CHEST

Original Research

DIFFUSE LUNG DISEASE

Effects of Lecithinized Superoxide Dismutase and/or Pirfenidone Against Bleomycin-Induced Pulmonary Fibrosis

Ken-Ichiro Tanaka, PhD; Arata Azuma, MD, PhD; Yuri Miyazaki; Keizo Sato, MD, PhD; and Tohru Mizushima, PhD

Background: **Idiopathic pulmonary fibrosis (IPF) involves lung injury induced by reactive oxygen species (ROS), such as superoxide anion, and fibrosis. Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion to hydrogen peroxide. We recently reported that inhalation of lecithinized SOD (PC-SOD) ameliorated bleomycin-induced pulmonary fibrosis. We here studied effects of PC-SOD on bleomycin-induced pulmonary fibrosis and lung dysfunction and compared the results to those obtained with pirfenidone, a newly developed drug for IPF.**

Methods: **Lung mechanics (elastance) and respiratory function (FVC) were assessed using a computer-controlled ventilator. Respiratory function was evaluated by monitoring percutaneous arterial oxygen saturation (Spo_2).**

Results: **Both inhalation of PC-SOD and oral administration of pirfenidone ameliorated bleomycin-induced pulmonary fibrosis and changes in lung mechanics. Administration of bleomycin produced a decrease in both FVC and Spo_2. PC-SOD treatment led to significant recovery of both parameters, whereas pirfenidone improved only Spo_2. PC-SOD suppressed the bleomycin-induced pulmonary inflammatory response and production of superoxide anions in the lung more effectively than pirfenidone. Furthermore, both PC-SOD and pirfenidone produced a therapeutic effect even when the drug was administered after the development of fibrosis. PC-SOD and pirfenidone also produced a synergistic therapeutic effect.**

Conclusions: **These results suggest that the superior activity of PC-SOD to pirfenidone against bleomycin-induced pulmonary fibrosis and lung dysfunction is due to its unique antioxidant activity. We propose that treatment of IPF with a combination of PC-SOD and pirfenidone could be therapeutically beneficial.** ***CHEST 2012; 142(4):1011–1019***

Abbreviations: BALF = BAL fluid; DPhPMPO = 2-diphenylphosphinoyl-2-methyl-3, 4-dihydro-2H-pyrrole N-oxide; ESR = electron spin resonance; IPF = idiopathic pulmonary fibrosis; KC = keratinocyte-derived chemokine; PC = phosphatidylcholine; PC-SOD = lecithinized human Cu/Zn-superoxide dismutase; ROS = reactive oxygen species; SMA = smooth muscle actin; SOD = superoxide dismutase; Spo_2 = percutaneous arterial oxygen saturation; TGF = transforming growth factor

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis.[1-3] Recent studies have suggested that IPF is triggered by repeated lung injury and promoted by abnormal wound repair and remodelling, resulting in fibrosis.[4,5] Both reactive oxygen species (ROS) and transforming growth factor (TGF)-β1 play important roles in IPF.[6,7] ROS that are released from the activated leukocytes cause not only lung injury but also fibrosis.[6,8] TGF-β1 also appears to stimulate the production of interstitial collagen.[9,10]

The cellular redox state, determined by the balance between ROS and antioxidant molecules (such as superoxide dismutase [SOD]), plays an important role in the pathogenesis of IPF.[11-17] Thus, antioxidant molecules have attracted considerable attention as therapeutic candidates for the treatment of IPF. Among the ROS, superoxide anions are believed to play a major role in numerous diseases, and SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide.[18]

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.[19-21] A phase 2 clinical study showed that IV administered PC-SOD (40 or 80 mg) significantly improved the symptoms of patients with IPF (Kamio D, Azuma A, Ohta K, et al, unpublished data, October 12, 2011). Furthermore, in consideration of the quality of life of patients, we have developed a method of PC-SOD administration by inhalation and have demonstrated that this procedure is effective against bleomycin-induced pulmonary fibrosis in mice.[22] However, the effect of PC-SOD inhalation on preexisting fibrosis and on lung mechanics and respiratory function has not been examined, although both are important considerations in the evaluation of compounds as candidate drugs for IPF.

Pirfenidone is a novel antifibrotic drug that has been demonstrated to have both preventive and therapeutic effects on bleomycin-induced pulmonary fibrosis in animals.[23-25] The antifibrotic effect of pirfenidone appears to be mediated by its inhibitory effect on TGF-β1 expression, TGF-β1-induced expression of collagen, and proliferation of various types of cells, including fibroblasts.[23,25] However, pirfenidone is pluripotent in its effects, exerting both antiinflammatory activity, due to downregulation of proinflammatory cytokine expression, and antioxidant activity. Together, these effects appear to play an important role in the suppression of IPF-related pulmonary fibrosis in animals.[23,25,26]

In the present study, we compared the effects of PC-SOD and pirfenidone on bleomycin-induced pulmonary fibrosis and alterations in lung mechanics and respiratory function. Our results revealed that both of these drugs have ameliorative and therapeutic effects, although PC-SOD showed more potent antiinflammatory and antioxidant activity than pirfenidone.

Manuscript received November 10, 2011; revision accepted February 22, 2012.
Affiliations: From the Department of Analytical Chemistry (Drs Tanaka and Mizushima), Faculty of Pharmacy, Keio University, Tokyo; the Graduate School of Medical and Pharmaceutical Sciences (Drs Tanaka, Sato, and Mizushima and Mr Miyazaki), Kumamoto University, Kumamoto; and the Department of Internal Medicine (Dr Azuma), Division of Respiratory, Infection, and Oncology, Nippon Medical School, Tokyo, Japan.
Funding/Support: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan [Grant H22-005], and the Japan Science and Technology Agency and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan [Grant 20015037].
Correspondence to: Tohru Mizushima, Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan; e-mail: mizushima-th@pha.keio.ac.jp

DOI: 10.1378/chest.11-2879

Materials and Methods

Measurement of Percutaneous Arterial Oxygen Saturation

Details are described in e-Appendix 1. Measurement of percutaneous arterial oxygen saturation (Spo_2) was performed with the MouseOx system (STARR Life Sciences Corp) as described previously.[27,28] Mice were anesthetized with chloral hydrate (500 mg/kg), and after 10 min, the sensor was attached to the thigh. All data were analyzed using MouseOx software (STARR Life Sciences Corp).

Measurement of Lung Mechanics and FVC

Measurement of lung mechanics was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ), as described previously.[29,30] 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 to 3 cm H_2O.

Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively. Determination of FVC was performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ), as described previously.[30] Details are described in e-Appendix 1.

Results

Effects of PC-SOD and Pirfenidone on Bleomycin-Induced Pulmonary Fibrosis

Pulmonary fibrosis was induced by giving mice a single (on day 0) intratracheal dose of bleomycin and confirmed 14 days later (Fig 1A). Hematoxylin and eosin staining revealed that severe pulmonary damage was induced by the bleomycin and that this damage was suppressed by inhalation of PC-SOD or oral administration of pirfenidone (Fig 1B). We determined the minimum dose of each drug required to achieve the maximum ameliorative effect (60 kU/chamber for PC-SOD and 400 mg/kg/d for pirfenidone) based on dose-response profile experiments (data not shown). Masson trichrome staining of collagen revealed that bleomycin-induced collagen deposition was clearly suppressed by administration of PC-SOD or pirfenidone (Fig 1B). We also found that the bleomycin-induced elevation of pulmonary hydroxyproline (an indicator of collagen levels) was significantly suppressed by both drugs (Fig 1C). Overall, these results about PC-SOD are consistent with our previously reported findings.[22]

Lung myofibroblasts produce considerable amounts of extracellular matrix components, such as collagen, and play an important role in pathogenesis of IPF.[31] We then examined the pulmonary level of myofibroblasts by immunohistochemical analysis with an antibody against α-smooth muscle actin (α-SMA), a marker for myofibroblasts.[31] As shown in Figure 1D, bleomycin administration increased the number of α-SMA-positive cells, whereas treatment with PC-SOD or pirfenidone restored this level to normal, suggesting

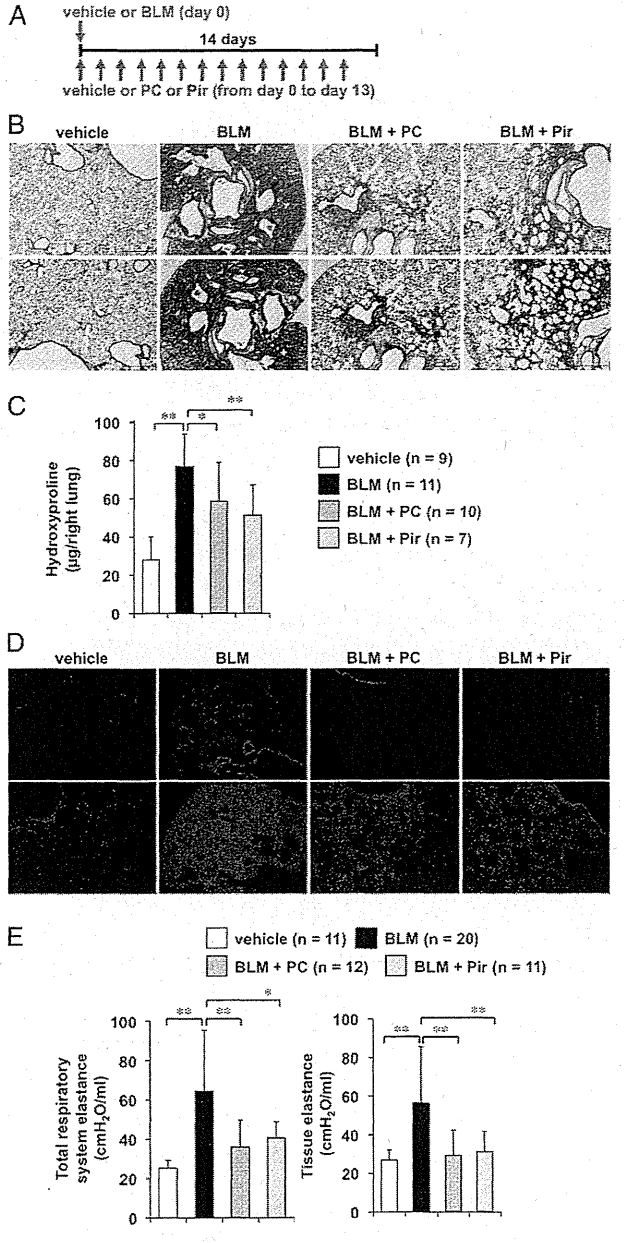

FIGURE 1. Effects of lecithinized human Cu/Zn-superoxide dismutase (PC-SOD) and pirfenidone against bleomycin-induced pulmonary fibrosis and alteration in lung mechanics. A, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were also treated by either inhalation of PC-SOD (PC, 60 kU/chamber) daily or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid for 14 days (from day 0 to day 13). B, Sections of pulmonary tissue were prepared on day 14 and subjected to histopathologic examination (hematoxylin and eosin staining [upper panels] and Masson trichrome staining [lower panels]). C, The pulmonary hydroxyproline level was determined on day 14 as described in the "Materials and Methods" section. D, The sections were subjected to immunohistochemical analysis with an antibody against α-smooth muscle actin (upper panels) and DAPI staining (lower panels). E, Total respiratory system elastance and tissue elastance were determined on day 14 as described in the"Materials and Methods" section. Values are mean ± SD. $*P < .05$; $**P < .01$. Scale bar, 500 μm.

that these drugs suppress the bleomycin-induced increase in lung myofibroblasts.

Effects of PC-SOD and Pirfenidone on Bleomycin-Induced Changes in Lung Mechanics and Respiratory Function

The changes in lung mechanics associated with pulmonary fibrosis are characterized by an increase in elastance.[32] Total respiratory system elastance (elastance of the total lung, including the bronchi, bronchioles, and alveoli) and tissue elastance (elastance of the alveoli) increased following bleomycin treatment, effects that were partially restored by administration of PC-SOD or pirfenidone (Fig 1E).

Using a computer-controlled ventilator and a negative pressure reservoir, we found that FVC clearly decreased in bleomycin-treated mice and that this

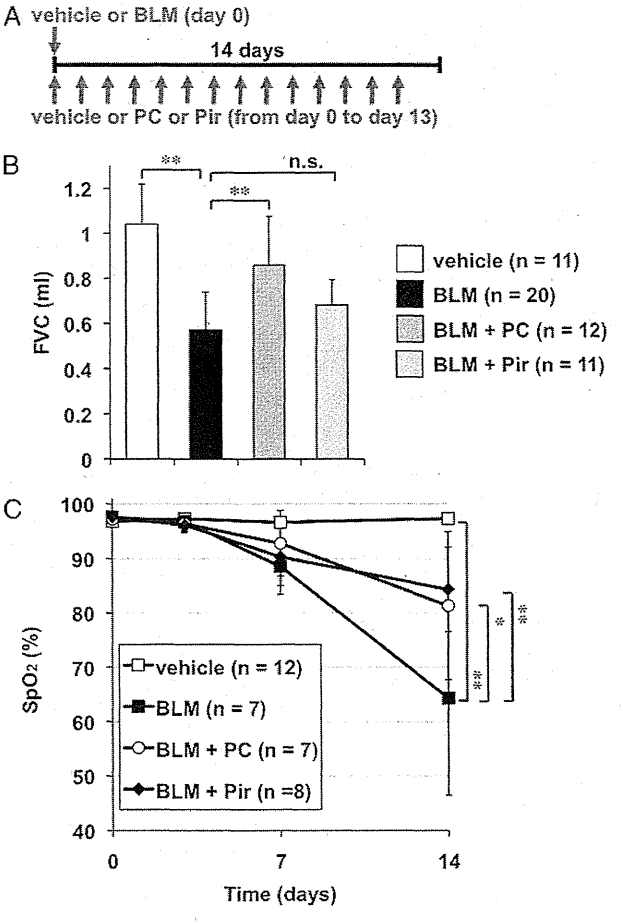

FIGURE 2. Effects of PC-SOD and pirfenidone against bleomycin-induced respiratory dysfunction. A, Outline for experimental design. Mice were treated with bleomycin (BLM, 5 mg/kg), PC-SOD (PC, 60 kU/chamber), and pirfenidone (Pir, 400 mg/kg/d) as described in the Fig 1 legend. B, FVC was determined on day 14, as described in the "Materials and Methods" section. C, SpO_2 was determined on days 0, 3, 7, and 14, as described in the "Materials and Methods" section. Values are mean ± SD. $*P < .05$; $**P < .01$. n.s. = not significant; SpO_2 = percutaneous arterial oxygen saturation. See Figure 1 legend for expansion of other abbreviations.

decrease was significantly suppressed by treatment with PC-SOD (Fig 2B). Although treatment with pirfenidone also produced a trend toward increased FVC in bleomycin-treated mice, this result was not statistically significant (Fig 2B). We also evaluated lung function by monitoring SpO_2. SpO_2 decreased over time in bleomycin-treated mice, an effect that was partially ameliorated by treatment with PC-SOD or pirfenidone (Fig 2C).

