Tiotropium bromide inhibits TGF-β-induced MMP production from lung fibroblasts by interfering with Smad and MAPK pathways in vitro

Kazuhito Asano1 Yusuke Shikama2 Naruo Shoji3 Kojiro Hirano3 Harumi Suzaki3 Hiroaki Nakajima2

1Division of Physiology, School of Nursing and Rehabilitation Sciences, Showa University, Yokohama, Japan; 2Department of Respiratory Diseases, Showa University Northern Yokohamashi Hospital, Yokohama, Japan; 3Department of Otorhinolaryngology, School of Medicine, Showa University, Tokyo, Japan

Background: Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation and structural alterations (ie, tissue remodeling) throughout the conducting airways, parenchyma, and pulmonary vasculature. Matrix metalloproteinases (MMPs) are extracellular degrading enzymes that play a critical role in inflammatory cell infiltration and tissue remodeling, but the influence of the agents that are used for the treatment of COPD on the production of MMPs is not well understood.

Purpose: The present study aimed to examine the influence of tiotropium bromide hydrate (TBH) on the production of MMPs from lung fibroblasts (LFs) induced by transforming growth factor (TGF)-β in vitro.

Methods: LFs, at a concentration of 5 × 10^5 cells·mL⁻¹, were stimulated with TGF-β in the presence of various concentrations of TBH. MMP-1 and MMP-2 levels in culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA), and MMP messenger ribonucleic acid (mRNA) expression was examined by real-time polymerase chain reaction (RT-PCR). The influence of TBH on TGF-β signaling pathways was also analyzed by examining Smad activation and signaling protein phosphorylation by ELISA.

Results: TBH at more than 15 pg·mL⁻¹ inhibited the production of MMP-1 and MMP-2, but not tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2, from LFs, after TGF-β stimulation. TBH also suppressed MMP mRNA expression through the inhibition of Smad activation and signaling protein, extracellular-signal-regulated kinase (ERK) 1 and 2, and c-Jun N-terminal kinase (JNK), phosphorylation.

Conclusion: These results may suggest that TBH suppresses MMP production from LFs, through interference of TGF-β-mediated signaling pathways and results in favorable modification of the clinical status of COPD.

Keywords: tiotropium bromide, matrix metalloproteinases, lung fibroblast, TGF-β, inhibition, in vitro

Introduction

Chronic obstructive pulmonary disease (COPD) is well accepted to be a major public health problem, associated with long-term exposure to toxic gases and particles and most often related to cigarette smoking.1,2 Histological observations of both biopsy specimens of patients with COPD show structural abnormalities such as increased smooth muscle mass; subepithelial fibrosis; and thickening of airway walls, with the intense infiltration of inflammatory cells, including macrophages and neutrophils.3,4 These cellular events are now called tissue remodeling and involve extensive alteration of extracellular matrix (ECM), which is essential for supporting normal lung functions.5
Matrix metalloproteinases (MMPs) are a family of Ca\(^{2+}\)-activated, Zn\(^{2+}\)-dependent proteases, which are secreted by a wide variety of various cells (e.g., inflammatory cells and fibroblasts). MMPs are also accepted to be broadly classified on the basis of substrate specificity into: collagenase (MMP-1 and MMP-8); gelatinase (MMP-2 and MMP-9); and elastase (MMP-7 and MMP-12), among others, and can degrade one or several constituents of ECM. In normal physiological conditions, MMPs promote remodeling of ECM, thus facilitating cell migration, and are implicated in the development of immune responses by cleaving the inactive forms of cytokines and chemokines. On the other hand, in pathological conditions, a switch in MMP production and activity occurs, which leads to excessive lung inflammation and abnormal tissue destruction. There is increasing evidence for a role of MMPs in COPD. In patients with emphysema, which is the most important histological finding of COPD, there is an increase in both bronchoalveolar lavage (BAL) fluid concentration and lung tissue expression of MMP-1 and MMP-9, and these are correlated with a decrease in forced expiratory volume in one second (FEV\(_1\)). It is also observed that desmosine, a degradation product of elastic fiber, which is one of the important components of ECM for lungs, is increased in the urine of subjects with COPD and correlates with the rate of lung function decline. Furthermore, MMP-2 expression in lung periphery progressively increases as lung function worsens and degree of emphysema increases. These reports may suggest that MMPs, including MMP-1, MMP-2, and MMP-9, play essential roles in the development of COPD, however very few studies have examined the influence of agents that are used for the treatment of COPD on MMP.

