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Regulating effect of glycyrrhetic acid on bronchial asthma smooth muscle proliferation and apoptosis as well as inflammatory factor expression through ERK1/2 signaling pathway

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ABSTRACT

Objective: To study the influence of glycyrrhetic acid (GA) on bronchial asthma (BA) smooth muscle proliferation and apoptosis as well as inflammatory factor expression and its molecular mechanism.

Methods: Male SD guinea pigs were selected and made into asthma models, bronchial asthma smooth muscle cells were cultured and divided into BA group, GA group and GA + LM group that were treated with serum-free RPMI1640 culture medium, serum-free RPMI1640 culture medium containing 50 ng/mL glycyrrhetic acid, serum-free RPMI1640 culture medium containing 50 ng/mL glycyrrhetic acid and 100 ng/mL LM22B-10 respectively; normal guinea pigs were collected and bronchial smooth muscle cells were cultured as control group. The cell proliferation activity as well as the expression of proliferation and apoptosis genes, inflammatory factors and p-ERK1/2 was determined.

Results: Proliferation activity value and mRNA expression of *Bcl-2*, *TNF-α*, *IL-4*, *IL-6*, *YKL-40*, protein expression of p-ERK1/2 of airway smooth muscle cell in BA group were significantly higher than those of control group while mRNA expression levels of *Bax*, *caspase-9* as well as *caspase-3* were significantly lower than that of control group ($P < 0.05$); proliferation activity value and mRNA expression of *Bcl-2*, *TNF-α*, *IL-4*, *IL-6*, *YKL-40*, protein expression of p-ERK1/2 of airway smooth muscle cell in GA group were significantly lower than those of BA group ($P < 0.05$) while the mRNA expression levels of *Bax*, *caspase-9* as well as *caspase-3* were significantly higher than those of BA group ($P < 0.05$); proliferation activity value and mRNA expression of *Bcl-2*, *TNF-α*, *IL-4*, *IL-6*, *YKL-40* of airway smooth muscle cell in GA + LM group were significantly higher than those of GA group ($P < 0.05$) while mRNA expression levels of *Bax*, *caspase-9* as well as *caspase-3* were significantly lower that of GA group ($P < 0.05$).

Conclusion: GA can inhibit the proliferation of bronchial smooth muscle cells and reduce the expression of inflammatory factors by inhibiting the phosphorylation of ERK1/2.

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1. Introduction

Bronchial asthma (BA) is the airway chronic disease which is characterized by incompletely reversible airway limitation, the main symptoms are shortness of breath and wheeze, and abnormal bronchial smooth muscle proliferation, massive inflammatory cell infiltration and massive inflammatory factor secretion are the important pathological characteristics in local airway [1–3]. Glycyrrhetic acid (GA) has the pharmacological activity in regulating inflammatory response, apoptosis and other biological processes [4,5]. In recent years, GA value for treatment of BA has received more and more attention, studies have confirmed that the drug has a delaying and inhibiting effect on the airway remodeling in animal models with asthma, but the specific molecular mechanism is still not clear. The abnormal airway smooth muscle proliferation and massive inflammatory cytokine infiltration mediated by extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway are the important molecular mechanisms which causing airway remodeling in the course of BA, and the regulating effect of GA on BA smooth muscle proliferation and apoptosis as well as inflammatory factor expression through ERK1/2 signaling pathway was analyzed in the following research.

2. Materials and methods

2.1. Experimental materials

The experimental animals were male SD guinea pigs that were provided by the Experimental Animal Center of Guangzhou University of Chinese Medicine, and the certificate number was 44005900002663; GA was purchased from Nanjing Jingzhu Bio-technology Co., Ltd., and ERK1/2 agonist LM22B-10 was purchased from MCE Company; the RPMI1640 culture medium and fetal bovine serum for cell culture were bought in Hyclone Company, RNA extraction kits, cDNA synthesis kits and fluorescence quantitative polymerase chain reaction kits were bought in Promega Company, and polyacrylamide, sodium dodecyl sulfate and other reagents for western blot were bought in Sigma Company. Animal experiments passed the ethics of the Guangzhou Yuexiu District Children's Hospital, and the animal experiment operation and animal treatment after execution were carried out according to the regulations.