Effects of PC-SOD and Pirfenidone on the Bleomycin-Induced Inflammatory Response and Production of Superoxide Anion and TGF-β1

Next, we examined the bleomycin-induced pulmonary inflammatory response. As shown in Table 1, the total number of leukocytes increased following bleomycin treatment, an effect that was partially suppressed by simultaneous treatment with PC-SOD. Similar results were observed in relation to the number of neutrophils (leukocytes that play an important role in IPF and bleomycin-induced pulmonary fibrosis) (Table 1). In the case of pirfenidone, although the same trends were observed, the results were not statistically significant (Table 1).

We also estimated the inflammatory response by determining the amounts of cytokines and chemokines in the BAL fluid (BALF). As shown in Table 1, the amount of each of all proteins tested (tumor necrosis factor-α, macrophage inflammatory protein-2, monocyte chemoattractant protein-1, and keratinocyte-derived chemokine [KC]) increased following bleomycin treatment, an effect that was partially suppressed by simultaneous treatment with PC-SOD. Pirfenidone suppressed the bleomycin-induced increase of KC but not those of other cytokines and chemokines (Table 1).

We next used electron spin resonance (ESR) analysis to monitor the level of superoxide anions in cells present in the BALF. Because we could not detect a clear ESR spectrum in cells prepared from mice treated with a single dose of bleomycin (5 mg/kg) (data not shown), we used cells prepared from animals treated once daily (5 mg/kg) for 2 days (both on day 0 and day 1), and ESR analysis was done on day 2 (Fig 3A). As shown in Figures 3B and 3C, 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 bleomycin-treated mice than in control cases. Simultaneous administration of PC-SOD but

Table 1—*Effects of PC-SOD and Pirfenidone on the Bleomycin-Induced Inflammatory Response and Production of TGF-β1*

	Vehicle	BLM	BLM + PC	BLM + Pir
Cells and Neutrophils[a]	n = 3	n = 7	n = 6	n = 6
Total cells, × 10^5 cells	1.33 ± 0.28	6.11 ± 1.06[b]	4.32 ± 0.67[c]	4.97 ± 1.10
Neutrophils, × 10^5 cells	0.03 ± 0.04	2.73 ± 1.26[b]	1.08 ± 0.49[c]	1.47 ± 0.72
Cytokines and chemokines[d]	n = 4	n = 8	n = 8	n = 8
TNF-α, pg/mL BALF	2.72 ± 2.72	50.5 ± 13.3[b]	33.8 ± 11.2[e]	45.2 ± 32.6
MIP-2, pg/mL BALF	2.84 ± 3.08	121 ± 49.7[b]	69.3 ± 26.8[e]	88.8 ± 42.3
KC, pg/mL BALF	17.1 ± 16.4	828 ± 333[b]	461 ± 202[e]	425 ± 144[e]
MCP-1, pg/mL BALF	2.27 ± 1.72	223 ± 84.0[b]	143 ± 54.3[e]	157 ± 88.5
TFG-β1[f]	n = 4	n = 7	n = 9	n = 5
Total TGF-β1, ng/lung	17.2 ± 2.64	48.8 ± 12.3[b]	33.3 ± 7.72[c]	28.1 ± 12.8[e]
Active TFG-β1[g]	n = 11	n = 7	n = 8	n = 9
Active TGF-β1, pg/lung	12.5 ± 2.53	19.3 ± 3.66[b]	14.1 ± 2.17[c]	9.97 ± 2.89[c]

Values are mean ± SD. ELISA = Enzyme-linked immunosorbent assay; KC = keratinocyte-derived chemokine; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; PC = phosphatidylcholine; PC-SOD = lecithinized human Cu/Zn-superoxide dismutase; TGF = transforming growth factor; TNF = tumor necrosis factor.

[a]Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on d 0. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) once or oral administration of pirfenidone (Pir, 400 mg/kg/d) twice on d 0. BALF was collected at d 1 and the total number of cells and number of neutrophils were determined as described in the "Materials and Methods" section.

[b]$P < .01$, vehicle vs BLM.

[c]$P < .01$, BLM vs BLM + PC or Pir.

[d]Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on d 0. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) once or oral administration of pirfenidone (Pir, 400 mg/kg/d) twice on d 0). BALF was collected at d 1 and the amounts of cytokines and chemokines were determined as described in the "Materials and Methods" section.

[e]$P < .05$, BLM vs BLM + PC or Pir.

[f]Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on d 0. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) once or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid for 14 d (from d 0 to d 13). The level of total TGF-β1 in pulmonary tissue on d 14 was determined by ELISA.

[g]Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on d 0. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) once or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid for 14 d (from d 0 to d 13). The level of active TGF-β1 in pulmonary tissue on d 14 was determined by ELISA.

not pirfenidone significantly decreased this peak (Figs 3B, 3C), suggesting that inhaled PC-SOD suppresses the bleomycin-induced production of superoxide anions in the lung.

The pulmonary level of TGF-β1 was measured using an enzyme-linked immunosorbent assay technique. As shown in Table 1, bleomycin treatment increased pulmonary total TGF-β1 and its active form only, and these increases were suppressed by simultaneous treatment of mice with PC-SOD or pirfenidone.

Effect of PC-SOD and Pirfenidone on Preexisting Fibrosis

We then tested the efficacy of PC-SOD and pirfenidone even when the treatment protocol was started after the development of fibrosis. The drug treatment was commenced 7 days after the administration of bleomycin, and pulmonary fibrosis and lung mechanics were assessed on day 14 (Fig 4A). We first confirmed the presence of pulmonary fibrosis on day 7 (data not shown). Treatment with PC-SOD or pirfenidone decreased the extent of pulmonary damage, pulmonary fibrosis, and lung elastance on day 14 (Figs 4B-4D), thus suggesting that PC-SOD and pirfenidone could be effective agents for the treatment of preexisting pulmonary fibrosis. We also examined the effect of combined treatment with PC-SOD and pirfenidone under the same experimental conditions; however, no additional effect was observed (Figs 4B-4D).

We then examined the effect of PC-SOD and pirfenidone on preexisting pulmonary fibrosis at a later stage. The drug treatment was commenced 14 days after the administration of bleomycin, and pulmonary fibrosis and lung mechanics were assessed on day 21 (Fig 5A). Although treatment with PC-SOD or pirfenidone produced a trend toward decreased pulmonary damage, pulmonary fibrosis, and lung elastance on day 21, this effect was not statistically significant (Figs 5B-5D). However, in this situation, treatment with a combination of the two drugs resulted in more pronounced amelioration of pulmonary fibrosis and lung mechanics than administration of either of the drugs alone (Figs 5B-5D). Finally, we examined FVC under the same experimental conditions (Fig 5A) and found that the combination of PC-SOD and pirfenidone suppressed decrease in FVC in a statistically significant manner (Fig 5E). Either of these drugs alone produced a trend toward ameliorated FVC (Fig 5E). These results suggest that PC-SOD and pirfenidone exert a synergistic effect on bleomycin-induced pulmonary fibrosis and lung dysfunction.

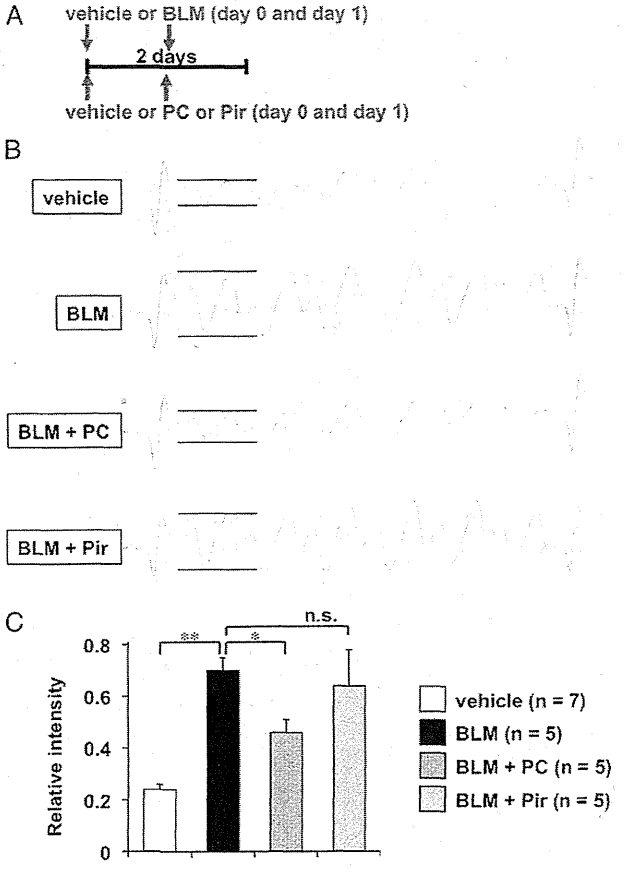

FIGURE 3. Effects of PC-SOD and pirfenidone on bleomycin-induced production of superoxide anion. A, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once daily on both day 0 and day 1. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) daily or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid on both day 0 and day 1. B, Cells in the BAL fluid were collected on day 2, incubated with a spin trap agent (DPhPMPO), and subjected to radical adduct electron spin resonance (ESR) spectrum analysis to determine the amount of superoxide anions present. C, 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 B) was determined. Values are mean ± SD. $*P < .05$; $**P < .01$. See Figure 1 and 2 legends for expansion of abbreviations.

DISCUSSION

SODs, and more particularly Cu/Zn-SOD, have attracted considerable attention as potential drugs for the treatment of IPF. This is because, of the three types of human SODs (Cu/Zn-SOD, mitochondrial manganese-SOD, and extracellular-SOD), Cu/Zn-SOD accounts for 80% of SOD activity within the lung.[33] However, the low stability of Cu/Zn-SOD in plasma and low affinity for tissue is an obstacle for its clinical use. PC-SOD, a derivative of SOD with higher stability in plasma and higher tissue affinity, offers an attractive alternative to Cu/Zn-SOD. In a phase 1 clinical study, IV administered PC-SOD (40-160 mg) had a terminal half-life of > 24 h, with good safety and tolerability.[34,35] A phase 2 clinical study showed that IV administered PC-SOD (40 or 80 mg) significantly

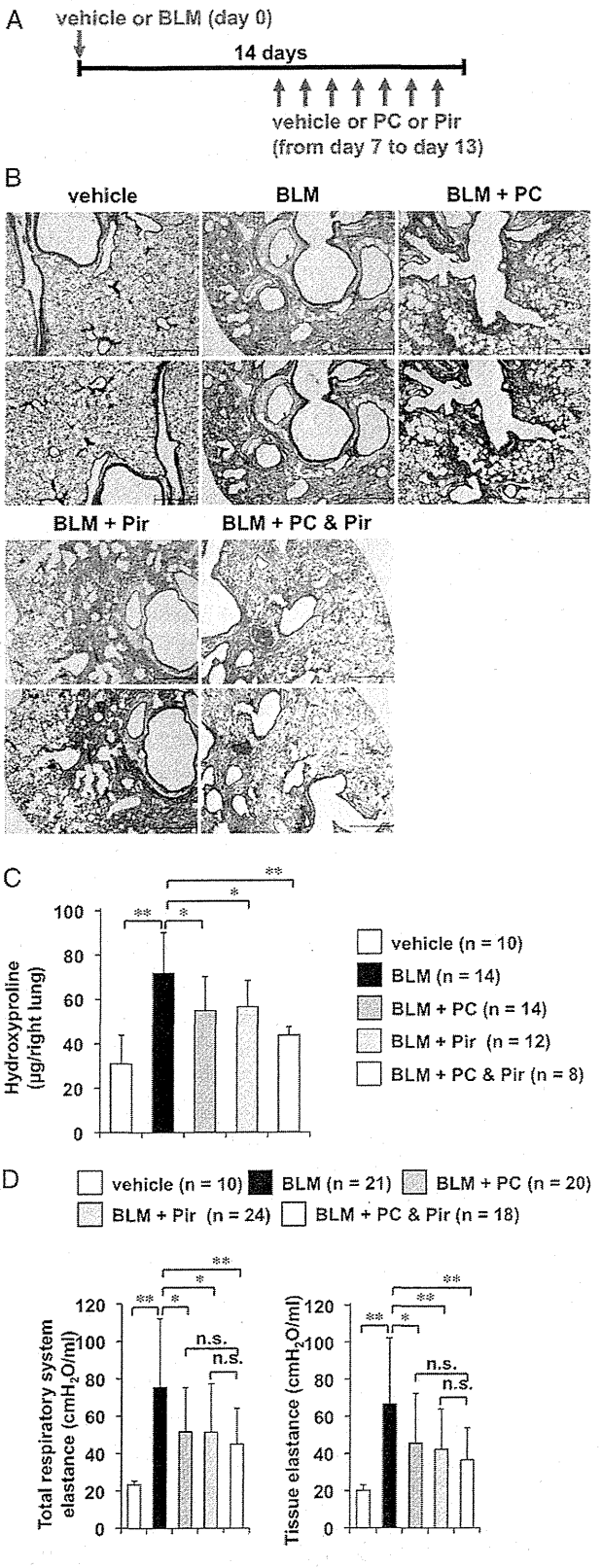

FIGURE 4. Therapeutic effects of PC-SOD and pirfenidone on bleomycin-induced pulmonary fibrosis and impaired lung mechanics. A, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were then subsequently treated by inhalation of PC-SOD (PC, 60 kU/chamber) daily and/or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid from day 7 to day 13. B, Histopathologic examination was done on day 14 as described in Figure 1 legend. C, Determination of pulmonary hydroxyproline level was done on day 14 as described in Figure 1 legend. D, Total respiratory system elastance and tissue elastance was done on day 14 as described in Figure 1 legend. Values are mean ± SD. $^{*}P < .05$; $^{**}P < .01$. Scale bar, 500 μm. See Figure 1 and 3 legends for expansion of abbreviations.

improved the symptoms of not only patients with ulcerative colitis[36] but also patients with IPF (Kamio K, Azuma A, Ohta K, et al, unpublished results, October 12, 2011). However, when considering the quality of life of patients, the present clinical protocol of PC-SOD administration based on daily IV infusion for 4 weeks needs to be improved. Given our recent finding that inhaled PC-SOD is effective against pulmonary fibrosis in mice,[22] we believe that inhalation of PC-SOD may provide a viable option for the treatment of patients with IPF. In this study, we performed a series of experiments to investigate the efficacy of this approach in an animal model.

