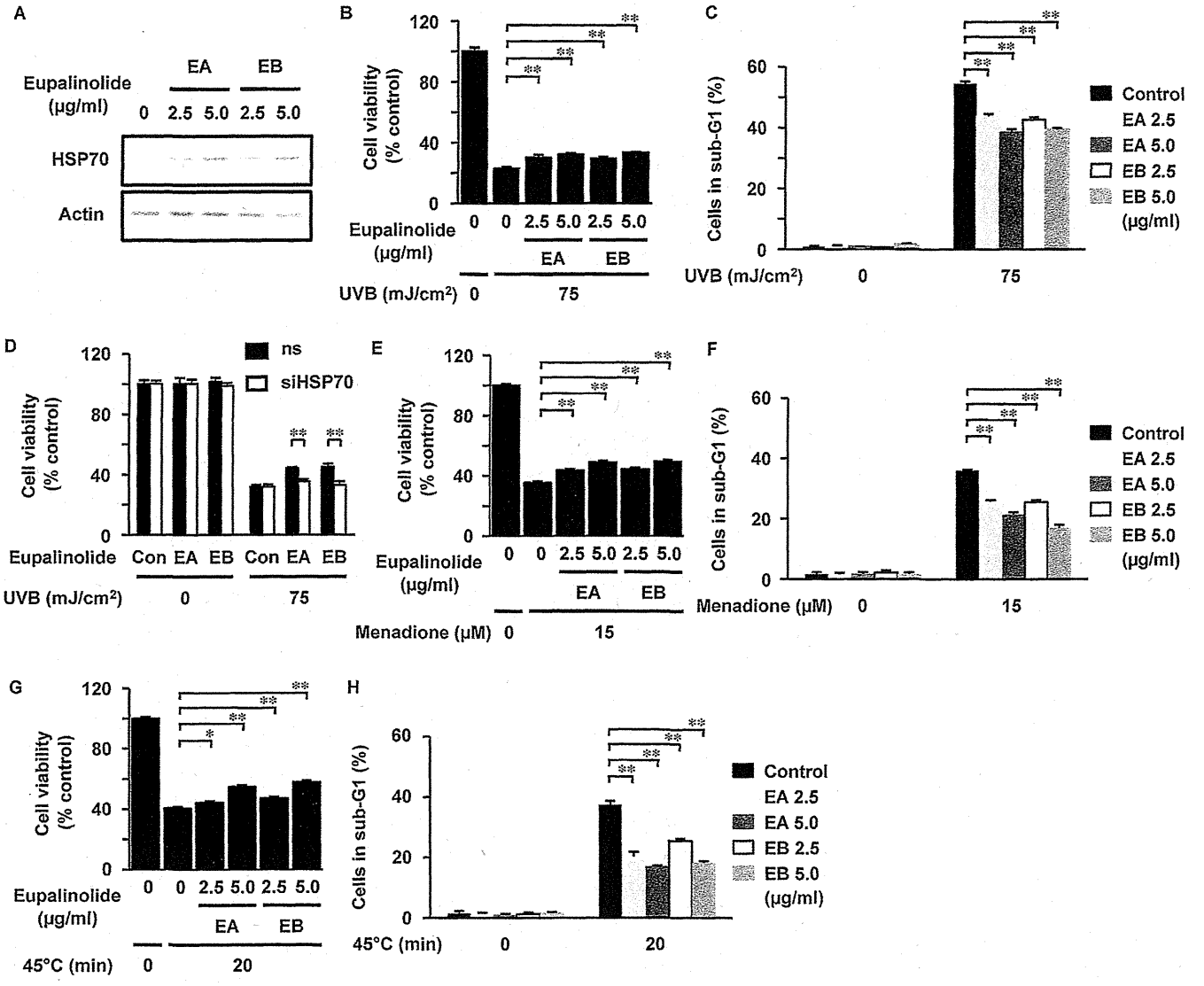

Fig. 6. Cytoprotective effects of EA and EB *in vitro*. PAM212 cells were pre-treated with indicated concentrations of EA or EB for 6 h, washed with fresh medium and cultured for 18 h. Cells were transfected with siRNA for HSP70 (siHSP70) or nonspecific siRNA (ns) and incubated for 48 h before the treatment with EA or EB described above (D). Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin (A). After treatment with EA or EB as described above, cells were irradiated with indicated doses of UVB and incubated for 24 h (B–D), incubated for 24 h with 15 μM menadione (E and F), or heat-shocked for 20 min at 45 °C followed by a recovery period at 37 °C for 24 h (G and H). Cell viability was determined by the MTT method (B, D, E and G). Apoptotic cells (cells in sub-G1) were counted by FACS as described in Section 2 (C, F and H). Values are given as mean ± S.D. ($n = 3$). $^{**}P < 0.01$; $^{*}P < 0.05$.

of EA and EB on melanin production (Fig. 7B), supporting the contribution of HSP70 to this EA- or EB-mediated inhibition of melanin production.

To determine the mechanism governing the inhibition of melanin production by EA or EB, we turned our attention to examining tyrosinase activity. As shown in Fig. 7C, treatment of cells with IBMX increased the tyrosinase activity of cell extracts, while pre-treatment of cells with EA or EB decreased this activity in the presence of IBMX. On the other hand, when EA or EB was added directly to cell extracts prepared from IBMX-treated B16 cells, the tyrosinase activity was not significantly affected (Fig. 7D). This finding indicates that EA and EB affect the expression of tyrosinase. To test this further, we treated cells with IBMX and observed an increase in the level of tyrosinase protein, which was suppressed in cells pre-treated with EA or EB (Fig. 7E). Similar results were observed at the mRNA level (Fig. 7F), suggesting that EA and EB inhibit transcription of the *tyrosinase* gene.

As described in the introduction, MITF plays a central role in the UVB-induced expression of various genes, including that for tyrosinase [48]. We examined the expression of two other MITF-regulated genes, tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (Dct). Expression of these genes was enhanced by IBMXin a manner that could be suppressed in cells pre-treated with either EA or EB (Fig. 7F). This finding suggests that EA and EB inhibit the MITF activity of cells.

We reported recently that overexpression of HSP70 in cells inhibited the transcriptional activity of MITF but not the expression of MITF [28]. We found here that treatment of cells with EA or EB affected neither protein nor mRNA levels of MITF (Fig. 7G and H). The results in Fig. 7 suggest that EA and EB inhibit the production of melanin by inducing the expression of HSP70.

3.4. Pharmacological effects of EA and EB in vivo

Finally, we tested the *in vivo* relevance of our *in vitro* results. To begin with, we examined the effect of EB on the expression of HSP70 in the skin by using immunohistochemical analysis with an antibody against HSP70 (we could not examine the effect of EA *in*