2.2. Experimental methods

2.2.1. Asthma model establishment

A total of 15 experimental animals were collected and made into asthma models as follows: 1 mL saline containing 1 mg ovalbumin and 100 mg aluminum hydroxide was intraperitoneally injected on the 1st and 8th day of the experiment. The experimental animals were put in atomization box for challenge from the 15th day, and ultrasonic nebulizer was used to spray into 8 mL saline solution containing 1% ovalbumin, which lasted for 30 min. It was done once every other day and continued for 8 wk. Another 15 experimental animals were collected, intraperitoneal injection of 1 mL saline was done on the 1st and 8th day of the experiment, and atomization of 8 mL saline solution was provided from the 15th day.

2.2.2. Cell culture

After animal models were made, the guinea pigs were executed to collect the airway smooth muscle tissue. It was cut into tissue blocks about 1 mm × 1 mm × 1 mm and inoculated in petri dishes. Tissue block adherence method was used to culture bronchial smooth muscle cells, and the cells were digested and sub-cultured with trypsin after the cell density reached about 80%. The 3–6 generation of cells were collected and inoculated within the culture plate and divided into control group, BA group, GA group and GA + LM group, and the treatment methods were as follows: Control group were bronchial smooth muscle cells of normal guinea pigs, inoculated in culture plate and then treated with serum-free RPMI1640 medium; BA group, GA group and GA + ERK1/2 agonist LM22B-10 group (LM group) were bronchial smooth muscle cells of guinea pigs with asthma, and the treatment methods were as follows: BA group were treated with serum-free RPMI1640 culture medium, GA group were treated with serum-free RPMI1640 culture medium containing 50 ng/mL GA and GA + LM group were treated with serum-free RPMI1640 culture medium containing 50 ng/mL GA and 100 ng/mL LM22B-10.

2.2.3. Cell viability detection

The density of digested cells was adjusted to 4×10^3 /mL, the cells were added in 96-well culture plate with 200 μ L/well and treated with different conditions for 24 h, then 10 μ L CCK-8 detection liquid was added in each culture well and evenly mixed. Cells were placed in the incubator and continuously incubated for 2 h. At last, the absorbance at 450 nm wavelength was measured at microplate reader and used as the cell proliferation activity value.

2.2.4. RNA expression detection

The density of digested cells was adjusted to 6×10^5 /mL, the cells were added in 96-well culture plate with 1.5 mL/well and treated with different conditions for 24 h, RNA extraction kit was used to separate the RNA in the cells, cDNA synthesis kit was used to synthesize the RNA into cDNA by reverse transcription, fluorescence quantitative polymerase chain reaction kit was used as last for the amplification of *Bcl-2*, *Bax*, *caspase-9*, *caspase-3*, *TNF- α* , *IL-4*, *IL-6* and *YKL-40*, and the mRNA expression of above genes were calculated according to amplification curve.

2.2.5. Protein expression detection

The density of digested cells was adjusted to 6×10^5 /mL, the cells were added in 96-well culture plate with 1.5 mL/well and treated with different conditions for 24 h. Then RIPA lysate was added to extract protein. The protein sample was mixed with loading buffer and added in polyacrylamide-sodium dodecyl sulfate gel sampling well, 5% skim milk was used to close NC membrane after the completion of vertical electrophoresis and electrophoretic transfer. The p-ERK1/2 and ERK1/2 antibodies were incubated for the night. The next day, the HRP-labeled second antibodies were incubated for 1 h and then developed on the visualizer to obtain the protein bands of p-ERK1/2 and ERK1/2 respectively. Image J software was used to calculate the gray value and the ratio of p-ERK1/2 and ERK1/2, the ratio of p-ERK1/2 and ERK1/2 of the control group was set as 1 to calculate the protein expression of p-ERK1/2 in other groups of cells.

2.3. Statistical approach

SPSS20.0 software was adopted for data input and analysis. Variance analysis was used to compare the measurement data among groups, LSD-*t* test was adopted for the pair-wise comparison, and $P < 0.05$ indicated that the differences were statistically significant.

