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The regulation mechanism of MUC5AC secretion in airway of obese asthma

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ABSTRACT

The purpose of this study was to establish a rat asthma model and extract MUC5AC to explore the mechanism of mucin 5AC (MUC5AC) signaling pathway regulating the function of asthmatic airway smooth muscle cells (ASMC) and participating in asthmatic airway remodeling. Western blot was used to detect β-catenin (β-catenin), glycogen synthase kinase-3β (GSK-3β), proto-oncogene MUC5AC and cyclin D1 (cyclin D1) in MUC5AC of asthmatic and normal groups. After inhibiting the interaction between β-catenin and transcription cofactor p300 / CBP in ASMC of the asthma group and control group, the cell viability and cycle changes of ASMC were detected by the CCK-8 method and flow cytometry. After inhibiting the activity of P38 mitogen-activated protein kinase (MAPK), the protein expression changes of c-Myc and cyclin D1 were detected by Western blot. Results showed that comprehensive HE staining results of lung tissue sections indicate that the experimental rat model of asthma airway remodeling was successfully established. Compared with the control group, 100 fxmol and L1 Efaroxan promoted insulin secretion ($P < 0.01$), and administration of the MUC5AC antagonist KU14R significantly inhibited the effect of MUC5AC.Western blot showed that the protein expression levels of β-catenin, c-Myc and cyclin D1 in ASMC of the obese asthma group were significantly higher than those of the control group ($P < 0.05$), while the protein expression level of GSK-3β was lower than Control group ($P < 0.05$). After inhibiting the interaction between β-catenin and p300 / CBP, the decrease in cell viability and the degree of cell cycle change of ASMC in the asthma group were more obvious than those in the control group ($P < 0.05$). After inhibiting the activity of P38 MAPK, the expressions of the target proteins c-Myc and cyclin D1 in the MUC5AC signaling pathway in ASMC model rats and control rats were down-regulated, and the difference was statistically significant ($P < 0.05$). The conclusion was that the Wnt/β-catenin signaling pathway can regulate the proliferation and differentiation of ASMC by up-regulating the expression level of cMyc. Cyclin D1 interacts with the MAPK signaling pathway, thereby affecting the function of ASMC and participating in asthma airway remodeling.

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Introduction

Bronchial asthma is a chronic airway inflammatory disease involving multiple cells and cellular components. It is characterized by respiratory tract inflammatory response, airway hyperresponsiveness, and airway remodeling. It is an important health problem facing the world today. Its morbidity and mortality are increasing year by year (1). The high secretion of asthma mucus is related to airway remodeling, which is the result of pathophysiological changes such as goblet cell hyperplasia and submucosal gland hypertrophy in asthmatic airway epithelium (2). High mucus secretion leads to airway obstruction, decreased lung function and increased infection are the primary problems facing severe asthma attacks (3).

Bronchial asthma (abbreviated as asthma) is a heterogeneous disease characterized by chronic airway inflammation, involving a variety of cells and cytokines. Airway remodeling is the most important pathophysiological feature of asthma, and its formation mechanism is very complex.

Asthma is a chronic respiratory disease with a high incidence in the clinic. Although most patients' conditions can be effectively controlled after effective treatment, some of them are still difficult to heal. How to improve the treatment effect of asthma is the focus of clinical research at present.

Although the current drug treatment has a certain effect on the clinical management of stable asthma, there is no specific effective drug treatment method for severe asthma and high secretion of airway mucus in asthma, so research is needed to find new drugs for the treatment of asthma with high secretion of airway mucus Treatment target (4). At present, basic research on asthma mucus mainly focuses on how inflammatory mediators and related signaling pathways lead to high mucus secretion, and whether it can reduce the clinical manifestations of mucus hypersecretion by inhibiting these inflammatory mediators and related signaling pathways. A review of the research progress (5-6).

Glycogen synthase kinase-3β (GSK-3β) regulates the expression of β-catenin in cells, while the nuclear proto-oncogene c-Myc and cyclin D1 (cyclin D1) are both Wnt / β -Target genes downstream of the catenin signaling pa-

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thway have been shown to participate in the airway remodeling of asthma. Therefore, the Wnt / β-catenin signaling pathway may participate in the formation and development of asthma airway remodeling by regulating the expression levels of its downstream target genes and affecting the proliferation and differentiation function of ASMC (7-8).

