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Effects of simvastatin on immunoreaction and inflammation in rats with asthma by regulating NOTCH signaling pathway

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ABSTRACT

To investigate the effect of simvastatin on the immunoreaction and inflammation in rats with asthma through the NOTCH signaling pathway, a total of 36 Sprague-Dawley (SD) rats were enrolled and randomly divided into the normal group (n=12), model group (n=12) and simvastatin group (n=12). The rats in the normal group were fed normally, those in the model group were prepared into models of asthma, and those in the simvastatin group were prepared into models of asthma and intervened with simvastatin. Next, the morphology of airway tissues was observed *via* hematoxylin-eosin (HE) staining assay. Besides, immunohistochemistry was employed to determine the expression of interferon-γ (INF-γ), and the relative protein expression levels of NOTCH2 and NOTCH3 were measured by Western blotting (WB). Additionally, enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR) assay were carried out to detect the content of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and the relative mRNA expression levels of INF-γ, IL-6 and TNF-α, respectively. HE staining results uncovered that the airway tissues displayed normal morphology in the normal group and disordered morphology and obvious inflammatory infiltration in the model group. In comparison with the model group, the simvastatin group exhibited significantly improved morphology of airway tissues. Based on immunohistochemistry, the average optical density of INF-γ positive expression was increased in the model group and simvastatin group compared with that in the normal group ($P<0.05$), and it was distinctly lower in the simvastatin group than that in the model group ($P<0.05$). The results of WB showed that compared with those in the normal group, the relative protein expression levels of NOTCH2 and NOTCH3 were elevated in model group and simvastatin group ($P<0.05$), whereas they were overtly reduced in simvastatin group compared with those in model group ($P<0.05$). It was found through ELISA that the model group and simvastatin group had raised content of IL-6 and TNF-α in comparison with the normal group ($P<0.05$), while the simvastatin group exhibited markedly decreased content of IL-6 and TNF-α in comparison with the model group ($P<0.05$). The results of qPCR revealed that the relative mRNA expression levels of INF-γ, IL-6 and TNF-α were distinctly up-regulated in the model group and simvastatin group compared with those in the normal group, displaying statistically significant differences ($P<0.05$), whereas they were markedly lowered in simvastatin group compared with those in the model group, showing statistically significant differences ($P<0.05$). Simvastatin represses the immunoreaction and inflammation in rats with asthma by down-regulating the NOTCH signaling pathway.

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Introduction

Asthma is a common chronic airway-reactive disease of the respiratory system. Besides, it is also deemed as a very harmful and complicated respiratory dysfunction caused by recurrent chronic inflammation of the airway, with high incidence and recurrence rates in clinic, severely affecting the quality of life, life and health of patients. Currently, it is believed that the pathogenesis and pathological reactions of asthma are relatively complex, and its major pathological reactions including immunoreaction and inflammation are the key pathological responses resulting in airway hyperresponsiveness and affecting airway remodeling (1, 2).

The NOTCH signaling pathway, one of the important signal transduction pathways in the body, is considered to play a vital role in the pathogenesis and pathological responses of asthma, and it participates in the pathological responses of asthma by modulating immunoreaction and inflammation (3-5). It has been now considered that interferon-γ (INF-γ) regulated by the NOTCH signaling pathway is one of the key environments and leading causes of immunoreaction during asthma. In addition, the inflammation modulated by the NOTCH signaling pathway is also one of the important pathological factors exacerbating the inflammation in asthma.

Simvastatin, a commonly used HMG-CoA reductase inhibitor, has a good anti-hyperlipidemic effect. Recent studies have manifested that simvastatin can also alleviate asthma to some extent and significantly mitigate the immunoreaction and inflammation in rats with asthma, but the related mechanisms are unclear (6,7). Therefore, this study aims to explore the effects of simvastatin on immunoreaction and inflammation in asthmatic rats by modulating the NOTCH signaling pathway.

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Materials and Methods

Laboratory animals

A total of 36 SPF Sprague-Dawley (SD) rats purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were adaptively fed in the Laboratory Animal Center for 7 d before experiments, and divided into normal group (n=12), model group (n=12) and simvastatin group (n=12) using a random number table. This study was approved by the Animal Ethics Committee of Hebei North University Animal Center.

Experimental reagents and instruments

Ovalbumin (OVA) and aluminum hydroxide (Sigma, St. Louis, MO, USA), simvastatin (Huayu Pharmaceutical, Chengdu, China), anti-INF-γ, anti-NOTCH2 and anti-NOTCH3 primary antibodies and secondary antibodies (Abcam, Cambridge, MA, USA), immunocytochemistry kits, enzyme-linked immunosorbent assay (ELISA) kits, kits for quantitative polymerase chain reaction (qPCR) (Vazyme, Nanjing, China), light microscopes (Leica, Wetzlar, Germany), and fluorescence qPCR instruments (ABI, Applied Biosystems, Foster City, CA, USA).

