

Research Paper

## Extracellular signal-regulated kinase activation in airway smooth muscle cell proliferation in chronic asthmatic rats

BAI Jing<sup>\*\*</sup>, LIU Xian-Sheng<sup>\*</sup>, XU Yong-Jian, ZHANG Zhen-Xiang, XIE Min, NI Wang

Department of Respiratory Medicine, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

**Abstract:** To investigate the regulatory effect of extracellular signal-regulated kinase (ERK) signaling pathway on airway smooth muscle cell (ASMC) proliferation in chronic asthmatic rats, the rat model of chronic asthma was established, and ERK agonist epidermal growth factor (EGF) and inhibitor PD98059 were used in the cell culture. ASMC proliferation was examined by flow cytometry analysis, methyl thiazolyl tetrazolium (MTT) colorimetric assay, [<sup>3</sup>H]-thymidine (TdR) incorporation and proliferating cell nuclear antigen (PCNA) immunocytochemical staining. The expressions of ERK mRNA, ERK protein, phosphorylated ERK1/2 (p-ERK1/2) protein were observed by RT-PCR and Western blot. The results showed that in chronic asthmatic group, compared with that in the control group, the percentage of cells at G<sub>0</sub>/G<sub>1</sub> phase was significantly decreased and the percentage of cells at S+G<sub>2</sub>/M phase was significantly increased. Absorbance (A<sub>490</sub>), DNA synthesis and the expression of PCNA protein in ASMCs in chronic asthmatic group were significantly increased. The expressions of ERK mRNA, ERK1/2 protein, p-ERK1/2 protein and the activation ratio of ERK in ASMCs in chronic asthmatic group were significantly increased compared with those in the control group. After treatment with PD98059, the percentage of cells at S+G<sub>2</sub>/M phase, A<sub>490</sub>, DNA synthesis and the expression of PCNA protein in ASMCs in chronic asthmatic group were significantly decreased; the expressions of ERK mRNA, ERK1/2 protein, p-ERK1/2 protein and the activation ratio of ERK in ASMCs in chronic asthmatic group were significantly decreased compared with those in the control group. After treatment with EGF, the percentage of cells at S+G<sub>2</sub>/M phase, A<sub>490</sub>, DNA synthesis and the expression of PCNA protein in ASMCs in chronic asthmatic group were significantly increased compared with those before treatment; and PD98059 markedly inhibited the effect of EGF. These results suggest that the endogenous proliferation activity of ASMCs in chronic asthmatic rats significantly increases compared with that in the control rats, and ERK1/2 participates in this process. The ERK signaling pathway might play an important role in regulating ASMC proliferation, leading to asthmatic airway remodeling.

**Key words:** asthma; extracellular signal-regulated kinase; smooth muscle cell; proliferation

## 细胞外信号调节激酶活化在慢性支气管哮喘大鼠气道平滑肌细胞增殖中的作用

白晶<sup>\*\*</sup>, 刘先胜<sup>\*</sup>, 徐永健, 张珍祥, 谢敏, 倪望

华中科技大学同济医学院附属同济医院呼吸内科, 武汉 430030

**摘要:** 本文旨在探讨细胞外信号调节激酶(extracellular signal-regulated kinase, ERK)在慢性支气管哮喘大鼠气道平滑肌细胞(airway smooth muscle cells, ASMCs)增殖中的作用。建立慢性哮喘大鼠模型, 用ERK激动剂表皮生长因子(epidermal growth factor, EGF)和抑制剂PD98059干预慢性哮喘大鼠ASMCs的培养。采用流式细胞仪、四甲基偶氮唑盐(MTT)法、<sup>3</sup>H-thymidine (TdR)掺入法和增殖细胞核抗原(proliferating cell nuclear antigen, PCNA)免疫组织化学法检测ASMCs增殖情况, 观察ERK信号通路对ASMCs增殖的影响。RT-PCR和Western blot检测ERK mRNA和ERK1/2、磷酸化ERK1/2 (p-ERK1/2)蛋白的表达。与正常对照组ASMCs比较, 慢性哮喘组ASMCs的G<sub>0</sub>/G<sub>1</sub>期细胞所占比例明显减少, S+G<sub>2</sub>/M期细胞所占比例增高; 吸光度(A<sub>490</sub>)值、细胞DNA合成量和PCNA阳性表达量均明显增加, ERK mRNA、ERK1/2蛋白、p-ERK1/2蛋白的表达量以及ERK活

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<sup>\*</sup>Corresponding author. Tel: +86-27-83663616; Fax: +86-27-83663616; E-mail: liuxiansheng@tjh.tjmu.edu.cn

<sup>\*\*</sup>Present address: Department of Respiratory Medicine, the First Affiliated Hospital of Guangxi Medical University, Nanning 510027, China.

