

RESEARCH ARTICLE

TRPC3 overexpression and intervention in airway smooth muscle of ovalbumin-induced hyperresponsiveness and remodeling

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Abstract

Transient receptor potential canonical channel 3 (TRPC3) proteins function as non-voltage-gated Ca^{2+} -permeable channels and play divergent roles in many processes of pathophysiology. The purpose of this study was to determine the relationship between TRPC3 expression and airway hyperresponsiveness and remodeling in ovalbumin-induced asthmatic Kunming mice. Mice were sensitized and challenged by ovalbumin to establish asthmatic model. Hematoxylin-eosin staining, hydroxyproline assay, and isometric tracheal ring force measurement were used to evaluate airway remodeling and hyperresponsiveness in asthmatic mice. Western blot was performed to detect the expression of TRPC3 proteins. MTT assay was used to measure the proliferation of airway smooth muscle cells. TRPC3 protein expression increased in airway smooth muscle of asthmatic mice. GdCl_3 , a nonspecific TRPC blocker, attenuated the contractile force of airway smooth muscle. Fetal bovine serum stimulated airway smooth muscle cells proliferation and augmented TRPC3 protein expression. Both TRPC3 blockade by GdCl_3 or specific TRPC3 antibodies and gene silencing by siRNA inhibited the proliferation of airway smooth muscle cells. In contrast, the current drugs treatment for asthma such as Dexamethasone and Aminophylline had no effects on TRPC3 protein overexpression. Therefore, TRPC3 protein overexpression may be involved in airway smooth muscle hyperresponsiveness and remodeling in asthmatic mice, providing evidence for a new direction of asthma pathogenesis research and a new target for drug intervention.

Keywords: airway smooth muscle cells; asthma; hyperresponsiveness; remodeling; transient receptor potential canonical channel 3

Introduction

The pathophysiology of asthma is characterized by airway hyperresponsiveness (AHR) and remodeling. The mechanism regulating airway AHR and remodeling is still unclear. Airway smooth muscle (ASM) is the main effector of AHR. Beyond its contractile function, hyperplasia of ASM cells is an important aspect of airway remodeling (Schuliga et al., 2013). The role of ASM cells in the pathogenesis of asthma has been paid much attention.

The free intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) plays the key role in the contraction and remodeling of ASM. $[\text{Ca}^{2+}]_i$

is controlled through two main pathways: extracellular Ca^{2+} influx and endoplasmic reticulum Ca^{2+} release. Extracellular Ca^{2+} influx occurs through three types of channels: voltage-dependent Ca^{2+} channels, receptor-operated Ca^{2+} channels and store-operated Ca^{2+} channels. Transient receptor potential canonical channel 3 (TRPC3) functions as non-voltage-gated Ca^{2+} -permeable channels and plays divergent roles in many pathophysiological processes.

TRPC3, among TRPC1-7 is expressed as the predominant protein in rodents and human ASM cells (Ong et al., 2003; Corteling et al., 2004; Manetsch et al., 2012). It has been found that TRPC3 regulates proliferation of another cell type

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Bo-Ming Xu and Jia-Hua Zhang contributed equally to this work.

Abbreviations: ACh, acetylcholine; AHR, airway hyperresponsiveness; ASM, airway smooth muscle; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; MTT, methyl thiazolyl tetrazolium; OVA, ovalbumin; TRPC, transient receptor potential canonical channel.

like cardiac fibroblasts (Zhu *et al.*, 2011). Following incubation with the inflammatory cytokine tumor necrosis factor, the TRPC3 protein expression in human ASM cells was markedly increased, accompanied by enhanced acetylcholine (ACh)-induced elevations in cytosolic Ca^{2+} concentration (White *et al.*, 2006). The molecular levels and functional activity of TRPC3 was upregulated in ASM, contributing to hyperresponsiveness in asthmatic mice (Xiao *et al.*, 2010). TRPC3 expression increased in ASM of a patient with asthma and in a mouse allergic model, and specific inhibition of TRPC3 with lentivirus-mediated shRNA and a selective TRPC3 antagonist could reduce ASM cells proliferation and migration (Song *et al.*, 2016; Wang *et al.*, 2017). However, there is limited evidence for its involvement in pathogenesis of asthma.

In this study, we established an asthmatic model in Kunming mice by ovalbumin (OVA) sensitization and challenge, and examined whether TRPC3 protein might be changed in freshly isolated ASM. We detected the proliferation of ASM cells and the expression levels of TRPC3 protein. We also determined the effect of TRPC3 blockade on ASM contraction and cell proliferation compared to the current drugs used for asthma treatment (Dexamethasone and Aminophylline) on TRPC3 expression.

