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ORIGINAL ARTICLE



Atrial natriuretic peptide inhibits epithelial-mesenchymal transition (EMT) of bronchial epithelial cells through cGMP/PKG signaling by targeting Smad3 in a murine model of allergic asthma

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ABSTRACT

Background: Atrial natriuretic peptide (ANP) inhibits TGF- β 1-induced epithelial-mesenchymal transition (EMT) in human airway cells. We aim to explore the role and mechanism of ANP on EMT of bronchial epithelial cells from murine model of allergic asthma in vitro. **Methods:** Murine model of allergic asthma was established with BALB/c mice using ovalbumin (OVA). Bronchial epithelial cells were isolated from OVA-exposed mice, and then were cocultured with TGF- β 1, ANP, natriuretic peptide receptor A antagonist, cGMP analog, cGMP inhibitor or/and protein kinase G (PKG) inhibitor, respectively. We assessed expressions of E-Cadherin, α -SMA, cGMP, Smad3 and p-Smad3 in the murine cells before and after Smad3 silence. **Results:** Compared with bronchial epithelial cells from controls and OVA-exposed mice without additional stimulation, the mRNA and protein expressions of E-Cadherin were decreased but α -SMA expressions were increased in cells with TGF- β 1 stimulation from OVA-exposed mice in vitro. That could be reversed by ANP. The effect of ANP could be mimicked by the cGMP analog, which could be reversed by cGMP or PKG inhibitor. Moreover, the phosphorylated Smad3 expression was consistent with that of α -SMA. When Smad3 was silenced, Smad3 was mostly expressed in cytoplasm. In contrast, it is mostly expressed in nucleus of non-silenced cells during EMT. **Conclusions:** In a murine model of allergic asthma, ANP could inhibit TGF- β 1-induced EMT of bronchial epithelial cells through cGMP/PKG signaling, targeting TGF- β 1/Smad3 via attenuating phosphorylation of Smad3 in vitro, which may provide potential of ANP in treating allergic asthma with airway remodeling.

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asthma; atrial natriuretic peptide; cGMP; epithelial-mesenchymal transition; Smad3

Introduction

Asthma is a respiratory disease that imposes a great health burden in worldwide. It affected about 300 million people globally in 2011 and accounts for 250,000–345,000 death each year.^{1,2} In our previous work, we found that the level of atrial natriuretic peptide (ANP) in serum was higher in asthmatic patients than healthy controls, and was increased when asthma was aggravated.³ We also validated that finding in the murine model of allergic asthma.⁴ ANP is a polypeptide hormone that controls blood pressure via

reducing water and sodium loads in circulatory system.⁵ It has been found to protect animal model against allergen-induced airway hyperresponsiveness,^{6,7} suggesting a protective role in allergic asthma. However, there is rare report on the precise mechanism how ANP plays a protective role in asthma. We further found that ANP could inhibit TGF- β 1-induced epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells.⁸ Airway EMT promotes mucosal remodeling, contributing to refractory asthma.⁹ Thus, we suppose that airway EMT of asthma

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may be interfered by ANP. In this study, we investigate the precise role of ANP in bronchial EMT of allergic asthma and its possible mechanism using bronchial epithelial cells from murine model of allergic asthma.

Methods

Mice

Female BALB/c mice (6–8 weeks old, weighing 20 ± 2 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd (Changsha, China) and housed in the SPF animal facility under a 12:12 h light/dark photcycle. 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.

Female BABL/c 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 the 13th day as previously described.¹⁰ 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 mice, normal saline replaced OVA in equal volume. Five mice were in each group and each set of experiments was repeated three times.

Cell culture

To isolate bronchial epithelial cells from mice, the bronchus was separated from the lobe of lung, and then was minced to small pieces and digested by 0.05% pronase (Sigma, MA, USA) in DMEM/F12 media (Hyclone, Utah, USA) at 4 °C overnight. Digestion was stopped by FBS (Gibco, BRL, USA). Bronchial epithelial cells were identified by CK-18 staining.

Seven days later, cells from OVA-exposed mice were subdivided into eight groups as follows: OVA-exposed group without any treatment, TGF- β 1 group was incubated with 10 ng/mL TGF- β 1 for 72 h, ANP group was with 1 μ M ANP for 30 min and followed by 10 ng/mL TGF- β 1, A71915 group was with 1 μ M A71915

(natriuretic peptide receptor A (NPRA) antagonist) for 30 min and followed by 10 ng/mL TGF- β 1 and 1 μ M ANP, 8-Br-cGMP group was with 1 mM 8-Br-cGMP (cGMP analogues) for 30 min and then 10 ng/mL TGF- β 1, Rp-8-Br-cGMP group was with 50 μ M Rp-8-Br-cGMP (cGMP inhibitor) for 30 min and then treated as 8-Br-cGMP group, KT + ANP group was with 1 μ M KT5823 (protein kinase G (PKG) inhibitor) for 30 min and then treated as ANP group, KT + 8-Br-cGMP group was with 1 μ M KT5823 for 30 min and then treated as 8-Br-cGMP group. Cell from mice without OVA exposure was the control group.

