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Anti-nerve growth factor antibody improves airway hyperresponsiveness by down-regulating RhoA

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ABSTRACT

Background: The pathogenesis of asthma is complex and continues to be considered as a challenging subject. Some studies have shown that nerve growth factor (NGF) participates in the pathogenesis of asthma, but the mechanism of airway contraction caused by NGF is still unclear. **Objective:** Our aim was to discuss the effect of anti-NGF antibody on RhoA expression, and further explore the role of NGF in airway hyperresponsiveness (AHR). **Methods:** Thirty female BALB/c mice were divided into three groups randomly: control group (group C, $n = 10$), asthma group (group A, $n = 10$) and anti-NGF antibody intervention group (group N, $n = 10$). The asthmatic mice were stimulated by OVA suspension, the intervention mice were given nasal instillation of anti-NGF antibody before the stimulation. Airway responsiveness, eosinophils, IL-13, IFN- γ were measured. The protein expression and mRNA level of NGF and RhoA were detected by immunohistochemical and Real Time-PCR (RT-PCR) analyses. **Results:** Airway responsiveness, eosinophils and IL-13 levels in group A were significantly increased compare with the other groups, and significantly decreased in group N than those in group A. IFN- γ level was significantly reduced in group A and increased in group N. Immunohistochemistry and RT-PCR analyses showed that the protein expression and mRNA level of NGF and RhoA were significantly increased in group A and significantly decreased in group N. **Conclusion:** NGF participates in the pathogenesis of asthma in mice. Anti-NGF antibody can inhibit airway inflammation and alleviate AHR by down-regulating the protein expression and mRNA level of RhoA.

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Nerve growth factor (NGF); RhoA; asthma; airway inflammation; airway hyperresponsiveness

Abbreviations

NGF nerve growth factor
AHR airway hyperresponsiveness
ASM airway smooth muscle

Introduction

Bronchial asthma (asthma) is a heterogeneous disease characterized by chronic inflammation (1,2). NGF (nerve growth factor) is a member of the neurologic factors family. The main function of NGF is control of the growth and differentiation of neurons (3), and it plays an important role in immunity, inflammation and the nervous system (4–6). As such, NGF has drawn attention in terms of the mechanisms for autoimmune diseases, immune deficiency disorders, chronic inflammatory diseases, allergies and asthma (7–11). Research has shown that NGF induces the generation of substance P by its high affinity receptor trkA (12), regulates the expression of SH2-B β (13) in airway smooth muscle (ASM), and plays a role

in asthma airway inflammation and airway hyperresponsiveness (AHR). Rho/ROCK signal transduction pathways play key roles in the mechanism of multiple smooth muscle and non-smooth muscle dysfunction-related diseases, such as hypertension, coronary heart disease, cerebrovascular disease, kidney disease and cancer metastasis. This broad impact has thus drawn widespread attention (14–17). The Rho/ROCK signaling pathway may cause ASM contraction and AHR through a Ca²⁺-dependent pathway (18); however, the exact mechanism is still unclear. The aim of this research was to explore the mechanism of NGF regulation of Rho/ROCK signal transduction pathways and its potential role in the pathogenesis of asthma.

Materials and method

Construction of the asthmatic model

The sample consisted of healthy female BALB/c mice (Beijing Weitong Lihua Experimental Animal Technological Company), aged from 4 weeks to 6 weeks, weighing

17 ± 2 g. The design and operation of the experiment were authorized by the Animal Ethics Committee, China Medical University. The rearing environment included a clean grade, 12-h light, a room temperature of 22~25°C, humidity at 40~70%, grain feeding and free drinking water. Thirty mice were divided into three groups randomly: control group (group C), asthma group (group A) and anti-NGF antibody intervention group (group N). On the 1st and 14th day, the mice in group A and group N were sensitized with an intra-peritoneal injection of OVA (ovalbumin, OVA, V grade, Sigma Company, USA) 20 µg together with 2 mg aluminum hydroxide; and at 25~29 days, the mice were stimulated with aerosolized 2% OVA in an atomization chamber, once a day, for 30 min each time. On the 1st and 14th day, the mice in group C were sensitized with an intra-peritoneal injection of the same volume of normal saline, at 25~29 days, the mice were stimulated with aerosolized normal saline. The mice in group N were given a drip of anti-NGF (Abcam Company, USA) antibody (1:50 dilution) 50 µL in the nasal cavity 30 min before each aerosolized stimulation, the mice in group A and group C had the same volume of normal saline injected intraperitoneally.