Materials

Modeling of asthma

A total of 100 mg of OVA and 100 mg of aluminum hydroxide were mixed, dissolved in 2 mL of normal saline and injected intraperitoneally into rats. After 1 week, the above operations were repeated once. Then, the rats were put in a box with a nebulizer to inhale aerosolized 3% OVA solution for 30 min once every other day for 4 weeks. Next, the respiratory symptoms of rats were observed, and polypnea, irritability, and decreased activity observed in rats indicated successful modeling.

Treatment in each group

The rats in the normal group received no treatment. In the model group, the models of asthma were constructed according to the above-mentioned modeling method, and normal saline was intraperitoneally injected into rats after successful modeling. The rats in the simvastatin group were prepared into the models of asthma and intraperitoneally injected with 40 mg/kg simvastatin at 0.5 h before aerosol inhalation. At 24 h after the last aerosol inhalation, samples were collected from rats in each group.

Collection of samples

After successful anesthesia, airway tissues were directly collected from 6 rats in each group, rinsed with normal saline, put into EP tubes and stored at -80°C for Western blotting (WB), qPCR assay and ELISA. Besides, for the remaining 6 rats, samples were collected through perfusion fixation. In other words, their chest cavity was cut open to expose the heart. Then, 400 mL of 4% paraformaldehyde was infused into the heart from the left atrial appendage, and then the airway tissues were taken out and fixed in 4% paraformaldehyde solution for immunohistochemistry and hematoxylin-eosin (HE) staining assay.

HE staining assay

After the prepared paraffin-embedded tissues were made into sections (5 μm in thickness), they were put in 42°C warm water for spreading, collected using slides, baked, and prepared into paraffin tissue sections. Next, the paraffin tissue sections were sequentially soaked in xylene solution and graded ethanol for conventional deparaffinization and hydration. Afterward, the HE staining kits were used. The sections were firstly stained with hematoxylin for 5 min, soaked in pure water for 10 min, put in 95% ethanol for color separation for 5 s, permeabilized with xylene for 10 s, and mounted with neutral gum.

Immunohistochemistry

The paraffin-embedded tissues prepared were cut into 5 μm-thick sections, placed in 42°C warm water for extension, collected using slides, baked, and prepared into paraffin tissue sections. Next, the paraffin tissue sections were sequentially soaked in an xylene solution and a gradient ethanol series for conventional deparaffinization and hydration, respectively. Thereafter, they were immersed in citric acid buffer and heated (3 min of heating + 5 min of simmer/each time) in a microwave 3 times for full antigen retrieval. Next, the specimens were rinsed, reacted with endogenous peroxidase blocker added dropwise for 10 min, rinsed, and blocked with goat serum added in drops for 20 min. Subsequently, the goat serum-blocking solution was removed, and the specimens were incubated with anti-INF-γ primary antibodies (1:200) in a refrigerator at 4°C overnight. The next day, the specimens were rinsed, dropwise added with secondary antibody solution for 10 min of incubation, thoroughly rinsed and added with streptavidin-peroxidase solution for the reaction for 10 min, followed by color development with dropwise adding of diaminobenzidine (DAB) (Solarbio, Beijing, China). Lastly, the nuclei were counterstained with hematoxylin, and the sections were mounted and observed.

WB

The cryopreserved airway tissues were added with lysis buffer, followed by an ice bath for 1 h and then centrifugation at 14,000 g for 10 min using a centrifuge. Next, the proteins were quantified via the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), and the protein concentration was calculated based on the absorbance values and standard curves obtained by a microplate reader. Thereafter, the proteins were denatured and separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) during which the position of the Marker protein was observed. When the Marker protein reached the bottom of the glass plate in a straight line, the separation was stopped. Then, the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), added with blocking solution for 1.5 h of reaction, and incubated with anti-NOTCH2 primary antibody (1:1000), anti-NOTCH3 primary antibody and (1:1000) and secondary antibody (1:1000) sequentially. After that, the membrane was rinsed and added with a chemiluminescent reagent for full development for 1 min in a dark place.

QPCR assay

The total ribonucleic acid (RNA) was extracted from fresh tissue samples and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA). With a reaction system (20 μL in volume), qPCR was conducted under the following conditions: reaction at 50°C for 2 min,

Table 1. Primer sequences.

