*) and $FEV_{0.05}\%$ (*C*) were determined as described in MATERIALS AND METHODS and Fig legends 1 and 2. Values are means ± SE. *$P < 0.05$; **$P < 0.01$.

drugs against PPE-induced pulmonary damage and dysfunction.

To further examine the mechanism for this superior protective effect of PC-SOD, particularly in light of the important role that pulmonary inflammation plays in the pathogenesis of COPD, we monitored the PPE-induced pulmonary inflammatory response by determining the number of leucocytes (alveolar macrophages, lymphocytes and neutrophils) in BALF 3 days after the administration of PPE (100 μg/mouse). As shown in Fig. 5, the total number of leucocytes and individual numbers of alveolar macrophages, lymphocytes, and neutrophils were all increased by the PPE treatment. This effect was partially, though significantly, suppressed by the simultaneous treatment of animals with PC-SOD (Fig. 5), a result that is consistent with a previous report (39). We also found that PPE-dependent increase in pulmonary level of proinflammatory cytokines and chemokines (TNF-α, macrophage inflammatory protein-2, monocyte chemoattractant protein-1, and keratinocyte-derived chemokine) were suppressed by simultaneous treatment of animals with PC-SOD (data not shown). On the other hand, treatment of animals with the other drugs did not suppress the PPE-induced increase in total number of leucocytes or individual numbers of alveolar macrophages and lymphocytes (Fig. 5). The administration of roflumilast and ipratropium bromide did decrease the level of neutrophils in BALF in PPE-treated mice; however, the extent of decrease was not as evident as that seen with PC-SOD (Fig. 5).

We also used ESR analysis to monitor the level of superoxide anions in cells present in BALF. The ESR spectrum was consistent with a previously reported DPhPMPO-OOH spectrum (a hyperfine coupling constant of $a^N = 1.24$ mT, $a_\beta^H = 1.16$ mT, $a^P = 3.95$ mT) (39). As shown in Fig. 6, *A* and *B*, the

peak amplitude of the radical spin adduct of the ESR spectrum corresponding to the superoxide anion level (DPhPMPO-OOH adduct) was higher in cells prepared from PPE-administered mice than those from control mice. Inhaled PC-SOD but not treatment with the other drugs significantly decreased this peak, suggesting that inhaled PC-SOD specifically suppresses the PPE-induced production of superoxide anions in the lung. The results shown in Figs. 5 and 6 thus suggest that the superior activity of PC-SOD compared with other drugs against PPE-induced pulmonary damage and dysfunction is attributable to its inhibitory activity on inflammation through its unique antioxidant activity.

To consider the clinical relevance, it is important to examine the effect of drugs on predeveloped lesions in an animal model. We previously reported that inhaled PC-SOD could suppress PPE-induced pulmonary emphysema even when the treatment protocol was started 3 days after the administration of PPE (39). However, because PPE-induced pulmonary dysfunction becomes clear 7–21 days after the PPE treatment (14), the therapeutic effect of the drug should be examined at stage later than *day 3*. Therefore, in the present study, PC-SOD treatment was commenced 14 days after the administration of PPE, and pulmonary emphysema and lung mechanics were assessed on *day 21*. Treatment with PC-SOD, but not with the other drugs, decreased the extent of pulmonary damage, emphysema, and alterations in lung mechanics on *day 21* (Fig. 7, *A* and *B*), thus suggesting that PC-SOD could be effective for the treatment of predeveloped pulmonary emphysema.