In this study, a rat model of chronic asthma was established and rat MUC5AC was obtained. The role of the Wnt/β-catenin signaling pathway in regulating MUC5AC function in the process of asthmatic airway remodeling was studied, and the Wnt/β-catenin signaling pathway was discussed. Specific mechanisms involved in asthmatic airway weight.

Materials and Methods

Establishment of animal models

70 SD rats of SPF grade, 8-9 weeks old, half male and half female batch No.: 20210411, provided by Speifu Biotechnology Co., Ltd.
Rat IgE ELISA kit, batch No.: KT32161, provided byRegbo.
Rat TNF-a ELISA kit, batch No.: KT32162, provided byRegbo.
Rat IL-4 ELISA kit, batch No.: KT32163, provided byRegbo.
Rat IL-5 ELISA kit, batch No.: KT32164, provided byRegbo.
Rat IL-17 ELISA kit, batch No.: KT32165, provided byRegbo.
402B Ultrasonic Nebulizer (Jiangsu Yuyue Medical Equipment Co., Ltd.);
Rat airway resistance and lung compliance invasive detection system (the United States, BUXCO company);
Automatic multi-function microplate reader (MULTISKAN MK3, Thereto, USA);
GL-21 M high-speed refrigerated centrifuge (Shanghai Luxiangyi Centrifuge Instrument Co., Ltd.);
Tissue-TEK VIP6 Biological Tissue Dehydrator (Sakura Company, Japan);
Tissue-TEK TEC biological tissue embedding machine (Japan Sakura Co., Ltd.);
Leica RM2235 tissue slicer (Leica, Germany);
Leica HI1210 film picker (Leica, Germany);
Leica HI1220 baker (Leica, Germany);
Leica ST5020 automatic dyeing machine (Leica, Germany);
Leica CV5030 automatic coverslipper (Leica, Germany);
Olympus BX51 microscope (Olympus, USA);
Path QC pathological quality control and data management system (China Langka Company).

The experimental animals were 16 male SD rats of SPF grade, aged 8 weeks, weighing 180-200 g. Replicate the model of chronic asthma airway remodeling with reference to the experimental group's previous successful model establishment method (9). The experimental animals were divided into the asthma group and the control group. The control was given accordingly Rats in the group were intraperitoneally injected with physiological saline 1. 5 mL. Starting from the first day of the third week, the next day using an ultrasonic nebulizer to atomize the asthmatic rats in a closed plastic box with 1% OVA physiological saline solution, each time for 30 minutes, a total of 8 weeks (10-11). And some rats in the asthma group were treated with weight gain to simulate the obesity effect. The model building process is divided into two stages, sensitization and excitation, for a total of 10 weeks. The sensitization phase lasted 2 weeks, and the asthmatic rats were intraperitoneally injected with OVA / Al (OH) 3 mixture 1.5 mL containing OVA 1 mg, Al (OH) 3100 mg on day 1 and day 8 respectively.

ASMC and lung tissue extraction

After the last atomization, the rats were killed by intraperitoneal injection of 10% chloral hydrate (4 mL/kg) within 16 to 24 hours, and the trachea and bronchus of the rats were obtained on the ultra-clean table (12). Operate under a microscope, cut a small piece of a lung lobe, immerse it in 4% paraformaldehyde to fix it, and use it for hematoxylin-eosin (HE) staining to observe the pathological structure of lung tissue (13-14). Continue to remove the remaining lung tissue, bronchial blood vessels, and fat tissue adhering to the trachea, cut the trachea and bronchus longitudinally, gently scrape the outer and inner membranes, and cut the tracheal segment into 1 mm or smaller tissue pieces. The collagenase-trypsin mixed digestion method was used to remove impurities such as epithelial cells and fibroblasts, and the ASMC with higher purity was obtained for subculture, and the 3rd to 6th generation ASMCs were used for experiments (15-16).

Identification of ASMC

Observe the cell morphology and take photos under an inverted phase contrast microscope. Digest the adherent ASMC to make a cell suspension, inoculate it on a sterile cell slide and place it in a 12-well plate, wait for the cell growth to converge to about 70% -80%, pour off the culture solution and wash the cells 3 times with PBS, Every 5 min (17). Fix with 4% paraformaldehyde for 15 min, and after washing and blocking, add diluted α-smooth actin (α-SMA) Ianti- (1: 100) 4 °C and incubate overnight. Then use the corresponding II anti- (1: 100) to incubate the cells at room temperature for 1 h, after washing, staining and other steps of microscopic examination (18).