The transforming growth factor (TGF)-\(\beta\) subfamily is critically involved in embryonic development (organogenesis) and act as multifunctional regulators of cell growth and differentiation. Several studies have reported an increased expression of TGF-\(\beta\) in the airway epithelium of COPD patients. It is also reported that increased TGF-\(\beta\), as well as TGF-\(\beta\) receptor, expression in the bronchial and alveolar epithelium of COPD patients is correlated with clinical future, such as lung function and histological changes of COPD, suggesting an impact of TGF-\(\beta\) signaling on the development and progression of COPD. Furthermore, there is much evidence that fibrotic alterations are crucial in airway remodeling in COPD and anti-obstructive agents such as muscarinic antagonists and \(\beta\)-adrenoceptor agonists appear to delay the decline in lung function through the inhibition of fibroblast functions, including proliferation and collagen synthesis.

Tiotropium bromide hydrate (TBH), the first choice agent in the treatment of COPD, is a once-daily inhaled anticholinergic bronchodilator, which binds to muscarinic receptor subtypes, especially M\(_{3}\). There is much evidence showing that administration of TBH in COPD patients could prevent airway smooth muscle remodeling. Although these reports may suggest that TBH exerts a suppressive effect on MMP production, or inhibition of MMP activation in airways, there is little evidence showing the influence of TBH on MMPs. In the present study, therefore, we examined the influence of TBH on the production of MMP-1, MMP-2, and MMP-9, from human lung fibroblasts (LFs), after TGF-\(\beta\) stimulation in vitro.

**Materials and methods**

**Agent**

TBH was kindly donated by Nippon Boehringer Ingelheim Co. Ltd. (Tokyo, Japan), as a preservative-free pure powder. The powder was dissolved in a Roswell Park Memorial Institute medium (RPMI-1640), supplemented with 10% fetal bovine serum (RPMI-FCS) at a concentration of 10 \(\mu\)g.mL\(^{-1}\). This solution was then sterilized by passing through 0.2 µm filters and stored at 4\(^\circ\)C as stock solution until used. All dilutions used in this study were prepared from this stock solution, by diluting with RPMI-FCS. Recombinant TGF-\(\beta\) (preservative-free) was purchased from R&D Systems Inc. (Temecula, CA, USA) and diluted with RPMI-FCS to give a concentration of 50 ng.mL\(^{-1}\).

**Cell source and induction of fibroblasts**

Tissue samples from patients without lung fibrosis or COPD were obtained from healthy tissue area, during pneumonectomy for tumor resection from a tumor-free area. All donors (3 female: 43–71 years; 2 male: 41 and 71 years) provided written informed consent, which was approved by the Ethics Committee of Showa University. The cells were induced from diced tissue specimens (approximately 1 mm\(^3\)) according to the method described previously. The cells were then characterized, and those with fibroblast purity of more than 99% used as LFs. LFs at five to six passages were used for the following experiments.

**Cell culture**

LFs were washed several times with RPMI-FCS and introduced into each well of 24-well culture plates in triplicate, at a concentration of \(5 \times 10^4\) cells.mL\(^{-1}\) in a volume of 1 mL. The cells were stimulated with 2.5 ng.mL\(^{-1}\) TGF-\(\beta\), in the presence of various concentrations of TBH, in a final volume...
of 2 mL. After 24 h, the culture supernatants were removed and stored at −40°C until used. To examine transcription factor activation and messenger ribonucleic acid (mRNA) expression, LFs were cultured in a similar manner for 4 h and stored at −80°C until used. In preparing cells to examine signaling protein phosphorylation, LFs were cultured in a similar manner, in 96-well flat-bottomed culture plates for 30 min. In all cases, TBH was added to cell cultures 2 h before the stimulation with TGF-β.

