

Original article:

Anti-fibrotic Effects of ONO-EF-345, a Specific Phosphodiesterase IV inhibitor, on Lung Fibroblasts

¹Masahiko Yanagita, ^{1*}Mitsuhiro Yoshida, ¹Shigenori Hoshino, ¹Yukihiro Yano, ¹Koji Inoue, ¹Takayuki Takimoto, ¹Haruhiko Hirata, ¹Yozo Kashiwa, ¹Toru Kumagai, ¹Tadashi Osaki, ¹Takashi Kijima, ¹Isao Tachibana, ¹Ichiro Kawase

¹Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, Phone: +81-6-879-3833, Fax: +81-6-879-3839, e-mail: hiroinosaka@hotmail.com (* corresponding author)

ABSTRACT

Phosphodiesterase (PDE) IV inhibitors have been shown to inhibit various inflammatory reactions in pulmonary diseases such as bronchial asthma and chronic obstructive lung diseases (COPD). However, there have been no studies evaluating the effect of PDE IV inhibitors on airway fibrosis, which is a critical feature of airway remodeling in asthma and COPD. We therefore examined whether ONO-EF-345 (ONO), a PDE IV inhibitor, affected the function of lung fibroblasts. ONO suppressed TGF- β -induced type I collagen (COL1) mRNA expression in lung fibroblasts and also inhibited TGF- β -induced α -smooth muscle actin (SMA) protein expression. ONO did not affect Smad2 phosphorylation or Smad7 expression. However, ONO reduced JNK and p38 activation, which regulates TGF- β -induced COL1 expression. These results indicate that PDE IV inhibitors exert anti-fibrotic effects through the JNK and/or p38 pathways.

Keywords: PDE IV; TGF- β ; collagen; fibroblast; JNK; p38

INTRODUCTION

Asthma and COPD are chronic inflammatory diseases of the conducting airways and lung parenchyma that are characterized by airflow obstruction. Accumulating evidence indicates that the structure of the airway wall is abnormal in asthmatic and COPD patients (Jeffery,

2001), and the term “airway remodeling” is now widely used for these structural changes. It is frequently assumed that airway remodeling is induced as a consequence of repeated injury and repair attributed to chronic inflammation within the airway wall (Bousquet *et al.*, 2000). However, the cellular and molecular

mechanisms underlying the remodeling process are not fully understood, and the precise relationship between the remodeling and inflammatory components in asthma and COPD still remains controversial (Jeffery, 2001).

The features of structural remodeling in airways include subepithelial fibrosis, myofibroblast accumulation, airway smooth muscle hyperplasia and hypertrophy, goblet cell hyperplasia, and epithelial disruption. Airway fibrosis, which is an irreversible component of airway obstruction (Gabbrielli *et al.*, 1994), involves the activation of myofibroblasts and the increased deposition of ECM constituents such as collagen and fibronectin (Redington, 2000). TGF- β induces the expression of collagen type I (COL1) and also stimulates the differentiation of fibroblasts into myofibroblasts. A significant correlation has been observed between TGF- β expression and the degree of airway fibrosis in both asthma and COPD (Antonio *et al.*, 1997), supporting the concept that TGF- β plays an important role in the development of airway fibrosis.

Recent evidence suggests that 3'5'-cyclic adenosine monophosphate (cAMP) functions as a second messenger which modulates fibroblast activation (Yano *et al.*, 2006; Liu *et al.*, 2004; Huang *et al.*, 2006). The cAMP concentration is regulated by adenylyl cyclase, which produces cAMP, and phosphodiesterases (PDEs), which hydrolyse cAMP. PDEs comprise 11 pharmacologically distinct

families of metallophosphohydrolases that degrade cAMP and/or 3'5'-cyclic guanosine monophosphate (cGMP), which results in the inactivation of their function. Among the members of the PDE family, PDE IV is regarded as a critical molecule in regulating the inflammatory cells associated with asthma and COPD (Lipworth, 2005). PDE IV is encoded by four genes and specifically hydrolyses cAMP. Recently, several PDE IV inhibitors have been developed, and cilomilast and roflumilast have already been evaluated in clinical trials.

