



ANP/NPRA Inhibits Epithelial-Mesenchymal Transition of Airway by Targeting Smad3 in Asthma

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Abstract

Atrial natriuretic peptide (ANP) has a protective effect on allergic disorders of airway. It could inhibit transforming growth factor beta 1 (TGF- β 1)-induced epithelial-mesenchymal transition (EMT) of human airway cells. In this study, we investigated the role and mechanism of ANP in airway EMT with asthma. Asthma model was established with BALB/c mice using ovalbumin. Asthmatic mice were respectively treated with ANP, NPRA antagonist (A71915) or ANP+A71915. The protein expressions of TGF- β 1, ANP, epithelial cadherin (E-Cadherin), α -smooth muscle actin (α -SMA), Smad3 and phosphorylated-Smad3 in lung tissues were detected by immunohistochemistry and Western blotting. The mRNA expressions of E-Cadherin, α -SMA, Smad3 in lung tissues were measured using real-time PCR. The levels of TGF- β 1, IL-4 and IL-5 in bronchoalveolar lavage fluid, cyclic guanosine monophosphate (cGMP) in lung tissues, and ANP in serum were assessed by ELISA. Compared with controls, mRNA and protein expressions of E-Cadherin were decreased, whereas α -SMA were increased in asthma group, which could be reversed by ANP. cGMP level was higher in ANP group than asthma group, whereas p-Smad3 expression was lower. TGF- β 1, IL-4 and IL-5 levels were higher in asthma group than controls, which could be reversed by ANP. The effect of ANP in asthma group could be inhibited by A71915. Therefore, in asthma, ANP/NPRA could inhibit airway EMT by targeting Smad3 via attenuating phosphorylation of Smad3. cGMP signaling may be involved into this process. This may provide a potential of ANP in treatment on refractory asthma with airway remodeling.

Keywords Atrial natriuretic peptide · Asthma · Epithelial-mesenchymal transition · Smad3

Introduction

Atrial natriuretic peptide (ANP) is a polypeptide hormone that exerts cardioprotective functions (Ferreira et al. 2017). ANP has a bronchoprotective effect against bronchoconstriction after allergic reaction in guinea pigs (Mizuguchi et al. 2000), showing a protective effect in allergic disorders of airway. It was further found to play a protective role against allergen-induced airway hyperresponsiveness in mice model (Kumar et al. 2002). Thus, we supposed that ANP/natriuretic peptide receptor A (NPRA) signaling may play a protective role in allergic asthma (Ma and Xiang 2011).

Asthma affected about 300 million people worldwide in 2011 and accounts for 250,000–345,000 death each year (Vos et al. 2012; Lozano et al. 2012). However, there is dilemma in the treatment that a number of patients can't control the disease according to recent strategy. It's a pity

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that the pathogenesis of asthma is still not very clarified (van Buul and Taube 2015). In our previous work, we found that ANP and NPRA were increasingly presented in blood from asthmatic patients compared with healthy controls, and increased when asthma aggravated (Yu et al. 2016). It has been confirmed in murine model (Ma et al. 2015). However, it remains unclear that how ANP/NPRA participate in the protection against asthma. In our previous work, we demonstrated that ANP could inhibit TGF- β 1-induced epithelial-mesenchymal transition (EMT) in both human bronchial and alveolar epithelial cells (Chu et al. 2017). Since airway EMT contributes to refractory asthma, we suppose that ANP may have a protective role on asthma via inhibiting airway EMT. In this study, we investigated the role of ANP in airway EMT of asthma and its possible mechanism using murine model.

Methods

Mice

Female BALB/c mice (6–8 weeks old, weighing 20 ± 2 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd (Hunan, China). They were provided with an ovalbumin (OVA) free diet and water ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of the Affiliated Hospital of Guilin Medical University. All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care and Use Committee of the Affiliated Hospital of Guilin Medical University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

To establish model, mice were sensitized by intraperitoneally injection of 0.01 mg OVA, which was (Grade V; Sigma, MA, USA) emulsified in 2 mg of aluminum hydroxide gel, in a total volume of 200 μ l on the first and 13th day as previously described (McMillan and Lloyd 2004). Mice were challenged with aerosolized 5% OVA for 30 min every day from 19th to 24th day, and three times every week from 25th to 56th day. In control group, normal saline replaces OVA in equal volume. Asthmatic mice were challenged with ANP (1 μ g/g mouse), and/or intraperitoneally injected exogenous NPRA antagonist (A71915, 0.5 μ g/g mouse, Bachem, CA, USA) 30 min before each OVA aerosol challenge from 25th to 56th day. Fifteen mice in each group.