3. Results

3.1. Effect of glycyrrhetic acid on airway smooth muscle cell proliferation activity

Airway smooth muscle cell proliferation activity value of control group, BA group and GA group were 0.52 ± 0.08 , 1.42 ± 0.20 and 0.85 ± 0.11 , respectively. Analysis of the proliferation activity of airway smooth muscle cells among three groups was as follows: Airway smooth muscle cell proliferation activity value of BA group was significantly higher than that of control group ($P < 0.05$), and airway smooth muscle cell proliferation activity value of GA group was significantly lower than that of BA group ($P < 0.05$).

3.2. Effect of glycyrrhetic acid on proliferation and expression of apoptosis gene in airway smooth muscle cells

The results showed that *Bcl-2* mRNA expression in BA group of airway smooth muscle cells was significantly higher than that in control group ($P < 0.05$), while *Bax*, *caspase-9* and *caspase-3* mRNA expression levels were significantly lower than those in control group ($P < 0.05$); the mRNA expression level of *Bcl-2* in GA group of airway smooth muscle cells was significantly lower than that in BA group ($P < 0.05$), while *Bax*, *caspase-9* and *caspase-3* mRNA expression levels were significantly higher than those in BA group ($P < 0.05$) (Table 1).

Table 1

Proliferation and mRNA expression of apoptosis gene in three groups of airway smooth muscle cells.

Groups	<i>n</i>	<i>Bcl-2</i>	<i>Bax</i>	<i>Caspase-9</i>	<i>Caspase-3</i>
Control group	5	1.00 ± 0.15	1.00 ± 0.14	1.00 ± 0.15	1.00 ± 0.17
BA group	5	$2.55 \pm 0.35^*$	$0.33 \pm 0.07^*$	$0.29 \pm 0.05^*$	$0.38 \pm 0.06^*$
GA group	5	$1.62 \pm 0.18^\#$	$0.79 \pm 0.10^\#$	$0.83 \pm 0.12^\#$	$0.75 \pm 0.08^\#$

Compared with control group, * $P < 0.05$; Compared with BA group, $^\#P < 0.05$.

Table 2

Inflammatory factor mRNA expression in three groups of airway smooth muscle cells.

Groups	<i>n</i>	<i>TNF-α</i>	<i>IL-4</i>	<i>IL-6</i>	<i>YKL-40</i>
Control group	5	1.04 ± 0.17	0.97 ± 0.14	0.99 ± 0.11	1.02 ± 0.15
BA group	5	$2.73 \pm 0.37^*$	$3.02 \pm 0.46^*$	$2.31 \pm 0.37^*$	$2.92 \pm 0.45^*$
GA group	5	$1.67 \pm 0.20^\#$	$1.77 \pm 0.27^\#$	$1.46 \pm 0.20^\#$	$1.62 \pm 0.18^\#$

Compared with control group, * $P < 0.05$; Compared with BA group, $^\#P < 0.05$.

3.3. Effect of glycyrrhetic acid on inflammatory factor expression in airway smooth muscle cells

TNF- α , *IL-4*, *IL-6* and *YKL-40* mRNA expression in BA group of airway smooth muscle cells were significantly higher than those in control group ($P < 0.05$); *TNF- α* , *IL-4*, *IL-6* and *YKL-40* mRNA expression in GA group of airway smooth muscle cells were significantly lower than those in BA group ($P < 0.05$) (Table 2).

3.4. Effect of glycyrrhetic acid on ERK1/2 expression in airway smooth muscle cells

p-ERK1/2 protein expression in control group, BA group and GA group of airway smooth muscle cells were 1.02 ± 0.15 , 2.79 ± 0.42 and 1.55 ± 0.20 , respectively. p-ERK1/2 protein expression in BA group of airway smooth muscle cells was significantly higher than that in control group ($P < 0.05$), and p-ERK1/2 protein expression in GA group of airway smooth muscle cells was significantly lower than that in BA group ($P < 0.05$).

3.5. Effect of ERK1/2 agonist combined with glycyrrhetic acid on proliferation activity and molecule expression

Airway smooth muscle cell proliferation activity value of GA group and GA + LM group were 0.85 ± 0.11 and 1.27 ± 0.19 , respectively. Airway smooth muscle cell proliferation activity value of GA + LM group was significantly higher than that in GA group ($P < 0.05$).