Despite numerous studies on the anti-inflammatory effects of PDE IV inhibitors, few studies have assessed the direct effects of PDE IV inhibitors on airway remodeling. It has recently been demonstrated that the inhibition of PDE IV reduces mucin production in bronchial cells (Mata *et al.*, 2005). We herein investigated whether a PDE IV inhibitor, ONO-EF-345 (ONO) described as ONO-12(b) in (Ochiai H, 2004), directly regulates lung fibroblast COL1 synthesis and α -SMA expression. We also investigated the mechanism of the anti-fibrotic effects of PDE IV inhibitors.

MATERIALS AND METHODS

Materials.

Human Transforming growth factor- β (TGF- β) was purchased from Sigma (St Louis, MO). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA). ONO-EF-345 (Ochiai H, 2004), a PDE IV inhibitor, was provided by ONO Pharmaceutical Co., Ltd (Osaka,

JAPAN). Isogen[®] was purchased from Nippon Gene (Tokyo, Japan). Antibodies against α -smooth muscle actin (α -SMA) and β -actin were purchased from American Research Products, Inc., (Belmont, MA) and MP Biochemicals (Irvine, CA), respectively. Antibodies against phospho-Smad2 and total Smad2 were obtained from Cell signaling technology (Beverly, MA). Fast Activated Cell-based ELISA (FACE[™]) Kits were purchased from Active Motif (Carlsbad, CA).

Cell culture.

Normal human lung fibroblast (NHLF) cells (Sanko Jyunyaku, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10 μ g/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. NHLF cells between passages 4 and 8 were used in the experiments.

TGF- β experiments.

NHLF cells were seeded on a 6-well dish and allowed to incubate overnight. After reaching subconfluence, the cells were starved for 24 h and were then treated with 10 ng/ml TGF- β . In pretreatment experiments, the cells were administered ONO at a concentration of 1 nM to 1 μ M 2 h before TGF- β stimulation. ONO has a specific PDE inhibitory activity and no cytotoxicity at these concentrations (Ochiai H, 2004). In posttreatment experiments, the cells were first incubated with TGF- β for 48 h and were then

stimulated by TGF- β with or without ONO. In some experiments, the cells were preincubated with SB203580, a specific p38 inhibitor, or SP600125, a specific JNK inhibitor, for 30 min before TGF- β administration.

Real-time PCR.

The cells were treated with TGF- β for 24 h before the extraction of total RNA from each well using Isogen[®] according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with SuperScripts III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was then conducted using ABI PRISM 7900 (Applied Biosystems, Foster City, CA) based on the ability of the 5' nuclease activity of Taq polymerase to cleave a labeled fluorogenic hybridization probe during DNA chain extension. The sequence-specific primers and probe mixtures for human COL1, human Smad7, and human GAPDH were obtained from TaqMan[®] Gene Expression Assays (Applied Biosystems, Foster City, CA). The relative mRNA expression levels of COL1 were normalized against that of the GAPDH gene from the same preparations.

Western blotting.

The cells were seeded on a 6-well dish. Before reaching confluence, the cells were starved for 24 h, then stimulated by TGF- β with or without ONO. The cells were lysed with RIPA buffer as described previously (Inoue *et al.*, 2005). Samples were separated on 10%

SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with primary antibodies against α -SMA (American Research Products, Inc., Belmont, MA), phospho-Smad2, or total Smad2 (Cell signaling technology, Beverly, MA). The membranes were then incubated with secondary HRP-conjugated antibodies. The membranes were visualized by a chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA), and the image was obtained by exposure to X-ray films. The relative protein loading was normalized after the membrane was stripped and re probed with anti- β -actin antibody (MP Biochemicals, Irvine, CA).

Phosphorylation of JNK and p38.

The amounts of JNK and p38 phosphorylation were determined by FACE™ Kits. Briefly, NHLF cells were cultured in 96-well plates at a density of 3,000 cells per well and stimulated by TGF- β with or without ONO. Following stimulation, the cells were rapidly fixed with 4% formaldehyde to preserve activation-specific protein modifications. Each well was then incubated with primary antibodies against phospho-JNK, phospho-p38, total JNK or total p38, and the cells were subsequently incubated with secondary HRP-conjugated antibodies and developing solution. The absorbance was read on a spectrophotometer at 450 nm with an optional reference wavelength of 655 nm. The relative number of cells in each well is then determined using the Crystal Violet

solution.