Quantitative Real-Time PCR

Total RNA from lung tissues was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's

protocol, and RNA was reverse transcribed into cDNA (Fermentas, Ontario, Canada). A quantitative real-time polymerase chain reaction (PCR) was performed with PIKO REAL 96 instrument (Thermo, MA, USA) and the SYBGREEN PCR Mix Mix (Applied Biosystems, CA, USA) in accordance with the manufacturer's protocol. Each set of experiments was repeated three times. The 30 μ l PCR reactions (with 2 μ l cDNA, 0.5 μ l (10 μ M) forward and 0.5 μ l (10 μ M) reverse primers, 15 μ l SYBR green, and 12 μ l PCR H₂O) were undergone 10 min at 95 $^{\circ}$ C, then 40 cycles of 15 s at 95 $^{\circ}$ C and 60 s at 60 $^{\circ}$ C. The primers were as follows: mouse NPRA F, 5'-TGCAGAATGAGCACTTGACCAG-3', and R, 5'-CGTTGTGTAGAAAGAGCATTCCT-3'; mouse epithelial cadherin (E-Cadherin) F, 5'-CGACCGGAAGTGACTCGAAAT-3', and R, 5'-CAGAACCACTGCCCTCGTAATC-3'; mouse α -smooth muscle actin (α -SMA) F, 5'-GCCGAGCGTGAGATTGTCC-3', and R, 5'-CCCCTGAGGCAGTTCGTAGC-3'; mouse Smad3 F, 5'-CTCCTGCTACCTGAGTGAAGATG-3', and R, 5'-GGCTGTGAGGCGTGGAATGTC-3'; mouse β -actin F, 5'-CATCCTGCCGTCTGGACCTGG-3', and 5'-TAATGTACGCACGATTTCC-3'. The level of mRNA expression was reported as fold change using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Western Blot Analysis

Murine lung tissues 0.25 g were lysed in 200 μ l RIPA Lysis Buffer (Welbio, Changsha, China), and then protein was extracted by centrifuging at 12,000 $\times$ g (4 $^{\circ}$ C) for 15 min. Each set of experiments was repeated three times. The loaded proteins (50–100 μ g) were separated on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto PVDF membranes. After blocking with 5% non-fat dried milk for 2 h at room temperature, the membranes were incubated with rabbit anti-mouse antibodies against NPRA (dilution 1:300, 55116-1-AP; Proteintech, IL, USA), E-cadherin (dilution 1:1000, 3195; CST, MA, USA), α -SMA (dilution 1:200 for tissues and 1:1000 for cells, 55135-1-AP; CST, MA, USA), Smad3 (dilution 1:1000, ab40854; Abcam, Cambridge, UK), phosphorylated-Smad3 (p-Smad3) (dilution 1:500 for tissues and 1:1000 for cells, ab63402; Abcam, Cambridge, UK), and mouse anti mouse β -actin antibody (dilution 1:5000 for tissues and 1:4000 for cells, 60008-1-Ig; Proteintech, IL, USA) overnight at 4 $^{\circ}$ C, and then were incubated with horseradish peroxidase conjugated goat anti-rabbit (dilution 1:6000 for tissues and 1:3000 for cells) or anti-mouse antibodies (dilution 1:5000 for tissues and 1:3000 for cells; Proteintech, IL, USA) for 120 min (tissues) or 60 min (cells), respectively. Finally, the blots were developed with the ECL Plus reagents (Thermo pierce, IL, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to measure the levels of TGF- β 1, IL-4 and IL-5 in bronchoalveolar lavage fluid (BALF), ANP in serum, and cGMP in lung tissue homogenate, according to the protocol of ELISA kit (Cloud-Clone Corp., Wuhan, China). 0.3 ml BALF and 1 ml blood collected from mice orbit were rested overnight at 4 °C, and then centrifuged at 3000 $\times$ g (4 °C) for 30 min. Each sample was repeated in three wells. Briefly, in 96-well plates, 100 μ l sample and 100 μ l biotinylated Detection Reagent A (50 μ l cells and 50 μ l Detection reagent A) were incubated for 1 h at 37 °C, followed by incubation with 100 μ l Detection reagent B conjugated by horseradish peroxidase for 1 h at 37 °C. Subsequently, plates were incubated with substrate solution as a chromogen for 2 min without light. The optical density (OD) was measured at 450 nm using a microplate reader (Huisong, Wuxi, China).

Histology and Immunohistochemistry

Lung tissue specimens were taken from the mice, and they were then transferred to 10% neutral buffer, formalin fixed and paraffin embedded. Deparaffinized sections (3- μ m) were stained with hematoxylin and eosin. Moreover, Periodic acid-Schiff (PAS) staining was applied to evaluate hyperplasia of goblet cells and secretion of mucus. MASSON staining was conducted to evaluate the collagen and extracellular matrix. The special staining methods are those described in previous study (Luna 1968).

Peribronchial infiltrates and goblet cell hyperplasia were assessed by semi-quantitative scores (0–5) as reported previously (Ma et al. 2015). Airway smooth muscle thickness was quantified by assessing diameter of the muscle layer surrounding the main bronchus based on α -SMA staining as previously described (Duechs et al. 2014). In brief, an inner line at the border of lumen and epithelium was drawn. In 300 μ m distances to the inner circle a second circle surrounding the main bronchus area was drawn. Vertical auxiliary lines in intervals of 2 μ m were drawn from the inner to the second circle. The length of stained auxiliary line was measured. The analyses were performed using a light microscope (Olympus Cor., Tokyo, Japan) connected to a video recorder that was linked to a computerized image system (Image-Pro Plus V6.0, Silver Spring, MD, USA). The measurements were made in a blinded fashion by two pathologists without knowledge of group for a given specimen. Disagreements were settled by consensus or adjudicated by a third reviewer.

