Mechanical strain-induced DNA synthesis and cell proliferation in human airway smooth muscle cells through extracellular signal-regulated kinase

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ORIGINAL ARTICLE

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ABSTRACT

An increased airway muscle content is one of the characteristic features of airway remodeling. Proinflammatory substances, growth factors and mechanical strain promote airway muscle cell proliferation and extracellular signal-regulated kinase (Erk) plays an important role in proinflammatory substance- and growth factor-induced airway smooth muscle (ASM) cell proliferation; however, little is known about intracellular signals that regulate mechanical strain-induced ASM cell proliferation. Therefore, in the present study we examined the role of Erk in mechanical strain-induced DNA synthesis and cell proliferation in human ASM cells. We examined the effect of mechanical strain on the threonine and tyrosine phosphorylation of Erk and activation of Erk and the effect of PD98059 on Erk activity, DNA synthesis and cell proliferation in mechanical strain-loaded human ASM cells. The results showed that mechanical strain-induced phosphorylation and activation of Erk in ASM cells and attenuation of Erk activation by PD98059 resulted in the inhibition of mechanical strain-induced Erk activity and increases in DNA synthesis and cell proliferation in ASM cells. These results indicate that Erk plays an important role in mechanical strain-induced increases in DNA synthesis and cell proliferation in human ASM cells.

INTRODUCTION

Airway remodeling has been documented to be a prominent feature of the bronchial wall of patients with chronic severe asthma.1,2 Many elements have been shown to contribute to the airway remodeling response, which is basement membrane thickening associated with subepithelial extracellular matrix deposition and increased airway muscle content occurring as a result of hyperplastic and/or hypertrophic growth.1,2

The mitogen-activated protein kinases (MAPK) are important mediators of signal transduction from the cell membrane to the nucleus. Several subgroups of mammalian MAPK have been characterized molecularly.3 The activation of extracellular signal-regulated kinase (Erk), which belongs to the mammalian MAPK superfamily, has been shown to play a central role in cell proliferation and differentiation in various cells.4,5 In airway smooth muscle (ASM) cells, proinflammatory substances, growth factors and mechanical strain have been shown to induce DNA and protein synthesis and cell proliferation.6–17 Analysis of intracellular signals has revealed that Erk plays an important role in regulating DNA and protein synthesis and cell proliferation in ASM cells in response to proinflammatory substances and growth factor.10–15 However, the role of Erk in mechanical strain-induced DNA and protein synthesis and cell proliferation in ASM cells has not been clarified.

The study reported herein was designed to clarify the intracellular signal by which mechanical strain induces DNA synthesis and cell proliferation in ASM cells. To this end, we analyzed the threonine and tyrosine...
phosphorylation and activation of Erk and the effect of PD98059 as the specific inhibitor of MAPK kinase-1 (MEK-1),\textsuperscript{18,19} which is upstream of Erk on mechanical strain-induced Erk activity and DNA synthesis and cell proliferation in human ASM cells.

**METHODS**

**Reagents**

PD98059 was obtained from New England BioLabs (Beverly, MA, USA) and was dissolved in dimethyl sulfoxide. Platelet-derived growth factor (PDGF) was obtained from Sigma Chemical Co. (St Louis, MO, USA).

**Cell culture**

Human bronchial smooth muscle (BSM) cells derived from normal healthy subjects used as ASM cells in this study were obtained from Clonetics (San Diego, CA, USA). We used silicone rubber culture dishes (Shin-Etsu Chemical, Tokyo, Japan) for this study, as described previously.\textsuperscript{20} Cells (1 $\times$ 10$^4$ cells/mL) were placed onto silicone rubber culture dishes coated with collagen type I (Sigma Chemical Co.) and cultured using BSM cell growth medium (SmBM; Clonetics) containing 5% fetal bovine serum (FBS), gentamicin-amphotericin B, epidermal growth factor (EGF), fibroblast growth factor (FGF) and dexamethasone (DEX) at 37°C in humidified 5% CO$_2$ atmosphere. In order to examine mechanical strain- and PDGF-induced threonine and tyrosine phosphorylation of Erk, activation of Erk DNA synthesis and cell proliferation, ASM cells were allowed to reach subconfluence (approximately 2 $\times$ 10$^4$ cells/mL) and the culture medium was then replaced with SmBM containing 0.1% FBS and cells were cultured for 16 h. At the end of depletion of growth factors, ASM cells were loaded by mechanical strain or stimulated with PDGF in the presence of SmBM containing 0.1% FBS. In the present study, ASM cells at passage numbers 3–5 were used for experiments. At confluence of each passage, using immunofluorescence techniques for both smooth muscle actin and myosin, more than 95% of cells displayed the characteristics of smooth muscle cells in culture.