**Assay for MMP and tissue inhibitor of matrix metalloproteinase (TIMP)**

MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in culture supernatants were assayed using commercially available human MMP and TIMP enzyme-linked immunosorbent assay (ELISA) test kits (Amersham Biosciences, Bucks, UK), according to the manufacturer’s recommendations. The results are expressed as the mean (ng·mL⁻¹) ± standard error (SE) of duplicate assays for five different subjects. According to the manufacturer’s data sheet, the ELISA kits used are specific for the corresponding enzymes and completely recognized free prometalloproteinase (proMMP) and that complexed with TIMP, but not the active form of MMP. The minimum detectable levels of these ELISA kits were 0.5 ng·mL⁻¹ for MMP-1; 2.0 ng·mL⁻¹ for MMP-2; and 0.5 ng·mL⁻¹ for MMP-9.

**Real-time polymerase chain reaction (PCR)**

mRNA was extracted from fibroblasts using magnetic-activated cell sorting (MACS) mRNA isolation kits (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The first-strand complementary deoxyribonucleic acid (cDNA) synthesis from 1.0 µg mRNA was performed using the SuperScript Preamplification System for cDNA synthesis (GIBCO BRL, Gaithersburg, MD, USA). PCR was then carried out using a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR mixture consisted of 2.0 µL of sample cDNA solution (10.0 ng·µL⁻¹); 25.0 µL of SYBR® Green Master Mix (Life Technologies Corporation, Carlsbad, CA, USA); 0.3 µL of both sense and antisense primers; and distilled water, to give a final volume of 50 µL. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 sec at 95°C, and 60 sec at 60°C. β-actin was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle (Ct) and normalized to β-actin. Oligonucleotide sequences of the primers used are shown in Table 1.

**Assay for Smad-2 and Smad-4 activities**

Smad-2 activity was analyzed with commercially available ELISA test kits (Active Motif, Carlsbad, CA, USA) that contained sufficient reagents, biotin-labeled double strand oligonucleotides with consensus sequence for Smad-2 (probe), and primary and secondary antibodies, according to the manufacturer’s recommendations.
manufacturer’s recommended procedure. In brief, nuclear extract (2.0 µg µL⁻¹ protein) from LFs was introduced into each well of 96-well microtiter plates, that contained 2.5 µL probe and 10.0 µL double strand oligonucleotide corresponding with probe, in a volume of 80 µL. These were then incubated for 30 min at 25°C. After washing three times, 45 µL samples were added to the appropriate wells of a 96-well plate coated with streptavidin and incubated for a further 1 h at 25°C. Primary antibody against Smad-2 protein in a volume of 50 µL was then added and incubated for 1 h at 25°C. After washing the plate, anti-immunoglobulin G horseradish peroxidase (anti-IgG HRP-conjugate) in a volume of 100 µL was then added and incubated for a further 1 h at 25°C. After addition of tetramethylbenzine solution, absorbance at 450 nm was measured with the ELISA plate reader. Smad-4 activity was also examined, using a commercially available ELISA test kit (Active Motif, Carlsbad, CA, USA) in a similar manner.

Assay for signaling protein phosphorylation
Phosphorylation of signaling proteins, extracellular-signal-regulated kinase (ERK) 1 and 2; p38 mitogen-activated protein kinases (p38 MAPK); and c-Jun N-terminal kinase (JNK), was analyzed with commercially available ELISA test kits (Active Motif, Carlsbad, CA, USA), according to the manufacturer’s recommendations. In brief, LFs stimulated with 2.5 ng mL⁻¹ TGF-β were fixed with 4% formaldehyde for 20 min at 25°C. After washing the plate, the primary antibody against phosphorylated-ERK1/2, p38 MAPK, or JNK was added into each well in a volume of 40 µL and incubated for 12 h at 4°C. After removing the antibody by washing the plate, 100 µL of HRP-conjugated secondary antibody was added. After 1 h at 25°C, tetramethylbenzine solution was added in a volume of 100 µL per well and absorbance at 450 nm was measured followed by addition of 2N Hydrogen Chloride (2N HCl).