We previously reported that intratracheal administration of PC-SOD suppressed the bleomycin-induced pulmonary inflammatory response.[22] In this study, we examined the effect of heat-denaturation of PC-SOD on this ameliorative effect. We confirmed that heat treatment of PC-SOD at 100°C for 60 min caused complete loss of SOD activity (data not shown). As shown in e-Table 1, intratracheal administration of intact PC-SOD but not that of heat-denatured PC-SOD suppressed bleomycin-induced increase in total number of leukocytes or the number of neutrophils in BALF. This result suggests that the ameliorative effect of PC-SOD on bleomycin-induced lung injury is mediated by its SOD activity but not by phospholipids in this drug.

As IPF is a disease that affects lung mechanics and respiratory function, it is important to examine the effect of candidate drugs on these parameters. Whereas in our previous study we only addressed the issue of pulmonary fibrosis,[22] the present results reveal that inhaled PC-SOD suppresses a bleomycin-induced increase in lung elastance and decrease in FVC and Spo_2, supporting the notion that such a treatment could be beneficial for the treatment of patients with IPF. We measured lung elastance or FVC, using a computer-controlled small-animal ventilator or this ventilator connected to a negative pressure reservoir. This invasive method is estimated to be accurate and reproductive; however, because this method requires anesthesia and causes death of the animal, this method could not measure these parameters under physiologic conditions.[30,37] In terms of clinical relevance, it is important to examine not only the preventive value of candidate compounds but also their therapeutic efficacy. Thus, we examined the effect of PC-SOD on preexisting pulmonary fibrosis

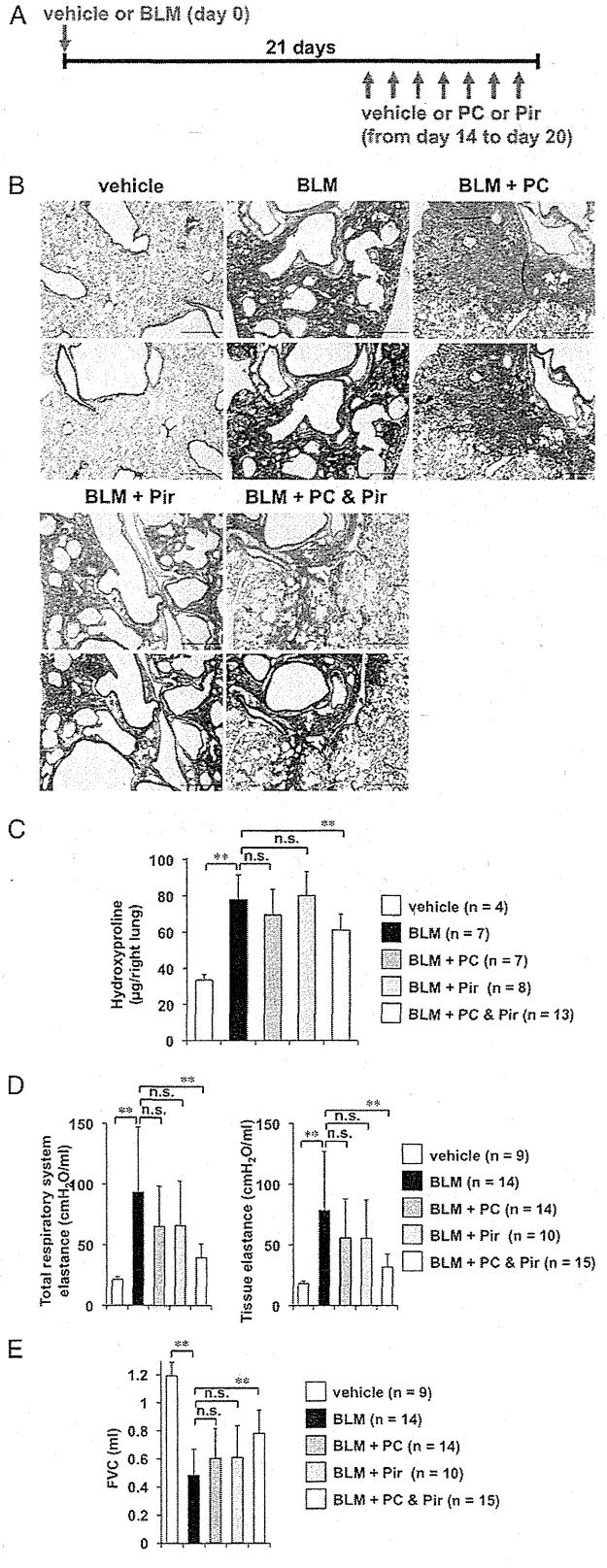

FIGURE 5. Synergistic effects of PC-SOD and pirfenidone on pre-existing pulmonary fibrosis. A, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were then subsequently treated by inhalation of PC-SOD (PC, 60 kU/chamber) daily and/or oral administration of pirfenidone (Pir, 400 mg/kg/d) bid from day 14 to day 20. B, Histopathologic examination was done at day 21 as described in Figure 1 legend. C, Determination of pulmonary hydroxyproline level was done at day 21 as described in Figure 1 legend. D, Total respiratory system elastance and tissue elastance was done at day 21 as described in Figure 1 legend. FVC was determined on day 21 as described in Figure 2E legend. Values are mean ± SD. $^{*}P < .05$; $^{**}P < .01$. Scale bar, 500 μm. See Figure 1 and 3 legends for expansion of abbreviations.

and again found that it was effective. It is interesting that the therapeutic effects were evident between days 7 and 13 but not between days 14 and 20. We consider that PC-SOD affects inflammatory responses rather than fibrosis.

In this study, we used pirfenidone as a control drug against which to evaluate the efficacy of PC-SOD, given that the beneficial effect of the former drug in patients with IPF has already been demonstrated in some clinical studies, and it has been approved in Japan and Europe for the treatment of IPF.[23,38,39] In terms of the various parameters examined (bleomycin-induced pulmonary fibrosis, increase in lung elastance, and decrease in FVC and Spo_2), PC-SOD produced equal or superior effects to pirfenidone, supporting the notion that PC-SOD could be clinically beneficial. It should be noted that, although effects of pirfenidone on bleomycin-induced pulmonary fibrosis in animals have been previously reported,[24,40] this is the first time that its effects on bleomycin-induced lung dysfunction are demonstrated in an animal model, thereby increasing our understanding of the mechanism governing the therapeutic effect of pirfenidone in patients with IPF.

When considering the mechanism regulating the ameliorative effects of PC-SOD and pirfenidone, we focused on ROS and TGF-β1, both of which play important roles in the pathogenesis of IPF. We have previously reported that inhaled PC-SOD suppresses the bleomycin-induced inflammatory response (as evidenced by an increase in the number of leukocytes in the BALF).[22] In this study, we confirmed this result and found that administration of PC-SOD to bleomycin-treated mice suppressed production of superoxide anions in cells present in the BALF, suggesting that the ameliorative effect of PC-SOD is mediated by its antioxidant activity. In contrast, administration of pirfenidone produced no significant effect, a finding that is inconsistent with the idea that this drug has antioxidant and antiinflammatory activity. This may be due to differences in experimental conditions and analytical methods.[41,42] On the other hand, administration of either PC-SOD or pirfenidone suppressed the bleomycin-induced increase in the pulmonary level of TGF-β1. In the case of pirfenidone, this is likely to represent direct suppression of the production of TGF-β1, as has been previously reported in cultured cells.[43] In contrast, the effect of PC-SOD may reflect its antioxidant activity, given

that it has been demonstrated that ROS stimulate the production of TGF-β1 in vitro.[44]

Oral administration of pirfenidone has been reported to slow disease progression in patients with IPF in some but not all studies, and the drug is licensed in Japan and recently in Europe as a treatment of IPF.[23,38,39] However, the Food and Drug Administration declined to approve pirfenidone because of inconclusive evidence of its clinical efficacy and severe side effects, such as photosensitivity in dermatitis, nausea, and anorexia.[23,45] Given that the outcomes of the present study suggest that the mechanism of action of pirfenidone is different from that of PC-SOD, combined treatment with the two drugs provides a potentially attractive option. This possibility is supported by our finding that PC-SOD and pirfenidone produce a synergistic therapeutic effect on bleomycin-induced pulmonary fibrosis and lung dysfunction. In conclusion, therefore, we consider that a regimen combining oral administration of pirfenidone with inhalation of PC-SOD may be therapeutically beneficial for the treatment of patients with IPF.

Acknowledgements

Author contributions: *Dr Tanaka:* contributed to analysis and interpretation of data and drafting the manuscript for important intellectual content.
Dr Azuma: contributed to drafting the manuscript for important intellectual content.
Mr Miyazaki: contributed to analysis and interpretation of data and drafting the manuscript for important intellectual content.
Dr Sato: contributed to analysis and interpretation of data and drafting the manuscript for important intellectual content.
Dr Mizushima: contributed to study conception and design and drafting the manuscript for important intellectual content.
Financial/nonfinancial disclosures: The authors have reported to *CHEST* the following conflicts of interest: Dr Azuma is a Steering Committee Member of Boehringer Ingelheim GmbH and Shionogi. Drs Tanaka, Sato, and Mizushima and Mr Miyazaki have reported that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.
Role of sponsors: The sponsor had no role in the design of the study, the collection and analysis of the data, or in the preparation of the manuscript.
Other contributions: We thank Shionogi & Co, Ltd (Osaka, Japan) and LTT Bio-Pharma Co, Ltd (Tokyo, Japan) for providing pirfenidone and PC-SOD, respectively.
Additional information: The e-Appendix and e-Table can be found in the "Supplemental Materials" area of the online supplement.

References

1. Nagai S, Kitaichi M, Hamada K, et al. Hospital-based historical cohort study of 234 histologically proven Japanese patients with IPF. *Sarcoidosis Vasc Diffuse Lung Dis*. 1999; 16(2):209-214.
2. Kim DS, Collard HR, King TE Jr. Classification and natural history of the idiopathic interstitial pneumonias. *Proc Am Thorac Soc*. 2006;3(4):285-292.
3. Raghu G, Collard HR, Egan JJ, et al; ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med*. 2011;183(6):788-824.
4. du Bois RM. Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug Discov*. 2010;9(2):129-140.
5. Maher TM, Wells AU, Laurent GJ. Idiopathic pulmonary fibrosis: multiple causes and multiple mechanisms? *Eur Respir J*. 2007;30(5):835-839.
6. Kinnula VL. Redox imbalance and lung fibrosis. *Antioxid Redox Signal*. 2008;10(2):249-252.
7. Sheppard D. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc*. 2006;3(5):413-417.
8. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*. 2005;436(7047):123-127.
9. Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol*. 2007;293(3):L525-L534.
10. Bartram U, Speer CP. The role of transforming growth factor beta in lung development and disease. *Chest*. 2004;125(2): 754-765.
11. Strausz J, Müller-Quernheim J, Steppling H, Ferlinz R. Oxygen radical production by alveolar inflammatory cells in idiopathic pulmonary fibrosis. *Am Rev Respir Dis*. 1990; 141(1):124-128.
12. Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG. Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. *J Clin Invest*. 1987;79(6):1665-1673.
13. Scheule RK, Perkins RC, Hamilton R, Holian A. Bleomycin stimulation of cytokine secretion by the human alveolar macrophage. *Am J Physiol*. 1992;262(4 pt 1):L386-L391.
14. Inghilleri S, Morbini P, Oggionni T, Barni S, Fenoglio C. In situ assessment of oxidant and nitrogenic stress in bleomycin pulmonary fibrosis. *Histochem Cell Biol*. 2006;125(6): 661-669.
15. Fattman CL, Chang LY, Termin TA, Petersen L, Enghild JJ, Oury TD. Enhanced bleomycin-induced pulmonary damage in mice lacking extracellular superoxide dismutase. *Free Radic Biol Med*. 2003;35(7):763-771.
16. Manoury B, Nenan S, Leclerc O, et al. The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. *Respir Res*. 2005;6:11.
17. Hoshino T, Nakamura H, Okamoto M, et al. Redox-active protein thioredoxin prevents proinflammatory cytokine- or bleomycin-induced lung injury. *Am J Respir Crit Care Med*. 2003;168(9):1075-1083.
18. Kruidenier L, Verspaget HW. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease—radicals or ridiculous? *Aliment Pharmacol Ther*. 2002;16(12): 1997-2015.
19. Igarashi R, Hoshino J, Takenaga M, et al. Lecithinization of superoxide dismutase potentiates its protective effect against Forssman antiserum-induced elevation in guinea pig airway resistance. *J Pharmacol Exp Ther*. 1992;262(3): 1214-1219.
20. Igarashi R, Hoshino J, Ochiai A, Morizawa Y, Mizushima Y. Lecithinized superoxide dismutase enhances its pharmacologic potency by increasing its cell membrane affinity. *J Pharmacol Exp Ther*. 1994;271(3):1672-1677.
21. Ishihara T, Tanaka K, Tasaka Y, et al. Therapeutic effect of lecithinized superoxide dismutase against colitis. *J Pharmacol Exp Ther*. 2009;328(1):152-164.
22. Tanaka K, Ishihara T, Azuma A, et al. Therapeutic effect of lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2010;298(3):L348-L360.

