Original Article
Effect of early inhalation of budesonide on cyclinD1, VEGF and α-SMA in asthmatic rats

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Abstract: Objective: This study aims to investigate the effect of early inhalation of budesonide on cyclinD1, VEGF, α-SMA, collagen deposition in lung tissues, and pulmonary function in asthmatic rats. Method: Twenty-four male Sprague Dawley rats were randomly divided into three groups: normal group, model group and treatment group (n=8, each group). Sensitization was induced by peritoneal cavity injection of ovalbumin. Then, the rat model of asthma was established by repeated inhalation of aerosolized ovalbumin. Rats in the treatment group inhaled 2 mg/4 ml of aerosolized budesonide, twice a day. Rats in other two groups inhaled aerosolized water for injection for control. Six weeks later, these rats were tested for pulmonary function, serum VEGF, collagen in the surrounding tissues of airway, cyclinD1 and α-SMA in lung tissues. Results: Basic airway resistance, airway reactivity and collagen deposition around the airway, as well as expression of cyclinD1, VEGF and α-SMA in lung tissues, significantly increased in rats in the model group. Inflammatory response, pulmonary function and airway remodeling were significantly better in the treatment group compared with that in the model group. Conclusion: Early inhaled budesonide can reduce airway inflammation, improve pulmonary function, inhibit cyclinD1 and VEGF expression, and reduce airway remodeling.

Keywords: Bronchial asthma, budesonide, cyclinD1, VEGF, α-SMA

Introduction

Airway remodeling refers to the formation of new structures through the repair of inflammation injury in the airway, which is an important pathological basis of the progressive decline of pulmonary function in patients with asthma [1]. It is also one of the important reasons for difficulties in the treatment of asthma. The mechanism of airway remodeling and the determination on how to inhibit airway remodeling have become new hot spots in the study of asthma [2, 3].

Vascular endothelial growth factor (VEGF) is one of the positive regulators of new blood vessels. VEGF not only promotes the formation of new blood vessels, but also promotes the growth of epithelial cells. A study suggests that VEGF is abundant in the lungs, and it plays an important role in the regulation of airway remodeling in asthma [4]. The cyclin system consists of cell cycle protein (cyclin), cyclin-dependent kinase (CDK) and cyclin-dependent-kinase inhibitor (CDKI). Cyclin binds with the corresponding CDK, and promotes various phases of the cell cycle to go forward through the phosphorylation of its substrate. However, CDKI can inhibit the above process. The relative contents of these three determine the progression or stagnation of the cell cycle.

Materials and methods

Establishment of the asthma model

Twenty-four clean-grade healthy male Sprague Dawley (SD) rats, weighing 130-170 g, were used in this study. These rats were randomly divided into three groups: control group, model group and treatment group. The method of model establishment was based on a related literature [5], which has been modified by the investigators of this study. At the 1st and 7th day, 2 ml of suspension containing 100 mg of ovalbumin and 100 mg of aluminum hydroxide were
Inhaled into the peritoneal cavity of rats; and 1 ml of pertussis vaccine (five billion bacteria) was simultaneously injected. From the 15th day, sensitized rats were placed in a self-made incomplete airtight fumigation box, and were allowed to inhale the aerosolized ovalbumin (the fumigation box volume was 50x40x30 cm). The concentration of the aerosolized ovalbumin solution gradually increased from 1% to 1.5%, 2%, 2.5% and 3%. The inhalation was conducted once every two days, and the aerosolized liquid concentration increased once after every four inhalations. Each inhalation lasted for 30 minutes, and a total of 20 inhalations were conducted. In the control group, rats inhaled aerosolized water instead. Rats in the treatment group inhaled 2 mg/4 ml of aerosolized budesonide, twice a day. Rats in the control and model groups inhaled the same dose of water for injection.

**Pulmonary function test**

Pulmonary function was detected 24 hours after the last inhalation of rats in each group. Anesthesia was performed using 1% sodium pentobarbital (90 mg/kg). A longitudinal incision was made in the middle of the neck, and the trachea was exposed and cut off. Mechanical ventilation was performed at a respiratory rate of 80 times/min, with an inspiratory-to-expiratory ratio of 1:1.5. Basic airway resistance was detected at four random points after the baseline of the ventilator was stabilized. A venous access was established in the jugular vein, and normal saline and acetylcholine (the doses of acetylcholine were 6.25, 12.5, 25, and 50 μg/kg, at 0.1-0.3 ml each time) were injected in that order. The interval between two infusions was five minutes. Changes in the area under the airway resistance curve (50 μS in width) after the administration of the drug were calculated (for detection of airway reactivity).