**Mechanical strain**

**Effects of stretch**

The ASM cells cultured on silicone culture rubber dishes were stretched by 20%, as described previously.\textsuperscript{20} Briefly, the silicone rubber culture dish was fixed to the sides of the stretch frame, which was designed so that the silicone rubber culture dish was mechanically expanded by 20%, thereby uniaxially increasing the length of the attached cells. The ASM cells were lysed just before stretching and at 5, 10, 15, 30 and 60 min after stretching of cells.

**Effects of contraction**

The ASM cells were cultured on the stretched silicone rubber culture dish that had been fixed to the sides of the stretch frame in order to stretch the silicone rubber culture dish by 20%. Cells were lysed just before detaching and at 5, 10, 15 and 30 min after detaching the stretched silicone rubber culture dish.

**Effects of stretch followed by contraction**

The ASM cells cultured on silicone rubber dishes were stretched by 20% for 10 min and then the silicone rubber culture dish was detached from the stretch frame to contract the cells. The ASM cells were lysed just before and at the end of stretching the cells and at 5, 10, 15 and 30 min after detaching the silicone rubber culture dish. Contraction, stretch and stretch followed by contraction were performed in a sterile laminar flowhood and, after completing these procedures, the silicone rubber dish fixed to the stretch frame and the silicone rubber dish kept in a Petri dish were incubated at 37°C in humidified 5% CO$_2$ atmosphere. In order to examine the effect of PD98059 on mechanical strain-induced Erk activity, DNA synthesis and cell proliferation, cells were preincubated with PD98059 (5 and 50 $\mu$mol/L) for 1 h followed by loading with mechanical strain.

**Platelet-derived growth factor stimulation**

We simultaneously stimulated ASM cells that were cultured on the silicone rubber culture dish with PDGF. Cells were stimulated with 10 ng/mL PDGF and lysed at 5, 15, 30, 60, 120 and 180 min after PDGF stimulation. For the determination of the effect of PD98059 on PDGF-induced Erk activity, DNA synthesis and cell proliferation, ASM cells were cultured with or without PD98059 (5 and 50 $\mu$mol/L) for 1 h and were stimulated with PDGF for 120 min to determine Erk activation, for 1 and 3 days to determine DNA synthesis and for 1–4 days to determine cell proliferation.
Western blot analysis of Erk

Analysis of threonine and tyrosine phosphorylation of Erk was performed using an antiphosphorylated threonine and tyrosine of p42/p44 MAPK antibody (antiphospho-specific p42/p44 MAPK antibody; New England Biolabs), which is specific for active p42/p44 MAPK and does not cross-react with p38 MAPK and c-Jun N-terminal kinase (JNK). The threonine and tyrosine phosphorylation of Erk was analyzed according to the manufacturer’s instructions. Briefly, after separating proteins from the cell lysate by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the cell lysate containing 10 µg protein was electrophoretically transferred to a membrane and the membrane was incubated with specific antibody to phosphorylated threonine and tyrosine of Erk (affinity purified rabbit polyclonal IgG) and was then incubated with horseradish peroxidase-conjugated antirabbit IgG antibody and horseradish peroxidase-conjugated antibiotin antibody to detect biotinylated protein markers. Blots were incubated with enhanced chemiluminescence solution (LumiGLO; New England Biolabs Inc., Beverly, MA, USA) for 1 min and exposed on Kodak XAR film (Kodak, Tokyo, Japan). In order to show the amounts of Erk precipitated, blots were stripped and reprobed using phosphorylation state-independent p42/p44 MAPK-specific antibody (affinity purified rabbit polyclonal IgG) to determine total p42/p44 MAPK levels.

