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Inhibition of nerve growth factor/tyrosine kinase receptor A signaling ameliorates airway remodeling in chronic allergic airway inflammation

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Abstract. – OBJECTIVE: The molecular mechanism leading to airway remodeling in patients with allergic asthma is not fully understood. We determined the role of nerve growth factor/tyrosine kinase receptor A signaling in airway remodeling in chronic allergic airway inflammation, and proved that inhibited nerve growth factor (NGF) production ameliorates airway remodeling during chronic allergic airway inflammation.

MATERIALS AND METHODS: Six- to eight-week-old female BALB/c mice were used in this study. Mice were randomized into four groups: phosphate buffer saline (PBS) control group (n = 10); chronic asthmatic group (n = 12); anti-NGF group (n=12); and anti-TrkA group (n=12). First, to determine the impact of NGF on airway remodeling, antibody-blocking experiments were performed in a chronic allergic murine model characterized by matrix deposition in the subepithelial. Secondly, the number of eosinophils, macrophages, neutrophils and the total number of cells in bronchoalveolar lavage fluid (BALF) was counted. Thirdly, growth-associated protein 43 (GAP43) and NGF protein expression was measured by western blot.

RESULTS: It was shown that the number of eosinophils and the total inflammatory cells, NGF and GAP43 protein expression in BALF were markedly higher in asthma group, compared to the other groups. And given anti-NGF or anti-TrkA antibody treatment can reduced GAP43 expression and collagen deposition in the airway.

CONCLUSIONS: NGF triggers wound healing process and airway remodeling by inducing GAP43 production dependent on TrkA in a mouse model of chronic experimental asthma. Controlling epithelial NGF production might be an efficient therapeutic target to prevent allergic asthma.

Key Words:
Chronic allergic airway inflammation, Airway remodeling, Growth-associated protein 43, Nerve growth factor.

Introduction

Asthma is pathophysiologically characteristic of chronic airway inflammation, airway hyper-responsiveness and airway remodeling. A sustained irritant stimulates the airway epithelial cells to produce growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines, which cause the excessive collagen deposition in subepithelial, following by airway remodeling and at last cause the function loss of airway[1,2]. The repeated airway remodeling, as a result of unbalance wound-healing processes aggravate the airway damage[3].

Nerve growth factor (NGF), as a prototypic member of the neurotrophic factor family, was originally found to promote survival and differentiation of neuronal cells. There is increasing evidence that NGF played a role in chronic allergic airway disease. It has recently been shown that NGF plays a critical role in inflammatory interactions among immune, neuronal, and structural cells in pathogenesis of chronic allergic asthma[4,5]; And the NGF functions by binding to tyrosine kinase receptor A (TrkA), the high-affinity receptor of NGF[6,7].

Growth-associated protein 43 (GAP43), a nervous tissue-specific cytoplasmic protein, is considered to play a key role in neurite formation, regeneration and plasticity. And normally, GAP43 expresses in a very low level in the peripheral nerves, and its overexpression prompts regeneration of peripheral nerve lesions. Therefore, GAP43 has been considered to be a well-established marker for nerve plasticity. Recent studies have shown that excessive NGF administration can promote GAP43 production and start the early phase of peripheral nerve regeneration and remodeling in several injury tissues[8].

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In this work, to determine whether the inhibited NGF production ameliorates airway remodeling during chronic allergic airway inflammation, we used a chronic allergic airway inflammation murine model after low-dose and repeated inhalation of OVA to demonstrate the NGF/TrkA signaling pathway induces GAP43 production and involveds in the airway wound healing.

Materials and Methods

Animal Experiments

Six- to eight-week-old female BALB/c mice from the Laboratory Animal Center, Dalian Medical University, were used in this study. Mice were randomized into four groups: phosphate buffered saline (PBS) control group (n = 10); chronic asthmatic group (n = 12); anti-NGF group (n=12); and anti-TrkA group (n=12). In the chronic asthmatic model group, mice were sensitized with an intraperitoneal injection of 10 μg ovalbumin (OVA; Grade V, Sigma, St Louis, MO, USA), adsorbed in 1.5 mg aluminium hydroxide (Pierce, Rockford, IL, USA), dissolved in 200 μL of phosphate buffered saline (PBS) at days 0, 14 and 21. Then, 1% OVA aerosol challenges were conducted three times a week, from 1 to 8 weeks. The control mice were conducted with PBS in a same way. All tests were conducted 24 hours post the last aerosol inhalation of OVA or PBS. Aerosolized OVA or PBS was generated using a jet nebulizer (PARI, Starnberg, Germany). Mice in anti-NGF group or anti-TrkA group were administered anti-NGF or anti-TrkA treatment, 3h before the OVA challenge. And the anti-NGF or anti-TrkA treatment was conducted with an injection of nasal cavity in a dose of 4 mL/kg of antibody (anti-NGF or anti-TrkA antibody was diluted to final concentration 0.1 mg/L or 0.2 mmol/L) (Figure 1). All of the animal experiments and operating procedures were approved by the Animal Ethics Committee.