For immunohistochemistry, formalin-fixed, paraffin-embedded tissue was cut in 3- μ m-thick sections. Antigen retrieval was achieved by treatment in a high-temperature cooker heated for 15–20 min in a citrate buffer, pH 6.0.

Sections were incubated in 3% H₂O₂ to quench endogenous peroxidase activity. The slides were then incubated with primary rabbit anti-mouse antibody against E-Cadherin (dilution 1:100, 20874-1-AP; Proteintech, IL, USA), or α -SMA (dilution 1:100, 14395-1-AP; Proteintech, IL, USA), for overnight at room temperature. The resulting slides were incubated for 30 min at 37 °C each with horseradish peroxidase conjugated anti-rabbit IgG antibody and subsequent rinses in PBS three times for 5 min. 3′3-diaminobenzidine-tetrahydrochloride was applied as a chromogen for 1–5 min. Sections were counterstained in haematoxylin for 5–10 min.

Statistical Analysis

Group data are expressed as the mean $\pm$ standard deviation (SD). Significant differences among multiple groups were evaluated using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test or the Games–Howell test. P values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 21.0 (IBM SPSS Inc., Chicago, IL, USA).

Results

ANP/NPRA Reduces Inflammation and Airway Remodeling in Lung Tissues of Asthmatic Mice

Figure 1 shows that both mRNA (Fig. 1a) and protein expressions (Fig. 1b, c) of NPRA were increased in asthma group and ANP group compared with controls. They were decreased in A71915 group when compared with ANP group. Those results suggest that NPRA could be induced by ANP during EMT in asthma but inhibited by A71915.

In lung tissues, asthmatic mice had an increased inflammation, goblet cell hyperplasia and smooth muscle thickening compared with controls. When ANP was treated in asthmatic mice, the inflammatory cells (Fig. 2a), goblet cells (Fig. 2b), mucous secretion (Fig. 2b) and extracellular matrix (Fig. 2c) in lung were decreased. Moreover, NPRA antagonist (A71951) could reduce the effect of ANP in lung tissues (Fig. 2). These findings suggest that ANP/NPRA could reduce inflammation and airway remodeling in lung tissues of asthmatic mice.

ANP/NPRA Reduces EMT in Lung Tissues of Asthmatic Mice

Immunohistochemistry staining shows that E-Cadherin distribution in lung was decreased and α -SMA distribution was increased in bronchus and alveolus of asthma group when

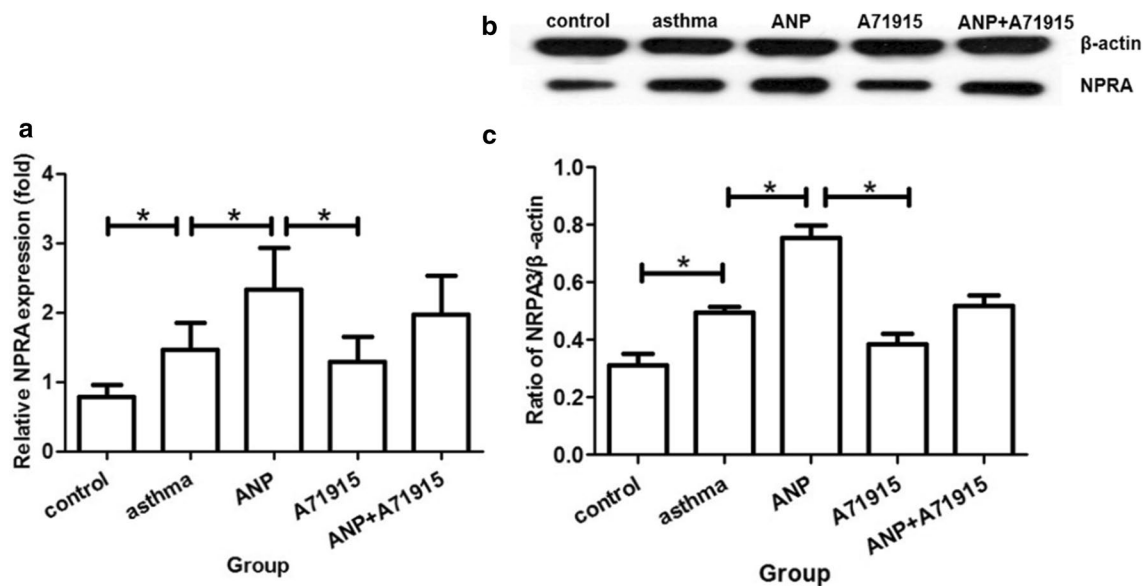


Fig. 1 The mRNA and protein expressions of NPRA in lung tissues. **a** The mRNA expressions of NPRA were increased in asthma group and ANP group compared with controls. They were decreased

in A71915 group when compared with ANP group. **b, c** The protein expressions of NPRA in lung tissues were consistent with the mRNA expressions. *P value < 0.05

compared with controls, which could be reversed by ANP. The effect of ANP could be inhibited by A71915 (Fig. 3a, b). Based on α -SMA staining, smooth muscle layer in lung was decreased in ANP group compared with controls, whereas they were increased in A71915 group and ANP+A71915 group (Fig. 3c).

