Inhibitory Effect of S100A11 on Airway Smooth Muscle Contraction and Airway Hyperresponsiveness*

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[Abstract] Objective: S100A11 is a member of the S100 calcium-binding protein family and has intracellular and extracellular regulatory activities. We previously reported that S100A11 was differentially expressed in the respiratory tracts of asthmatic rats as compared with normal controls. Here, we aimed to analyze the potential of S100A11 to regulate both allergen-induced airway hyperresponsiveness (AHR) as well as acetylcholine (ACh)-induced hypercontractility of airway smooth muscle (ASM) and contraction of ASM cells (ASMCs). **Methods:** Purified recombinant rat S100A11 protein (rS100A11) was administered to OVA-sensitized and challenged rats and then the AHR of animals was measured. The relaxation effects of rS100A11 on ASM were detected using isolated tracheal rings and primary ASMCs. The expression levels of un-phosphorylated myosin light chain (MLC) and phosphorylated MLC in ASMCs were analyzed using Western blotting. **Results:** Treatment with rS100A11 attenuated AHR in the rats. ASM contraction assays showed that rS100A11 reduced the contractile responses of isolated tracheal rings and primary ASMCs treated with ACh. In addition, rS100A11 markedly decreased the ACh-induced phosphorylation of the myosin light chain in ASMCs. Moreover, rS100A11 also suppressed the contractile response of tracheal rings in calcium-free buffer medium. **Conclusion:** These results indicate that S100A11 protein can relieve AHR by relaxing ASM independently of extracellular calcium. Our data support the idea that S100A11 is a potential therapeutic target for reducing airway resistance in asthma patients.

Key words: S100A11; asthma; airway hyperresponsiveness; airway smooth muscle; relaxation

Airflow obstruction and shortness of breath, the cardinal clinical features of asthma, are mainly caused by excessive contraction of airway smooth muscle (ASM) in response to a variety of stimuli[1]. Phosphorylation of the 20-kD regulatory light chain of myosin by calcium/calmodulin-dependent myosin light chain kinase (MLCK) plays a critical role in ASM contraction. β_2-adrenoceptor agonists are the most commonly used bronchodilators currently available, and they can activate G-protein signaling and increase cyclic adenosine monophosphate (cAMP) by binding to β_2-adrenergic receptors on ASM cells (ASMCs). In turn, cAMP reduces the intracellular calcium concentration and activates protein kinase A, which ultimately decreases MLCK activity and leads to ASM relaxation[2]. However, tolerance and resistance to β_2-adrenoceptor agonists in asthmatic patients is a major clinical limitation in the treatment of asthma[3]. Thus, there is an urgent need to design new bronchodilator drugs that relax ASM and reduce pulmonary flow resistance (RL) in asthmatic patients.

S100 proteins are a large family of EF-hand (helix E-loop helix F) calcium-binding proteins that play critical roles in various biological and pathological processes. When these EF-hand proteins bind to calcium, a conformational change is triggered, allowing the proteins to interact with the appropriate target receptors and regulate various cellular processes, including smooth muscle cell contraction. S100A1, which is highly expressed in striated muscle, inhibits isometric tension and stiffness in skinned rabbit skeletal muscle at physiological concentrations[4]. Another S100 family member, S100A2, enhances calcium cycling to improve myocyte contractility and increases the relaxation performance in normal and failing cardiac myocytes[5]. A recent study has also reported that S100A4 plays a critical role in tension development in ASM by catalyzing nonmuscle myosin filament assembly[6]. These results uncover differential effects of S100 proteins on smooth muscle cell contraction function and point to S100 proteins as

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potential candidates for regulating ASM contraction in patients with asthma.