Materials and methods

Preparation of OVA-sensitized/-challenged mouse model

All animal studies were approved by the Animal Ethics Committee of Huazhong University of Science and Technology. Kunming mice (6- to 7-week-old) were randomly divided into control and model groups. Mice in model group were sensitized by intraperitoneal injection of 0.2 mL OVA and aluminum hydroxide mixture containing 3 mg OVA and 2 mg aluminum hydroxide in 1 mL 0.9% saline solution. On days 15 through 19, animals were challenged by intranasal instillation of 50 μL 0.9% saline solution containing OVA. Control mice were treated with 0.9% saline solution alone. After the last intranasal exposure to the OVA or saline, mice were sacrificed for the subsequent experiments.

Histological examination

Lung tissue sections stained with hematoxylin-eosin in each group were selected and analyzed under an optical microscope (Olympus DP72, Japan). Bronchi with about 100 μm diameters were chosen to measure airway wall thickness with Image-Pro Plus software.

Hydroxyproline measurement

The content of ASM hydroxyproline was assayed by chloramine-T method. The ASM tissues were hydrolyzed

in 1 mL 6 mol/L HCl at 121°C for 3 h. Then, the samples were neutralized with 6 mol/L NaOH to pH = 7. Hydroxyproline oxidation was initiated by adding 1 mL of 0.5 mol/L chloramine-T reagents to each sample. After standing 10 min at room temperature, each sample was added 1 mL of 3.15 mol/L perchloric acid and dimethylaminobenzaldehyde, then mixed and placed in a 100°C bath for 3 min. Finally, the samples were cooled for 15 min and the absorption value was read at 562 nm with a microplate reader (Tecan, Switzerland).

Isometric tracheal rings force measurement

The tracheal ASM rings including 3–4 C-shape bones were isolated and fixed in the organ bath containing physiological saline solution (in mM: 130 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1 MgSO_4 , 10 HEPES, 10 Glucose, pH value was adjusted to 7.4 with NaOH). The rings were held at a resting tension of 0.5 g and equilibrated for 1 h. During the equilibration period, the solution was exchanged every 15 min. Contractile force was recorded with a force transducer (Chengyi, China). The concentration-effect curves of acetylcholine (ACh, 0.01–100 μM) were constructed. And concentration for 50% of maximal effect ($\sim\text{EC}_{50}$) of ACh was determined for each tracheal ring based on these concentration-effect curves. Tracheal rings were contracted to the determined $\sim\text{EC}_{50}$ of ACh and force was allowed to plateau, and then 5 or 10 μM GdCl_3 was added to test its influence on airway contraction. The contractile force was expressed as mg force/mg tracheal ASM rings.

Western blot

Isolated ASM tissues or ASM cells were homogenized in ice-cold radioimmunoprecipitation assay buffer. The homogenate was centrifuged to obtain total cell lysate. The protein concentration was determined by Bradford assay. Proteins were separated by 8% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. The nonspecific binding sites on the membrane were blocked by 5% non-fat milk in Tris-buffered saline with Tween-20. The membrane was incubated with specific antibodies against TRPC3 (1:200 dilution, Alomone Laboratories, Israel) or actin (1:1,000 dilution, Santa Cruz Biotechnology, USA). Using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents, protein bands were shown. TRPC3 protein expression levels were normalized to actin expression levels.

Cell culture

The tracheas dissected from the surrounding tissues were washed three times in precooled phosphate buffer saline

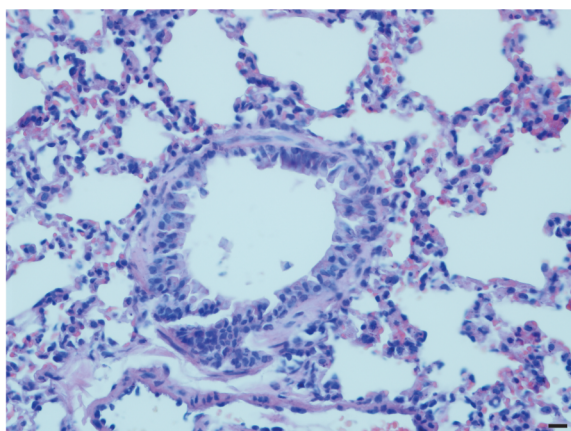
solution (in mM: 137 NaCl, 2.7 KCl, 10 NaH₂PO₄, 2 KH₂PO₄, pH value was adjusted to 7.4 with NaOH) containing penicillin G 100 U/mL and streptomycin 100 U/mL. Then, ASM strips were isolated, cut into small pieces, digested by Collagenase II (Worthington Biochemical Corporation, USA). Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA), 100 U/mL penicillin and streptomycin, in a 5% CO₂ humidified incubator at 37°C. Cells were passaged until cell confluence occurred. The culture medium was replaced every 48 h. Cells between passages 3 and 6 were used for experiments.