Bronchial Responsiveness

Mice responsiveness to β-methacholine (MCh) was assessed by whole-body plethysmography (EMKA, Paris, France) and by measuring increases in enhanced pause (Penh) as an index of airway obstruction. PBS or various doses of MCh (1-100 mg/mL) were nebulized into the nasal chamber for 1.5 min. Airway reactivity was expressed as the fold increase in Penh for each MCh concentration, compared with the Penh value after PBS challenge.

Bronchoalveolar Lavage Fluid (BALF) Collection

BALF was collected three times with 0.8 mL of saline. 70 to 80% of the liquid was routine recovered. The number of total or different type of cells were calculated. The concentrations of IL-4, IL-5 and IL-13 were determined by ELISA(BD, Franklin Lakes, NJ, USA) in the cell-free fluid, according to the manufacturer's instructions. Sensitivity for all of the assays was 1 pg/mL.

Histopathology

In order to make the alveolar expansion and flattening before fixing, the right lung of each

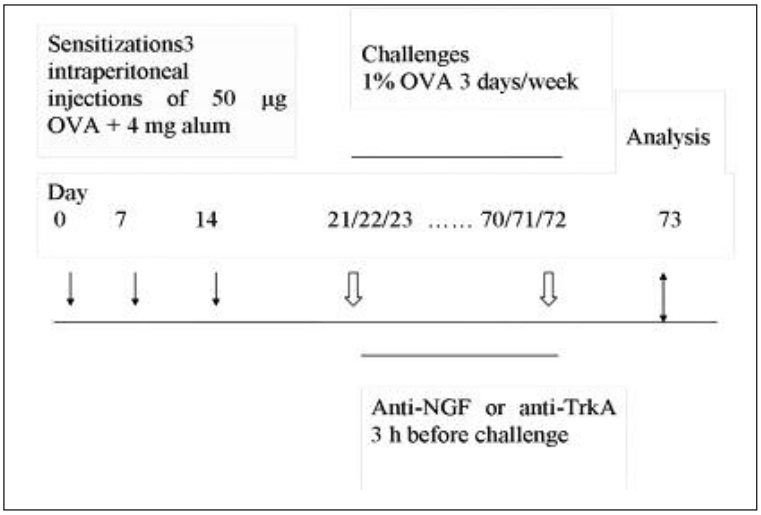


Figure 1. Neutralization of nerve growth factor (NGF) in experimental chronic asthma. Mice were immunized with three intraperitoneal injections of OVA/AL (OH) on days 0, 7 and 14. OVA challenge was performed three consecutive times per week at the indicated time points. Antibodies were applied 3 h before each challenge period by intraperitoneal injection. Animals were analysed 24 h after the final challenge. TrkA, tyrosine kinase receptor A.

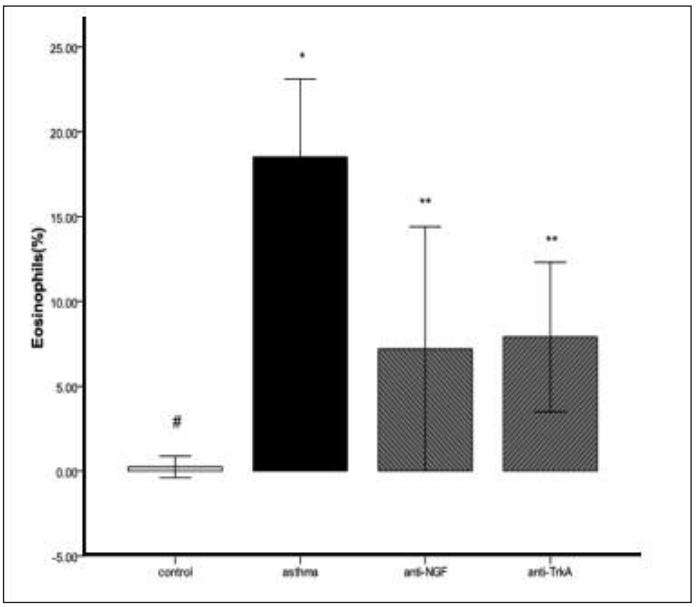


Figure 2. Eosinophil cell percentages in bronchoalveolar lavage fluid (BALF). Eosinophil counts in BALF were significantly greater in the chronic experiment asthma group than in the control group and mice treated with anti-nerve growth factor (NGF), anti-tyrosine kinase receptor A (TrkA) antibodies (n = 6 per group). *$p < 0.01$ versus control; **$p < 0.05$ versus asthma.