Results in Fig. 7 showed that the MLI or elastance on *day 21* was higher or lower, respectively, than that on *day 14* in mice treated with PPE alone. On the other hand, the MLI or
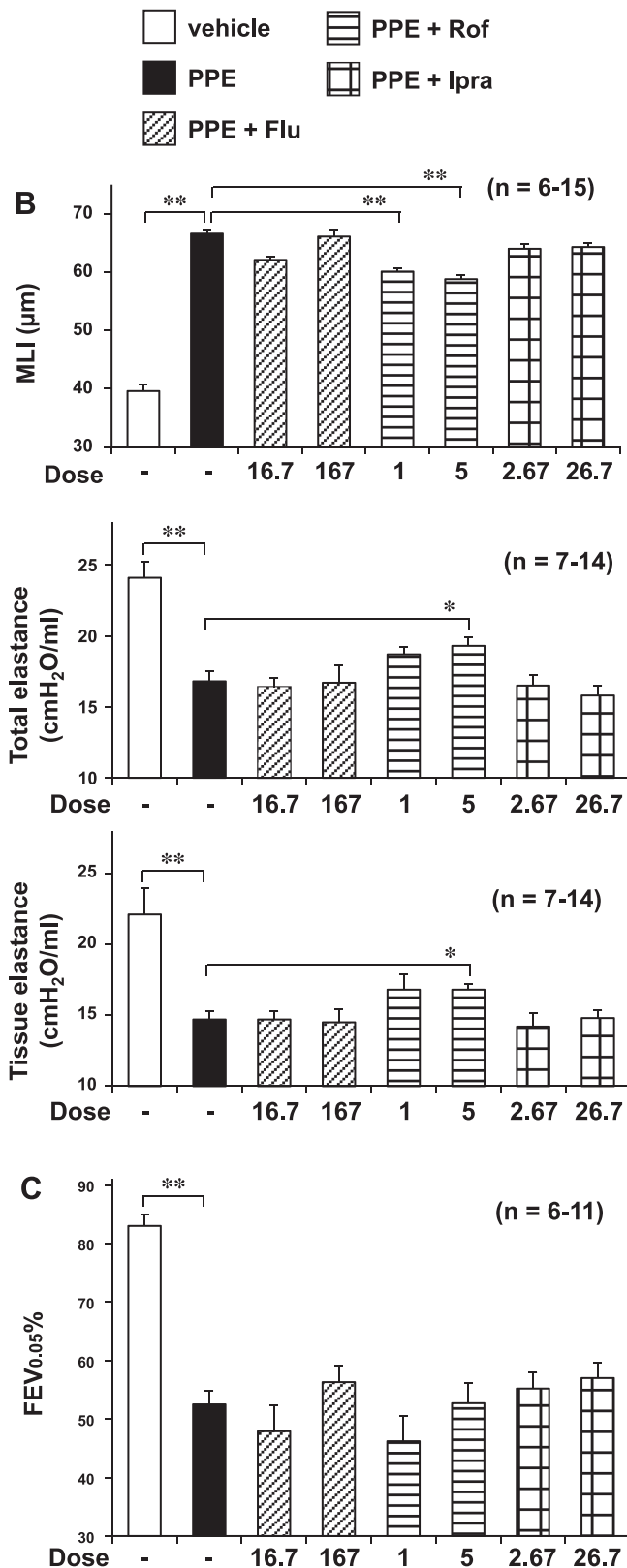
Fig. 4—*Continued*

elastance on *day 21* in mice treated with both PPE and PC-SOD was similar to that on *day 14* in mice treated with PPE alone (Fig. 7). These results suggest that the pulmonary emphysema and alteration of lung mechanics progress from *day 14* to *day 21* in this model. Thus we examined the effect of treatment with PC-SOD from *day 14* to *day 16* on inflammatory response on *day 17*. Total number of leucocytes and individual numbers of alveolar macrophages, lymphocytes, and neutrophils in BALF were lower in mice treated with PC-SOD than in nontreated mice although the differences for lymphocytes and neutrophils were not statistically significant (data not shown). This result suggests that PC-SOD ameliorates the pulmonary inflammatory response even if the drug was administered after development of emphysema and that this effect is involved in the suppression by this drug of progression of pulmonary emphysema and alteration of lung mechanics from *day 14* to *day 21* in Fig. 7.

Growth factors play important roles in pulmonary emphysema; preadministration of keratinocyte growth factor (KGF) suppressed elastase-induced pulmonary emphysema and administration of hepatocyte growth factor (HGF) after establishment of pulmonary emphysema stimulated the repair process (17, 31). Thus we examined the mRNA expression of these growth factors. *Kgf* mRNA expression was higher in mice treated with both PPE and PC-SOD than in those treated with PPE alone (data not shown). However, treatment with PC-SOD did not affect the *Hgf* mRNA expression (data not shown). The upregulation of *Kgf* mRNA expression in the presence of PC-SOD may be involved in the therapeutic effect of PC-SOD against PPE-induced pulmonary emphysema and alteration of lung mechanics in Fig. 7.