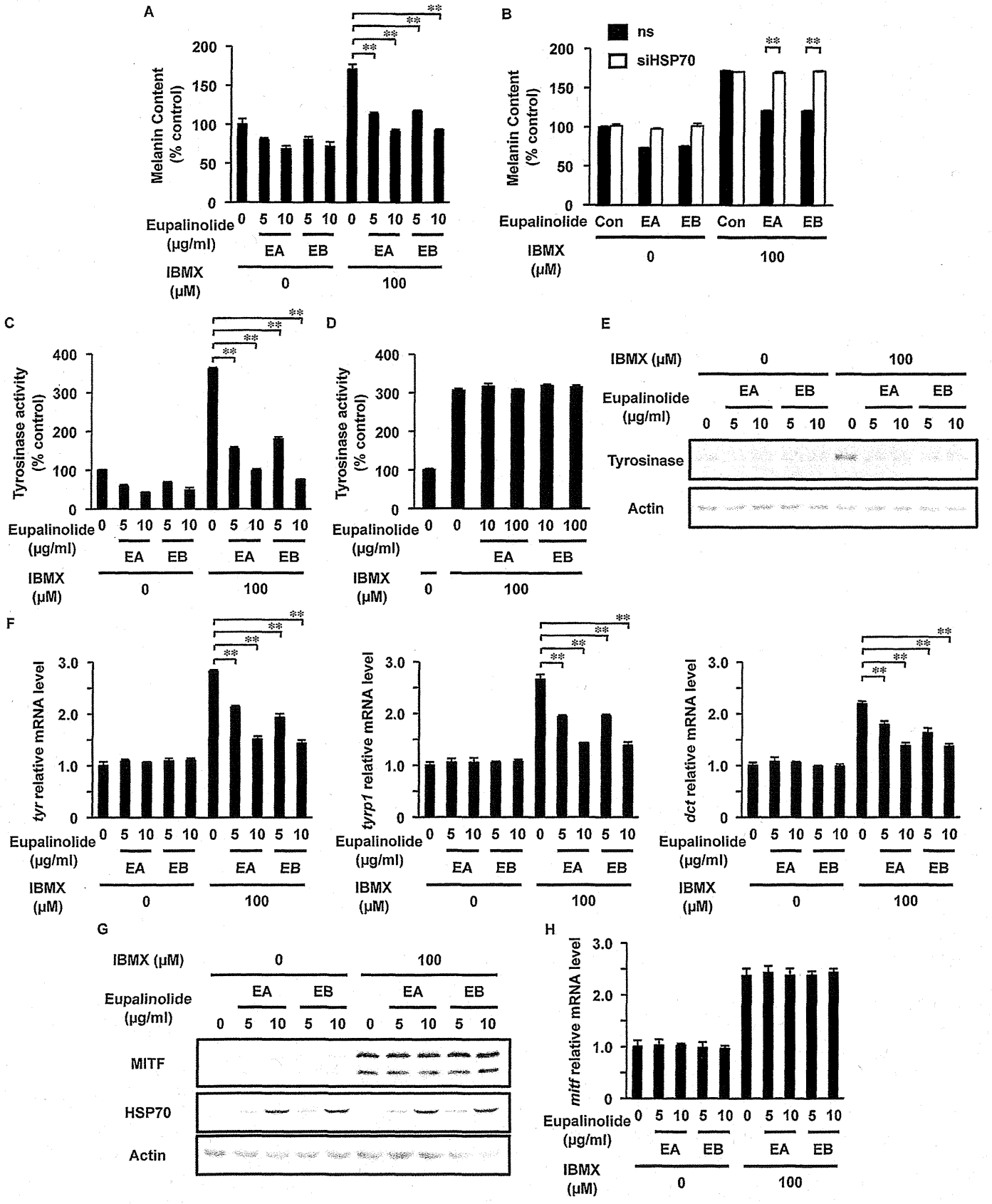

Fig. 7. Effects of EA and EB on melanin production *in vitro*. B16 cells were pre-incubated with indicated concentrations of EA, EB or ethanol extract of *E. lindleyanum* for 24 h (A–C, E–H). Cells were transfected with siRNA for HSP70 (siHSP70) or nonspecific siRNA (ns) and incubated for 48 h before the pre-treatment with EA or EB described above (B). After the pre-treatment procedure, cells were further incubated with or without 100 μM IBMX for 72 h (A, B), 48 h (C, E and F), 3 h (G) or 1.5 h (H). B16 cells were incubated with 100 μM IBMX for 48 h, and prepared whole cell extracts were mixed with EA or EB to obtain the indicated final concentrations (D). The amount of melanin in cell extracts was determined and is expressed relative to the control (A and B). Tyrosinase activity was determined and expressed relative to the control (C and D). Whole cell extracts were analyzed by immunoblotting with an antibody against each protein (E and G). mRNA expression was monitored as described in the legend of Fig. 2 (F and H). Values are given as mean ± S.D. ($n = 3$). $^{**}P < 0.01$.

vivo due to the limited amount of EA available). As shown in Fig. 8A, the expression of HSP70 in mouse skin was induced in a dose-dependent manner by the percutaneous administration of EB. The level of expression induced with 1 mg/cm^2 EB was similar to that observed in transgenic mice expressing HSP70 (Fig. 8A). The induction of HSP70 expression by EB in the skin was also observed at the mRNA level (Fig. 8B). Immunohistochemical analysis also demonstrated that the expression of HSP70 was higher in the epidermis than in the dermis, as described previously [22]

We then examined the effect of EB on UVB-induced epidermal damage. Eighteen hours after the percutaneous administration of EB, mice were subjected to UVB radiation and UVB-induced epidermal damage was estimated by histological observations and measurement of MPO activity, an indicator of the inflammatory infiltration of leukocytes. Histological observations revealed the extensive infiltration of leukocytes and epidermal disruption in skin sections prepared from UVB-irradiated control mice. In mice pre-administered with EB, however, the cutaneous damage was suppressed in a dose-dependent manner (Fig. 8C). MPO activity was increased in control mice in response to the UVB radiation and this activation was suppressed in UVB-irradiated mice pre-treated with EB (Fig. 8D). The level of skin damage and MPO activity in mice pre-treated with 1 mg/cm^2 EB was similar to that seen in transgenic mice expressing HSP70 (Fig. 8C and D). As described above, HSP70 was reported to suppress the activation of NF-κB through various mechanisms such as suppression of the inflammatory stimuli-induced degradation of IκB-α (an inhibitor of NF-κB) [15]. We therefore examined the effect of EB on the level of IκB-α *in vivo*. As shown in Fig. 8E and F, UVB irradiation decreased the cutaneous level of IκB-α, although the level remained significantly higher in mice pre-treated with EB or in transgenic mice expressing HSP70. These results suggest that percutaneously administered EB protects the skin against UVB-induced damage by inducing the expression of HSP70.

Finally, we examined the effect of EB on melanin production in the skin. After the percutaneous administration of EB, mice were exposed to UVB radiation. This cycle was repeated once every 2 days for 8 days and the melanin content was estimated by Fontana-Masson staining of sections or by a narrow-band simple reflectance meter (Mexameter). For this we used the murine tail skin, which resembles human skin in terms of the presence of epidermal melanocytes and UV-dependent melanin production [49]. As shown in Fig. 8G and H, an increase in melanin staining in the basal layer of the epidermis (the dermal/epidermal border) was observed in control mice after UVB irradiation but this increase was significantly suppressed in sections prepared from the mice pre-treated with EB. Measurement of the melanin content with a Mexameter also showed that the content was lower in the UVB- and EB-treated mice than in control mice (mice treated with UVB only) (Fig. 8I). The level of melanin in the skin of mice pre-treated with 1 mg/cm^2 EB was similar to that seen in transgenic mice expressing HSP70 (Fig. 8G–I). These results suggest that percutaneously administered EB suppresses UVB-induced melanin production by inducing HSP70 expression.

4. Discussion

In this study we purified compounds with HSP70-inducing ability from an ethanol extract of *E. lindleyanum*. Two compounds, EA and EB, were identified, which are optical isomers that have similar pharmacological activities (induction of HSP70 expression, cytoprotection, and inhibition of melanin production). EA and EB were previously identified as major sesquiterpenes in *E. lindleyanum* [33], and although no biological activity was reported for them, a range of biological activities, such anti-tumor, anti-allergic and anti-inflammatory activities have been reported for other sesquiterpenes [50,51]. This report marks the first time that sesquiterpenes have been described to induce the expression of HSPs. *E. lindleyanum* has been used in China and other Asian countries for the treatment of cough, fever and tracheitis based on its anti-microbial and anti-inflammatory activities [30–32]. Since HSP70 suppresses inflammation through the inhibition of NF-κB (an inflammation-inducing transcription factor) [13,15], it is reasonable to speculate that the anti-inflammatory effect of *E. lindleyanum* is due to the EA- or EB-mediated induction of HSP70 expression.

A number of compounds have been shown to induce the expression of HSP70; however, in most cases the induced expression is accompanied by a simultaneous decrease in cell viability. These cytotoxic effects of HSP70-inducers may cause side effects, resulting in their unsuccessful development and use in medicines and cosmetics. Therefore, in our previous report where we screened for HSP70-inducers from Chinese herbal extracts [29], we focused our search not only on the capacity of compounds to induce HSP70 expression, but also on their reduced cytotoxic activity. Compared to other Chinese herbal extracts, *E. lindleyanum* extract showed less toxicity at concentrations high enough to induce HSP70 expression; however, as shown in Fig. 1D, at a concentration of 150 μg/ml, *E. lindleyanum* extract reduced cell viability to 85% and induced a less than 8-fold increase in HSP70 expression. On the other hand, when EA and EB were employed at a concentration of 10 μg/ml, cell viability remained higher than 98% and HSP70 expression was induced more than 13-fold (Fig. 1D). This could be due to the fact that, in addition to EA and EB, the *E. lindleyanum* extract contains cytotoxic molecules that were separated from EA and EB in the purification process. This low cytotoxicitiy of EA and EB could be highly advantageous for their future use in medicines and cosmetics.

We showed here on the basis of several different results that the induction of HSP70 expression by EA or EB is mediated by the activation of HSF1. In this way, (i) the induction of expression was also observed for HSPs other than HSP70, (ii) the suppression of HSF1 expression by siRNA inhibited the EA- or EB-mediated induction of HSP70 expression, and (iii) the treatment of cells with EA or EB induced both the phosphorylation and re-localization of HSF1 into the nucleus. Furthermore, EA and EB seem to activate HSF1 by binding to HSP90, thereby inhibiting the interaction between HSP90 and HSF1. This conclusion was arrived at on the basis of the following results: (i) the co-immunoprecipitation of HSP90 with HSF1 was suppressed in extracts prepared from cells treated with EA or EB, and (ii) EB-fixed beads bound to HSP90 but not HSF1, and this binding was suppressed by excess amounts of free EB.

