Tetomilast Attenuates Elastase-Induced Pulmonary Emphysema through Inhibition of Oxidative Stress in Rabbits

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Tetomilast was originally identified as a potent inhibitor of superoxide production in human neutrophils, and is of interest because it may relieve oxidative stress related to chronic obstructive pulmonary disease (COPD). Our objective was to determine whether tetomilast effectively protects against the development of porcine pancreatic elastase (PPE)-induced emphysema in rabbits. Rabbits were divided into three groups (sham n=19, PPE n=19, PPE/Tetomilast n=18). The rabbits were once daily orally administered vehicle solution or tetomilast 5 d/week for 4 weeks before the PPE instillation. We compared pulmonary function, inflammatory cell infiltration, oxidative stress, and the incidences of apoptosis among the three groups.

Tetomilast suppressed PPE-induced increases in the incidence of apoptosis and the production of 8-hydroxydeoxyguanosine (8-OHdG) in lung tissues. PPE-instilled rabbits treated with tetomilast showed significantly less mean linear intercept and significantly better pulmonary function than rabbits administered PPE alone. Tetomilast may inhibit the development of emphysema by attenuating pulmonary inflammation and apoptosis caused by PPE-induced oxidative stress.

Key words chronic obstructive pulmonary disease; tetomilast; oxidative stress; 8-hydroxydeoxyguanosine

Pulmonary emphysema is a chronic, progressive obstructive pulmonary disease pathologically defined as “dilation of the alveolar space without desmoplasia by breakdown of alveolar walls and degradation of gas exchange.” Among the numerous factors known to play key roles in the pathogenesis of the disease are inflammation, reactive oxygen species (ROS), smoking, α1-antitrypsin defects and disequilibrium between elastase and anti-elastase, as well as between matrix metalloproteases (MMPs) and tissue MMP inhibitor. Airflow limitation in chronic obstructive pulmonary disease (COPD) patients results from mucosal inflammation and edema, bronchoconstriction, increased secretions in the airways and loss of elastic recoil. Phosphodiesterase 4 (PDE4) is expressed in almost all inflammatory cell types. While PDE4 inhibitors are very efficacious at inhibiting pro-inflammatory mediator release from certain cell types (e.g. neutrophils, eosinophils), there is evidence to suggest that dual inhibition of PDE4 is additive or synergistic at suppressing the activation/functions of other cell types, which are thought to play a role in COPD (e.g. macrophages, dendritic cells, epithelial cells, lymphocytes, airway smooth muscle cells and endothelial cells).

Tetomilast (6-[(2-(3,4-dioxyphenyl)thiazol-4-yl)pyridine-2-carboxylic acid], a PDE4 inhibitor was initially isolated in vitro as the lead compound in a series of thiazole derivatives with inhibitory effects on superoxide production by human neutrophils. Tetomilast exerts both vasoprotectant and anti-inflammatorily effects, mediated by inhibition of PDE4 and superoxide production. Tetomilast also attenuates acute lung injury and its anti-inflammatory effects have been noted in both the liver and colon. In the present study, we determined whether tetomilast could be an effective treatment for PPE-induced emphysema in rabbits.

MATERIAL AND METHODS

Experimental Animals Male Japanese white rabbits (2.5 to 3.0 kg; Chubu Kagaku, Japan) were used for these experiments. Rabbits were housed in separate cages according to treatment protocol. Food and water were provided ad libitum. All of the rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethics Committee of Gifu University School of Medicine, Gifu, Japan.

