Inflammation and remodelling in experimental models of COPD - Mechanisms and therapeutic perspectives

Pera, Tonio

Chapter 3

TAK1 PLAYS A MAJOR ROLE IN GROWTH FACTOR-INDUCED PEHENOTYPIC MODULATION OF AIRWAY SMOOTH MUSCLE

Tonio Pera, Riham Sami, Johan Zaagsma, Herman Meurs

Submitted (2011)

Abstract

Increased airway smooth muscle (ASM) mass is a major feature of airway remodeling in asthma and COPD. Growth factors induce an ASM phenotype, characterized by an increased proliferative state and a decreased contractile protein expression, reducing contractility of the muscle. TGF-β-activated kinase 1 (TAK1), a mitogen-activated protein kinase kinase kinase (MAP3K7), is a key enzyme in pro-inflammatory signaling in various cell types; however, its function in ASM is unknown. The aim of this study was to investigate the role of TAK1 in growth factor-induced phenotypic modulation of ASM. Using bovine tracheal smooth muscle (BTSM) strips and -cells, as well as human tracheal smooth muscle cells, we investigated the role of TAK1 in growth factor-induced proliferation and hypocontractility. Platelet-derived growth factor (PDGF; 10 ng/ml)- and fetal bovine serum (FBS; 5%)-induced increases in DNA synthesis and cell number in bovine and human cells were significantly inhibited by pretreatment with the specific TAK1 antagonist LL-Z-1640-2 (5*Z*-7-oxozeaenol; 100 nM). PDGF-induced DNA synthesis and ERK 1/2 phosphorylation in BTSM cells were strongly inhibited by both LL-Z-1640-2 pretreatment and transfection of dominant-negative TAK1. In addition, LL-Z-1640-2 inhibited PDGF-induced reduction of BTSM contractility and smooth muscle α-actin expression. The data indicate that TAK1 plays a major role in growth factor-induced phenotypic modulation of ASM.

Introduction

Airway smooth muscle (ASM) thickening is a pathological feature of asthma and chronic obstructive pulmonary disease (COPD) which may contribute to airflow limitation and airway hyperresponsiveness (1). The mechanisms underlying ASM remodeling have not been fully elucidated; however, there is evidence that ASM cell proliferation (2, 3), which can be induced by increased expression of growth factors in the airway wall (4, 5) may be involved. Growth factors induce a proliferative ASM phenotype, which is characterized by increased ASM cell proliferation and a decreased expression of contractile proteins, leading to decreased contractility (6-9). Previous studies have shown that extracellular signal-regulated kinase (ERK) 1/2 is a key enzyme in platelet-derived growth factor (PDGF)-induced proliferation (6, 7, 10, 11) and decrease in contractility of ASM (6, 7). Indeed, induction of sustained ERK 1/2 phosphorylation is required for cell cycle progression and proliferation of ASM cells (12, 13).

TGF-β-activated kinase 1 (TAK1), a serine/threonine kinase, is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family (MAP3K7). Initially, TAK1 was identified as a mediator of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (14), but has since emerged as a key player in interleukin-1 (IL-1) –receptor (15), Toll-like receptor (TLR) (16) and tumor necrosis factor-α (TNF-α)-receptor (17) signaling. TAK1 activates both nuclear factor - kappa B (NF-κB) (17, 18) and MAP kinase (14) pathways, including the ERK 1/2 pathway (19-22).TAK1 has been found to play a major role in various immune responses (16, 23-25) and embryonal development (26-29). In addition, accumulating evidence suggests that TAK1 plays a role in cardiac muscle hypertrophy (30, 31), indicating that this enzyme may also be involved in the pathogenesis of tissue remodeling. The role of TAK1 in ASM function is currently unknown. In this study, we present evidence that TAK1 plays a major role in growth factor-induced proliferation and reduced contractility of ASM.

Materials and Methods

Isolation of tracheal smooth muscle cells

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), and fetal bovine serum (FBS, 0.5%) (all purchased from GIBCO BRL Life Technologies, Paisley, UK). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml, Boehringer, Mannheim, Germany), papain (1 mg/ml, Boehringer), and Soybean trypsin inhibitor (1 mg/ml, Sigma Chemical, St. Louis, MO, USA). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over a 50 μm gauze, cells were washed three times in supplemented DMEM containing 10% FBS. This isolation method results in a cell population positive for smooth muscle α-actin and smooth muscle myosin heavy chain (6, 32).

