The effect of formoterol on airway goblet cell hyperplasia and protein Muc5ac expression in asthmatic mice

Y.-F. TAN, W. ZHANG, L. YANG, S.-P. JIANG

Department of Respiratory Medicine, the Second Affiliated Hospital, Sun Yat-sen University, Guangzhou (China)

Abstract. – *Objectives:* Aim for this study was to investigate the effect of long-acting β2-adrenoceptor agonist formoterol on airway goblet cell hyperplasia and protein Muc5ac expression in asthmatic mice.

Materials and Methods: Forty female BABL/c mice were randomly divided into four groups with 10 mice in each. Mice in group A were treated with saline as control, and mice in group B, group C and group D were sensitized by intraperitoneal injection of 10 μg alum precipitated chicken egg ovalbumin (OVA) to establish asthmatic model, but group C were pretreated with formoterol and group D were pretreated with dexamethasone. All mice were killed 24 hours after the final OVA challenging. The left lung tissue sections were stained with periodic acid Schiff (PAS) for identification of goblet cell hyperplasia. Immunohistochemistry was used to identify the protein of Muc5ac. The right lung was isolated for detecting Muc5ac mRNA by the method of real-time fluorescence quantitative reverse transcription Polymerase Chain Reaction (real-time qRT-PCR).

Results: The number of the goblet cells, the percentage of goblet cell to total cell, the transcription and the expression of Muc5ac were significantly higher in group B than those in group A [(163.63±16.68) *vs* (0.46±0.16), (77.36±5.05) % vs (0.03±0.01) % (10.31±0.73) vs (1.00±0.13), (0.64±0.03) vs (0.19±0.03) respectively, all *P*<0.05]. The number of the goblet cells, the percentage of goblet cell to total cell, the transcription and the expression of Muc5ac were significantly lower in group C than those in group B [(52.04±4.60) *vs* (163.63±16.68), (30.05±3.72) % vs (77.36±5.05) %, (1.64±0.14) *vs* (10.31±0.73), (0.26±0.01) vs (0.64±0.03) respectively, all *P* <0.05] The number of the goblet cells, the percentage of goblet cell to total cell, the transcription and the expression of Muc5ac were significantly lower in group D than those in group B [(63.41±6.39) *vs* (163.63±16.68), (38.52±3.83)% *vs* (77.36±5.05) %, (1.72±0.10) vs (10.31±0.73), (0.31±0.01) *vs* (0.64±0.03) respectively, all *P*<0.05]. For mentioned above, no significant differences were found between group C and group D [(52.04±4.60) *vs* (63.41±6.39), (30.05±3.72) % *vs* (38.52±3.83) %, (1.64±0.14) *vs* (1.72±0.10), (0.26±0.01) *vs* (0.31±0.01) respectively, all *P* <0.05].

Conclusions: This study demonstrates that the long-acting β2-receptor agonist formoterol may inhibit airway goblet cell hyperplasia and protein Muc5ac expression in asthmatic mice.

Key Words:

Asthma, Formoterol, Goblet cell, Protein Muc5ac, Mice.

Introduction

Airway hypersecretion is one of the most important pathophysiological changes of asthmatic airway, that also contributes to the high incidence and mortality of asthmatic patients[1]. Goblet cell is the main producer and secretor of mucus in airway epithelium. Its hyperplasia is not only a part of the airway remodeling[2], but also an important mechanism leading to deterioration of asthma when degranulation happens severely[3]. The main ingredient of mucus is mucin. Protein mucin5ac (Muc5ac) exists mainly in asthmatic epithelium. Its gene transcription and expression are significantly increased in asthmatic airway[4]. This study was designed to detect the level of airway goblet cell hyperplasia and protein Muc5ac expression in asthmatic mice after treated with the long-acting β_2-adrenoceptor agonist formoterol.

Materials and Methods

Specific pathogen-free female BABL/c mice aged 6-8 weeks (16-20 g) were purchased and

housed in the laboratory Animal Center of Sun Yat-sen University (Certificate Number: 2007A045). All mice were maintained in a laminar flow holding unit on a standard of 12 hours light/dark cycle. Autoclaved food and acidified water were provided all time. The experimental and animal handling procedures were approved by the Institutional Animal Ethics Committee. Ovalbumin (OVA) was purchased from SigmaAldrich Company (St Louis, MO, USA). Formoterol was purchased from Astra Zeneca Company (WuXi, JiangSu, China).