Quantitative real-time PCR

Total RNA from cells was extracted using the TRIzol reagent (Invitrogen, CA, USA) 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. 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) underwent 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The primers used were as follows: mouse E-cadherin F, 5'-CGACCGGAAGTGACT CGAAAT-3', and R, 5'-CAGAACCACTGCCCT CGTAATC-3'; α -SMA F, 5'-GCCGAGCGTGAGATTGTCC-3' and R, 5'-CCCGTCAGGCAGTTCGTAGC-3'; Smad3 F, 5'-CTCCTGGCTACCTGAGTGAAGATG-3', and R, 5'-GGCTGTGAGGCGTGGAATGTC-3'; β -actin F, 5'-CATCCTGCGTCTGGACCTGG-3', and 5'-TAATGTCACGCA CGATTTC-3'. Level of mRNA expression for each gene was reported as fold change using the $2^{-\Delta\Delta CT}$ method.¹¹

Western blot analysis

The bronchial epithelial cells were treated with 60 μ L RIPA for 10 min on ice, and then centrifuged at $12,000 \times g$ (4 °C) for 15 min. The loaded

proteins (50–100 µg) were separated on a 10% SDS-polyacrylamide gel electrophoresis, followed by transferring onto PVDF membranes. After blocking with TBS-Tween 20 containing 5% skim milk for 2 h at room temperature, the membranes were incubated with rabbit anti-mouse antibodies against mouse E-cadherin (dilution 1:1000, 3195; CST, MA, USA), α -SMA (dilution 1:1000, 55135-1-AP; CST, MA, USA), Smad3 (dilution 1:1000, ab40854; Abcam, Cambridge, UK), phosphorylated-Smad3 (p-Smad3) (dilution 1:1000, ab63402; Abcam, Cambridge, UK) and mouse anti mouse β -actin antibody (dilution 1:4000, 60008-1-Ig; Proteintech, IL, USA) overnight at 4 °C. Following that, they were incubated with horseradish peroxidase conjugated goat anti-rabbit (dilution 1:3000; Proteintech, IL, USA) or anti-mouse antibodies (dilution 1:3000; Proteintech, IL, USA) for 60 min, respectively. Finally, blots were developed with the ECL Plus reagents (Thermo pierce, IL, USA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to detect levels of cGMP in bronchial epithelial cells, according to the protocol of ELISA kit (Cloud-Clone Corp., Wuhan, China). Briefly, in 96-well plates, 100 µL sample and 100 µL biotinylated Detection reagent A (50 µL cells and 50 µL Detection reagent A) were incubated for 1 h at 37 °C, followed by incubating with 100 µL Detection reagent B conjugated by horseradish peroxidase for 1 h at 37 °C. Then, plates were incubated with substrate solution as a chromogen for 2 min without light. The optical density was measured at 450 nm using a microplate reader (Huisong, Wuxi, China).

Transfection of small interfering RNA (siRNA)

The Smad3 siRNA and scramble siRNA were synthesized by Ribobio (Guangzhou, China). The siRNAs were transfected with riboFECT™ CP Reagent according to the manufacturer's protocol. Bronchial epithelial cells from OVA-exposed mice and controls were transiently transfected with 20 nM scramble or siRNA. After transfection for 24 h at 37 °C in 5% CO₂, the cells from OVA-

exposed mice were divided into eight groups' undergone similar treatment as described before.

Immunohistochemistry

The slides of cells were fixed by 4% paraformaldehyde for 30 min and incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. The slides were incubated with primary rabbit anti-mouse antibody against CK-18 (dilution 1:100, 10830-1-AP; Proteintech, IL, USA) at 4 °C overnight, and then were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (PV-9000, Zisbio, Beijing, China) at 37 °C for 30 min. After rinsing with PBS for three times, 3',3'-diaminobenzidine-tetrahydrochloride was applied on the slides as a chromogen for 1–5 min. Slides were counterstained in hematoxylin for 5–10 min.

The siRNA transfected cells were cultured for 72 h at 37 °C, and then crawled onto slides for immunohistochemical staining. The slides were fixed by 4% paraformaldehyde for 30 min and incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. The slides were incubated with primary rabbit anti-mouse antibody against Smad3 (dilution 1:100, 25494-1-AP; Proteintech, IL, USA) at 4 °C overnight, and then were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (PV-9000, Zisbio, Beijing, China) at 37 °C for 30 min. The slides were rinsed with PBS for three times and subsequent applied 3',3'-diaminobenzidine-tetrahydrochloride as a chromogen for 1–5 min, followed by hematoxylin for 5–10 min.