In the lung tissues of asthma group, the mRNA expression of E-Cadherin was lower than controls, which could be reversed by ANP. In A71915 group and ANP+A71915 group, E-Cadherin expressions were lower than that in ANP group (Fig. 4a). The protein expressions of E-Cadherin in lung tissues were consistent with the mRNA expressions (Fig. 4b, c).

In contrast, the mRNA expression of α -SMA in asthma group was higher than controls, which could be reversed in ANP group. In A71915 group and ANP+A71915 group, α -SMA mRNA expressions were higher than that in ANP group (Fig. 4d). The protein expressions of α -SMA in lung tissues were consistent with the mRNA expressions (Fig. 4e, f). These results indicate that ANP/NPRA could inhibit EMT in lung.

ANP/NPRA Inhibits EMT by Targeting on Smad3 in Lung Tissues of Asthmatic Mice

The Smad3 mRNA expression in asthma group was higher than controls, but was similar with other groups (Fig. 5a). In contrast, although both Smad3 and p-Smad3 protein levels in asthmatic mice were higher than those in controls,

Smad3 levels weren't significantly changed after ANP or/and A71915 treated, whereas p-Smad3 levels was significantly changed. When treated with ANP, p-Smad3 level was decreased compared with asthma group, which could be reversed by A71915. Thus, ANP may reduce EMT of lung by attenuating phosphorylation of Smad3 in lung tissues. (Fig. 5b–d).

ANP may Reducing EMT Through cGMP in Lung Tissues of Asthma

The ELISA showed that the ANP level in serum of ANP group was higher than asthmatic group and A71915 group. It was increased in ANP+A71915 group compared with A71915 group (Fig. 6a). Thus, exogenous ANP had been successfully administrated into mice.

Figure 6b illustrates that the level of cGMP in the lung tissues of asthmatic mice was higher than controls. This level in ANP group was higher than asthma group. The effect of ANP could be reversed by A71915. cGMP levels in lung tissues was consistent with ANP levels in serum. These suggest that cGMP may be involved into the process of ANP reducing EMT.

ANP Inhibits Th2-Type Inflammation in Bronchus of Asthma

In the BALF of asthma group, the protein levels of IL-4, IL-5 and TGF- β 1 were increased when compared with controls.

