

Figure 3. Anti-inflammatory activities of loxoprofen (**1**), 2-fluoroloxoprofen (**2**), and the latter's derivatives (**11a**, **14**, **21a**, and **22a**). Rats were orally administered 3.73, 11.2, or 37.3 $\mu\text{M}/\text{kg}$ test compound and 1 h later received an intradermal injection of carrageenan (1%) into the left hindpaw. Footpad edema was measured 3 h (A) and 6 h (B) after the administration of carrageenan, and the relative inhibition of the increase in edema volume by each compound was determined. Values are the mean \pm SEM ($n = 3-6$): (*) $P < 0.05$.

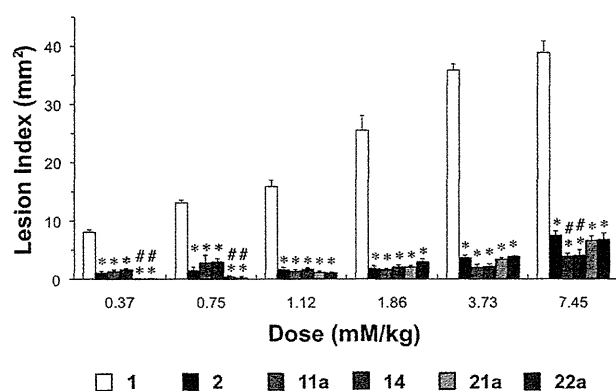


Figure 4. Production of gastric lesions in the presence of loxoprofen (**1**), 2-fluoroloxoprofen (**2**), and the latter's derivatives (**11a**, **14**, **21a**, and **22a**). Rats were orally administered 0.37, 0.75, 1.12, 1.86, 3.73, and 7.45 mM/kg test compound, and their stomachs were removed after 8 h. Stomachs were scored for hemorrhagic damage. Values are the mean \pm SEM ($n = 3-6$): (*) $P < 0.05$ (vs **1**); (#) $P < 0.05$ (vs **2**).

active metabolite (2-[2-fluoro-4-((2-hydroxycyclopentyl)methyl)phenyl]propanoic acid) was subjected to the analysis. We recently reported results for **1** by this analysis, which showed that the cyclopentanone ring interacts with Y385 and S530, whereas propanoic acid interacts with R120 and Y355.¹⁶ All of these amino acids were reported to be important for the interaction between COXs and NSAIDs.^{25,26} Similar orientation and interactions were observed for **2** and its selected derivatives in this study (Figure 5).

We have thus not only identified here interesting and beneficial NSAIDs (see Conclusion) but also suggested structure-activity relationships of **2** for COX inhibition and anti-inflammatory effects, as follows:

For type-A derivatives, as described above, **2** is a prodrug and the trans-alcohol form of **2** showed a more potent inhibitory effect on both COX-1 and COX-2 activity than **2**.¹⁵ However, **13** and **16**, corresponding to the trans-alcohol form of **12** and **15**, respectively, showed a weaker inhibitory effect on both COX-1 and COX-2 than **12** and **15**, respectively (Table 1). Thus, the alteration in bridge heteroatom (O or S) between the two rings may result in the disappearance of **2**'s property as a prodrug.

For type-B derivatives, **21a** and **22a** showed COX-inhibition and anti-inflammatory effects equivalent to **2**, suggesting that the furan ring can become a bioisostere of the cyclopentanone ring of **2**. On the other hand, **23** and **24** showed very weak COX-inhibition and anti-inflammatory effects, suggesting that the closed circular ring in **2** is important for its COX-inhibition and anti-inflammatory effects.

We previously reported that, as well as **2**, oral administration into rats of 2-bromoloxoprofen or 2-*p*-hydroxyphenylloxoprofen produced fewer gastric lesions but showed an equivalent anti-inflammatory effect compared to **1**.^{15,16} Therefore, we examined here the effect of a similar modification of the aromatic ring of **2** by F, Br, or *p*-phenol on the anti-inflammatory effect of **2** (type-C derivatives). However, **33a**, **33b**, **39**, and **40** showed weaker COX inhibition and anti-inflammatory effects than **2**, suggesting that the introduction of a substituted group into the aromatic ring of **1** should be restricted to one position in order to maintain its anti-inflammatory activity.

CONCLUSION

Compound **11a** was found to have a more potent anti-inflammatory effect and an equivalent gastric ulcerogenic activity compared with **2**. Furthermore, as for **2**, **11a** has no apparent selectivity for COX-2. Thus, we consider that **11a** could be therapeutically beneficial for clinical use as an NSAID.

EXPERIMENTAL SECTION

The purity of the final compounds was greater than 95% as judged by HPLC (for details, see Supporting Information).

2-[4-(Cyclopentylamino)-2-fluorophenyl]propanoic Acid (11a). To a solution of **7** (1.50 g, 7.6 mmol) in a mixture of MeOH (10 mL) and AcOH (0.2 mL) were added cyclopentanone (1.4 mL, 15.2 mmol) and sodium cyanoborohydride (NaBH₃CN) (0.96 g, 15.2 mmol). The solution was stirred at room temperature for 12 h. The reaction mixture was evaporated to dryness, extracted with CH₂Cl₂, dried over anhydrous Na₂SO₄, and filtered. The filtrate was evaporated to dryness and the residue was purified on silica gel chromatography (*n*-hexane/AcOEt, 3:2) to afford the methyl ester precursor of **11a**. Hydrolysis with NaOH was done to give final compound **11a** as a brown powder solid (1.09 g, 54%). ¹H NMR (CDCl₃) δ : 1.28 (6H, d, $J = 7.0$ Hz), 1.33–1.68 (6H, m), 1.79–1.91 (2H, m), 3.56–3.64 (1H, m), 3.70 (1H, q, $J = 7.3$ Hz), 6.17–6.30 (2H, m), 6.90 (1H, t, $J = 8.8$ Hz). ¹³C NMR (CDCl₃) δ : 18.1, 25.0, 33.9, 39.0, 55.7, 100.3, 110.4, 116.1, 116.3, 129.8, 130.0, 150.8, 161.2