MTT assay

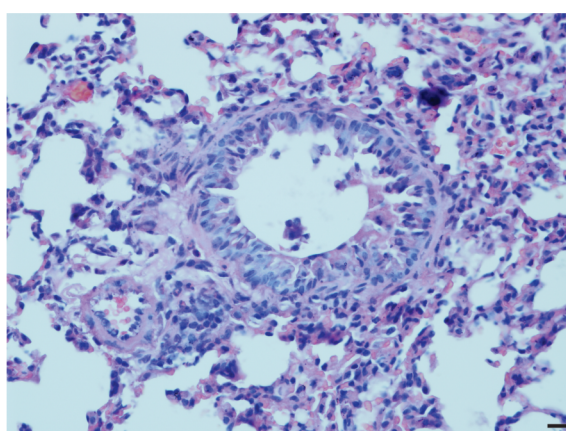
ASM cells were digested with 0.25% trypsin and then seeded into 96-well culture plate with the density of 0.5×10^4 /well. Cells were cultured in DMEM containing 10% FBS for 24 h and then arrested in FBS-free DMEM for another 24 h. ASM cells were stimulated with 10% FBS or 0% FBS again. When necessary, TRPC3 channel blockers or TRPC3 antibodies were included in the medium. After 24 h, methyl thiazolyl tetrazolium (MTT) (Sigma, USA) was added to each well incubating for 4 h at 37°C. The medium was removed from the wells, and 150 μ L dimethyl sulfoxide (DMSO, Sigma)

A

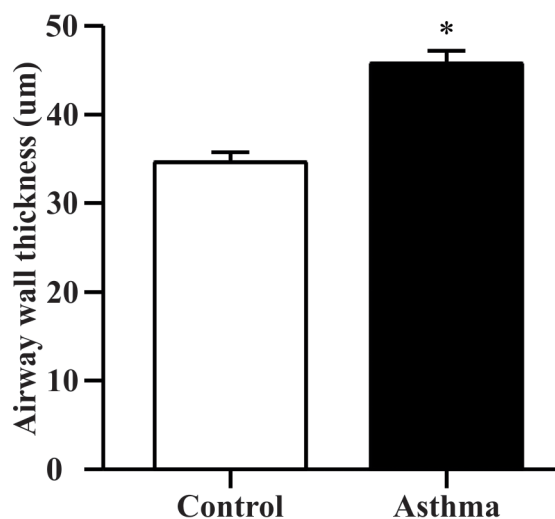
Control



Asthma



B



C

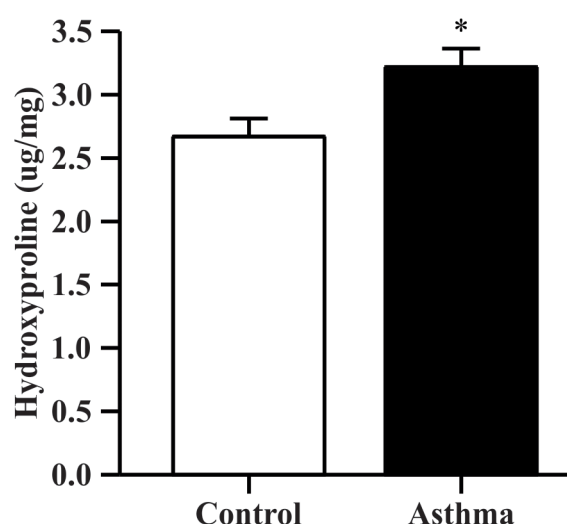


Figure 1 Histological changes in airway smooth muscle of mice. (A) Hematoxylin-eosin staining of the lung tissue sections. Scale bar: 50 μ m. (B) Airway wall thickness of mice ($n = 5$). (C) Hydroxyproline contents in airway smooth muscle of mice (control group $n = 20$, asthma group $n = 16$). Data are present as mean $\pm$ S.E.M, * $P < 0.05$, compared with control group.

was added to dissolve the colored formazan crystals. After mixing thoroughly with the oscillator for 20 min, absorbance values at 490 nm wavelength were recorded with a microplate reader (Tecan). The value of absorbance is proportional to the number of living cells. Each group had at least six wells and repeated three times.