Statistics.

Data are expressed as the means \pm SEM. The statistical significance of the differences was evaluated by ANOVA. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

ONO decreases TGF- β -induced COL1 mRNA expression in lung fibroblasts.

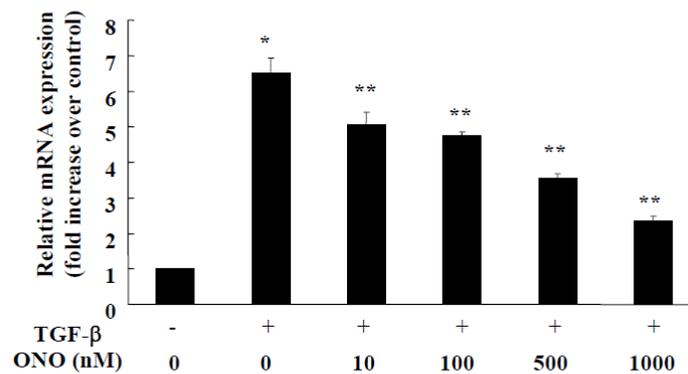
TGF- β is a potent inducer of COL1 and is involved in the pathogenesis of airway fibrosis in asthma and COPD (Takizawa *et al.*, 2001; Postma *et al.*, 2006). To investigate the inhibitory effect of ONO on TGF- β -induced COL1 mRNA expression, NHLF cells were treated with TGF- β in the presence or absence of ONO. The amount of COL1 mRNA expression was determined by the real-time PCR method. As expected, we observed a 6-fold increase in TGF- β -enhanced COL1 mRNA expression in comparison to the control. ONO decreased TGF- β -induced COL1 mRNA expression in a dose-dependent manner (Fig.1A).

ONO decreases TGF- β -induced α -SMA protein expression in NHLF cells.

The transformation of fibroblasts into myofibroblasts, a prominent feature of fibroblast activation, is often observed in airway fibrosis. α -SMA is a myofibroblast marker that is also induced by TGF- β . Therefore, we next investigated the effect of ONO on TGF- β -induced α -SMA expression. NHLF cells were treated with TGF- β in the presence or absence of ONO

for 48 h, and the amount of α -SMA protein was determined by Western blotting. TGF- β markedly enhanced α -SMA protein expression. ONO significantly decreased TGF- β -induced α -SMA expression in a dose-dependent manner (Fig.1B).

A



B

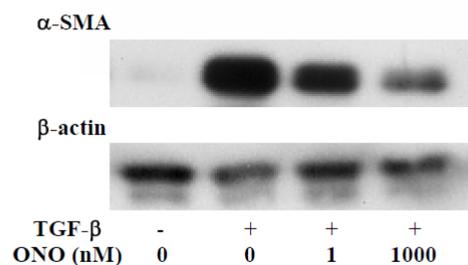


Figure 1: Effects of ONO on TGF- β -induced COL1 mRNA expression and α -SMA protein expression. The cells were treated with 10 ng/ml TGF- β in the presence or absence of the indicated concentrations of ONO. (A) The amount of COL1 mRNA expression was evaluated by real-time PCR. The relative levels of COL1 mRNA expression were normalized by assessing the ratio of COL1 against GAPDH. Data are expressed as the ratio of the control value of COL1mRNA/GAPDH mRNA from the vehicle treatment. Data represent the means \pm SEM of results from three independent experiments. * p <0.0001 control vs. TGF- β , ** p <0.05 TGF- β vs. TGF- β plus ONO. (B) The amount of TGF- β -induced α -SMA was determined by Western blotting. The result is representative of three separate experiments with similar results.

Posttreatment with ONO inhibits TGF- β -induced COL1 mRNA expression and α -SMA protein expression in NHLF cells. In this study we demonstrated that pretreatment with ONO inhibited TGF- β -induced COL1 mRNA expression and α -SMA protein expression in NHLF

cells. However, in clinical practice, the process of airway fibrosis has already commenced before treatment is initiated. Therefore, we investigated whether treatment of NHLF cells with ONO after their stimulation with TGF- β also exerted

inhibitory effects. Treatment with ONO even 48 h after TGF- β stimulation significantly decreased TGF- β -induced COL1 mRNA expression and α -SMA protein expression (Fig.2).