Extracellular signal-regulated kinase kinase assay

The activity of Erk was analyzed by commercially available kits (MAPK Assay Kit; New England Biolabs). The kit uses two different antibodies, antiphospho-specific p42/p44 MAPK antibody, which is specific for active p42/p44 MAPK and does not cross react with p38 MAPK or JNK, and antiphospho-specific Elk-1 antibody to detect p42/p44-induced phosphorylation of Elk-1. Extracellular signal-regulated kinase activity was analyzed by specific immunoprecipitation with antiphospho-specific p42/p44 kinase antibody followed by an in vitro kinase assay of its substrate, Elk-1, according to manufacturer’s instructions, as described previously.21 Briefly, the cell lysate, containing 200 µg protein was incubated with antiphospho-specific p42/p44 MAPK antibody to selectively immunoprecipitate active p42/p44 MAPK from cell lysates and the immunoprecipitates were incubated with Elk-1 fusion protein in the presence of ATP, a process that allowed immunoprecipitated active p42/p44 to phosphorylate its substrate, Elk-1. The samples were separated by 15% SDS-PAGE, transferred to membranes and blotted with antiphospho-specific Elk-1 antibody. The membrane was incubated with horseradish peroxidase (HRP)-conjugated antirabbit IgG antibody and HRP-conjugated antibiotin antibody and the membrane was then incubated with 10 µL enhanced chemiluminescence (ECL) solution and exposed on Kodak XAR film for 1 min.

DNA synthesis

DNA synthesis in ASM cells was measured by [3H]-thymidine incorporation. The ASM cells were preincubated with or without PD98059 (5 and 50 µmol/L) for 1 h followed by stretching of cells on the silicone rubber culture dish for 10 min. After completion of stretching of the cells, the silicone rubber culture dish was detached from the stretch frame and the silicone rubber culture dish kept in Petri dish was incubated at 37°C in humidified 5% CO2 atmosphere for 1 and 3 days. The ASM cells were preincubated with or without PD98059 (5 and 50 µmol/L) for 1 h and were stimulated with PDGF (10 ng/mL) at 37°C in humidified 5% CO2 atmosphere for 1 and 3 days. [3H]-Thymidine was added to cell culture dishes for the last 4 h of the incubation period. After incubation, cells were trypsinized and transferred to a flat-bottomed 96-well culture plate and harvested onto glass fiber strips with a cell harvester and retained radioactivity was counted in a scintillation counter.

Cell number determination

Airway smooth muscle cells were preincubated with or without PD98059 (5 and 50 µmol/L) for 1 h followed by stretching the cells on a silicone rubber culture dish for 10 min. After completion of stretching of the cells, the silicone rubber culture dish was detached from the stretch frame and the silicone rubber culture dish kept in a Petri dish was incubated at 37°C in humidified 5% CO2 atmosphere for 1–4 days. The ASM cells were preincubated with or without PD98059 (5 and 50 µmol/L) for 1 h and were stimulated with PDGF (10 ng/mL) at 37°C in a humidified 5% CO2 atmosphere for 1–4 days. In order to determine cell proliferation, cells were trypsinized and counted at each time point of the culture period.