mouse was routinely perfused with 4% paraformaldehyde then the whole lung was fixed in 4% paraformaldehyde. After paraffin embeding, the tissues were sectioned (5 μm) for HE and Masson staining for pathological assessment by microscopy.

Western Blotting Analysis

A total of 100 μg of lung homogenates was prepared in dodecyl sulfate, sodium salt (SDS) sample buffer and subjected to (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were transferred onto a nitrocellulose membrane and immunostained with rabbit polyclonal monoclonal antibodies against mouse NGF, GAP43 and β-actin (ab6199, ab11136, ab15263, Abcam, Cambridge, UK). Goat goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody, conjugated to horseradish peroxidase and enhanced chemiluminescence detection systems (Super Signal West Femto; Pierce) were used for detection.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 Edition software (IBM, Armonk, NY, USA). Differences among groups were determined using Friedman's one-way ANOVA for multiple comparisons between groups. Data were expressed as means±SE and $p < 0.05$ for the difference was statistically significant.

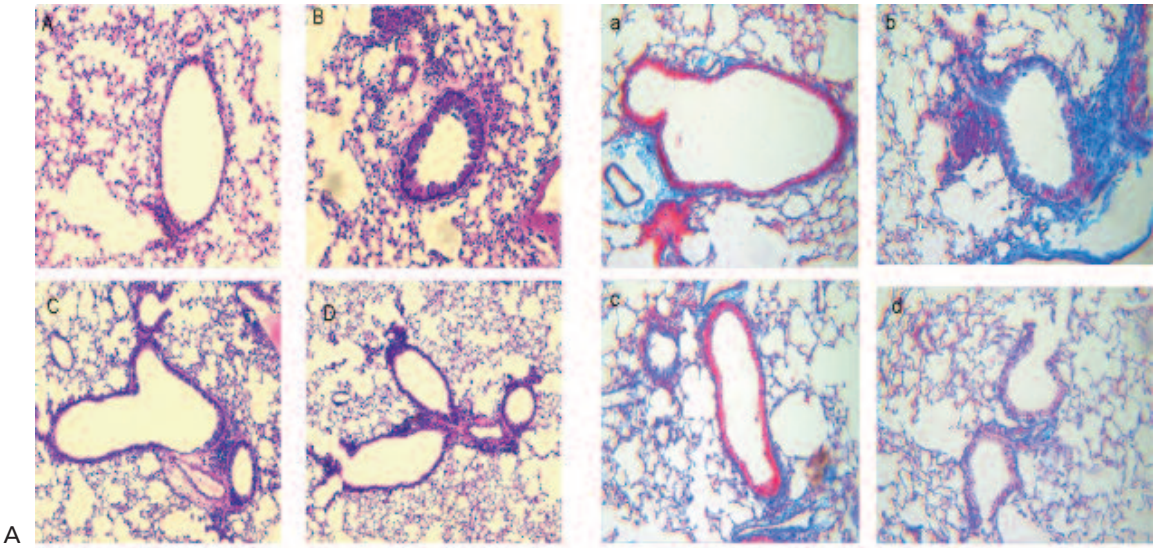
Results

Chronic Asthma of Mice After OVA Aerosol Challenges

OVA challenges caused a thickening of the subepithelial wall of bronchi and inflammatory cell infiltration, as observed in HE and Masson staining (Figure 1), which is typical of airway inflammation. The number of total or different type of cells in mice BALF were calculated, and it was shown that lymphocytes and eosinophils in BALF were significantly increased in the chronic asthma group than in control group (Figure 2). And also, there were pathological change of chronic asthma in the airway of mice in chronic asthma group: subepithelial collagen deposition and subepithelial fibrosis were significant (Figure 3).

Anti-NGF and Anti-TrkA Treatment Markedly Prevented the Airway Inflammatory Response

IL-4, IL-5 and IL-13 concentration released into BALF were measured by ELISA. The OVA inhalation can remarkable increase their concentration in BALF, and intraperitoneal injection of anti-NGF and anti-TrkA pretreatment can prevent this change (Figure 4). We measured Penh as an indicator of bronchial responsiveness with unrestrained body plethysmography. Bronchial hyperresponsiveness was augmented in the asthma group. the data showed that anti-NGF and anti-TrkA treatment could alleviate bronchial hyperresponsiveness due to OVA inhalation to the basal level (Figure 5).