Human tracheal sections from anonymized lung transplantation donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen, and transported to the laboratory in ice-cold Krebs-Henseleit KH buffer. Human tracheal smooth muscle (HTSM) layer was prepared as described for BTSM and chopped using a McIlwain tissue chopper. Tissue slices were washed once with supplemented DMEM, placed in culture flasks and allowed to adhere. Upon reaching confluence, cells were passaged by trypsinization. Cells from passages 1-5 were used for the present study.

[^{3}H]-Thymidine incorporation

BTSM or primary HTSM cells were plated in 24-well cluster plates at a density of 30,000 cells per well, and were allowed to attach overnight in 10% FBS-containing DMEM in a humidified 5% CO_2 incubator at 37°C. Cells were washed twice with sterile phosphate-buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; Na_2HPO_4, 8.1; pH 7.4) and made quiescent by incubation in FBS-free DMEM supplemented with apo-transferrin (5 μg/ml, human, Sigma), ascorbate (100 μM, Merck, Darmstadt, Germany), and insulin (1 μM, bovine pancreas, Sigma), for 72 h. Cells were then washed with PBS and stimulated with PDGF (10 ng/ml) or FBS (5%) in FBS-free DMEM for 28 h, in the presence or absence of the TAK1 antagonist LL-Z1640-2 (100 nM, Bioaustralis, Smithfield NSW, Australia), which was added 30 min before stimulation. Treatment of cells lasted 28 h, the last 24 h in the presence of [^{3}H]-thymidine (0.25 μCi/ml, Amersham, Buckinghamshire, UK) after which the cells were washed twice with PBS and incubated with ice-cold 5% trichloroacetic acid (TCA) on ice for 30 min. Subsequently, the acid-insoluble fraction was dissolved in 0.5 ml NaOH (1 M). Incorporated [^{3}H]-thymidine was quantified by liquid-scintillation counting.

Alamar blue assay

BTSM cells were plated, cultured and made quiescent as described above. Cells were then stimulated with PDGF (10 ng/ml) or FBS (5%) for 4 days. After 4 days of stimulation, cells were washed twice with PBS and incubated with Hanks' balanced salt solution containing 5% Alamar blue (BioSource, Camarillo, CA) solution for 45 minutes. Proliferation was assessed by conversion of Alamar blue, as indicated by the manufacturer.

Transfection of plasmid DNA

For proliferation studies, cells were grown to 95% confluence on 24-well plates, washed twice with PBS and then transfected using a mixture of 2 μl Lipofectamine 2000 and 0.4 μg DNA encoding a hemmagluttinine (HA)-tagged,

kinase dead TAK1 mutant (TAK1 K63W) or GFP, as control, for 6 h in 200 µl DMEM without serum and antibiotics. After 6 h cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and insulin (1 µM) and the cells were then cultured for another 72 h.

For western blotting, cells were grown to 95% confluence on 6-well plates and transfected using a mixture of 10 µl Lipofectamine 2000 and 2 µg DNA encoding TAK1 K63W or GFP for 6 h in 600 µl DMEM without serum and antibiotics. After 6 h cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and 10% FBS and the cells were then cultured for another 18 h. Dominant-negative TAK1 was a generous gift from Dr. B.J.L. Eggen (33), (Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands), with kind permission of Prof. K. Matsumoto, (Department of Molecular Biology, Graduate School of Science, Nagoya University, Japan).

Western blot analysis

BTSM cells were grown to confluence on 6-well cluster plates, using DMEM containing 10% FBS. Cells were then washed twice with sterile PBS and made quiescent by incubation in FBS-free DMEM, supplemented with insulin (1 µM), apo-transferrin (5 µg/ml) and ascorbate (100 µM) for 24 h. Cells were then washed with PBS and stimulated with PDGF (10 ng/ml), for up to 2 h, in FBS-free medium. Subsequently, cells were washed once with ice-cold PBS and then lysed in ice-cold RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 5 mM 2-glycerophosphoric acid, pH 8.0). Lysates were stored at –80 °C until further use. Protein content was determined according to Bradford (34). Total cell homogenates were then subjected to immunoblot analysis using antibodies against p-ERK 1/2 (Thr^{202}/Tyr^{204}), ERK 1/2 (Cell Signaling Technology, MA, USA), smooth muscle α-actin (Sigma), GAPDH (Santa Cruz Biotechnology, CA, USA) or HA (Roche, IN, USA). The antibodies were visualized using enhanced chemiluminescence. Photographs of the blots were analyzed by densitometry.