Experimental Design Pulmonary emphysema was induced in rabbits as described previously. We previously showed that daily oral administration of tetomilast dose-dependently reduces airway resistance and neutrophil accumulation in the lungs of cigarette smoke-induced pulmonary inflammation, and that dose of 10 mg/kg tetomilast is highly effective. Moreover, it has been reported that tetomilast (10 mg/kg) suppressed cytokine production in a murine chronic colitis model. Therefore, we decided the dose of tetomilast as 10 mg/kg in the present study. The rabbits were divided into three groups. In the sham group, the rabbits were intratracheally instilled with 2 mL of phosphate buffered saline (PBS) (1×) and then administered a once daily oral gavage of 10 mL of vehicle solution (distilled water with 0.5% tragacanth; Suzu and Co., Japan) 5 d/week for 4 weeks. In the porcine pancreatic elastase (PPE) group, the rabbits were intratracheally instilled with (PPE: 200 U/kg) diluted in 2 mL of PBS and then administered using the same oral gavage protocol used for the sham animals. In the PPE/Tetomilast group, the rabbits were orally administered 10 mg/kg tetomilast (Otsuka Pharmaceutical Co., Ltd., Japan) suspended in 10 mL of vehicle solution 2 h before the intratracheal instillation of PPE (200 U/kg). Tetomilast was then continued with once daily oral administration 5 d/week.

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for 4 weeks. In all cases, the rabbits were anesthetized by intravenous injection with 30mg/kg sodium pentobarbital prior to intratracheal instillation of PPE or PBS.

Two sets of experiments were performed. In one set, the rabbits (sham, n=11; PPE, n=11; PPE/Tetomilast, n=10) were used for measurement of pulmonary function, analysis of bronchoalveolar lavage fluid (BALF), histological evaluation and immunohistochemical staining. In the other set, the rabbits (n=8 in each group) were used for measurement of myeloperoxidase (MPO) activity and glutathione assays. Samples of lung tissues were snap-frozen in liquid nitrogen immediately after sacrifice.

**Bronchoalveolar Lavage** After measuring pulmonary function 4 weeks after PPE instillation, the rabbits were sacrificed under pentobarbital anesthesia and exsanguinated by puncture of the carotid artery. The trachea, lungs and heart were then removed en masse, a soft rubber tracheal tube (Toray Medical, Japan) was inserted into the trachea of the resected organs, and saline was infused and immediately aspirated. After staining with Wright’s stain total cell and macrophage counts in the BALF were determined.

**Myeloperoxidase Activity** MPO activity was measured as previously described. Briefly, lung tissues from each lobe (approximately 3x3x2 mm) in whole lung were homogenized and centrifuged. The supernatant was then allowed to react and degradation of H2O2 was measured spectrophotometrically as a change in absorbance at 650 nm.

**Glutathione Assay** Total glutathione levels were measured as described previously. Briefly, lung tissues from each lobe (approximately 3x3x2 mm) in whole lung were homogenized and centrifuged. The supernatant was incubated in 0.2 mg/mL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1.67 U/mL glutathione reductase and the rate of the DTNB reduction was measured spectrophotometrically at 405 nm.

**Histological Evaluation** After collecting the BALF, the lungs were fixed by infusing 10% buffered formalin into the trachea for 24 h at a pressure of 25 cmH2O. The fixed lung tissues (from each lobe, approximately 10x10x6 mm) was embedded in paraffin, cut into 4-µm-thick sections. Using the hematoxylin and eosin (H&E)-stained sections, the mean linear intercept was calculated using the method as previously described. Mean linear intercept was a more sensitive measure of destruction than destructive index in PPE-induced emphysema model but not in human smokers.

**Immunohistochemistry and Immunofluorescence** We used the indirect immunoperoxidase method for immunohistochemical staining of Ki-67 (anti-rat Ki-67 antibody, Daka, Denmark) to assess cell proliferation and terminal deoxynucleotidyl transferase biotin-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL; TACS 2 TdT DAB kit; Trevigen, U.S.A.) to detect apoptotic cells. We counted the numbers of Ki-67-TUNEL-positive cells from eight fields randomly distributed across the slide of each lobe, and the result was expressed as the number of Ki-67-TUNEL-positive cells.