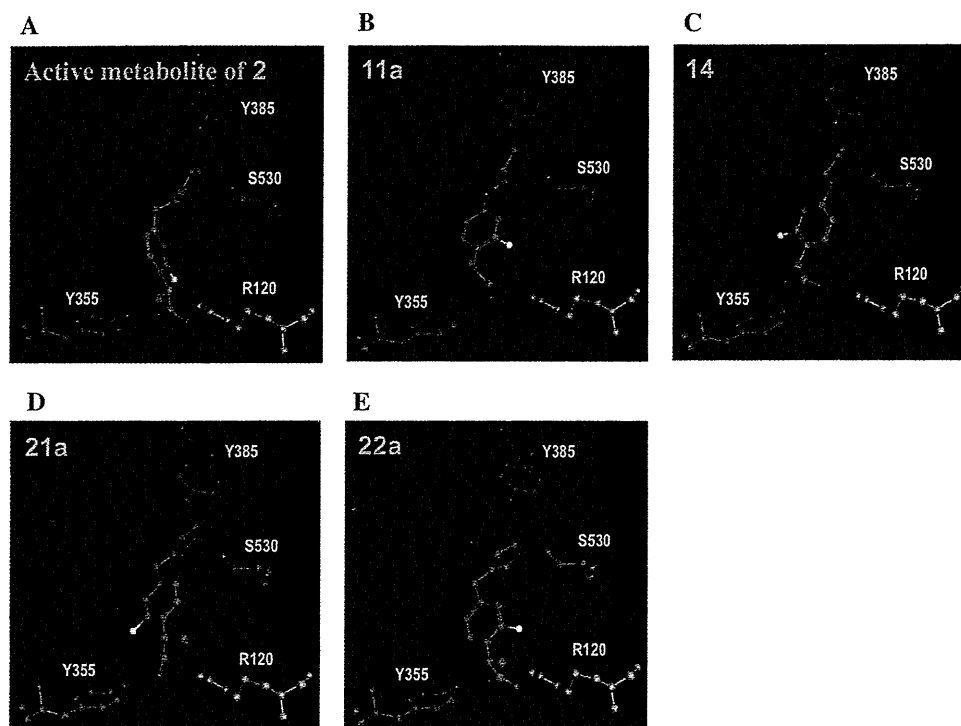


Figure 5. Potential binding mode of the active metabolite of **2** (A), **11a** (B), **14** (C), **21a** (D), and **22a** (E) to the active site of murine COX-2. Hydrogen atoms of the amino acid residues and the ligand have been removed.

(d, $J_{C-F} = 242$ Hz), 178.4. HR-FAB-MS (m/z): 251.1324 (M^+ , calcd for $C_{14}H_{18}FNO_2$, 251.1322). Anal. Calcd for $C_{14}H_{18}FNO_2$: C, 66.91; H, 7.22; N, 5.57. Found: C, 67.05; H, 7.24; N, 5.46.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone and fax: 81-3-5400-2628. E-mail: mizushima-th@pha.keio.ac.jp.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; NBS, *N*-bromosuccinimide; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran

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Development of NSAIDs with Lower Gastric Side Effect

Tohru Mizushima

Faculty of Pharmacy, Keio University, Tokyo, Japan

Abstract

The anti-inflammatory action of nonsteroidal anti-inflammatory drugs (NSAIDs) is mediated through their inhibitory effects on cyclooxygenase (COX) activity. On the other hand, NSAIDs use is associated with gastrointestinal complications. The inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs. In this article, I review our recent work on the COX-independent mechanism involved in NSAID-induced gastric lesions. Using DNA microarray analysis, we found that NSAIDs affect expression of various genes in a COX-independent manner and found that membrane permeabilization activity of NSAIDs and resulting NSAID-induced apoptosis are involved in NSAID-induced gastric lesions. These results suggest that NSAIDs with lower membrane permeabilization activity would be safer on stomach tissue and we found that loxoprofen, a clinically used NSAID, has relatively lower membrane permeabilization activity than other NSAIDs. We synthesized derivatives of loxoprofen and found that fluoro-loxoprofen has lower membrane permeabilization activity and their oral administration produced fewer gastric lesions but showed an equivalent anti-inflammatory effect. These results suggest that fluoro-loxoprofen is likely to be therapeutically beneficial as safer NSAIDs.

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world. An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions because COX is an enzyme essential for the synthesis of prostaglandins (PGs), such as PGE₂, which have a strong capacity to induce inflammation. However, NSAIDs administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding, which sometimes become life-threatening diseases [1]. Therefore, the molecular mechanism governing NSAID-induced gastrointestinal damage needs to be elucidated in order to develop new NSAIDs that do not have these side effects.