Effect of PC-SOD on the CS-induced inflammatory response and airway hyperresponsiveness. We recently reported that inhalation of PC-SOD suppressed the CS-induced pulmonary inflammatory response (39). Here, we extended that work to compare the effect of various drugs on the CS-induced pulmonary inflammatory response following periodic exposure to CS over a 3-day period (see MATERIALS AND METHODS). CS treatment induced an inflammatory response (increase in the total number of leucocytes in BALF) that could be suppressed by PC-SOD but not by the other drugs (Fig. 8*A*).

In a final experiment, we examined the effect of these drugs on CS-induced airway hyperresponsiveness to methacholine. As shown in Fig. 8*B*, treatment of mice with CS stimulated the methacholine-dependent increase in airway resistance (airway hyperresponsiveness to methacholine), as previously reported (5, 27), and this response could be suppressed by the concomitant treatment of animals with PC-SOD (Fig. 8*B*). A similar effect was observed with the higher dose of fluticasone propionate but not with any doses of roflumilast or ipratropium bromide (Fig. 8*B*). These results suggest that PC-SOD is protective against CS-induced inflammation and airway hyperresponsiveness.

DISCUSSION

Among the oxidative radicals generated in physiological processes, superoxide anions are believed to play a major role in numerous inflammatory diseases. This is because they are the primary molecules produced by the reduction of oxygen to water and can produce other potent oxidant molecules, such as
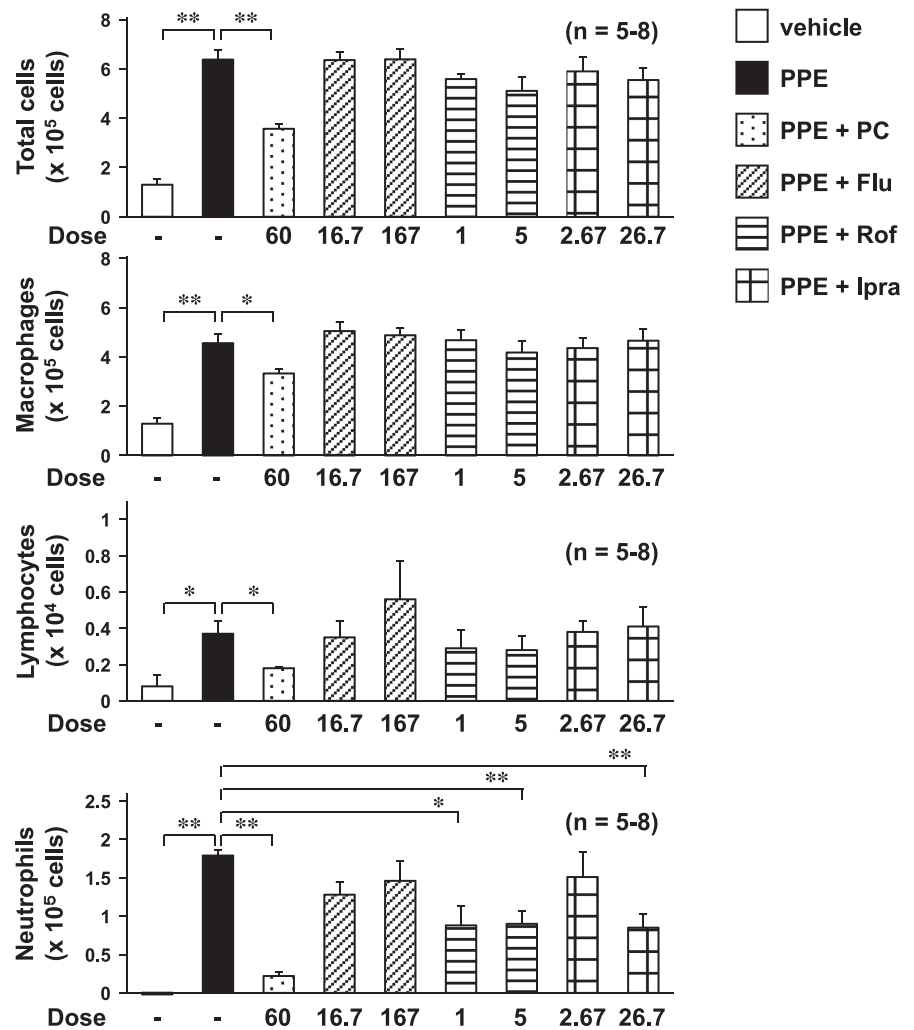
Fig. 5. Effect of different drugs on the PPE-induced inflammatory response. Mice were treated with or without (vehicle only) PPE (100 µg/mouse) once only on *day 0*. PC-SOD (PC; kU/chamber), fluticasone propionate (Flu; µg/kg), ipratropium bromide (Ipra; µg/kg), or roflumilast (Rof; mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once daily for 3 days (from *day 0* to *day 2*). The total cell number and numbers of alveolar macrophages, lymphocytes, and neutrophils were determined on *day 3* as described in MATERIALS AND METHODS. Values are means ± SE. *$P < 0.05$; **$P < 0.01$.