23. Maher TM. Pirfenidone in idiopathic pulmonary fibrosis. *Drugs Today (Barc)*. 2010;46(7):473-482.
24. Kakugawa T, Mukae H, Hayashi T, et al. Pirfenidone attenuates expression of HSP47 in murine bleomycin-induced pulmonary fibrosis. *Eur Respir J*. 2004;24(1):57-65.
25. Oku H, Shimizu T, Kawabata T, et al. Antifibrotic action of pirfenidone and prednisolone: different effects on pulmonary cytokines and growth factors in bleomycin-induced murine pulmonary fibrosis. *Eur J Pharmacol*. 2008;590(1-3): 400-408.
26. Mitani Y, Sato K, Muramoto Y, et al. Superoxide scavenging activity of pirfenidone-iron complex. *Biochem Biophys Res Commun*. 2008;372(1):19-23.
27. Pilling D, Roife D, Wang M, et al. Reduction of bleomycin-induced pulmonary fibrosis by serum amyloid P. *J Immunol*. 2007;179(6):4035-4044.
28. Davis IC, Lazarowski ER, Chen FP, Hickman-Davis JM, Sullender WM, Matalon S. Post-infection A77-1726 blocks pathophysiologic sequelae of respiratory syncytial virus infection. *Am J Respir Cell Mol Biol*. 2007;37(4):379-386.
29. Tanaka K, Tanaka Y, Miyazaki Y, et al. Therapeutic effect of lecithinized superoxide dismutase on pulmonary emphysema. *J Pharmacol Exp Ther*. 2011;338(3):810-818.
30. Shalaby KH, Gold LG, Schuessler TF, Martin JG, Robichaud A. Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness. *Respir Res*. 2010;11:82.
31. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. *Am J Pathol*. 2007;170(6):1807-1816.
32. Tanaka K, Tanaka Y, Namba T, Azuma A, Mizushima T. Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice. *Biochem Pharmacol*. 2010;80(6): 920-931.
33. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med*. 2003; 167(12):1600-1619.
34. Suzuki J, Broeyer F, Cohen A, Takebe M, Burggraaf J, Mizushima Y. Pharmacokinetics of PC-SOD, a lecithinized recombinant superoxide dismutase, after single- and multiple-dose administration to healthy Japanese and Caucasian volunteers. *J Clin Pharmacol*. 2008;48(2):184-192.
35. Broeyer FJ, van Aken BE, Suzuki J, et al. The pharmacokinetics and effects of a long-acting preparation of superoxide dismutase (PC-SOD) in man. *Br J Clin Pharmacol*. 2008;65(1): 22-29.
36. Suzuki Y, Matsumoto T, Okamoto S, Hibi T. A lecithinized superoxide dismutase (PC-SOD) improves ulcerative colitis. *Colorectal Dis*. 2008;10(9):931-934.
37. Vanoirbeek JA, Rinaldi M, De Vooght V, et al. Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases. *Am J Respir Cell Mol Biol*. 2010;42(1):96-104.
38. Azuma A, Nukiwa T, Tsuboi E, et al. Double-blind, placebo-controlled trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2005;171(9): 1040-1047.
39. Taniguchi H, Ebina M, Kondoh Y, et al; Pirfenidone Clinical Study Group in Japan. Pirfenidone in idiopathic pulmonary fibrosis. *Eur Respir J*. 2010;35(4):821-829.
40. Iyer SN, Wild JS, Schiedt MJ, Hyde DM, Margolin SB, Giri SN. Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. *J Lab Clin Med*. 1995; 125(6):779-785.
41. Giri SN, Leonard S, Shi X, Margolin SB, Vallyathan V. Effects of pirfenidone on the generation of reactive oxygen species in vitro. *J Environ Pathol Toxicol Oncol*. 1999;18(3):169-177.
42. Oku H, Nakazato H, Horikawa T, Tsuruta Y, Suzuki R. Pirfenidone suppresses tumor necrosis factor-alpha, enhances interleukin-10 and protects mice from endotoxic shock. *Eur J Pharmacol*. 2002;446(1-3):167-176.
43. Tokura T, Oku H, Tsukamoto Y. [Pharmacological properties and clinical effects of the antifibrotic agent pirfenidone (Pirespa) for treatment of idiopathic pulmonary fibrosis]. *Nippon Yakurigaku Zasshi*. 2009;134(2):97-104.
44. Bellocq A, Azoulay E, Marullo S, et al. Reactive oxygen and nitrogen intermediates increase transforming growth factor-beta1 release from human epithelial alveolar cells through two different mechanisms. *Am J Respir Cell Mol Biol*. 1999; 21(1):128-136.
45. Noble PW, Albera C, Bradford WZ, et al; CAPACITY Study Group. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. *Lancet*. 2011; 377(9779):1760-1769.

Biochemical Pharmacology 84 (2012) 1470–1481

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Identification of a unique nsaid, fluoro-loxoprofen with gastroprotective activity☆,☆☆

Shintaro Suemasu [a,b], Naoki Yamakawa [a,b], Tomoaki Ishihara [a], Teita Asano [a], Kayoko Tahara [a], Ken-ichiro Tanaka [a,b], Hirofumi Matsui [c], Yoshinari Okamoto [b], Masami Otsuka [b], Koji Takeuchi [d], Hidekazu Suzuki [e], Tohru Mizushima [a,b,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, Tokyo 105-8512, Japan
[b] Faculty of Life Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[c] Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8575, Japan
[d] Division of Pathological Sciences, Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Kyoto 607-8414, Japan
[e] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 6 August 2012
Accepted 18 September 2012
Available online 26 September 2012

Keywords:
NSAIDs
Gastrointestinal complications
Mucus

ABSTRACT

We previously proposed that direct cytotoxicity of NSAIDs due to their membrane permeabilization activity, together with their ability to decrease gastric prostaglandin E_2, contributes to production of gastric lesions. Compared to loxoprofen (LOX), fluoro-loxoprofen (F-LOX) has much lower membrane permeabilization and gastric ulcerogenic activities but similar anti-inflammatory activity. In this study, we examined the mechanism for this low ulcerogenic activity in rats. Compared to LOX, the level of gastric mucosal cell death was lower following administration of F-LOX. However, the gastric level of prostaglandin E_2 was similar in response to treatment with the two NSAIDs. Oral pre-administration of F-LOX conferred protection against the formation of gastric lesions produced by subsequent administration of LOX and orally administered F-LOX resulted in a higher gastric pH value and mucus content. In the presence of a stimulant of gastric acid secretion, the difference in the ulcerogenic activity of F-LOX and LOX was less apparent. Furthermore, an increase in the mucus was observed in gastric cells cultured in the presence of F-LOX in a manner dependent of increase in the cellular level of cAMP. These results suggest that low ulcerogenic activity of F-LOX involves its both low direct cytotoxicity and protective effect against the development of gastric lesions. This protective effect seems to be mediated through an increase in a protective factor (mucus) and a decrease in an aggressive factor (acid).

1. Introduction

The balance between aggressive and defensive factors determines the development of gastric lesions, with either a relative increase in aggressive factors or a decrease in protective factors resulting in lesions. The gastric mucosa can be challenged by a variety of both endogenous and exogenous factors, including gastric acid, reactive oxygen species, ethanol, *Helicobacter pylori* and non-steroidal anti-inflammatory drugs (NSAIDs) [1]. In order to protect the mucosa, the body relies on defence systems such as the production of surface mucus and bicarbonate, and the regulation of gastric mucosal blood flow. Prostaglandin E_2 (PGE_2) also exerts a strong protective effect, inhibiting the secretion of gastric acid and stimulating the production of mucus [2].

NSAIDs, such as indomethacin, comprise a therapeutically valuable family of drugs [3]. An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions, COX being an enzyme that is essential for

☆ We thank Dr. H. Sakai (University of Toyama, Toyama, Japan) for helpful discussion on the membrane preparation and enzyme assay.
☆☆ This work was supported by Grants-in-Aid of Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grants-in-Aid of the Japan Science and Technology Agency.

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH_4; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; COX, cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; EIA, enzyme immunoassay; ELLA, enzyme-linked lectin-binding assay; F-LOX, fluoro-loxoprofen; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H & E, hematoxylin and eosin; IBMX, 3-isobutyl-1-methylxanthine; LOX, loxoprofen; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; RGM1, rat normal gastric epithelial cell line; SD, Sprague-Dawley; SBA, soybean agglutinin; TCA, trichloroacetic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

* Corresponding author at: Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Tel./fax: +81 3 5400 2628.
E-mail address: mizushima-th@pha.keio.ac.jp (T. Mizushima).

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2012.09.016

the synthesis of prostaglandins, which have a strong capacity to induce inflammation. However, as described above, NSAID use is also associated with gastrointestinal complications [4–7], which was thought to result from the inhibition of COX and a decrease in gastric PGE_2 level. In fact, NSAIDs have been reported to stimulate the secretion of gastric acid and inhibit the production of mucus through decreasing gastric PGE_2 level [8,9]. However, it is now believed that the production of gastric lesions by NSAIDs involves additional mechanisms, given that the increased incidence of gastric lesions and the decrease in PGE_2 levels induced by NSAIDs do not always occur in parallel [10,11]. We have recently demonstrated that NSAIDs induce cell death (apoptosis) in cultured gastric mucosal cells and at the gastric mucosa in a manner independent of COX inhibition [12–16]. With regards to the molecular mechanism governing this apoptosis, we have proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx and increases intracellular Ca^{2+} levels, which in turn induces the endoplasmic reticulum stress response [12,17,18]. During the course of this response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor, is induced and, as we have previously shown, this protein is essential for NSAID-induced apoptosis [13,19]. Furthermore, we have proposed that both COX inhibition and gastric mucosal cell death are important for the formation of NSAID-induced gastric lesions *in vivo* [16,20].

In 1991, two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissues subject to inflammation, respectively, were identified [21]. It is therefore not surprising that a reduced incidence of gastroduodenal lesions has been reported following treatment with selective COX-2 inhibitors [22–24]. However, a recently raised issue concerning the use of selective COX-2 inhibitors is the potential risk of cardiovascular thrombotic events [25,26]. This may be due to the fact that prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 [27–29]. Therefore, in order to minimize clinical complications, gastric safe NSAIDs other than selective COX-2 inhibitors need to be developed. Based on the hypothesis outlined above, we believe that NSAIDs with lower membrane permeabilization activity would represent an efficacious alternative, even if they had no selectivity for COX-2 [14].

In order to investigate this possibility, we screened for such compounds from a range of clinically used NSAIDs without COX-2 selectivity, and found that the membrane permeabilization activity and direct cytotoxicity of loxoprofen (LOX) (Fig. 1A) was relatively lower than that of the other NSAIDs tested [30]. LOX is a leading NSAID on the Japanese market, being widely used because clinical studies have suggested that it is safer than other traditional (non-selective) NSAIDs [31,32]. LOX is a pro-drug, which is converted (by reduction of the cyclopentanone moiety) to its active metabolite (the *trans*-alcohol metabolite, LOX-OH) by aromatic aldehyde-ketone reductase (Fig. 1A) [33]. We therefore synthesized a series of LOX derivatives, demonstrating that fluoroloxoprofen (F-LOX) (Fig. 1A) has much lower membrane permeabilization and gastric ulcerogenic activities than LOX, but similar anti-inflammatory activity, suggesting that it is likely to be a therapeutically viable drug [34]. We suggested that F-LOX is also a pro-drug, which is converted to its active metabolite (the *trans*-alcohol metabolite, F-LOX-OH (Fig. 1A)), because the inhibitory effect of F-LOX-OH on COX is much more potent than that of F-LOX *in vitro* [34]. Although we concluded that the low membrane permeabilization activity of F-LOX is responsible for its low ulcerogenic activity [34], it remained possible that other mechanisms could also be involved. In this study, we therefore examined the mechanism governing the low ulcerogenic activity of F-LOX. Our results suggest that this effect is mediated not only by the low direct cytotoxicity due to its low membrane permeabilization activity, but also by protection of the gastric mucosa. In contrast to LOX and other NSAIDs, oral administration of F-LOX led to an increase in gastric pH value and mucus, suggesting that these effects are also involved in the low ulcerogenic activity of this drug.