Statistical analysis
The statistical significance of the difference between the control and experimental data was analyzed using ANOVA followed by Fisher’s protected least significant difference (PLSD) test. A P value <0.05 was accepted as statistically significant.

Results
Influence of TGF-β stimulation on MMP production from LFs
The first experiments were undertaken to examine the optimal dose of TGF-β on MMP production from LFs in vitro. LFs (5 × 10⁵ cells mL⁻¹) were stimulated with various concentrations of TGF-β for 24 h. MMP-2 and MMP-9 levels in culture supernatants were examined by ELISA. Data are expressed as the mean (pg mL⁻¹) ± standard error (SE), for five different subjects.

As shown in Figure 1, stimulation of LFs with 1.0 ng mL⁻¹ TGF-β significantly increases MMP-2 levels in culture supernatants, as compared with non-stimulated control. The ability of LFs to produce MMP-2 was further increased when 2.5 ng mL⁻¹ TGF-β was used for stimulation, and culture supernatants contained much higher levels of MMP-2 than that in supernatants stimulated with 1.0 ng mL⁻¹ TGF-β (Figure 1). However, TGF-β stimulation exerts a suppressive effect on MMP-2 production when the agent at 5.0 ng mL⁻¹ or more was used for stimulation (Figure 1). On the other hand, TGF-β stimulation could not induce MMP-9 production from LFs: culture supernatants contained undetectable levels of MMP-9 (<0.5 ng mL⁻¹) by ELISA (data not shown).

Influence of TBH on MMP-1, MMP-2, TIMP-1 and TIMP-2 production from LFs
The second set of experiments was undertaken to examine the influence of TBH on MMPs production from LFs in response to TGF-β stimulation. LFs (5 × 10⁵ cells mL⁻¹) were stimulated with 2.5 ng mL⁻¹ TGF-β in the presence of 0, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, or 30.0 pg mL⁻¹ TBH for 24 h. MMP-1 and MMP-2 levels in culture supernatants were assayed by ELISA. Data are expressed as the mean ng mL⁻¹ ± SE, for five different subjects. As shown in Figure 2A, treatment of LFs with TBH at more than 15.0 ng mL⁻¹
caused significant suppression of MMP-1 production from cells, which was increased by TGF-β stimulation. TBH also suppressed TGF-β-induced MMP-2 production from LFs (Figure 2B). The minimum concentration of the agent which caused significant suppression of MMP-2 production was 15.0 pg·mL⁻¹ (Figure 2B). We next examined the influence of TBH on the enzymatic activities of MMP-1 and MMP-2. The data in Figures 2C and 2D clearly show that addition of TBH into cell cultures at more than 15.0 pg·mL⁻¹ could significantly suppress the activity of both MMP-1 and MMP-2 in culture supernatants. The final experiments in this section were undertaken to examine the production of both TIMP-1 and TIMP-2 from LFs, even when 30.0 pg·mL⁻¹ of the agent was added to cell cultures.

Influence of TBH on MMP and TIMP mRNA expression in LFs

The third set of experiments was undertaken to examine the influence of TBH on mRNA expression for MMP-1, MMP-2, TIMP-1, and TIMP-2 in LFs. LFs (5 × 10⁵ cells·mL⁻¹) were stimulated with 2.5 ng·mL⁻¹ TGF-β in the presence of 0, 5.0, 15.0, 20.0, or 25.0 pg·mL⁻¹ TBH for 4 h. Levels of mRNA expression were evaluated by real-time PCR. Data are expressed as the mean % ± SE, for five different subjects. Addition of TBH at more than 15.0 pg·mL⁻¹ into cell cultures significantly suppressed mRNA expression for MMP-1 and MMP-2 in LFs (Figure 4). However, TBH could not reduce levels of TIMP-1 and TIMP-2 mRNA expression in LFs (Figure 4).