Statistical analysis

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

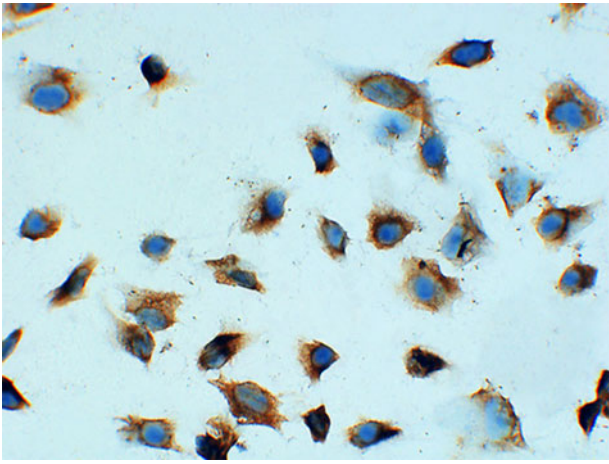


Figure 1. Cells identification. When bronchial epithelial cells were isolated and cultured, cells were identified by staining of CK-18. Cells were mainly CK-18+ staining ($\times 400$ magnification).

Results

Cells identification

When bronchial epithelial cells were isolated from mice, they were identified by immunohistochemical staining of CK-18. Bronchial epithelial cells were mainly CK-18+ stained (Figure 1).

mRNA and protein expressions of E-Cadherin in bronchial epithelial cells from mice

In OVA-exposed mice, the mRNA expression of E-Cadherin in bronchial epithelial cells was lower than that in controls. When cells from OVA-exposed mice were treated with TGF- β 1, E-Cadherin mRNA expression was reduced in TGF- β 1 group. In contrast, E-Cadherin mRNA expression in ANP group was significantly higher than that in TGF- β 1 group. But in A71915 group, that expression was lower than that in ANP group (Figure 2A). These suggest that ANP could inhibit the decrease of E-Cadherin mRNA expression, which was induced by TGF- β 1 in bronchial epithelial cells from OVA-exposed mice.

Moreover, when TGF- β 1-induced cells were treated with 8-Br-cGMP (a cGMP analogues), E-Cadherin mRNA expression was enhanced. In contrast, that expression in Rp-8-Br-cGMP group was lower than that in 8-Br-cGMP group (Figure 2A). These suggest that cGMP may contribute to

inhibiting the decrease of E-Cadherin mRNA expression, which was induced by TGF- β 1.

When ANP group were treated with KT5823 (a PKG inhibitor), E-Cadherin mRNA expression was reduced, suggesting that PKG may be the downstream of ANP. When 8-Br-cGMP group were treated with KT5823, E-Cadherin mRNA expression was reduced, further indicating that PKG may be the downstream of cGMP (Figure 2A).

When Smad3 was silenced, E-Cadherin mRNA expressions in all groups were increased, and the changes among groups were consistent with those without Smad3 silence (Figure 2A).

The protein expressions of E-Cadherin in bronchial epithelial cells were consistent with mRNA expressions (Figure 2B–D).

mRNA and protein expressions of α -SMA in bronchial epithelial cells from mice

In OVA-exposed group, the mRNA expression of α -SMA in bronchial epithelial cells was higher than controls. When cells from OVA-exposed mice were treated with TGF- β 1, α -SMA mRNA expression was enhanced. In contrast, α -SMA mRNA expression in ANP group was significantly lower than that in TGF- β 1 group. However, that expression in A71915 group was higher than that in ANP group (Figure 3A). These suggest that ANP could inhibit the increase of TGF- β 1-induced α -SMA mRNA expression in bronchial epithelial cells from OVA-exposed mice.

Moreover, when TGF- β 1-induced cells were treated with 8-Br-cGMP (a cGMP analogues), α -SMA mRNA expression was reduced. In contrast, that expression in Rp-8-Br-cGMP group was higher than that in 8-Br-cGMP (Figure 3A). These suggest that cGMP may contribute to inhibiting the increase of TGF- β 1-induced α -SMA mRNA expression.

When ANP group were treated with KT5823 (a PKG inhibitor), α -SMA mRNA expression was enhanced, suggesting that PKG may be the downstream of ANP. When 8-Br-cGMP group were treated with KT5823, α -SMA mRNA expression was increased, indicating that PKG may be the downstream of cGMP (Figure 3A).