Evaluation of mice airway responsiveness

Using a non-invasive pneumatometer, the BALB/c mice were placed in body plethysmography in a natural state, and were first measured for pulmonary enhanced pause (Penh), then challenged with normal saline and concentrations of methacholine (Mch) aerosol ranging from 3.12, 6.25, 12.5, 25 to 50 g/L. The enhanced value of Penh was recorded, with every 30 seconds for an inhalation, at a 1-min interval, recorded over 2 min and traced in a real-time respiratory waveform.

Collection and detection of serum and BALF

Serum was extracted from the blood, which was obtained from the eyeballs of the mice. The left lung was lavaged with sterile saline three times and bronchoalveolar lavage fluid (BALF) was recycled (recoveries of 75~80%). We counted the percentage of eosinophils in a blood smear and BALF sediment smear. We also detected the concentration of IL-13, IFN-γ (Shanghai Lanji Biological Technology Company) in the blood smear and BALF by ELISA (Sandwich).

HE staining and immunohistochemistry of lung tissue

The upper lobe of the right lung was fixed with 4% paraformaldehyde for 4–6 h, embedded in paraffin and cut into slices. Lung tissue pathology changes were observed under a normal optical microscope through

Hematoxylin-eosin (HE) staining. With the immunohistochemical Strept Avidin-Biotin Complex (SABC) method, antigen was retrieved by a high-pressure method, using serum as a blocking agent and dropping an appropriate proportion of diluted first antibody (NGF first antibody working concentration is 1:100, the RhoA first antibody working concentration is 1:100) (Santa Cruz Company, USA). Primary antibody incubation was at 4°C overnight, where we added the biotin-labeled second antibody, and performed diaminobezidin (DAB) stain, counter stain and sealed the slides.

Detecting levels of NGF and RhoA mRNA in mice lung tissue by RT-PCR

Following the introduction of a high-purity total RNA quick extraction kit and one-step retroviral RT-PCR kit (Beijing Tiangen Biochemical Technology Company), we measured the Ct values of the sample. RNA template primer and probe sequences are NGF-F 5'-3' GATCGGC-GTACAGGCAGAAC, NGF-R 5'-3' TGGGC TTCAGGG-ACAGAGTCT, NGF-P 5'-3' ACACAGATAGCAATGTC; RhoA- 5'-3' TCCCGCCTGAGGCAGTTATA, RhoA-R 5'-3' ACCCTGCAGTCCAGGTGAGA, RhoA-P 5'-3' T-TTGAGGTGGCATAGC; GAPDH-F 5'-3' GATGCCC-CCATGTTTGTGA, GAPDH-R 5'-3' GCATTGCTGAC-AATCTTGAGTGA, GAPDH-P 5'-3' TGAA CCAC-GAGAAATA. We assessed $2^{-\Delta\Delta CT}$ in the untreated group and treatment group for comparison. $\Delta\Delta CT = (CT_{\text{targetgene}} - CT_{\text{internal}})_{\text{treatment}} - (CT_{\text{targetgene}} - CT_{\text{internal}})_{\text{targetgene}}$.

Statistical analysis

All results have been expressed as mean ± standard deviation (± SD). Graph Pad Prism software was used to analyze the normal distribution and equal variance of the data, a *t*-test was selected for comparison between the two groups, and analysis of variance was used in pair-wise comparisons among groups. Non-parametric tests were used for non-normal distribution. *p* < 0.05 was statistically significant for the difference.