化率显著增高。经PD98059干预之后,慢性哮喘组ASMCs的S+G<sub>2</sub>/M期细胞所占比例、A<sub>490</sub>值、细胞DNA合成量和PCNA阳性表达量明显降低,ERK mRNA、ERK1/2蛋白、p-ERK1/2蛋白的表达量以及ERK活化率显著降低。经EGF干预后,慢性哮喘组ASMCs的S+G<sub>2</sub>/M期细胞所占比例、A<sub>490</sub>值、细胞DNA合成量和PCNA阳性表达量进一步增高,而这一作用可以被PD98059抑制。以上结果提示,慢性哮喘大鼠ASMCs内源性增殖活性增加,ERK1/2参与其增殖活性的调控,ERK信号通路在哮喘气道重建的ASMCs增殖调控中具有重要作用。

**关键词:** 哮喘; 细胞外信号调节激酶; 平滑肌细胞; 增殖

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Airway remodeling plays an important role in initiating irreversible airflow obstruction and persistent airway hyperresponsiveness in severe asthma<sup>[1]</sup>. It has been previously reported that airway smooth muscle cells (ASMCs), as the main component of airway remodeling, participate in the whole process of asthmatic airway remodeling through abnormal proliferation<sup>[2,3]</sup>. However, the intracellular signal regulation mechanism of the abnormal proliferation in asthmatic ASMCs remains unclear. Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are primarily distributed in ASMCs. The ERK signaling pathway has also been reported as a vital signaling pathway mediating vascular smooth muscle cell (VSMC) abnormal proliferation<sup>[4]</sup>. However, whether the ERK signaling pathway is involved in the chronic asthmatic ASMC abnormal proliferation is not yet known. Previous studies have demonstrated that ERK is required for the normal ASMC proliferation. In this study, we focused on the endogenous proliferation and the regulation of ASMC proliferation by the ERK signaling pathway in chronic asthmatic rats.

## 1 MATERIALS AND METHODS

### 1.1 Materials

Inactivated bordetella pertussis vaccine was from the Institute of Biological Products in Chengdu; ERK agonist epidermal growth factor (EGF) was from Peprotech-BRL Inc., USA; Inhibitor of ERK PD98059 was from CST-BRL Inc., USA; Caroid, trypsin, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), methyl thiazolyl tetrazolium (MTT) and TRIZOL reagent were from Gibco-BRL Inc., USA; Mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) was from NeoMarkers-BRL Inc., USA; Rabbit monoclonal anti-ERK1/2 and mouse monoclonal anti-phosphorylated ERK1/2 (p-ERK1/2) antibodies purchased from R&D Biotechnology Inc., USA; Ovalbumin (OVA), propidium iodide (PI), rat monoclonal antibody against  $\alpha$ -smooth muscle actin ( $\alpha$ -actin) were from Sigma Biotechnology Inc., USA; Fluorescein isothiocyanate (FITC) labeled-goat to mouse and

streptavidin peroxidase immunocytochemical kit were from Zhongshan Biotechnology Co., Beijing, China; *Taq* DNA polymerase, *M-MuLV*, OligodT, RNasin and gelose were from Promega Biotechnology Inc., USA; PCR reaction kit was from BMI Biotechnology Inc., USA; Enhanced chemiluminescence (ECL) detection kit was from Pierce Biotechnology Inc., USA; <sup>3</sup>H-thymidine (TdR) was purchased from the Institute of Atomic Energy, China; The primers of ERK and  $\beta$ -actin were synthesized by Aoke Biotechnology Co., Beijing, China.

### 1.2 Animals and sensitization

A rat asthmatic model was established by the methods of Kumar *et al*<sup>[5]</sup>. All rats were sensitized with subcutaneous injection of 1 mL sterile suspension with 10 mg OVA and 200 mg aluminum hydroxide in saline, and 1 mL inactivated bordetella pertussis vaccine (6 $\times$ 10<sup>9</sup> heat-killed bacillin) was administered intraperitoneally as an adjuvant on the first day (day 1). The rats were sensitized again on day 8. From day 15 to day 56 (6 weeks), all rats were challenged with aerosole of OVA solution [2% (W/V) in saline] for 30 min and repeated three times a week. After challenged, rats presented asthmatic symptom such as agitation, cough, tachypnoea, cyanosis, and so on. Thirty male rats weighing 150-200 g obtained from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, were randomly divided into 2 groups: (1) chronic asthmatic group; (2) normal group: rats were sensitized and challenged with equal amounts of liquor natrii chloridi otonicus instead of OVA. The rats were killed within 18-24 h after the final challenge in each group. The left lungs were fixed with 4% formaldehydum ispolymeratum for 4 h after adequate inflation, then dehydrated and embedded routinely with paraffin. Sections (5  $\mu$ m in thickness) from midsagittal and hilar slices of the lungs were stained with HE. The right lung lobes were used to cell culture.