Transfection of siRNA

ASM cells were seeded into 96-well plates and cultured with DMEM containing 10% FBS in a 5% CO₂ incubator at 37°C and transfected with 80 nM TRPC3 (catalog number: J-049680-00-05, Dharmacon, Chicago, IL) or nontarget siRNAs (catalog number: D-001810-01-05) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according the manufacturer's instructions. The efficiency of

siRNA-mediated gene silencing was analyzed by Western blot. Proliferation of ASM cells after transfection of siRNA for 72 h was evaluated by MTT assay.

Drugs treatment

Kunming mice (6–7 weeks old) were randomly divided into four groups of Control, Asthma, Dexamethasone, and Aminophylline, six mice per group. Asthmatic model mice were established according to the above method. In Dexamethasone and Aminophylline groups, 1 h before OVA challenge, the mice were treated with 3 mg/kg Dexamethasone or 20 mg/kg Aminophylline by intraperitoneal injection for 5 consecutive days. In Control and Asthma groups, the mice were treated with 0.9% saline solution by intraperitoneal injection. After treatment, mice were sacrificed to determine changes of hydroxyproline and TRPC3 protein expression in ASM.

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical analysis was performed using Student's *t*-test or the one-way ANOVA test followed by Tukey's post hoc test. A value of *P* < 0.05 was considered statistically significant.

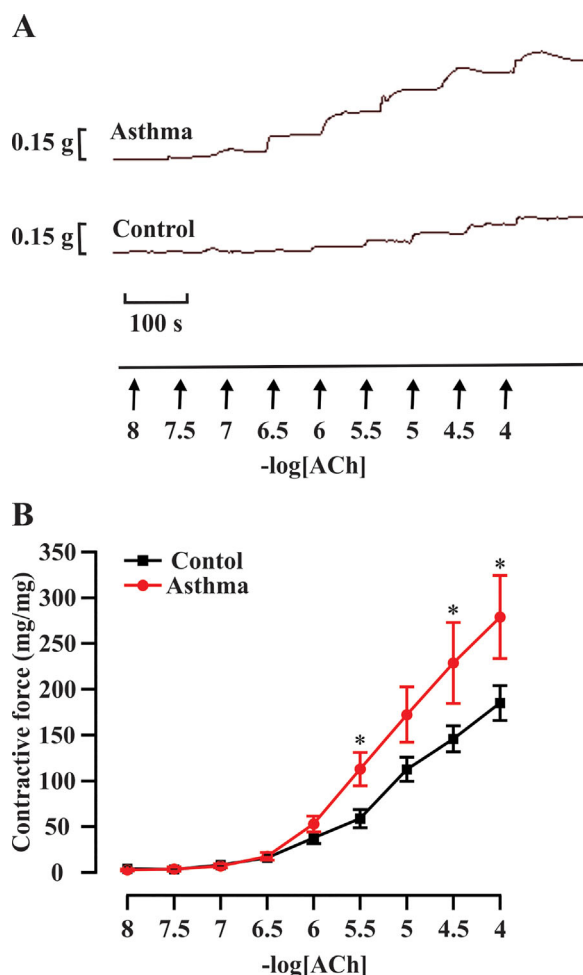


Figure 2 Contractile changes of isolated tracheal rings induced by acetylcholine (ACh). (A) Typical curve of the effect of ACh on isolated tracheal rings, arrows indicate the point at which ACh was added. (B) Concentration-response curve of ACh. Data are present as mean $\pm$ S.E.M, *n* = 10. **P* < 0.05, compared with control group.

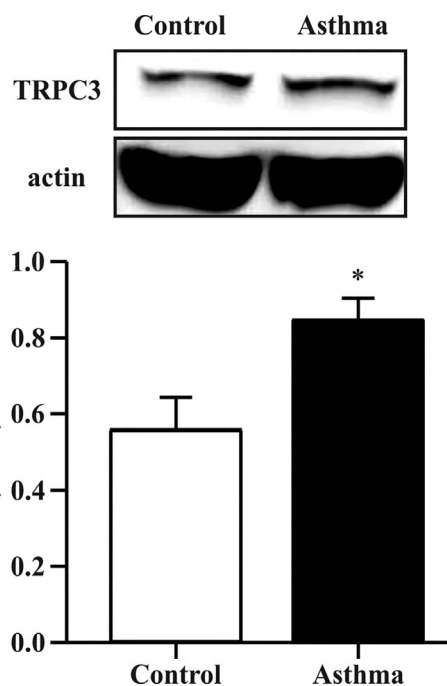


Figure 3 Western blot analyses of TRPC3 protein expression in airway smooth muscle of mice. TRPC3 expression levels were normalized to actin expression levels. Data were obtained from at least three different experiments. Data are present as mean $\pm$ S.E.M. **P* < 0.05, compared with control group.