Inhibition of COX by NSAIDs was previously thought to be fully responsible for their gastrointestinal side effects [2], because PGE₂ has a strong protective effect

on gastrointestinal mucosa [2]. There are at least two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastric mucosa and tissues with inflammation, respectively [3, 4]. Therefore, it is reasonable to speculate that selective COX-2 inhibitors have anti-inflammatory activity without gastrointestinal side effects. In fact, a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib) both in animal and clinical data [5]. However, the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other. For example, higher doses of NSAIDs were required for producing gastric lesions than were required for inhibiting COX at the gastric mucosa [6]. Understanding the additional mechanisms is necessary in order to establish an alternative method for development of gastrointestinally safe NSAIDs other than simply increasing their COX-2 selectivity. This new class of NSAIDs may be clinically beneficial because clinical disadvantages (i.e. risk of cardiovascular thrombotic disease) of selective COX-2 inhibitors were recently suggested [7, 8].

In this study, I review our recent work on COX-independent actions of NSAIDs in order to understand the molecular mechanism for NSAID-associated gastrointestinal complications. Results suggest that NSAIDs affect expression of various genes in a COX-independent manner, which is involved in NSAID-associated gastrointestinal complications. These studies also provide useful information about development of new types of NSAIDs with lower gastrointestinal side effects.

Results and Discussion

Direct Cytotoxic Effect of NSAIDs

A number of previous reports suggested that NSAIDs are cytotoxic. Thus, using the primary culture of guinea pig gastric mucosal cells that well mimic the gastric mucosal cells in vivo, we examined effect of NSAIDs on cell death. We found that short-term or long-term treatments of gastric mucosal cells with NSAIDs induce necrosis or apoptosis, respectively [9, 10]. In order to test whether the cytotoxic effect of NSAIDs (necrosis and apoptosis) is dependent of their ability to inhibit COX, we examined the effect of exogenously added PGE₂ on necrosis and apoptosis induced by NSAIDs. Exogenously added PGE₂ did not affect the extent of NSAID-induced necrosis or apoptosis even at higher concentrations of PGE₂ than is present endogenously in medium [11]. Results suggest that the cytotoxic effect of NSAIDs (necrosis and apoptosis) is independent of their ability to inhibit COX [11].

Membrane Permeabilization Activity of NSAIDs

Based on structure of NSAIDs, we hypothesized that NSAIDs have membrane permeabilization activity and this activity is involved in the cytotoxic effect of NSAIDs, in

other words, the primary target of NSAIDs is the membrane. We examined membrane permeabilization activity of more than 10 NSAIDs (nimesulide, celecoxib, mefenamic acid, flufenamic acid, flurbiprofen, indomethacin, diclofenac, etodolac, ibuprofen and ketoprofen) using calcein-loaded liposomes [12, 13]. Calcein fluoresces very weakly at high concentrations due to self-quenching, so the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting out the calcein [12]. All of the NSAIDs tested increased the calcein fluorescence, suggesting that they have membrane permeabilization activity [12, 13]. We also examined the necrosis- and apoptosis-inducing ability of these NSAIDs. To examine the relationship between NSAID-induced necrosis or apoptosis and membrane permeabilization, we determined ED_{50} values of the 10 NSAIDs for necrosis or apoptosis (concentrations of NSAIDs required for 50% inhibition of cell viability by necrosis or apoptosis) and ED_{20} values for membrane permeabilization (concentration of NSAIDs required for 20% release of calcein). Plotting ED_{50} values for necrosis or apoptosis versus ED_{20} values for membrane permeabilization (calcein release) yielded an r^2 value of 0.94 or 0.93, respectively, which suggests that NSAID-induced necrosis and apoptosis is mediated by their ability to permeabilize membranes.

DNA Microarray Analysis

It is believed that necrosis is induced by drastic permeabilization of cytoplasmic membranes; however, the mechanism how membrane permeabilization activity of NSAIDs induces apoptosis remains unclear. In order to understand the molecular mechanism governing this apoptosis, we searched for genes whose expression is induced by indomethacin using DNA microarray analysis and found that CHOP, a transcription factor with apoptosis-inducing ability is induced by various NSAIDs [14–16]. In order to test whether the induction of CHOP by indomethacin is involved in indomethacin-induced apoptosis, we used CHOP-deficient mice. Indomethacin-induced chromatin condensation was observed in peritoneal macrophages from wild-type mice but not so apparently in those from CHOP-deficient mice [15]. This result strongly suggests that the induction of CHOP is involved in NSAIDs-induced apoptosis.

Contribution of the Increase in Intracellular Ca^{2+} Level and Mitochondrial Dysfunction to NSAID-Induced Apoptosis

Permeabilization of cytoplasmic membranes causes an increase in intracellular Ca^{2+} levels by stimulating Ca^{2+} influx across the cytoplasmic membrane and we showed that all of the NSAIDs tested increase the intracellular Ca^{2+} level [13]. We used BAPTA-AM, an intracellular Ca^{2+} chelator that is permeable for cytoplasmic membranes to test the contribution of the increase to NSAID-induced apoptosis. BAPTA-AM inhibited NSAID-induced cell death, apoptotic chromatin condensation and induction of CHOP [13], suggesting that the increase in intracellular