To examine the development of DNA oxidative injury, the lungs were stained immunohistochemically and immunofluorescence with monoclonal antibody N4S3.1. For immunohistochemically staining, we used an method as previously described. Sections were incubated with monoclonal anti-8-OHdG antibody (Japan Institute for Control of Aging, Japan), and the staining was developed using 3,3'-diaminobenzidine (DAB). The immunoreactivities of 8-OHdG from eight fields randomly distributed across the slide of each lobe were scored using the following scheme: 0=none; 1=weak; 2=moderate; and 3=strong immunoreactivity. Morphometric analyses carried out by two persons blinded to the conditions. For immunofluorescent staining, sections were heated in the Target Retrieval Solution (Dako) in a boiling water bath for 10 min in an antigen retrieval step, then incubated in blocking buffer (Vectastain ABC kit; Vector) for 60 min, and then with anti-8-OHdG monoclonal antibody overnight at 4°C. Following the washing step, sections were incubated with Alexa 568 (red; Molecular Probe) for 1 h at room temperature. These sections were then counterstaining with Hoechst 33342 and photographed with a laser scanning confocal microscope equipped with multiphoton technology (LSM-510, Zeiss). Then, 8-OHdG-positive cells were counted by free NIH ImageJ software.

**Measurement of Pulmonary Function** Pulmonary function was assessed before and 4 weeks after PPE (or PBS) instillation. To make the measurements, rabbits were connected to a respiratory function machine via a soft rubber tracheal tube inserted under anesthesia. The rabbits were slowly inspired to a tracheal pressure of 20 cmH2O and then forced to expire. From the expiration waveform, we measured the forced vital capacity (volume expired during the fast expiration; FVC), the volume expired in first 100 ms of the fast expiration (FEV100), and the volume expired at the peak expiratory flow (FEVPEF). After the expiration, we measured the functional residual capacity (FRC). These pulmonary function parameters were assessed using an EMMS forced maneuver system (Bordon, U.K.) as previously described. Mean pulmonary function changes in pulmonary function parameters using paired t-tests. Multiple comparisons among groups were made using one-way analysis of variance (ANOVA) with post hoc Tukey’s tests. Values of p<0.05 were considered significant.

**RESULTS**

**Effect of Tetomilast on Inflammatory Response in PPE-Induced Emphysema Rabbit Model** As shown in Fig. 1A, the total cell and macrophage counts were significantly higher in BALF from the PPE group than from the sham and PPE/Tetomilast groups. In addition, there was no significant difference in cell counts between the sham and PPE/Tetomilast groups. Lung MPO activity was measured in lung tissue. The results demonstrated an increase in lung MPO activity at days 28 after PPE instillation and Tetomilast treatment potentially inhibits PPE-induced MPO activity in lungs. (Fig. 1B).

**Antioxidant Effect of Tetomilast in PPE-Induced Emphysema Lungs** An important effect of oxidative stress and inflammation is the up-regulation of protective antioxidant genes. Among antioxidants, glutathione have an important protective role in the airspaces and intracellularly in lung epithelial cells. Glutathione levels in the lungs of rabbits in the PPE group were significantly lower than in the sham or PPE/Tetomilast groups, but there was no significant difference in glutathione levels between the sham and PPE/Tetomilast groups. (Fig. 1C). To determine the extent to which oxidative stress contributes to the emphysema seen in this model,
Fig. 1. (A) Total Cell and Macrophage Counts in BALF 4 Weeks after the Indicated Treatment (B) Myeloperoxidase Activity in Samples of Lung Tissue 4 Weeks after the Indicated Treatment (C) Glutathione Levels in Samples of Lung Tissue 4 Weeks after the Indicated Treatment

(A) The total cell and macrophage counts were significantly higher in the PPE group than in the PPE/Tetomilast group. (B) The levels of MPO activity were significantly higher in the PPE group than in the PPE/Tetomilast group. (C) Glutathione levels in the PPE group were significantly lower than in the PPE/Tetomilast group. Data are means±S.D.; **p<0.01 vs. sham, †p<0.05 vs. PPE, ††p<0.01 vs. PPE.