### 1.3 Pathomorphological image analysis of airway remodeling

Bilateral lung tissue sections stained with HE in each rat

group were selected and analyzed with HPIAS-1000 Type High-resolution Medical Colorful Image Analyzing System (Qianping Image Co., Wuhan). We defined the intact airways with microscope<sup>[6]</sup>; and image analysis system was used to measure the airway internal perimeter (Pi), wall area (WA), external perimeter (Pe), the area of bronchial smooth muscle (S), the number of bronchial smooth muscle nucleus (N) and calculated percentages of Pi according to formula, including the airway wall thickness (WA/Pi), the bronchial smooth muscle area (S/Pi), the number of bronchial smooth muscle nucleus (N/Pi), respectively.

#### 1.4 Cell culture

Primary cultures of ASMCs were established as previously reported<sup>[7]</sup>. The identification of ASMCs was confirmed by the presence of positive immunofluorescent staining for  $\alpha$ -actin. 99% purity of ASMCs were yielded. Cells between passages 3 and 5 were used for experiments. ASMCs were seeded at a density of  $1 \times 10^5$  cells/mL in 100-cm<sup>2</sup> tissue culture flasks or 96-well plates at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> and were cultured in DMEM with 10% FBS for 24 h, then in serum-free DMEM for another 24 h to arrest the cell growth at G<sub>0</sub> phase. Based on treatment with or without PD98059 (10  $\mu$ mol/L) and EGF 20 (ng/mL), ASMCs were divided into 4 groups randomly: (1) blank control group: ASMCs were cultured in 10% FBS group without any intervention; (2) FBS + PD98059 group (PD group): ASMCs were cultured in 10% FBS with 10  $\mu$ mol/L PD98059; (3) FBS + EGF group (EGF group): ASMCs were cultured in 10% FBS with 20 ng/mL EGF; (4) FBS + PD98059 + EGF group (PD + EGF group): Cells pretreated with 10  $\mu$ mol/L PD98059 for 1 h, and then 20 ng/mL EGF was added. Every group had 4 replicated wells. Culture lasted another 24 h. Proliferation of ASMCs was detected by the following methods. The same test repeated 3 times.

#### 1.5 Proliferation assay

##### 1.5.1 Analysis of cell cycle by flow cytometry

Collected ASMCs were prepared at a density of  $1 \times 10^6$  cells/mL in cell suspension and were fixed in 75% cold ethanol at 4 °C, and then treated with 1 mg/mL RNase (TaKaRa, Japan) for 30 min at 37 °C. Then cells were stained with 0.5 mL of 10 mg/mL PI avoiding light for 30 min at 4 °C. The cell cycle was detected on a flow cytometer. Each group was analyzed in triplicate. Analysis was carried out with a FACSort Plus (Becon-Dicknson Co., USA), and the data were analyzed with Cellquest software.

##### 1.5.2 MTT colorimetric assay

ASMCs were seeded into 96-well plates, cultivated and

divided into 4 groups as described before. MTT (5 mg/mL) was added to the wells (20  $\mu$ L/ well) for the final 4 h and incubated at 37 °C. At the end of the experimental period, media were removed from the wells and dimethylsulfoxide was added to the wells (150  $\mu$ L/ well). The plates were agitated at room temperature for 30 min. Absorbance at 490 nm ( $A_{490}$  value) of every well was read on an enzyme-linked immunosorbent assay reader (Bio-tek ELX800, Japan).

##### 1.5.3 <sup>3</sup>H-TdR incorporation

<sup>3</sup>H-TdR incorporation was used as an indicator of cell proliferation. Cells were plated in 96-well cluster plates. For the final 6 h, 1  $\mu$ Ci/well of <sup>3</sup>H-TdR (final concentration  $3.7 \times 10^7$  kBq/mL) was added to each well to measure DNA synthesis. Cell proliferation was arrested by 10% trichloroacetic acid (TCA) freezing and 1 mol/L sodium hydroxydatum, and the cells were harvested into scintillation vial. After 500  $\mu$ L scintillation fluid was added, the samples were counted with a multifunction liquid scintillation counting meter and the square pulse per minute was measured. The results were expressed as counts per minute (cpm)/ $1 \times 10^6$  cells.