In addition to inducers of HSPs, co-inducers of HSPs (such as bimoclomol and indomethacin) have been paid considerable attention [11]. Co-inducers of HSPs cannot induce the expression of HSPs by themselves, but stimulate the expression achieved by other inducers. Interestingly, some molecules (such as GGA) possess properties of both inducers and co-inducers of HSPs [19,46]. The results of this study suggest that EA and EB may also belong to this category; 1 or 2 μg/ml EA (or EB) or 3.5% ethanol alone did not induce the expression of HSP70, but when used in combination the EA (or EB) and ethanol clearly induced the expression of HSP70. We also found that treatment of cells with 1 or 2 μg/ml EA (or EB) likely induced the phosphorylation of HSF1 given the shift in HSF1 protein migration, suggesting that the co-inducer-like actions of these compounds could be due to their capacity to phosphorylate HSF1. A similar mechanism for co-induction has been proposed for indomethacin [52].

With regards to the *in vitro* pharmacological activity of EA and EB, we showed that both exerted cytoprotective effects in addition to an inhibitory effect on melanin production. Pre-treatment of

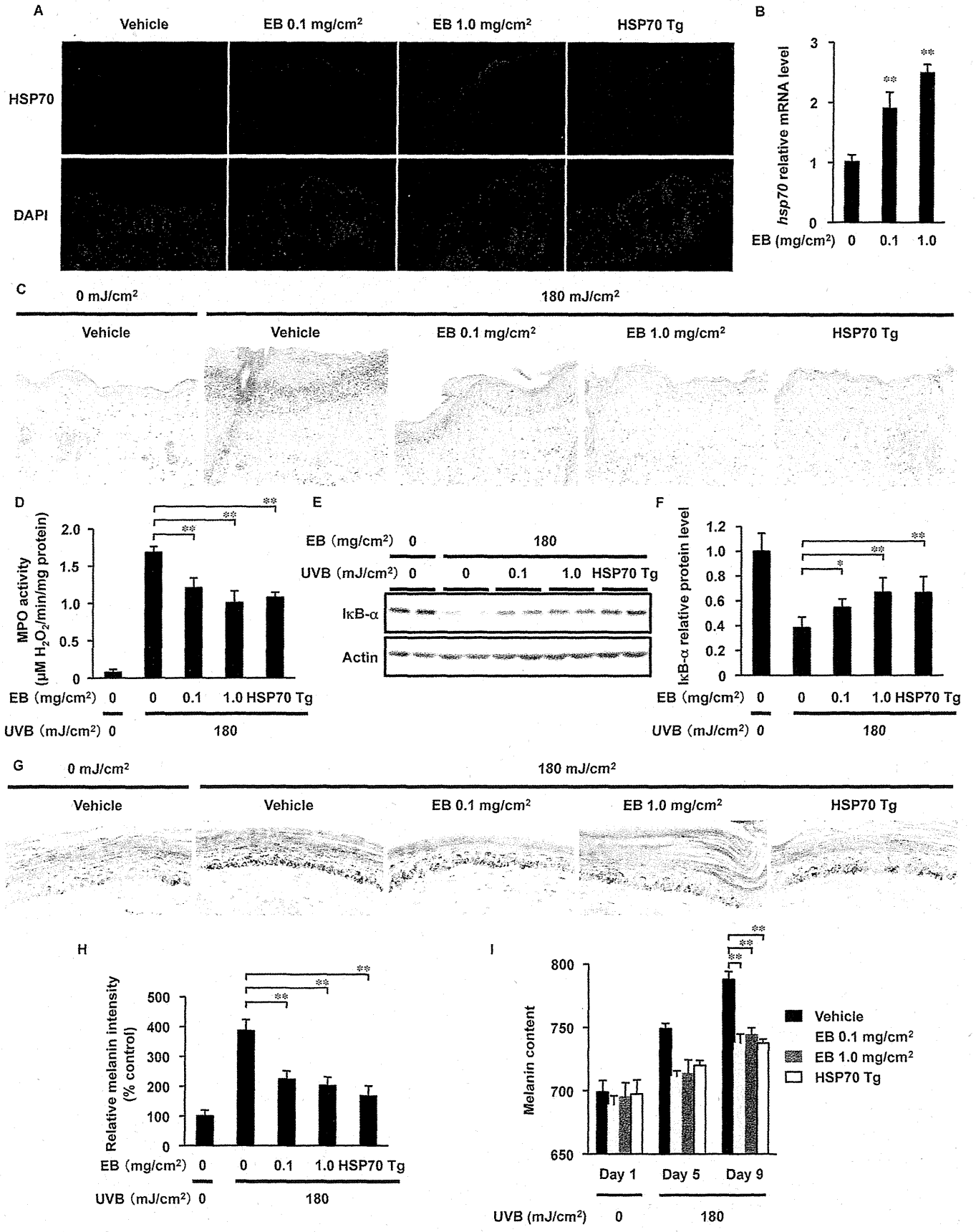

Fig. 8. *Effects of EA and EB on UVB-induced skin damage and melanin production in vivo.* (A–F) The dorsal skin of C57BL/6 (wild-type) mice was percutaneously administered with the indicated doses of EB. Mice were maintained for 18 h (A–F) before sacrifice and the dorsal skin was removed (A and B). Mice were irradiated with indicated doses of UVB (C–F) and sacrificed just prior to dorsal skin removal after 48 h (C, E and F) or 24 h (D). Transgenic mice expressing HSP70 (HSP70 Tg) were treated similarly but without EB administration. Sections of dorsal skin were prepared and subjected to immunohistochemical analysis with an antibody against HSP70 and DAPI staining (A) or hematoxylin and eosin staining (C). The *hsp70* mRNA expression was monitored as described in the legend of Fig. 2 (B). MPO activity was measured as described in Section 2

cells with EA or EB made cells resistant to the induction of apoptosis by subsequent treatment with various stressors (UVB, menadione and heat-shock). In relation to the UVB treatment, we also showed that suppression of HSP70 expression by siRNA suppressed this cytoprotective effect of EA and EB. It has been suggested that HSP70 suppresses a number of steps in the molecular pathways governing apoptosis, including p53 activation, which plays an important role in UVB-induced apoptosis [53,54].

We recently reported that overexpression of HSP70 in B16 cells suppresses IBMX-induced melanin production and increases the activity and expression of tyrosinase, but does not affect the expression of MITF. As a mechanism to explain this phenomenon, we showed that HSP70 physically interacts with MITF and suppresses MITF's transcription activity on the *tyrosinase* gene [28]. To this extent, we showed here that EA and EB decrease the activity and expression of tyrosinase without decreasing the expression of MITF in IBMX-treated cells.

As for *in vivo* relevance of these *in vitro* results, we showed that the percutaneous administration of EB increased HSP70 expression in the skin in a dose-dependent manner. Although we did not show the penetration of EB into epidermis, the result suggests that EB could reach the region and induce HSP70 expression. We also suggest that the percutaneous administration of EB suppresses UVB-induced skin damage, inflammatory responses and melanin production by inducing HSP70 expression *in vivo*. Since the use of hypopigmenting reagents could be useful as drugs and cosmetics, a number of compounds that inhibit tyrosinase and/or MITF have been discovered; however, most of their cosmetic and pharmaceutical applications have not been successful due to skin irritation side effects [55]. This seems to be due to the fact that UV-induced melanogenesis plays a protective role against UV-induced skin damage. Based on the results of this study, we propose that EA or EB could be cosmetically and pharmaceutically beneficial because HSP70 protects cells from UV-induced damage in conjunction with inhibition of melanin production.

An ameliorative effect of HSP70 due to its cytoprotective, anti-inflammatory and molecular chaperone (quality control of proteins) properties has been reported for various diseases. For example, we have shown using transgenic mice and GGA that HSP70 protects against irritant-produced lesions in the stomach and small intestine, as well as in inflammatory bowel disease-related experimental colitis, pulmonary fibrosis and in Alzheimer's disease-related phenomena in mice [18,20,35,56–58]. EA or EB could thus be therapeutically beneficial for use in these diseases.

Acknowledgments

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(D). Whole cell extracts were analyzed by immunoblotting with an antibody against IκB-α or actin (E). The intensity of the IκB-α band relative to the actin band is shown (F). Values are given as mean ± S.E.M. (n = 5–6). $^{**}P < 0.01$. (G–I) The tail skin of C57BL/6 (wild-type) mice was percutaneously administered with indicated doses of EB and, after 18 h, mice were irradiated with 180 mJ/cm^2 UVB. This cycle was repeated once every 2 days for 8 days (*i.e.*, a total of 4 times, on days 2, 4, 6, 8). Transgenic mice expressing HSP70 (HSP70 Tg) were treated similarly but without EB administration. Sections were prepared from the tail skin on day 9 and subjected to Fontana-Masson staining (G). The intensity of epidermal melanin in each section was determined and is shown (H). The amount of melanin in the tail skin was also measured by Mexameter (day 1, 5, 9) (I). Values are given as the mean ± S.E.M. (n = 9–10). $^{**}P < 0.01$.