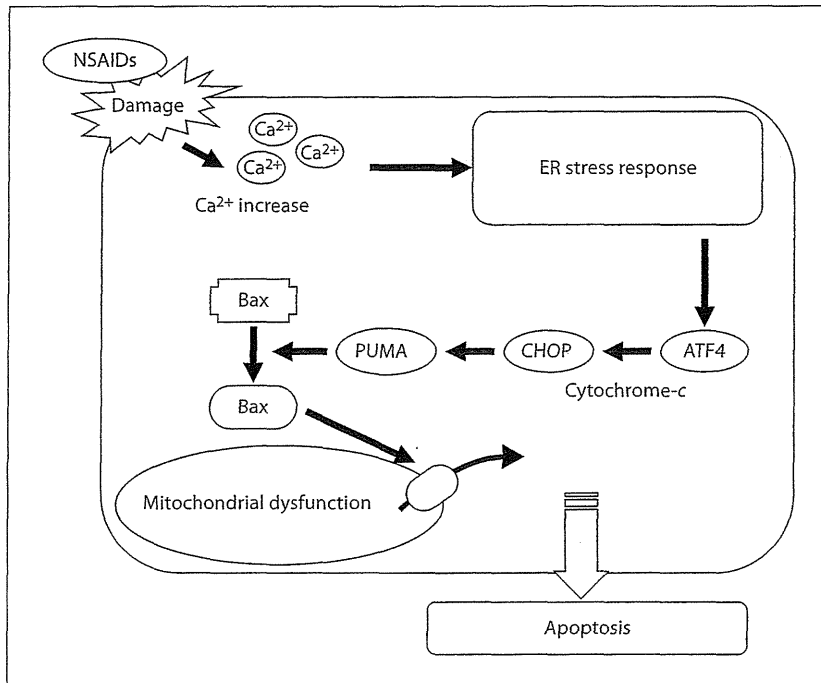


Fig. 1. Molecular mechanism for NSAID-induced apoptosis.

Ca²⁺ levels caused by NSAIDs is involved in NSAID-induced CHOP induction and resulting apoptosis.

CHOP is induced by endoplasmic reticulum (ER) stress response. Accumulation of unfolded protein in the ER induces the ER stress response. On the other hand, PUMA (p53 upregulated modulator of apoptosis) is a BH3 only domain protein with potent apoptosis-inducing activity [17, 18]. PUMA stimulates conformational change, translocation and multimerization of Bax that permeabilizes the mitochondrial outer membrane, resulting in mitochondrial dysfunction and apoptosis [19]. We found that various NSAIDs upregulate PUMA [20]. Furthermore, we suggested that NSAID-induced upregulation of PUMA is mediated through an increase in the intracellular Ca²⁺ level, upregulation of ATF4 and CHOP [20].

Based on these results, we proposed the following pathway for NSAID-induced apoptosis (fig. 1). Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca²⁺ influx and increases intracellular Ca²⁺ levels, which in turn induces the ER stress response. In the ER stress response, expression of CHOP and ATF4 are induced to induce the expression PUMA and the resulting translocation and activation of Bax. Bax plays an important role in NSAID-induced mitochondrial dysfunction, activation of caspases and apoptosis.

Contribution of Mucosal Cell Death to NSAID-Induced Gastric Lesions

We considered that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastrointestinal lesions in vivo. For testing this idea by pharmacological experiments, it is necessary to separate these two properties of NSAIDs (i.e. COX inhibition and direct cytotoxicity) in the model of NSAID-induced gastric lesions in vivo. We tried to achieve this by employing intravenous administration of a nonselective NSAID (indomethacin) and oral administration of cytotoxic selective COX-2 inhibitors (such as celecoxib) in rats. Intravenous administration of nonselective NSAIDs may cause inhibition of both COX-1 and COX-2 (thus inhibition of PG synthesis) at the gastric mucosa without any direct cytotoxicity to the gastric mucosa, because the concentration of NSAIDs at the gastric mucosa following intravenous administration is much lower compared to when NSAIDs are orally administered. On the other hand, oral administration of celecoxib may cause direct cytotoxicity to the gastric mucosa without inhibition of COX-1 and thus PG synthesis may be maintained.

Intravenously administered indomethacin, which completely inhibited COX activity at the gastric mucosa, did not produce gastric lesions. Orally administered celecoxib did not also produce gastric lesions. Interestingly, a combination of the oral administration of celecoxib with the intravenous administration of indomethacin clearly produced gastric lesions [11]. These results suggest that in addition to COX-inhibition by NSAIDs, direct cytotoxicity of NSAIDs is involved in NSAID-induced gastric lesions.

Development of NSAIDs with Lower Gastrointestinal Side Effects

The results described above suggest that NSAIDs without membrane permeabilizing activity have reduced gastrointestinal side effects. Loxoprofen (LOX) has been used clinically for a long time as a standard NSAID in Japan, and clinical studies have suggested that it is safer than other NSAIDs, such as indomethacin [21, 22]. We found that LOX has relatively lower membrane permeabilization activity than other NSAIDs [23].

We then synthesized a series of LOX derivatives, examined their membrane permeabilization activities and selected fluoro-loxoprofen (F-LOX). Membrane permeabilization assay by use of calcein-loaded liposomes revealed that F-LOX has much lower membrane permeabilization activity than LOX [24].

The COX inhibition assay in vitro revealed that F-LOX showed IC_{50} values for COX-1- or COX-2-derived PG biosynthesis that are similar to LOX and that as for LOX, F-LOX did not exhibit apparent selectivity for COX-2 [24].