**Sampling and staining**

Sampling was conducted after the end of the pulmonary function test. The upper lobe of the right lung was preserved in 4% formaldehyde, embedded with paraffin, sliced, and underwent hematoxylin-eosin (H&E) staining and Masson staining.

**Western blot of cyclinD1**

Approximately 500 mg of the upper lobe of the left lung was obtained from each rat in all groups, 1 ml of extract was added, and this was centrifuged at 12,000 rpm for five minutes at 4°C. The supernatant was obtained, and two folds of sodium dodecyl sulfate (SDS) buffer were added and boiled at 100°C for five minutes. The supernatant was obtained and preserved at -20°C. Next, the sample underwent gel electrophoresis, and the protein was transferred onto the film by water-bath electrophoresing. The blocking membrane was shaken for two hours, and the first antibody diluent with a dilution of 1:250 was added, shaken at room temperature for two hours. Then, the membrane was washed three times with phosphate-buffered saline with tween 20 (PBST), the second antibody diluent with a dilution of 1:8,000 was added, and shaken at room temperature for one hour. After the membrane was fully washed with PBST, the membrane was immersed in diaminobenzidine (DAB) color-substrate solution for complete coloration at room temperature. The membrane was washed with distilled water, and transferred in PBS for observation.

**Serum VEGF detection**

The detection procedures were in reference with the instructions of the ELISA kit.

**Airway α-smooth muscle actin (α-SMA) detection**

The sections of the lower lobe of the right lung were dewaxed and dehydrated, incubated with 0.3% methanol-hydrogen peroxide at room temperature for 20 minutes, washed three times with distilled water, placed in 0.1 mmol citrate buffer (pH 6.0) for repair in a microwave oven, placed in 10% goat serum and incubated for 30 minutes at a constant temperature of 37°C in a humidity box, and rabbit anti-IgG polyclonal antibody and mouse anti-rat IgG monoclonal antibody were added (1:100 dilution). Next, biotinylated secondary antibody (goat anti-rabbit and goat anti-mouse) working fluids were added, and incubated for 30 minutes at a constant temperature of 37°C in a humidity box. Then, horseradish peroxidase-labelled streptavidin working fluid was added, incubated for 30 minutes, colored by DAB for 2-5 minutes, rinsed, coloration was terminated, and gradiently dehydrated with alcohol.

**Airway cyclinD1 detection**

The sections were treated with xylene I and immunohistochemistry II and III for 15 minutes.
until it became transparent, and sealed with neutral gum. The sections were routinely de-waxed, and washed three times with 0.01 M PBS for five minutes each time. Then, the sections were incubated with 0.3% methanol-hydrogen peroxide at room temperature for 20 minutes, washed three times with distilled water for five minutes each time, placed in 0.1 M citrate buffer (pH 6.0) for repair in a microwave oven, and maintained at 98°C for 20 minutes. After allowing to cool down to room temperature, the sections were placed in 10% goat serum and incubated for 30 minutes at a constant temperature of 37°C in a humidity box. The excess serum was removed with filter paper, rabbit anti-IgG polyclonal antibody was added (1:100 dilution), washed three times with 0.01 M PBS for five minutes each time. Then, the biotinylated secondary antibody (goat anti-rabbit) working fluid was added, incubated for 30 minutes at a constant temperature of 37°C in a humidity box. Subsequently, the sections were washed three times with 0.01 M PBS for five minutes each time, horseradish peroxidase-labelled streptavidin working fluid was added, incubated for 30 minutes, washed three times with 0.01 M PBS for five minutes each time, colored by DAB for 2.5 minutes, fully rinsed with running water, and coloration was terminated. Next, the sections were gradiently dehydrated with alcohol: treated with 70%, 80% and 90% alcohol for five minutes each, with 95% alcohol for 10 minutes, and with 100% alcohol I and II for 15 minutes each. The sections were treated with xylene I, II and III for 15 minutes each to induce the sections to become transparent, and sealed these with neutral gum. In the negative control group, the first antibody was replaced by PBS, and other procedures were the same as above.

Collagen deposition and α-SMA results were analyzed using Motic 6.0 digital medical image analysis system, while cyclinD1 protein blot results were analyzed using the Bio-1D analysis software in the BIO-ROIFIF gel image analysis system (VL, French).