S100A11, also known as S100C or calgizzarin, is an important member of the S100 protein family; it has been proposed to play specific biological roles in the processes of cell growth[7], cell migration[8], cell apoptosis[9], cell membrane repair[10], and inflammation[11]. This protein is localized in the nucleus, cytoplasm, or outside the cell and is most abundant in smooth muscle tissues. In previous studies, it has been shown that S10011 regulates smooth muscle function by interacting with target molecules mainly in a calcium-dependent manner. For example, S100A11 inhibits the actin-activated myosin Mg^{2+}-ATPase activity of smooth muscle through its calcium-dependent interaction with actin filaments[12]. Also, calcium is involved in S100A11-mediated hypoxia-induced mitogenic factor (HIMF)-induced migration of smooth muscle cells[13]. However, calcium-independent interactions have also been recently described for some S100 proteins[14]. Our previous study demonstrated increased S100A11 expression in the lung tissue of a rat model of asthma[15]. Here, we aimed to investigate the effects of S100A11 on both allergen-induced airway hyperresponsiveness (AHR) in an asthma model and acetylcholine (ACh)-induced hypercontractility of tracheal rings and ASMCs to evaluate the potential of S100A11 to reduce ASM contraction in patients with asthma.

1 MATERIALS AND METHODS

1.1 Expression and Purification of Recombinant Rat S100A11 Protein

The rat S100A11 genomic sequences were obtained from Gene Bank (www.ncbi.nlm.nih.gov) and amplified via RT-PCR using the following sequence-specific primers: sense, 5′-GGAATTCCATATGCCTA CAGAGACTGAGA-3′; anti-sense, 5′-CCGCTCGAG GATACGCTTCTGGGAAGTC-3′. The recombinant pET-22b-rS100A11 plasmids containing the synthetic S100A11 gene were cloned through the *Nde* I and *Xho* I restriction sites and were transformed into BL21 (DE3) cells by calcium chloride transformation. Colony PCR and sequencing analysis were carried out to screen the positive colonies containing the recombinant pET-22b-rS100A11 plasmids. Expression of the S100A11 gene on the positive pET-22b-rS100A11 plasmid was then induced by 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant rat S100A11 protein (rS100A11) was then purified by affinity chromatography purification using Ni^{2+} Sepharose 6 Fast Flow beads (GE Healthcare, Sweden) and ion-exchange chromatography purification using Q Sepharose Fast Flow beads (GE Healthcare, Sweden), according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to calculate the purity of rS100A11. Specifically, the optical density value of the protein band was divided by that of the whole lane and multiplied by 100 to obtain purity percentages. The antigenic activity of rS100A11 was analyzed by Western blotting using anti-rat S100A11 antibody (Abcam, ab180593, 1:2000, UK).

1.2 Ovalbumin Sensitization and Challenge Model

Male, 6-week-old Sprague-Dawley rats were purchased from the Shanghai Laboratory Animal Center (China) and were randomly divided into five groups (n=8 per group). Animals were housed in a specific pathogen-free laboratory animal facility maintained at a controlled temperature of 20–22°C and relative humidity of 45%–55% with a 12-h light/dark cycle. Animal experiments were reviewed and approved by the Ethics Committee of Animal Experiments of Shanghai University of Traditional Chinese Medicine (approval ID: 08001) and were carried out according to "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health as well as the guidelines of the Animal Welfare Act. The rats were sensitized and challenged with ovalbumin (OVA, grade V, Sigma, Germany), according to a previously described method[15]. Briefly, the rats were sensitized by intraperitoneal (i.p.) injections with 1 mg of OVA in 10 mg of aluminum hydroxide (Pierce, Rockford, IL, USA) at day 0. On day 14, the rats were anesthetized with 1% sodium pentobarbitone (50 mg/kg body weight, i.p.), which was followed by pretreatment with rS100A11 protein (100 ng/kg and 1000 ng/kg), terbutaline sulfate (TB, 55 μg/kg body weight) or saline solution (vehicle control) injection into the external jugular vein. Finally, the rats were challenged with 1 mL/kg 5% OVA in saline by injection into the external jugular vein over a 10-s period. Control rats were injected with normal saline (0.9% NaCl) during both the sensitization and challenge phases.

1.3 Measurement of Airway Hyperresponsiveness Induced by Allergens

Invasive measurements of dynamic airway resistance after the allergen challenge were performed as previously described[15]. Briefly, anesthetized rats were tracheotomized and immediately intubated with a T-shaped cannula that was directly attached to a heater-controlled pneumotachograph (Godart 17212, The Netherlands). The tidal volume and flow rate were measured by connecting the pneumotachograph to a differential pressure transducer (AutoTran, model 600D-011, ±2 cm H_2O). A water-filled PE-90 tube was inserted into the esophagus to the mid-thorax level and was coupled to a pressure transducer (PT14MX, Jialong Teaching Equipment; China) to measure the transpulmonary pressure, which is the difference in pressure measured between the alveoli and the

esophagus at the end of inhalation or exhalation. The RL was calculated over a complete respiratory cycle using an integration method over airway flows (F) and transpulmonary pressures (P_{TP}) and was defined as $RL=\Delta P_{TP}/\Delta F$.