Airway smooth muscle cell proliferation activity value as well as *Bcl-2*, *TNF- α* , *IL-4*, *IL-6* and *YKL-40* mRNA expression in GA + LM group of airway smooth muscle cells were significantly higher than those in GA group ($P < 0.05$) (Tables 3 and 4), while the *Bax*, *caspase-9* and *caspase-3* mRNA expression were significantly lower than those in GA group ($P < 0.05$) (Tables 3 and 4).

Table 3

Proliferation and mRNA expression of apoptosis gene in GA group and GA + LM group of airway smooth muscle cells.

Groups	<i>n</i>	<i>Bcl-2</i>	<i>Bax</i>	<i>Caspase-9</i>	<i>Caspase-3</i>
GA group	5	1.62 ± 0.18	0.79 ± 0.10	0.83 ± 0.12	0.75 ± 0.08
GA + LM group	5	2.28 ± 0.34	0.43 ± 0.06	0.37 ± 0.05	0.48 ± 0.07
<i>t</i>		7.382	8.798	12.375	8.273
<i>P</i>		<0.05	<0.05	<0.05	<0.05

Table 4

Inflammatory factor mRNA expression of apoptosis gene in GA group and GA + LM group of airway smooth muscle cells.

Groups	<i>n</i>	<i>TNF-α</i>	<i>IL-4</i>	<i>IL-6</i>	<i>YKL-40</i>
GA group	5	1.67 ± 0.20	1.77 ± 0.27	1.46 ± 0.20	1.62 ± 0.18
GA + LM group	5	2.41 ± 0.34	2.55 ± 0.35	2.03 ± 0.31	2.27 ± 0.31
<i>t</i>		7.182	7.938	8.582	6.797
<i>P</i>		<0.05	<0.05	<0.05	<0.05

4. Discussion

Airway remodeling is the outstanding pathological feature of patients with BA, the continuous infiltration of inflammatory cells in local airway can stimulate the proliferation of bronchial smooth muscle and induce the occurrence of airway remodeling, and the degree of airway remodeling is directly related to the condition as well as the prognosis and outcome of BA [6]. In the study, analysis of the changes in the bronchial smooth muscle cell proliferation activity in the guinea pig models with asthma showed that airway smooth muscle cell proliferation activity value of BA group was significantly higher than that of control group. This indicated that there was the excessive proliferation of airway smooth muscle cells in the course of asthma, and also showed that inhibiting the proliferation of bronchial smooth muscle is an important strategy to treat BA. In recent years, GA value for BA has received more and more attention, study has confirmed that the drug has a delaying and inhibiting effect on the airway remodeling in animal models with asthma [7], but there is still no clear report about the GA effect on the airway remodeling caused by the excessive proliferation of bronchial smooth muscle cells in the course of asthma. Further analysis of the GA effect on the proliferation of bronchial smooth muscle cells in the models with asthma showed that airway smooth muscle cell proliferation activity value of GA group was significantly lower than that of BA group. This indicated that GA can effectively inhibit the excessive proliferation of bronchial smooth muscle cells in the course of asthma.

Apoptosis of mitochondrial pathway is an important mechanism for regulating cell proliferation and apoptosis, and the excessive proliferation of smooth muscle cells in local airway is closely related to the blocked mitochondrial pathway apoptosis [8]. Bax and Bcl-2 are the key molecules in regulating mitochondrial pathway apoptosis, both are located in the mitochondrial membrane, the former can promote the mitochondrial cytochrome C to enter into the cytoplasm via mitochondrial membrane, and the latter can block the release of cytochrome C [9,10]; the cytochrome C in the cytoplasm can activate caspase-9 at first, then induce caspase-3 activation and mediate apoptosis through a series of cascade activation reactions [11]. The analysis of the expression of mitochondrial apoptotic molecules in bronchial smooth muscle of guinea pig models with asthma in the study showed that the mRNA expression level of *Bcl-2* in BA group of airway smooth muscle cells was greatly higher than that in control group, while the mRNA expression levels of *Bax*, *caspase-9* and *caspase-3* were significantly lower than that in control group. This indicated that mitochondrial pathway apoptosis was blocked in the airway smooth muscle of BA, and the abnormal apoptosis will further cause the abnormal proliferation of bronchial smooth muscle cells. Further analysis of the GA effect on mitochondrial pathway apoptosis molecules in bronchial smooth muscle of guinea pig models with asthma showed that *Bcl-2* mRNA expression level in GA group of airway smooth muscle cells was significantly lower than that in BA group; while *Bax*, *caspase-9* as well as *caspase-3* mRNA expression levels were significantly higher than those of BA group. This indicated that GA can regulate the Bax/Bcl-2 balance in local airway in the course of BA and thus promote the mitochondrial pathway apoptosis of bronchial smooth muscle cells.