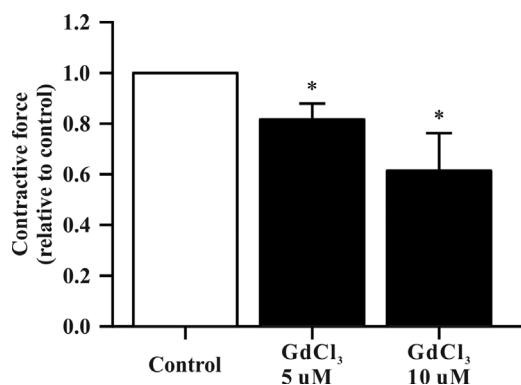


Figure 4 Effect of GdCl₃ on the contraction of airway smooth muscle of asthmatic Kunming mice. Data are present as mean $\pm$ S.E.M, $n = 4$. * $P < 0.05$, compared with control.

Results

Histological changes in ASM in Kunming mice

Airways from asthmatic mice revealed severe peribronchial inflammatory infiltrate compared with control mice (Figure 1A). We chose the bronchi with about 100 μ m diameter to measure airway wall thickness with Image-Pro Plus software. Airway walls obviously thickened in asthmatic mice compared with control mice (Figure 1B). Hydroxyproline content is an important indicator of tissue collagen deposition. Hydroxyproline content significantly increased

in ASM from asthmatic mice compared with control mice (Figure 1C).

Contractile changes of isolated tracheal rings induced by ACh

Asthmatic mice showed airway hyperresponsiveness. ACh (0.01–100 μ M) elicited a dose-dependent contraction in isolated tracheal rings (Figure 2A). The contractile response of tracheal rings to ACh markedly augmented in asthmatic mice (Figure 2B).

Changes of TRPC3 protein expression in ASM

Previous studies have shown that TRPC3 molecule is present in isolated C57/B6, Swiss Webster and BALB/c mouse ASM tissues, we examined whether TRPC3 changed in asthmatic Kunming mice using Western blot. TRPC3 was detected in ASM, and TRPC3 protein expression significantly increased in ASM from asthmatic Kunming mice (Figure 3), which was consistent with previous experimental results, suggesting its importance of potential function in ASM.

Effects of GdCl₃ on the contraction of ASM

AHR is associated with abnormal contraction of ASM. To further confirm the functional importance of TRPC3 in ASM, we observed the effect of GdCl₃, a nonspecific blocker

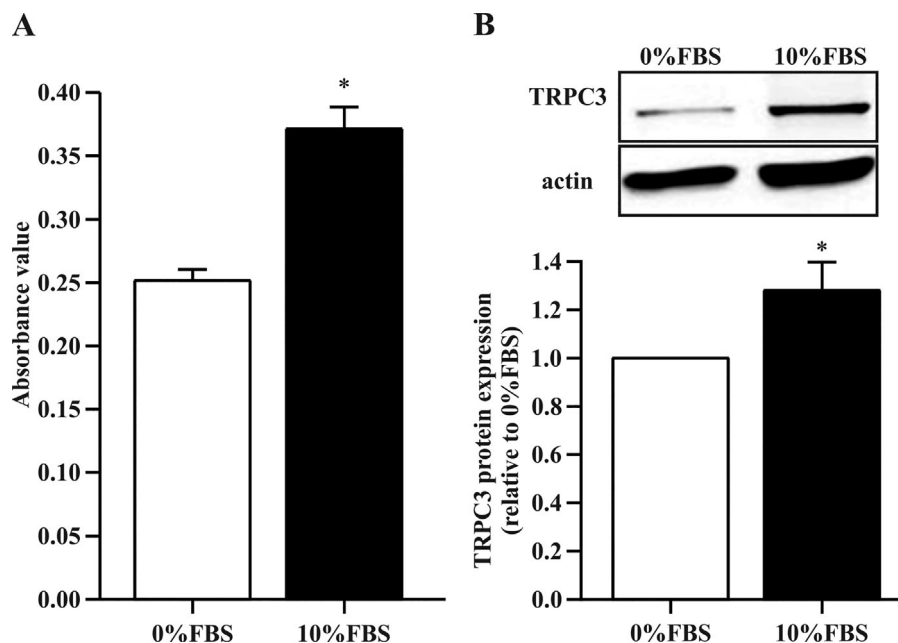


Figure 5 Effects of 10% fetal bovine serum (FBS) on the airway smooth muscle cell proliferation and TRPC3 protein expression. (A) The effect of 10% FBS on proliferation of airway smooth muscle cells. (B) Western blot analyses of TRPC3 protein expression in airway smooth muscle cells of mice. TRPC3 expression levels were normalized to 0% FBS group. Data are present as mean $\pm$ S.E.M, $n = 6$. * $P < 0.05$, compared with 0% FBS group.