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Effects of β-(1,3–1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity

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β-Glucan

ABSTRACT

Irritable bowel syndrome (IBS) is a gastrointestinal disorder characterized by chronic abdominal pain associated with altered bowel habits. Since the prevalence of IBS is very high and thus, involves elevated health-care costs, treatment of this condition by methods other than prescribed medicines could be beneficial. β-(1,3)-D-glucan with β-(1,6) branches (β-glucan) has been used as a nutritional supplement for many years. In this study, we examined the effect of β-glucan on fecal pellet output and visceral pain response in animal models of IBS. Oral administration of β-glucan suppressed the restraint stress- or drug-induced fecal pellet output. β-Glucan also suppressed the visceral pain response to colorectal distension. These results suggest that β-glucan could be beneficial for the treatment and prevention of IBS.

1. Introduction

IBS is a functional gastrointestinal disorder characterized by chronic and recurrent abdominal pain and discomfort (colonic hypersensitivity) that are associated with altered bowel habits but not with any detectable structural or biochemical abnormality [1,2]. IBS is categorized into subtypes according to the predominant bowel habit: diarrhea-predominant IBS, constipation -predominant IBS, and mixed pattern IBS [1]. In spite of the significant impact that IBS has on patient quality-of-life, currently available clinical treatments for IBS have proved unsatisfactory, mainly due to the difficulty in suppressing the visceral pain associated with IBS.

IBS is one of the most common gastrointestinal disorders, estimated to affect 7–15% of the general population in the USA and 6–12% in Asian countries [2,3]. Considering the health-care costs associated with treating the condition, the identification of effective therapies (such as the taking of supplements) that do not involve prescription drugs is beneficial [4,5].

Although the pathogenesis of IBS is not completely understood, studies have suggested that genetic factors, previous inflammation, mental stressors and microbiota play important roles [6]. A number of animal models for IBS has been established and used to evaluate clinical protocols designed to treat the condition. Mental stressor- or drug-induced alterations in defecation have been used as a model for defecation disorders related to IBS in animals [7–9]. Since hypersensitivity to colorectal distension (CRD) was observed in IBS patients [10], monitoring the electrical activity of the abdominal muscles (visceromotor response) in response to CRD is a standard procedure to detect IBS-related abdominal pain (visceral pain) in animals [11,12]. Furthermore, based on the increased colonic level of butyrate in IBS patients [13,14], butyrate enema-induced hypersensitivity to CRD is also considered as a useful animal model for IBS [15,16].

β-Glucans are naturally-occurring polysaccharides found in the cell walls of yeast, fungi, cereal plants and certain bacteria [17,18]. As suggested by the fact that various foods contain β-glucans, they are known to have few toxic and adverse effects [18]. β-Glucans from mushrooms have been used in Japan as anti-tumor drugs due to their immunostimulating activities [17]. In addition, β-(1,3)-D-glucans with β-(1,6) branches have been reported to have various clinically beneficial effects, such as enhancing the

Abbreviations: AUC, area under the curve; β-glucan, β-(1,3)-D-glucan with β-(1,6) branches; CRD, colorectal distention; 5-HT, 5-hydroxytryptamine hydrochlor ide; IBS, irritable bowel syndrome; LMW, low-molecular-weight; PBS, phosphate-buffered saline; S.E.M, standard error of the mean.

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bio-defense activity against bacterial, viral, fungal and parasitic challenge, increasing hematopoiesis and radioprotection, stimulating the wound healing response, and decreasing serum lipid levels [17–20]. Interestingly, it was recently reported that β-glucans suppress inflammatory responses in some animal models [21–26], suggesting that β-glucan could be an interesting immunomodulator, causing opposing effects on different aspects of the immune system.

We succeeded in the purification and industrial-scale production of low-molecular-weight β-(1,3–1,6)-D-glucan from *Aureobasidium pullulans* (*A. pullulans*) GM-NH-1A1 strain (LMW β-glucan) [27,28]. The characteristic features of LMW β-glucan are its low molecular weight (about 100 kDa), low viscosity, high water-solubility and high level of β-(1–6) branching (50–80%) [27,28]. We previously reported that LMW β-glucan has various clinically beneficial effects, such as suppression of the allergic response, suppression of restraint stress-induced immunosuppression and anti-tumor and anti-metastatic actions [27–29]. Moreover, we recently reported that LMW β-glucan protects the gastric mucosa against the formation of irritant-induced lesions by increasing levels of defensive factors such as heat shock protein 70 and gastric mucin [30]. In the present study, we use different animal models for IBS to test the hypothesis that LMW β-glucan could be effective in the treatment of this condition. Our results suggest that the oral administration of LMW β-glucan suppresses not only fecal pellet output but also the visceromotor response to CRD (visceral pain response). These findings suggest that LMW β-glucan could be therapeutically effective for the treatment of IBS.

2. Materials and methods

2.1. Chemicals and animals

LMW β-glucan was prepared from the conditioned culture medium of *A. pullulans* GM-NH-1A1, as described previously [27,28]. Analysis of ^{1}H and ^{13}C NMR spectra and gel-filtration chromatography revealed that the LMW β-glucan contains approximately 70% β-(1–6) branches and an average molecular weight of 100 kDa, as described previously [27,28]. Clonidine hydrochloride and castor oil were from WAKO Pure Chemicals (Osaka, Japan). Sodium butyrate, brewer's yeast and carbamyl- β-methylcholine chloride (bethanecol) were obtained from Sigma (St. Louis, MO). Loperamide hydrochloride and 5-hydroxytryptamine hydrochloride (5-HT) were purchased from Nacalai Tesque (Kyoto, Japan). Wild-type mice (C57/BL6, 6–8 weeks of age) and Wistar rats (4–6 weeks of age) were obtained from Charles River (Yokohama, Japan). Wistar-Imamichi rats (4 weeks of age) were purchased from the Institute for Animal Reproduction (Kasumigaura, 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 Committees of Keio University and Kumamoto University.

2.2. Analysis of fecal pellet output in mice

Female mice were subjected to restraint stress by being placed individually into a 50 ml Falcon tube (Becton Dickinson, Franklin Lakes, NJ) for 1 h, as described previously [31]. These tubes are small enough to restrain a mouse so that it is able to breathe but unable to move freely. Control mice were left to move freely in the cage. The number of fecal pellets excreted during the 1-h restraint stress period was measured. β-Glucan was dissolved in phosphate-buffered saline (PBS) and administered orally 2 h before animals were subjected to the restraint stress. Control animals were administered PBS.

In a separate experiment, mice were administered one of different drugs that stimulate intestinal motility (bethanecol and 5-HT), cause diarrhea (castor oil) or cause constipation (loperamide and clonidine). Animals were then placed in a cage and the number or wet weight of fecal pellets excreted in the subsequent 1-, 2- or 24-h period determined. Drugs administered subcutaneously were bethanechol (3 mg/kg) and 5-HT (3 mg/kg), while those administered orally were loperamide (10 mg/kg), clonidine (3.5 mg/kg) and castor oil (300 μl/mouse).

β-Glucan was dissolved in PBS and administered orally 2 h before animals were subjected to the restraint stress or drug-treatment. Control animals were administered PBS.

2.3. Electromyography and CRD

Rats were deeply anaesthetized with pentobarbital sodium (40 mg/kg) and then electromyography electrodes (Star Medical, Tokyo, Japan) sutured into the external oblique muscle of the abdomen for electromyogram recording. Electrode leads were tunneled subcutaneously and exteriorized at the nape of the neck for future access. After surgery, rats were housed individually and allowed to recuperate for 6 days before being used for visceromotor response testing.

Repeated CRD was performed as described previously [32]. Rats were restrained in a plastic conical-shape tube (diameter, 6 cm; height, 15 cm), 15 min before electromyography. To reduce confounding effects due to restraint stress, rats were habituated to the tube 30 min per day for 3 days prior to the experiment. A polyethylene bag (length 2 cm) was inserted in the distal colon, positioned 1 cm proximal to rectum, and connected to a balloon catheter which was anchored with tape to the base of the tail. The pressure and volume of the balloon were controlled and monitored by a pressure controller-timing device (Distender Series II; G & J Electronics, Toronto, Canada), connected to the balloon. Rats were subjected to repeated CRD (80 mm Hg, 30 s, 5-min interstimulus interval, 12 times) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

In separate experiments, CRD associated with the use of butyrate enemas was examined as described previously [15]. Rats were instilled with 1 ml sodium butyrate (110 mg/ml, pH 6.9) or saline into the colon twice daily for 3 days (day 1, 2 and 3). Rats were subjected to CRD (10, 20, 40 60 and 80 mm Hg, 20 s, 150-s interstimulus interval) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

Visceromotor responses were monitored by electromyography, as described previously [11,33], 12 h after the last administration of β-glucan. Electromyograph data were collected and analyzed using 8 STAR software (version 6.0–19.2 for Windows; Star Medical, Tokyo, Japan). Responses evoked by contraction of the external oblique musculature were quantified by calculating the area under the curve (AUC) of the voltage alteration graph. The baseline was determined by data collected 20 s (butyrate enema) or 30 s (repeated CRD) before each distention.