Results

Evaluation of airway responsiveness in mice

Results from the spirometer showed that with increasing concentrations of Mch, the breathing curve of mice in each group demonstrated a prolonged expiratory phase. Group A was significantly longer than group C, and group N improved significantly compared with group A. Meanwhile, the Penh value for each group of mice rose with an increased concentration of Mch. The Penh value of

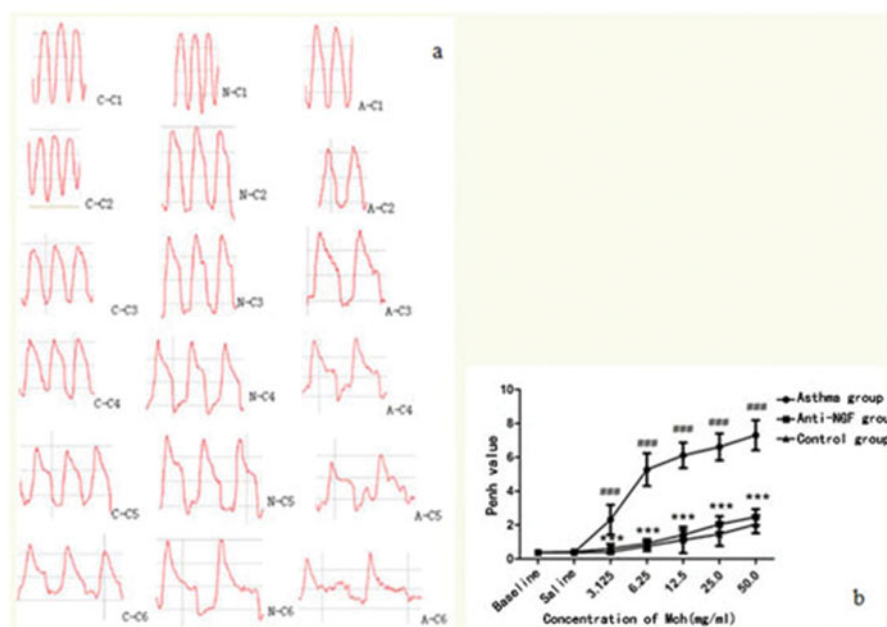


Figure 1. Mch breathing curve at different concentrations in mice (a). C represents control group, N represents anti-NGF intervention group, A represents asthma group, C1~C6 represents the different concentration of Mch, respectively, is 3.12, 6.25, 12.5, 25 and 50 g/L. Pneumatometer shows that with increasing concentrations of Mch, the breathing curve of mice in each group prolonged the expiration phase, group A was significantly longer than group C, and group N improved significantly compared with group A. Airway responsiveness Penh value curve with the increasing concentration of Mch in mice (b). ### The Penh value of group A increased significantly compared with group C ($p < 0.001$), *** the Penh value of group N decreased significantly compared with group A ($p < 0.001$).

group A increased significantly compared with group C ($p < 0.001$), and that of group N decreased significantly compared with group A ($p < 0.001$). The differences were statistically significant (Figure 1).

Serum and BALF eosinophil counts and ELISA detection

Compared with group C, the percentage of eosinophils in the blood smear and BALF sediment smear of group A was significantly increased ($p < 0.001$), and that of group N decreased significantly compared with group A ($p < 0.001$) (Figure 2). ELISA detection indicated that IL-13 levels in serum and BALF of group A were obviously

higher than group C ($p < 0.001$), while that of group N decreased significantly compared with group A ($p < 0.001$). IFN- γ levels in serum of group A were significantly lower than group C ($p < 0.001$), and that of group N increased significantly compared with group A ($p < 0.001$), IFN- γ levels in BALF of group A were lower than group C ($p < 0.01$), and that of group N increased significantly compared with group A ($p < 0.05$) (Figure 3).

HE staining and immunohistochemistry of lung tissue

HE staining of murine lung tissue showed that the lung tissue structure of the mice in group C was normal.