Tissue culture

After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, BTSM strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile FBS-free DMEM, supplemented with apo-transferrin (5

μg/ml) and ascorbate (100 μM). Next, the tissue strips were transferred into suspension culture flasks containing 7.5 ml FBS-free DMEM, and were cultured for 4 days in the absence or presence of PDGF (10 ng/ml) and/or LL-Z-1640-2 (100 nM).

Isometric tension measurements

Tissue strips, collected from the suspension culture flasks, were washed with several volumes of KH buffer pregassed with 5% CO_2 and 95% O_2, pH 7.4 at 37°C. Subsequently, the strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20-ml water-jacked organ baths containing KH buffer at 37°C, continuously gassed with 5% CO_2 and 95% O_2, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3g. Subsequently, the muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two washouts, maximal relaxation was established by the addition of 0.1 μM (-)-isoprenaline (Sigma). In most of the experiments, no basal myogenic tone was detected. Tension was readjusted to 3 g, immediately followed by three washes with fresh KH buffer. After another equilibration period of 30 min, cumulative concentration response curves were constructed using stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM–100 μM; ICN Biomedicals, Costa Mesa, CA, USA). When maximal tension was obtained, the strips were washed several times, and maximal relaxation was established using 10 μM (-)-isoprenaline.

Data analysis

All data represent means ± s.e.mean from separate experiments. The statistical significance of differences between data was determined either by one-way ANOVA, followed by Bonferroni multiple comparison test, or by the Student's t-test, as appropriate. Differences were considered to be statistically significant when $P<0.05$.

Results

TAK1 regulates proliferation of BTSM and HTSM cells

In order to investigate the role of TAK1 in the proliferation of BTSM cells, [^{3}H]-thymidine incorporation and Alamar blue assays were performed. Stimulation of these cells with PDGF (10 ng/ml) or FBS (5%) resulted in an increase in [^{3}H]-thymidine incorporation (3.0- and 3.7-fold, respectively, after 28h Figure 1A), as well as an increase in cell numbers (1.4- and 2.4-fold, respectively, after 4 days; Figure 1B). Pretreatment of these cells with the specific TAK1 inhibitor, LL-Z-1640-2 (5Z-7-oxozeaenol; 100 nM) (19, 35), fully inhibited the PDGF-induced

increase in [^{3}H]-thymidine incorporation, whereas the FBS-induced response was inhibited by 69% (Figure 1A). Similarly, LL-Z-1640-2 pretreatment abolished the PDGF-induced increase in cell number and inhibited the FBS-induced increase by 55% (Figure 1B). As in BTSM cells, pretreatment with LL-Z-1640-2 abolished the PDGF-induced increase in [^{3}H]-thymidine incorporation (Figure 1C) and inhibited the FBS-induced increase in cell number by 62% (Figure 1D) in primary HTSM cells. Transfection of BTSM cells with dominant-negative TAK1 (TAK1 DN) resulted in a significant decrease of PDGF-induced [^{3}H]-thymidine incorporation, compared to GFP-transfected cells (Figure 2). Collectively, these data suggest that TAK1 plays a major role in growth factor-induced proliferation both in bovine and human ASM cells.