hydrogen peroxide, hydroxyl radicals, and peroxynitrite (21). Thus SODs, and more particularly Cu/Zn-SOD, have been paid much attention as potential drugs for the treatment of inflammatory diseases. However, the low stability of Cu/Zn-SOD in plasma and its low affinity for cells form an obstacle for its clinical development. PC-SOD, a derivative of SOD with higher stability in plasma and higher affinity for tissue, thus offers an attractive alternative to Cu/Zn-SOD, and its heightened therapeutic actions were demonstrated in animal models of various inflammatory diseases such as idiopathic pulmonary fibrosis (IPF), colitis, focal cerebral ischemic injury, and spinal cord injury-induced motor dysfunction (20, 37, 38, 40). 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 (7, 35). Moreover, intravenously administered PC-SOD significantly improves the symptoms of ulcerative colitis (36) and IPF (K. Kamio, A. Azuma, K. Ohta, Y. Sugiyama, T. Nukiwa, and S. Kudoh, unpublished results). However, when considering the quality of life of patients, the present clinical protocol of PC-SOD administration based on daily intravenous infusion for 4 wk needs to be improved. Given our recent reports that inhaled PC-SOD is effective against pulmonary fibrosis (38) and elastase- and CS-induced pulmonary emphysema (39) in mice, we believe that inhalation

may provide a viable option for administering PC-SOD to patients. In this study, we performed several lines of experiments that can be considered important for the future development of PC-SOD to be administered via inhalation to treat patients with COPD.