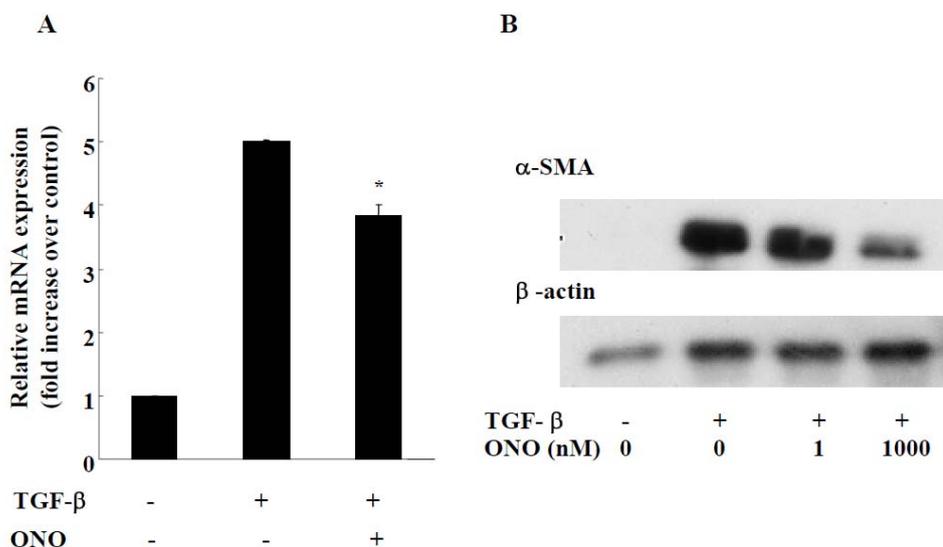


Figure 2: Effects of posttreatment with ONO on TGF- β -induced COL1 mRNA expression and α -SMA protein expression. After 48 h stimulation with 10 ng/ml TGF- β , NHLF cells were treated with TGF- β in the various concentrations of ONO. (A) The amount of COL1 mRNA expression was evaluated by real-time PCR. The relative levels of COL1 mRNA expression were normalized by assessing the ratio of COL1 against GAPDH. Data are expressed as the ratio of the control value of COL1mRNA/GAPDH mRNA from the vehicle treatment. Data represent the means \pm SEM of results from three independent experiments. * $p < 0.05$ TGF- β vs. TGF- β plus ONO. (B) The amount of TGF- β -induced α -SMA was determined by Western blotting. The result is representative of three separate experiments with similar results.

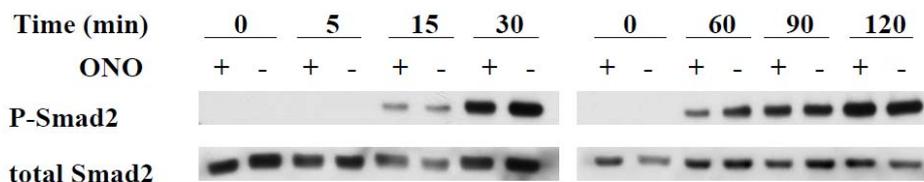
ONO does not inhibit the TGF- β -mediated phosphorylation of Smad2 in NHLF cells

Many of the effects of TGF- β are mediated through Smad2/3 pathways (Derynck *et al.*, 2003). To determine the extent of Smad2 phosphorylation by

TGF- β , NHLF cells were treated with TGF- β in the presence or absence of ONO, and a Western blot analysis was conducted. As shown in Fig. 3A, TGF- β markedly induced Smad2 phosphorylation. However, ONO did not decrease the amounts of

TGF- β -mediated Smad2 phosphorylation.