CCK-8 experiment to detect cell viability

The Asthma group and control rats were divided into groups: (i) negative control (negative control, NC) group: normal cultured cells without any treatment; (ii) DMSO group: added 0. 5% DMSO, check whether the concentration of 0.5 ‰ DMSO affects cell viability; (iii) β-catenin / CBP inhibitor ICG-001 group: 2 kinds of ASMC with specific inhibitor ICG-001 at 100 μmol / L, 50 μmol / L, 25 μmol / L and 5 μmol / L concentrations were incubated for 24 h; (iv) β-catenin / p300 inhibitor IQ1 group: 2 specific inhibitors of IQ1 IQ1 at 100 μmol / L, 50 μmol / L, 25 μmol / L and 5 μmol / L concentrations were cultivated for 24 h. 2 inhibitors ICG-001 and IQ are dissolved in DMSO, and according to the conditions of each treatment group, the corresponding concentration of the drug is configured, and finally the concentration of DMSO in the culture medium of each treatment group is fixed at 0.5 ‰. Each treatment group was set up with 6 complex wells. After removing the maximum and minimum values, the average value was taken as the final experimental result. Each group of experiments was repeated 3 times (19-20).

Digest the adherent cultured asthma group and control group ASMC with 0.25% trypsin, make a single cell

suspension with high glucose DMEM medium containing 10% fetal bovine serum, and make each group MUC5AC at a concentration of 1 × 108 / L Inoculate in a 96-well plate with a volume of 100 μL per well and place in a constant temperature incubator (37 °C, 5% CO2) for about 1 day. When the cell growth converges to about 50% ~ 60%, change the serum-free DMEM medium to culture the cells for 24h, so that all cells are in the same growth stage, and then carry out drug intervention on the above-grouped cells (21). After culturing the cells under the influence of drugs for 24 hours, aspirate the medium, wash the plate twice with PBS, re-add the culture solution containing 10% CCK-8 reagent, protect from light for 2 hours at 37 °C, use a multifunctional microplate reader at 450 The absorbance (A) value of the cells in each well was measured under nm, and the growth of cells in each group was judged according to the calculation result of the formula. The cell viability of the negative control group without treatment was 100%.

Flow cytometry to detect cell cycle distribution

The asthma group and the control group MUC5AC are divided into (i) NC group: normal cultured cells do not add any treatment; (ii) ICG-001 group: use 50 μμmol / L ICG-001 to cultivate ASMC 24; (iii) IQ1 group: Use 50 μmol / L IQ1 to cultivate ASMC 24h. Digest each adherent group of ASMC with 0.25% trypsin, make a single cell suspension and collect the cells to each 15mL centrifuge tube, centrifuge to collect the cells; wash the cells with pre-chilled PBS by pipetting, 1 000 r / min Discard the supernatant after centrifugation for 5 min and repeat once; add pre-cooled 70% ethanol and fix at 4 °C overnight (more than 18 h); collect the fixed cells by centrifugation, wash the cells once with PBS, centrifuge again for 5 min and discard Clear; add 500 μL of cell cycle detection reagent propidium iodide (PI) to each sample, stain and incubate at room temperature in the dark for 20 minutes; use flow cytometry standard procedures to detect cells, count 2-3 million cells, the results Analysis using cell cycle fitting software. Set the dilution coefficient of the antibody in four gradient settings, which are determined by a laboratory staining kit: 0.2X, 2X, 3X, 1X (1X is the correct nominal dosage). HE staining of lung tissue The fixed lung tissue was routinely embedded in paraffin, sectioned (4um), and the pathological structure of the lung tissue was observed after HE staining.