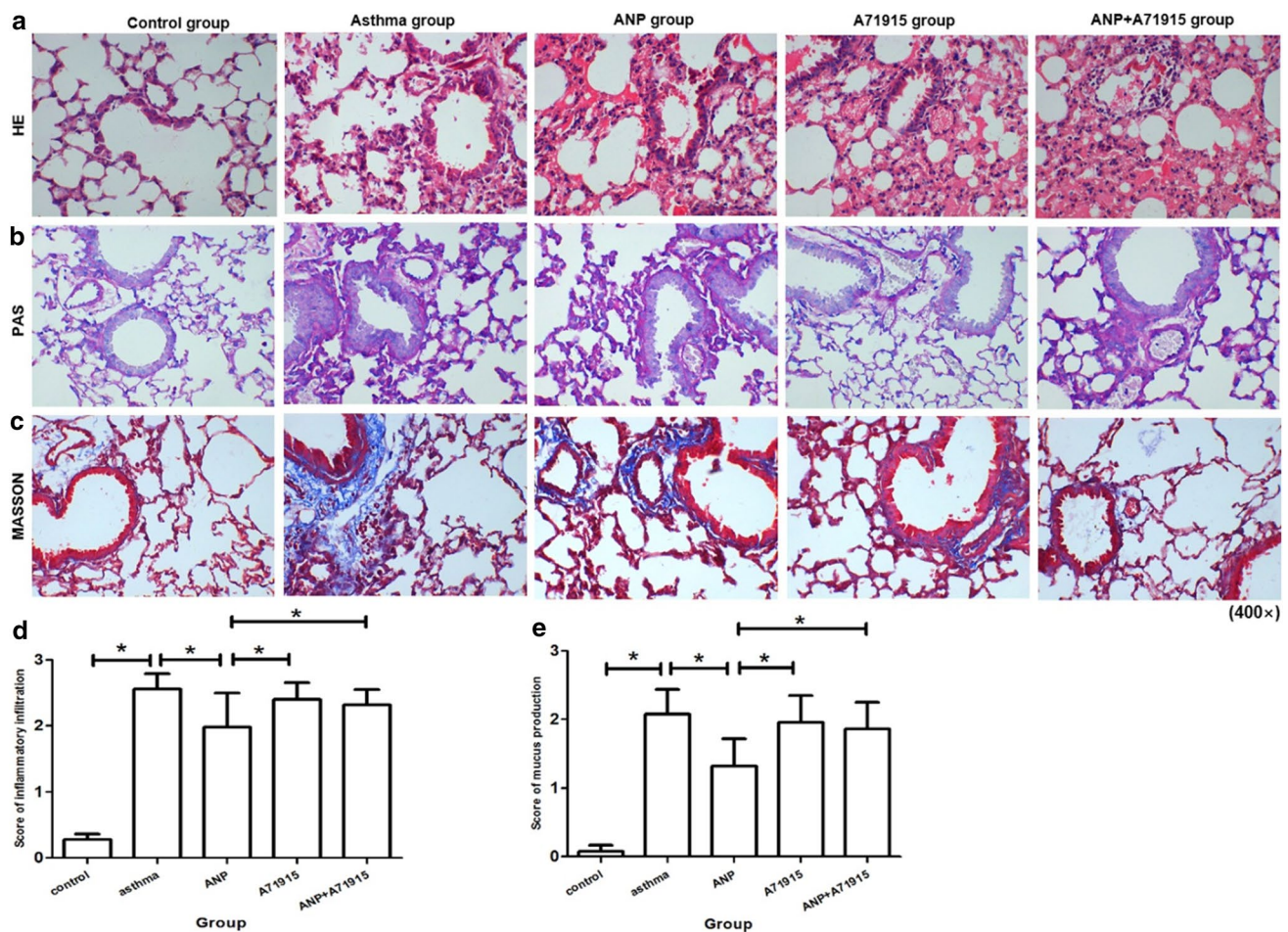


Fig. 2 Histology of lung tissues. **a** HE staining. **b** PAS staining. **c** MASSON staining. **d** Score of inflammatory infiltration. **e** Score of mucus production. **f** Smooth muscle thickening. Asthmatic mice have an increased inflammation (**a**, **d**), goblet cell hyperplasia (**b**, **e**) and extracellular matrix (**c**) than controls. The inflammatory cells,

goblet cells, mucous secretion and extracellular matrix in lung were decreased in ANP group compared with controls, whereas they were increased in A71915 group and ANP+A71915 group. Four mice were in each group and each set of experiments was repeated three times. ($\times 400$ magnification)

When administrated with ANP, the levels were decreased, which could be reversed by A71915 (Fig. 7). These results indicated that ANP/cGMP may inhibit TGF- β 1-induced Th2-type inflammation in bronchus.

Discussion

In this study, we found that ANP/NPRA could inhibit EMT in airway of asthmatic mice by targeting Smad3 via attenuating phosphorylation of Smad3. cGMP signaling may be involved into this process.

The HE, PAS and MASSON staining showed that the inflammatory cells, goblet cells, mucous secretion and smooth muscle layer in lung were decreased in ANP group

compared with asthma group, which could be reversed by antagonist of ANP receptor. This confirms a protective effect of ANP in asthma that reduce inflammation and airway remodeling.

We explored the mechanism how ANP inhibits airway EMT in asthma. We detected the protein expression of Smad3 and p-Smad3. In asthma group, both Smad3 and p-Smad3 expression was increased compared with controls, suggesting that Smad3 and p-Smad3 may be involved into asthma. However, only p-Smad3 expression was decreased when ANP treated, which could be reversed by NPRA antagonist. That change was consistent with airway EMT in asthma. Previous studies in lung and heart found that ANP could inhibit nuclear translocation of p-Smad3, and then reducing extracellular matrix