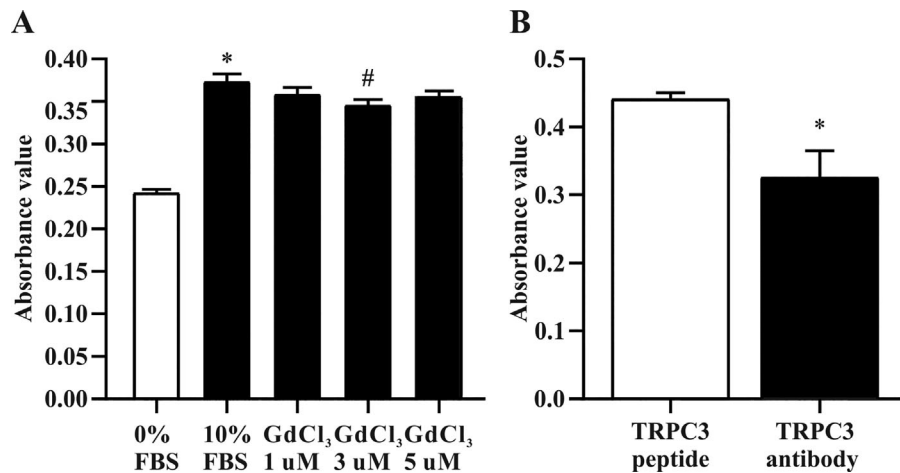


Figure 6 Effect of TRPC3 blockade on the airway smooth muscle cells proliferation. (A) Effect of nonspecific TRPC3 blocker GdCl₃ on the proliferation of airway smooth muscle cells. Data are present as mean $\pm$ S.E.M, $n = 5$. * $P < 0.05$, compared with 0% FBS group; # $P < 0.05$, compared with 10% FBS group. (B) Effect of specific TRPC3 antibodies (1:200) on the proliferation of airway smooth muscle cells. Data are present as mean $\pm$ S.E.M, $n = 5$. * $P < 0.05$, compared with TRPC3 peptide group.

of TRPC3, on airway contraction induced by ACh. A total of 5, 10 μ M GdCl₃ attenuated contractile force of ASM induced by ACh, and the contractile force reduced to 81.6 and 61.3%, respectively, compared with before the blocker added (Figure 4).

Effects of 10% FBS on the ASM cells proliferation and TRPC3 protein expression

ASM cell proliferation is an important aspect of airway remodeling. ASM was digested with enzyme for cell culture. The proliferation rate of ASM cells was measured by MTT assay. Western blot was performed to detect the expression levels of TRPC3 protein in cultured ASM cells. A total of 10% FBS significantly stimulated ASM cells proliferation (Figure 5A) and augmented TRPC3 protein expression (Figure 5B).

Effects of TRPC3 blockade and gene silencing on the ASM cells proliferation

To further investigate the role of TRPC3 in cell proliferation, we then examined the effects of TRPC3 blockade on 10% FBS-induced cell proliferation using MTT assay. As we predicted, 3 μ M GdCl₃, a nonspecific blocker of TRPC3, inhibited the proliferation of ASM cells (Figure 6A). Similarly, both specific TRPC3 antibodies (1:200) and TRPC3 gene silencing by siRNA significantly inhibited the proliferation of ASM cells (Figures 6B and 7).

Effects of Aminophylline and Dexamethasone on TRPC3 protein expression

Aminophylline and Dexamethasone are commonly used drugs to treat asthma in the clinical. So we wonder whether