2.4. Inflamed paw pressure nociception test

The pain threshold in Wistar-Imamichi rats was measured using a Randall–Sellito test with an analgometer (Ugo basile, Comerio, Italy), as described previously [34]. Brewer's yeast (20%, 1 ml) was injected into one of the hind paws. Seven hours later, an increasing pressure was applied to the underside of the hind limb and the pain threshold was defined as the pressure in grams eliciting a cry from the animal.

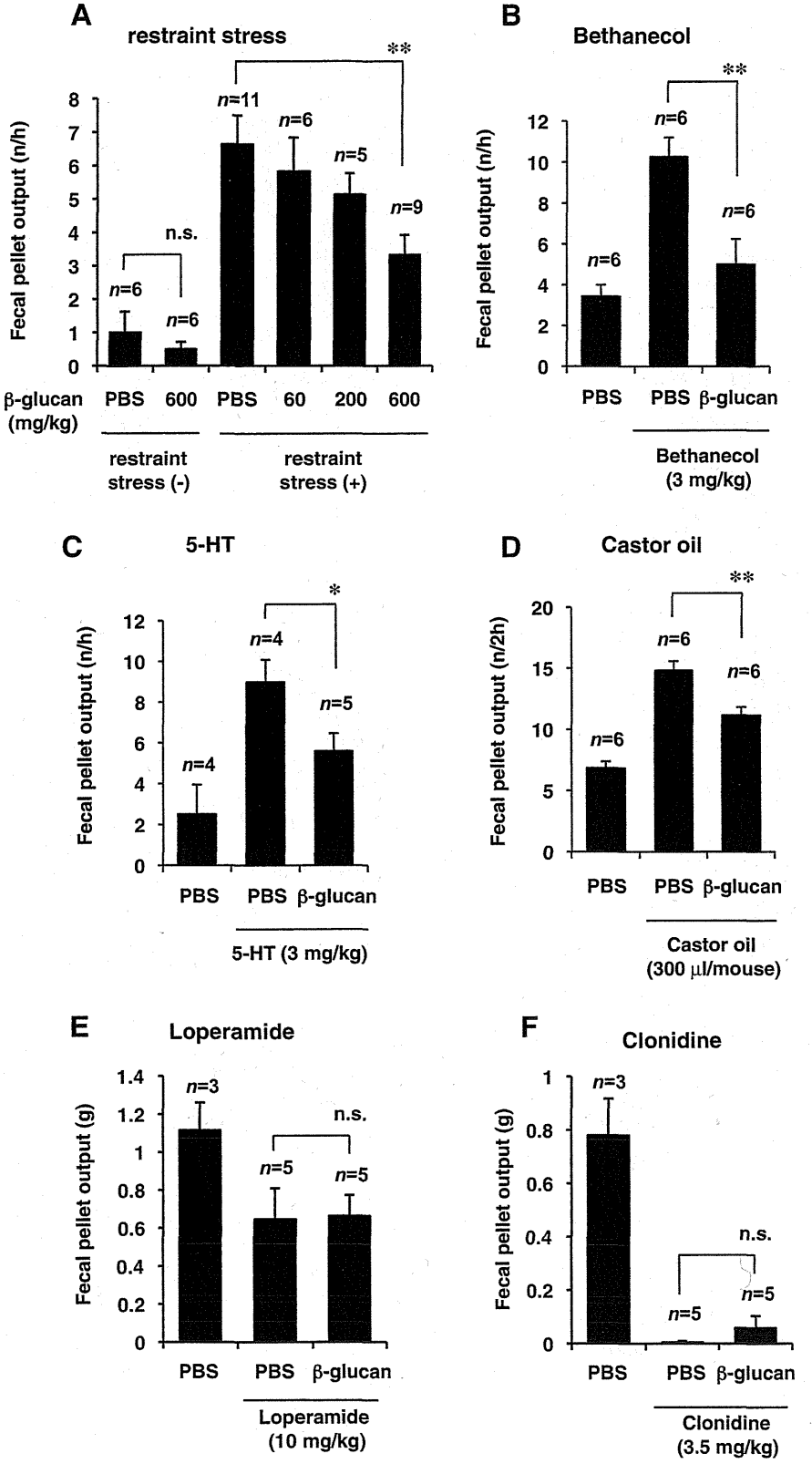

Fig. 1. Effects of LMW β-glucan on restraint stress- or drug-induced alteration of fecal pellet output in mice. Mice were orally administered indicated doses (A) or 600 mg/kg (B–F) of LMW β-glucan or vehicle (PBS). Two hours later, mice were exposed to restraint stress (A) or administered bethanecol (3 mg/kg, *s.c.*) (B), 5-HT (3 mg/kg, *s.c.*) (C), castor oil (300 μl/mouse, *p.o.*) (D), loperamide (10 mg/kg, *p.o.*) or clonidine (3.5 mg/kg, *p.o.*). The number (A–D) or wet weight (E and F) of fecal pellets excreted in the subsequent 0–1 h (A–C), 0–2 h (D) or 0–24 h (E and F) period was determined. Values are mean ± S.E.M $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

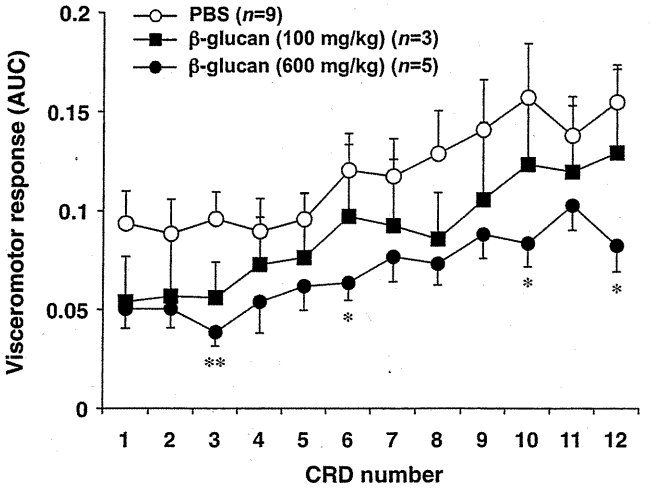

Fig. 2. Effect of LMW β-glucan on the viscermotor response to CRD in rats. The indicated doses (mg/kg) of β-glucan or PBS were orally administered to female Wistar rats once daily for 7 days. Twelve hours after the last administration of LMW β-glucan, rats were subjected to repetitive CRD and the visceromotor response was recorded and analysed as described in Section 2. Values are mean ± S.E.M $^{*}P < 0.05$; $^{**}P < 0.01$.

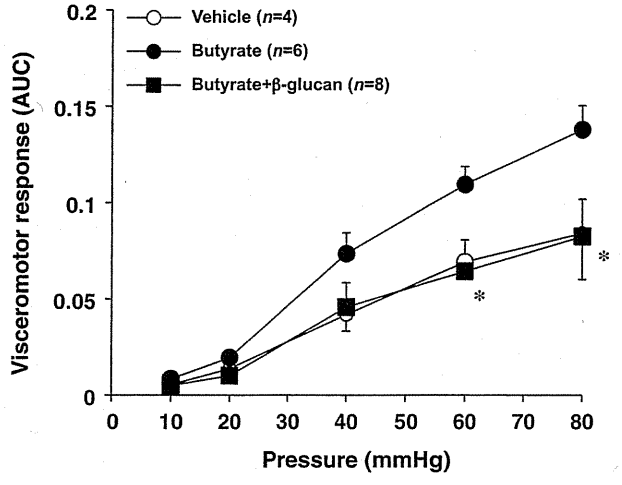

Fig. 3. Effect of LMW β-glucan on butyrate enema-induced colonic hypersensitivity to CRD in rats. Butyrate enemas were administered twice daily on days 1, 2 and 3. Administration of LMW β-glucan (600 mg/kg) (once daily from day 0 to day 6) and monitoring and analysis of the visceromotor response to CRD (on day 7) were performed as described in the legend of Fig. 2. Values are mean ± S.E.M $^{*}P < 0.05$.

2.5. Statistical analysis

All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or a Student's *t* test for unpaired results was used to evaluate differences between more than two groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1. Effect of LMW β-glucan on fecal pellet output in mice

We first examined the effect of a once-only oral administration of LMW β-glucan on restraint stress-induced fecal pellet output in mice. In untreated mice (administered PBS vehicle only), restraint stress (restricted movement by placement of mouse in a 50 ml plastic tube) caused a more than 5-fold increase in fecal pellet output per hour compared to unrestrained mice (Fig. 1A), as described previously [35]. The once-only oral pre-administration of LMW β-glucan suppressed this increase in a dose-dependent manner without affecting the basal level (without restraint stress) of fecal pellet output (Fig. 1A). Similar results were observed in response to a once-daily oral administration of LMW β-glucan for 7 days (data not shown). The LMW β-glucan-dependent suppression of restraint stress-induced fecal pellet output was also confirmed in rats (data not shown).