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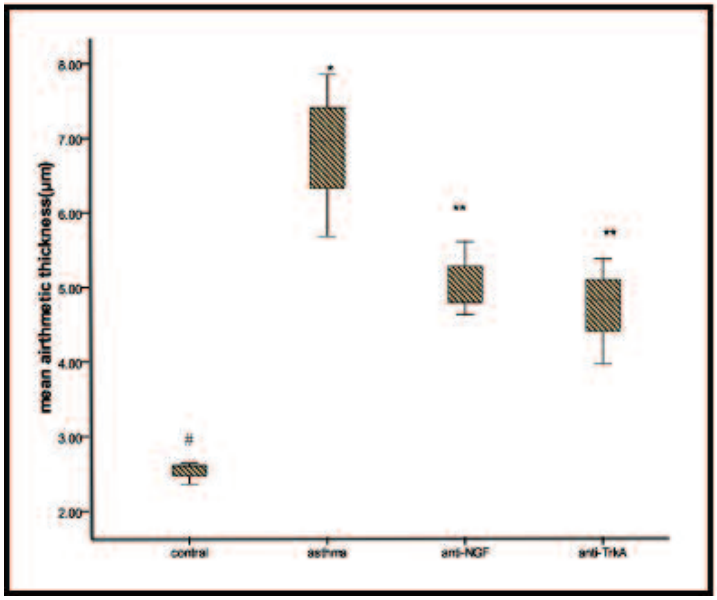

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Figure 3. Neutralization of nerve growth factor (NGF) in ovalbumin (OVA)-induced experimental chronic asthma suppressed airway inflammation and subepithelial collagen deposition. **A**, Lung sections stained for HE (magnification ×200) and Masson (magnification × 200). **B**, Stereological quantification of subepithelial collagen layer thickness (**C**) (n = 6 per group). *p < 0.01 versus control; **p < 0.05 versus asthma. TrkA, tyrosine kinase receptor A.

Anti-NGF and Anti-TrkA Treatment Markedly Inhibited Subepithelial Fibrosis in Experimental Chronic Asthma

Nerve growth factor levels were increased in the chronic asthma group, accompanied by subepithelial collagen deposition, peribronchial fibrosis (Figure 6). Nerve growth factor also mediated TrkA-dependent increase in GAP43 in experimental chronic asthma (Figures 6, 7). To investigate the role of NGF, we treated chronic allergic mice with two different antibodies: anti-NGF and anti-TrkA. It was shown that anti-NGF or anti-TrkA treatment could markedly inhibit airway remodeling, by ameliorating subepithelial collagen deposition, peribronchial fibrosis (Figure 3), as confirmed through quantification of peribronchial collagen layer thickness (Figure 3).

Discussion

The 5-10% of patients suffering from severe asthma and accordingly bring higher disease-related mortality[9,10]. Airway remodeling was found to occur in all serious asthma, and the degree of reticular basement membrane collagen deposition was correlated with the severity of the dis-