##### 1.5.4 Detection of PCNA protein in ASMCs by immunohistochemical staining

ASMCs were fixed with cool acetone. The expression of PCNA protein in ASMCs was detected by SP immunocytochemical staining. Immunostaining was carried out by standard procedures. SP method referring to kit instructions was used. Cells were incubated with the primary antibody at 4 °C overnight. The same process without the primary antibody was used as a control. After incubation with the secondary antibody labeled with fluorescein at 37 °C for 1 h and DAB staining, ASMCs were observed under microscope at once. The cells positive for PCNA staining were brownish-yellow in nuclei. Five high-power fields were randomly selected and the gray value was semi-quantitatively analyzed with HPIAS-1000 Type High-resolution Medical Colorful Image Analyzing System. The gray value inversely correlated with the expression of PCNA.

#### 1.6 Western blot

The whole-cell proteins were extracted, and then protein concentration was measured by BCA protein assay kit to ensure the fixed quantity in each hole. Protein extracts (20  $\mu$ g) were combined with a homo-volum gel loading buffer and heated to 100 °C for 10 min. Proteins were subjected to SDS-polyacrylamide gel electrophoresis [12% (W/V)] at 120 V for 2 h, and then transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered

saline (TBS, 20 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk at room temperature for 2 h. After a brief rinse with TBS-T, the membrane was incubated with the primary antibody (ERK1/2 1: 1 000, p-ERK1/2 1:1 000 in TBS-T and 0.5% BSA) at 4 °C overnight. The membrane was rinsed three times with TBS-T before being incubated with horseradish peroxidase-conjugated secondary antibody (1:2 000 in TBS-T and 0.5% BSA) for 2 h. The membrane was then rinsed three times with TBS-T and developed with an ECL assay kit before exposure to Kodak X-OMAT film. The bands of interest were confirmed by comparison with molecular mass markers. The blots were quantified by computerizing the gray scale values with GDS8000 Image Analysis System (UVP Corporation, UK). Expression of protein was normalized to the expression of  $\beta$ -actin detected in the same membrane. Activation ratio of ERK was calculated as the ratio of p-ERK1/2 protein to ERK1/2 protein.

#### 1.7 Expression of ERK mRNA in ASMCs

Total RNA was extracted by the TRIzol procedure following the manufacturer's instructions. 1~2  $\mu$ g of total RNA was reversely transcribed. Semi-quantitative PCR was performed by using 4  $\mu$ L of reverse transcript sample per reaction mixture, 200 nmol/L of each primer, and 1 U of *Taq* polymerase. The thermal cycling conditions included an initial denaturation step (95 °C for 5 min) and each cycle consisted of 94 °C for 30 s, 52.7 °C for 30 s, and 72° for 45 s. The final products were subjected to agarose gel electrophoresis [2% (W/V)]. Experiments were performed in triplicate. The sequences of the different primers used were as follows: ERK (F), 5'-GACACAGCACCTCAGCAA-3'; ERK (R), 5'-GGAGATCCAAGAATACCC-3';  $\beta$ -actin (F), 5'-GTCACCAACTGGGACGATA-3';  $\beta$ -actin (R), 5'-AGGTCTTACGGATGTCAACG-3'. Amplifications were quantified by computerizing A values with GDS8000 Image Analysis System. The expression level of ERK mRNA was expressed as the ratio of amplification to  $\beta$ -actin.

#### 1.8 Statistics

The data were analyzed by SPSS software (version 11.5) to assess the statistical difference and expressed as mean $\pm$ SD. *t* test was used for comparison. Differences between groups were analyzed by using one-way analysis of variance (ANOVA) with Student-Newman-Keuls *post hoc* test. Equal variance not assumed was analyzed by using Games-Howell test. Differences were considered significant at  $P<0.05$ .

## 2 RESULTS

### 2.1 Pathomorphological image of airway remodeling

In chronic asthmatic rats, the mucosal fold membranes of bronchiole were increased and broken; a large number of eosinophils infiltrated into the bronchial submucosa; airway wall and mucus plugs were observed in airway lumen; and the thickness of airway wall and bronchial smooth muscle were significantly increased (Fig.1). WA/Pi (12.57 $\pm$ 1.53), S/Pi (3.69 $\pm$ 0.62), and N/Pi (0.0329 $\pm$ 0.0174) were significantly increased by 62.57%, 38.64% and 75% ( $P<0.05$ ), respectively, compared with those in the control group, which were 7.71 $\pm$ 0.24, 2.65 $\pm$ 0.25 and 0.0188 $\pm$ 0.0044, respectively.