### Statistics processing

Data were analyzed using SPSS 13.0 statistics software. Data were expressed as mean ± standard deviation. Multi-group comparison was conducted using univariate analysis of variance. Pairwise comparison was conducted using Q test. The correlation between two variables was analyzed using linear correlation analysis. P<0.05 was considered statistically significant.

### Results

#### Pulmonary function test

Compared with the control group, basic airway resistance increased in the model group and treatment group; and this increase was more significant in the model group. In rats in all three groups, airway resistance increased after intravenous injection of acetylcholine; but this increase was more significant in the model group and treatment group, and airway reactivity was smaller in the treatment group than in the model group (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Basic airway resistance (cmH2O/ml)</th>
<th>0.9% NaCl</th>
<th>6.25</th>
<th>12.50</th>
<th>25</th>
<th>50 Ach (uk/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>1.11±0.18</td>
<td>9.86±1.73</td>
<td>16.53±2.64</td>
<td>29.63±2.62</td>
<td>46.4±3.19</td>
<td>99.34±8.30</td>
</tr>
<tr>
<td>Model group</td>
<td>1.88±0.31*</td>
<td>10.04±1.98</td>
<td>19.66±1.70</td>
<td>59.57±2.86*</td>
<td>107.44±17*</td>
<td>197.25±25.98*</td>
</tr>
<tr>
<td>Treatment group</td>
<td>1.22±0.21</td>
<td>9.88±1.16</td>
<td>17.26±1.64</td>
<td>40.32±4.21</td>
<td>68.22±10.28*</td>
<td>140.33±14.68*</td>
</tr>
<tr>
<td>F</td>
<td>8.30</td>
<td>3.06</td>
<td>5.98</td>
<td>30.36</td>
<td>26.30</td>
<td>27.58</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: The area under the airway is a ratio, and without unit of measurement.
found to infiltrate into the surrounding tissues of the airway and alveolar septa, which were mainly eosinophils. Compared with the model group, the inflammatory cells around the airway decreased in the treatment group, the luminal morphology was relatively regular, and smooth muscle cells mildly proliferated (Figure 1).

**Expression of collagen around the airway**

In the control group, a small number of collagen III was observed in the airway, and collagen III deposition increased in the model group and the treatment group. This increase was more significant in the model group, while collagen expression was lesser in the treatment group than in the model group (Figure 2).

**Serum VEGF results**

Compared with the control group, serum VEGF was elevated in the model group and treatment group, and VEGF level was lower in the treatment group than in the model group (Figure 3). VEGF expression was positively correlated with collagen III levels ($r=0.67$, $P<0.05$).

**Detection results of cyclinD1 in lung tissue**

Compared with the control group, cyclinD1 expression was significantly higher in the model group and treatment group; and optical density was lower in the treatment group than in the
model group. The same results were also presented in immunohistochemical staining (Figure 4).

Alpha-SMA expression in the surrounding tissues of airway

Compared with the control group, α-SMA expression was significantly higher in the model group and treatment group, but was lower in the treatment group than in the model group (Figure 5).

In the model group and treatment group, cyclinD1 expression in lung tissues and airway α-SMA expression significantly increased, while the expression of both in serum were lower in the treatment group than in the model group. CyclinD1 (Western blot) was positively correlated with α-SMA expression ($r=0.71$, $P<0.05$).
Discussion

Airway remodeling is a repair to injury in the airway. However, the structures and functions of these repaired tissues are often different from those of the normal airway. These often manifest as epithelial damage, subepithelial fibrosis, smooth muscle cell proliferation, collagen deposition and neovascularization in the airway [6]. Therefore, airway remodeling often results in increased airway resistance [7] and the chronic and progressive decline in lung function. At present, it has been considered that airway remodeling is one of the reasons for recurrent attack and poor response to treatment in patients with asthma [8]. In the past, it was believed that asthma mainly affected the large airway, and the airway lumen can completely recover in the remission stage. However, at present, people have realized that the peripheral small airways can also be affected; and the clinical performances were similar to the performance of chronic obstructive pulmonary disease (COPD) due to airway remodeling [9]. The important features of airway remodeling include the proliferation of smooth muscle cells and the deposition of collagen in the airway. These play essential roles in the occurrence and development of airway remodeling [10]. If doctors can effectively inhibit the proliferation of the airway smooth muscle cells and collagen deposition in the basement membrane, airway remodeling can be reduced and the progression of the disease can be delayed.