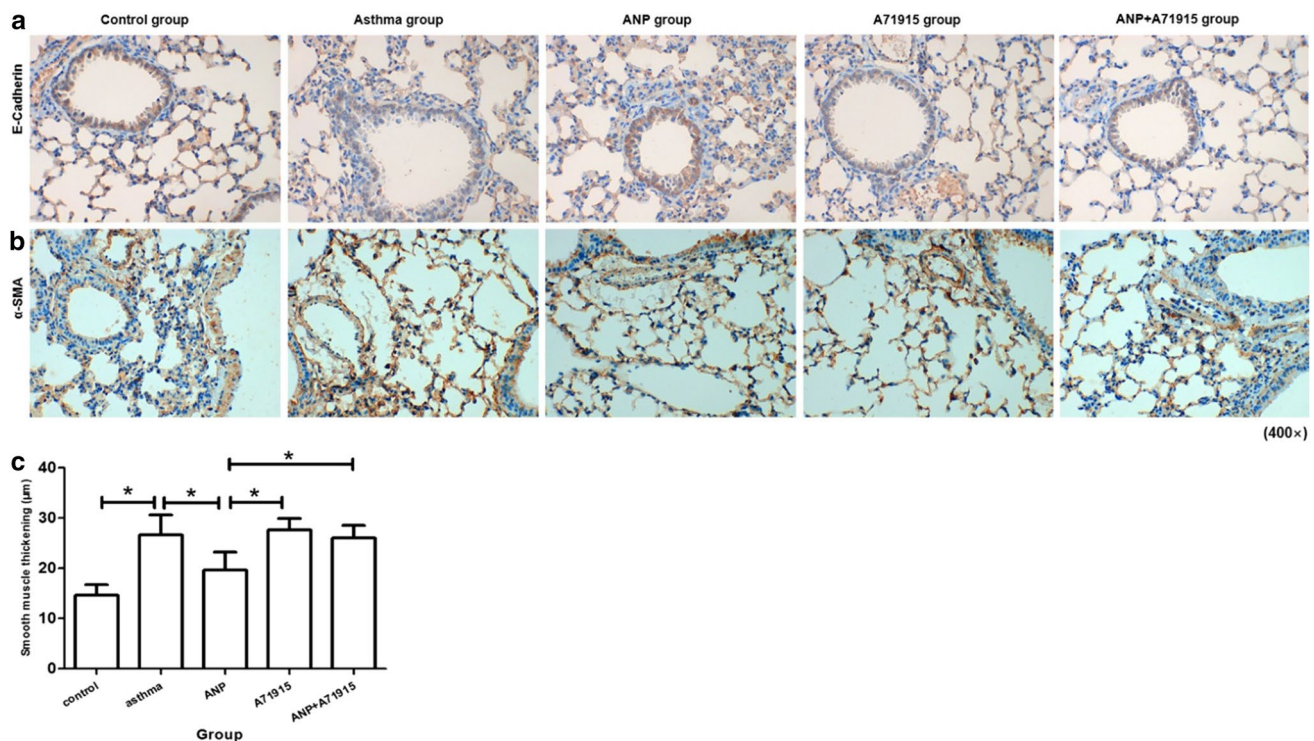


Fig. 3 Immunohistochemistry of E-Cadherin+ and α -SMA+ cells in lung tissues. **a** E-Cadherin+ cells distribution was decreased in asthma group compared with controls, but was increased in ANP group compared with asthma group. E-Cadherin+ cells distribution was decreased in A71915 group and ANP+A71915 group when compared with ANP group. **b** α -SMA+ cells distribution was increased in asthma group compared with controls, but was decreased in ANP

group compared with asthma group. It was increased in A71915 group and ANP+A71915 group when compared with ANP group. **c** Smooth muscle layer in lung was assessed by α -SMA staining. It was decreased in ANP group compared with controls, whereas they were increased in A71915 group and ANP+A71915 group. *P value < 0.05. ($\times 400$ magnification)

expression (Li et al. 2007, 2008; Gong et al. 2011). Our findings in asthma was consistent with that previous founding. Thus, we supposed that ANP may target on Smad3 via attenuating phosphorylation of Smad3 when reducing airway EMT in asthma. Moreover, TGF- β 1 levels in BALF could be reduced by ANP in our study, suggesting that ANP might attenuate p-Smad3 via inhibiting TGF- β 1.

Interestingly, our study found that cGMP level in lung tissues was consistent with ANP levels. cGMP level was increased in asthma group compared with controls, which even more increased in ANP group and could be reduced by NPRA antagonist. Previous study with rat cardiac myocytes found that ANP/cGMP signaling could reduce proliferation and function of ventricular fibroblasts (Moubarak et al. 2015). These indicated that cGMP may be activated by ANP/NPRA when it reduces airway EMT in

asthma. Since the protective role of ANP/cGMP signaling against cardiac injury and remodeling has been found (Inserte and Garcia-Dorado 2015; Chen et al. 2017), our study demonstrated its protective role in airway remodeling. Previous study on cardiac fibroblasts in mice demonstrated that ANP/cGMP signaling could inhibit nuclear translocation of p-Smad3 and downstream events, such as myofibroblast transformation and proliferation (Li et al. 2008), suggesting that ANP/cGMP signaling could reduce myofibroblast via inhibiting p-Smad3. Since the p-Smad3 expression was decreased and was in consistent with EMT of airway when treated with ANP in our study, we proposed that ANP/NPRA may inhibit airway EMT by targeting on Smad3 via attenuating phosphorylation of Smad3. cGMP activation by ANP may be involved into that process.