Experimental Design

Forty mice were randomly divided into four groups with 10 mice in each, including group A (saline control group), group B (asthmatic group), group C (formoterol pretreated group) and group D (dexamethasone pretreated group). The mice in Group B, group C and group D were sensitized by intraperitoneal injection of 10 μg alum precipitated chicken egg ovalbumin (OVA) (grade V, >98% pure, Sigma, St Louis, MO, USA) and 20 μg aluminum hydroxide (Chemical Reagent Factory, Guangzhou, China)in the first day and fourteenth day. From the twenty-first day, all the mice were exposed to 2.5% ovalbumin for 30 min/day on three days/week in a case (size about 0.5 m × 0.5 m × 0.5 m) for totally 6 weeks. Group C was intraperitoneal injected with 1 mg/kg formoterol 10 min before OVA exposure, and Group D was intraperitoneal injected with 0.5 mg/kg dexamethasone before exposure. Saline solution was used instead of ovalbumin in group A when sensitized and aerosol inhalation challenged. All mice were anesthetized by intraperitoneal injection with 1% pentobarbital (40 mg/kg) 24 hours after the last inhalational exposure. Then we killed all mice and got the lungs. The right lungs were kept in liquid nitrogen tanks for extracting mRNA. The left lungs were perfused with 4% phosphate buffered saline (PBS) (pH7.4) under a pressure of 20 cm H_2O (1 cm H_2O=0.098 kPa) for 24 hours.

Lung Histology and Quantitative Image Analysis

After fitting in 4% PBS and embedded in paraffin, tissue sections were stained with diastase-periodic acid-Schiff (PAS) for the detection of goblet cell by image-pro plus 6.0 analysis software. Quantitative analyses of PAS-stained

lung sections were performed as following. Five lung tissue sections was randomly selected of each mouse, where were observed five bronchi (relatively circular cross-section and diameter of 100 μm above) each in a single-blind randomized way. Then, the areas of goblet cell dyed purple in bronchi of each lung tissue sections were measured by image-pro plus 6.0 software. The purple areas were expressed as a percentage of the total areas and the numbers of goblet cells were calculated.

Real-Time Reverse Transcription-Polymerase Chain Reaction (Real-Time rtPCR)

For real-time rtPCR, primer sequences for Muc5ac were selected to minimize primer dimerization which was designed and synthesized by GenePharma Company Ltd (Shanghai, China). The primer sequences (amplicon size in parentheses)were as following: for Muc5ac upper primer, 5'-ACC ACT TTC TCC TTC TCC ACAC-3' and for Muc5ac lower primer, 5'-AAC AGG GCT CTT CAC AGA CAA TA-3' (150 bp). 18s rRNA was used to control endogenously to evaluate threshold cycle and served as an internal positive control. Total RNA was extracted from the lung tissue kept in liquid nitrogen by the way of Trizol based on the procedure described by Chomczynski and Sacchi[5]. After estimation of the purity, all RNA was turned into cDNA after using M-MLV reverse transcriptase kit. The cDNA was placed in −20°C refrigerator for spare. In SYBR Green fluorescent dye reaction, fluorescence was collected during the whole process, including pre-denaturation, denaturation, annealing, extension and re-extension in a total of 40 cycles. All Muc5ac mRNA expression data were normalized to 18s rRNA expression level from the same individual samples. After amplification of Muc5ac mRNA and 18s rRNA (each sample repeated 3 times), the original copies were calculated by the way of $2^{-\Delta Ct}$ method[6]. Appropriate software was used for amplification curves of each group samples comparing with 18s rRNA. Ct values, ΔCt values and $\Delta\Delta$Ct values were calculated and eventually came to $2^{-\Delta\Delta Ct}$ value. Ct values means the amplification cycle times of the PCR products' fluorescent signal that reached the threshold set in the amplified process. ΔCt value means the difference of the average Ct value between

the tested gene and the reference gene in one sample. $^{\Delta\Delta}$Ct value means the difference of $^{\Delta}$Ct values between gene in certain samples and other samples. $2^{-\Delta\Delta Ct}$ value means the multiples compared with reference gene. From the $2^{-\Delta\Delta Ct}$ value, the relative quantitative comparison of Muc5ac mRNA was obtained in four groups. In another words, group A (saline control group) was taken as a reference, the relative expressions of Muc5ac mRNA in other groups were expressed by $2^{-\Delta\Delta Ct}$ value.