Western blot method to detect changes in protein levels of C-Myc, GSK-3β, β-Catenin and Cyclin D1

Put the culture dishes of smooth muscle cells covered with a normal control group and asthma group on the ice, wash the cells with pre-chilled PBS 3 times, add cell lysis solution RIPA, lyse for 30 min, scrape off the cells with cells, collect the liquid to EP tube, ultrasonic cracking 5 seconds, 3 times, move the EP tube to a high-speed centrifuge, centrifuge at 4 °C 12 000 / min 20 min, take supernatant. Use the BCA kit to determine the protein concentration, configure an equal volume and equal concentration protein system, use 8% SDS-PAGE to separate the protein, 300 mA constant flow transfer membrane and 5% skim milk at room temperature for 2 hours, then add the corresponding monoclonal antibody 4 Incubate overnight at °C. 16-18 After immersing the membrane in TBST, rinsing it on a shaker 3 times, adding II anti-incubation 2h, and performing chemiluminescence, color development and development after washing TBST.The experimental results will be given in the subsequent experiments.

Statistical processing

The experimental data were analyzed by SPSS 22.0 statistical software, and the measurement data were expressed by mean ± standard (mean ± SD); the difference between the mean was compared by independent sample t-test; the correlation analysis between the two variables was by linear correlation analysis. P <0.05 was considered statistically significant.

Results

Effect of MUC5AC

MUC5AC detection technology is an experimental diagnostic method with high sensitivity, strong specificity and good repeatability. Although the operation steps of the ELISA kit are simple, if the experimental operation is not standardized, it will affect the stability and reliability of the experimental results. The expression level of MUC5AC in the airway of asthmatic mice is positively correlated with the degree of inflammatory cell infiltration, and MUC5AC can directly enhance the proliferation, adhesion and migration of T lymphocytes and other cells. Under the condition of 8.3 mmol/L glucose concentration, the effect of MUC5AC on insulin secretion was observed. The results showed that KU14R had no obvious effect on insulin secretion (P> 0.05 vs control) o given the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methyl yellow whine, 50 jxmol / L'1) significantly promoted insulin secretion (P <0.01 vs control). When MUC5AC was administered, the insulin secretion promoting effect of IBMX was significantly inhibited (P <0.01). IBMX works by inhibiting phosphodiesterase to reduce the degradation of cAMP, thereby increasing the content of cAMP in pancreatic islet P cells. The effect of Efaroxan on insulin secretion Under the condition of different glucose concentrations, it was found that 100ummol/L Efaroxan had obvious glucose concentration-dependent characteristics in promoting insulin secretion. Compared with its parallel control group (control), Efaroxan had no significant effect on insulin secretion at lower glucose concentrations; while at higher glucose concentrations, Efar-oxan significantly promoted insulin secretion (P<0.01). Therefore, the results of this experiment also showed that MUC5AC played a

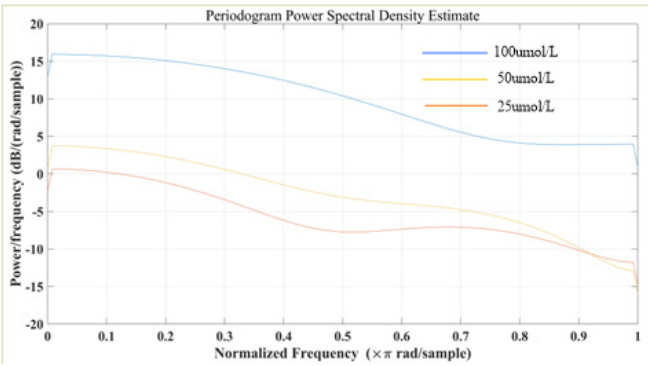

Figure 1. MUC5AC inhibits insulin secretion by inhibiting the cAMP signaling pathway. The blue, yellow and brown lines represent the effects of different concentrations on inhibiting insulin secretion.

Table 1. MUC5AC inhibits insulin secretion by inhibiting the cAMP signaling pathway.

Glucose concentration/	Insulin release/ng	MUC5AC	*t*	*p*-value
mmol - L-1	Control	Efaroxan		
0	0.43 ±0.04	0.44±0. 04	0.986	0.713
2.8	0.52 ±0.05	0.56 ±0.05	10.375	0.005
8.3	1.03 ±0.06	1.89 ±0.23	13.755	0.002
11.1	1.85 ±0.14	2.57 ±0.36	11.533	0.003

role in inhibiting insulin secretion by inhibiting the cAMP signaling pathway. Among them, the relationship between glucose concentration and insulin release and MUC5AC dose as shown in Table 1 and Figure 1.