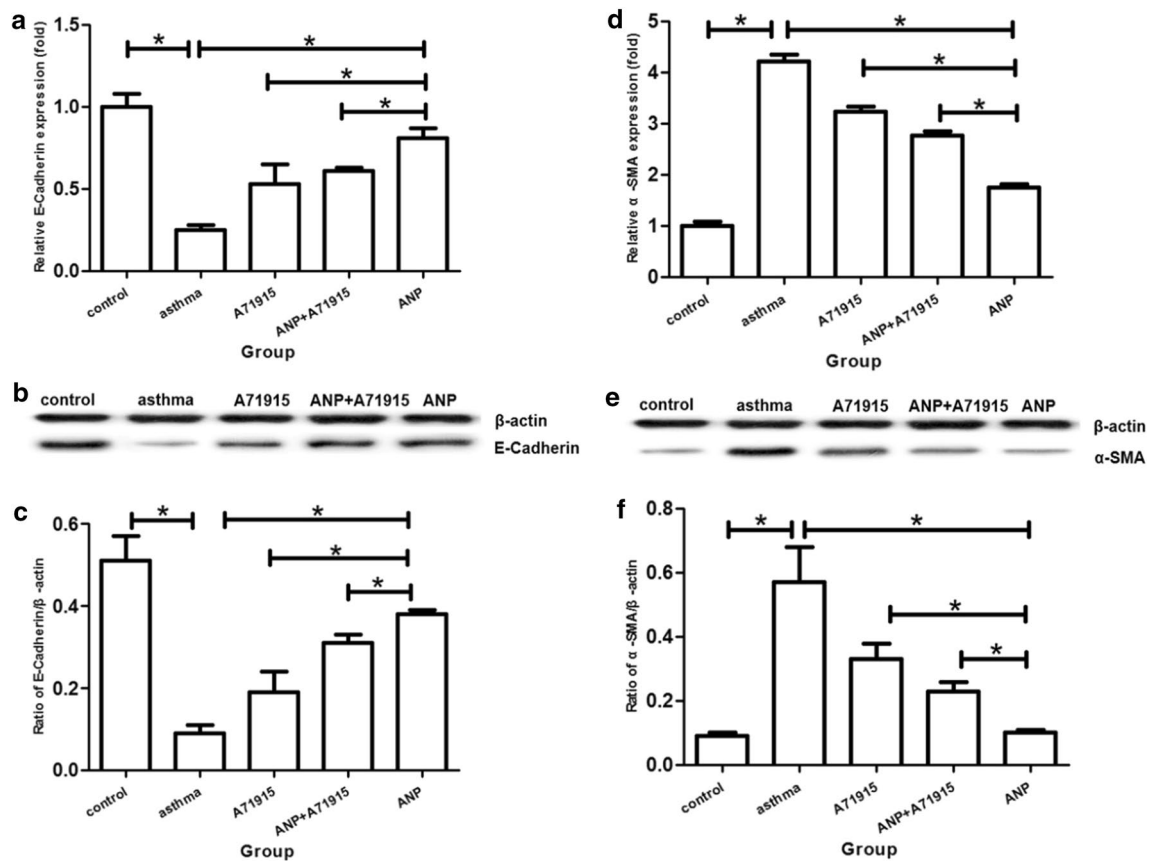


Fig. 4 The mRNA and protein expressions of E-Cadherin and α -SMA in murine lung tissues. **a** The mRNA expression of E-Cadherin was decreased in asthmatic group when compared with controls. It's increased when treated with ANP. In A71951 group and ANP+A71951 group, E-Cadherin levels were lower than that in ANP group. **b, c** The protein expressions of E-Cadherin in lung tissues were consistent with the mRNA expressions. **d** The mRNA

expression of α -SMA was increased in asthma group, which could be reduced by ANP. In A71951 group and ANP+A71951 group, α -SMA expressions were higher than that in ANP group. **e, f** The protein expressions of α -SMA in lung tissues were consistent with the mRNA expressions. Five mice were in each group and each set of experiments was repeated three times. *P value < 0.05

Moreover, ELISA showed that the increased TGF- β 1, IL-4 and IL-5 levels in BALF of asthma group could be reduced by ANP, which could be reversed by NPRA antagonist. Thus, ANP may inhibit Th2-type inflammation in airway. Since ANP was increasingly expressed in asthma (Yu et al. 2016; Ma et al. 2015), we supposed that the increased ANP level might be a response to asthma. However, there's difference in the level of Th2-type inflammation and mucus production between this study and the previous one (Ma et al. 2015). It may be partly due to a longer period of ANP and/or A71951 treatment in model establishment. This suggests that the chronic effect of ANP on inflammation in asthma may be protective. Therefore, ANP plays a protective

role not only in EMT but also in Th2-type inflammation of airway in asthma.

Conclusions

In asthmatic mice, ANP/NPRA could inhibit EMT of airway by targeting Smad3 via attenuating phosphorylation of Smad3. cGMP signaling may be involved into this process. This may provide a potential of ANP in the treatment on refractory asthma with airway remodeling.