As pulmonary inflammation is believed to play an important role in the progression of COPD (32), anti-inflammatory drugs are necessary for the treatment of patients with this condition. However, characteristics of inflammation in patients with COPD are different from those in patients of other inflammatory diseases, such as asthma, and COPD is poorly responsive to standard anti-inflammatory drugs such as steroids (3, 16). On the other hand, to increase the quality of life of patients with COPD, it is essential to improve the symptoms of COPD related to airflow limitations (such as dyspnea), thus necessitating the concomitant use of a bronchodilator. Indeed, the standard regime for the treatment of COPD is the combination application of anti-inflammatory and bronchodilator drugs (13, 26). Because PC-SOD has no bronchodilator activity (Fig. 3), the combination application of PC-SOD with a bronchodilator might be necessary, and it is important to ensure that the bronchodilator drug does not reduce the clinical efficacy of the PC-SOD. We suggest here that the combination application of PC-SOD with the bronchodilator ipratropium bromide, a short-
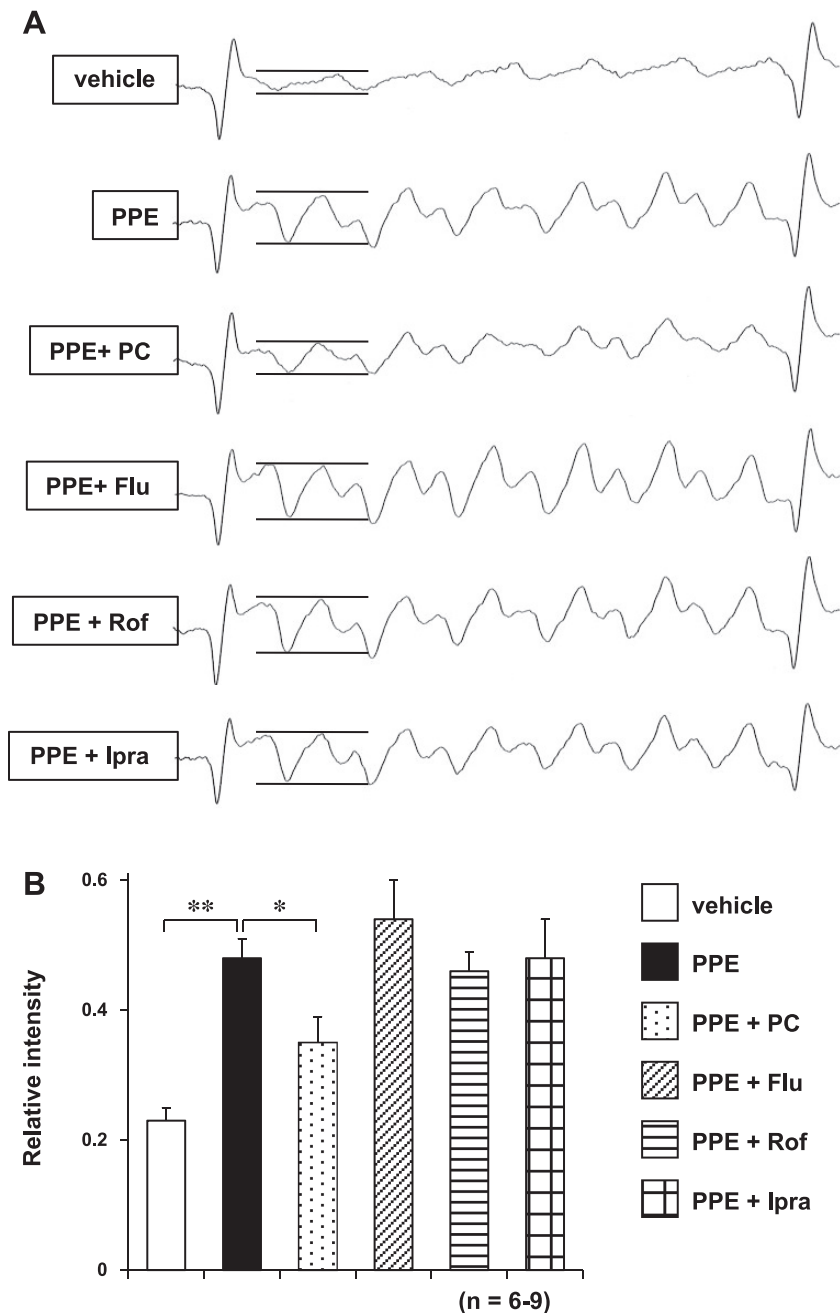
A

B

Fig. 6. Effect of different drugs on the PPE-induced production of superoxide anions. Mice were treated with or without (vehicle only) PPE (100 µg/mouse) once only on *day 0*. PC-SOD (PC; 60 kU/chamber), fluticasone propionate (Flu; 167 µg/kg), ipratropium bromide (Ipra; 26.7 µg/kg), or roflumilast (Rof; 5 mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once only on *day 0*. Cells in bronchoalveolar lavage fluid were collected on *day 1*, incubated with a spin trap agent 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2H-pyrrole *N*-oxide (DPhPMPO), and subjected to radical adduct electron spin resonance (ESR) spectrum analysis to determine the amount of superoxide anions present. The intensity of the ESR signal of the superoxide anion adduct (DPhPMPO-OOH adduct shown by the separation between the bars in the spectra shown in *A*) was determined (*B*). Values are means $\pm$ SE. *$P < 0.05$; **$P < 0.01$.

acting anticholinergic drug, is clinically useful because neither drug perturbed the pharmacological activity of the other. Because there are a number of bronchodilator types used clinically (such as long-acting anticholinergics and long-acting and short-acting β_2-agonists), the combination application of PC-SOD with these drugs should be also examined in future studies.

As the diagnosis of COPD in human patients is confirmed by a decrease in $FEV_1\%$, it is important to examine the effect of candidate drugs on respiratory function related to $FEV_1\%$ in animal models of COPD. Given that such a system has not yet been established in animal models, we here established such a system in mice by using a computer-controlled ventilator and negative pressure reservoir and found that the $FEV_{0.05}\%$ was

clearly decreased in PPE-administered mice compared with control mice. We found that PC-SOD partially restored the $FEV_{0.05}\%$ in PPE-administered mice, supporting the notion that inhaled PC-SOD could be beneficial for the treatment of patients with COPD. We propose that this technique used here could also be valuable for evaluating other candidate drugs for use in the treatment of COPD.