The proliferation and differentiation of human cells are regulated by the cell cycle. The core of the cyclin system is CDK, and its activity is dependent on the regulation by cyclin and CDK. The regulation of the whole cell cycle is carried out at the control points of every phase. Among these, control points in the G1/S and G2/M phases are the two most important control points. If the positive regulation factor in the G1 phase reaches a certain level, the cell cycle moves across the G1/S junction point. Then, these cells can complete the whole cell cycle, and is no longer dependent on extracellular growth promoting factors [11]. Hence, the G1/S phase is an important “restriction point (R point)” in the process of mitosis of eukaryotic cells. It determines whether the cell will go into the cycle of mitosis. In addition, the G1/S control point is also the key to control the cell cycle. CyclinD1 is an important positive regulatory protein that promotes the cell cycle to move across the R point, converting from the G1 phase to the S phase; thus, promoting the mitosis of the cell [8, 12]. If cyclinD1 is overexpressed, the G1 phase would be shortened, and cell division would be sped up. Therefore, cyclinD1 is closely related to the occurrence of tumors [13]. A study revealed that an imbalance in the regulation of cyclin was found in tumor cells [14]. CyclinD1 is highly expressed in airway smooth muscle. Thus, it is possible for it to be involved in the development of airway remodeling [15]. A study revealed that cyclinD1 expression in airway smooth muscles significantly increased, and the proportion of airway smooth muscle cells in the phases of DNA synthesis and cell division increased. In addition, cyclin E and cyclinD1 expression in CD4+ and CD8+ T lymphocytes in patients with bronchial asthma were significantly higher than in the control group [16]. Therefore, cyclin may be involved in cell mitosis and promote cell proliferation in patients with asthma. Some scholars have considered that extracellular signal-regulated protein kinase (ERK) may affect the proliferation of cells by regulating cyclinD1 in smooth muscle cells in patients with asthma [17].

Airway smooth muscles play an important role in airway contraction. Infection, allergy, and environmental factors can all induce changes in smooth muscles, affecting the normal structures and function of the airway [7]. Smooth muscle actin is an important component of the airway smooth muscle, which involves in the contraction and relaxation of the airway and the maintenance of cell structures. Smooth muscle actin consists of three subtypes: α, β and γ. Among these, α-SMA accounts for the highest proportion. At present, it is considered that α-SMA is an important component involved in the contraction of airway smooth muscles, and is also an important part that is involved in airway remodeling. Increases in α-SMA and other components that involve in smooth muscle contraction are helpful to the proliferation and migration of airway smooth muscle cells. This is crucial for airway remodeling in patients with asthma [7].

A small amount of α-SMA was expressed around the airway in normal rats. The α-SMA mRNA expression in lung tissues can increase...
at one week after asthma is induced in rats, and this increase was more significant at two weeks after induction. Under the action of mitogenic substances, airway smooth muscle cells can not only proliferate, enlarge and promote airway remodeling, but also cause the conversion of airway smooth muscle phenotypes from a contractile phenotype to a synthetic secretion phenotype. These cells secrete cytokines such as IL-1, IL-2 and IFN-γ and inflammatory mediators such as histamine and thromboxane-A2. In autocrine or paracrine manners, these substances stimulate the PI3K signaling pathway and promote cyclinD1 expression in the cell through G protein-coupled receptors [29], in order to increase the number of cells and the volume of smooth muscle, and enhance airway inflammatory response and airway remodeling [7, 18]. In addition, changes in the structures of the airway can lead to a sustained increase in airway resistance and airway reactivity, and a sustained decrease in forced expiratory volume in one second (FEV1) [19-21]. Bradding P revealed that not only the fibroblasts in bronchial smooth muscle significantly increased in patients with asthma, and smooth muscle fibroblasts could also promote the excessive deposition of type I collagen in the extracellular matrix, aggravating subepithelial fibrosis [22]. Hence, the role of airway smooth muscle cell proliferation in airway remodeling has attracted more and more attention [23]. Thus, airway smooth muscles have the function of contracting the airway, promote inflammatory response, and are involved in airway remodeling. In view of the important role of airway smooth muscles in the pathogenesis of asthma, regarding it as the target of asthma treatment has become possible [24]. A study revealed that the formation of fibronectin is possibly correlated to smooth muscle secretion induced by vascular growth factors through the ERK signaling pathway [34]. VEGF widely exists in the lung [35]. A study revealed that VEGF levels were higher in serum and sputum in patients with asthma and in asthma animal models, compared with the controls. This suggests that VEGF may be involved in the pathogenesis of asthma [36].