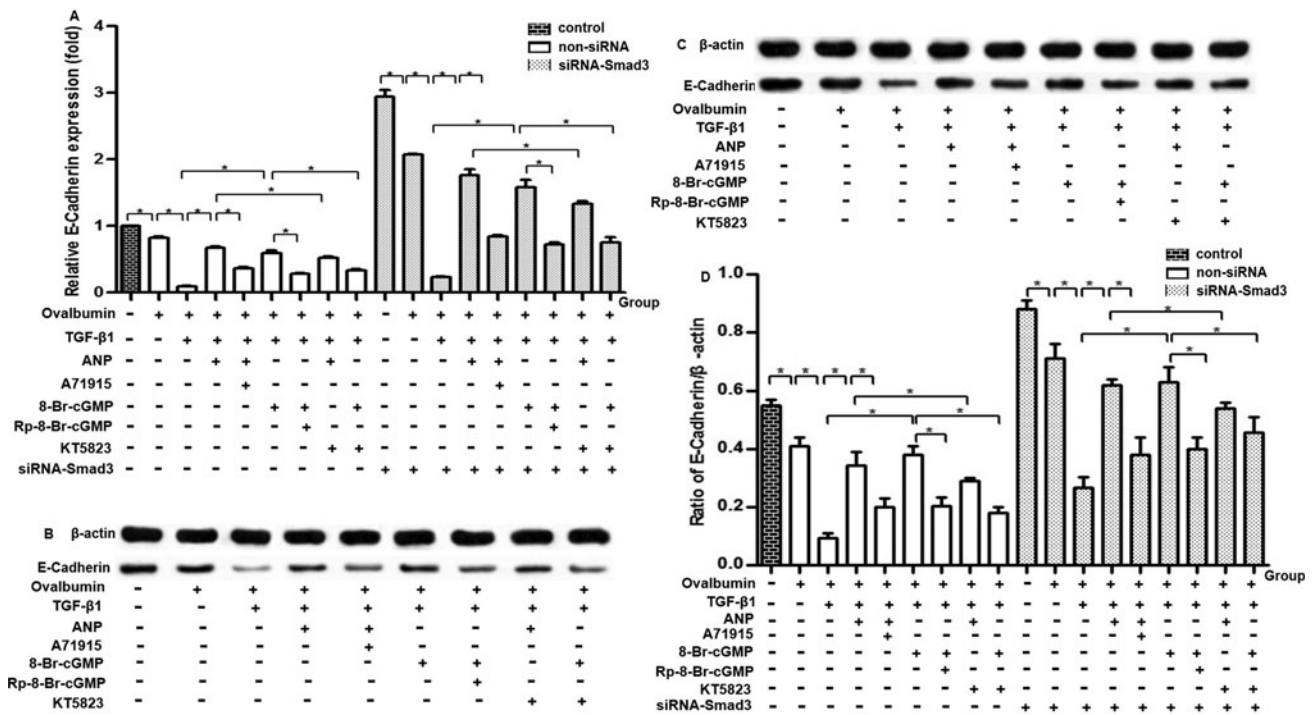


Figure 2. The mRNA and protein expressions of E-Cadherin in bronchial epithelial cells before and after Smad3 silenced. Cells from OVA-exposed mice were subdivided into eight groups as follows: OVA-exposed group without any treatment, TGF- β 1 group (10 ng/mL TGF- β 1), ANP group (1 μ M ANP + 10 ng/mL TGF- β 1), A71915 group (1 μ M A71915 + 10 ng/mL TGF- β 1 + 1 μ M ANP), 8-Br-cGMP group (1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), Rp-8-Br-cGMP group (50 μ M Rp-8-Br-cGMP + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), KT + ANP group (1 μ M KT5823 + 1 μ M ANP + 10 ng/mL TGF- β 1), KT + 8-Br-cGMP group (1 μ M KT5823 + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1). Cells from control mice without OVA treatment were the control group. Bronchial epithelial cells from OVA-exposed mice and controls were transfected with 20 nM siRNA. After transfection, the cells from OVA-exposed mice were divided into eight groups undergone similar treatment as described before. The mRNA expression was assessed by real time PCR, and the protein expression was detected by western blotting. (A) mRNA expressions of E-Cadherin. (B) Western blotting showing E-Cadherin expressions in bronchial epithelial cells. (C) Western blotting showing E-Cadherin expressions in bronchial epithelial cells with Smad3 silence. (D) Quantitation of protein bands showing E-Cadherin expressions with and without Smad3 silence. *: P value < 0.05.

When Smad3 was silenced, α -SMA mRNA expressions in all groups were decreased, and the difference among groups was consistent with those without Smad3 silence (Figure 3A).

The protein expressions of α -SMA in bronchial epithelial cells were consistent with mRNA expressions (Figure 3B–D).

cGMP levels in bronchial epithelial cells from mice

Figure 4 illustrates that in bronchial epithelial cells of ANP group, cGMP level was significantly higher than that in TGF- β 1 group. It was decreased when treated with A71915. The cGMP levels in cells transfected with scrambled Smad3-siRNA were similar with those without transfection. When cells transfected with siRNA to silence Smad3, cGMP levels were not significantly

decreased. These suggesting that cGMP maybe upstream of Smad3.

Smad3 and p-Smad3 expressions in bronchial epithelial cells from mice

In bronchial epithelial cells, the mRNA expression of Smad3 in OVA-exposed group was higher than that in controls. It is even higher in TGF- β 1 group than that in OVA-exposed group (Figure 5A). When Smad3 was silenced, mRNA expressions of Smad3 in all group were significantly decreased, suggesting a successful silence of Smad3 (Figure 5A).

The protein expression of Smad3 was consistent with mRNA expression (Figure 5B–D).