We then evaluated the activities of F-LOX in vivo. LOX (40 or 50 mg/kg) and equivalent molar amounts of F-LOX were orally administered to rats and the lesion index was calculated. Administration of LOX produced gastric lesions in a dose-dependent manner (fig. 2a). F-LOX produced fewer gastric lesions than

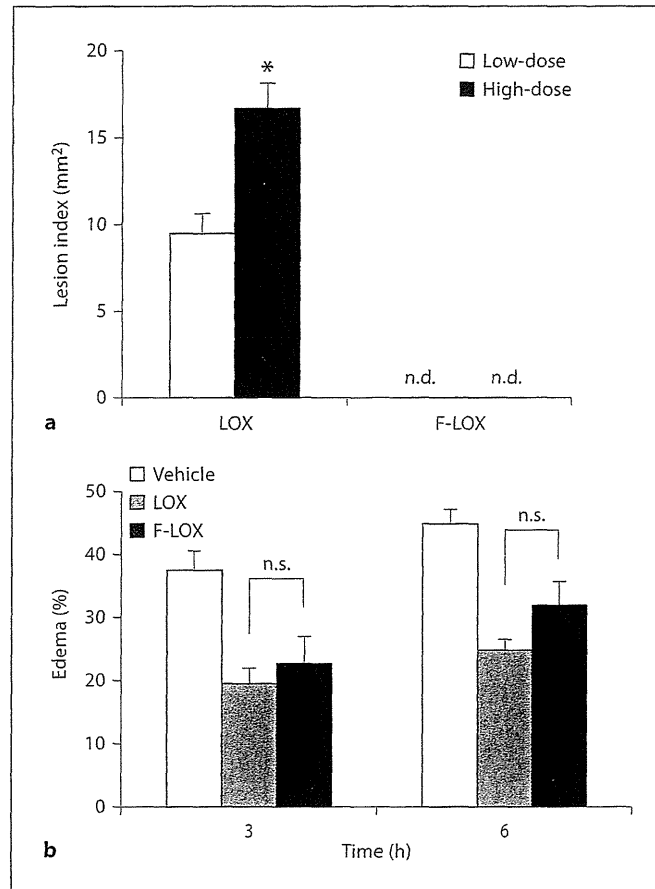


Fig. 2. a,b Ulcer-inducing and anti-inflammatory effects of LOX and F-LOX.

LOX (fig. 2a). This is chemical evidence in support of our proposal that the membrane permeabilization activity of NSAIDs is involved in their induction of gastric lesions.

Finally, we compared the anti-inflammatory effects of F-LOX to LOX by employing a rat carrageenan-induced footpad edema assay. As shown in figure 2b, the volume of carrageenan-induced footpad edema was significantly decreased after oral administration of LOX. The effects of LOX were much the same as that of LOX, showing that F-LOX has anti-inflammatory activity equivalent to LOX.

Conclusion

The results of our recent studies reviewed in this article suggest that both COX-dependent and COX-independent mechanisms are involved in NSAID-induced gastrointestinal complications. We also found that F-LOX showed very low gastric

lesion-inducing activity in rats, although it has no apparent selectivity for COX-2. Thus, we consider that F-LOX has likely to be therapeutically beneficial NSAID in terms of gastrointestinal and cardiovascular safety.

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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 (SpO₂).

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 SpO₂. PC-SOD treatment led to significant recovery of both parameters, whereas pirfenidone improved only SpO₂. 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.

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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; SpO₂ = 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)- β 1 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- β 1 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- β 1 expression, TGF- β 1-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.

MATERIALS AND METHODS

Measurement of Percutaneous Arterial Oxygen Saturation

Details are described in e-Appendix 1. Measurement of percutaneous arterial oxygen saturation (SpO₂) 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 H₂O.

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

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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.

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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

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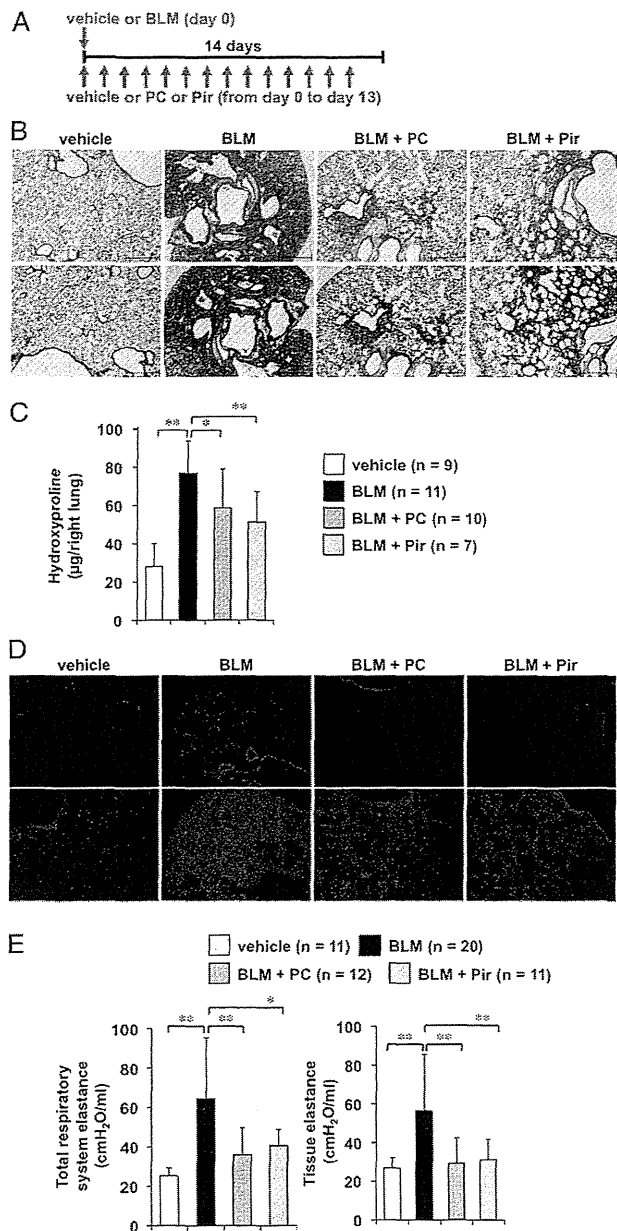


FIGURE 1. Effects of lecithinized human Cu/Zn-superoxide dismutase (PC-SOD) and pirfenidone against bleomycin-induced pulmonary fibrosis and alteration in lung mechanics. **A**, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were 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 \pm SD. * $P < .05$; ** $P < .01$. Scale bar, 500 μ m.