We recently reported that inhaled PC-SOD is protective against PPE-induced pulmonary emphysema (airspace enlargement) and alteration of lung mechanics (decrease in elastance). We also reported that inhaled PC-SOD is effective for treating predeveloped pulmonary emphysema (39). Because these protective and therapeutic effects were more apparent than those seen with other types of drugs studied in previous reports (6,
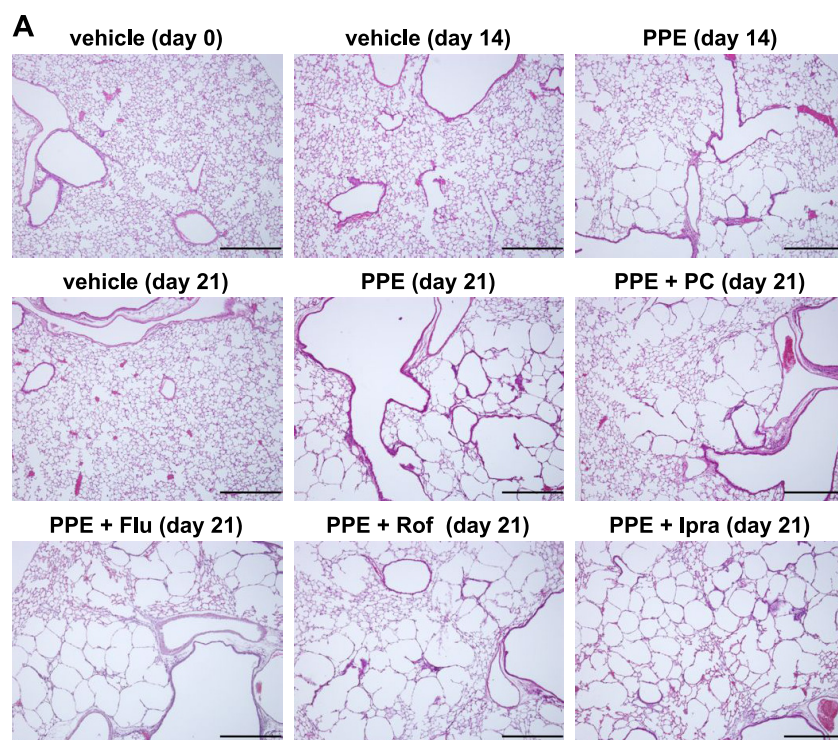
Fig. 7. Effect of different drugs on predeveloped pulmonary emphysema. Mice were treated with or without (vehicle only) PPE (100 µg/mouse) once only on *day 0*. PC-SOD (PC; 60 kU/chamber), fluticasone propionate (Flu; 167 µg/kg), ipratropium bromide (Ipra; 26.7 µg/kg), or roflumilast (Rof; 5 mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once daily from *day 14* to *day 20*. Histopathological examination (scale bar = 500 µm) (*A*), determination of the MLI (*B*), and measurement of total respiratory