In addition, p-Smad3 protein expression in OVA-exposed group and TGF- β 1 group was consistent with Smad3. Moreover, in A71915 group,

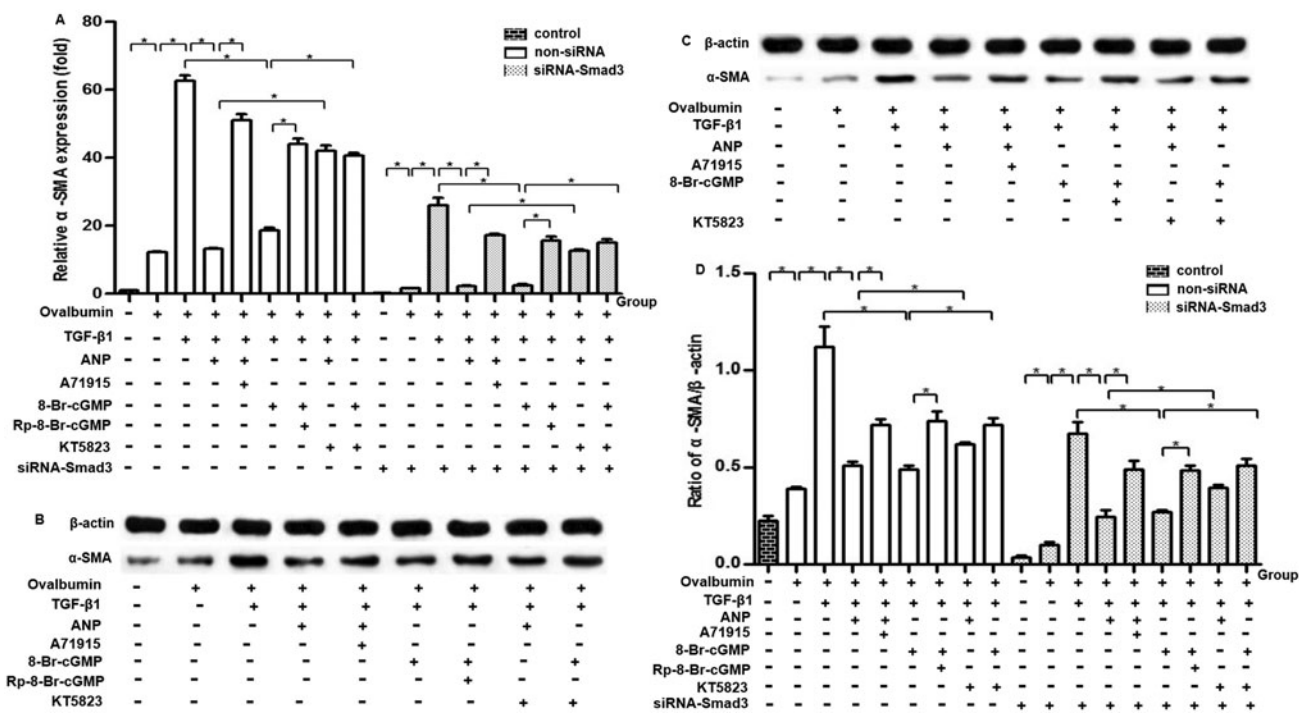


Figure 3. The mRNA and protein expressions of α -SMA in bronchial epithelial cells before and after Smad3 silenced. Cells from OVA-exposed mice were subdivided into eight groups as follows: asthma group without any treatment, TGF- β 1 group (10 ng/mL TGF- β 1), ANP group (1 μ M ANP + 10 ng/mL TGF- β 1), A71915 group (1 μ M A71915 + 10 ng/mL TGF- β 1 + 1 μ M ANP), 8-Br-cGMP group (1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), Rp-8-Br-cGMP group (50 μ M Rp-8-Br-cGMP + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), KT + ANP group (1 μ M KT5823 + 1 μ M ANP + 10 ng/mL TGF- β 1), KT + 8-Br-cGMP group (1 μ M KT5823 + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1). Cells from control mice without asthma were the control group. Bronchial epithelial cells from OVA-exposed mice and controls were transiently transfected with 20 nM siRNA. After transfection, the cells from OVA-exposed mice were divided into eight groups' undergone similar treatment as described before. The mRNA expression was assessed by real time PCR, and the protein expression was detected by western blotting. (A) mRNA expressions of α -SMA. (B) Western blotting showing α -SMA expressions in bronchial epithelial cells. (C) Western blotting showing α -SMA expressions in bronchial epithelial cells with Smad3 silence. (D) Quantitation of protein bands showing α -SMA expressions with and without Smad3 silence. *: P value < 0.05.

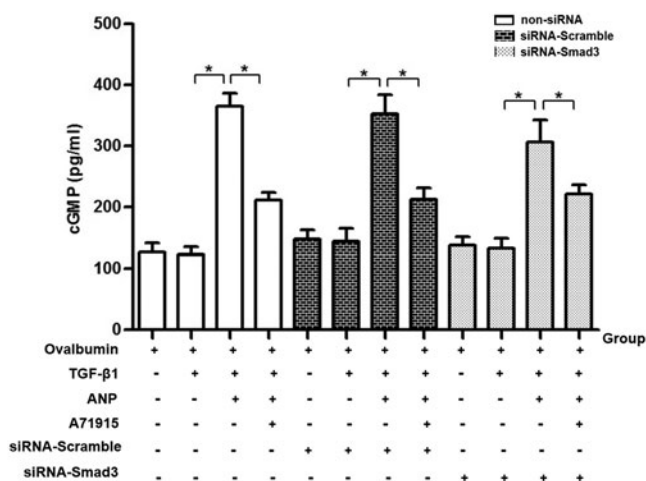


Figure 4. cGMP level in murine bronchial epithelial cells assessed by ELISA. In ANP group, cGMP level was higher than that in TGF- β 1 group. It was decreased when treated with A71915. The cGMP levels in cells transfected with Smad3-siRNA was similar with those without transfection. *: P value < 0.05.