### 2.2 Cell identification

Under inverted phase contrast microscope, the cells showed spindle-shape, having central oval nuclei with prominent nucleoli, as well as the typical "hill and valley" appearance. Immunofluorescence of  $\alpha$ -actin was positive in cytoplasm (Fig.2). Thus, the cultured cells were identified as ASMCs.

### 2.3 Effects of ERK on ASMCs

#### 2.3.1 Cell proliferation assay

##### 2.3.1.1 Flow cytometry analysis in ASMCs

As shown in Table 1, compared with that in the blank control of normal rats, the percentage of cells at  $G_0/G_1$  phase was significantly decreased ( $P<0.05$ ), and that at S+ $G_2/M$  phase was manifestly elevated in the blank control of chronic asthmatic rats ( $P<0.05$ ). No matter Whether in the normal or chronic asthmatic rats, the percentage of cells at S+ $G_2/M$  phase in EGF group was significantly increased, compared with that in the corresponding blank control group ( $P<0.01$ ), while this effect of EGF was suppressed by PD98059 pretreatment. There were no significant difference in the cell percentages at  $G_0/G_1$  and S +  $G_2/M$  phase between the control and EGF + PG groups.

##### 2.3.1.2 MTT colorimetric assay in ASMCs

As shown in Table 1, compared with that in the blank control of normal rats,  $A_{490}$  was manifestly elevated in the blank control of chronic asthmatic rats ( $P<0.01$ ). No matter in the normal or chronic asthmatic rats,  $A_{490}$  values in EGF group were significantly increased compared with that in the corresponding blank control group ( $P<0.01$ ), while those were not significantly changed in PD + EGF group.

##### 2.3.1.3 $^3H$ -TdR incorporation in ASMCs

As shown in Table 1, compared with that in the blank control of normal rats, cellular DNA synthesis quantity was manifestly elevated in the blank control of chronic asthmatic rats ( $P<0.05$ ). No matter in the normal or chronic

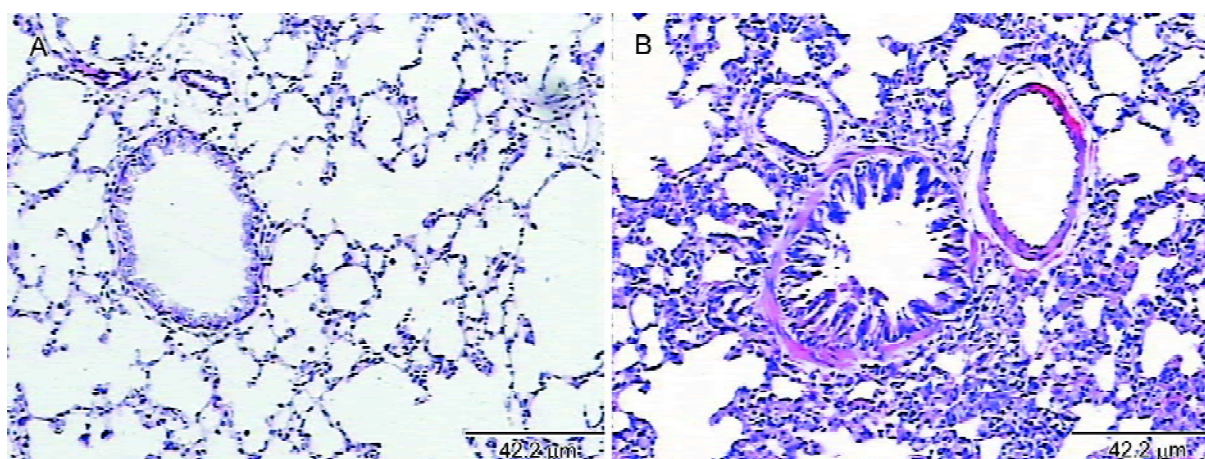


Fig.1. The bronchial smooth muscles stained by HE in the control rats and chronic asthmatic rats. A: Control group; B: Chronic asthmatic group. Scale bar, 42.2  $\mu\text{m}$ .