Rat lung tissue experiment control

HE staining of lung tissue in normal control rats showed that the normal small airways and alveoli were intact, the mucosal epithelium was intact, the bronchial lumen was regular, and there was no infiltration of inflammatory cells around the bronchi and blood vessels.HE staining of rat lung tissue is shown in Figure 2.

Comprehensive HE staining results of lung tissue sections indicate that the experimental rat model of asthma airway remodeling was successfully established. The model of rat asthma airway remodeling is shown in Figure 2. In the asthma group, HE staining of lung tissue showed significant thickening of the airway wall, irregular thickening of the basement membrane, increased mucosal folds, mucous gland hyperplasia, mucus plug formation and submucosal edema, exudate and inflammation in the lumen Cells increase, and eosinophilia can be seen under the bronchial mucosa, bronchi and blood vessels. Extensive infiltration of inflammatory cells such as cells, neutrophils and lymphocytes.

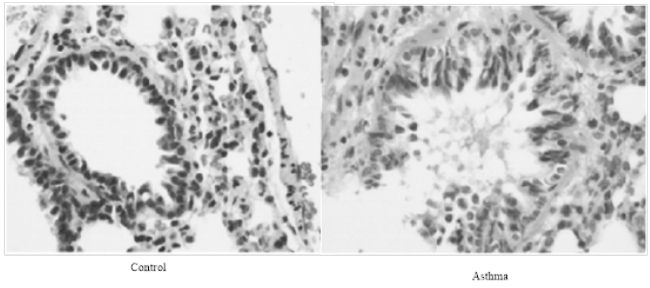

Figure 2. HE staining of rat lung tissue.

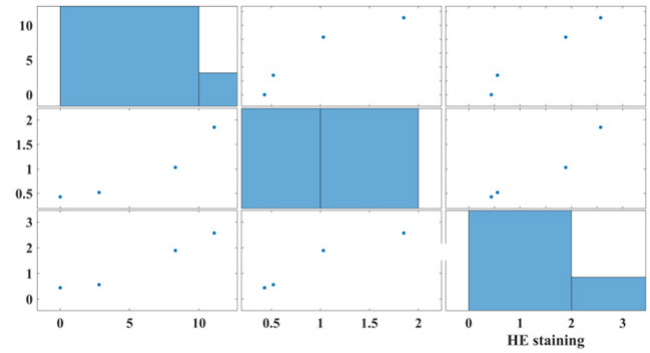

Figure 2. Rat asthma airway remodeling model.

Identification of rat MUC5AC

The morphology of the isolated and cultured cells was observed under the inverted phase MUC5AC differential microscope. Under the microscope, a single smooth muscle cell was seen to be a spindle-shaped or irregular triangle, with multiple cell protrusions, abundant cytoplasm, high density, centered round core and multiple core benevolence. When the cells grow densely, they are arranged in parallel and bunched, partially overlapping, showing typical " valley " shapes / shaped. After the specific α-SMA, immunocytochemical staining of MUC5AC using the SP method, more than 99% of the cells were strongly positively stained, and the cytoplasm was brownish yellow or brownish red, as shown in Figure 3.

The results of HE staining of lung tissue sections showed that the asthma airway remodeling model was successfully established in this experiment.

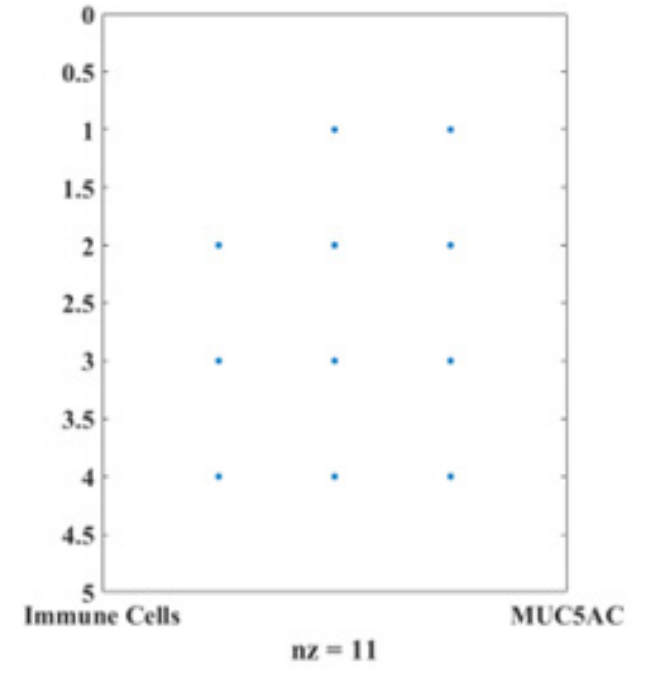

Figure 3. Confirmed as MUC5AC.