1.4 Measurement of Tracheal Smooth Muscle Contractility

Animals were euthanized by rapid intravenous injection with an overdose of sodium pentobarbital. The trachea was excised, the surrounding connective tissues and fat were removed, and then the trachea was immediately placed in ice-cold Krebs-Henseleit solution (pH 7.4, 119 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, and 11 mmol/L D-glucose). Each trachea was then cut transversally into a ring of 3–4 segments in length before measuring the contraction force in response to the smooth muscle agonist ACh. To begin an experiment, the tracheal ring was hung on two L-shaped platinum hooks in organ bath chambers containing oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution that was maintained at 37°C. The upper end of the tracheal ring was attached to a tension transducer (Jialong, China). Following equilibration for 30 min under a resting tension of 1 g, the tracheal ring was pretreated with rS100A11 (100 ng/mL, 1000 ng/mL) or TB (1 μg/mL) for 15 min and then was stimulated with ACh (10 μmol/L). The contraction force of each treated tracheal ring was continuously monitored with MFLab 3.01 software (Fudan University, China) for 10 min, and the final value was calculated from the corresponding baseline values subtracted from each recorded value. In the experiments exploring the effect of rS100A11 on tracheal contraction in calcium-free buffer medium, tracheal rings were incubated in Krebs-Henseleit solution containing ethylene glycol tetraacetic acid (EGTA, 1 mmol/L) before the addition of ACh.

1.5 Measurement of ASMC Contraction

Primary rat ASMCs were isolated and then cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone Labs, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (HyClone Labs, USA). All experiments were performed on cells cultured between passages 5 and 7, as described previously[16]. ASMCs were cultured in glass-bottom culture dishes (NEST, China) and kept at 37°C in a CO_2 incubator until 50% confluence was reached. The culture medium was then removed and replaced with fresh medium. Each dish was placed on a live cell imaging system (IX81, Olympus, Japan) equipped with a CO_2 incubator that maintained the culture dishes at 37°C in an atmosphere containing 5% CO_2 during the measurements. ASMCs were treated with or without rS100A8 (1 μg/mL) for 10 min and then stimulated with ACh (10 μmol/L) for 15 min. Cells in the control group were pretreated and stimulated with PBS. The morphology of the live cells was continuously monitored in assigned fields under differential interference contrast optics. Cell images were captured at a rate of 1 frame per min. Changes in the cell surface area were used to evaluate the cell contraction responses. Specifically, the cell surface area before stimulation was designated as A, and the cell surface area after stimulation was designated as B. The cell contraction rate for a given time point was calculated as $(A–B)/A\times100\%$[16].

1.6 Western Blotting

ASMCs were treated with rS100A11 protein (1 μg/mL) for 1 h, followed by stimulation with ACh (10 μmol/L) for 30 min. Total cell protein was extracted and separated by SDS-PAGE. Immunoblotting was performed with antibodies against myosin light chain (MLC; Abcam, UK, 1:1000 dilution) and phospho-myosin light chain 2 (Ser19) (p-MLC; Cell Signaling Technology, USA, 1:1000 dilution). Immunocomplexes were visualized using the enhanced chemiluminescence and imaged on an Amersham Imager 600 (GE Healthcare, USA). The house-keeping protein β-actin was used as loading control. Determination of band densities was performed by using ImageJ software and presented as p-MLC/MLC.

1.7 Statistical Analysis

All data are presented as the mean±standard deviation (SD). Comparisons among groups were performed using one-way ANOVA. Differences between two groups at each time point were analyzed by Bonferroni's post hoc test. Mann-Whitney U tests were used to compare mean values between two experimental groups. A P-value of less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Purification of Recombinant rS100A11 Protein

SDS-PAGE analysis showed that the rS100A11 protein was obtained with >95% purity after affinity chromatography purification (fig. 1A). Western blotting assays with a specific antibody against rat S100A11 (1:3000, Santa Cruz Biotechnology, USA, sc-8113) demonstrated the antigenic activity of the rS100A11 protein (fig. 1B).