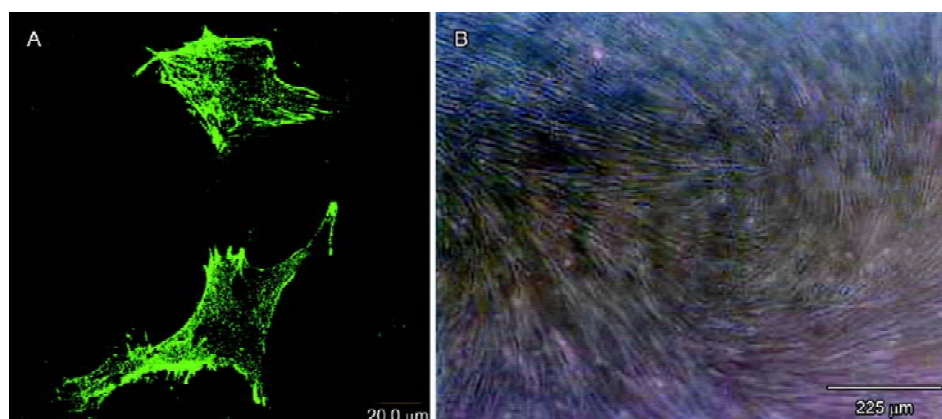


Fig. 2. Identification of ASM cells. A: Positive expression of  $\alpha$ -actin by immunofluorescent staining in ASM cells. Scale bar, 20  $\mu\text{m}$ . B: ASM cells displayed the typical "hill and valley" appearance under phase contrast microscope. Scale bar, 225  $\mu\text{m}$ .

asthmatic rats, DNA synthesis values in EGF group were significantly increased compared with that in the corresponding blank control group ( $P < 0.01$ ), while those were not significantly changed in PD + EGF group.

#### 2.3.1.4 Expression of PCNA protein in ASMCs

There was a similar trend in the expression of PCNA protein (Table 1).

#### 2.3.2 Regulation of ASMC proliferation by ERK

After treatment with EGF, no matter in the normal or chronic asthmatic rats, compared with that in the corresponding blank control group, the percentage of cells at S + G<sub>2</sub>/M phase, A value, DNA synthesis and PCNA expression were significantly increased ( $P < 0.01$ ). In chronic asthmatic rats, they were increased by 63.40%, 41.03%, 35.07%, 12.05%, respectively, which were more signifi-

cant than those in the normal rats, which were 56.18%, 25.01%, 16.61%, 7.73%, respectively (Table 1).

After treatment with ERK inhibitor PD98059, no matter in the normal or chronic asthmatic rats, compared with that in the corresponding blank control group, the percentage of cells at S+G<sub>2</sub>/M phase, A value, DNA synthesis and PCNA expression were significantly decreased. In the chronic asthmatic rats, they were inhibited by 35.40%, 30.77%, 41.03%, 11.23%, respectively, which were more significant than those in the normal rats, which were 11.41%, 16.61%, 25.01%, 6.58%, respectively (Table 1).

#### 2.4 Expression of ERK mRNA in ASMCs

Compared with the blank control in normal rats, there was a significant increase of ERK mRNA expression in the blank control chronic asthmatic rats ( $0.78 \pm 0.12$  vs  $1.54 \pm$

Table 1. ERK effect on cell proliferation of ASMCs

| Groups         | Percentage of cells at G <sub>0</sub> /G <sub>1</sub> phase (%) |              | Percentage of cells at S+G <sub>2</sub> /M phase (%) |              | A <sub>490</sub> |             |
|----------------|-----------------------------------------------------------------|--------------|------------------------------------------------------|--------------|------------------|-------------|
|                | Normal                                                          | Asthmatic    | Normal                                               | Asthmatic    | Normal           | Asthmatic   |
| Control        | 84.15±2.90                                                      | 73.23±5.19*  | 15.85±2.90                                           | 26.84±5.23*  | 0.24±0.04        | 0.39±0.05** |
| PD group       | 85.96±1.63                                                      | 83.29±1.34#  | 14.04±1.63#                                          | 17.31±1.29#  | 0.20±0.01#       | 0.27±0.02## |
| EGF group      | 75.15±4.27##                                                    | 56.12±4.22## | 24.85±4.27##                                         | 43.88±4.22## | 0.30±0.03##      | 0.55±0.04## |
| PD + EGF group | 83.06±2.45                                                      | 73.72±1.92   | 16.94±2.44                                           | 26.83±1.92   | 0.26±0.02        | 0.40±0.04   |

| Groups         | <sup>3</sup> H-TdR (cpm/1×10 <sup>6</sup> cells) |                   | Gray scale of PCNA |               |
|----------------|--------------------------------------------------|-------------------|--------------------|---------------|
|                | Normal                                           | Asthmatic         | Normal             | Asthmatic     |
| Control        | 1 139.50±65.36                                   | 1 465.28±98.56*   | 198.15±8.73        | 174.44±6.13** |
| PD group       | 850.14±96.44##                                   | 849.00±75.72##    | 211.20±3.32#       | 194.47±2.84#  |
| EGF group      | 1 329.42±110.68##                                | 1 987.17±134.59## | 182.84±5.67##      | 153.38±4.78## |
| PD + EGF group | 1 150.83±94.51                                   | 1 674.25±137.64   | 193.78±9.70        | 170.37±7.56   |