Influence of TBH on transcription factor activation in LFs

The fourth set of experiments was designed to examine the influence of TBH on transcription factor (Smad-2 and Smad-4) activation in LFs. LFs (5 × 10⁵ cells·mL⁻¹) were stimulated with 2.5 ng·mL⁻¹ TGF-β in the presence of 0, 5, 10, 15, 20, or 25 pg·mL⁻¹ TBH for 4 h. The nuclear extract was prepared and Smad activity was assessed by ELISA. Data are expressed as the mean OD at 450 nm ± SE, for five different subjects. As shown in Figure 5, addition of TBH at more than 15 pg·mL⁻¹ caused significant suppression of Smad activation in LFs, which was induced by TGF-β stimulation.

---

**Figure 2** Suppressive activity of tiotropium bromide hydrate (TBH) on the production of MMP-1 and MMP-2 by TGF-β stimulation from lung fibroblasts and on their activities.
Influence of TBH on the phosphorylation of signaling proteins

The final set of experiments was undertaken to examine the influence of TBH on signaling protein phosphorylation after TGF-β stimulation in LFs. To do this, LFs were stimulated with 2.5 ng·mL⁻¹ TGF-β, in the presence of 5, 15, 20, and 25 pg·mL⁻¹ TBH for 30 min, and phosphorylation of p38 MAPK, JNK, and ERK1/2 were examined by ELISA. Data are expressed as the mean OD at 450 nm ± SE, for five different subjects. Treatment of LFs with TBH could not
suppress p38 MAPK phosphorylation, which was increased by TGF-β stimulation of LFs: the levels of p38 MAPK phosphorylation in LFs treated with 25 pg·mL⁻¹ TBH was nearly identical (not significant; \( P > 0.05 \)) to that observed in TGF-β-stimulated cells (Figure 6). On the other hand, treatment of cells with TBH at 15 pg·mL⁻¹ or more caused significant suppression of phosphorylation of both JNK and ERK1/2, which were increased by TGF-β stimulation (Figure 6).

**Discussion**

The present results clearly show that TBH at more than 15 pg·mL⁻¹, which is extremely low compared with therapeutic blood levels,²⁵ could suppress the production of both MMP-1 and MMP-2 from LFs, with no detectable effect on the production of TIMP-1 and TIMP-2. In addition, this inhibitory action of TBH on MMP-1 and MMP-2 production is due, in part, to its suppressive effect on MMP mRNA expression,
through the inhibition of both Smads signaling pathways and signaling protein phosphorylation, especially JNK and ERK1/2, induced by TGF-β stimulation.

COPD is characterized by the presence of a partially reversible airflow obstruction. This pathology is also associated with an airway inflammation process characterized by intense accumulation of inflammatory cells, such as macrophages and neutrophils, in airways and lung. It is believed that the development of emphysema, which is the most important histological finding of COPD, reflects the relative excess of proteases that degrade the connective tissues of the lung and a relative paucity of anti-proteolytic defenses. This concept is often referred to as the protease-antiprotease imbalance hypothesis and involves mainly serine proteases, including MMPs. Immunohistochemical analysis of collagenase and gelatinase expression in COPD, MMP-1, -2, -8, and -9 were found to be upregulated. In a study combining RT-PCR, ELISA, immunohistochemistry, and a collagen degradation assay, to analyse MMP-1, -9, and -12, increased MMP-1 expression was observed in patients with COPD. It is also reported that sputum from patients with COPD showed an increased gelatinolytic activity, which linked to MMP-2. Furthermore, it is reported that the activated forms of MMP-9 and prometalloproteinase-2 (proMMP-2) were found in the sputum of 85% and 25% of COPD patients, respectively. From these reports, it is possible that the attenuating effect of TBH on MMP-1 and MMP-2 production from LFs induced by TGF-β stimulation, may underlie the therapeutic mode of action of the agent on COPD. The extracellular activity of MMPs is regulated by TIMPs that form a 1:1 complex with MMPs. The present results clearly show that TBH could not inhibit TIMP-1 and TIMP-2 production from LFs after TGF-β stimulation, suggesting that MMP-1 and MMP-2, secreted in small amounts from LFs during TBH treatment, are inactivated by TIMPs, resulting in modification of clinical symptoms derived from ECM remodeling and accumulation of inflammatory cells in COPD.