Statistical analysis

Statistical significance was analyzed by using analysis of variance (ANOVA). P < 0.05 was considered significant.
RESULTS

Mechanical strain induces phosphorylation of Erk

First, we examined whether stretch could induce threonine and tyrosine phosphorylation of Erk in ASM cells. To this end, ASM cells cultured on a silicone rubber culture dish were stretched and incubated for 5, 10, 15, 30 and 60 min. Amounts of phosphorylated Erk increased at 5 min and were maximal at 10 min and, thereafter, returned to near basal levels (Fig. 1a). Second, we examined whether the contraction could induce Erk phosphorylation in ASM cells. To this end, ASM cells cultured on a stretched silicone rubber culture dish were detached from the stretch frame to contract the cells and they were then incubated for 5, 10, 15, and 30 min. Amounts of phosphorylated Erk increased at 5 min and, thereafter, returned to near basal levels at 30 min (Fig. 1b). Finally, we examined the effect of stretch followed by contraction on Erk phosphorylation in ASM cells. To this end, ASM cells cultured on a silicone rubber culture dish were stretched and incubated for 10 min. Thereafter, the silicone rubber culture dishes were detached from the stretch frame to contract the cells and they were then incubated for 5, 10, 15, and 30 min. Amounts of phosphorylated Erk increased at the end of stretching (lane S) and were maximal at 5 min after detaching, and then returned to near basal levels at 15 min (Fig. 1c). Also shown in Fig. 1a–c is the fact that equal amounts of Erk protein were immunoblotted with Erk-specific antibody regardless of the time of culture periods, indicating that mechanical strain-induced Erk phosphorylation occurred in the absence of changes in Erk protein levels.

Platelet-derived growth factor induces Erk phosphorylation

We simultaneously stimulated ASM cells with PDGF and examined Erk phosphorylation and activation. Amounts of phosphorylated Erk increased at 5 min, were maximal at 30 min and were sustained between 60 and 120 min; thereafter, they returned to near basal levels at 180 min (Fig. 2). Also shown in Fig. 2 is the fact that equal amounts of Erk protein were immunoblotted with Erk-specific antibody regardless of the time of culture periods, indicating that mechanical strain-induced Erk phosphorylation occurred in the absence of changes in Erk protein levels.
Mechanical strain and PDGF induce Erk activation and PD98059 inhibits Erk activity

We used three different regimens of mechanical strain that were stretch, contraction and stretch followed by contraction in order to examine whether mechanical strain could induce threonine and tyrosine phosphorylation of Erk. Extracellular signal-regulated kinase phosphorylation was induced by the three different regimens, but Erk was profoundly phosphorylated in stretched followed by contracted ASM cells. Therefore, the stretch followed by contraction regimen was used in order to examine the effect of mechanical strain on Erk activation and cell proliferation. Activation of Erk is mediated by dual phosphorylation of threonine and tyrosine by MEK. Extracellular signal-regulated kinase activation culminates in the downstream cytosolic and nuclear factors that regulate various cellular responses. In addition to western blot analysis of phosphorylated threonine and tyrosine of Erk, we next examined whether mechanical strain could induce Erk activation and whether the specific inhibitor for MEK-1, PD98059, could inhibit mechanical strain-induced increases in Erk activity. As shown in Fig. 3a, mechanical strain activated Erk, as demonstrated by an increased phosphorylation of Elk-1. Pretreatment of cells with PD98059 attenuated mechanical strain-induced increases in Erk activity in a dose-dependent manner. Platelet-derived growth factor activated Erk, as demonstrated by an increased phosphorylation of Elk-1. Pretreatment of cells with PD98059 attenuated PDGF-induced increases in Erk activity in a dose-dependent manner (Fig. 3b).

DNA synthesis and cell proliferation in ASM cells and the effect of PD98059

DNA synthesis in ASM cells was measured by [3H]-thymidine incorporation on days 1 and 3 after completing the stretch and the contraction. Unstretched and uncontracted control ASM cells were simultaneously cultured on a silicone rubber culture dish in the presence or absence of PD98059. Compared with unstretched and uncontracted control cells, mechanical strain induced increases in DNA synthesis on day 3. PD98059 abolished mechanical strain-induced increases in DNA synthesis in ASM cells in a dose-dependent manner (Fig. 4). We also stimulated ASM cells with PDGF in order to compare the rate of DNA synthesis between the two groups. Platelet-derived growth factor induced DNA synthesis and PD98059 attenuated PDGF-induced DNA synthesis in a dose-dependent manner. When the rate of DNA synthesis was compared between the two groups, PDGF-induced DNA synthesis was much higher than mechanical strain-induced DNA synthesis, regardless of culture periods (Fig. 4). In order to determine cell proliferation, we counted the cell number on days 1–4 after loading of mechanical strain or PDGF stimulation. The number of mechanical strain-loaded cells was slightly higher than that of unstimulated control cells on day 2, but this was not statistically significant. The number of mechanical strain-loaded cells was statistically higher than that of unstimulated control cells after 3 and 4 days. In PDGF stimulation, the number of PDGF-stimulated cells was higher than that of unstimulated control cells regardless of culture periods (Fig. 5). PD98059 attenuated...
mechanical strain- and PDGF-induced increases in cell proliferation (Fig. 5).