2.2 rS100A11 Reduces Allergen-induced RL

AHR to an OVA challenge mainly manifested as exaggerated RL, which appeared 2 min after the intravenous challenge with OVA in OVA-sensitized rats (AS group). Pretreatment with TB, a well-known β_2-adrenergic receptor agonist, significantly suppressed the RL for 2–4 min after the OVA challenge as compared with that of the allergen-challenged control (all $P<0.05$). Moreover, OVA-induced AHR was conspicuously inhibited in a dose-dependent

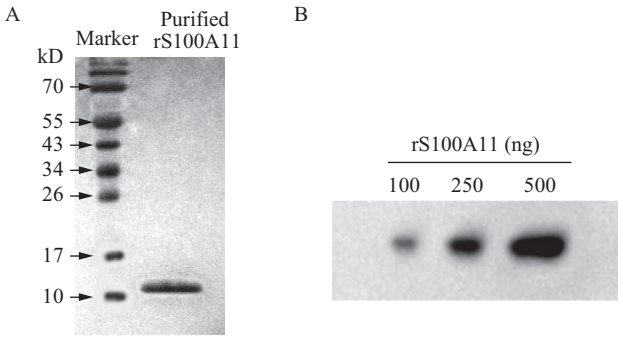

Fig. 1 Purification of recombinant rat S100A11 protein (rS100A11)
A: SDS-PAGE analysis of rS100A11, which was purified by affinity chromatography and ion exchange chromatography. B: Western blot analysis showed the antigenic activity of the rS100A11 protein.

manner by pretreatment with rS100A11. As shown in fig. 2, rS100A11 at 1000 ng/kg produced the maximum inhibitory effect on OVA-induced AHR in sensitized animals (P<0.05 *vs.* the AS group).

2.3 rS100A11 Suppresses ACh-induced Hypercontractility of ASM

AHR in asthma is mainly due to increased ASM contractility. Thus, we analyzed the potential of rS100A11 to control AHR in tracheal ring contractions *ex vivo*. ACh is a muscarinic agonist that induces dose-dependent (10^{-8} to 10^{-4} mol/L) contractions in isolated rat tracheal rings[17]. TB is one of several sympathomimetic agents that act by selectively stimulating β_2-adrenergic receptors to relieve bronchospasms associated with dyspnea. As expected, treatment with TB at a concentration of 1 μg/mL significantly reduced the contraction force of tracheal rings induced by treatment with 10 μmol/L ACh. Additionally, the preincubation of tracheal rings with rS100A11 (1000 ng/mL) significantly inhibited ASM contractions, with an inhibitory effect equivalent to an average of 64% of that induced by TB 2–10 min after ACh stimulation (fig. 3A).

2.4 rS100A11 Suppresses ACh-induced Hypercontractility of ASMs in an Extracellular Calcium-free Manner

Extracellular calcium ion was removed from the buffer to test for the possibility that rS100A11 may induce airway relaxation in a calcium-independent manner. When tracheal rings were rinsed and incubated in calcium-free Krebs-Henseleit solution (supplemented with 1 mmol/L EGTA), the addition of 10 μmol/L ACh still produced an increase in ASM contraction (fig. 3B), although the contractility was less than that observed in standard Krebs-Henseleit solution. This could be due to the fact that ACh