that expression was higher than that in ANP group, suggesting that p-Smad3 may be downstream of ANP. When TGF- β 1-induced cells

were treated with 8-Br-cGMP (a cGMP analogues), p-Smad3 protein expression was decreased compared with TGF- β 1 group. In

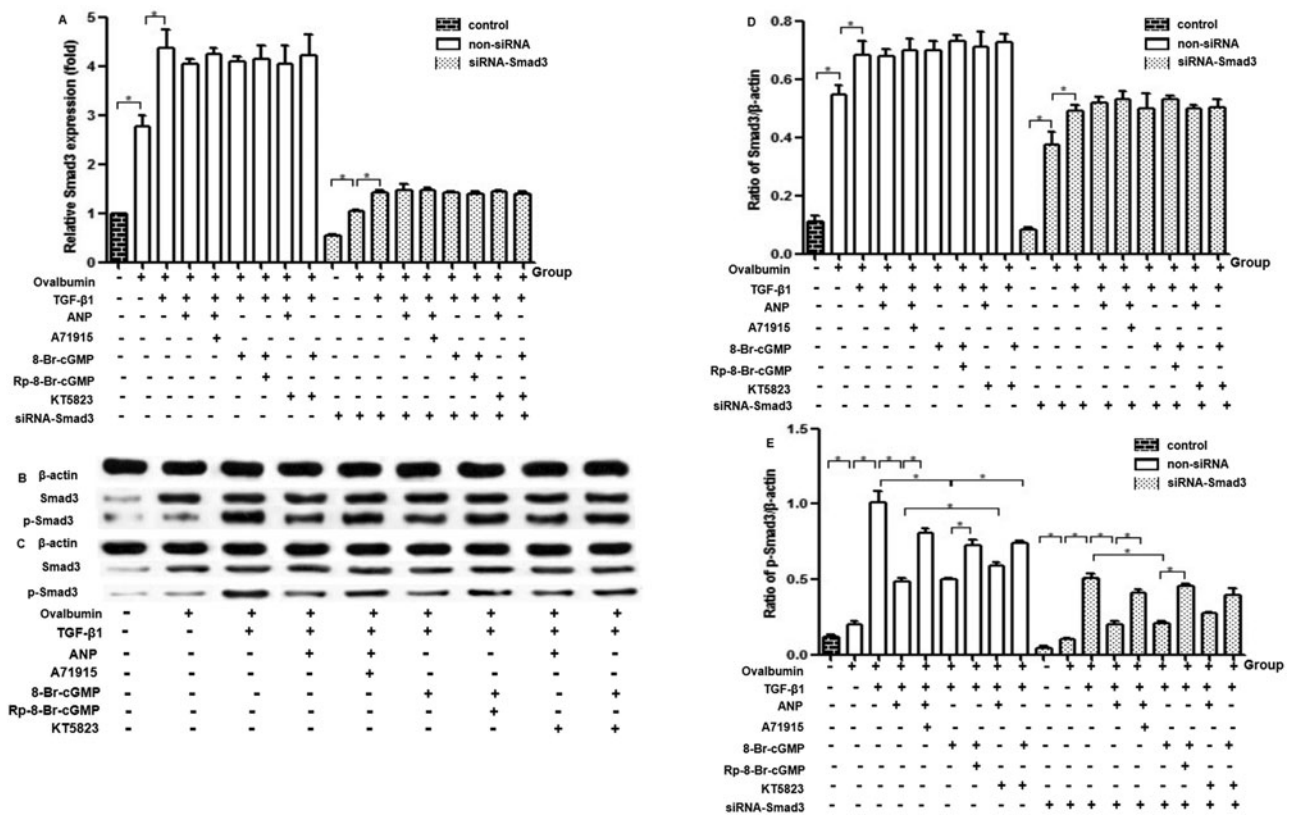


Figure 5. The mRNA and protein expressions of Smad3 and p-Smad3 in bronchial epithelial cells before and after Smad3 silenced. Cells from OVA-exposed mice were subdivided into eight groups as follows: asthma group without any treatment, TGF- β 1 group (10 ng/mL TGF- β 1), ANP group (1 μ M ANP + 10 ng/mL TGF- β 1), A71915 group (1 μ M A71915 + 10 ng/mL TGF- β 1 + 1 μ M ANP), 8-Br-cGMP group (1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), Rp-8-Br-cGMP group (50 μ M Rp-8-Br-cGMP + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1), KT + ANP group (1 μ M KT5823 + 1 μ M ANP + 10 ng/mL TGF- β 1), KT + 8-Br-cGMP group (1 μ M KT5823 + 1 mM 8-Br-cGMP + 10 ng/mL TGF- β 1). Cells from control mice without asthma were the control group. Bronchial epithelial cells from OVA-exposed mice and controls were transiently transfected with 20 nM siRNA. After transfection, the cells from OVA-exposed mice were divided into eight groups' undergone similar treatment as described before. The mRNA expression was assessed by real time PCR, and the protein expression was detected by western blotting. (A) mRNA expression of Smad3. (B) Western blotting showing Smad3 and p-Smad3 expressions in bronchial epithelial cells. (C) Western blotting showing Smad3 and p-Smad3 expressions in bronchial epithelial cells with Smad3 silence. (D) Quantitation of protein bands showing Smad3 expressions with and without Smad3 silence. (E) Quantitation of protein bands showing p-Smad3 expressions with and without Smad3 silence. *: P value < 0.05.