system elastance (total elastance) and tissue elastance (*B*) were determined as described in Fig. legend 1. Values are means ± SE. *P < 0.05; **P < 0.01.
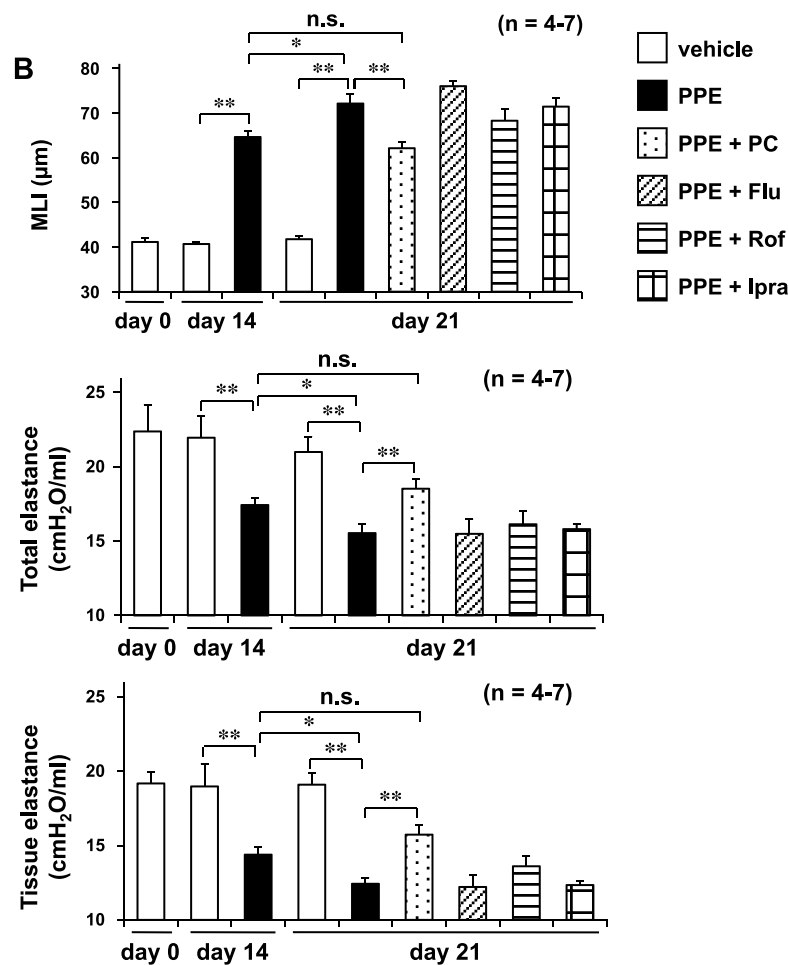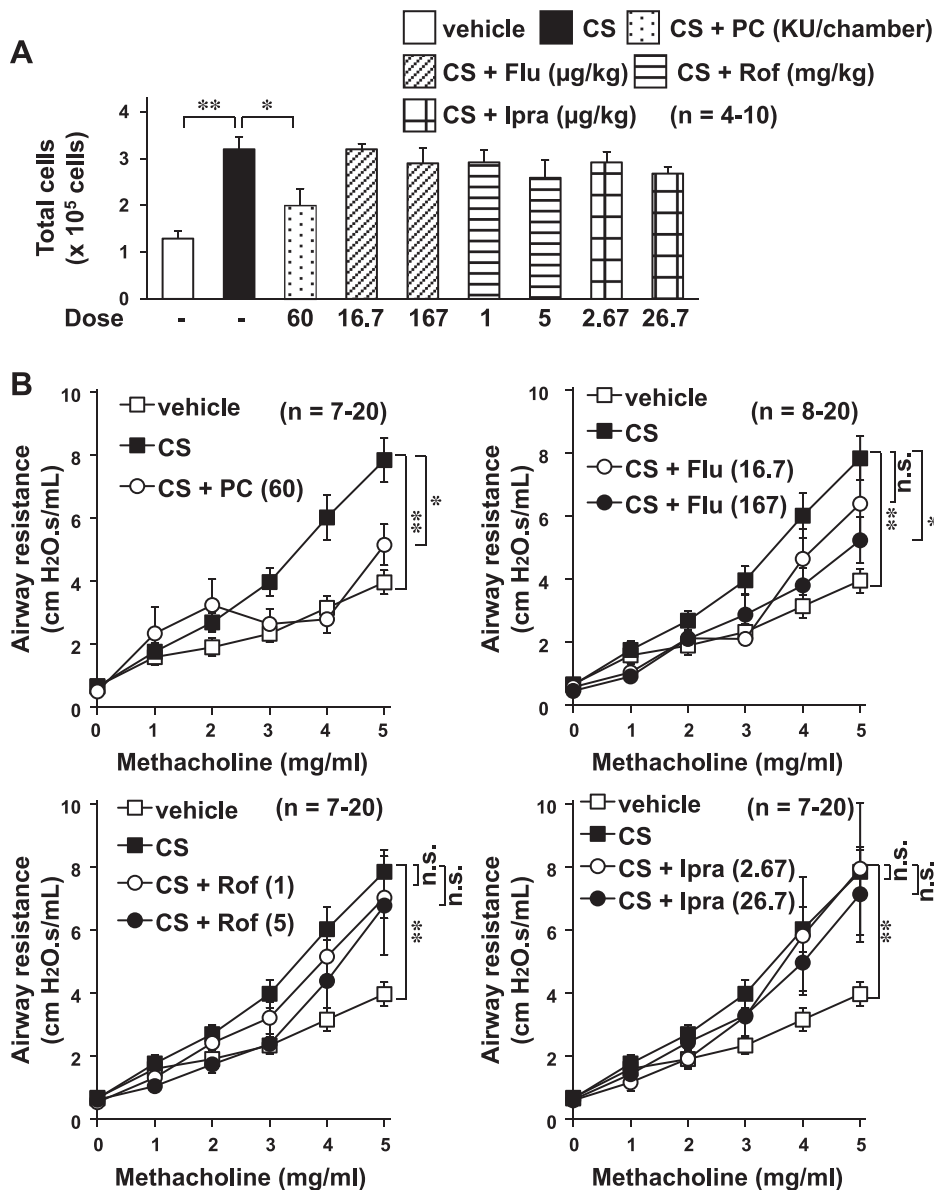
Fig. 8. Effect of different drugs on the CS-induced inflammatory response and hyperresponsiveness to methacholine. Mice were exposed to cigarette smoke (CS) for 3 days as described in MATERIALS AND METHODS. PC-SOD (PC; kU/chamber), fluticasone propionate (Flu; μg/kg), ipratropium bromide (Ipra; μg/kg), or roflumilast (Rof; mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once daily for 3 days (from *day 0* to *day 2*). Inflammatory response (*A*) and airway resistance (*B*) were assessed on *day 3* (26 h after the final administration of each drug) as described in Fig. legends 3 and 5 (*A*). Same data for vehicle and CS are used in all panels in *B*. Values are means $\pm$ SE. *$P < 0.05$; **$P < 0.01$; n.s., not significant.