A



B

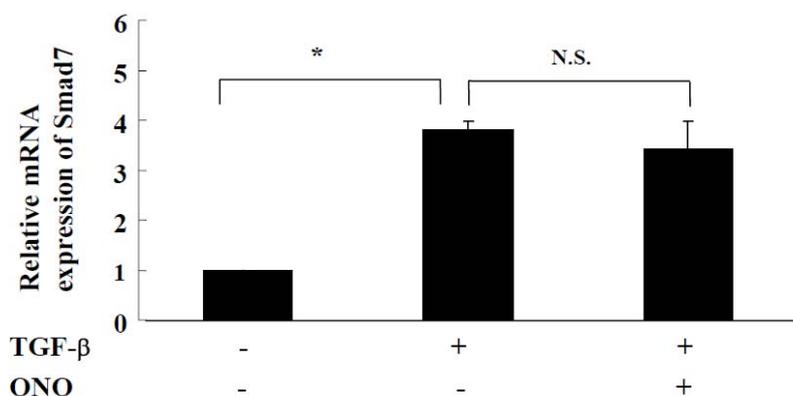


Figure 3: Effects of ONO on TGF- β -mediated Smad signaling. (A) Effects on TGF- β -induced Smad2 phosphorylation. NHLF cells were treated with 10 ng/ml TGF- β in the presence or absence of 1 μ M ONO. The amounts of phospho-Smad2 were evaluated by Western blotting. (B) Effects on TGF- β -mediated mRNA expression of Smad7. NHLF cells were treated with 10 ng/ml TGF- β in the presence or absence of 1 μ M ONO for 24 h. The relative levels of Smad7 mRNA expression were normalized by assessing the ratio of Smad7 against GAPDH. Data are expressed as the ratio of the control value of Smad7 mRNA/GAPDH mRNA from the vehicle treatment. Data represent the means \pm SEM from three independent experiments. * $p < 0.001$ control vs. TGF- β , N.S. TGF- β vs. TGF- β plus ONO. N.S. stands for Not Significant.

ONO does not affect the TGF- β -induced upregulation of Smad7 mRNA expression in NHLF cells.

Smad7 is an intracellular antagonist for TGF- β signaling and inhibits Smad2 activation (Derynck *et al.*, 2003). To investigate whether ONO could affect the TGF- β -mediated Smad7 pathway, NHLF cells were treated with TGF- β in the

presence or absence of ONO for 24 h. The amounts of Smad7 mRNA were determined by real-time PCR. As shown in Fig.3B, TGF- β increased the expression of Smad7 mRNA. However, ONO did not affect the TGF- β -induced upregulation of Smad7 mRNA expression. Collectively, these results indicate that ONO does not affect TGF- β -mediated Smad pathways.