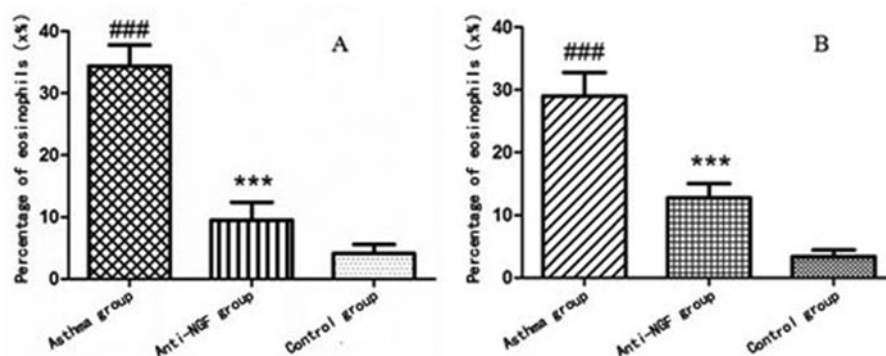


Figure 2. The percentage of eosinophils in the blood smear (A) and BALF smear (B). ### The percentage of eosinophils increased significantly in group A ($p < 0.001$), *** decreased significantly in group N ($p < 0.001$).

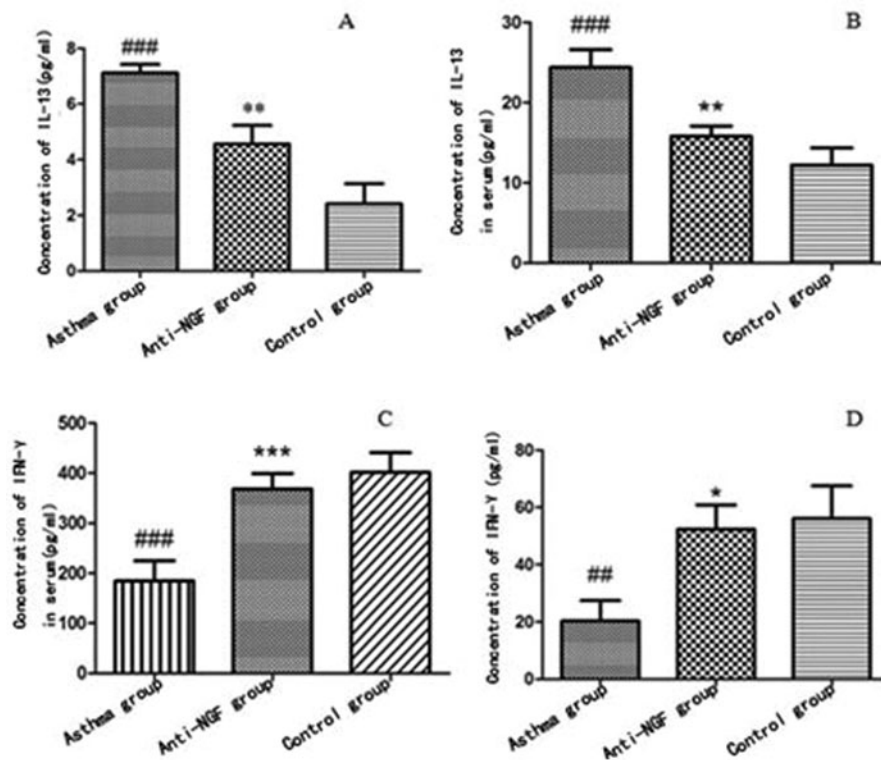


Figure 3. The IL-13 levels in serum (A) and BALF (B). ### Group A was much higher than group C ($p < 0.001$), ** group N was significantly lower than group A ($p < 0.01$). IFN- γ levels in serum (C) and BALF (D). ### Group A was significantly lower than group C ($p < 0.001$), ## Group A was obviously lower than group C ($p < 0.01$), *** Group N was much higher than group A ($p < 0.001$), * Group N was much higher than group A ($p < 0.05$).

However, the mice in group A had bronchial wall thickening, mucosal swelling, epithelial cell exfoliation, increased mucus secretion, and lumen stenosis. The pulmonary vascular wall was thickened with lumen stenosis. Around the airway, the epithelial cells, bronchi and blood vessels showed macrophages, eosinophils, lymphocytes and neutrophil infiltration. Compared with group A, the inflammation in Group N was significantly alleviated. Immunohistochemistry showed that NGF and RhoA were positively expressed in cytoplasm, showing brownish yellow, and were mainly located in bronchial epithelium cells, alveolar epithelial cells and macrophages in the murine lung tissues. The results showed that the expression of NGF and RhoA in group A lung tissue was significantly higher than group C ($p < 0.001$) and group N ($p < 0.001$) (Figure 4).