11, 25, 43), we suggested that PC-SOD may be superior to these drugs for the treatment of COPD (39). In this study, we compared the protective and therapeutic effects of various drugs, including PC-SOD, under the same conditions. We used not only anti-inflammatory drugs (fluticasone propionate and roflumilast) but also ipratropium bromide, given the recent report concerning the anti-inflammatory effects of bronchodilators (43). For fluticasone propionate and ipratropium bromide, we used both clinical and higher doses (16.7 μg/kg and 167 μg/kg, respectively, for fluticasone propionate and 2.67 μg/kg and 26.7 μg/kg, respectively, for ipratropium bromide). In most previous animal studies, each drug was used within these dose ranges (24, 29, 41). For roflumilast, although the clinical dose is 500 μg/body (8.3 μg/kg), doses of 1–5 mg/kg have been used in previous animal studies (25, 43). Thus we used roflumilast doses of 1 and 5 mg/kg in this study. Under these conditions, neither fluticasone propionate nor ipratropium bromide showed any protective or therapeutic effects with respect to PPE-induced pulmonary emphysema, altera-

tions in lung mechanics or respiratory dysfunction (decrease in FEV$_{0.05}$%). On the other hand, roflumilast showed a protective effect against PPE-induced pulmonary emphysema and alteration of lung mechanics; however, the degree of protection was less than that afforded by PC-SOD. Furthermore, roflumilast did not exhibit any protective effect against PPE-induced respiratory dysfunction or any therapeutic effect against PPE-induced pulmonary damage. At present, it is not clear why roflumilast is positive for some indexes but not for other ones in this animal model. These results suggest that inhaled PC-SOD could be superior to these other drugs for the treatment of patients with COPD. We also found that the PPE-induced pulmonary inflammatory response and the production of superoxide anions were suppressed more clearly in mice concomitantly treated with PC-SOD compared with those treated with other drugs, suggesting that the antioxidant activity provided by PC-SOD is responsible for its superior therapeutic effects in this animal model. We also found that inhaled PC-SOD suppressed the CS-induced airway hyperresponsive-

ness to methacholine, which was previously suggested to involve the infiltration of leucocytes into the lung (5, 27). These results also suggest the clinical benefit of this treatment method.

In conclusion, we consider that a combination regime of administration of a bronchodilator along with inhaled PC-SOD may be therapeutically beneficial for patients with COPD.

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