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

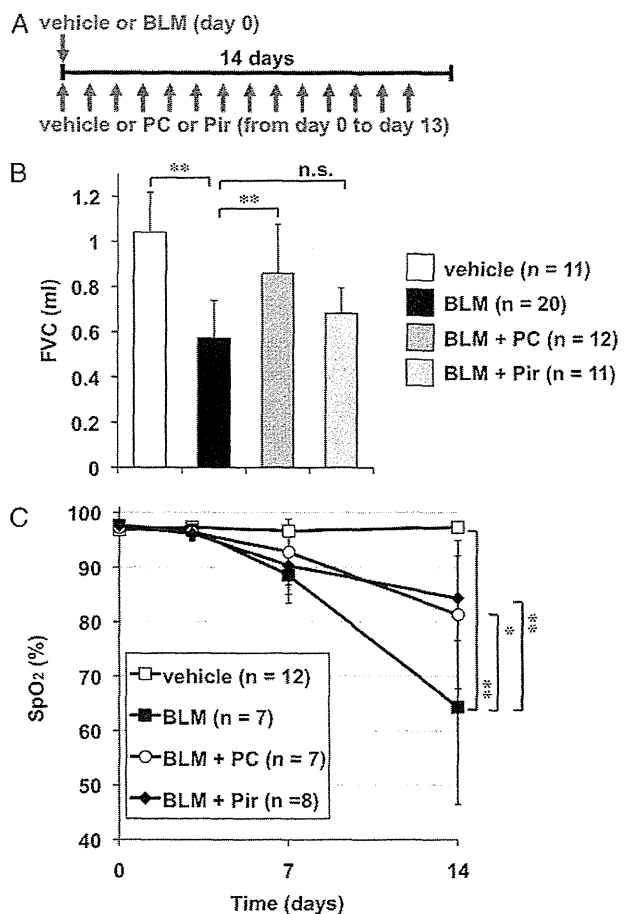


FIGURE 2. Effects of PC-SOD and pirfenidone against bleomycin-induced respiratory dysfunction. **A**, Outline for experimental design. Mice were treated with bleomycin (BLM, 5 mg/kg), PC-SOD (PC, 60 kU/chamber), and pirfenidone (Pir, 400 mg/kg/d) as described in the Fig 1 legend. **B**, FVC was determined on day 14, as described in the "Materials and Methods" section. **C**, SpO₂ was determined on days 0, 3, 7, and 14, as described in the "Materials and Methods" section. Values are mean \pm SD. * $P < .05$; ** $P < .01$. n.s. = not significant; SpO₂ = 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 SpO₂. SpO₂ 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
TGF-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 ^e	28.1 ± 12.8 ^e
Active TGF-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.

^aMice 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.

^bP < .01, vehicle vs BLM.

^cP < .01, BLM vs BLM + PC or Pir.

^dMice 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.

^eP < .05, BLM vs BLM + PC or Pir.

^fMice 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.

^gMice 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- β 1 was measured using an enzyme-linked immunosorbent assay technique. As shown in Table 1, bleomycin treatment increased pulmonary total TGF- β 1 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

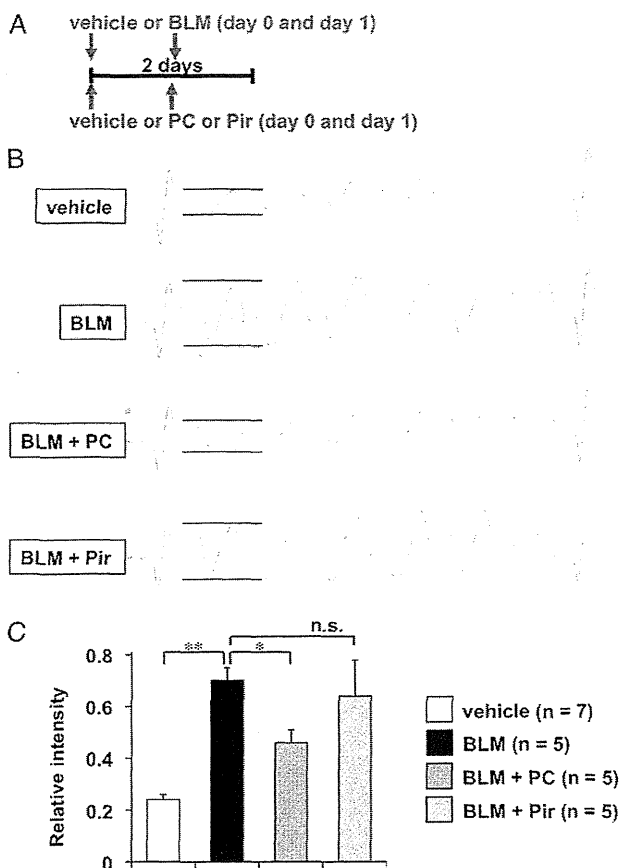


FIGURE 3. Effects of PC-SOD and pirfenidone on bleomycin-induced production of superoxide anion. A, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once daily on both day 0 and day 1. They were also treated by inhalation of PC-SOD (PC, 60 kU/chamber) 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 \pm SD. * P < .05; ** P < .01. See Figure 1 and 2 legends for expansion of abbreviations.