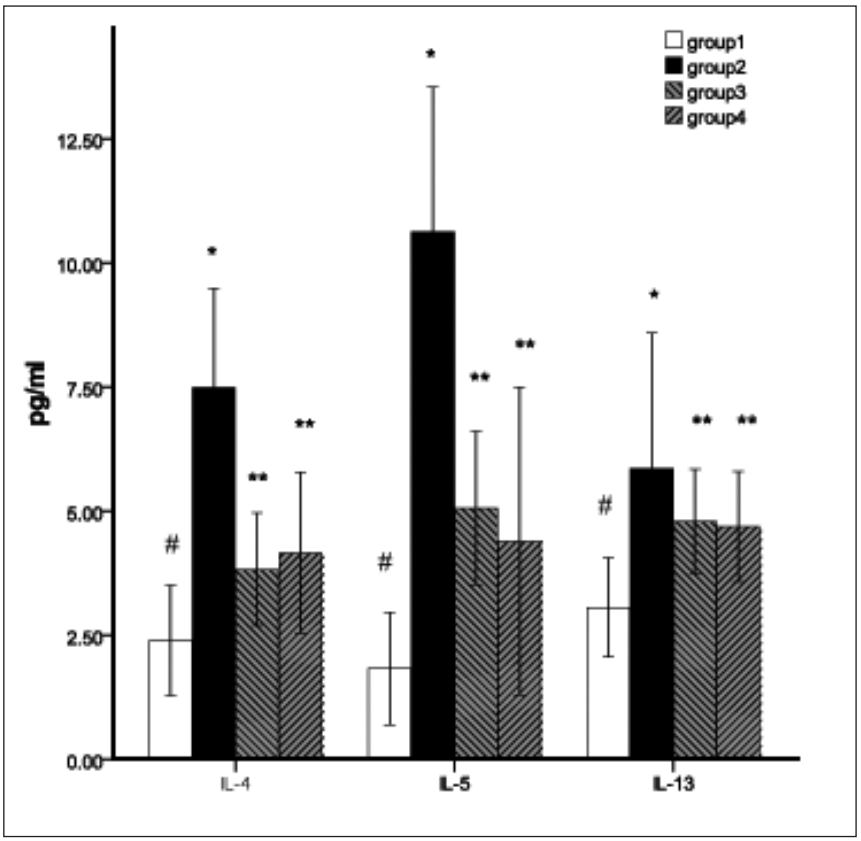

Figure 4. T helper 2 (Th2)cytokines released into bronchoalveolar lavage fluid were measured by ELISA. Interleukin (IL)-4, IL-5 and IL-13 were significantly increased by ovalbumin (OVA) inhalation, and these increases were significantly attenuated by anti-nerve growth factor (NGF) and anti-tyrosine kinase receptor A (TrkA) pretreatment (n = 6 per group). *p < 0.01 versus control; **p < 0.05 versus asthma.

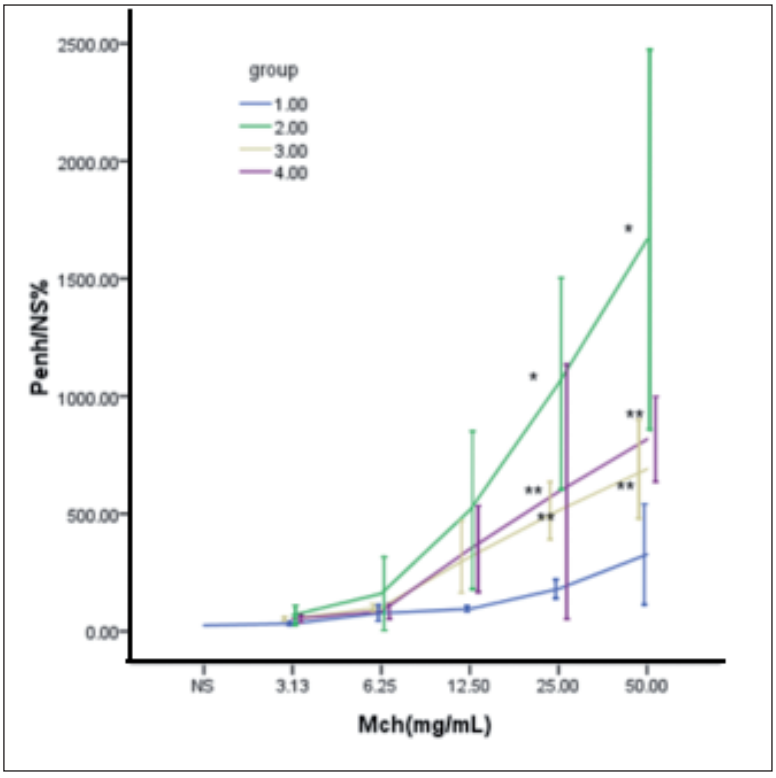

Figure 5. Nerve growth factor (NGF) inhibitors reduce methacholine (MCh)-induced bronchial hyperresponsiveness in ovalbumin (OVA)-challenged mice. (a) The dose-response curves of MCh-induced bronchoconstriction in each group animals were expressed as fold increase in enhanced pause (Penh) for each concentration of MCh (n = 6 per group). *p < 0.01 versus asthma; ** p < 0.05 versus control.