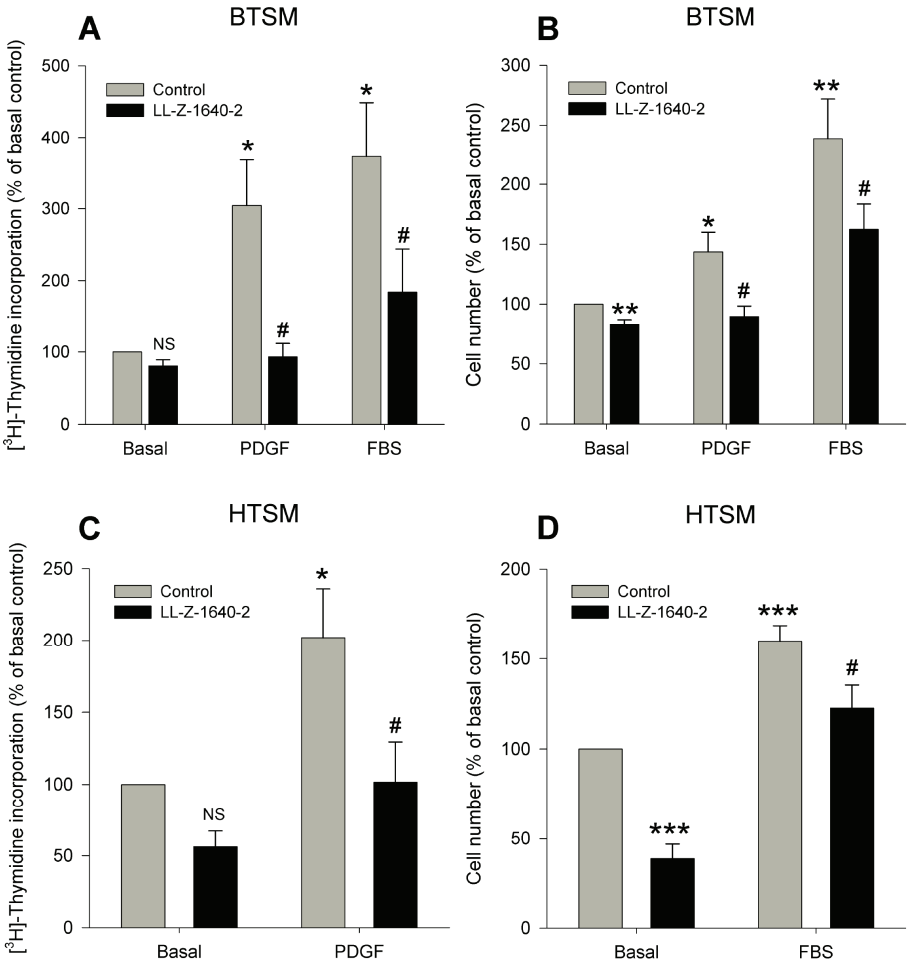

Figure 1: Growth factor-induced proliferation is inhibited by the TAK1 inhibitor LL-Z-1640-2. (A) [3H]-Thymidine incorporation in BTSM cells. (B) BTSM cell numbers determined by Alamar Blue. (C) [3H]-Thymidine incorporation in primary HTSM cells. Cells were stimulated with PDGF (10 ng/ml) or FBS (5%) in the presence or absence of LL-Z-1640-2 (100 nM). Data are expressed as % of basal controls and are means ± S.E.M. of 6-7 experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs untreated control, #P<0.05 vs treatment in the absence of inhibitor, NS: not significant vs untreated control.

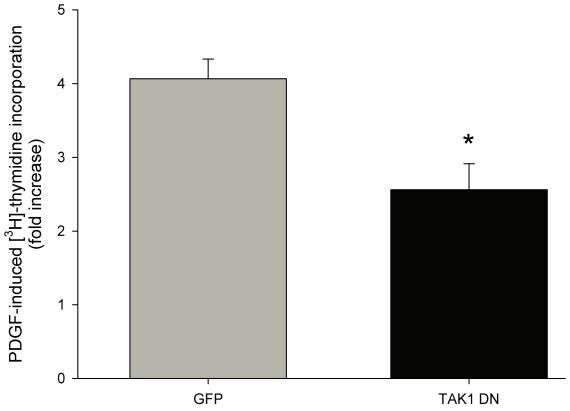

Figure 2: PDGF-induced [3H]-thymidine incorporation is inhibited by dominant-negative TAK1 (TAK1 DN). BTSM cells transfected with GFP (control) or TAK1 DN were stimulated with PDGF (10 ng/ml). Data are expressed as fold increase from respective unstimulated, transfected cells and are means of 5-7 experiments, each performed in triplicate. *P<0.05 vs GFP

TAK1 regulates ERK 1/2 phosphorylation in BTSM cells

In order to investigate the role of TAK1 in growth factor-induced ERK 1/2 phosphorylation, western blot analysis was performed. Stimulation of BTSM cells with PDGF for 5 min and 2 h resulted in a 6.4- and 3.7-fold increase in ERK 1/2 phosphorylation, respectively (Figure 3). LL-Z-1640-2 pretreatment inhibited the PDGF-induced increase at 5 min and 2 h by 68% and 73%, respectively (Figure 3).