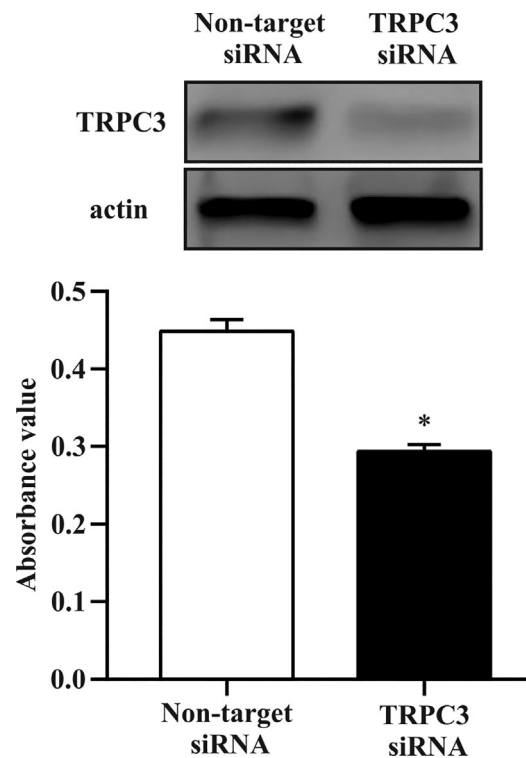


Figure 7 Effect of TRPC3 gene silencing by siRNA on the airway smooth muscle cells proliferation. (A) Western blot analyses of the efficiency of siRNA-mediated TRPC3 gene silencing. (B) The proliferation of airway smooth muscle cells after transfection of TRPC3 siRNA for 72 h. Data are present as mean $\pm$ S.E.M, $n = 5$. * $P < 0.05$, compared with Nontarget siRNA group.

these commonly used drugs can influence the TRPC3 protein expression. First, we observed the effects of Aminophylline and Dexamethasone on contraction of isolated tracheal rings. Aminophylline not Dexamethasone relieved the contraction induced by ACh (Figure 8A). Then, we used Aminophylline and Dexamethasone to treat asthmatic mice for 5 consecutive days. Continuous administration of Dexamethasone for 5 days not Aminophylline reduced hydroxyproline content in ASM from asthmatic mice (Figure 8B). TRPC3 protein expression in ASM was not affected by both Aminophylline and Dexamethasone (Figure 8C).

Discussion

Asthma is a chronic inflammatory airway disease, with AHR and remodeling as its main features (Girodet *et al.*, 2011).

Kunming mice were sensitized/challenged by OVA to establish asthmatic model. Deepened breathing, die blausucht and nodding breathing were observed during challenge phase in OVA-sensitized mice. Airways from asthmatic mice revealed severe peribronchial inflammatory infiltrate and airway walls obviously thickened. Hydroxyproline content increased in ASM from asthmatic mice. The contractile response of asthmatic tracheal rings to ACh markedly augmented. The above results demonstrated that the model we established had airway inflammation, hyperresponsiveness and remodeling which are characteristics of allergic asthma.

Previous studies have found that many TRPCs proteins are expressed in ASM, such as TRPC1, 3, 4 in pig ASM, TRPC1-6 in guinea pig ASM, TRPC1, 3, 4, 5, 6 in human ASM (Sweeney *et al.*, 2002; Corteling *et al.*, 2004; Manetsch *et al.*, 2012), suggesting some certain relationship between