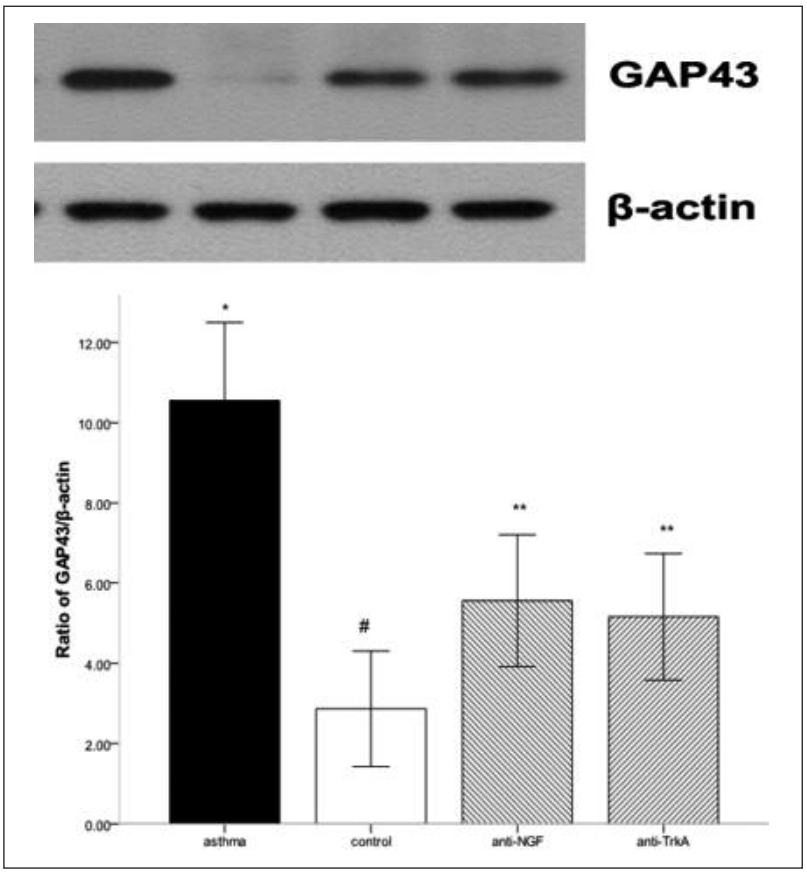

Figure 6. Expression of growth-associated protein 43 (GAP43) in lung tissue of mice detected by western blotting ($n = 6$ per group). *$p < 0.01$ versus control; **$p < 0.05$ versus asthma. NGF, nerve growth factor; TrkA, tyrosine kinase receptor A.

ease[11]. In the process of inflammatory response and tissue damage, growth factors secreted increasingly from the inflammatory cells or tissue cells and started subsequent wound healing process[12]. Therefore, a balance of inflammatory interactions among immune, neuronal, and local tissue cells is necessary for an appropriate process of recuperation and repair[13]. However, during chronic persistent inflammation and injury, this balance is disrupted lead to exaggerated pathological processes, causing airway remodeling[14,15]. Previous studies have found that the concentration of NGF was increased in the serum and BALF of patients with chronic allergic asthma, and this increase was positively correlated with disease severity[16]. Airway epithelial cells were the major source of NGF in the chronic airway inflammation. NGF production can be unregulated by IL-1β, TNF-α, and TH2 cytokines[17]. And NGF and its receptor has been described to play an important role in the early responses during the process of wound healing[18,19].

In this research, we first built an OVA-induced mice model of experimental asthma. It was shown by others[20] that NGF played a role in the airway remodeling of asthma, and GAP43 production and participates in airway remodeling. And the role of NGF is achieved by binding to TrkA, NGF/TrkA singling pathway can induce GAP43 production then regulate neuronal plasticity[21]. In our model, OVA challenge can cause nerve growth factor and its receptor TrkA protein expression in bronchial. We also found a corresponding increase in GAP43 protein expression and subepithelial collagen deposition, and peribronchial fibrosis and other pathological changes of airway remodeling.

To further prove the role of NGF in the airway remodeling of asthma, the treatment of anti-NGF and anti-TrkA were conducted, and both treatments could inhibit GAP43 production and the subsequent airway remodeling. In this work and our previous studies, we found that NGF expression in the immune and structural cells of the airways, such as bronchial epithelial cells, airway smooth muscle cells and alveolar macrophages[22]. All the evidence demonstrates that NGF signaling pathway mediated airway remodeling of