contrast, p-Smad3 expression in Rp-8-Br-cGMP group was higher than that in 8-Br-cGMP, suggesting that p-Smad3 may be downstream of cGMP. Furthermore, when ANP group was treated with KT5823 (a PKG inhibitor), p-Smad3 protein expression was enhanced, indicating that p-Smad3 may be the downstream of PKG (Figure 5B,E). Therefore, p-Smad3 may be the downstream of ANP/cGMP/PKG pathway.

When Smad3 was silenced, protein expressions of p-Smad3 in all groups were decreased (Figure 5C,E).

Figure 6 illustrates the expressions of Smad3 in murine bronchial epithelial cells identified by using immunohistochemistry. In non-Smad3-

silenced cells, Smad3 in control group was located mostly in cytoplasm, and it becomes expressed in nucleus in OVA-exposed group. In the TGF- β 1 group, Smad3 was predominantly expressed in nucleus. Cells in ANP, 8-Br-cGMP or KT + ANP group manifested a cytoplasmic translocation for Smad3 as compared with that of cells in TGF- β 1 group. In contrast, Smad3 was highly expressed in the nucleus after co-cultured with A71915, Rp-8-Br-cGMP or KT + 8-Br-cGMP when compared with ANP group. Once Smad3 was silenced by siRNA, it was decreasingly expressed, and was mostly expressed in the cytoplasm in all groups. These results confirm that ANP could inhibit TGF- β 1-induced nuclear

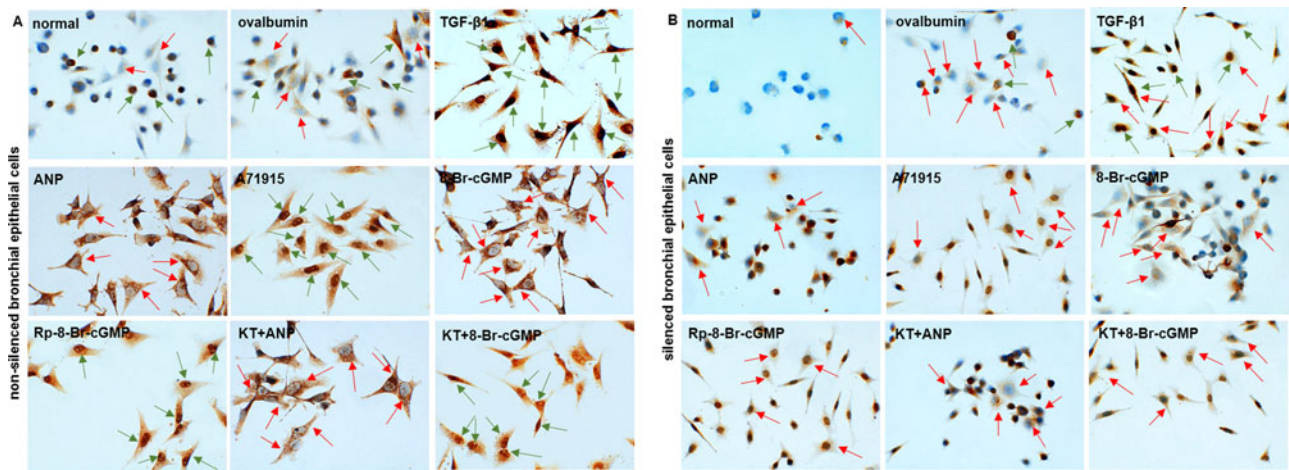


Figure 6. The expressions of Smad3 in murine bronchial epithelial cells identified by using immunohistochemistry. (A) Smad3 in control group was expressed mostly in cytoplasm of non-Smad3-silenced cells, whereas it was mainly expressed in nucleus in TGF- β 1 group. When administrated with ANP, 8-Br-cGMP, or KT + ANP, Smad3 became more expressed in cytoplasm than that with TGF- β 1. In contrast, Smad3 was more expressed in nucleus after co-cultured with A71915, Rp-8-Br-cGMP, or KT + 8-Br-cGMP than that with ANP. (B) In Smad3-silenced cells, Smad3 was mostly expressed in cytoplasm in all groups. Red arrow: Smad3 was expressed in cytoplasm. Green arrow: Smad3 was expressed in nucleus ($\times 400$ magnification).

translocation of p-Smad3 in bronchial epithelial cells through cGMP/PKG signaling, and then reducing EMT of airway.