TGF-β is a pleiotropic cytokine that functions both during development and in the adult, by affecting cell differentiation, growth, apoptosis, and immune responses. Upon TGF-β stimulation, the type I and type II receptors form a stable complex, in which the constitutive serine-threonine kinase of the type II receptor activates the type I receptor. The activated type I receptor phosphorylates Smad-2 and Smad-3 at C-terminal serines. These receptor-activated Smads then form trimers with Smad-4 that migrate to the nucleus, where they associate with sequence-specific transcription factors, such as AP-1, at regulatory sequence of target genes. There are also other pathways that have been implicated in TGF-β signaling: tyrosine phosphorylation of both type I and type II receptors can induce activation of the p38 MAPK pathway, as well as the ERK and JNK MAPK pathways. We then examined the possible mechanisms by which TBH could inhibit MMP-1 and MMP-2 production from LFs in response to TGF-β stimulation. The data clearly showed that pre-treatment of LFs with TBH decreased Smad-2 and Smad-4 levels in the nucleus, which are increased by TGF-β stimulation in LFs. It is also observed that TBH could suppress phosphorylation of JNK and ERK1/2, but not p38 MAPK, induced by TGF-β stimulation, suggesting that TBH inhibits the activation of signal transducers and transcriptional modulators, especially Smad-2 and Smad-4 pathways, and JNK and ERK1/2 pathways. This also resulted in the suppression of MMP-1 and MMP-2 mRNA expression in LFs by TGF-β stimulation, which is responsible for inhibition of MMP-1 and MMP-2 production at protein levels. On the other hand, TGF-β stimulation has been reported to increase the levels of intracellular Ca²⁺, which is essential for TGF-β receptor phosphorylation and activation of signal transducers and transcriptional modulators. Solifenacin succinate, a newly developed muscarinic receptor antagonist, could inhibit carbachol-induced intracellular Ca²⁺ mobilization in guinea pig smooth muscle cells and murine submandibular gland cells in vitro. Solifenacin succinate and the other antimuscarinic drugs, such as darifenacin and oxybutynin chloride, which have higher affinity for muscarinic M₄ receptor, are also reported to inhibit an increase in intracellular Ca²⁺ levels in rat salivary gland cells induced by carbachol stimulation. Furthermore, otilonium bromide, a muscarinic receptor antagonist, could inhibit increases in intracellular calcium levels in cells from rat colon by blocking L-type calcium channel. Judging from these reports, there is another possibility that TBH blocks an increase in Ca²⁺ in cytosole and results in inhibition of MMP-1 and MMP-2 production after TGF-β stimulation through the suppression of Smad signaling pathways.

In addition to MMP-1 and MMP-2, MMP-9 and MMP-12 have been the focus of interest in several studies. An increase in MMP-9 activity in the lung parenchyma of patients with emphysema is reported. MMP-9 and the MMP-9/TIMP-1 ratio are also reported to be increased in induced sputum from patients with COPD. Alveolar macrophages from patients with COPD express more MMP-9 than those from healthy volunteers and the ability of macrophages to produce MMP-9 is further increased in response to inflammatory stimulation.
MMP-12 is able to degrade elastine, which is distributed widely throughout the lungs, and is responsible for the development of emphysema in COPD. Immunochemical analysis of BAL and bronchial biopsy samples from COPD patients revealed the presence of much higher numbers of MMP-12-expressing macrophages, as compared with those from normal subjects. Therefore, to further elucidate the therapeutic mechanisms of TBH on COPD via suppression of MMP production, it is necessary to examine whether TBH could also suppress the production of MMP-9 and MMP-12 by using macrophages and other inflammatory cells such as neutrophils.

In conclusion, TBH exerts suppressive effects on MMP production from LFs, through interference of TGF-β-mediated signaling pathways and results in favorable modification of the clinical status of COPD.

Acknowledgment
The authors and thank Nippon Boehringer Ingelheim Co. Ltd. (Tokyo, Japan) for the kind donation of pure powder of TBH.

Disclosure
The authors report no conflicts of interest in this work.

References