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.

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

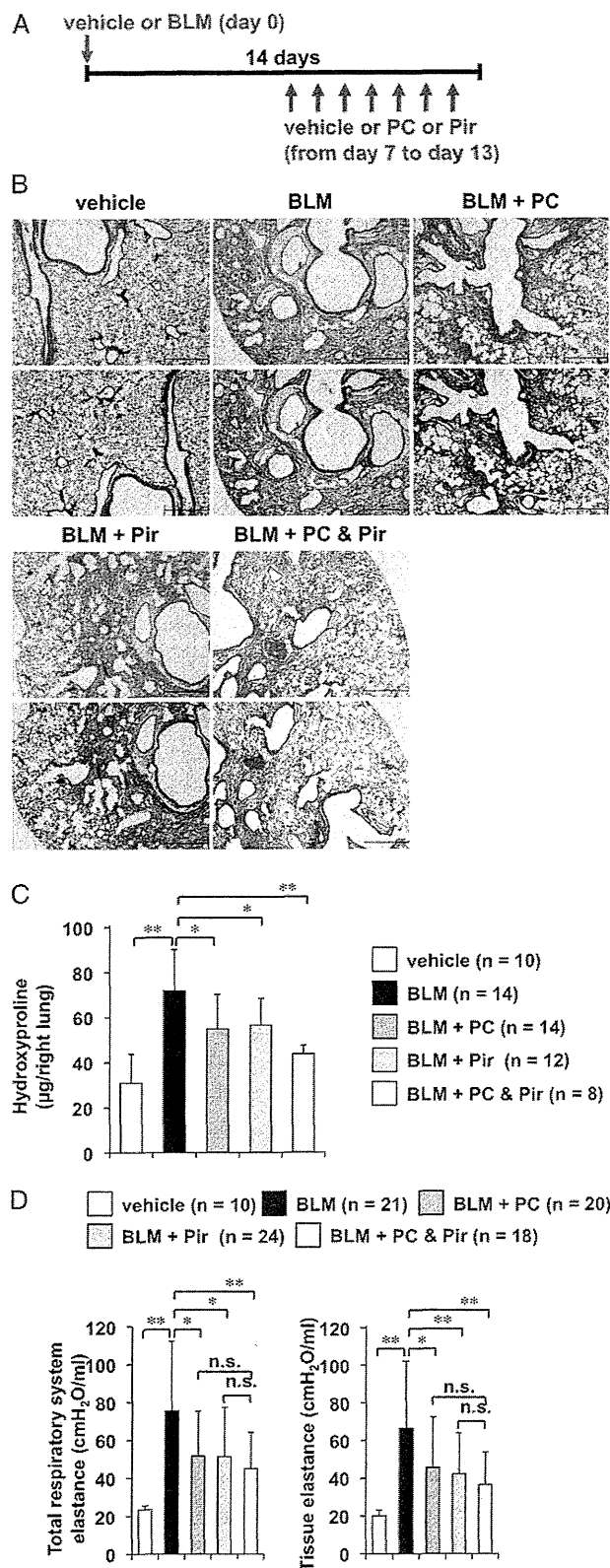


FIGURE 4. Therapeutic effects of PC-SOD and pirfenidone on bleomycin-induced pulmonary fibrosis and impaired lung mechanics. **A**, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were then subsequently treated by inhalation of 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

improved the symptoms of not only patients with ulcerative colitis³⁶ 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 SpO₂, 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

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.