Fig. 2. Immunostaining for 8-OHdG (×400) (A–C) and Scoring of Morphometrical Changes (D) in Rabbit Lung 4 Weeks after the Indicated Treatment

PPE significantly increased numbers of 8-OHdG-positive cells in the alveolar walls, and this effect was significantly reduced by tetomilast. Data are means±S.D.; *p<0.05 vs. sham, †p<0.05 vs. PPE.
April 2012 497

lung sections were stained using an antibody against 8-OHdG (oxidative marker), which labels oxidized DNA. We found that PPE significantly increased levels of 8-OHdG in the alveolar walls and that this effect was significantly reduced by tetomilast (Figs. 2D, 3B).

**Effect of Tetomilast on Air Space Enlargement in PPE-Induced Emphysema Lungs.** Histological assessment was performed on day 28 after PPE administration. Examination of samples of lung tissue from the PPE group revealed marked expansion of the alveolar spaces caused by destruction of the alveolar walls (Fig. 4B). Similar destruction of alveolar walls was not seen in the sham group (Fig. 4A). Moreover, these

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Fig. 3. Immunofluorescent straining for 8-OHdG (A) and 8-OHdG-positive alveolar cells in lung (B) 4 weeks after the indicated treatment. PPE significantly increased numbers of 8-OHdG-positive cells in the alveolar walls, and this effect was significantly reduced by tetomilast. Data are means±S.D.; *p<0.05 vs. sham, †p<0.05 vs. PPE.

Fig. 4. Photomicrograph showing enlargement of the airspace and emphysematous changes in a region of representative lung tissue from a rabbit in the PPE group (B) (H&E ×200). Such changes are clearly absent in the sham (A) and PPE/tetomilast groups (C). Mean linear intercepts (D). Data are means±S.D.; **p<0.01 vs. sham, ††p<0.01 vs. PPE.
lesions appeared to be greatly attenuated by treatment with tetomilast (Fig. 4C). The observation was confirmed by our finding that the mean linear intercepts of the alveoli were significantly smaller in the PPE/Tetomilast group than in the PPE group (Fig. 4D).

**Tetomilast Prevented PPE-Induced Damage in Alveolar Cells** We determined by the TUNEL method whether the protective effect of tetomilast was related to a reduction in damage in alveolar cells on the 28th day after elastase instillation. The possible occurrence of cell proliferation events was evaluated via analysis of the expression of Ki-67. The numbers of Ki-67-positive cells (the incidence of proliferative cells) in the alveolar walls was significantly lower in the PPE group than the sham group. By contrast there was no significant difference between the PPE and PPE/Tetomilast groups (Fig. 5D). This was corroborated in the similar distribution of Ki-67-positive cells between PPE and PPE/Tetomilast groups. The pulmonary instillation of PPE led to a significant increase in the number of TUNEL-positive cells (the incidence of apoptotic cells) within the alveolar walls. Treatment with tetomilast significantly suppressed that apparent PPE-induced increase in apoptosis (Fig. 6D).

**Effect of Tetomilast on Pulmonary Function in PPE-Induced Emphysema Rabbit Model** The results of the pulmonary functions tests are summarized in Fig. 7. In the sham group, FVC, FEV100 and FEVPEF were all significantly higher 4 weeks after PBS instillation than before it (Figs. 7A–C). There was also an increase in FRC, but the change did not reach significance (Fig. 7D). The significant increases in FVC, FEV100 and FEVPEF observed in the sham group were not seen in the PPE group, but FRC was significantly increased in the PPE group. Rabbits receiving tetomilast showed significant increases in FVC, FEV100 and FEVPEF similar to those seen in the sham group, and the FRC were significantly increased in the PPE group.

