

Original article

Role of the extracellular signal-regulated kinase 1/2 signaling pathway in regulating the secretion of bronchial smooth muscle cells in a rat model of chronic asthma

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Background Although it is recognized that bronchial smooth muscle cells (BSMCs) play a key role in airway remodeling during chronic asthma, it is not well understood how BSMCs exert their inflammatory functions. The extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway is an important signaling pathway in chronic asthma, but its influence on secretion by BSMCs has not been well-studied. We investigated the impact of ERK1/2 signaling pathway on secretion by BSMCs in a rat model of chronic asthma in this study.

Methods To create a rat model of chronic asthma, Wistar rats underwent ovalbumin (OVA) injection and eight weeks of inhalation. BSMCs were isolated and cultured *in vitro*. Epidermal growth factor, PD98059 and ERK1/2 antisense oligonucleotide were used to explore the role of ERK1/2 signaling pathway. The expression of P-ERK1/2 (phospho-ERK1/2) in BSMCs was analyzed by Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR). Secretion of BSMCs was detected by enzyme-linked immunosorbent assay (ELISA).

Results Phospho-ERK1/2 expression was increased in BSMCs of chronic asthmatic rats compared with the controls. PD98059 inhibited expression of phospho-ERK1/2 protein, while treatment with an antisense oligonucleotide inhibited the expression of P-ERK1/2 mRNA and protein. BSMCs obtained from the chronic asthma group secreted significantly greater quantities of growth factors (transforming growth factor (TGF)- β_1 , vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF)), cytokines (regulated upon activation, normal T cell-expressed and secreted (RANTES) and eotaxin), and extracellular matrix (fibronectin and collagen I) compared with normal controls. Epidermal growth factor stimulated secretion in both groups, but the response of the chronic asthma group was more intense. Both PD98059 and antisense oligonucleotide suppressed secretion by BSMCs in chronic asthmatic rats. Antisense oligonucleotide reduced the level of RANTES nearly to that of normal controls, while PD98059 could not.

Conclusion These results suggest that ERK1/2 signaling pathway may play an important role in the augmented secretion of BSMCs in chronic asthmatic rats, and ERK1/2 antisense oligonucleotide effectively inhibits the process.

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Asthma is a chronic inflammatory disorder characterized by reversible airway obstruction. However, it is increasingly clear that irreversible obstruction can develop in chronic asthma because of airway remodeling that might be due to poor therapy in some persistent and severe asthmatics.¹ Several studies report that bronchial smooth muscle cells (BSMCs) play a critical role in airway remodeling, not only through their contraction function, but more importantly, via the multiple active mediators that they secrete in chronic asthmatic status.² However, the underlying mechanisms have not been identified.

Our previous study suggested that the ERK1/2 signaling pathway may be implicated in the pathophysiology of chronic asthma.³ However, few reports have addressed the role of ERK1/2 signaling pathway in the secretion functions of BSMCs in chronic asthmatics. Therefore, the aim of this study was to determine the influence of ERK1/2 signaling on the secretion of BSMCs in a rat model of chronic asthma.

METHODS

Animal model

Twenty male Wistar adult rats (Animal Apply Center, Tongji Medical College, China), weighing 180–200 g, were randomly divided into normal control and chronic asthma groups (10 rats per group). The rats in chronic asthma group were sensitized twice by ovalbumin (OVA, Sigma, USA) and Al(OH)₃ (Sigma), then challenged with aerosolized OVA for 30 min/d 3 days per week for 8 weeks as described.⁴ Control rats were sensitized and

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challenged by phosphate buffered saline (PBS).

Histological examination

The right middle lobes of the lungs were harvested 24 hours after the last OVA challenge, fixed in 4% paraformaldehyde, then imbedded in paraffin. Airway wall (Wat) and smooth muscle (WAs) content were determined by morphometric analysis (HMIAS 2000, Qianping Systems, Wuhan, China) of 5- μ m transverse sections after HE staining. Basement membrane perimeter (Pbm) was measured for normalization of WAs and Wat.

Cell culture and identification

According to the method of Liu et al,⁵ the rest of the lobes of the lungs were excised and transferred to a dish of ice old physiological salt solution (PSS) that was gently bubbled with 5% CO₂ in oxygen. The smooth muscle layer was obtained, dissected free of parenchyma and connective tissue, and then minced into 1 mm³ fragments. The tissues were incubated for 20–30 minutes at 37°C in Ca²⁺ free PSS containing 2 g/L collagenase, 5 g/L papain, and 2 g/L bovine serum albumin. After digestion, the separated cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 20% fetal bovine serum (FBS).