Immunohistochemistry

Lung tissues were perfused and immersed in the paraformaldehyde. Paraffin sections were made 5 μm in thickness preparing for immunohistochemistry. For protein MUC5ac immunostaining, a monoclonal anti-MUC5ac antibody (Abcam Company, Cambridge, UK) and Histostain-Plus Kits (ZSGB-BIO, Beijing, China) were used. After 30 min baking, paraffin sections were washed by xylene gradient before 20 min incubation by periodates. Then the sections were placed in 0.01 mol/L citric acid buffer (pH 6.0) for microwave antigen retrieval before treatment with 3% H_2O_2 for 30 min (37°C) and pre-incubation with goat serum (1:10). The sections were then incubated with anti-MUC5ac antibody (1:200) overnight at 4°C. After 15 min rinse in PBS, the sections were incubated with biotinylated secondary antibody and avidin-biotin peroxidase complex for 30 min each at a room temperature. Immunoreactions were visualized in diaminobenzidine (DBA) for 2 min and then hematoxylin for dyeing. The positive MUC5ac protein was dyed brown in bronchial epithelia and was measured by image-pro plus 6.0 software which was corrected by the average optical density. Cumulative optical density (IOD SUM) divided by the area of the selected region (area SUM) leads to average optical density.

Statistical Analysis

All data were expressed by arithmetic means ± SD (±) and were processed by Statistical Package of Social Sciences (SPSS Inc.) version 13 (Chicago, IL, USA) statistical software. Measuring data of each group was used univariate analysis of variance (ANOVA), comparison between groups was used LSD method. Statistical differences were considered to be significant when $P<0.05$. Two variables were used for rank correlation analysis Pearson correlation.

Results

Goblet Cell Hyperplasia in Airway

There were obvious differences of purple-stained areas representing numbers of goblet cells in airway epithelium among 4 groups (Figure 1). After analysis by image-pro plus 6.0 software, we got data in Table I. Test showed that the differences of the numbers of goblet cells between Group A and Group B, Group C, Group D were all significant (*t* values were 163.17, 51.58 and 62.96 respectively, all $P<0.05$); the number of goblet cells of group C and D were significantly less than those of group B (*t* values were 111.59 and 100.22 respectively, all $P<0.05$); whereas, the difference between group C and D was not significant (*t* = 1.32, $P>0.05$). At the same time, the difference of the percentage of goblet cells to total cells between group A and group B, group C, group D were all significant (*t* values were 77.33, 30.02 and 38.50 respectively, all $P<0.05$); the percentage of goblet cells to total cells of group C and D were significantly less than those of group B (*t* values were 47.31 and 38.84 respectively, all $P<0.05$); the difference between group C and D was not significant (*t* = 0.47, $P>0.05$).

Analysis of MUC5ac mRNA Relative Expression

The state of MUC5ac mRNA expression was evaluated by real-time rtPCR method. Taking Group A (saline control group) a standard, we calculated the Ct values, $^{\Delta}$Ct values, $^{\Delta\Delta}$Ct values and $2^{-\Delta\Delta Ct}$ values of Group B, Group C and Group D (see Table II). Test showed that the differences of the MUC5ac mRNA expression were significant between group A and group B, group C, group D (*t* values were 9.31, 0.65 and 0.72 respectively, all $P<0.05$). The transcription levels of group B were significantly higher than those in group C and D (*t* values were 8.66 and 8.60 respectively, all $P<0.05$). Differences of the transcription levels between group C and D were not significant (*t* = 0.07, $P>0.05$).