**DISCUSSION**

In the present study, we examined the signal transduction pathway in mechanical strain-induced DNA synthesis and cell proliferation in human ASM cells. The results showed that mechanical strain induced increases in DNA synthesis and cell proliferation in ASM cells. Analysis of the signal transduction pathway regulating mechanical strain-induced increases in DNA synthesis and cell proliferation in ASM cells revealed that the threonine and tyrosine phosphorylation of Erk and activation of Erk were induced by mechanical strain and PD98059, as the specific inhibitor for MEK-1, which is the upstream regulator of Erk, $^{18, 19}$ attenuated mechanical strain-induced Erk activity, increases in DNA synthesis and cell proliferation in ASM cells. Addition of dimethylsulfoxide vehicle alone did not affect Erk activity.
ASM cells. These results indicate that Erk plays an important role in the mechanical strain-activated signaling pathway that regulates DNA synthesis and cell proliferation in human ASM cells.

Proinflammatory substances, growth factors and mechanical strain have been shown to induce DNA and protein synthesis and promote cell proliferation in ASM cells.6–17 With regard to proinflammatory substances and growth factors, several studies have suggested that Erk plays an important role in regulating DNA and protein synthesis and cell proliferation in ASM cells.10–15 However, the intracellular signal that regulates mechanical strain-induced DNA synthesis and cell proliferation in ASM cells has not been determined. The specific inhibitor of the Erk signaling pathway has been identified, providing an effective tool for the investigation of the role of Erk in cellular signaling. In the present study, PD98059 was used as the specific inhibitor of MEK-1 activity in order to elucidate the biologic function of Erk. We used concentrations of 5 and 50 µmol/L PD98059 in the present study, because in a previous study 50 µmol/L PD98059 was found to be an optimal concentration with which to examine the role of Erk in cellular signaling.18,19 We showed that PD98059 attenuated Erk activity, DNA synthesis and cell proliferation in ASM cells in response to mechanical strain and PDGF in a dose-dependent

Fig. 4  a) Mechanical strain and (b) platelet-derived growth factor (PDGF) induce increases in DNA synthesis in airway smooth muscle (ASM) cells and PD98059 inhibits increases in DNA synthesis. The ASM cells that had been preincubated with or without PD98059 (5 or 50 µmol/L) were stretched for 10 min and, thereafter, contracted and cultured for 1 and 3 days. The ASM cells that had been preincubated with or without PD98059 (5 or 50 µmol/L) were stimulated with PDGF (10 ng/mL) and cultured for 1 and 3 days. Unstretched and uncontracted, and unstimulated control ASM cells preincubated with or without PD98059 were simultaneously cultured on the silicone rubber culture dish for 1 and 3 days. DNA synthesis in ASM cells was measured by [3H]-thymidine incorporation. Results are expressed as the mean±SD in three different experiments. *P < 0.01 compared with control cells; †P < 0.05 compared with mechanical strain-loaded cells preincubated without PD98059; ‡P < 0.01, §P < 0.05 compared with PDGF-stimulated cells preincubated without PD98059. The addition of dimethylsulfoxide vehicle alone did not affect [3H]-thymidine incorporation.
manner and that 50 μmol/L PD98059 almost completely attenuated DNA synthesis and cell proliferation. These results indicate that Erk plays an important role in inducing DNA synthesis and cell proliferation in ASM cells in response to mechanical strain as well as growth factors.