Detection of NGF and RhoA mRNA levels in murine lung tissue by RT-PCR

RT-PCR testing found that the level of NGF and RhoA mRNA was different in each group murine lung tissue. The level of group A was significantly higher than group C ($p < 0.001$), while that of group N was significantly lower than group A ($p < 0.001$) (Figure 5).

Discussion

Asthma is a chronic inflammatory airway disease, which is accompanied by a variety of cell and cellular components, characterized by reversible airflow limitation and AHR, and has a complicated pathogenesis. Recent studies have shown that the occurrence and persistence of asthma are closely related to immune, inflammatory and neurological mechanisms. NGF works as a contact of the three intermediary substances and has gained widespread attention in the pathogenesis of asthma. Studies by Hahn C (19) and other research have shown that high expression levels of NGF could enhance the vitality of eosinophils, and thus enhance eosinophil inflammation in the airway caused by allergens, and participate in the development of AHR in mice. Ogawa HC (20) found that in a chronic asthmatic murine model constructed by dust mite, the high expression of NGF and substance P in bronchial and alveolar epithelial cells could be inhibited by NGF targeted siRNA. Studies also found that increased expression of NGF mRNA in asthmatic rat cells was always accompanied by a high level of α -SMA mRNA, and the immune response of fibroblasts and smooth muscle cells was intensified (21). However, the specific mechanistic role of NGF is still unclear. In the Ras super family, Rho family proteins are the first