2. Experimental procedures

2.1. Chemicals and animals

LOX, LOX-OH, F-LOX and F-LOX-OH (Fig. 1A) were synthesized in our laboratory as previously described [34]. Methylcellulose and RPMI1640 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Formaldehyde, paraformaldehyde, Alcian blue 8GX, mucin, cycloheximide, histamine, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile (SCH 28080; an inhibitor of gastric H^+,K^+-ATPase), 3-isobutyl-1-methylxanthine (IBMX), omeprazole, forskolin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Mayer's hematoxylin, 1% eosin alcohol solution and mounting medium for histochemical analysis (malinol) were from MUTO Pure Chemicals (Tokyo, Japan). Terminal transferase was obtained from Roche Diagnostics (Mannheim, Germany). Biotin 14-ATP and streptavidin-conjugated Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA). Mounting medium for the TdT-mediated biotinylated UTP nick end labeling (TUNEL) assay (VECTASHIELD) was from Vector Laboratories, Inc. (Burlingame, CA). 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) were obtained from Dojindo (Kumamoto, Japan). SQ22536 was from CALBIOCHEM (San Diego, CA). ONO-8711 and ONO-AE2-227 were from our laboratory stocks. The prostaglandin E_2 enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI). cAMP complete ELISA kit was from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase-labeled soybean agglutinin (SBA-HRP) was from Seikagaku Biobusiness Co. (Tokyo, Japan). 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH_4 (ABTS) was obtained from KPL (Gaithersburg, MD). The RNeasy Mini kit was from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was obtained from Takara (Kyoto, Japan), and iQ SsoFast EvaGreen Supermix was purchased from Bio-Rad (Hercules, CA). Wistar rats (3-week-old males) and Sprague-Dawley (SD) rats (6-week-old males) were obtained from Charles River Laboratories Japan (Yokohama, Japan). Guinea pigs (3-week-old males) were obtained from Japan SLC (Shizuoka, Japan). Animals were housed under conditions of controlled temperature (22–24 °C) and illumination (12 h light cycle starting at 8:00 AM) for 1 week before experiments. The experiments and procedures described here were performed 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 Keio University and Kumamoto University. Totally, we used 287 Wistar rats, 182 SD rats and 4 guinea pigs for all experiments in this study.

2.2. Gastric and small intestinal damage assay

The gastric ulcerogenic response was examined as described previously [20,35] with some modifications. Wistar or SD rats fasted for 18 h were orally administered each NSAID and, after 8 h or 4 h, respectively, the animals were sacrificed, their stomachs were removed, and the gastric mucosal lesion area measured by an observer unaware of the treatment that the animals had received. For Wistar rats, calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the

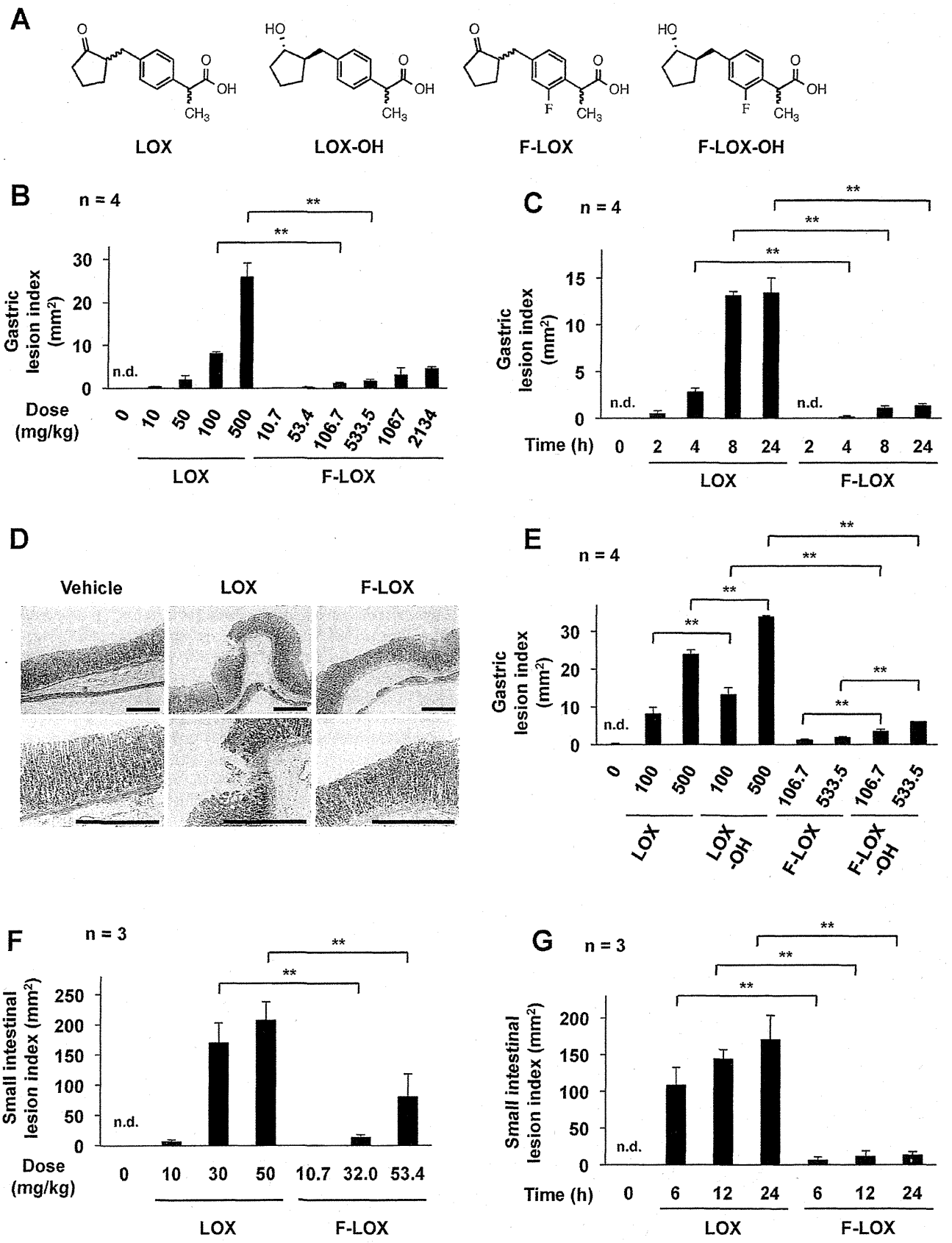

Fig. 1. Production of lesions in the stomach and small intestine by oral administration of F-LOX or LOX. Structures of drugs used in this study were shown (A). Wistar rats were orally administered either the indicated doses (B, E, –F), 200 or 213.4 mg/kg (C and D), or 30 or 32.0 mg/kg (G) of LOX or F-LOX, respectively. Rats were similarly administered LOX-OH or F-LOX-OH (E). Their stomachs (B–E) or small intestines (F, G) were then removed either 8 h later (B, D, E), at the indicated time-points (C, G), or 24 h later (F). The stomach and small intestine were scored for damage (B, C, E, F and G) and sections of gastric tissues were prepared and subjected to H & E staining. Images are magnified 2.5 times (lower panels) (D). Values are mean ± S.E.M. $^{**}P < 0.01$; n.d., not detected. Scale bar, 50 μm.

values to give an overall lesion index. For SD rats, calculation of the scores involved measuring the length of all the lesions in millimeters and summing the values to give an overall gastric lesion index.

The gastric PGE_2 level was determined by EIA according to the manufacturer's instructions.

The intestinal ulcerogenic response was examined as described previously [36,37], with some modifications. LOX or F-LOX was orally administered to unfasted rats and the animals were sacrificed 24 h later. Both the jejunum and ileum were removed and treated with formalin for fixation. Samples were opened along the antimesenteric attachment and the areas of the small intestinal lesions were measured by an observer unaware of the treatment that the animals had received. Calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the values to give an overall small intestinal lesion index.

2.3. Histochemical analysis and TUNEL assay

Wistar rats which had been fasted for 18 h were orally administered LOX or F-LOX and, 8 h later, the animals were sacrificed and their stomachs were removed. Samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4-μm sections.

For histological examination (hematoxylin and eosin (H&E) staining), the sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. The samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope (Tokyo, Japan).

For the TUNEL assay, the sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with TdT and biotin 14-ATP for 1 h at 37 °C, and finally with streptavidin-conjugated Alexa Fluor 488 for 1 h. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (KEYENCE BIOREVO, Osaka, Japan).

2.4. Measurement of gastric content volume, gastric pH value and contents of gastric mucin

The gastric pH value and mucus level were measured as previously described [38–40]. SD rats which had been fasted for 18 h were orally administered LOX or F-LOX and, 1 h later, the abdomen was opened and the pylorus was ligated under ether anesthesia. Three hours later, the animals were killed by deep ether anesthesia, the stomach was removed, the gastric contents were collected and its volume was determined. The gastric contents titrated with 10 mM NaOH to pH 7.0 using Twin pH (Horiba, Kyoto, Japan). The gastric acid output was calculated based on the volume of 10 mM NaOH required for neutralization and the gastric content volume.

For determination of mucus content, the gastric contents were incubated with 0.4 mg/ml (final concentration) Alcian blue 8GX for 24 h at 20 °C and then centrifuged. The concentration of Alcian blue in the supernatant was estimated by measuring the optical density at 615 nm. The amount of mucus adhering to the gastric mucosa was also determined, and the sum of the two values used as a measure of the total mucus content.

2.5. Real-time RT-PCR analysis

Total RNA was extracted from gastric tissues and cells using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg of RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Bio-Rad Chromo 4 system) experiments using SsoFast EvaGreen Supermix, and analyzed with Opticon Monitor software according to the manufacturer's instructions. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin cDNA was used as an internal standard.

Primers were designed using the Primer3 website. The primers used were (name, forward primer and reverse primer): *muc1*, 5′-agagaccgctactgccattg-3′ and 5′-cagctggacctctttccaac-3′; muc5ac, 5′-aactctgcccaccacaagc-3′ and 5′-tgccatctatccaatcagtccaat-3′; *muc6*, 5′-tgctgtctccagcacaaaac-3′ and 5′-tcagaagtctgcgtcactgc-3′; *gapdh*, 5′-atgtatccgttgtggatctgac-3′ and 5′-cctgcttcaccaccttcttg-3′; *actin*, 5′-gtcgtaccactggcattgtg-3′ and 5′-gctgatcttgcccttgagac-3′.

2.6. Cell culture

A rat normal gastric epithelial cell line (RGM1) [41] was provided by the Riken Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham containing 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin on plastic culture plates without collagen coating. Cells were exposed to LOX or F-LOX by replacement of the entire bathing medium. The cAMP level in cells was measured by EIA according to the manufacture's instructions.

2.7. Determination of mucin level in culture medium

The amount of mucin in the culture medium was determined by an enzyme-linked lectin-binding assay (ELLA) as described previously [42]. The culture medium was loaded on polystyrene 96-well ELISA plates (Iwaki) and incubated at 4 °C for 12 h. After washing, each well was incubated with blocking buffer (1% BSA in phosphate-buffered saline) for 2 h at 37 °C. After washing, each well was incubated with SBA solution (1 μg/ml SBA-HRP (lectin) in phosphate-buffered saline) for 1 h at 37 °C. After further washing, each well was finally incubated with 100 μl ABTS solution (1 mM ABTS, 0.1 M citrate buffer (pH 4.0) and 0.03% hydrogen peroxide) for 15 min at room temperature. The optical density at 405 nm was then measured.

2.8. Determination of activities of H^+,K^+-ATPase and adenylate cyclase in membrane fraction

Cytoplasmic membrane fractions were prepared from guinea pig gastric mucosa as described previously [43]. Briefly, the fundic region of the mucosa was scraped and homogenized in 5 mM Tris-HCl (pH 7.4) buffer containing 250 mM sucrose and 1 mM EGTA. The suspension was centrifuged at 800 × *g* for 10 min, and the resultant supernatant was further centrifuged at 100, 000 × *g* for 90 min. The pellet was re-suspended in PBS and used for the assay as membrane fraction.

H^+,K^+-ATPase activity was measured as described previously [44]. Briefly, membrane fraction (100 μg protein) was diluted with 40 mM Tris-HCl (pH 6.8) buffer containing 3 mM $MgSO_4$, 15 mM KCl, 5 mM NaN_3 and 2 mM ouabain and pre-incubated with each tested chemical in the presence or absence of 50 μM SCH 28080 for 30 min at 37 °C. Omeprazole was activated by treatment with Tris-PIPES (pH 5.7) buffer before this incubation. Then, ATP solution (1 mM at the final concentration) was added, incubated for 10 min at 37 °C and the reaction was terminated by the addition of ice-cold stop solution (12% perchloric acid and 3.6% ammonium molybdate). Inorganic phosphate released was measured as previously described [45]. The H^+,K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of SCH 28080.

Adenylate cyclase activity was measured as described previously [46]. Briefly, membrane fraction (100 μg protein, 75 μl) was pre-incubated with each tested chemical in 96 well plates for 5 min at room temperature. Then 25 μl adenylate cyclase assay buffer (final concentrations; 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.25 mM ATP and 1 mM IBMX) was added, incubated for 30 min at 37 °C and the reaction was terminated by the addition of 100 μl 0.2 N HCl. The cAMP level was measured by EIA according to the manufacture's instructions.

2.9. Statistical analysis

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

3. Results

3.1. Comparison between the ulcerogenic response of F-LOX and LOX

The development of gastric lesions following oral administration of F-LOX and LOX to Wistar rats was compared. LOX produced gastric lesions in a dose-dependent manner but F-LOX produced fewer lesions (Fig. 1B). The level of gastric lesions caused by 2134 mg/kg F-LOX (corresponding to 2000 mg/kg LOX, in terms of number of molecules) was lower than that observed with 100 mg/kg LOX, demonstrating that the ulcerogenic activity of F-LOX was less than one-twentieth that of LOX. The time-course for the effect of the two NSAIDs was similar (Fig. 1C), showing that the difference in ulcerogenic activity illustrated in Fig. 1B was not a function of time. Histochemical analysis of gastric sections also revealed that F-LOX caused less gastric mucosal damage than LOX (Fig. 1D). We also examined the ulcerogenic activity of LOX-OH and F-LOX-OH. As shown in Fig. 1E, the ulcerogenic activity of F-LOX-OH was much lower than that of LOX-OH and the activity of LOX-OH or F-LOX-OH was a little more potent than that of LOX or F-LOX, respectively.

We also compared the production of lesions in the small intestine. As shown in Fig. 1F and G, oral administration of F-LOX produced fewer lesions than LOX at all examined time-points. However, the difference between the effect of the two drugs in this case was not as marked as that seen for gastric lesions (Fig. 1B and F).