Name	Primer sequence
INF-γ	Forward primer: 5' AATAGGCGCCAATTATAGC 3' Reverse primer: 5' GCGCGAATGCATTCAATGC 3'
IL-6	Forward primer: 5' GGGATTAGCTCAGAATCTGCCG 3' Reverse primer: 5' TATATTAGCTATCCTAGGCTAGC 3'
TNF-α	Forward primer: 5' TTGCATAATTGCTCCGCTGATCG 3' Reverse primer: 5' CCGCTTGCTCCTTAGTAATCGCC 3'
GAPDH	Forward primer: 5' ACGGCAAGTTCAACGGCACAG 3' Reverse primer: 5' GAAGACGCCAGTAGACTCCACGAC 3'

pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, and annealing at 50°C for 30 s, for 45 cycles. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression level of related messenger RNAs (mRNAs) was calculated. The primer sequences are shown in Table 1.

ELISA

The airway tissues freshly collected were minced. Then, ELISA was performed as per the instructions of the ELISA kits. Briefly, the samples were loaded and added with standard substance, biotinylated antibody working solution and enzyme conjugate working solution, followed by washing of the plate. Lastly, the microplate reader was utilized for detection at 450 nm.

Statistical analysis

In this study, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were expressed as mean ± standard deviation. t-test was utilized for data in line with normal distribution and homogeneity of variance, corrected t-test for those conforming to normal distribution and heterogeneity of variance, and non-parametric test for those not in line with normal distribution and homogeneity of variance. The rank sum test was applied for ranked data. For enumeration data, a chi-square test was employed.

Results

Morphology of airway tissues observed through HE staining assay

The airway tissues in the normal group had normal morphology and complete structure, without obvious abnormality and inflammatory infiltration. In the model group, there were irregular airway tissues, disorderly arranged cells that were necrotic and damaged, and evident inflammatory infiltration. In the simvastatin group, some airway tissues were disordered, and the inflammatory infiltration was mild, which was improved compared with those in the model group. INF-γ-positive expression was tan and scattered in the airway tissues, and it was lower in the normal group and higher in the model group. The statistical analysis results of mean optical density showed that in comparison with the normal group, the model group and simvastatin group had a remarkably increased mean optical density of INF-γ-positive expression, and the difference was of statistical significance (P<0.05), while the mean optical density of INF-γ-positive expression was overtly lower in simvastatin group than that in the model group, showing a statistically significant difference (P<0.05).

Relative protein expression level measured through WB

The protein expression levels of NOTCH2 and NOTCH3 were detected in all groups, and the results showed that they were lower in the normal group and higher in the model group. The statistical analysis results (Figure 1) showed that the relative protein expression levels of NOTCH2 and NOTCH3 were higher in model group and simvastatin group than those in normal group, with statistically significant differences (P<0.05), while they were remarkably lower in simvastatin group than those in model group, and the differences were statistically significant (P<0.05).

The content of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) was higher in the model group and simvastatin group than in the normal group, and the difference was statistically significant (P<0.05), and it notably declined in simvastatin group compared with that in the model group, demonstrating a statistically significant difference (P<0.05) (Figure 2).

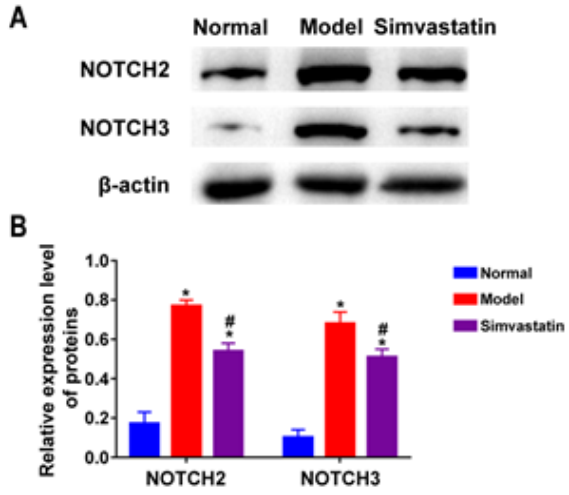

Figure 1. Protein expressions determined via WB. The relative expression level of proteins in each group. *P<0.05 vs. normal group, #P<0.05 vs. model group.

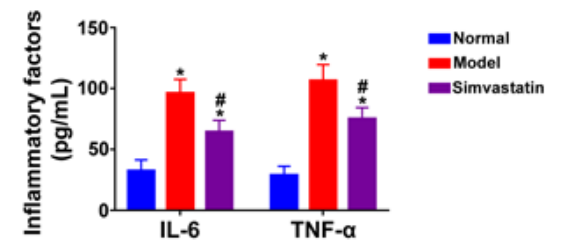

Figure 2. Content of inflammatory factors detected via ELISA. Note: *P<0.05 vs. normal group, #P<0.05 vs. model group.