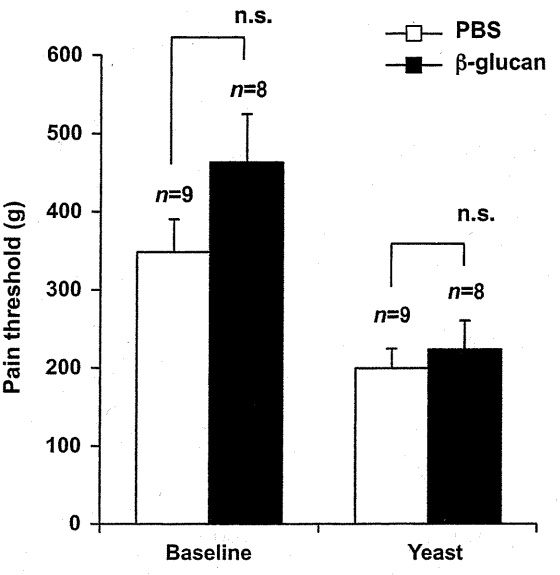

Fig. 4. Effect of LMW β-glucan on the pain response of rats in the inflamed paw pressure test. LMW β-glucan (600 mg/kg) was administered orally as described in the legend of Fig. 2. Twelve hours after the last administration of β-glucan, the inflamed paw pressure test was performed as described in Section 2. The pain threshold before (baseline) and after (yeast) the yeast injection was determined. Values are mean ± S.E.M n.s., not significant.

We also examined the effect of LMW β-glucan on the fecal pellet output induced by drugs that increase intestinal motility (bethanecol and 5-HT) or cause diarrhea (castor oil) [8,36]. As shown in Fig. 1B–D, the oral administration of LMW β-glucan (600 mg/kg) to mice suppressed the fecal pellet output induced by each of these drugs.

We then examined the effect of LMW β-glucan on drug-induced constipation. As shown in Fig. 1E and F, administration of loperamide or clonidine to mice decreased fecal pellet output, as described previously [36]. The oral pre-administration of LMW β-glucan did not alter the fecal pellet output. The results in Fig. 1 thus suggest that orally administered LMW β-glucan suppresses the restraint stress- or drug-induced stimulation of intestinal motility but does not affect the motility in the absence of these stimuli or in presence of constipation-inducing drugs. The mechanism underlying the LMW β-glucan-dependent suppression of intestinal motility is not clear at present.

3.2. Effect of LMW β-glucan on the visceromotor response to CRD in rats

In addition to alterations of fecal pellet output, hypersensitivity to visceral pain is one of the principle pathogenetic pathways for IBS. To study this phenomenon, we examined the effect of LMW β-glucan on visceromotor response to CRD, which has been used as an index of visceral pain response [33]. Rats were used for this analysis since the techniques for measuring the visceromotor response and CRD were established with these animals. As a single oral administration of LMW β-glucan did not significantly affect the visceromotor response to CRD (data not shown), we decided

to determine the effect of LMW β-glucan administered orally once-daily for 7 days. In control rats (PBS-treated), CRD evoked a visceromotor response which increased in amplitude in response to repeated CRDs (Fig. 2), as described previously [32]. Oral pre-administration of LMW β-glucan (600 mg/kg) to animals significantly decreased the visceromotor response to CRD not only after repetitive CRDs but also upon the first CRD (Fig. 2). Pre-administration of LMW β-glucan (100 mg/kg) also showed a tendency to decrease the visceromotor response to CRD, however the effect was not statistically significant (Fig. 2). These results indicate that oral pre-administration of high dose of LMW β-glucan suppresses the visceral pain response to CRD.

Since the visceromotor response to the first CRD was reduced by the pre-administration of LMW β-glucan, the results in Fig. 2 can be interpreted to indicate that LMW β-glucan suppresses the visceral pain response to CRD itself, but does not affect the repeated CRD-induced hypersensitivity to visceral pain. However, although we tried to habituate rats to the tube used for CRD experiment (see Section 2), it is possible that the animals entered into a state of restraint-like stress. Thus, it is also possible that LMW β-glucan suppresses the restraint stress-induced hypersensitivity to visceral pain.

We then examined the effect of LMW β-glucan on the visceral pain response in another animal model, butyrate-induced hypersensitivity to CRD. The butyrate enema is known to reduce the threshold of the visceromotor response to CRD [15,16]. We confirmed that twice-daily butyrate enemas (on days 1, 2 and 3) stimulated the visceromotor response to CRD on day 7 and found that when LMW β-glucan was orally pre-administered once daily from day 0 to day 6, the visceromotor response to CRD was similar to that measured in control rats (not given butyrate enemas) (Fig. 3). This result suggests that LMW β-glucan suppresses butyrate-induced hypersensitivity to CRD.

Finally, we tested whether the inhibitory effect of LMW β-glucan on the pain response is specific for visceral pain. For this purpose, we used the inflamed paw pressure test in which a yeast solution was administered to one of hind paws of rats to induce inflammation and the pressure-induced pain response was subsequently determined. As shown in Fig. 4, oral administration of LMW β-glucan once daily for 7 days did not affect the paw pressure required to elicit a nociception response (pain threshold) in both presence and absence of yeast injection. This finding suggests that LMW β-glucan does not affect the pain response in general but specifically affects the visceral pain response.

In conclusion, we have shown here that the oral administration of LMW β-glucan suppresses not only restraint stress- or drug-induced fecal pellet output, but also suppresses the visceral pain response. The difficulty associated with therapeutic management of IBS can be attributed to the fact that both abdominal pain and bowel habit disorders must be addressed. The results presented in this study thus suggest that LMW β-glucan could prove therapeutically beneficial for the prevention and treatment of IBS, especially in relation to the diarrhea-predominant IBS.

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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 cmH_2O 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 cmH_2O.

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 cmH_2O 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

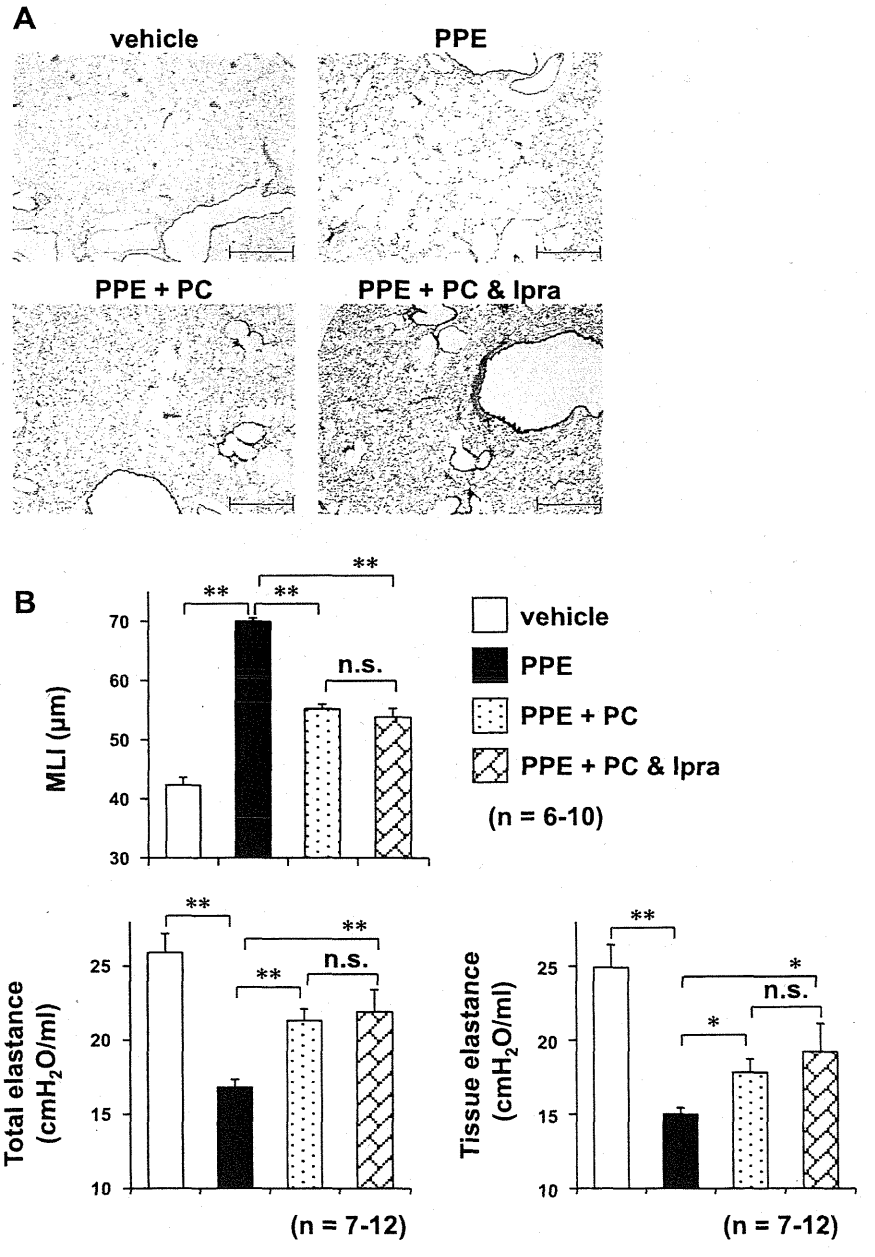

Fig. 1. Effect of ipratropium bromide on the protective effects of lecithinized superoxide dismutase (PC-SOD) against porcine pancreatic elastase (PPE)-induced pulmonary emphysema and alteration of lung mechanics. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. 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.