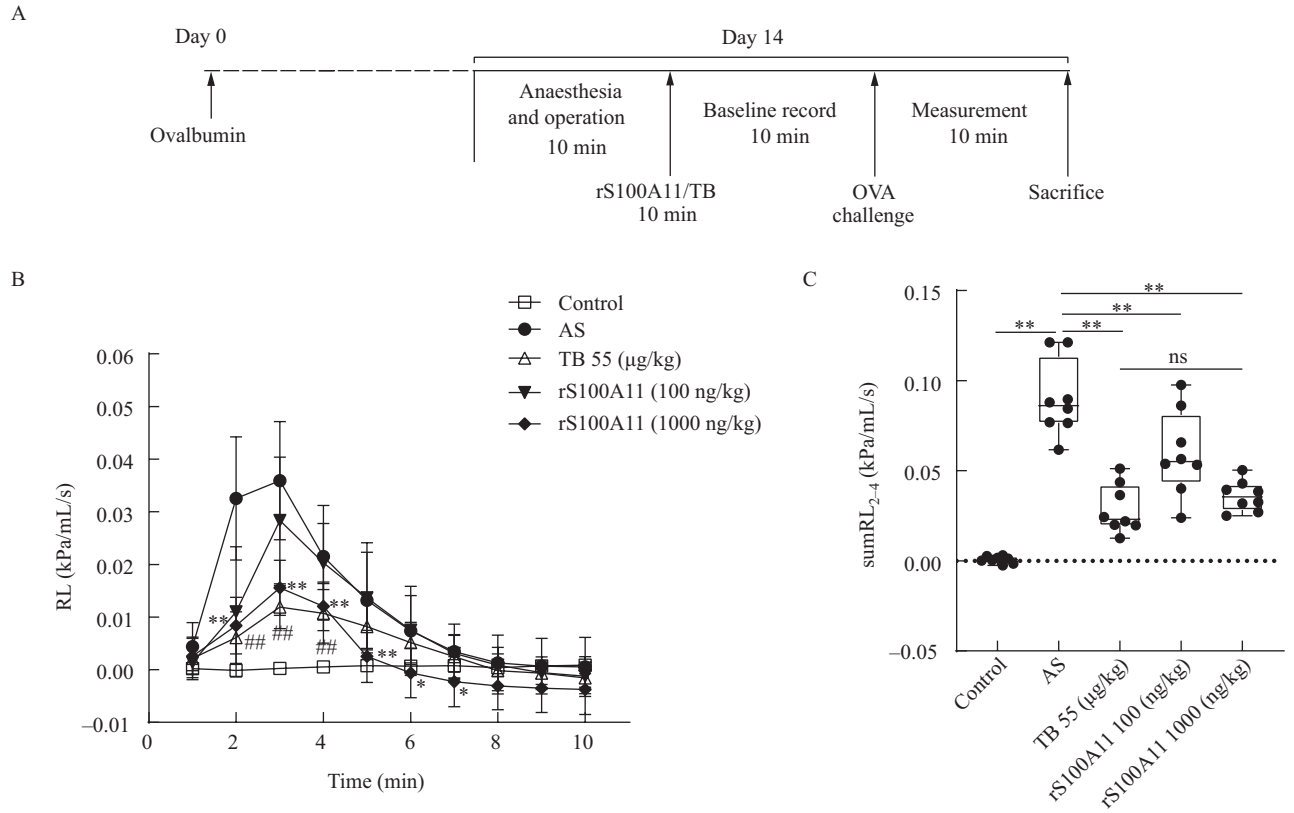

Fig. 2 rS100A11 attenuates allergen-induced AHR after OVA sensitization and challenge
A: the schematic timeline of the experimental procedure. B: time-response curve of AHR. RL is expressed as the differential value subtracted from the corresponding baseline value. The data are presented as mean±SD [n=8 in each group, $^{*}P$<0.05, $^{**}P$<0.01 for the rS100A11 (1000 ng/kg)-treated group *vs.* the AS group; $^{\#\#}P$<0.01 for the TB-treated group *vs.* the AS group]. C: Boxplot analysis of the peak value sum of the RL during the 2–4-min period of the early airway response ($sumRL_{2-4}$) among the treatment groups ($^{**}P$<0.01 *vs.* AS group). OVA: ovalbumin