BSMCs after passage 3 were used for the experiment. The cells were seeded onto six-cell plates with 10% FBS-DMEM, and then changed to serum free DMEM for 24 hours synchronization when nearly 70% confluent. After that, BSMCs were cultured in serum-free DMEM with 10 ng/ml epidermal growth factor (EGF, Accuratechemical, USA), 100 μ mol/L PD98059 (Biosource, USA) or antisense oligonucleotide (ODN). Twenty-four hours later, both medium and cells were collected.

For identification of BSMCs, immunofluorescent cytochemical staining was performed with anti- α -actin primary antibody (1:100) and visualized using a fluorescence microscope.

ERK1/2 ODN transfection

The antisense phosphorothioate-modified ODN to ERK1/2 was a 17-mer (5'-GCCGCCGCCGCCGCC-AT-3'). Sense ODN (5'-ATGGCGGCGGCGGCGGC-3') was used as control. Cells were treated with an ODN-lipofectin mixture at a ratio of 1:2 (0.5 μ mol/L ODN) and incubated for 6 hours. The cells were then washed to remove the ODN-lipofectin mixture and incubated in DMEM containing the appropriate concentration of ODN alone. Part of the ODN was labeled with fluorescein isothiocyanate (FITC) to confirm transfection with ODN.

Western blot analysis

Proteins were extracted from cells with sodium dodecylsulfate (SDS) sample buffer, then separated in 8%

SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Anti-phospho ERK1/2 (R&D Systems, USA, 1:5000) Ab was used to detect expression of ERK1/2. Antibodies against connective tissue growth factor (CTGF, Abcam England, 1:2500), fibronectin (Abcam England, 1:400) and collagen I (Abcam England, 1:500) were also used. Antibodies against reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) (KangChen Biotech, Shanghai, China, 1:1000) were used for protein control. Protein band intensity was quantified by scanning densitometry.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, USA). RT-PCR was performed as recommended in the RT-PCR kit (TaKaRa, Japan). GAPDH (140 bp) served as housekeeping gene loading control. The primers of ERK1 (373 bp) gene were 5'-GCT GAA TCA CAT CCT GGG TAT-3' and 5'-AGA 2TCT GTA TCC TGG CTG GAA-3'. The primers of GAPDH were 5'-GCA AGT TCA ACG GCA CAG -3' and 5'-GCC AGT AGA CTC CAC GAC A-3'. The cycling parameters were as follows: 94°C 3 minutes, 94°C 30 seconds, 58°C 1 minute, 72°C 1 minute, for 35 cycles, followed by the final step at 72°C for 10 minutes.

Enzyme linked immunosorbent assay (ELISA)

Cell culture supernatants were collected. Transforming growth factor (TGF- β_1), RANTES, VEGF, and eotaxin were quantified using ELISA kits (R&D, USA) according to the procedure recommended by the manufacturer. Obtained values were normalized to cell counts.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) and *q* test using SPSS 11.5. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Histologic changes in chronically OVA-challenged rats

In addition to the marked increase of inflammatory cells in the lung mucosal and submucosal areas, the eight weeks OVA-challenged rats presented the thicker airway wall ((60.21 $\pm$ 3.07) vs (29.92 $\pm$ 2.36) μ m²/ μ m, *P*<0.01) and the increased smooth muscle layer ((16.93 $\pm$ 0.73) μ m²/ μ m vs (9.47 $\pm$ 0.31) μ m²/ μ m, *P*<0.01) than that of normal control rats after correction for airway basement perimeter (Figure 1).

Expression of ERK1/2 in BSMCs

By Western blot analysis, protein level of phospho-ERK1/2 in BSMCs of chronic asthma group was markedly increased compared to that of normal controls (*P*<0.05). EGF stimulated the synthesis of ERK1/2 in both control and chronic asthmatic rats (*P*<0.05), and PD98059 decreased the expression of ERK1/2 and