The airway wall is thickened by 25-150% in patients with common asthma, while the proportion of airway wall thickening can reach 50-230% in patients with severe or fatal asthma. Thus, the degree of airway wall thickening is closely correlated to the severity of the disease [37]. Compared with normal subjects, the components and proportion of the extracellular matrix in asthma patients undergo a significant change [38]. The thickening of the basement membrane and the subepithelial fibrosis in the airway in patients with asthma are mainly caused by the precipitation of the extracellular matrix. Its components are mainly collagen I, III and V. The thickness of the collagen reticular layer is within 4-5 μm in normal individuals, and is often within 7-23 μm in asthma patients [39]. The thickening of the basement membrane and the formation of subepithelial fibrosis cause irreversible changes in the structures of the airway. Therefore, reducing the deposition of col-

VEGF is a protein with a molecular weight of 34,000-42,000 Dalton. Eosinophils, fibroblasts, neutrophils and many other cells can release VEGF. VEGF consists of different types such as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, etc. Among these, VEGF-A mainly regulates neovascularization and vascular permeability. VEGF is one of the strongest factors that promote neovascularization [31]. It not only plays an important role in tumor progression and lymph node metastasis [32, 33], but also increases the permeability of the venulae and venules; causing the extravasation of proteins and other macromolecular substances from the blood vessels. Part of these exosmic proteins can form fibrin. At present, it is considered that the formation of fibronectin is possibly correlated to smooth muscle secretion induced by vascular growth factors through the ERK signaling pathway [34]. VEGF widely exists in the lung [35]. A study revealed that VEGF levels were higher in serum and sputum in patients with asthma and in asthma animal models, compared with the controls. This suggests that VEGF may be involved in the pathogenesis of asthma [36].

Influence of inhalation of budesonide to asthma
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Colagen and the thickening of the basement membrane have important significance for reducing airway remodeling and delaying the progression of the disease. In this study, collagen deposition increased in the model group and the treatment group, and the increase in the model group was more significant. In addition, collagen deposition was positively correlated with VEGF expression. Therefore, it is considered that VEGF may be involved in the regulation of collagen expression.

This study revealed that airway basic resistance was significantly higher in the model group than in the control group, and the change in airway resistance after intravenous injection of acetylcholine was significantly higher in the model group than in the normal group. Airway basic resistance and changes in airway resistance after inhalation were lower in the treatment group than in the model group. Therefore, we considered that inhalation of aerosolized budesonide can effectively improve pulmonary function, reduce airway inflammatory response, reduce airway remodeling, and reduce airway reactivity. This study revealed that cyclinD1 expression in lung tissues was positively correlated with α-SMA expression, suggesting that cyclinD1 may be involved in the regulation of α-SMA. In addition, this study revealed that VEGF expression significantly increased in serum in rats with asthma, and the degree of increase was positively correlated with the degree of collagen deposition surrounding the airway, suggesting that VEGF may be involved in the process of collagen deposition surrounding the airway. Therefore, we hypothesized that early inhalation of budesonide can reduce airway remodeling and improve the prognosis of asthma by inhibiting the VEGF and cyclinD1 expression.

To date, glucocorticoids are the most effective drugs to inhibit airway inflammation. Since the systemic application of glucocorticoids may bring about many adverse reactions, the clinical application of glucocorticoids is severely restricted. Hence, at present, the local inhalation of hormones are often recommended, which can reduce inflammatory response and the safety problems caused by the single use of β2-receptor agonist [40]. However, it remains unclear whether inhaled corticosteroid therapy can effectively inhibit airway remodeling. In the present study, the collagen deposition and smooth muscle cell proliferation in airway wall were significantly decreased after early inhaled corticosteroid therapy, which suggests that although early inhaled corticosteroids cannot completely inhibit airway remodeling, it can significantly slow down its progression. This pathway may be related to the inhibition of VEGF and cyclinD1 expression.

Conclusion

At present, the global incidence of asthma continues to increase [41]. Determining how to effectively control asthma has been a serious challenge for respiratory physicians. Airway remodeling is one of the important pathological basis of refractory asthma in clinical practice. Determining how to effectively reduce airway remodeling has been an important target to effectively delay the progression of the disease and treat bronchial asthma. It requires further studies and reviews to investigate how to inhibit airway remodeling in asthma, and how to effectively delay the progression of the disease.

Disclosure of conflict of interest

None.

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