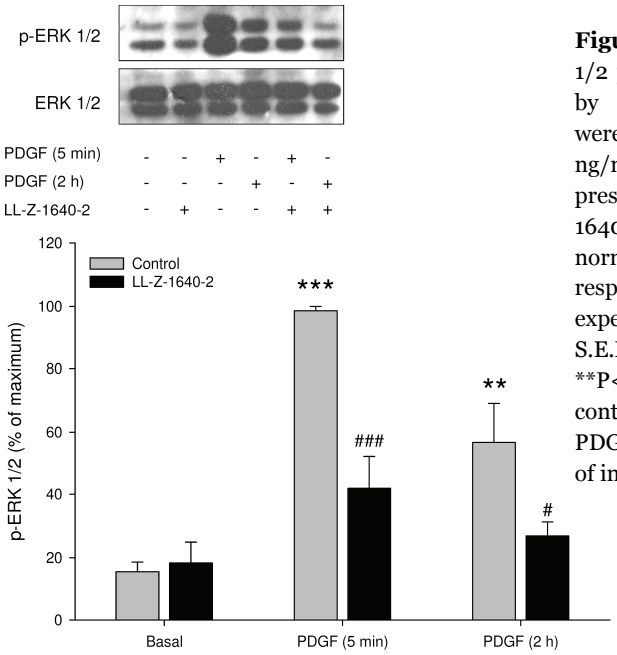

Figure 3: PDGF-induced ERK 1/2 phosphorylation is inhibited by LL-Z-1640-2. BTSM cells were treated with PDGF (10 ng/ml) for 5 min or 2 h in presence or absence of LL-Z-1640-2. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 5 experiments. **P<0.01, ***P<0.001 vs basal control, #P<0.05, ###P<0.001 vs PDGF treatment in the absence of inhibitor.

In addition, the ability of PDGF to induce ERK 1/2 phosphorylation was also evaluated in cells transfected with dominant-negative TAK1 or GFP, as a control. Stimulation of GFP-transfected cells with PDGF for 5 min and 2 h resulted in a 5.4- and 3.5-fold increase in ERK 1/2 phosphorylation. In dominant-negative TAK1-transfected cells, the PDGF-induced ERK 1/2 phosphorylation was inhibited by 55% at 5 min and 85% at 2 h, compared to GFP-transfected cells (Figure 4). Neither LL-Z-1640-2 pretreatment nor dominant negative TAK1 expression had a significant effect on basal ERK 1/2 phosphorylation. These data indicate that TAK1 plays a major role in PDGF-induced ERK 1/2 phosphorylation in ASM cells.

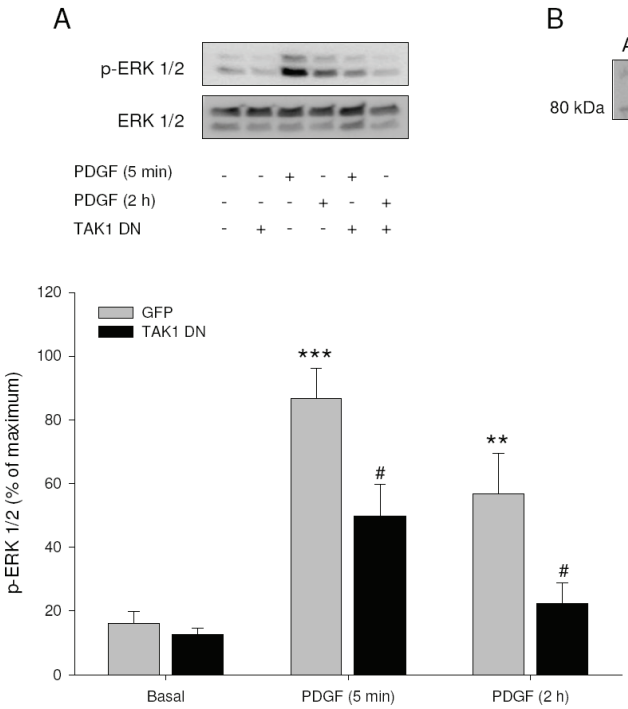

Figure 4: PDGF-induced ERK 1/2 phosphorylation is inhibited by dominant negative TAK1 (TAK1 DN) protein expression. BTSM cells transfected with TAK1 DN or GFP were treated with PDGF for 5 min or 2 h (A). Cell lysates were analyzed by immunoblotting for p-ERK 1/2 and total ERK 1/2. Expression of TAK1 DN was confirmed by immunoblotting for HA (B). Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 7 experiments. **P<0.01, ***P<0.001 vs untreated GFP-transfected control cells, #P<0.05 vs PDGF-treated, GFP-transfected control cells.

TAK1 regulates contractility of BTSM tissue preparations

Previous studies have shown that growth factor-induced ASM cell proliferation strongly correlates with the capacity of growth factors to induce a decrease in contractility of ASM tissue preparations (6), which is associated with a decrease in contractile protein expression. In order to investigate the potential role of TAK1 in the development of growth factor-induced hypocontractile phenotype, we performed isometric contraction experiments using BTSM strip preparations. After 4 days of tissue culture in the presence of PDGF there was a significant decrease in maximal contraction to both methacholine (Figure 5A) and KCl (Figure 5B). The decrease in contractility to both stimuli was strongly inhibited in the presence of LL-Z-1640-2 (Figures 5A and 5B). Accordingly, LL-Z-1640-2 abolished the PDGF-induced decrease in smooth muscle α-actin expression in these tissue preparations. Collectively, our data indicate that TAK1 plays a major role in the induction of a proliferative, hypocontractile ASM phenotype by PDGF.