**DISCUSSION**

COPD is an inflammatory lung disease that is characterized by systemic and chronic localized inflammation and oxidative stress. Sources of oxidative stress arise from the increased burden of inhaled oxidants, as well as elevated amounts of ROS. The current generally accepted hypothesis of cigarette smoke-induced emphysema is the protease-antiprotease hypothesis. Experimental emphysema models developed from this hypothesis was caused by administration of elastolytic enzymes, such as PPE, either by intratracheal instillation or aerosol inhalation. Nonetheless, the precise mechanisms by which oxidative stress contributes to the pathogenesis of COPD remain controversial, as do the mechanisms by which antioxidants exert beneficial effects against COPD. It also remains unclear whether oxidative stress plays a significant part in the pathogenesis of elastase-induced emphysema in animal models, which have proven to be a useful and simple means of studying the development of emphysema over the period of a few weeks. Tetomilast was originally discovered as a potent inhibitor of superoxide production in human neutrophils. Although its mechanism of action is not completely understood, it is thought to have multiple molecular targets, including PDE4. The therapeutic effect of PDE4 inhibitors
on COPD has been studied in cigarette smoke-exposure models, but not in elastase instillation models. In the present study, tetomilast also exhibited potent action against the development of PPE-induced emphysema in rabbits.

In the sham group, FVC, FEV100, and FEVPEF were all significantly increased 4 weeks after PBS instillation, which is consistent with the normal growth of the rabbits in their average body weight change +26% (range, 10–43%); data not shown) over the 4-week period of the experiment. Despite similar changes in average body weight [+29% (range, 18–42%); data not shown], FVC, FEV100 and FEVPEF did not increase significantly in the PPE group. In addition to the significant enlargement of alveolar air spaces observed in the PPE group, the significant increases in FRC may indicate that PPE instillation leads to hyperinflation of the lung. In accord with these findings, tetomilast showed significantly increases in FVC, FEV100 and FEVPEF and also mitigated the increase in alveolar mean linear intercept and decreased in FRC in our present study, suggesting it suppresses the development of PPE-induced emphysema and hyperinflation.

The beneficial effects of tetomilast were associated with desirable changes in parameters of inflammation, oxidative stress, proteolytic enzyme expression. However, tetomilast does not inhibit the enzymatic activity of elastase (Nagamoto personal communication).

There is a 5- to 10-fold increase in the numbers of macrophages in the airways, lung parenchyma, and BALF in patients with COPD. Macrophage numbers in the airways correlate with the severity of COPD. Macrophages also secrete proteases, including MMP-2, MMP-9, and MMP-12; cathepsins K, L, and S; and neutrophil elastase, taken up from neutrophils. Compared with macrophages from normal smokers, those from patients with COPD are more activated, secrete more inflammatory proteins, and have greater elastolytic activity. A previous study implicated macrophages rather than neutrophils as the critical pathogenic factor in cigarette smoke-induced emphysema in rats. The PPE-induced emphysema rabbit model we used has limited clinical relevance because the major environmental risk factor for human COPD is cigarette smoking. Although these two emphysema models are basically different, they share common mechanisms such as pulmonary inflammation with activated macrophages. After elastase administration in elastase-induced emphysema model, there is an inflammatory exudate which includes macrophages in the lower respiratory tract, and there is up-regulation of a variety of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8, and there are increased numbers of apoptotic parenchymal cells. Inflammatory processes clearly play an important role since mice lacking both TNF-α and IL-1β receptors are 80% protected against PPE-driven emphysema and inflammation, as are mice overexpressing CuZnSOD and mice given the anti-inflammatory agent heme oxygenase-1 (HO-1) by adenoviral transfer. Tetomilast exert an anti-inflammatory effect in the present study, as evidenced by the diminished total cell and macrophage counts in the bronchoalveolar lavage fluid of Tetomilast/PPE group.