Analysis of Relative Protein Muc5ac Expression

Protein Muc5ac mainly expressed in cytoplasm of airway epithelial cells. There were obvious differences of brown-stained areas representing protein Muc5ac in airway epithelium
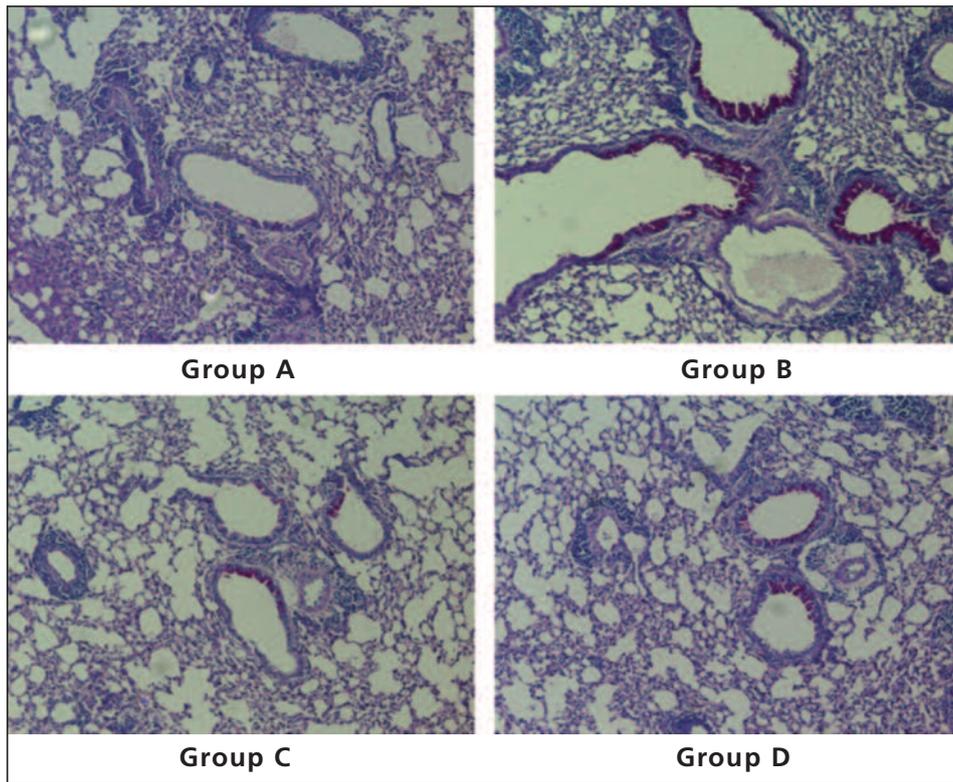
Figure 1. PAS staining studies of purple-stained airway epithelial goblet cells in 4 groups. *Group A* (saline control group): few goblet cells in airway epithelium; *Group B* (asthmatic group): thickening of airway epithelial mucosa and proliferation of large amount of purple goblet cells; *Group C* (formoterol pretreated group): compared with asthmatic group, fewer goblet cells in airway epithelium; *Group D* (dexamethasone pretreated group): compared with asthmatic group, fewer goblet cells in airway epithelium, but no obvious different from formoterol pretreated group. (PAS staining × 200).

among 4 groups (Figure 3). Optical density was measured by software for relative protein Muc5ac expression (Table III). It showed that there were significant differences between Group A and Group B, group C, group D (*t* value were 0.4500, 0.0680 and 0.1180 respectively, all $P<0.05$). The optical density measurement of B group was significantly higher than those of group C and D (t values were 0.3820 and 0.3320 respectively, all $P<0.05$). However, the difference between group C and D was not significant ($t = 0.050$, $P>0.05$).

Table I. Comparison of airway epithelial goblet cells in different groups (±).

Group	Cases	Number of goblet cells	Area percentage of goblet cell to total cell (%)
Saline control group	10	0.46 ± 0.16	0.03 ± 0.01
Asthmatic group	10	163.63 ± 16.681	77.36 ± 5.051
Formoterol pretreated group	10	52.04 ± 4.602	30.05 ± 3.722
Dexamethasone pretreated group	10	63.41 ± 6.393	38.52 ± 3.833
F value		546.89	750.28
P value		<0.05	< 0.05

*Significantly different (*P*<0.05).

Table II. Comparison of MUC5ac mRNA relative expression of different groups (±).

Group	Case	$2^{-\Delta\Delta Ct}$ values
Saline control group	10	1.00 ± 0.130
Asthmatic group	10	10.31 ± 0.731
Formoterol pretreated group	10	1.64 ± 0.142
Dexamethasone pretreated group	10	1.72 ± 0.103
F value		1508.46
P value		< 0.05

*Significantly different ($P<0.05$).