The proliferation of airway smooth muscle cells in BA is not only associated with abnormal apoptosis, but also associated with

the persistent chronic inflammatory response in airway [12]. The infiltration of inflammatory cells as well as the secretion of various inflammatory factors such as TNF- α , IL-4, IL-6 and YKL-40 are the main features of chronic airway inflammation. TNF- α is mainly secreted by activated mononuclear macrophages in airway, which can induce airway inflammation cascade activation, and also stimulate airway smooth muscle cell proliferation [13]; IL-4 and IL-5 are secreted by the mature Th2 cells in the airway, which can promote the infiltration of eosinophils and the activation of mast cells to increase the sensitivity of airway [14,15]; YKL-40 is a chitinase derivative secreted by airway epithelial cells and smooth muscle cells, which can directly participate in the Th2 cell activation and airway remodeling [16]. In the research, the analysis of inflammatory cytokine expression in the bronchial smooth muscle of guinea pig models with asthma showed that *TNF- α* , *IL-4*, *IL-6* and *YKL-40* mRNA expression in BA group of airway smooth muscle cells were significantly higher than those in control group. It illustrated that there was excessive activation of inflammation in the airway smooth muscle of BA and various inflammatory cytokines are highly expressed. Further analysis of the GA effect on inflammatory cytokines in bronchial smooth muscle of guinea pig models with asthma showed that *TNF- α* , *IL-4*, *IL-6* and *YKL-40* mRNA expression in GA group of airway smooth muscle cells were significantly lower than those in BA group. It showed that GA can inhibit the local airway inflammation and reduce the expression of various inflammatory cytokines in the course of BA.

After it was confirmed that GA could inhibit BA smooth muscle proliferation and airway inflammation factor expression, the molecular mechanisms of GA to exert the above regulating effect were further explored in the study. ERK1/2 pathway is an important signaling pathway in cells, ERK1/2 itself has the phosphorylation function of both threonine and tyrosine, which is activated after phosphorylation and can regulate the expression of multiple downstream genes to participate in apoptosis, inflammation and other processes [17,18]. In the research, analysis of the changes of ERK1/2 expression in airway smooth muscle showed that p-ERK1/2 protein expression in BA group of airway smooth muscle cells was quite higher than that in control group, and p-ERK1/2 protein expression in GA group of airway smooth muscle cells was significantly lower than that in BA group. This means that within the excessive ERK1/2 activation in airway is associated with the occurrence of BA and GA has obvious inhibiting effect on the activation of ERK1/2, and it also indicated that inhibiting ERK1/2 activation may be the molecular pathway of GA to adjust BA smooth muscle proliferation and airway inflammation. To test the regulating mechanism of GA, GA combined with ERK1/2 agonist was used in the study to treat BA airway smooth muscle cells, and the comparison with GA treatment alone showed that airway smooth muscle cell proliferation activity value as well as *Bcl-2*, *TNF- α* , *IL-4*, *IL-6* and *YKL-40* mRNA expression in GA + LM group of airway smooth muscle cells were significantly higher than those in GA group, while *Bax*, *caspase-9* as well as *caspase-3* mRNA expression were significantly lower than those in GA group. This means that the combination with ERK1/2 agonist can reverse the regulating effect of GA on BA airway smooth muscle cell proliferation activity as well as the expression of apoptosis genes and inflammatory markers, and it also confirmed that the GA inhibited the phosphorylation of ERK1/2 to exert the regulating effect on BA airway smooth muscle cells.

In conclusion, We believed that there are mitochondrial pathway apoptosis disorder and excessive activation of inflammation in the airway smooth muscle of BA; GA can inhibit the phosphorylation of ERK1/2 to promote apoptosis and inhibit the secretion of inflammatory cytokines in BA airway smooth muscle.

Conflict of interest statement

We declare that we have no conflict of interest.

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