Both gastric mucosal cell death and a decrease in the gastric level of PGE_2 have been shown to play an important role in the production of gastric lesions by NSAIDs, leading us to compare these processes after oral administration of F-LOX and LOX. The number of TUNEL-positive gastric mucosal cells (level of cell death) was lower in the F-LOX-treated rats (Fig. 2A and B). However, both the dose-response and time-course profiles of the gastric PGE_2 level were similar between the two treatment groups (Fig. 3A and B), suggesting that mucosal cell death rather than gastric PGE_2 level is involved in the lower ulcerogenic activity of F-LOX.

To test this idea, we compared the production of gastric lesions between F-LOX-treated and LOX-treated rats in which the gastric PGE_2 level had been lowered by pre-administration of aspirin (10 mg/kg). This regimen reduced PGE_2 to a negligible level, with subsequent administration of F-LOX or LOX having no effect (Fig. 3 C). As shown in Fig. 3D, the production of gastric lesions in the two treatment groups of rats pre-administered with aspirin was similar to that of control rats, supporting the idea that gastric PGE_2 level is not responsible for the lower ulcerogenic activity of F-LOX compared with LOX.

3.2. Protective effect of orally administered F-LOX on the gastric mucosa

The lower ulcerogenic activity of F-LOX was also observed in SD rats (Fig. 4A), which we used in the following experiments due to

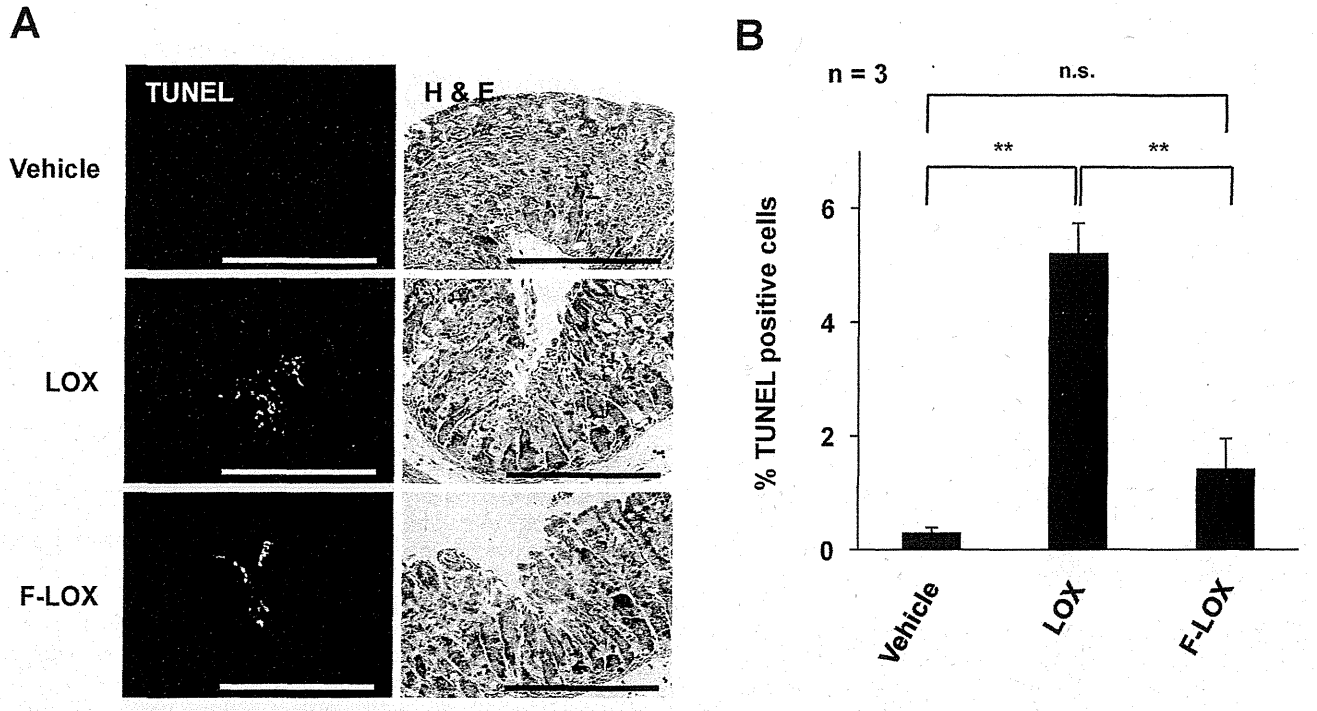

Fig. 2. Gastric mucosal cell death following oral administration of F-LOX or LOX. Wistar rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and their stomachs were removed 8 h later. A, sections of gastric tissues were prepared and subjected to TUNEL assay, DAPI staining and H & E staining. B, the ratio of TUNEL-positive cells to total cells was determined. Values are mean ± S.E.M. $^{**}P < 0.01$; n.s., not significant. Scale bar, 50 μm.

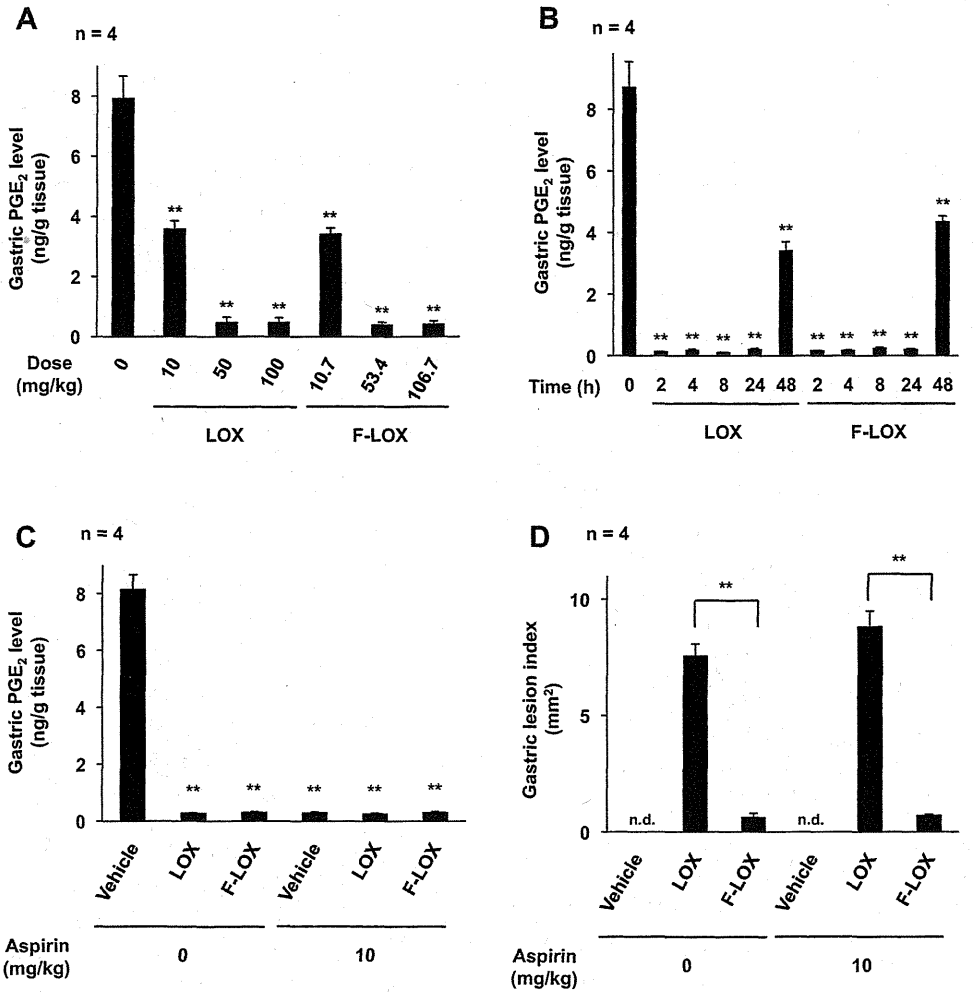

Fig. 3. Decrease in gastric PGE2 level in response to oral administration of F-LOX or LOX. Wistar rats were orally administered either the indicated doses (A), 200 or 213.4 mg/kg (B), or 100 or 106.7 mg/kg (C, D) of LOX or F-LOX, respectively. Rats were orally pre-administered the indicated dose of aspirin, 1 h before NSAID administration (C, D). The stomach was removed either 8 h (A, C, D) or at the indicated time-points (B) after the administration of LOX or F-LOX. A-C, the gastric PGE_2 level was determined by EIA. D, the stomach was scored for damage. Values are mean ± S.E.M. $^{**}P < 0.01$; n.d., not detected.

the availability of established protocols for monitoring gastric pH and mucus level in this strain.

We found that there was no significant difference in the gastric lesions produced by subcutaneous administration of F-LOX and LOX (Fig. 4B). In contrast to treatment with LOX, subcutaneous administration of F-LOX produced more gastric lesions than oral administration (Fig. 4B), although the gastric PGE_2 level in both cases was similar to that obtained following oral administration of the drugs (Fig. 4 C). Surprisingly, we found that oral pre-administration of F-LOX suppressed the production of gastric lesions induced by subsequent oral administration of LOX (Fig. 4D). A similar protective effect of F-LOX was observed for indomethacin-induced gastric lesions (data not shown). As shown in Fig. 4E and F-LOX-OH also showed such a protective effect against LOX-induced gastric lesions. We also found that this protective effect of F-LOX did not occur following its subcutaneous administration (data not shown). These results suggest that the direct interaction of F-LOX (at high concentrations) with the gastric mucosa is somehow protective against NSAID-induced gastric lesions.

3.3. Mechanism for the protective effect of F-LOX on the gastric mucosa

To understand the mechanism responsible for the protective effect of F-LOX, we examined the outcome of oral administration of F-LOX or LOX on gastric levels of aggressive (acid) and defensive (mucus) factors. LOX lowered the gastric pH and mucus content (Fig. 5A), phenomena that have been reported with various NSAIDs [8,9]. Surprisingly, oral administration of F-LOX shifted these indexes in the opposite direction, elevating the gastric pH value and mucus content (Fig. 5A). However, subcutaneous administration of F-LOX produced no such effects; subcutaneous administration of both LOX and F-LOX lowered the gastric pH value and mucus content (Fig. 5B).

To understand the mechanism responsible for the increase in the gastric pH value after oral administration of F-LOX, we measured the gastric content volume and determined the gastric acid output. As shown in Fig. 5A, oral administration of F-LOX but not that of LOX increased the gastric content volume, however, both of these drugs similarly increased the gastric acid output. On the other hand, subcutaneous administration of both LOX and F-LOX increased the gastric acid output but did not affect the gastric content volume (Fig. 5B). These results suggest that oral administration of F-LOX increases the gastric pH value through increasing the gastric content volume rather than decreasing the gastric acid output. Supporting this notion, we found that neither LOX nor F-LOX affected the H^+,K^+-ATPase activity in membrane fraction prepared from guinea pig gastric mucosa (Fig. 5C).

To test the contribution of the F-LOX-dependent increase in gastric pH value to the low ulcerogenic activity of this NSAID, we

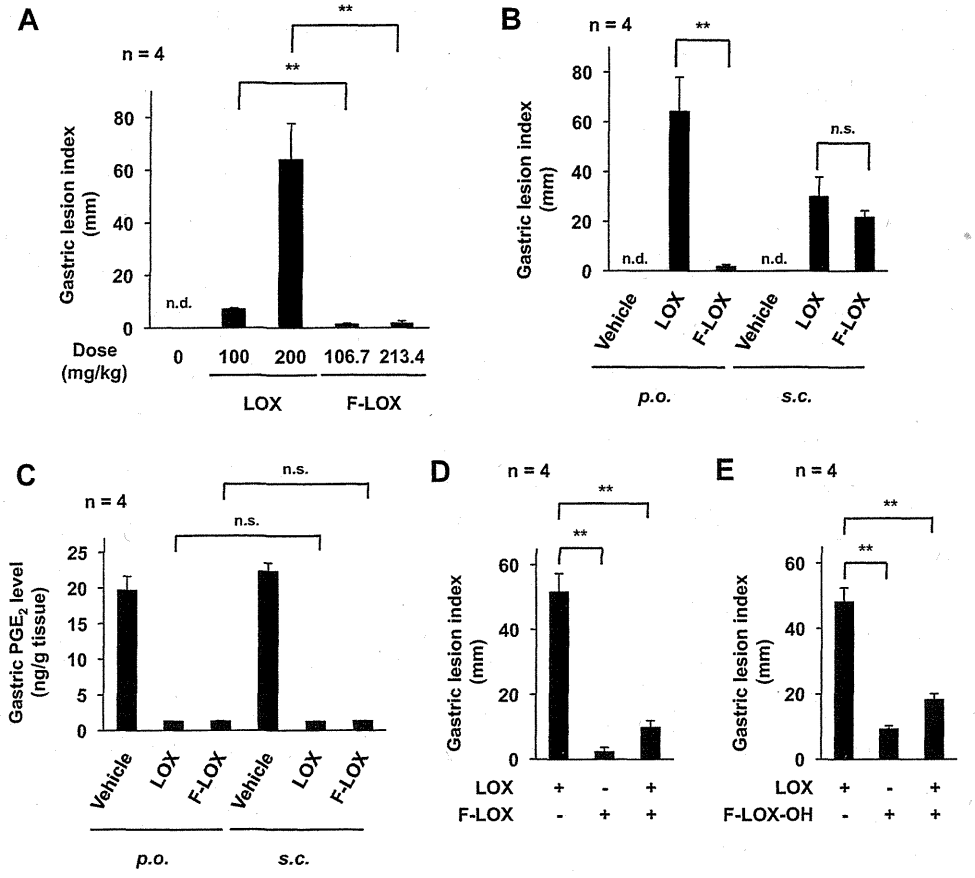

Fig. 4. Protective effect of orally administered F-LOX on the gastric mucosa. (A) SD rats were orally administered the indicated doses of LOX or F-LOX. (B and C) SD rats were orally (*p.o.*) or subcutaneously (*s.c.*) administered 200 or 213.4 mg/kg of LOX or F-LOX, respectively. (D and E) SD rats were orally pre-administered 106.7 mg/kg F-LOX (D) or F-LOX-OH (E), 1 h after which they were orally administered 200 mg/kg of LOX. A, B, D and E, the stomach was removed 4 h after the final administration of the NSAID and scored for damage. C, the gastric PGE_2 level was determined by EIA 4 h after the administration of LOX. Values are mean ± S.E.M. $^{**}P < 0.01$; n.s., not significant; n.d., not detected.

examined the effect of a stimulator of gastric acid secretion (histamine). Pre-administration of 5 mg/kg histamine decreased the pH value in both the presence and absence of subsequent oral administration of F-LOX or LOX (Fig. 6A), as a result of which the extent of the difference in the gastric pH value between the F-LOX- and LOX-treated groups became less apparent (Fig. 6A). As shown in Fig. 6B, F-LOX produced fewer gastric lesions than LOX, even following pre-administration of histamine; however, the extent of this difference was less marked than in the absence of histamine treatment. These results suggest that the higher gastric pH value observed after oral administration of F-LOX is partially responsible for the lower ulcerogenic activity of this NSAID compared with LOX.