Correlation Analysis

The transcription and expression levels of Muc5ac were positively correlated with the numbers of bronchial goblet cells. (Correlation coefficient were 0.878 and 0.962 respectively, all $P<0.01$).

Discussion

Airway mucus hypersecretion is one of the most important pathophysiology features of asthma, which can not only lead to airway obstruction causing infection but also is the main reason for asthma deterioration and fatal asthma[7]. Previous research showed that epithelial goblet cells are the major producer and secretor of mucus in airway[8]. With increasing goblet cell number and volume, hyperplasia changes play an important role of airway remodeling in asthma[9]. In this study, mouse asthma model was built in Temelkovski et al[10] way. After pathological staining of airway epithelia, the percentage of goblet cell to total cell and the number of goblet cells were differently significant between asthma group (group A) and saline control group (group B), which means there were goblet cell hyperplasia changes. It was consistent with what reported.
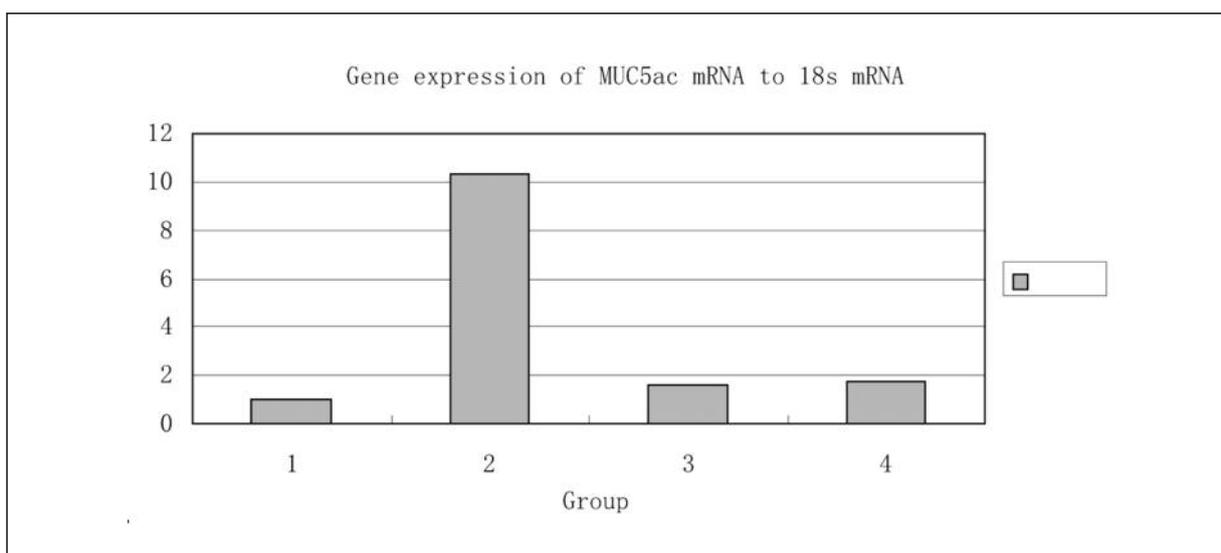