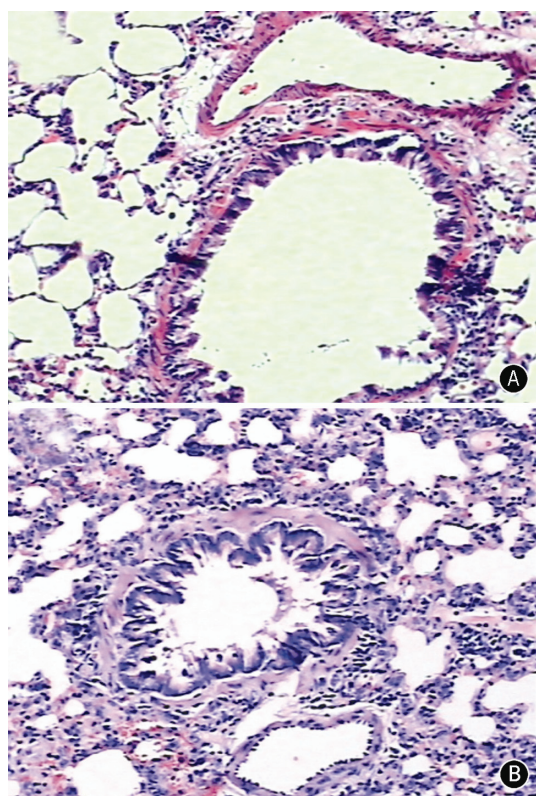


Figure 1. HE staining of bronchial lung sections from Wistar rats (original magnification $\times 100$). The photomicrographs are representative of lungs removed from normal controlled rats (A) and ovalbumin (OVA)-challenged rats (B). In OVA-exposed rats, increases in the thickness of airway wall and smooth muscle layers are apparent.

blocked the effect of EGF ($P < 0.05$). ERK1/2 antisense ODN inhibited protein expression ($P < 0.05$), while sense ODN had no effect (Figure 2A).

By RT-PCR, mRNA levels of ERK1 BSMCs in the chronic asthma group was significantly higher than that of normal controls. EGF stimulated the transcription of ERK1 ($P < 0.05$). PD98059 had no obvious impact on mRNA levels of ERK1 in either chronic asthmatic BSMCs or EGF stimulated cells. On the other hand, ERK1/2 antisense ODN decreased levels of ERK1 mRNA in BSMCs from chronic asthmatic rats ($P < 0.05$), while sense ODN had no effect (Figure 2B).

Secretion of BSMCs in chronic asthmatic rats

ELISA assays showed that both TGF- $\beta 1$ and VEGF increased dramatically in the chronic asthmatic group compared to normal control ($P < 0.05$), and VEGF was barely detectable in control rats. EGF stimulated secretion of these proteins in both groups ($P < 0.05$), but the response in chronic asthmatic BSMCs was much more intense. PD98059 inhibited expression of both TGF- $\beta 1$ and VEGF in the chronic asthmatic group ($P < 0.05$), and partially inhibited the stimulating effect of EGF on chronic asthmatic BSMCs ($P < 0.05$). ERK1/2 antisense ODN reduced secretions in the chronic asthmatic group ($P < 0.05$), but sense ODN showed little effect (Figure 3A).