\* $P<0.05$ , \*\* $P<0.01$  compared with normal rats; # $P<0.05$ , ## $P<0.01$  compared with control rats.

0.32,  $P<0.05$ ). Compared with that in the blank control of chronic asthmatic rats, the expression of ERK mRNA was significantly decreased in PD group (0.88±0.05), significantly increased in EGF group (2.14±0.43) ( $P<0.05$ ), while there were no significant changes in PD + EGF group (1.61±0.30) (Fig.3).

## 2.5 Expression of ERK protein in ASMCs

Compared with that in the blank control of normal rats (0.069±0.017, 0.172±0.015, 50.21±3.07, respectively), the protein expressions of ERK1/2 (0.172±0.015), p-ERK1/2

(0.140±0.012) and activation ratio of ERK (81.40±5.95) were significantly increased in the blank control of chronic asthmatic rats. Compared with that in the blank control of chronic asthmatic rats (0.172±0.015, 0.140±0.012, 81.40±5.95, respectively), the expressions of ERK1/2, p-ERK1/2 protein and activation ratio of ERK were significantly decreased in PD group (0.104±0.006, 0.057±0.028, 55.03±3.78, respectively), and they were significantly increased in EGF group (0.361±0.003, 0.321±0.003, 91.57±5.32, respectively) ( $P<0.05$ ), while there were no significant

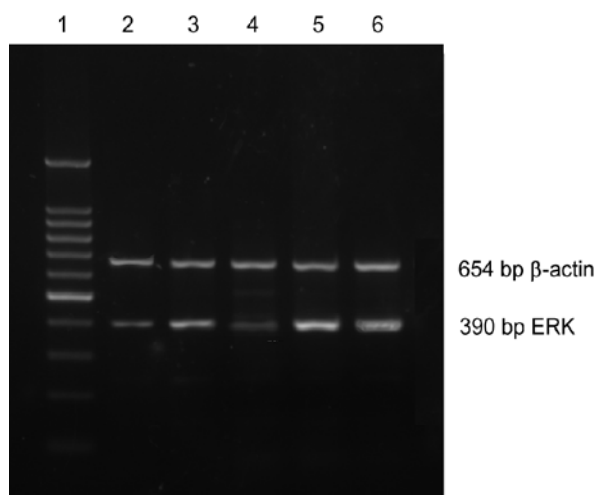


Fig.3. Expression of ERK mRNA in ASMCs. Lane 1, marker; Lane 2, blank control of normal rats; Lane 3, blank control of chronic asthmatic rats; Lane 4, PD group of chronic asthmatic rats; Lane 5, EGF group of chronic asthmatic rats; Lane 6, PD + EGF group of chronic asthmatic rats.

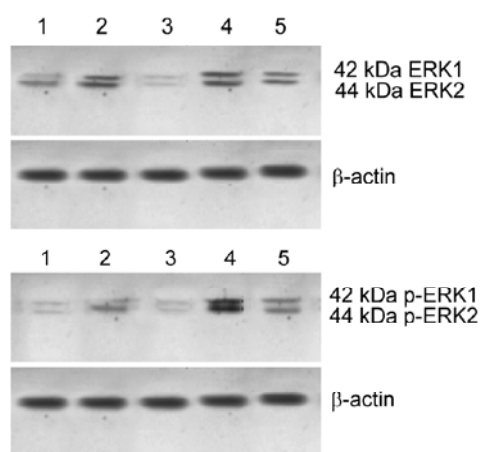


Fig.4. Expressions of ERK1/2 and p-ERK1/2 protein in ASMCs. Lane 1, blank control of normal rats; Lane 2, blank control of chronic asthmatic rats; Lane 3, PD group of chronic asthmatic rats; Lane 4, EGF group of chronic asthmatic rats; Lane 5, PD + EGF group chronic asthmatic rats.

changes in PD + EGF group ( $0.155 \pm 0.020$ ,  $0.125 \pm 0.012$ ,  $80.22 \pm 7.06$ , respectively) (Fig.4).