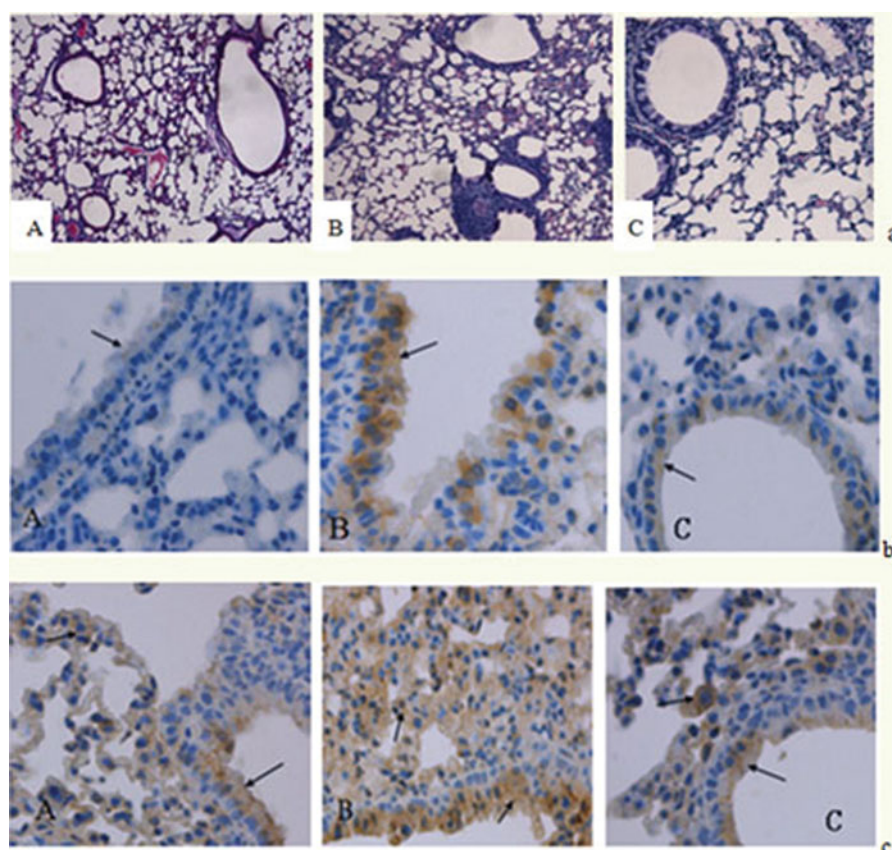


Figure 4. HE staining of mice lung tissue (a). Control group (A) had normal lung tissue structure; asthma group (B) showed bronchial wall thickening, mucosal swelling, lumen stenosis, and macrophages, eosinophils, lymphocyte and neutrophil infiltration could be seen around the bronchial. Anti-NGF intervention group (C) was much lower than asthma group (light microscope $\times 100$). Immunohistochemistry showed NGF (b), RhoA (c) positively expressed in the cytoplasm, tan, mainly distributed in the bronchial epithelium and macrophages. The control group (A) expressed weakly, asthma group (B) expressed strongly, anti-NGF intervention group (C) was much lower than asthma group ($\times 400$).

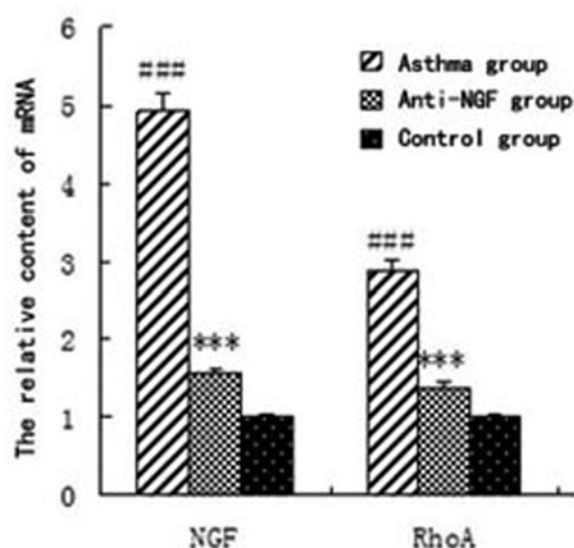


Figure 5. Detect the relative content of NGF and RhoA mRNA by RealTime RT-PCR. ***Asthma group significantly increased compared with control group ($p < 0.001$), ***anti-NGF antibody intervention group was much lower than asthma group ($p < 0.001$).

proteins to be cloned. RhoA is one of the members of the Rho GTP enzyme family, which is a key molecule in cell signal transduction pathways and act as a bridge in intracellular signal transduction. Studies have also found that the expression of RhoA increased in OVA sensitized asthmatic guinea pigs. Rho kinase inhibitors can inhibit its expression and improve airway inflammation and the contraction of smooth muscle (22).

Our data showed an increased level of IL-13 and eosinophils, and a decreased level of $\text{INF-}\gamma$ in the serum and BALF of the OVA-induced asthmatic mice. Immunohistochemistry and RT-PCR analysis demonstrated that the protein expression and mRNA level of NGF and RhoA were significantly increased in the lung tissue of the asthmatic mice model. OVA caused an imbalance ratio of Th1 and Th2 cells in mice which resulted in airway inflammation, and elevated the expression of NGF and RhoA, which increased the activity of its downstream effector Rho-associated kinase (ROCK). ROCK can phosphorylate MLCP and induce ASM contraction (23). OVA may potentially lead to asthma by Rho/ROCK pathway (24).

Our study suggests that the percentage of eosinophils and level of IL-13 have declined in murine serum and BALF after nasal instillation of anti-NGF antibody. Meanwhile, INF- γ level has increased significantly, the protein expression and mRNA level of RhoA have significantly decreased in the murine lung tissue. In summary, our study illustrates that anti-NGF antibody may inhibit the overexpression of RhoA at the gene transcription level, preventing the contraction of ASM (25) by adjusting the balance of Th1 and Th2 cells.

In conclusion, our study provides evidence that NGF participates in the pathogenesis of OVA-induced murine asthma. In addition, it suggests that anti-NGF antibody may improve AHR, relieve the onset of asthma in mice by down-regulating the RhoA pathway. Currently, our pharmacological data were obtained from mice which also have limitations to human, it still needs further confirmations.

Declaration of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

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