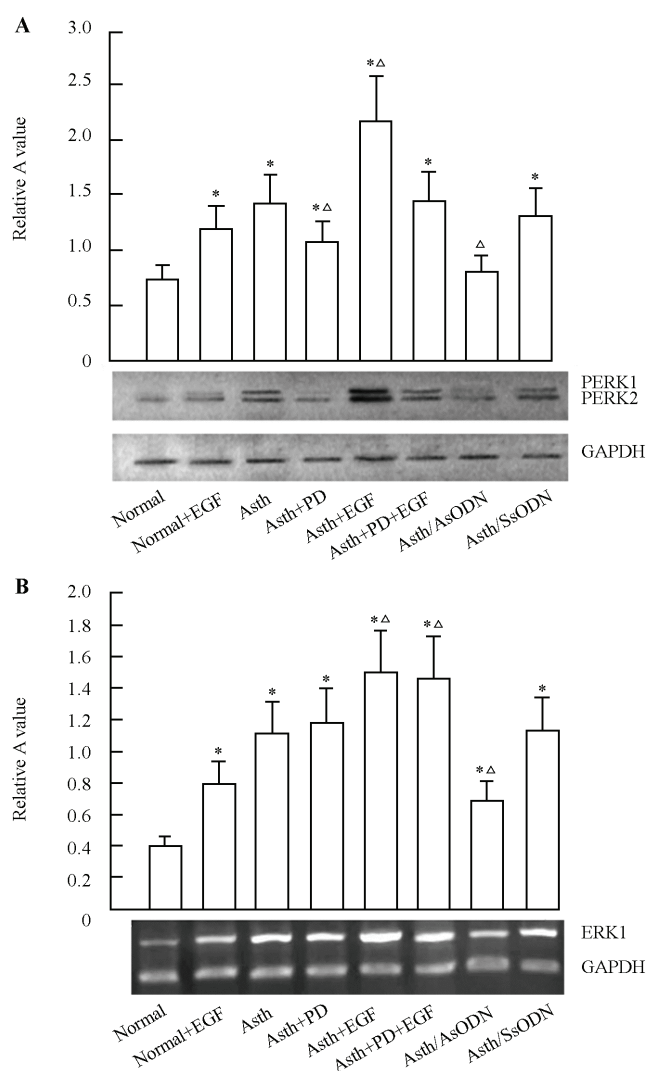


Figure 2. Expressions of ERK1/2 in bronchial smooth muscle cells detected by Western blot (A) and RT-PCR (B). Results shown are representatives of ten different subjects for each condition, and analyses in each subject was performed in triplicate. Values were normalized to GAPDH and are expressed as the mean $\pm$ standard error of the ten experiments. ERK: extracellular signal-regulated kinase. Asth: chronic asthmatic group. PD: 100 $\mu\text{mol/L}$ PD98059. EGF: 10 ng/ml epidermal growth factor. AsODN: 0.5 $\mu\text{mol/L}$ ERK1/2 antisense oligonucleotide. SsODN: 0.5 $\mu\text{mol/L}$ ERK1/2 sense oligonucleotide. *Significant difference from normal controls, $P < 0.05$. Δ Significant difference from chronic asthmatic cells incubated in serum free medium, $P < 0.05$.

Similarly, BSMCs of chronic asthmatic rats showed more active secretion of RANTES and eotaxin compared to normal controls ($P < 0.05$). EGF increased expression of the two cytokines in both normal control and chronic asthmatic groups ($P < 0.05$). Although PD98059 inhibited the secretions of RANTES and eotaxin in BSMCs of chronic asthmatic rats ($P < 0.05$), there was still a significant difference in the levels of RANTES in the pretreated asthmatic group compared with normal controls ($P < 0.05$). Antisense ODN strongly repressed secretion of these cytokines ($P < 0.05$), and nearly restored RANTES to normal levels. In contrast, sense ODN did not change the levels of the two cytokines (Figure 3B).

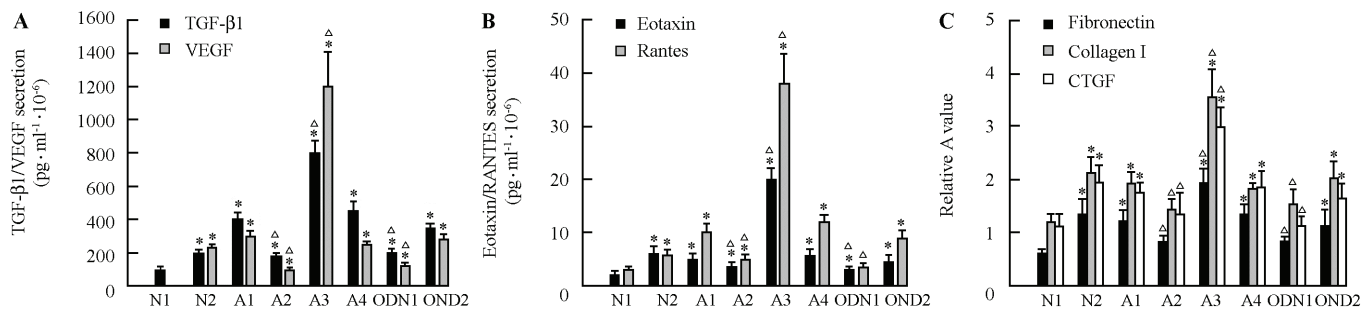


Figure 3. Secretion of BSMCs in chronic asthmatic rats. Graph A shows expression of TGF- β_1 and VEGF detected by ELISA. Graph B shows expression of RANTES and eotaxin detected by ELISA. Graph C shows expression of CTGF, fibronectin and collagen I detected by Western blot. Results shown are representatives of the ten animals in each condition, with analyses in each animal performed in triplicate. Values shown are mean $\pm$ standard error of the ten experiments. N1 indicates controls group, and N2 is the group stimulated by EGF. A1 is chronic asthmatic group, A2, A3, and A4 denote experiments involving treatment with PD98059, EGF, and EGF plus PD98059 respectively. ODN1 indicates chronic asthmatic BSMCs transfected with ERK1/2 antisense oligonucleotide, whereas ODN2 indicates BSMCs transfected with sense oligonucleotide. *Significant difference from normal controls, $P < 0.05$. Δ Significant difference from chronic asthmatic cells incubated in serum free medium, $P < 0.05$.