### 3 DISCUSSION

By the application of chronic asthmatic rat model, we found that the airway wall and the area of airway smooth muscles in chronic asthmatic rats were significantly thickened. After the standardization of the area of airway wall, the area of bronchial smooth muscle and the number of bronchial smooth muscle nucleus, WA/Pi, S/Pi and N/Pi in chronic asthmatic rats were significantly higher than those in the normal rats. Thus our findings indicated that airway remodeling had been developed in this chronic asthmatic rat model and the remodeling was characterized by an increase in the airway smooth muscle proliferation.

Respecting the difference in the phenotype and function of the normal and asthmatic ASMCs<sup>[8]</sup>, more accurate information could be obtained from asthmatic subjects *in vivo* to reflect the characteristics and signal transduction pathway in asthmatic ASMC proliferation. Our results showed that, compared with that in the blank control of normal rats, the percentage of cells at G<sub>0</sub>/G<sub>1</sub> phase was significantly decreased and the percentage of cells at S + G<sub>2</sub>/M phase, A<sub>490</sub> value, cellular DNA synthesis and PCNA-positive expression were manifestly elevated in the blank control of chronic asthmatic rats. Those demonstrated that asthmatic ASMCs were significantly increased from G<sub>0</sub>/G<sub>1</sub> into S phase.

In order to further elucidate the role of ERK in controlling ASMC proliferation, ERK agonist EGF and selective inhibitor PD98059 were selected to treat ASMCs. Whether in the normal or chronic asthmatic rats, compared with that in the corresponding blank control group, the percentage of cells at S + G<sub>2</sub>/M phase, A<sub>490</sub> value, cellular DNA synthesis and PCNA-positive expression were significantly increased in EGF group, while those were not significantly changed in PD + EGF group. Values of proliferation index, A<sub>490</sub>, DNA synthesis and the expression of PCNA protein in chronic asthmatic rats were markedly increased compared with those in the normal rats. It revealed that endogenous proliferation was significantly increased in chronic asthmatic rat ASMCs. PD98059 inhibited the effects of EGF, which indicated that ERK may play a critical role leading in promoting endogenous proliferation of ASMCs in chronic asthmatic rats. After treatment with PD98059, the afore-mentioned items could be predominantly suppressed by PD98059 and they were inhibited more significantly in chronic asthmatic rats than

those in the normal rats. Thus, the remarked endogenous proliferation of ASMCs in chronic asthmatic rats was possibly related to the ERK signaling pathway.

A critical role for ERK activation in VSMC abnormal proliferation has been suggested in vessel remodeling<sup>[9,10]</sup>. In our study, the expressions of ERK mRNA, ERK1/2, p-ERK1/2 proteins and activation ratio of ERK in the blank control of chronic asthmatic rats were significantly elevated compared with those in the blank control of normal rats, and they were decreased after PD98059 intervention. It showed that the expression and activation of ERK1/2 were predominantly up-regulated during chronic asthma and the intensified asthmatic ASMC proliferation activity was possibly related to ERK activation. Thus the results further provided evidence for increased endogenous proliferation of ASMCs in chronic asthmatic rats through the ERK-dependent signal pathway.

In addition, although PD98059 could inhibit asthmatic rat ASMC proliferation, several proliferation values in PD group of chronic asthmatic rats were yet higher than those in the blank control of normal rats. So there might exist other ERK-independent pathways leading to asthmatic ASMC proliferation. Black *et al.*<sup>[11]</sup> showed that phorbol ester could irritate the normal ASMC proliferation in human or pigs by the activated protein kinase C (PKC) signal pathway, and R0318220 could inhibit the normal ASMC proliferation induced by FBS in rabbits. Krymskaya *et al.*<sup>[12]</sup> found that the PI3K signaling pathway possibly partly participated in regulation of ASMC proliferation.

In conclusion, as described in advance, our study showed endogenous proliferation of ASMCs in chronic asthmatic rats was significantly enhanced, and ERK1/2 expression and activation in asthmatic ASMCs were also manifestly elevated. Thus the abnormal proliferation of ASMCs in chronic asthmatic rats may be regulated via the up-regulated ERK signaling pathway. In the level of intracellular signal transduction, our study revealed that ERK is required for chronic asthmatic ASMC proliferation. Thus targeting the control of ERK activation may provide a new therapeutic approach for airway remodeling in asthma. In recent studies on other cells, plenty of transcription factors have been involved in the downstream of ERK signaling pathway, so the further studies on special kind of transcription factors participating in regulating ASMC proliferation would be required.

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