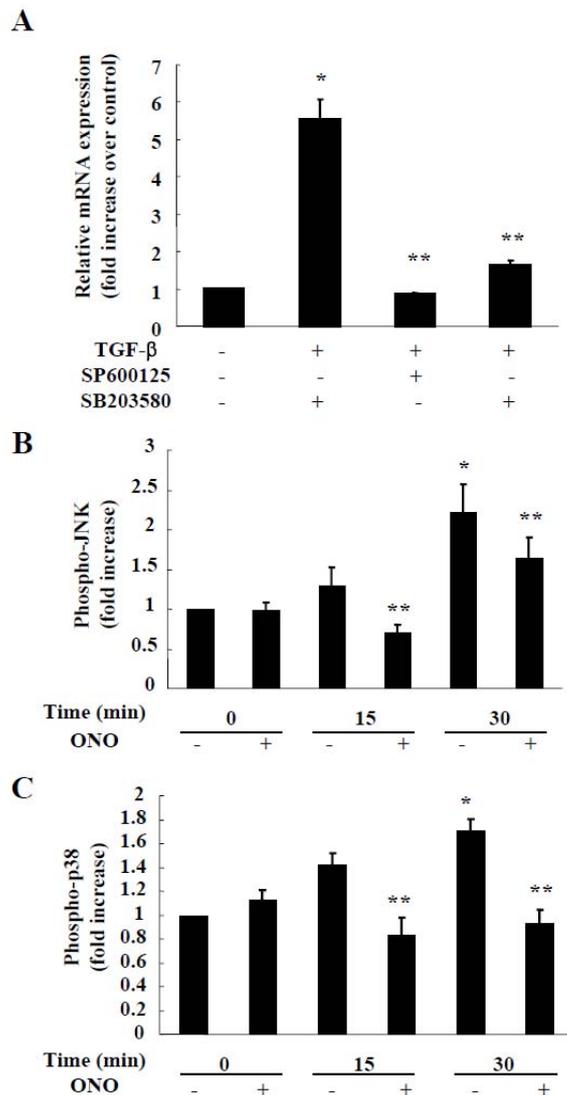


Figure 4: Effects of ONO on TGF- β -mediated JNK and p38 activation. NHFL cells were treated with 10 ng/ml TGF- β in the presence or absence of SP600125, a specific JNK inhibitor, SB203580, a specific p38 inhibitor, or ONO. (A) The amount of COL1 mRNA expression was evaluated by real-time PCR. The relative levels of COL1 mRNA expression were normalized by assessing the ratio of COL1 against GAPDH. Data are expressed as the ratio of the control value of COL1mRNA/GAPDH mRNA from the vehicle treatment. Data represent the means \pm SEM of results from three independent experiments. * p <0.0001 control vs. TGF- β , ** p <0.0001 TGF- β vs. TGF- β plus SP600125 or SB203580. (B, C) The levels of TGF- β -induced JNK (B) and p38 (C) phosphorylation were determined by FACE™ ELISA Kits. The relative levels of phosphorylated JNK and p38 were normalized by assessing the ratio against total JNK and p38, respectively. Data are expressed as the ratio of the control value of phosphorylated JNK and p38/total JNK and p38, respectively, from the vehicle treatment. Data represent the means \pm SEM of results from six and four independent experiments, respectively. * p <0.0001 control vs. TGF- β , ** p <0.05 TGF- β vs. TGF- β plus ONO.

ONO inhibits the TGF- β -mediated phosphorylation of JNK and p38 in NHLF cells

In addition to classical Smad pathways, mitogen-activated protein kinase (MAPK) pathways function as downstream of TGF- β signaling (Derynck *et al.*, 2003). We and other investigators have shown that TGF- β induces JNK and p38 phosphorylation and that TGF- β induces COL1 mRNA expression and α -SMA expression via the JNK and/or p38 pathways (Inoue *et al.*, 2005; Hu *et al.*, 2006). To confirm that inhibition of JNK or p38 suppresses TGF- β -induced COL1 mRNA expression, we used selective and specific inhibitors of JNK and p38. As expected, both inhibitors significantly decreased TGF- β -induced COL1 mRNA expression (Fig. 4A). Then we determined whether ONO could block the TGF- β -mediated JNK and p38 pathways. The cells were treated with TGF- β in the presence or absence of ONO, and the cell-based ELISA system for detecting the phosphorylation of JNK and p38 was conducted. As shown in Fig. 4B and 4C, TGF- β induced the phosphorylation of JNK and p38. ONO significantly suppressed the extent of TGF- β -induced JNK and p38 phosphorylation.

DISCUSSION

PDE IV inhibitors have been known to exert anti-inflammatory effects. However, the direct effect of PDE IV inhibitors on airway fibrosis, one of the critical features of airway remodeling, had not previously been assessed. Our examination of whether ONO, a specific PDE IV inhibitor,

affected the function of lung fibroblasts revealed that it inhibited TGF- β -induced COL1 mRNA expression in lung fibroblasts and also reduced TGF- β -induced α -SMA protein. However, ONO did not affect Smad2 phosphorylation or Smad7 expression induced by TGF- β . ONO suppressed JNK and p38 phosphorylation, which regulate TGF- β -induced COL1 mRNA expression and α -SMA protein expression. Collectively, these results indicate that PDE IV inhibitors exert anti-fibrotic effects, at least partly, through the JNK and/or p38 pathways.