Effects of causing secretion of MUC5AC

The experiments in this group were completed under 8.3 mmol-L'1 glucose concentration. Compared with the control group, 100 fxmol and L1 Efaroxan significantly promoted insulin secretion (P <0.01); administration of the MUC5AC antagonist KU14R significantly inhibited the effect of MUC5AC. Application of adenylate cyclase agonist also significantly promoted insulin secretion, which was significantly different from the control group (F <0.01). It is worth noting that this effect can also be significantly inhibited by MUC5AC. MUC5AC can increase the intracellular adenosine cyclic adenosine content by agonizing adenylate cyclase (Table 2 and Fig 4), so this

Table 2. Effects of causing secretion of MUC5AC.

Group	Insulin release/ng • islet -1	Protein expression level
Control	0.83 ±0.06	0.73 ±0.052
Efaroxan	1.52 ±0.15	1.53 ±0.053
MUC5AC	0.90 ±0.07	0.82 ±0.13
Forskolin	1.75 ±0. 13	1.82 ±0.05
Forskolin + MUC5AC	0.96 ±0. 17	0.97 ±0.03

experiment suggests that Efaroxan may promote insulin secretion and MUC5AC.

The mechanism of action of MUC5AC was investigated. The results showed that KU14R could significantly inhibit the promoting effect of MUC5AC on insulin secretion. At the same time, we found that KU14R significantly inhibited the promoting effect of forskolin on insulin secretion. Therefore, our results suggest that MUC5AC may also regulate insulin secretion through the cAMP signaling pathway.

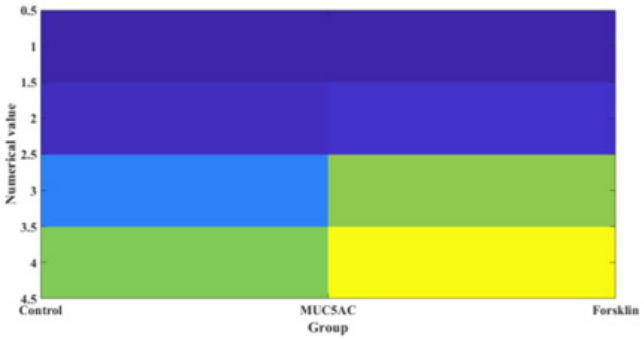

Figure 4. Effects of causing secretion of MUC5AC.

Discussion

At present, the goal of asthma treatment is to control symptoms, and glucocorticoids are regarded as the first-line drug for asthma. However, recent studies have shown that despite the optimal hormone dose, 5% to 10% of asthma patients are not well controlled. The role of airway remodeling and high secretion of airway mucus in the pathogenesis of asthma is increasingly valued and is considered to be one of the pathological basis and important causes of irreversible airway obstruction and refractory asthma. Therefore, its pathogenesis, related inflammatory cells and cytokines involved in this pathophysiological process, and inflammatory mediators have gradually become research hotspots to find potential drug targets for asthma treatment (22). This study was to explore the mechanism of mucin 5AC (MUC5AC) signaling pathway regulating the function of asthma airway smooth muscle cells (ASMC) and participating in asthma airway remodeling, so as to provide scientific data and a basis for improving the therapeutic effect and prognosis of asthma.