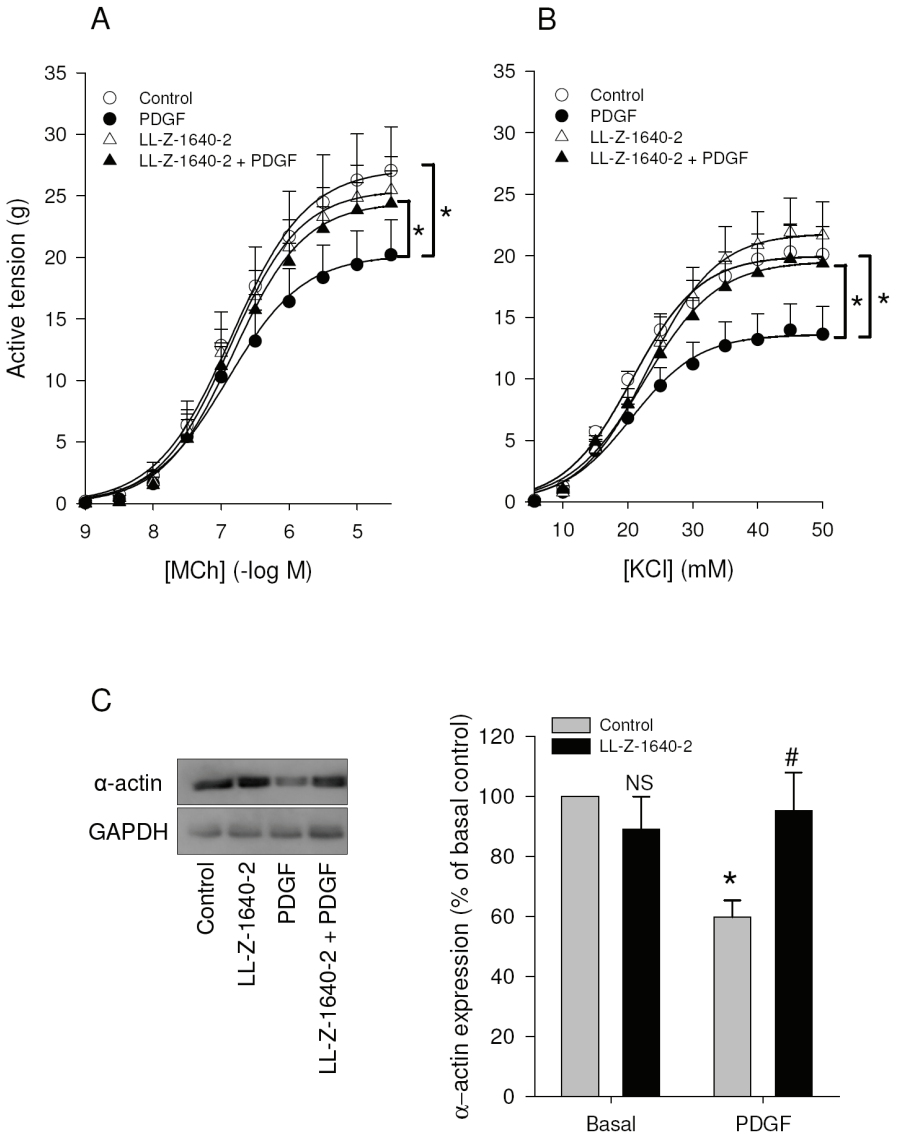

Figure 5: PDGF-induced decrease in contractility and contractile protein expression in BTSM tissue are inhibited by LL-Z-1640-2. Methacholine (MCh) (A)- and KCl (B)-induced contractions of BTSM strips cultured for 4 days in the absence or presence of PDGF (10 ng/ml), with or without LL-Z-1640-2 (100 nM). Contractile protein expression in cultured BTSM strips was determined by immunoblotting for α-actin and GAPDH as loading control (C). α-Actin blots were analyzed by densitometry. A representative blot is shown. Data are means ± S.E.M. of 4-6 experiments. *P<0.05 vs basal control, #P<0.05 vs PDGF, NS: not significant vs basal control.

Discussion

This is the first study addressing the role of TAK1 in ASM. In this study, we demonstrated that TAK1 is importantly involved in growth factor induced ASM cell proliferation, ERK 1/2 phosphorylation, hypocontractility and reduced contractile protein expression. Collectively, the data indicate that TAK1 plays a major role in the induction of a proliferative, hypocontractile ASM phenotype by growth factors such as PDGF.