We demonstrated the anti-fibrotic effects of ONO on NHLF cells in this study. We also investigated whether rolipram, another PDE IV inhibitor, also exerts an inhibitory effect on TGF- β -induced COL1 mRNA expression with the same results (personal observation). Our findings indicate that ONO suppresses TGF- β -induced COL1 mRNA expression as a specific PDE IV inhibitor via the cAMP pathway. This result is supported by previous reports demonstrating that PGE₂ and other adenylyl cyclase activators, which increase intracellular cAMP, inhibit collagen synthesis (Liu *et al.*, 2004; Huang *et al.*, 2006). It has also been demonstrated that cAMP inhibits myofibroblast differentiation (Kolodsick *et al.*, 2003). It is therefore likely that ONO also reduces TGF- β -induced α -SMA expression through the cAMP pathway. Collectively, these results support the concept of the anti-fibrotic effects of PDE IV inhibition via the cAMP

pathway.

Smad pathways have been recognized as the main mediator of TGF- β -induced gene expression, including the expression of COL1 (Derynck *et al.*, 2003). The phosphorylation of Smad2/3 causes it to associate with Smad4. The complex translocates into the nucleus, where it regulates transcriptional responses together with additional DNA binding cofactors. Inversely, Smad7 regulates fibrotic reactions induced by TGF- β (Nakao *et al.*, 1999). Smad7 is induced by TGF- β to negatively regulate TGF- β signaling by interrupting Smad2/3 activation. However, ONO did not influence either the Smad7 gene expression or Smad2 phosphorylation in this study. These results are consistent with a previous report demonstrating that pentoxifylline, a xanthine derivative that increases intracellular cAMP, does not interfere with TGF- β signaling to Smad2/3 activation controlled by Smad7 (Lin *et al.*, 2005). Furthermore, the cAMP pathway has been demonstrated to inhibit TGF- β -induced Smad-dependent transcription by the disruption of transcription cofactor binding (Schiller *et al.*, 2003). These reports support our results that the suppressive effects of PDE IV inhibitors on TGF- β signaling are not achieved through the direct modulation of Smad pathways.

In addition to the classical Smad pathways, the MAPK pathways are involved in the TGF- β signaling (Derynck *et al.*, 2003). MAPK regulates collagen gene expression

by various stimuli, including TGF- β (Chen *et al.*, 1999; Varela-Rey *et al.*, 2002). We have recently demonstrated that IL-10 inhibits TGF- β -induced COL1 mRNA expression through the JNK and/or p38 pathways in lung fibroblasts, but not through the Smad pathways (Inoue *et al.*, 2005). It has also been shown that α -SMA expression induced by TGF- β is regulated by the JNK and/or p38 pathways (Inoue *et al.*, 2005; Hu *et al.*, 2006). In our results, ONO suppressed TGF- β -induced JNK and p38 activation, which suggests that ONO exerts anti-fibrotic effects through the regulation of the JNK and/or p38 pathways. Our results are consistent with the results of recent reports demonstrating that the JNK pathway is negatively regulated by the cAMP cascade (Delgado, 2002), which is activated by the inhibition of PDE IV.

Our results have also showed that even the treatment of cells with the PDE IV inhibitor after their stimulation by TGF- β resulted in the inhibition of TGF- β -induced COL1 mRNA expression and α -SMA expression. This finding is notable because in the clinical setting, airway remodeling and fibrosis have already commenced before treatment is started. Our results strongly indicate that PDE IV inhibition has suppressive as well as preventive effects on airway fibrosis. Furthermore, the administration of PDE IV inhibitors for the treatment of airway remodeling had a clinical advantage related to the two hypotheses about the mechanism of airway remodeling. Recent evidence suggests that airway remodeling

is induced as a consequence of repeated injury and repair attributed to chronic inflammation within the airway wall (Bousquet *et al.*, 2000). Another equally plausible hypothesis is that the processes of chronic inflammation are distinct from those of remodeling (Jeffery, 2001). PDE IV inhibitors were originally regarded as anti-inflammatory drugs. Our data demonstrate that the inhibition of PDE IV also exerts direct anti-fibrotic effects. Therefore, PDE IV inhibitor administration could regulate airway remodeling by modulating both inflammation and fibroblast activation. To date, no simple or combination therapy is available for completely inhibiting airway inflammation and remodeling. Therefore, treatment with PDE IV inhibitors could be a feasible therapy for chronic airway diseases that are characterized by both inflammation and airway remodeling. Future studies are needed to determine whether these anti-fibrotic effects of PDE IV inhibitors are also observed *in vivo*.

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