Figure 2. Histogram of gene expression of MUC5ac mRNA of different groups (to 18s rRNA). Take group 1 (saline control group) as control, the MUC5ac mRNA expression level of group 2 (asthmatic group) significantly increased. MUC5ac mRNA expression levels of group 3 (formoterol group) and group 4 (dexamethasone group) increased to group 1 but significantly decreased to group 2.
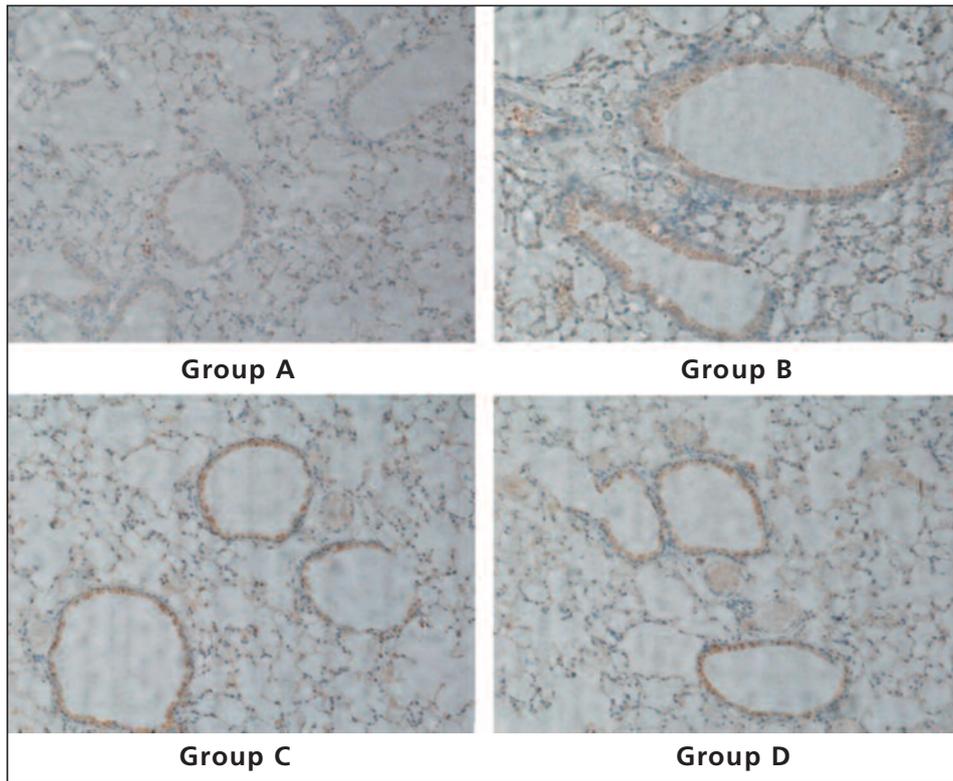
Figure 3. Immuno-histochemical study of brown-stained protein Muc5ac expression in different groups. *Group A* (saline control group): few brown-stained protein Muc5ac in airway epithelium; *Group B* (asthmatic group): thickening of airway epithelial mucosa and proliferating of large amount of brown protein Muc5ac; *Group C* (formoterol pretreated group): compared with group B, fewer protein Muc5ac in airway epithelium but more than group A; *Group D* (dexamethasone pretreated group): compared with asthmatic group, fewer protein Muc5ac in airway epithelium, but no obvious different from group C. (Immuno-histochemical staining × 200).

Mucin (MUC), polymer glycosylation, is the main component for airway mucus elasticity and adhesion. Protein Muc5ac is the major mucin in adult respiratory tract[11]. In asthmatic airway, goblet cell hyperplasia together with increased transcription and expression of Muc5ac suggest that Muc5ac may be the major sign of goblet cell hyperplasia and secretion[12]. Our results showed that transcription and expression of Muc5ac increased in Group B increased with

Table III. Comparison of MUC5ac protein relative expression of different groups (±).

Group	Case	Muc5ac mucin protein expression
Saline control group	10	0.1903 ± 0.0312
Asthmatic group	10	0.6389 ± 0.03082
Formoterol pretreated group	10	0.2606 ± 0.0074
Dexamethasone pretreated group	10	0.3131 ± 0.01268
F value		256.980
P value		< 0.05

*Significantly different ($P<0.05$).

748

Group A. At the same time, the transcription and expression levels of Muc5ac were positively correlated with the number of bronchial goblet cells, which was consistent with the reported. Thus, inhibition of goblet cell hyperplasia and Muc5ac expression can effectively control the airway hypersecretion and is important for asthma prevention and treatment.