Western blot detected expression of CTGF, fibronectin and collagen I by BSMCs. Expression of these products was increased in the chronic asthmatic group when compared with normal control ($P < 0.05$), and EGF stimulated their expression in BSMCs from both chronic asthmatic and normal rats ($P < 0.05$). Pretreatment with PD98059 decreased the expression of all three proteins ($P < 0.05$). Antisense ODN produced an effect similar to that of PD98059, while sense ODN had little impact on expression of these proteins (Figure 3C).

DISCUSSION

The pathophysiologic mechanism of airway remodeling is complex.⁶ Many recent studies have focused on the secretory ability of BSMCs that enable the cells to become a rich source of many cytokines, chemokines, and inflammatory mediators; these factors may modulate airway inflammation and influence airway remodeling in asthma.^{2,7} Both TGF- β_1 and VEGF are critical factors that mediate airway inflammation and remodeling.⁸ RANTES and eotaxin are important chemotactic factors for eosinophils.⁹ Moreover, fibronectin, collagen I and CTGF importantly participate in the increased production of extracellular matrix in the airway.¹⁰ Our study demonstrated that BSMCs of chronic asthmatic rats not only produced much more of these proteins than controls, but also reacted more intensely to stimulation of the ERK1/2 pathway by EGF. These results suggest that BSMCs of chronic asthmatic rats possessed a strong ability to secrete multiple cytokines, growth factors and components of the extracellular matrix.

ERK1/2 signaling pathway is implicated in airway inflammation of asthma,^{11,12} but its role in secretion by BSMCs has not been well-studied. We used ERK1/2 stimulator EGF, a pharmacologic inhibitor (PD98059), and ERK1/2 antisense ODN to explore the regulatory action of ERK1/2 signaling pathway in this process.¹³ Our results demonstrated that EGF dramatically stimulated the secretion of BSMCs, while PD98059

suppressed the secretory ability of BSMCs whether the cells were stimulated by EGF or not. ERK1/2 antisense ODN effectively inhibited expression of ERK1/2 protein and mRNA, and it also strongly suppressed the secretory function of BSMCs in chronic asthmatic rats. These data suggest that stimulation of the ERK1/2 signaling pathway induced greater secretion by BSMCs, whereas inhibition of the pathway suppressed secretion. Therefore, the ERK1/2 signaling pathway appears to play an important role in regulating the secretory function of BSMCs in chronic asthma.

We noted a difference between the effect of PD98059 and that of antisense ODN. Antisense ODN not only decreased protein expression of ERK1/2, but also inhibited ERK1/2 mRNA levels. However, PD98059 inhibited only phospho-ERK1/2 protein levels, but had no effect on mRNA levels. The probable reason is that PD98059 inhibits ERK kinase (MEK) and can therefore only inhibit phosphorylation of ERK1/2, but has no effect on transcription of ERK1/2. Furthermore, antisense ODN reduced the secretion of RANTES to nearly the normal levels while PD98059 could not, suggesting that antisense ODN more strongly inhibits the increased secretion of BSMCs in chronic asthmatic rats. Might there be a role for ERK1/2 antisense ODN in treating chronic asthmatic patients? Are there any toxic effects or side effects of ODN?^{14,15} Our data here suggest that these questions may warrant further consideration and preclinical or clinical evaluation.

Although we recognize the importance of the ERK1/2 signaling pathway in regulating the secretory function of BSMCs in chronic asthma, a more comprehensive knowledge of the cytokine network may nevertheless help unravel the complex intracellular events that occur in chronic asthma.^{16,17} Also, other signaling pathway likely mediate the mechanism of secretion of BSMCs as well.^{18,19} Which is the predominant signaling pathway that regulates the process? Or do multiple signaling pathways operate cooperatively? These questions remain

to be explored.

It is thought that products secreted by BSMCs have effects on nearby cells and the extracellular matrix, but also may mediate the function of BSMCs in an autocrine fashion. Of the seven products in this study, TGF- β 1 is the most likely candidate to act on BSMCs by autocrine mechanisms.²⁰ It will be particularly interesting to determine whether ERK1/2 signaling might importantly mediate these autocrine mechanisms.

In conclusion, this study demonstrates that the ERK1/2 signaling pathway plays an important role in regulating the increased secretion of BSMCs in chronic asthmatic rats, and suggests that ERK1/2 antisense ODN effectively inhibits the process. Our results therefore suggest that further preclinical evaluation of ERK1/2 antisense ODN might now be warranted.

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