Phenotypic modulation of ASM to a proliferative, hypocontractile phenotype occurs in response to a variety of mitogens, including growth factors (6, 7), G-protein-coupled receptor agonists (36-38), extracellular matrix proteins (39, 40) and, as recently shown, cigarette smoke and lipopolysaccharide (LPS) (41). The capacity of mitogens to induce ASM cell proliferation and decreased expression of contractile proteins has been found to correlate strongly with their capacity to decrease contractility of the ASM tissue preparations (6, 39). In addition, ERK 1/2 signaling induced by these mitogens has been shown to be fundamental to ASM cell proliferation (6, 7, 10-13) and the associated decrease in ASM contractility (6, 7). Induction of a proliferative phenotype of ASM may cause ASM thickening (42, 43) and thus contribute to decline in lung function and airway hyperresponsiveness in chronic pulmonary diseases like asthma and COPD (1). Our present data therefore indicate that TAK1 could be importantly involved in the progression of asthma and COPD.

TAK1 has previously been shown to play a role in cardiac hypertrophy as well. TAK1 activity was increased in mouse myocardium after pressure overload (31) as well as in the noninfarcted cardiomyocytes, in a rat model of myocardial infarction (30). In both cases, this was associated with the subsequent development of cardiac hypertrophy. Indeed, the sole expression of activated TAK1 in the myocardium of transgenic mice was sufficient to induce cardiac hypertrophy (31). Interestingly, in addition to airway and cardiac remodeling, TAK1 has also been involved in embryonal development. Thus, in the mouse embryo, TAK1 is highly expressed in a variety of tissues (26). TAK1-deficient mouse embryos are not viable (21, 27) and show an abnormal, undeveloped vasculature, lacking vascular smooth muscle (27). Moreover, *Tab1* gene mutant mouse embryos, which lack a functional TAK1-binding protein (TAB1) protein and TAK1 activity, also demonstrated abnormal development of the vasculature and the lung (28). Remarkably, a recent study in a Dutch birth cohort (44) has identified single nucleotide polymorphisms (SNPs) located in the *Tab1* and *Tab2* genes, encoding TAK1-binding proteins which regulate TAK1 activation, which

were associated with asthma. Although the functional consequence of these SNPs remains to be determined, this finding could implicate TAK1 signaling in the pathogenesis of asthma.

In addition to its inhibitory effect on ASM cell proliferation and ERK 1/2 phosphorylation, the TAK1 inhibitor LL-Z-1640-2 also inhibited the PDGF-induced decrease in maximal contraction to both a receptor-dependent stimulus, methacholine, and a receptor-independent stimulus, KCl. This suggests that PDGF induces a downstream effect and is confirmed by the decreased expression of the contractile protein, sm-α-actin. These data are consistent with the inhibition of a growth factor-induced shift to a proliferative, hypocontractile phenotype.

Our data identify TAK1 as a novel mediator of growth factor-induced proliferation of ASM cells. TAK1 has thus far not been implicated in PDGF-induced receptor tyrosine kinase signaling. In our study, PDGF-induced DNA synthesis and cell proliferation were fully inhibited, whereas the FBS-induced effects were only partially reduced by the TAK1 inhibitor LL-Z-1640-2. This observation suggests that mitogenic components of FBS may also increase cell proliferation via signaling pathways independent of TAK1. Although PDGF is one of the major mitogenic components of FBS, differences in pro-proliferative signaling induced by PDGF and FBS in ASM cells have been reported previously (7, 43, 45). For example, Rho-kinase was shown to play a role in FBS-induced proliferation of ASM cells (45), whereas it was not involved in proliferation or hypocontractility induced by PDGF (7).

TAK1 has been shown to play a role in ERK 1/2 phosphorylation induced by BMP in mouse chondrocytes (21, 22), IL-1β in mouse embryonal fibroblasts (21, 22), LPS and TNF-α in human neutrophils (19, 46) and LPS and phorbol-12-myristate-13-acetate (PMA) in human peripheral blood monocytes (19, 46). Interestingly, in HeLa cells TAK1 was shown to mediate TNF-α- but not EGF-induced ERK 1/2 phosphorylation (20), indicating that the contribution of TAK1 to ERK 1/2 signaling may be stimulus-specific. TAK1 was found to associate with Ras in caveolae of rat hepatic macrophages (47) and was also found to mediate resistance to apoptosis in H-Ras-transformed human bladder carcinoma T24 cells (48). However, at present it is still unknown at which signaling level PDGF may cross over to TAK1.