Formoterol is a commonly used long-acting β_2 agonist that has high selectivity, strong onset and a few systemic side effects. It has been proved to effectively prevent and control asthma attack. Previous researches about formoterol focusing on its mechanism of distending bronchi and its anti-inflammatory role rather to its influence on airway mucin secretion. It is not reported whether formoterol could inhibit goblet cell hypertrophy and Muc5ac expression. However, Kaur et al[13] research showed that formoterol could control the inflammatory gene expression; Maneechotesuwan et al[14] found that formoterol reduced the number of IL-8 and neutrophils inhibiting inflammatory mediator release. Meanwhile, Basbaum et al[15] showed that the release of various inflammatory mediators can induce airway goblet cell hyperplasia and mucin gene expression, so it is possible that formoterol has the inhibition effect on airway goblet cell hyperplasia and mucin gene expression in mechanism. In this study, we intervened asthmatic mice by intraperitoneal injection of formoterol. The results showed that the number of goblet cells, the percentage of goblet cell to total cell, the transcription and the expression of protein MUC5ac in formoterol pretreated group (group C) were significant fewer in asthmatic group (group B). This prompted formoterol could reduce airway mucus secretion to some extent, but its mechanism remains to be further studied. Dexamethasone is a commonly used glucocorticoid, whose inhibition effect has been proved on airway goblet cells hyperplasia and Muc5ac expression[16]. In our study, the number of goblet cells, the percentage of goblet cells to total cells, the transcription and the expression of MUC5ac in dexamethasone pretreated group (group D) were significant fewer than in asthmatic group (group B), consistent with the report, but there were no significant differences between formoterol pretreated group (group C) and dexamethasone pretreated group (group D). It prompted that formoterol had similar effect on airway hypersecretion as dexamethasone. It also need further study if it is the same in human airway.

References

1) EVANS CM, KOO JS. Airway mucus: The good, the bad, the sticky. Pharmacol Ther 2009; 121: 332-348.

2) SIDDIQUI S, MARTIN JG. Structural aspects of airway remodeling in asthma. Curr Allergy Asthma Rep 2008; 8: 540-547.

3) ORDONEZ CL, KHASHAYAR R, WONG HH, FERRANDO R, WU R, HYDE DM, HOTCHKISS JA, ZHANG Y, NOVIKOV A, DOLGANOV G, FAHY JV. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med 2001; 163: 517-523.

4) EVANS CM, KIM K, TUVIM MJ, DICKEY BF. Mucus hypersecretion in asthma: causes and effects. Curr Opin Pulm Med 2009; 15: 4-11.

5) CHOMCZYNSKI P, SACCHI N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extration: twenty-something years on. Nat Protoc 2006; 1: 581-585.

6) LIVAK KJ, SCHMITTGEN TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods 2001; 25: 402-408.

7) HAYS SR, FAHY JV. The role of mucus in fatal asthma. Am J Med 2003; 115: 68-69.

8) FICKER JH. Physiology and pathophysiology of bronchial secretion. Pneumologie 2008; 62(suppl 1): S11-S13.

9) BERGERON C, TULIC M, HAMID Q. Airway remodeling in asthma: from bench to clinical practice. Can Respir J 2010; 17: 85-94.

10) TEMELKOVSKI J, HOGAN SP, SHEPHERED DP, FOSTER PS, KUMAR RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax 1998; 53: 849-856.

11) KIRKHAM S, SHEEHAN JK, KNIGHT D, RICHARDSON PS, THORNTON DJ. Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins Muc5ac and Muc5b. Biochem J 2002; 361: 537-546.

12) ZUHDI ALIMAM M, PIAZZA FM, SELBY DM, LETWIN N, HUANG L, ROSE MC. Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. Am J Respir Cell Mol Biol 2000; 22: 253-260.

13) KAUR M, HOLDEN NS, WILSON SM, SUKKAR MB, CHUNG KF, BARNES PJ, NEWTON R, GIEMBYCZ MA. Effect of beta2-adrenoceptor agonists and other

cAMP-elevating agents on inflammatory gene expression in human ASM cells: a role for protein kinase A. Am J Physiol Lung Cell Mol Physiol 2008; 295: L505-L514.

14) Maneechotesuwan K, Essilfie-Quaye S, Meah S, Kelly C, Kharitonov SA, Adcock IM, Barnes PJ. Formoterol attenuates neutrophilic airway inflammation in asthma. Chest 2005; 128: 1936-1942.

15) Basbaum C, Gallup M, Gum J, Kim Y, Jany B. Modification of mucin gene expression in the airways of rats exposed to sulfur dioxide. Biorheology 1990; 27: 485-489.

16) Chen Y, Nickola TJ, DiFronzo NL, Colberg-Poley AM, Rose MC. Dexamethasone-mediated repression of MUC5AC gene expression in human lung epithelial cells. Am J Respir Cell Mol Biol 2006; 34: 338-347.