3.4. Mechanism for the stimulative effect of F-LOX on the production of mucus

We next examined the effect of oral administration of F-LOX or LOX on the expression levels of mRNAs corresponding to mucin proteins. As shown in Fig. 7A, oral administration of F-LOX or LOX either up-regulated or down-regulated, respectively, the mRNA expression of the *muc1*, *muc5ac* and *muc6* genes (although the down-regulation of *muc6* mRNA by LOX was not statistically significant). We also examined the effect of the two NSAIDs on the production and secretion of mucin *in vitro*, using a rat normal gastric epithelial cell line (RGM1 cells). Treatment of these cells with F-LOX or LOX increased or decreased, respectively, the mucin content in the culture medium (Fig. 7B). Treatment of cells with F-LOX-OH also increased the mucin content in the culture medium (data not shown). To detect the secretion of pre-produced mucin, we examined the effect of F-LOX on the mucin secretion from cells whose protein synthesis was inhibited. Even in this situation, an increase in the mucin content in the culture medium was observed following pre-treatment with a protein synthesis inhibitor, cycloheximide (Fig. 7C), suggesting that the secretion of mucin was stimulated by F-LOX treatment of the cells. On the other hand, treatment of the cells with F-LOX up-regulated the expression levels of mRNAs corresponding to mucin proteins (Fig. 7D). Although there was a trend towards down-regulation of the mRNA expression of these genes following LOX treatment, this effect was not statistically significant (Fig. 7D). Taken together, these results suggest that direct interaction of F-LOX with the gastric mucosa directly stimulates the production and secretion of mucin.

Finally, we addressed the molecular mechanism governing these phenomena. PGE_2 stimulates the production of mucus through both EP_1 and EP_4 receptors [47]. As shown in Fig. 8A, pre-treatment of RGM1 cells with antagonists for EP_1 and EP_4 receptors suppressed PGE_2-induced production of mucin but not F-LOX-induced one (Fig. 8A). EP receptor subtypes are coupled to different intracellular signaling pathways. The EP_1 receptor is coupled to Ca^{2+} mobilization and activation of EP_4 receptor causes activation of adenylate cyclase activity and an increase in the cellular level of cAMP [48]. Thus, we examined the effect of an intracellular Ca^{2+} chelator that is permeable for cytoplasmic membranes (BAPTA-AM) or an inhibitor of adenylate cyclase (SQ22536) on F-LOX-induced production of mucin. As shown in Fig. 8B, pre-treatment of RGM1 cells with SQ22536 but not with BAPTA-AM

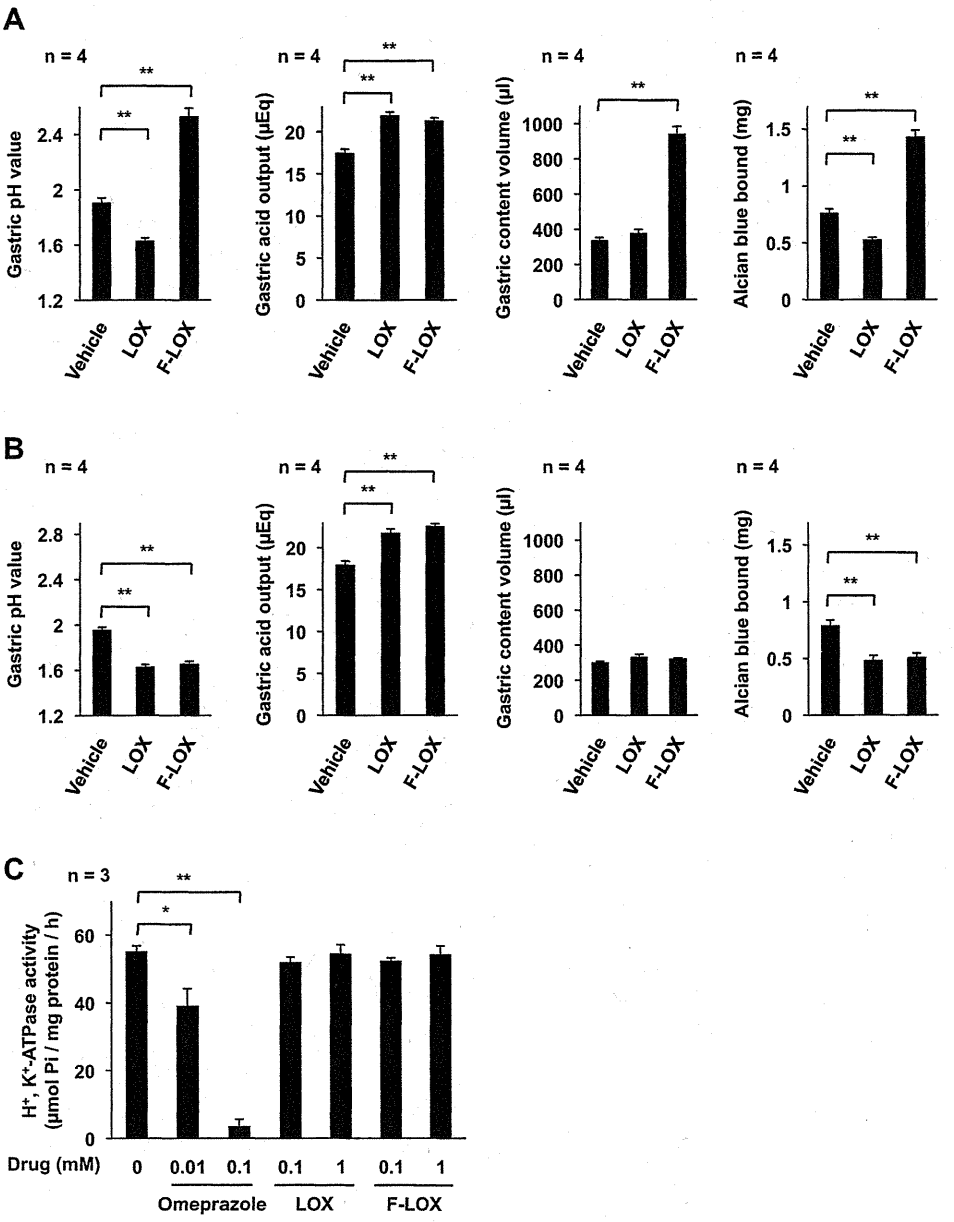

Fig. 5. Effect of F-LOX and LOX on gastric pH and mucus content. SD rats were orally (A) or subcutaneously (B) administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, 1 h after which the pylorus was ligated and the rats were maintained for a further 3 h. The gastric pH value, gastric acid output, gastric content volume and the amount of mucus in the gastric contents were measured as described in the experimental procedures. (C) Activity of H^+,K^+-ATPase in membrane fraction prepared from guinea pig gastric mucosa was measured in the presence of indicated concentrations of omeprazole (an inhibitor of H^+,K^+-ATPase), LOX or F-LOX as described in the experimental procedures. Values are mean ± S.E.M. $^{**}P < 0.01$; $^{*}P < 0.05$.

suppressed F-LOX-induced production of mucin. We also found that treatment of RGM1 cells with F-LOX increased the cellular level of cAMP to the extent similar to that induced by PGE_2 (Fig. 8C). Since neither LOX nor F-LOX activated adenylate cyclase activity in membrane fraction prepared from guinea pig gastric mucosa (Fig. 8D), F-LOX seems to activate adenylate cyclase indirectly. Furthermore, pre-treatment of cells with SQ22536 suppressed not only PGE_2- but also F-LOX-induced expression levels of mRNAs corresponding to mucin proteins (Fig. 8E). Results in Fig. 8 suggest that direct interaction of F-LOX with the gastric mucosa increases the level of mucin through increase in the cellular level of cAMP.

4. Discussion

F-LOX is a derivative of LOX, which is a leading NSAID on the Japanese market due to its relatively lower incidence of gastrointestinal complications than other traditional NSAIDs [31,32]. The results of a previous study, in which we demonstrated that, compared to LOX, F-LOX has lower ulcerogenic activity but similar anti-inflammatory activity, led us to propose that F-LOX is likely to represent a safer NSAID. However, before this compound can be developed for clinical use, it is first necessary to understand the molecular mechanism governing its low ulcerogenic activity.

In order to address this, we initially determined the dose-response profile of F-LOX, which revealed that its ulcerogenic activity is less than one-twentieth that of LOX when orally administered. Furthermore, the fact that the time-course for the production of gastric lesions observed with F-LOX was similar to that obtained with LOX demonstrated that the former NSAID has lower rather than slower ulcerogenic activity.

We have previously suggested that both gastric mucosal cell death due to the membrane permeabilization activity of NSAIDs and a decrease in the gastric level of PGE_2 due to COX inhibition are

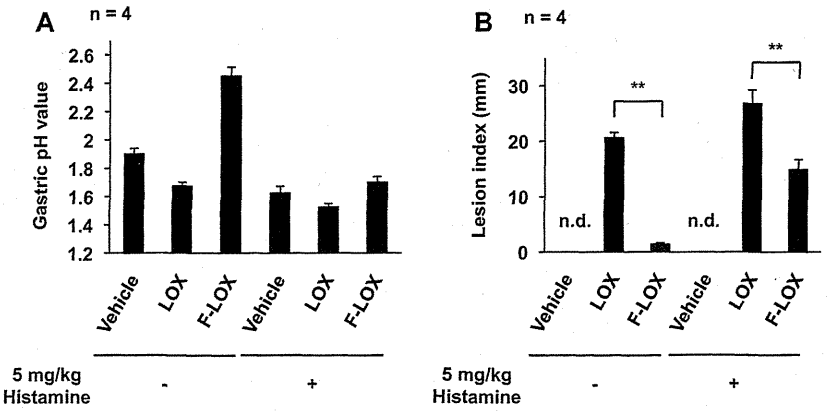

Fig. 6. Effects of pre-administration of histamine on the production of gastric lesions by F-LOX and LOX. SD rats were orally pre-administered the indicated dose of histamine, 1 h after which they were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively. The stomach was then removed 4 h later. A, gastric pH value was determined as described in the legend of Fig. 5. B, the stomach was scored for damage. Values are mean ± S.E.M. $^{**}P < 0.01$; n.d., not detected.

key factors in the production of gastric lesions *in vivo* [16,20]. Based on this hypothesis, NSAIDs without membrane permeabilization activity or those without the ability to decrease the gastric level of PGE_2 (such as COX-2 selective NSAIDs) would represent a therapeutically beneficial option. Among the clinically used NSAIDs that we tested, LOX had the weakest membrane permeabilization activity [30] and, among its derivatives, F-LOX had the weakest such activity [34]. Therefore, it is not surprising that in the current study less gastric mucosal cell death was observed with F-LOX than LOX. Furthermore, as neither LOX nor F-LOX display COX-2 selectivity [34], it is not unexpected that F-LOX decreased the gastric level of PGE_2 to a similar extent to LOX. We also showed that the lower ulcerogenic activity of F-LOX occurred even in the presence of an inhibitor of prostaglandin synthesis (aspirin). Taken together these results led us to conclude that the lower ulcerogenic activity of F-LOX compared with LOX involved gastric mucosal cell death rather than the gastric level of PGE_2.

In contrast to the above findings following oral NSAID administration, the ulcerogenic activities of F-LOX and LOX were indistinguishable when the drugs were administered subcutaneously. It is known that most NSAIDs produce more gastric lesions when administered orally rather than subcutaneously, which is in accordance with what we observed in the case of LOX in the present study. However, F-LOX produced more gastric lesions following subcutaneous administration than following oral administration of the drug. By way of explanation for the opposite effect of F-LOX, we consider a possibility that direct interaction of this NSAID with the gastric mucosa somehow confers a protective effect, but that this protection requires relatively higher concentrations of F-LOX, which can only be achieved by its oral administration. In support of this idea, we found that oral but not subcutaneous pre-administration of F-LOX protected against the formation of gastric lesions induced by subsequent administration of LOX or indomethacin.

A possible explanation for the lower ulcerogenic activity of F-LOX with oral administration than that with subcutaneous administration is that F-LOX but not its active metabolite (F-LOX-OH) confers a protective effect, because we recently found that the conversion of F-LOX to F-LOX-OH occurred very rapidly and the serum level of F-LOX-OH peaked within 1 h after either oral or intravenous administration of F-LOX [49]. However, this idea was ruled out by following observations in this study; the ulcerogenic activity of F-LOX-OH was much lower than that of LOX with their oral administration; oral pre-administration of F-LOX-OH also suppressed the production of gastric lesions induced by subsequent oral administration of LOX; as well as F-LOX (see below), F-LOX-OH increased the level of mucin in culture medium *in vitro*.