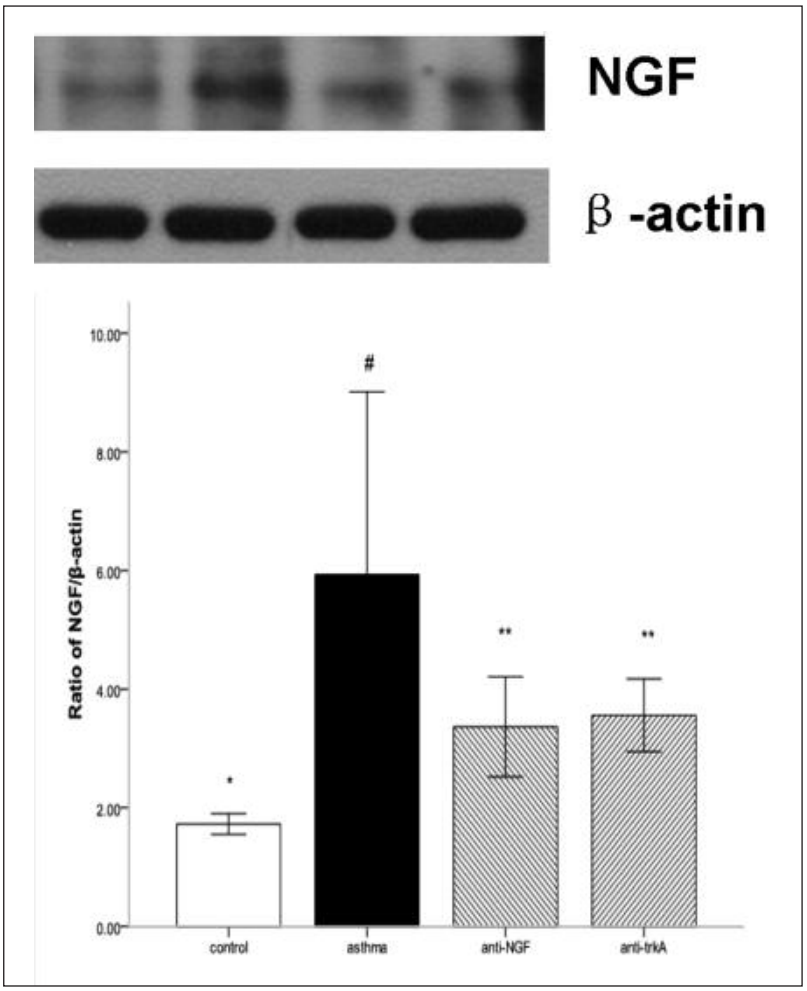

Figure 7. Expression of nerve growth factor (NGF) protein in lung tissue of mice as detected by western blotting ($n = 6$ per group). *$p < 0.01$ versus control; **$p < 0.05$ versus asthma. TrkA, tyrosine kinase receptor A.

chronic bronchial asthma, and interrupted the uncontrolled regulation may be an effective method for the treatment of bronchial asthma[23,24].

Conclusions

We demonstrated a novel function and singling pathway of NGF in allergic asthma, that contributes a key role to airway remodeling. Chronic allergic airway inflammation can lead to further changes in the structure of the airway, and this change is associated with increased NGF levels[25-27]. In chronic experimental asthma model, the concentration of NGF was significantly increased. This change not only causes chronic allergic airway inflammation, stimulates airway hyperresponsiveness but also leads to further airway remodeling[28-31]. Our data provide evidence of a novel therapeutic approach to airway remodeling in chronic allergic airway inflammation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Ta Wynn. Cellular and molecular mechanisms of fibrosis. J Pathol 2008; 214: 199-210.
2) Halwan R, Al-Muhsen S, Hamid Q. Airway remodeling in asthma. Curr Opin Pharmacol 2010; 10: 236-245.
3) Sonar SS, Schwinge D, Kolic A, Yildirim AO, Conrad ML, Seidler K, Muller B, Renz H, Nockher WA. Nerve growth factor enhances Clara cell proliferation after lung injury. Eur Respir J 2010; 36: 105-111.
4) Hahn C, Islamian AP, Renz H, Nockger WA. Airway epithelial cells produce neurotrophins and pro mote the survival of eosinophils during allergic airway inflammation. J Allergy Clin Immunol 2006; 117: 787-794.
5) NGEOW WC, Scar less: a review of methods of scar reduction at sites of peripheral nerve. Oral Maxillofac Surg 2010; 109: 357-366.