Studies have shown that MUC5AC ligands are expressed in the airway epithelium and regulate their proliferation, differentiation, and repair. The expression of MUC5AC and its ligands in chronic airway inflammatory response is increasing. There is a positive correlation between the expression of EGF-R and pathological hyperplasia of airway mucus goblet cells. Many stimuli produced by mucus are cleaved by precursor MUC5AC by increasing matrix metalloproteinases on the membrane surface. Therefore, the activation of MUC5AC plays an important role in the regulation of mucin synthesis (23). The PI3K / Akt and ERK1 / 2 pathways are also necessary to regulate mucin production. The activation of PI3K / Akt and ERK1 / 2 is regulated by EGFR activity. The MUC5AC-specific inhibitor AG1478 can completely prevent the expression of p-ERK1 / 2, PI3K, and p-Akt proteins stimulated by human neutrophil elastin. ERK pharmacological inhibitors can completely prevent the expression of MUC5AC mRNA and the activation of the MUC5AC promoter in the EGFR-regulated airway MUC5AC protein expression experiment. The ERK pathway regulates the transcriptional activation of the MUC5AC gene, which must be initiated by SP-1 and his in MUC5AC The action elements of the child combine to function. All in all, the Ras / Raf / ERK / SP-1 signaling pathway plays an important role in regulating the expression of MUC5AC in the EGFR pathway, but it is not the only pathway. Studies have shown that the Notch signaling pathway stimulates the expression of MUC5AC and the EGFR signaling pathway. Notch plays a decisive role in the cell fate of various organ systems (24).

NF-kB plays an important role in regulating apoptosis, viral replication, tumorigenesis and many autoimmune diseases. NF-kB can regulate the expression of many genes, and the molecules encoded by these genes play an important role in the inflammatory response. Recent studies have shown that oxidative stress and NF-kB can increase MUC5AC protein expression in chronic airway inflammation. NF-kB can promote the transcription of the MUC2 mucin gene together with the TGF-p-SMAD signaling pathway (25). Some current studies have shown that RV-14 up-regulates MUC5AC through Src / MEK / NF-kB, implying that NF * B activation energy directly increases MUC5AC release, and Hewson et al. Confirmed that, as previously described, the EGFR-MEK / ERK signal. The pathway is through SP-1 binding to the MUC5AC promoter to initiate transcription and translation. RV stimulation of MUC5AC protein expression is NF-kB dependent, but it does not directly bind to the MUC5AC promoter to function. NF-kB is through the increase of matrix metalloproteinase Transcription, and NF-kB activation promotes MUC5AC protein expression is the release of matrix metalloproteinase (MMP) -dependent EGFR ligand TGF-a. MMPS can cleave TGF-a precursor, activated TGF-a is Released on the surface of the cell membrane to bind to EGFR and activate EGFR. Therefore, it interacts with the above-mentioned epidermal factor receptor pathway. Comprehensive HE staining results of lung tissue sections indicate that the experimental rat model of asthma airway remodeling was successfully established (26).

Many cytokines and related signaling pathways are involved in the transcription and release of mucin genes. This article describes the research progress of molecular mechanisms of high secretion of asthma airway mucus

from several important signal transduction pathways and aims to explore whether it can be by inhibiting the activation of these inflammatory mediators or their signaling pathways has become a reasonable choice for the treatment of clinical symptoms of airway mucus hypersecretion, but currently, more in vitro and animal experiments have found or found a certain inflammatory mediator and its related signaling pathways and airway remodeling are related to the high secretion of airway mucus, but its specific mechanism of action and the interaction between various mechanisms and evaluation of how to use these signaling pathways as a drug target for the treatment of chronic airway inflammation with the high secretion of airway mucus still needs further a lot of experimental research.

Conclusion

In summary, this study established a rat model of chronic asthma and obtained rat ASMCs to explore the relevant mechanism of the Wnt/β-catenin signaling pathway involved in asthma airway remodeling. The results suggest that the Wnt/β-catenin signaling pathway may be upregulated by The expression of cMyc and cyclin D1 interacts with the MAPK signaling pathway and regulates the proliferation and differentiation of ASMC, which affects the function of ASMC and participates in airway remodeling in asthma.

Declaration

Ethics approval and consent to participate

All animal experiments have met with the approval of Animal Care and Use Guidelines of the Qingdao Municipal Hospital, School of Medicine Qingdao University.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, ST, upon reasonable request.

Competing interests

The authors declare no competing interests.

Funding

The authors declare that they have not taken any funding.

Authors' contributions

TL,YW, SH, and HT contributed equally to this work, contributed to the study design, statistical analysis, interpretation of data and wrote the manuscript. All authors reviewed the manuscript and gave final approval the manuscript.

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