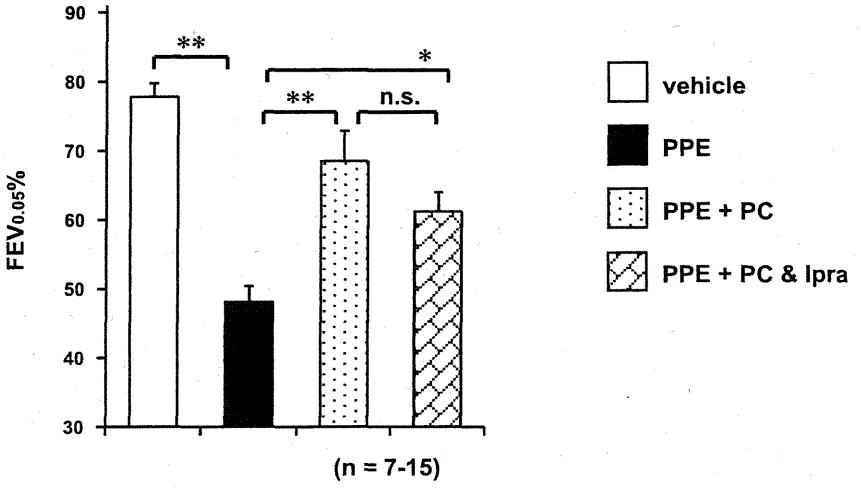

Fig. 2. Effect of ipratropium bromide on the protective effects of PC-SOD against the PPE-induced reduction of forced expiratory volume in the first 0.05 s/forced vital capacity ($FEV_{0.05}$%). Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. Mice were then treated 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. 1*B*). 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 ipratropium 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. 4*B*, 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. 1*B*). Furthermore, none of these drugs affected PPE-induced respiratory dysfunction (decrease in $FEV_{0.05}$%) (Fig. 4*C*). The results in Fig. 4 thus suggest that treatment with PC-SOD offers a greater protective effect than other types of

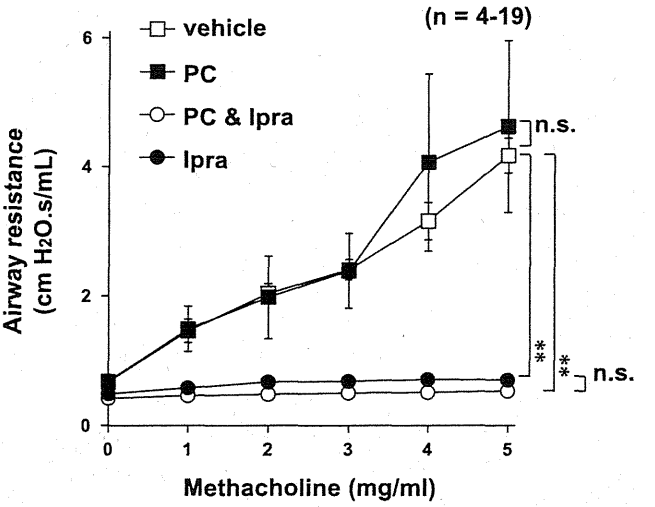

Fig. 3. Effect of PC-SOD on the ipratropium bromide-dependent decrease in airway resistance. Mice were treated with PC-SOD (PC; 60 kU/chamber) by inhalation and/or intratracheal administration of ipratropium bromide (Ipra; 26.7 μg/kg). After 1 h, mice were exposed to nebulized methacholine 5 times, and 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.

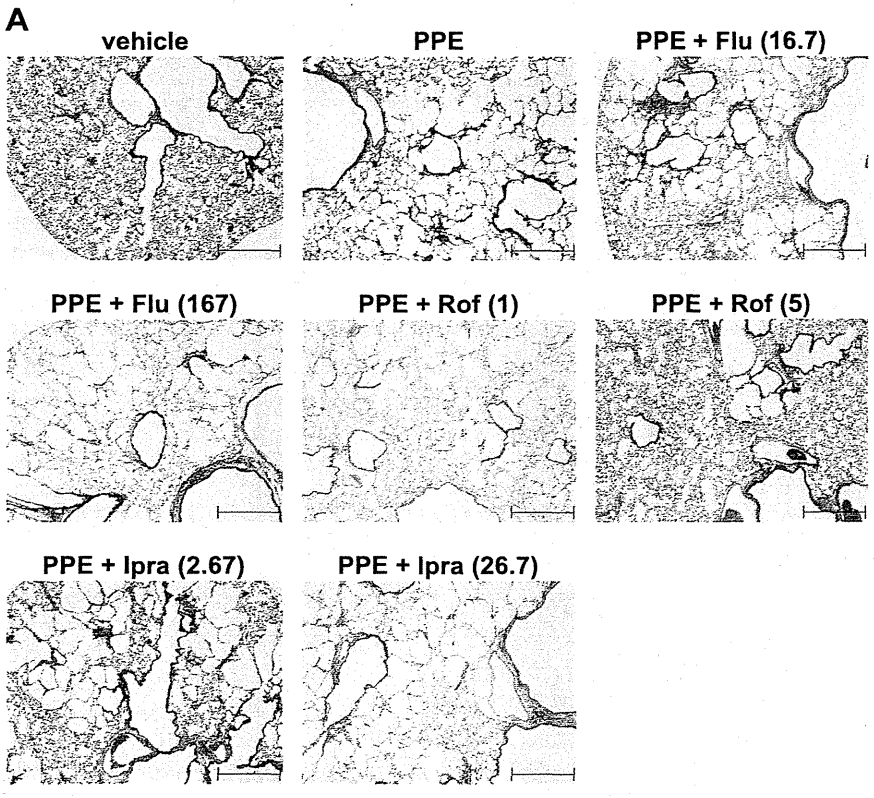

Fig. 4. Effect of different drugs on PPE-induced pulmonary emphysema. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. Fluticasone propionate (Flu; μg/kg) and ipratropium bromide (Ipra; μg/kg) or roflumilast (Rof; mg/kg) were administered intratracheally or orally, 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^H_β = 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