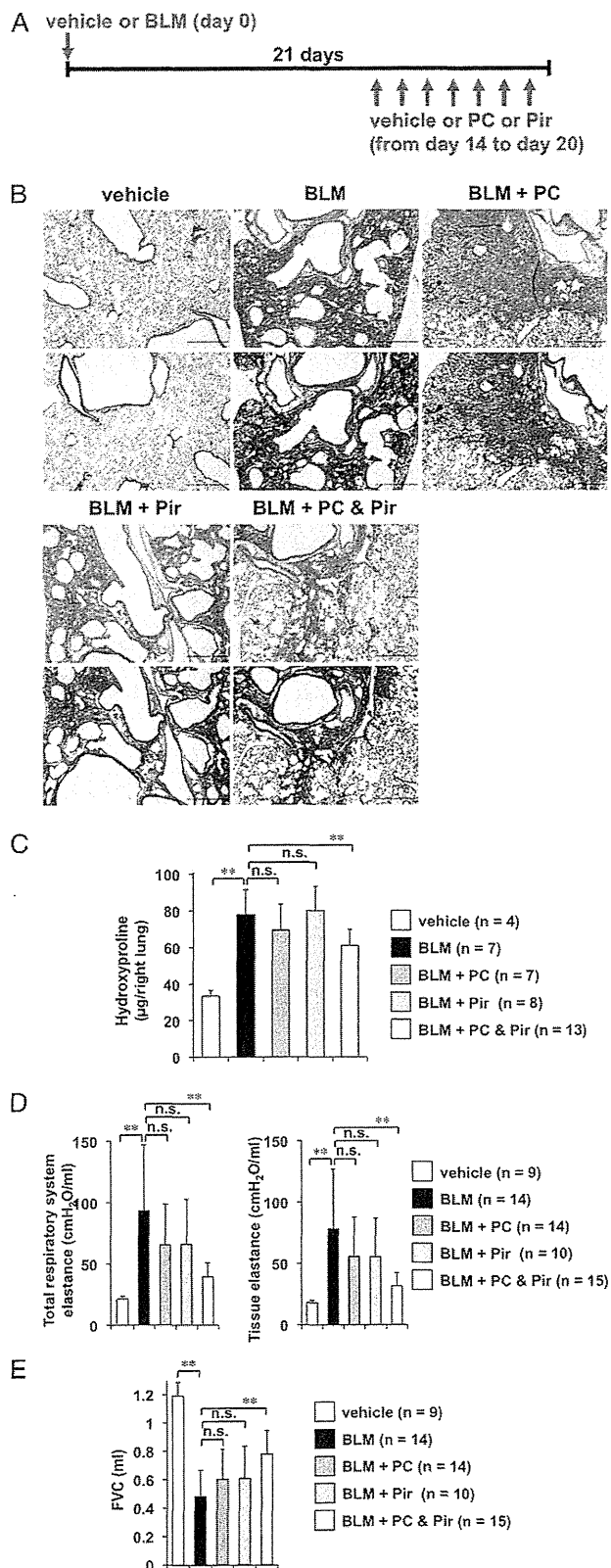


FIGURE 5. Synergistic effects of PC-SOD and pirfenidone on pre-existing pulmonary fibrosis. **A**, Outline for experimental design. Mice were treated with or without (vehicle) bleomycin (BLM, 5 mg/kg) once on day 0. They were then subsequently treated by inhalation of PC-SOD (PC, 60 kU/chamber) daily 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

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 SpO₂), 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

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.

that it has been demonstrated that ROS stimulate the production of TGF- β 1 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.

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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.

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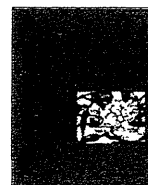
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Expression of 150-kDa oxygen-regulated protein (ORP150) stimulates bleomycin-induced pulmonary fibrosis and dysfunction in mice

Ken-Ichiro Tanaka^{a,b}, Ayano Shirai^a, Yosuke Ito^b, Takushi Namba^b, Kayoko Tahara^a, Naoki Yamakawa^{a,b}, 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

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) involves pulmonary injury associated with inflammatory responses, fibrosis and dysfunction. Myofibroblasts and transforming growth factor (TGF)-β1 play major roles in the pathogenesis of this disease. Endoplasmic reticulum (ER) stress response is induced in the lungs of IPF patients. One of ER chaperones, the 150-kDa oxygen-regulated protein (ORP150), is essential for the maintenance of cellular viability under stress conditions. In this study, we used heterozygous ORP150-deficient mice (ORP150^{+/-} mice) to examine the role of ORP150 in bleomycin-induced pulmonary fibrosis. Treatment of mice with bleomycin induced the expression of ORP150 in the lung. Bleomycin-induced inflammatory responses were slightly exacerbated in ORP150^{+/-} mice compared to wild-type mice. On the other hand, bleomycin-induced pulmonary fibrosis, alteration of lung mechanics and respiratory dysfunction was clearly ameliorated in the ORP150^{+/-} mice. Bleomycin-induced increases in pulmonary levels of both active TGF-β1 and myofibroblasts were suppressed in ORP150^{+/-} mice. These results suggest that although ORP150 is protective against bleomycin-induced lung injury, this protein could stimulate bleomycin-induced pulmonary fibrosis by increasing pulmonary levels of TGF-β1 and myofibroblasts.

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1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis; the mean length of survival from the time of diagnosis ranges from 2.8 to 4.2 years. No treatment has been shown to improve the prognosis for IPF patients [1]. Recent studies have suggested that lung injury associated with inflammatory responses, transforming growth factor (TGF)-β1 and myofibroblasts play important roles in the pathogenesis of IPF [1,2].

An increase in lung myofibroblasts, an intermediate cell type between fibroblasts and smooth muscle cells, has been suggested to play an important role in the atypical fibrosis and collagen deposition that observed in IPF patients [2]. It was previously thought

that the origin of myofibroblasts was solely peribronchiolar and that perivascular fibroblasts transdifferentiate to myofibroblasts in response to various stimuli, in particular TGF-β1 [3]. However, recently it was revealed that lung epithelial cells undergo epithelial–mesenchymal transition (EMT) to become myofibroblasts after treatment with TGF-β *in vitro* [4,5] and that EMT of epithelial cells is induced in the lungs of IPF patients and animals with pulmonary fibrosis (bleomycin-induced pulmonary fibrosis) [4,6,7]. These results suggest that some myofibroblasts in IPF patients are derived from the EMT of lung epithelial cells.

The endoplasmic reticulum (ER) stress response is induced by the accumulation of unfolded and misfolded proteins in the ER [8,9]. ER stress response-related proteins contain not only ER chaperones (such as glucose-regulated protein (GRP)78), that confer protection against stressors by refolding unfolded and misfolded proteins in the ER, but also C/EBP homologous transcription factor, a transcription factor with apoptosis-inducing activity [10]. Since the ER stress response is induced by pathogenic conditions such as hypoxia, inflammation and toxic chemicals, it is not surprising that recent studies have suggested that the ER stress response plays an important role in diseases such as gastric ulcer and Alzheimer's disease [11,12].

Abbreviations: BALF, bronchoalveolar lavage fluid; CHOP, C/EBP homologous protein; EMT, epithelial–mesenchymal transition; FVC, forced vital capacity; GRP, glucose-regulated protein; IPF, idiopathic pulmonary fibrosis; MPO, myeloperoxidase; ORP150, 150-kDa oxygen-regulated protein; α-SMA, α-smooth muscle actin; SP-C, surfactant protein C.

* Corresponding author at: Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Fax: +81 3 5400 2628.

E-mail address: mizushima-th@pha.keio.ac.jp (T. Mizushima).

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