In order to understand the molecular mechanism governing the protective activity of F-LOX, we examined the effect of its oral administration on gastric levels of aggressive (acid) and defensive (mucus) factors, demonstrating that the gastric pH value and mucus content were higher following oral administration of the drug. This result is surprising because it is known that PGE_2 inhibits the secretion of acid and stimulates the production of mucus, and therefore that NSAIDs affect these responses in the opposite direction through their inhibitory effects on COX and prostaglandin synthesis [8,9]. This was reflected by the finding that both oral and subcutaneous administration of LOX caused a decrease in gastric pH and mucus. Similarly, in contrast to its oral administration, subcutaneous administration of F-LOX lowered the gastric pH and mucus content. However, the F-LOX-dependent decrease in the gastric level of PGE_2 was indistinguishable following oral and subcutaneous administration, suggesting that orally administered F-LOX, in other words, direct interaction of relatively higher concentrations of F-LOX with the gastric mucosa exerts its protective effects through a COX-independent mechanism.

Oral administration of F-LOX but not that of LOX increased the gastric content volume, however, both of these drugs increased the gastric acid output. On the other hand, subcutaneous administration of both LOX and F-LOX increased the gastric acid output but did not affect the gastric content volume. We also found that neither LOX nor F-LOX affects the H^+,K^+-ATPase activity *in vitro*. These results suggest that direct interaction of relatively higher concentrations of F-LOX with the gastric mucosa increases the gastric pH value through increasing the gastric content volume. However, the mechanism for this increase is unclear at present.

In order to test the contribution of gastric pH value to the production of lesions after oral administration of F-LOX, we examined the effect of a stimulator for gastric acid secretion, histamine. Following oral pre-administration of 5 mg/kg histamine, the difference in gastric pH value in response to oral F-LOX and LOX treatment became less apparent. Similarly, the difference in the production of gastric lesions was reduced, suggesting that the higher gastric pH value contributes to the lower gastric lesion index after oral administration of F-LOX.

We also found that the expression of mRNAs corresponding to mucin proteins was up-regulated not only at the gastric mucosa after oral administration of F-LOX but also in cultured RGM1 cells treated with this NSAID. Furthermore, a F-LOX-dependent increase in the level of mucin was observed in the culture medium, these

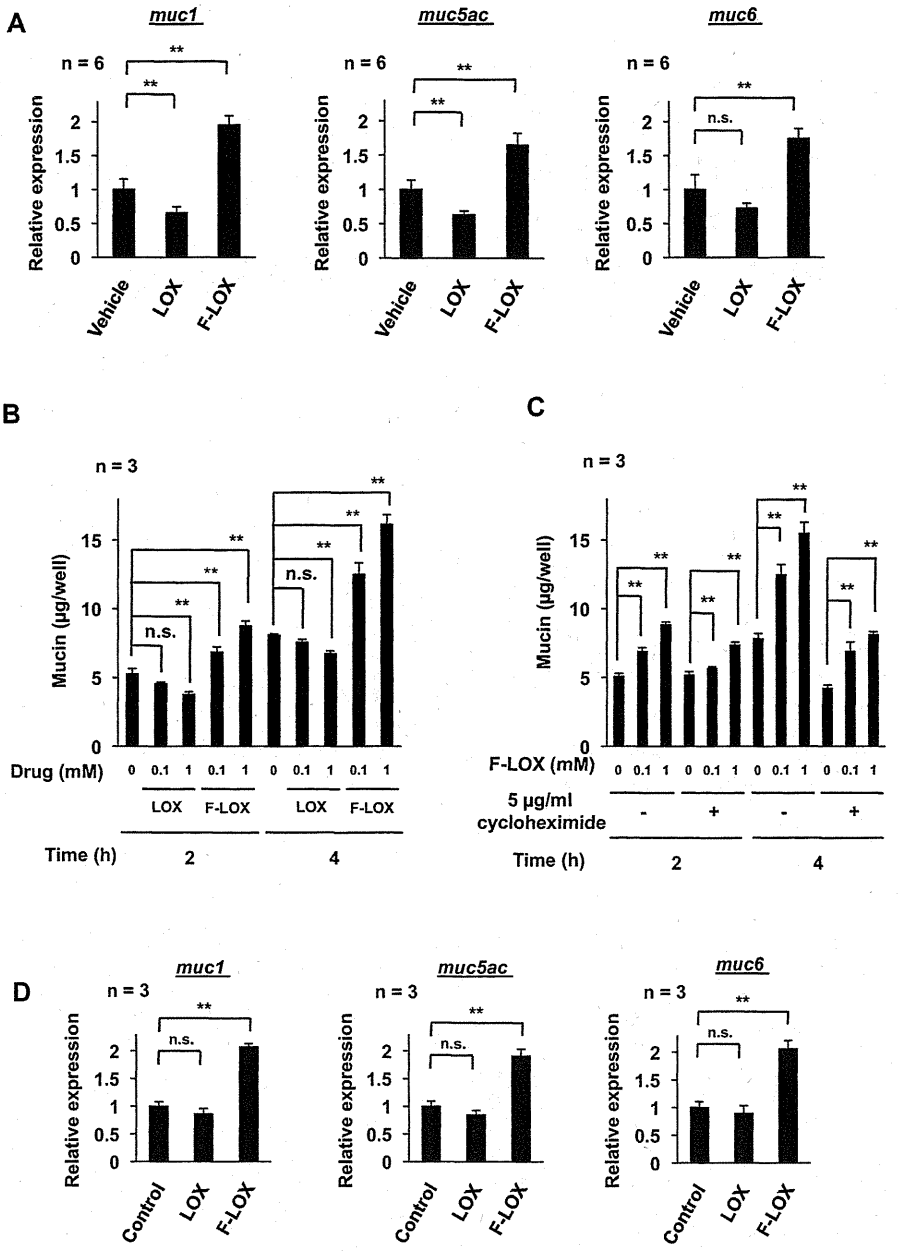

Fig. 7. Effect of F-LOX and LOX on the production and secretion of mucin. (A) SD rats were orally administered 100 or 106.7 mg/kg of LOX or F-LOX, respectively, and the gastric mucosa was removed 4 h later. Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the *gapdh* gene are expressed relative to the control sample. (B) RGM1 cells were incubated with the indicated concentrations of LOX or F-LOX for the indicated periods. (C) RGM1 cells were pre-incubated with 5 μg/ml of cycloheximide for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured the indicated periods. (B and C) The amount of mucin in the culture medium was determined by ELLA. (D) RGM1 cells were incubated with 1 mM of LOX or F-LOX for 4 h. Expression of each mRNA was examined as described above. Values normalized to the *actin* gene are expressed relative to the control sample. Values are mean ± S.E.M. $^{**}P < 0.01$; n.s., not significant.

changes occurring even when the cells were pre-treated with an inhibitor of protein synthesis. These results suggest that F-LOX affects both production and secretion of mucin.

Since PGE_2 stimulates production of mucin through both EP_1 and EP_4 receptors [47], we consider the possibility that F-LOX is an agonist for these receptors. However, this idea was ruled out by the observation that pre-treatment of RGM1 cells with antagonists for EP_1 and EP_4 suppressed PGE_2-induced production of mucin but not F-LOX-induced one. We then tested whether F-LOX directly affects the intracellular signalling pathway coupled with EP_1 or EP_4 receptor (Ca^{2+} mobilization or activation of adenylate cyclase and resulting increase in the cellular level of cAMP, respectively) and found that pre-treatment of RGM1 cells with an inhibitor of adenylate cyclase suppresses F-LOX-dependent increase in the level of mucin and that treatment of cells with F-LOX increases the cellular level of cAMP. Furthermore, we found that pre-treatment of cells with SQ22536 also suppressed F-LOX-induced expression levels of mRNAs corresponding to mucin proteins. These results suggested that F-LOX increases the level of mucin through increase in the cellular level of cAMP.

In line with improvements in diagnostic procedures, it has become clear that NSAIDs induce lesions not only in the stomach but also in the small intestine. However, clinical protocols for the treatment of NSAID-induced lesions of the small intestine have not been established. This is because acid secretion is not as important in the development of these lesions compared with gastric lesions,

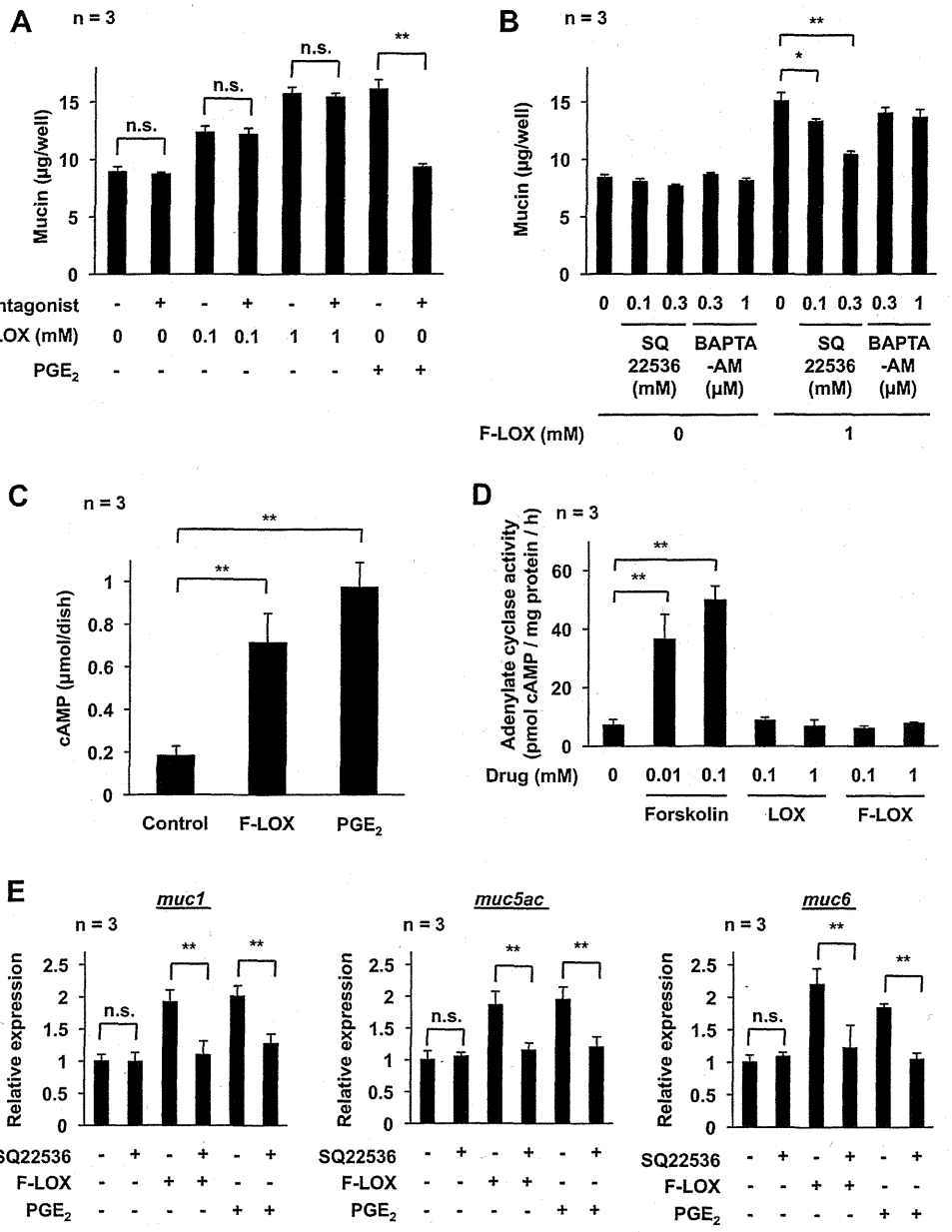

Fig. 8. Molecular mechanism for F-LOX-mediated alteration of production of mucin. RGM1 cells were pre-incubated with 1 μg/ml ONO-8711 (EP_1 antagonist) and 0.1 μg/ml ONO-AE2-227 (EP_4 antagonist) for 0.5 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h (A). RGM1 cells were pre-incubated with the indicated concentrations of SQ22536 (an inhibitor of adenylate cyclase) or BAPTA-AM for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h (B). (A and B) The amount of mucin in the culture medium was determined by ELLA. (C) RGM1 cells were incubated with the indicated concentrations of F-LOX for 0.5 h and cellular cAMP levels were determined by EIA. (D) The activity of adenylate cyclase in membrane fraction prepared from guinea pig gastric mucosa was measured in the presence of indicated concentrations of forskolin (an activator of adenylate cyclase), LOX or F-LOX as described in the experimental procedures. (E) RGM1 cells were pre-incubated with the indicated concentrations of SQ22536 for 1 h, after which the medium was exchanged to one containing indicated concentrations of F-LOX and cultured for 4 h. Expression of each mRNA was examined as described in the legend of Fig. 7. Values are mean ± S.E.M. $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant.

as a result of which acid-controlling drugs are not efficacious [50,51]. In this study, we found that orally administered F-LOX produced fewer small intestine lesions than LOX. Given that the direct cytotoxicity of NSAIDs seems to be involved in, and mucus is protective against, NSAID-induced damage of the small intestine [52,53], this reduced ulcerogenic activity of F-LOX may be the result of its lower cytotoxicity and ability to stimulate mucus synthesis; the development of F-LOX for clinical use is therefore worth considering. However, the extent of the difference between F-LOX and LOX was not as apparent in the small intestine as in the stomach. This may be due to the fact that the pH-increasing activity by F-LOX does not contribute to the suppression of lesion production in the small intestine.

In conclusion, this study has revealed that F-LOX is an unique NSAID, because this NSAID has protective effect against the gastric mucosa and this unique activity contribute to the ulcerogenic activity of this NSAID.

References

[1] Holzer P. Neural emergency system in the stomach. Gastroenterology 1998;114:823–39.
[2] Miller TA. Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 1983;245: G601–23.
[3] Smalley WE, Ray WA, Daugherty JR, Griffin MR. Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. Am J Epidemiol 1995;141:539–45.