Previous studies have demonstrated that cyclic stretch of canine ASM cells induces increases in DNA and protein synthesis and cell proliferation and induces activation and/or phosphorylation of several biochemical enzymes and proteins, such as myosin light chain kinase, focal adhesion kinase pp125 focal adhesion kinase (pp125FAK) and paxillin. In the present study, we used three different regimens, namely stretch, contraction and stretch followed by contraction. Human ASM cells
were stretched by 20%, contracted by 20% or stretched by 20% followed by contraction. The results showed that these different regimens of mechanical strain induced threonine and tyrosine phosphorylation of Erk and phosphorylation of Erk was profoundly induced by stretch followed by contraction. Therefore, we examined the stretch followed by contraction-induced Erk activation and the effect of PD98059 on the stretch followed by contraction-induced Erk activation, DNA synthesis and cell proliferation. The results showed that the stretch followed by contraction induced Erk activation and increases in DNA synthesis and cell proliferation in ASM cells. These results indicate that mechanical strain-induced increases in DNA synthesis and cell proliferation in ASM cells are mediated through the Erk pathway and that the regimen used in this study provided a sufficient mechanical strain for the activation of intracellular signals and the induction of DNA synthesis and cell proliferation in ASM cells. In addition, although the regimen used in this study is different from the physiologic situation and other factors, such as proinflammatory substances and growth factors, may contribute to inducing DNA synthesis and cell proliferation in ASM cells, the mechanical strain regimen used in this study is thought to be a good model with which to examine the mechanism in DNA synthesis and cell proliferation in ASM cells induced by mechanical strain.

It has been shown that the duration of activation of Erk may be critical for determining cellular response. In neuronal cells, sustained activation of Erk results in cell differentiation, while transient activation of Erk is associated with cell proliferation. In other cell types, such as fibroblasts, sustained Erk activation in the cell is associated with proliferation, not differentiation. In ASM cells, PDGF-induced DNA synthesis and cell proliferation has been shown to be associated with the sustained activation of Erk. The present study showed that the transient activation of Erk induced by mechanical strain was associated with increases in DNA synthesis and cell proliferation in ASM cells. The stretch regimen used in this study was performed according to methods described in previous studies, which showed that the transient activation of Erk induced increases in protein synthesis and the expression of immediate early genes, such as c-fos in stretched cardiac myocytes. Therefore, it is conceivable that the transient activation of Erk in ASM cells induced by mechanical strain in the present study may be sufficient for inducing increases in DNA synthesis and cell proliferation. In the present study, we also stimulated ASM cells with PDGF in order to determine a possible association between the duration of Erk activation and the rate of DNA synthesis and cell proliferation. Platelet-derived growth factor-induced Erk activation was sustained, whereas mechanical strain-induced Erk activation was transient. DNA synthesis and cell proliferation were much higher in PDGF-stimulated cells than in mechanical strain-loaded cells, regardless of culture periods. These observations indicate that the duration of Erk activation may be a rate-limiting factor in DNA synthesis and cell proliferation in ASM cells.

There are several possible mechanisms by which mechanical strain induces phosphorylation and activation of Erk in ASM cells. One possible mechanism is thought to be integrin-mediated phosphorylation and activation of Erk. Several lines of evidence may support this hypothesis, including the observations that mechanical strain of integrin induces increased tyrosine phosphorylation of Erk in osteosarcoma cells and that mechanical strain induces tyrosine phosphorylation of pp125FAK in canine ASM cells and mesangial cells. However, signal transduction pathways in mechanical strain-induced phosphorylation and activation of Erk remain to be determined, because other possible mechanisms have been described.

From the data presented here, we conclude that mechanical strain induces DNA synthesis and cell proliferation in human ASM cells mediated through Erk. These results provide new evidence on the mechanism in mechanical strain-induced increases in DNA synthesis and cell proliferation in ASM cells. However, the precise role of Erk-mediated mechanical strain-induced increases in DNA synthesis and cell proliferation in ASM airway cells in airway remodeling remains to be determined.

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