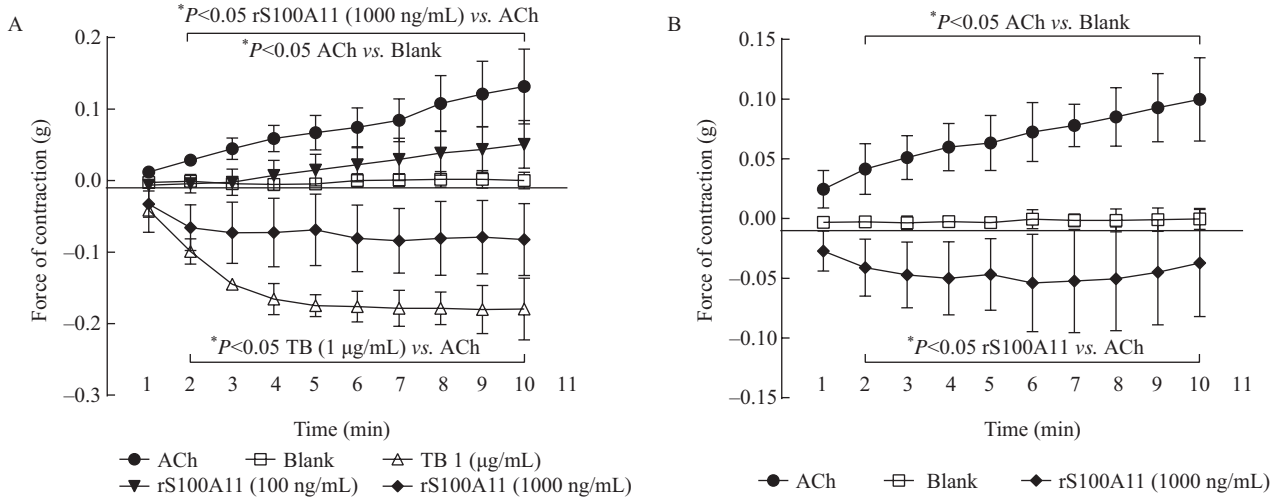

Fig. 3 rS100A11 suppresses ACh-induced hypercontractility in tracheal rings
A: Time-response curves of the contraction force of tracheal rings in standard Krebs-Henseleit solution. The data are presented as mean±SD (n=5 in each experimental group). B: Time-response curves of the contraction force of tracheal rings in calcium-free Krebs-Henseleit solution. The data are presented as mean±SD (n=5 for each experimental group).

provokes muscle contraction through the release of intracellular stores of calcium from the sarcoplasmic reticulum rather than calcium influx. However, rS100A11 could still induce a relaxation of the ACh-contracted airway in calcium-free solution. There was no significant difference in the effect of rS100A11 protein on relaxing ASM in standard and calcium-free Krebs-Henseleit solutions.

2.5 rS100A11 Inhibits the ACh-induced Contraction Response of ASMCs

ASMCs are the main effector cells that cause airway stenosis and dyspnea in patients with asthma, so we examined the inhibitory effect of rS100A11 on ACh-induced contractile responses of ASMCs. Taking into account our initial experiment as well as other related studies, we used an ACh concentration of 10 μmol/L to induce the contractile responses of ASMCs. As anticipated, ACh stimulation for 15 min resulted in significant contraction of ASMCs, which was manifested as a significant decrease in the cell surface area [(40.6±12.4)%]. However, ASMCs treated with PBS for 15 min displayed no apparent contractile responses. In contrast, pretreatment of ASMCs with rS100A11 (1000 ng/mL) for 15 min significantly