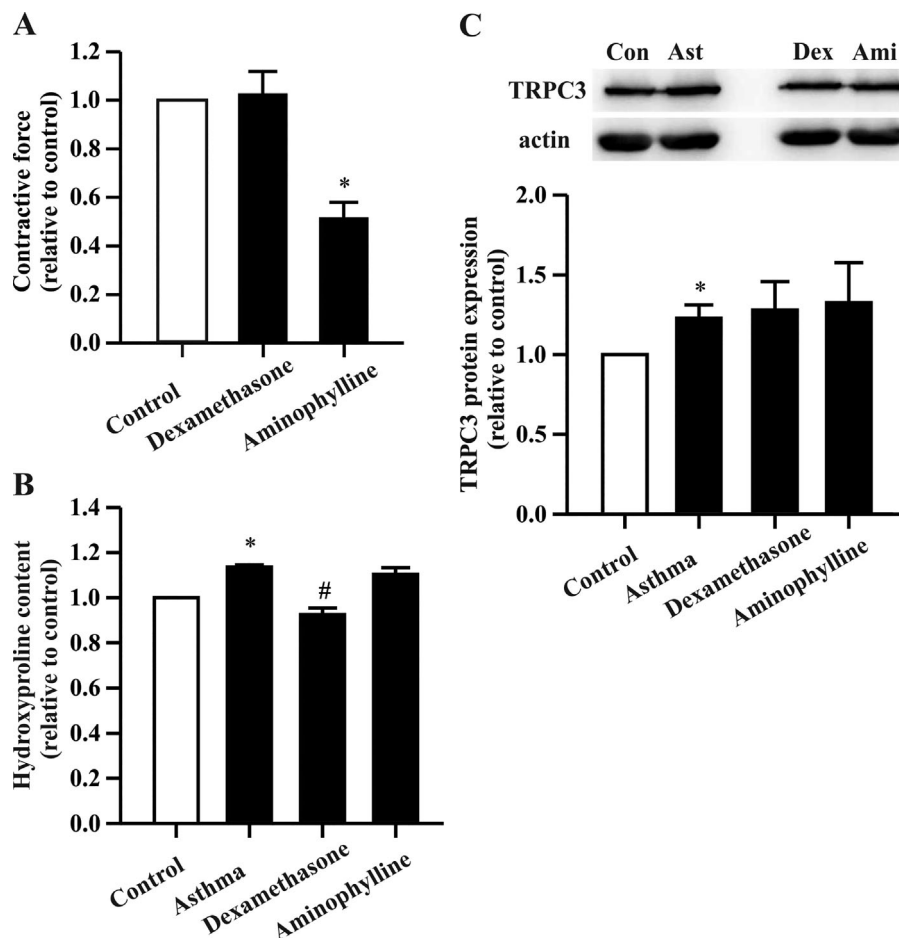


Figure 8 Effects of Dexamethasone and Aminophylline on contractility, hydroxyproline content, and TRPC3 protein expression of airway smooth muscle. (A) Effects of Dexamethasone and Aminophylline on contractile force of airway smooth muscle induced by ACh EC₅₀. (B) Effects of Dexamethasone and Aminophylline on hydroxyproline content. (C) Effects of Dexamethasone and Aminophylline on TRPC3 protein expression. Data are present as mean $\pm$ S.E.M, $n = 4-6$. * $P < 0.05$, compared with control group; # $P < 0.05$, compared with asthma group.

the changes of TRPCs expression and ASM dysfunction. Methacholine-induced AHR and agonist-induced contractility of tracheal rings were increased in TRPC6(-/-) mice compared with wild-type mice, most probably due to compensatory up-regulation of TRPC3 in ASM (Sel *et al.*, 2008). TRPC3 has higher level of basic activities than TRPC6 (Dietrich *et al.*, 2003). Expression level of TRPC3 may affect ASM cell membrane potential. Expression and activity of TRPC3 significantly up-regulated in OVA-sensitized/challenged ASM cells, contributing to membrane depolarization and airway hyperresponsiveness (Xiao *et al.*, 2010; Song *et al.*, 2016; Wang *et al.*, 2017). In our study, TRPC3 protein increased in ASM from asthmatic Kunming mice, suggesting that the airway hyperresponsiveness and remodeling induced by OVA may be associated with TRPC3 protein overexpression in ASM.

To further confirm the relevance of TRPC3 overexpression to airway hyperresponsiveness, we used GdCl₃, a nonspecific blocker of TRPC3 (Mahn *et al.*, 2009). We found that 5, 10 μ M GdCl₃ attenuated contractile force of ASM, and the contractile force reduced to 81.6 and 61.3%, respectively. Our data in asthmatic Kunming mice were consistent with previous reports in asthmatic Swiss Webster and BALB/c mice (Song *et al.*, 2016; Wang *et al.*, 2017).

Hyperplasia of ASM cells is an important aspect of airway remodeling. Compelling evidence suggests an excessive ASM cell proliferation in asthma (Johnson *et al.*, 2001; Paulino *et al.*, 2003; Mahn *et al.*, 2009). Intracellular Ca²⁺ is a critical signal transduction element in regulating cell proliferation (Sil'vestrova *et al.*, 2011; Aravamudan *et al.*, 2016). TRPC3 has higher permeability to this ion (Dietrich *et al.*, 2006) among TRPC1-7. Furthermore, TRPC3 protein increased in ASM from asthmatic mice. Thus, TRPC3 maybe participate in ASM cell proliferation. In our study, we compared the changes of TRPC3 protein expression in 10% FBS-treated ASM cells with 0% FBS-treated ASM cells and observed the effects of TRPC3 blockade on 10% FBS-induced cell proliferation. The data indicated that TRPC3 protein expression increased in proliferative ASM cells, and nonspecific TRPC3 blocker GdCl₃, specific TRPC3 antibodies and TRPC3 gene silencing by siRNA inhibited the proliferation of ASM cells. We also found that Aminophylline not Dexamethasone, the current commonly used drugs for asthma, relieved the contraction induced by ACh. Continuous administration of Dexamethasone for 5 days not Aminophylline reduced hydroxyproline content in ASM from asthmatic mice. TRPC3 protein expression in ASM was not affected by both Aminophylline and Dexamethasone. Our data further supported that TRPC3 protein overexpression in asthmatic ASM may contribute to the ASM cells hyperplasia.

Taken together, overexpression of TRPC3 protein may be involved in AHR and remodeling in asthmatic mice,

providing evidence for a new direction of asthma pathogenesis research and a new target for drug intervention.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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