Discussion

In this study, we found that ANP could inhibit TGF- β 1-induced EMT of bronchial epithelial cells from murine model of allergic asthma. We further demonstrated that ANP could inhibit that TGF- β 1-induced EMT through cGMP/PKG signaling, targeting TGF- β 1/Smad3 via attenuating phosphorylation of Smad3 in vitro.

ANP/NPRA inhibits TGF- β 1-induced EMT of murine bronchial epithelial cells

In the present study, mRNA and protein expressions of E-Cadherin were decreased with TGF- β 1 inducing in bronchial epithelial cells from OVA-exposed mice, whereas α -SMA expression is increased, demonstrating that TGF- β 1 successfully induced EMT in murine bronchial epithelial cells. The altered expressions of E-Cadherin and α -SMA in TGF- β 1-induced bronchial epithelial cells from OVA-exposed mice could be reversed by ANP. Moreover, the effect of ANP could be inhibited by NPRA antagonist. These results were consistent with our previous findings in human airway epithelial cells,⁸ and suggest that ANP/

NPRA could inhibit TGF- β 1-induced EMT of bronchial epithelial cells from murine model of allergic asthma. We further demonstrated the potential therapeutic role of ANP in airway EMT of asthma in this study.

ANP/NPRA inhibits TGF- β 1-induced EMT of murine bronchial epithelial cells through cGMP/PKG pathway

We further explored the mechanism for how ANP inhibits airway EMT. We found that in TGF- β 1-induced bronchial epithelial cells from murine model of allergic asthma, the EMT could be inhibited using cGMP analog, which could be reversed by cGMP inhibitor. The cGMP level in the TGF- β 1-induced bronchial epithelial cells was increased when treated with ANP, which could be reversed by NPRA antagonist. Those results indicate that cGMP expression could be induced by ANP/NPRA signaling. Thus, ANP/NPRA could inhibit airway EMT of allergic asthma model through inducing cGMP expression. Previous study on rat cardiac myocytes found that ANP/cGMP signaling could reduce proliferation and function of ventricular fibroblasts.¹² Our study shows similar role of ANP/cGMP signaling in bronchial epithelial cells, and suggests that ANP/cGMP signaling could inhibit TGF- β 1-induced EMT on asthma.

Furthermore, our study shows that PKG inhibitor could inhibit the effect of cGMP analog, indicating that PKG is the downstream of cGMP signaling. A protective role of ANP/cGMP/PKG signaling in cardiac injury and remodeling has been reported previously.^{13,14} Our study suggests its protective role in airway remodeling of asthma. Therefore, ANP/NPRA may inhibit TGF- β 1-induced EMT of murine bronchial epithelial cells through the cGMP/PKG signaling pathway in asthma.

ANP/cGMP/PKG inhibits TGF- β 1-induced EMT of murine bronchial epithelial cells by targeting Smad3

TGF- β 1 could induce EMT by targeting Smad3 in several kinds of epithelial cells.^{15–17} This present study confirmed that effect of TGF- β 1 in murine bronchial epithelial cells. In our study, Smad3 was mostly expressed in nucleus of bronchial epithelial cells when it is induced by TGF- β 1, which could be reduced by ANP, cGMP analog or PKG inhibitor. When administrated with ANP, 8-Br-cGMP or KT + ANP, Smad3 became more expressed in cytoplasm than that with TGF- β 1. After silenced with Smad3, Smad3 was mostly expressed in cytoplasm in all groups. These results confirm our previous findings on human airway cells,⁸ and further indicate that TGF- β 1 may induce Smad3 activation in EMT, which could be inhibited by ANP/cGMP/PKG signaling activation. This could be confirmed with findings that EMT was decreased after Smad3 inhibition in vitro. Moreover, in our study, compared with Smad3 protein expression, p-Smad3 was showed a significantly difference of protein expression among all groups. This confirms that Smad3 promotes EMT in airway when it is phosphorylated, which may be a target of ANP/cGMP/PKG signaling in inhibiting EMT.

Moreover, it may be related with TGF- β 1/Smad3 signaling for possible mechanisms through which ANP/cGMP/PKG signaling alters Smad3 phosphorylation. The ANP/cGMP/PKG could act to limit the effects of TGF- β signaling by diverting phosphorylated Smad3 away from the nucleus so that it can no longer act as a transcription factor and offer support for ANP/

cGMP/PKG as a transcriptional regulator of Smad3-dependent signal transduction.^{18–21} In our study, we compared TGF- β 1-induced E-Cadherin and α -SMA expressions to those with ANP and NPRA antagonist administration, and found that ANP/NPRA could inhibit TGF- β 1-induced EMT of murine bronchial epithelial cells. Thus, our findings support the mechanism that ANP/cGMP/PKG signaling targets on TGF- β 1/Smad3 via attenuating phosphorylation of Smad3 in vitro.

Conclusions

In a murine model of allergic asthma, ANP could inhibit TGF- β 1-induced EMT of bronchial epithelial cells through activating cGMP/PKG signaling, targeting on TGF- β 1/Smad3 via attenuating phosphorylation of Smad3 in vitro, which may provide the potential of ANP in treating refractory asthma with airway remodeling.

Declaration of interest

The authors report no conflict of interest.

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