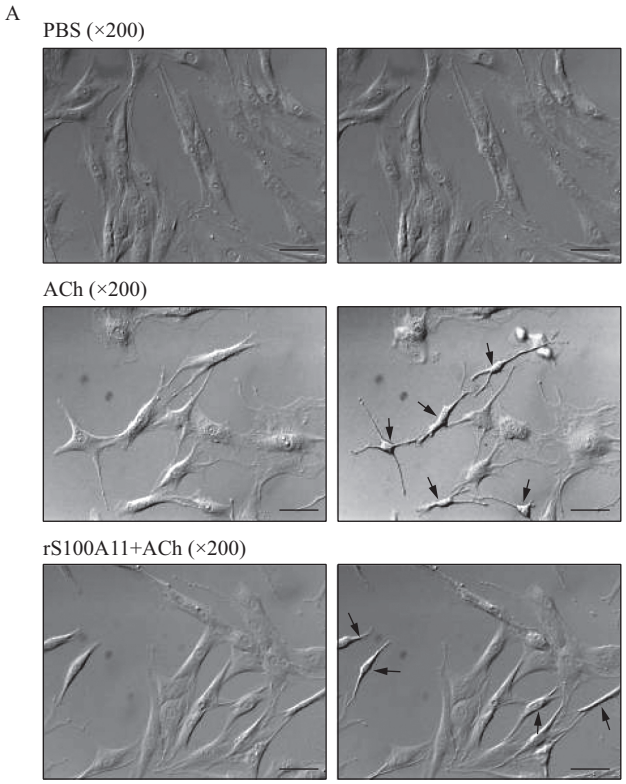

Fig. 4 rS100A11 inhibits ACh-induced ASMC contraction
A: representative images showing the contractile response of ASMCs to ACh (magnification, 200×). The left panel depicts cells pretreated with PBS (PBS and ACh groups) and rS100A11 (1000 ng/mL, rS100A11+ACh group) for 15 min, and the right panel shows cells stimulated with PBS (PBS group) and ACh (10 μmol/L, ACh and rS100A11+ACh groups) for another 15 min. Scale bar=100 μm. B: Boxplot analysis of the changes in the cell surface area (%, contraction rate, n=20 cells in each group, $^{**}P$<0.01 *vs.* the ACh group).

inhibited the contractile responses induced by ACh [(17.4±6.8)%, P<0.01 *vs.* the responses of the ACh group] (fig. 4).

2.6 rS100A11 Inhibits ACh-induced Phosphorylation of MLC in ASMCs

Given that ASM contraction is regulated by the phosphorylation of the MLC, we further performed Western blotting to analyze whether rS100A11 can affect MLC phosphorylation. ACh stimulation significantly induced MLC phosphorylation (p-MLC/MLC=0.350±0.088) as compared with the untreated control samples (p-MLC/MLC=0.075±0.008, P<0.01). Pretreatment with rS100A11 significantly inhibited ACh-induced MLC phosphorylation as compared with that of the ACh group (p-MLC/MLC=0.109±0.036, P<0.01, fig. 5).

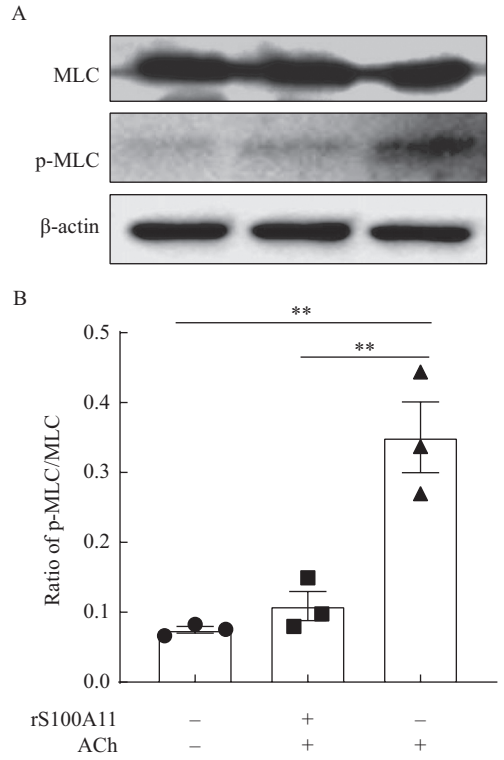

Fig. 5 rS100A11 inhibits the ACh-induced phosphorylation of MLC in ASMCs
The phosphorylation levels of MLC in ACh-stimulated ASMCs that were pretreated with or without rS100A11 (1 μg/mL) were detected by Western blotting. A: A representative immunoblot shows nonphosphorylated MLC and phosphorylated MLC (p-MLC) induced by ACh. β-actin was used as a loading control. B: The ratio of the absorbance values of p-MLC relative to that of MLC was calculated, and data are presented as mean±SD of three separate experiments, $^{**}P$<0.01