TAK1 has been shown to play a role in the proliferation of various cell types. Thus, in cultured TAK1-deficient mouse B cells, TLR- as well as B cell receptor- and

CD40-mediated proliferation and cell cycle progression were impaired (23). Similarly, in cultured TAK1-deficient mouse effector T cells, cytokine-induced proliferation and cell cycle progression proved also to be dependent on TAK1 (25). In a study using a xenograft model of breast cancer in mice, it was demonstrated that tumors originating from breast cancer cells expressing a dominant-negative TAK1 had a lower growth rate and a decreased expression of the proliferative marker Ki-67 compared to tumors originating form cells expressing wild type TAK1 (49). Studies in human hematopoietic stem cells have shown that a low concentration (10 pg/ml) of TGF-β - which was associated with TAK1 and ERK 1/2 activation but not SMAD or p38 MAP kinase pathway activation - did induce cell proliferation, whereas a high concentration of TGF-β (10 ng/ml), which strongly activated the SMAD and p38 MAP kinase pathways, inhibited cell proliferation (50, 51). These data clearly suggest that at low concentrations of TGF-β TAK1 may play a role in ERK 1/2-dependent, SMAD-independent signaling, causing cell proliferation.

In contrast, several studies have reported an inhibitory role for TAK1 in cell proliferation. Thus, expression of constitutively active TAK1 decreased DNA synthesis, cyclin D1 expression and cell cycle progression in porcine renal proximal tubular cells, whereas the expression of dominant negative TAK1 in these cells was shown to increase DNA synthesis and cyclin D1 expression (52). Dominant negative TAK1 expression in rat liver *in vivo* resulted in G_0 exit and cell cycle progression during regeneration after partial hepatectomy (53). These data indicate that the role of TAK1 in proliferation may be cell type specific.

In ASM cells, our data clearly indicate that TAK1 mediates PDGF-induced proliferation, since both proliferation and ERK 1/2 phosphorylation are inhibited by the TAK1 inhibitor LL-Z-1640-2. LL-Z-1640-2 has previously been identified as a specific inhibitor of TAK1 (35). In an *in vitro* kinase assay, this small molecule inhibitor was found to inhibit TAK1 with a high selectivity (IC_{50}=8 nM) over mitogen-activated protein kinase kinase 1 (MEK1; IC_{50}=411 nM) and MEK kinase 1 (MEKK1; IC_{50}=268 nM) (35). In other *in vitro* kinase assays, LL-Z-1640-2 (300 nM) inhibited TAK1-mediated MAP kinase kinase 6 (MKK6) phosphorylation, but had no effect on MEKK1-, MEKK4- or apoptosis signal-regulating kinase 1 (ASK1)-mediated phosphorylation (35). *In vitro* kinase assays performed on lysates from TNF-α-stimulated neutrophils have also indicated that LL-Z-1640-2 (1 μM) strongly inhibits TAK1, but does not affect MEKK1 or MEKK3 kinase activity (19). In accordance with the observation that EGF does not activate TAK1 (14), LL-Z-1640-2 (300 and 500 nM) had no effect on EGF induced ERK 1/2 phosphorylation in HEK293 (20) or HeLa cells (54), indicating

that the inhibitor is specific for TAK1 rather than a general inhibitor of MAP kinase signalling. In addition, LL-Z-1640-2 was shown to inhibit LPS-induced TNF-α, IL-1β and IL-6 production by human peripheral monocytes with an IC_{50} range (10 - 25 nM) similar to the values observed in the *in vitro* kinase assay mentioned above. Moreover, pretreatment of mice with LL-Z-1640-2 (ip injection; 3 - 30 mg/kg body mass) dose-dependently inhibited the LPS-induced increase of serum TNF-α concentration, indicating the potential for use of this inhibitor *in vivo*. In the present study, the specificity of LL-Z-1640-2 was confirmed by the inhibition of PDGF-induced [^{3}H]-thymidine incorporation and ERK 1/2 phosphorylation in cells transfected with dominant-negative TAK1.

In conclusion, our study has identified TAK1 as a novel regulator of growth factor-induced proliferation in ASM cells. TAK1 may therefore regulate phenotypic modulation of ASM and could contribute to the development of ASM remodeling in obstructive airways diseases.

Acknowledgements
The authors would like to thank Dr. B.G.J. Dekkers for technical assistance.

Funding
This study was financially supported by Boehringer Ingelheim Pharma GmbH & Co. KG and the Graduate School of Behavioral and Cognitive Neurosciences, University of Groningen, The Netherlands.

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