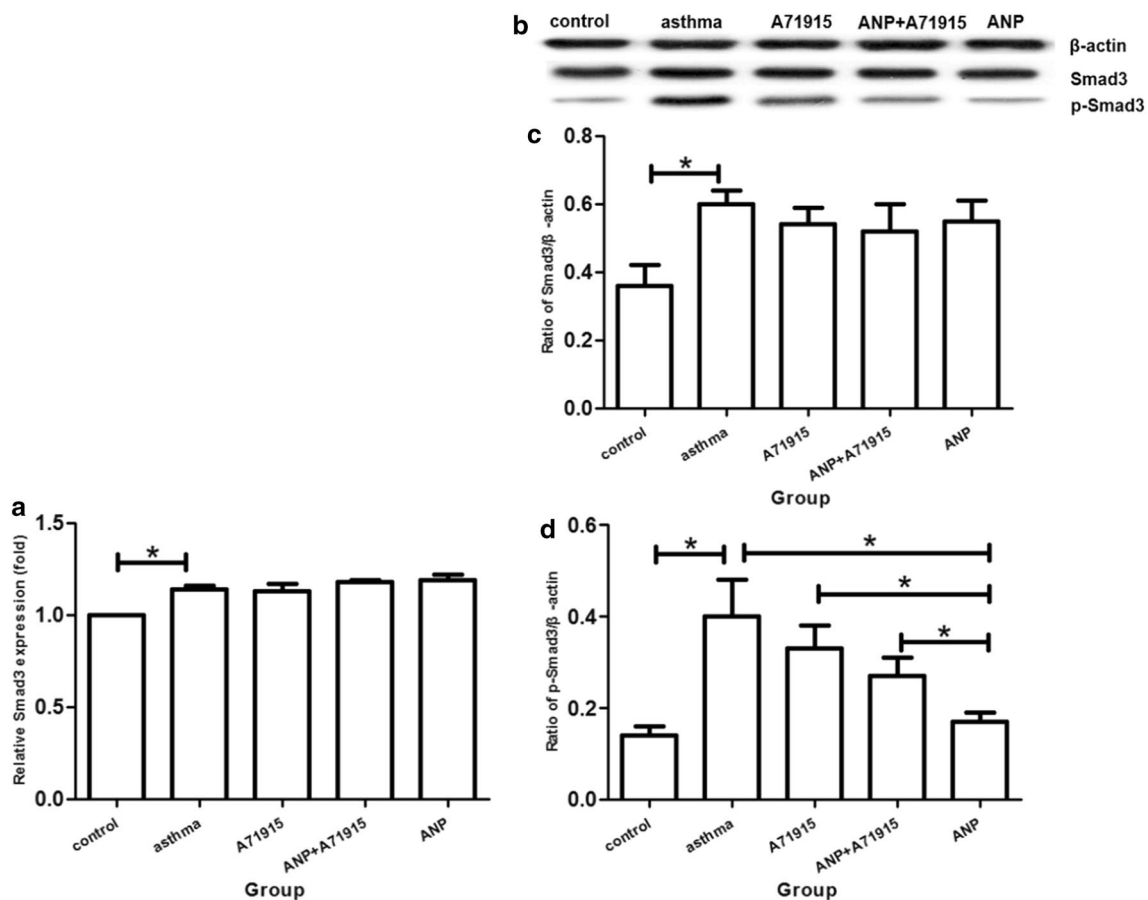
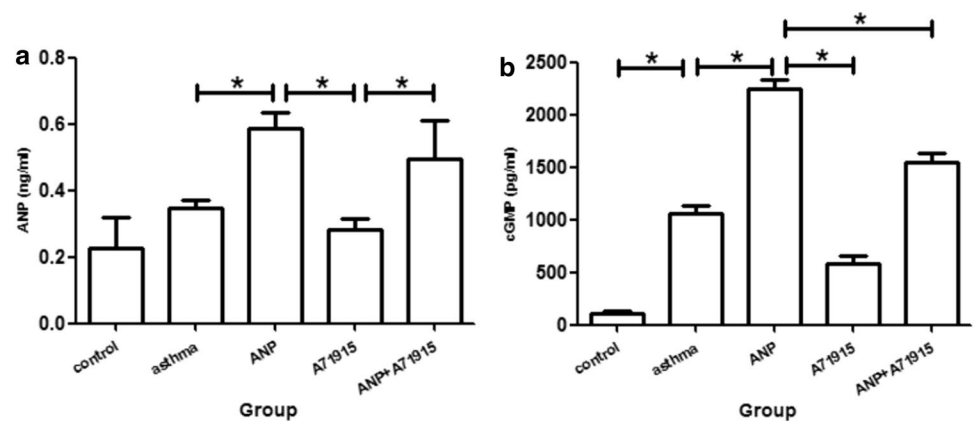


Fig. 5 PCR and Western blotting showing differentially expressed Smad3 and p-Smad3 in murine lung tissues. The mRNA expression of Smad3 was increased in asthmatic mice when compared with controls (a). Smad3 protein level in asthma group was higher than in controls, but not significantly different among other groups (b, c). In

contrast, p-Smad3 protein levels were different among groups and were consistent with α -SMA protein level (b, d). Five mice were in each group and each set of experiments was repeated three times. p-Smad3: phosphorylated Smad3. *P value < 0.05

Fig. 6 ELISA showing differential levels of ANP in serum (a) and cGMP in lung tissues (b). ANP and cGMP levels in ANP group were higher than asthmatic group and A71915 group. In A71915 group and ANP+A71915 group, cGMP levels were lower than that in ANP group. Five mice were in each group and each sample was repeated in three wells. *P value < 0.05



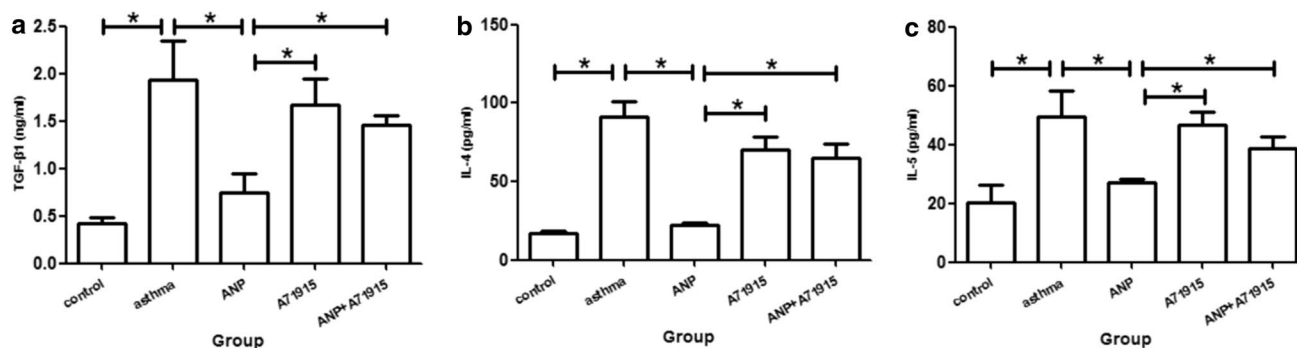


Fig. 7 ELISA showing differential levels of TGF- β 1 (a), IL-4 (b) and IL-5 (c) in bronchoalveolar lavage fluid (BALF). The levels of TGF- β 1, IL-4 and IL-5 in BALF were higher in asthma group than that in controls. When administrated with ANP, the levels were decreased.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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