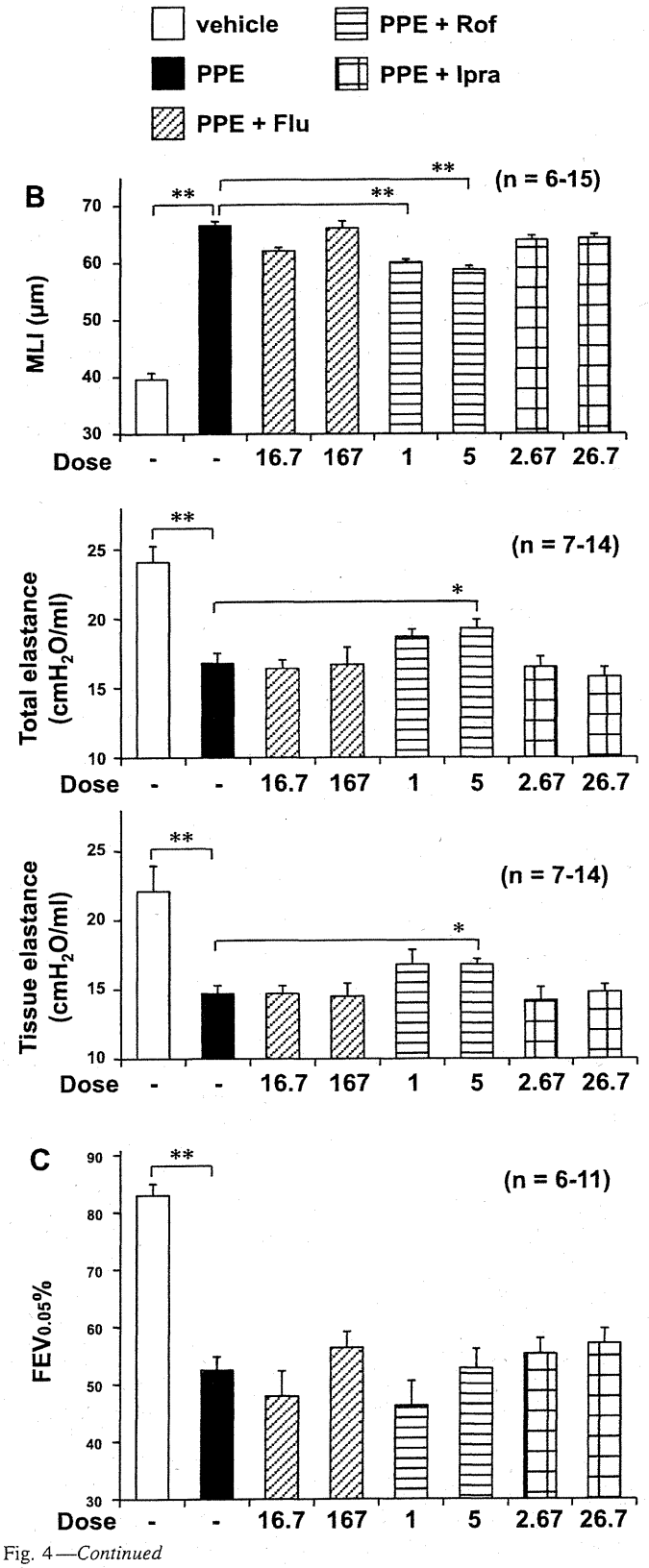

Fig. 4—*Continued*

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

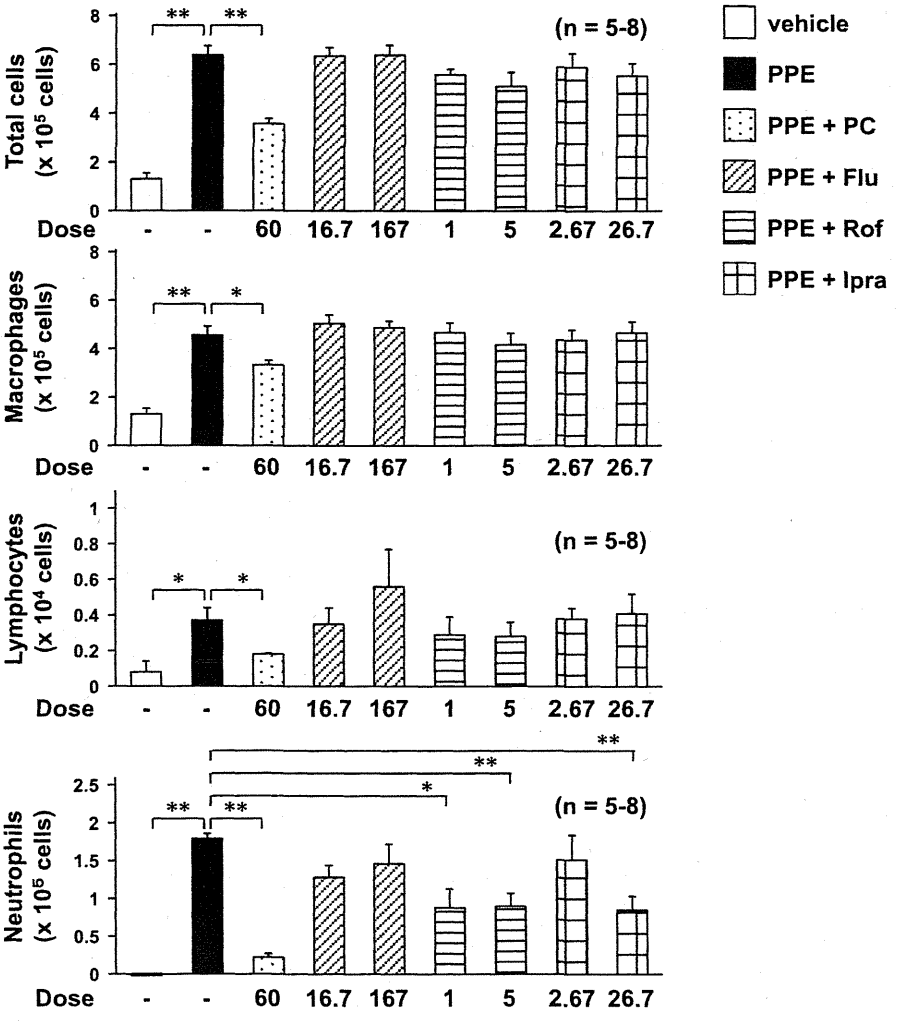

Fig. 5. Effect of different drugs on the PPE-induced inflammatory response. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. PC-SOD (PC; kU/chamber), fluticasone propionate (Flu; μg/kg), ipratropium bromide (Ipra; μg/kg), or roflumilast (Rof; mg/kg) were administered 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-

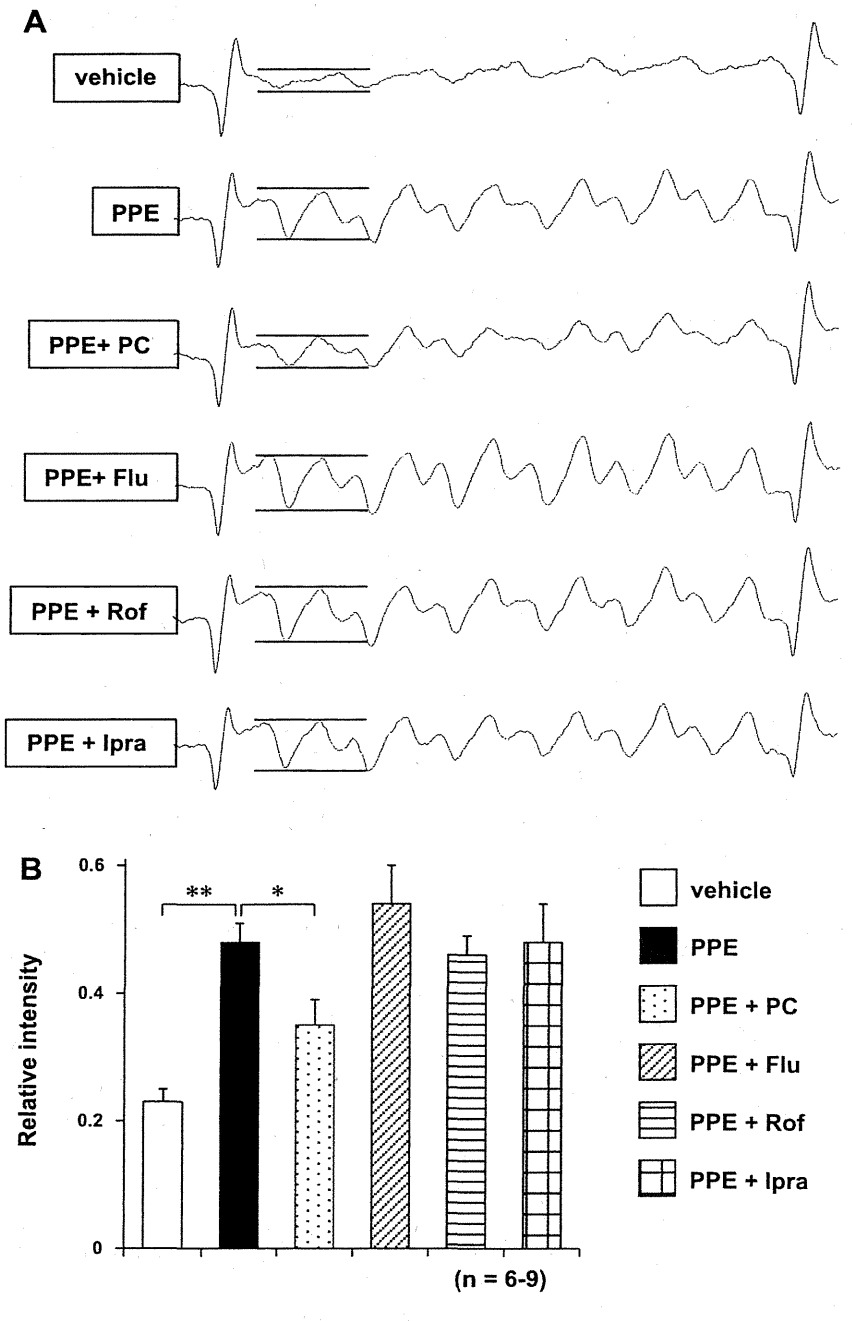

Fig. 6. Effect of different drugs on the PPE-induced production of superoxide anions. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. PC-SOD (PC; 60 kU/chamber), fluticasone propionate (Flu; 167 μg/kg), ipratropium bromide (Ipra; 26.7 μg/kg), or roflumilast (Rof; 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 ± 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,