6) Freund-Michel V, Bertrand C, Frossard N. TrkA signalling pathways in human airways mooth muscle cell proliferation. Cell Signal 2006; 18: 621-627.
7) Nakamura T, Komiyam, Sone K, Hirose E, Gotoh N, Morii H, Ohta Y, Mori N. Grit a GTPase-activating protein for the Rho family, regulates neurite extension through association with the TrkA receptor and N-Shc and CrkL/Crk adapter molecules. Mol Cell Biol 2002; 22: 8721-8734.
8) Perrone-Bizzozero NI, Neve RL, Irwin N, Lewis S, Fischer I, Benowitz LI. Post-transcriptional regulation of GAP-43 mRNA levels during neuronal differentiation and nerve regeneration. Mol Cell Neurosci 1991; 2: 402-409.
9) Kilic A, Sonar SS, Yildirm AO, Fehrenbach H, Nockher WA, Renz H. Nerve growth factor induces type III collagen production in chronic allergic airway inflammation. J Allergy Clin Immunol 2011; 128: 1058-1066.
10) Päth G, Braun A, Meents N, Kerzel S, Quarcoo D, Raap U, Hoyle GW, Nockher WA, Renz H. Augmentation of allergic early-phase reaction by nerve growth factor. Am J Respir Crit Care Med 2002; 166: 818-826.
11) Yildirim AO, Veith M, Rausch T, Müller B, Kilb P, Van Winkle LS, Fehrenbach H. Keratinocyte growth factor protects against Clara cell injury induced by naphthalene. Eur Respir J 2008; 32: 694-704.
12) Alcorn JF, van der Velden J, Brown AL, McElhinney B, Irvin CG, Janssen-Heininger YM. c-Jun N-terminal kinase 1 is required for the development of pulmonary fibrosis. Am J Respir Cell Mol Biol 2009; 40: 422-432.
13) Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. FASEB J 2003; 17: 1576-1578.
14) Allodi I, Udina E, Navarro X. Specificity of peripheral nerve regeneration: interactions at the axon level. Progr Neurobiol 2012; 98: 16-37.
15) Olgart C Frossard N. Human lung fibroblasts secrete nerve growth factor: effect of inflammatory cytokines and glucocorticoids. Eur Respir J 2001; 18: 115-121.
16) Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). J Exp Med 2001; 194: 809-821.
17) Virchow JC, Julius P, Lommatzsch M, Luttmann W, Renz H, Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am J Respir Crit Care Med 1998; 158: 2002-2005.
18) Nithya M, Suguna L, Rose C. The effect of nerve growth factor on the early responses during the process of wound healing. Biochim Biophys Acta 2003; 1620: 25-31.
19) Kassel O, De Blay F, Duvernelle C, Olgart C, Israel-Biet D, Krieger P, Moreau L, Muller C, Pauli G, Frossard N. Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated in halation of allergen at low-dose. Clin Exp Allergy 2001; 31: 1432-1440.
20) John B Denny. Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43 current. Neuropharmacol 2006; 4: 293-304.
21) Freund-Michel V, Bertrand C, Frossard N. TrkA signalling pathways in human airway smooth muscle cell proliferation. Cell Signal 2006; 18: 621-627.
22) Li L, Kong L, Fang X, Jiang C, Wang Y, Zhong Z, Sun Q, Gu G, Zheng D, Meng R, Kang J. SH2-B beta expression in alveolar macrophages in BAL fluid of asthmatic guinea pigs and its role in NGF-TrkA-mediated asthma. Respirology 2009; 14: 60-68.
23) Kobayashi H, Gleich GJ, Butterfield JH, Kita H. Human eosinophils produce neurotrophins and secrete nerve growth factor on immunologic stimuli. Blood 2002; 99: 2214-2220.
24) Freund V, Pons F, Joly V, Mathieu E, Martinet N, Frossard N. Upregulation of nerve growth factor expression by human airway smooth muscle cells in inflammatory conditions. Eur Respir J 2002; 20: 458-463.
25) Hasan W, Zhang R, Liu M, Warn JD, Smith PG. Coordinate expression of NGF and alpha-smooth muscle actin mRNA and protein in cutaneous wound tissue of developing and adult rats. Cell Tissue Res 2000; 300: 97-109.
26) Nassenstein C, Braun A, Erpenbeck VJ, Lommatzsch M, Schmidt S, Krug N, Luttmann W, Renz H, Virchow JC Jr. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 are survival and activation factors for eosinophils in patients with allergic bronchial asthma. J Exp Med 2003; 198: 455-467.
27) Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosc 2003; 4: 299-309.
28) Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. N Engl J Med 2001; 345: 517-552.
29) Lutz M, Krieglstein K, Schmitt S, Ten Dijke P, Sebald W, Wizenmann A, Knaus P. Nerve growth factor mediates activation of the S mad pathway in PC12 cells. Eur J Biochem 2004; 271: 920-931.
30) Wegmann M, Fehrenbach H, Fehrenbach A, Held T, Schramm C, Garn H, Renz H. Involvement of distal airways in a chronic model of experimental asthma. Clin Exp Allergy 2005; 35: 1263-1271.
31) Pardo A, Selman M. Matrix metalloproteases in aberrant fibrotic tissue remodeling. Proc Am Thorac Soc 2006; 3: 383-338.