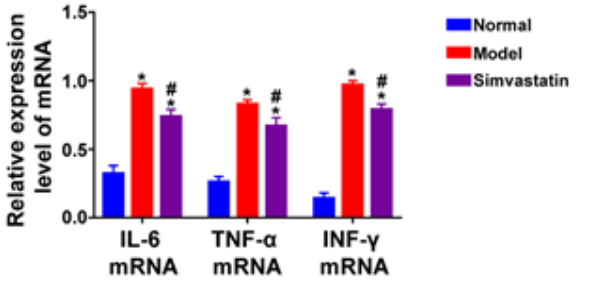

Figure 3. Results of qPCR assay. Note: *P<0.05 vs. normal group, #P<0.05 vs. model group.

Results of qPCR assay

The relative mRNA expression levels of INF-γ, IL-6 and TNF-α were distinctly up-regulated in the other two groups compared with those in the normal group, displaying statistically significant differences (P<0.05), and they were markedly reduced in the simvastatin group compared with those in model group, showing statistically significant differences (P<0.05) (Figure 3).

Discussion

As a common chronic inflammatory respiratory disease in clinical practice, asthma is mainly characterized by airway inflammation and hyperresponsiveness, and its clinical treatment has always been a clinical difficulty. Patients with asthma are prone to persistent and recurrent respiratory symptoms, such as polypnea, gasping, oppression in the chest and significantly decreased activity. In severe cases, patients may have dyspnea or even die from suffocation. Studies have demonstrated that asthma has a high clinical incidence rate, with unique characteristics, including seasonal and recurrent onset, often induced by allergens (8-10). At present, the treatment of asthma is a challenge, and there are no ideal drugs for its treatment. As asthma is deeply studied, the pathogenesis and pathological responses of asthma have been gradually understood. Studies have denoted that persistent, chronic and recurrent airway inflammation and hyperresponsiveness are pathological features of asthma (11-13). Immunoreaction and inflammation are closely related to the development and progression of asthma. Studies have shown that in the case of asthma, immunoreaction and inflammation are the important pathological reactions, and the relieved infiltration of a large number of inflammatory cells leads to abnormally high expression of a large number of INF-γ and synthesis and secretion of the inflammatory factors IL-6 and TNF-α in quantity, further aggravating the immunoreaction and inflammation (14,15). INF-γ, the main effector of immunoreaction, can trigger the immunoreaction in the body, promote the aggregation of immune cells, and lead to the imbalance of immune cells Th1/Th2, thereby inducing immunoreaction, and it can also mediate inflammation by causing the synthesis and secretion of a large number of inflammatory factors, exacerbating inflammation and injury (16,17). However, excessive immunoreaction and inflammation will further damage cells and tissues and exacerbate injuries, forming a vicious cycle of immunoreaction-inflammation damage. As one of the important signaling pathways in the body, the NOTCH signaling pathway has crucial regulatory effects on the physiological and pathological processes such as proliferation, differentiation, apoptosis, and autophagy of cells and also plays an important role in mediating inflammation and immunoreaction. It is discovered in a study that NOTCH2 and NOTCH3, the key molecules in the NOTCH signaling pathway, are important members of the NOTCH family, which can be up-regulated after activation by massive inflammatory and cellular factors and other pathological factors and are involved in regulating various downstream substances including INF-γ, IL-6 and TNF-α and pathophysiological responses such as inflammation and immunoreaction (18). In this study, it was further confirmed that the NOTCH signaling pathway was closely associated with inflammation and immunoreaction during asthma. The activation of the NOTCH signaling pathway due to the abnormally high expressions of NOTCH2 and NOTCH3 in the airway tissues of asthmatic rats is one of the major pathological causes of immunoreaction and inflammation in airway tissues of rats with asthma.

As a commonly used hypolipidemic drug, simvastatin is widely applied in the prevention and treatment of cardiovascular and cerebrovascular diseases. Moreover, further studies have shown that simvastatin exerts a good regulatory effect on airway inflammation and immunoreaction in asthmatic rats, and can significantly improve lung function in such rats (19,20). The results of this study further verified that simvastatin had a good effect in improving the morphology of airway tissues of asthmatic rats, and effectively reduced the content of INF-γ, IL-6 and TNF-α, indicating that simvastatin plays a good inhibitory role in the immunoreaction and inflammation of airway tissues of asthmatic rats. Besides, the levels of NOTCH2 and NOTCH3, important molecules in the NOTCH signaling pathway, in airway tissues of asthmatic rats were measured after intervention with simvastatin, and it was uncovered that simvastatin significantly reduced the expressions of NOTCH2 and NOTCH3, thereby inhibiting the NOTCH signal pathway. This may be a possible reason why simvastatin inhibits the immunoreaction and inflammation of airway tissues of asthmatic rats.

It is concluded that simvastatin down-regulates the NOTCH signaling pathway to suppress the immunoreaction and inflammation in rats with asthma.

Conflict of Interest

The authors declared no conflict of interest.

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