3 DISCUSSION

S100A11 is an important member of the family of S100 proteins, which are involved in the regulation of a variety of cellular processes, such as motility, cell growth, and differentiation[18]. It has been reported that the expression of S100A9 and S100A12, two other members of the S100 calcium-binding protein family, is significantly upregulated during asthma exacerbation[19]. In addition, we previously reported the induction of S100A8, S100A9, and S100A11 protein expression in the lungs of an OVA-induced rat model of asthma. S100A8, S100A9, and S100A12 can activate airway epithelial cells to produce MUC5AC, a major mucin protein in the respiratory tract, suggesting that they all serve as key mediators linking neutrophil-dominant airway inflammation to mucin hyperproduction[20]. Moreover, our previous studies showed that S100A8 and S100A9 proteins also inhibited ASM contraction and ASMC migration[21, 22]. Nevertheless, there is no clear understanding of the functional role of S100A11 in asthma. Herein, we demonstrated that intravenous injection of rS100A11 attenuated AHR in an OVA-sensitized and challenged rat asthma model. It is noteworthy that the inhibitory effects of rS100A11 on AHR are more efficient than those of TB within 5–8 min after the OVA challenge; however, TB was more effective than rS100A11 in the initial phase (2–3 min) of the allergen challenge. TB is an agonist that is selective for β_2-adrenoceptor and is used in the treatment of asthma because it specifically mediates relaxation of ASM of the bronchioles in the lungs. The time phase difference between S100A11 and TB in reducing airway resistance indicates that S100A11 may induce a mechanism that is distinct from that of TB in acute AHR.

In vitro experiments demonstrated that rS100A8 markedly suppressed ACh-induced contractions in isolated tracheal rings and primary ASMCs, suggesting that the effects of S100A8 on AHR are likely due to the suppression of ASM contractions. Muscarinic agonists such as ACh increase the cytosolic calcium concentration through the release of intracellular stores of calcium from the sarcoplasmic reticulum to cause a chain reaction that regulates muscle contraction. The combination of calcium and calmodulin activates MLCK and causes the phosphorylation of MLC20, which subsequently activates myosin ATPase and leads to the formation of actin-myosin cross-bridges and the generation of contraction forces. Although evidence has shown that S100A11 inhibits the myosin ATPase activity of smooth muscle by binding to actin filaments[12], here, our results suggest that S100A11 can also decrease the phosphorylation of MLC20 to further suppress the activation of myosin ATPase and inhibit smooth muscle cell contraction. It has been well established that some functions of S100A11, such as translocation[23] and regulation of cytoskeleton components[24], are dependent on binding with calcium. Therefore, we further tested whether exogenous S100A11 protein relaxes ASM in a calcium-

dependent manner. To clarify the dependence of S100A11-induced relaxation on extracellular calcium, we tested the effects of rS100A11 on the contractile force of tracheal rings in standard Krebs-Henseleit solution and EGTA-treated calcium-free Krebs-Henseleit solution. The results showed that rS100A11 still significantly inhibited the ACh-induced ASM contractile responses in calcium-free Krebs-Henseleit solution, suggesting that the relaxation effect induced by rS100A11 seemed to be independent of extracellular calcium. Thus, further research is needed to determine whether S100A11 inhibits ASM contraction caused by MLC phosphorylation by regulating the release of intracellular calcium.

Several studies have demonstrated specific interactions between S100A11 protein and its receptors annexin 2[10], annexin 6[25], and receptor for advanced glycation end products (RAGE)[26–28] in a variety of cell types. Although the interactions between S100A11 and annexins are calcium-dependent, it has been shown that S100A11 interacts with RAGE and other target proteins in a calcium-independent manner[27]. Further studies are needed to determine the role of the S100A11/RAGE signaling pathway and other possible mechanisms in S100A11-induced relaxation of ASM. Even though the antigen-induced ASM contraction is strongly associated with airway inflammation in asthma[1], the effect of S100A11 on pulmonary inflammation was not assessed in this current study due to the lack of obvious inflammatory damage in the AHR animal model. Notably, previous studies have defined S100A11 protein as a proinflammatory mediator involved in inflammatory diseases such as rheumatoid arthritis[11], inflammatory myopathies[29], and osteoarthritis[30], so it is rational to assume that the inhibition effect of S100A11 in ASM contraction may not be attributed to the attenuation of airway inflammation. However, the specific role of S100A11 in the airway inflammatory responses of asthma needs to be further studied.

Collectively, the present study demonstrated that rS100A11 can attenuate allergen-induced AHR by suppressing the hypercontractility of ASM. The relaxation effect induced by exogenous S100A11 protein may be independent of extracellular calcium. Our results support the idea that targeting S100A11 protein and the S100A11-mediated pathway may be an effective approach for reducing airway resistance in asthma.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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