Oxidative stress has been attributed a important role in the pathogenesis of COPD because, in addition to causing direct injury to the respiratory tract, oxidative stress triggers and ex-
acerbates the other mechanisms including inflammation; protease-antiprotease imbalance; and apoptosis. Oxidants present can stimulate alveolar macrophages to produce ROS and to release a host of mediators, some of which attract neutrophils and other inflammatory cells into the lungs. Macrophages, which are known to migrate in increased numbers into the lungs of COPD patients, compared with non-smokers, can generate ROS via the NADPH oxidase system. The ROS activity is that smokers and patients with COPD have higher levels of exhaled H₂O₂ than non-smokers. This increase in H₂O₂ is in part derived from increased release of superoxide from alveolar macrophages in smokers. In present study, increase in superoxide thought to be mostly derived from increased alveolar macrophages. MPO, an oxidizing enzyme is synthesized by promyelocytes and stored in azurophil granules in neutrophils and to some extent in circulating monocytes. MPO mainly released from neutrophil, also released from alveolar macrophages. Therefore, increase in MPO also thought to be mostly derived from increased alveolar macrophages in present study. Superoxide from inflammatory cells is a major ROS and can be converted to H₂O₂, which can in turn be used by MPO expressed in macrophages and neutrophils to produce the strong oxidant hypochlorite. MPO generated oxidants cause damage to tissues at inflammatory sites. Oxidative stress has been shown to correlate inversely with the percent predicted FEV₁ in a population study, and MPO activity also has a negative correlation with FEV₁ in patients with COPD, suggesting that MPO and/or oxidative stress may play a role in the pathogenesis of COPD. Counteracting these effects are superoxide dismutase (SOD), catalase and the glutathione system, which provide protection against ROS-induced damage. Direct oxidative damage to components of the lung matrix (such as elastin and collagen) can results from oxidants in cigarette smoke and elastin synthesis and repair can also be impaired by ROS from cigarette smoke, suggesting which can augment proteolytic damage to matrix components and thus enhance the development of emphysema. In the present study, our findings that the lungs from rabbits in the PPE group show elevated 8-OHdG levels, MPO activity, as well as reduced glutathione levels, 4 weeks after PPE-instillation suggest that PPE stimulates ROS production, and that the effect of this oxidative stress persists for at least 4 weeks. Tetomilast reduced in 8-OHdG levels, MPO activity, and increased in glutathione levels, in the PPE-induced emphysema lungs. Tetomilast likely acts by further inhibiting ROS production in macrophages infiltrating the lungs in this study. It is known that tetomilast inhibits superoxide production in both neutrophils and monocytes, though it does not scavenge superoxide. In this way tetomilast may mitigate the development of emphysema by reducing oxidative damage of alveolar extracellular matrix.

cAMP has largely inhibitory effects on a variety of com-

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**Fig. 7. Pulmonary Function: Measurements of FVC (A), FEV100 (B), FEVPEF (C) and FRC (D) Were Made before and 4 Weeks after the Indicated Treatment**

The significant increases in FVC, FEV100 and FEVPEF observed in the sham group were not seen in the PPE group, but FRC was significantly increased in the PPE group. Rabbits receiving tetomilast showed significant increases in FVC, FEV100 and FEVPEF similar to those seen in the sham group, and the FRC were significantly increased in the PPE group. Data are means±S.D., *p<0.05, **p<0.01.
ponents of cell activation, including phagocytosis,\textsuperscript{59} reactive oxygen intermediate (ROI) generation,\textsuperscript{54} and the production of inflammatory mediators such as TNF-\(\alpha\).\textsuperscript{55} Tetomilast reportedly reduces apoptosis among hepatocytes by reducing oxidation stress\textsuperscript{56} and TNF-\(\alpha\) production.\textsuperscript{79} Our TUNEL data show that tetomilast reduces the incidence of PPE-induced apoptosis in the alveolar wall. On the other hand, we found no tetomilast-induced effect on numbers of Ki-67-positive cells, suggesting that tetomilast does not affect alveolar cell proliferation.

CONCLUSION

Tetomilast exerts a protective effect against the development of PPE-induced emphysema in rabbits by suppressing macrophages inflammation, excessive oxidative stress and apoptosis. These results suggest tetomilast is potentially useful for the treatment of COPD.

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REFERENCES