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S100A8 protein attenuates airway hyperresponsiveness by suppressing the contraction of airway smooth muscle

Yu-Dong Xu^a, Yu Wang^a, Lei-Miao Yin^a, Gyoung-Hee Park^a, Luis Ulloa^{a, b, **}, Yong-Qing Yang^{a, *}

^a Shanghai Research Institute of Acupuncture and Meridian, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China

^b Center of Immunology and Inflammation, Rutgers-New Jersey Medical School, Rutgers University, Newark, NJ 07101, USA

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ABSTRACT

Airway hyperresponsiveness (AHR) is a major clinical problem in allergic asthma mainly caused by the hypercontractility of airway smooth muscles (ASM). S100A8 is an important member of the S100 calcium-binding protein family with a potential to regulate cell contractility. Here, we analyze the potential of S100A8 to regulate allergen-induced AHR and ASM contraction. Treatment with recombinant S100A8 (rS100A8) diminished airway hyperresponsiveness in OVA-sensitized rats. ASM contraction assays showed that rS100A8 reduced hypercontractility in both isolated tracheal rings and primary ASM cells treated by acetylcholine. rS100A8 markedly rescued the phosphorylation level of myosin light chain induced by acetylcholine in ASM cells. These results show that rS100A8 plays a protective role in regulating AHR in asthma by inhibiting ASM contraction. These results support S100A8 as a novel therapeutic target to control ASM contraction in asthma.

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1. Introduction

Airway hyperresponsiveness (AHR) is the main pathophysiological hallmark of allergic asthma causing airflow obstruction and dramatic airway narrowing in response to a variety of stimuli. AHR is mainly due to the contraction of the airway smooth muscle (ASM) cells [1,2]. Previous studies suggested that AHR can be caused by muscarinic M2 receptors because their activation inhibits β -adrenoceptor-induced ASM relaxation [3]. Thus, the release of muscarinic agonists such as acetylcholine (ACh) by parasympathetic nerves during airway inflammation is considered to be the main cause of excessive ASM contraction and AHR [4]. Inhaled β 2-adrenoceptor agonists are the most widely used bronchodilators currently available. However, the development of tolerance

and resistance to β 2-adrenoceptor agonists in asthmatic patients is a major clinical limitation for the treatment of asthma [5,6]. Thus, it is critical to design novel therapeutic strategies to control ASM contraction in asthmatic patients.

The S100 proteins are a unique family of EF-hand (helix E-loop-helix F) calcium-binding proteins with increasing interest in recent years due to their role in several human disorders including cardiomyopathy, cancer, and inflammatory diseases [7]. The binding of S100 to calcium allows them to interact with multiple proteins to regulate cellular events including muscle contractility [8]. One critical example is that S100A1, which is highly expressed in striated muscle, inhibits isometric tension and stiffness in skinned rabbit skeletal muscle at physiological concentrations [9]. Another S100 family member S100A2 enhances Ca^{2+} cycling to improve contraction and relaxation performance in normal and failing cardiac myocytes [10]. The above studies indicate distinct functions of highly homologous S100 family members in the pathophysiology of muscle contractility.

S100A8 (also known as MRP8 and calgranulin A) is an important member of the S100 protein family that has both intra and extracellular biological functions [11,12]. Although S100A8 and S100A9 normally exist as homodimers, S100A8 has been found to be the active component of the S100A8/A9 complex [13]. We recently

* Corresponding author. Laboratory of Molecular Biology, Shanghai Research Institute of Acupuncture and Meridian, Shanghai University of Traditional Chinese Medicine, 650 South Wanping Road, Shanghai, 200030, China.

** Corresponding author. Center of Immunology and Inflammation, Rutgers-New Jersey Medical School, Rutgers University, 185 South Orange Ave., Newark, NJ 07101, USA.

E-mail addresses: Luis.Ulloa@Rutgers.edu (L. Ulloa), yyq@shutcm.edu.cn (Y.-Q. Yang).

reported that S100A8 is highly expressed in lung of asthmatic rats [14]. Moreover, recombinant S100A8 inhibits ASM cell-migration induced by platelet-derived growth factor *in vitro* [15]. These results concurs with several recent studies suggesting the anti-inflammatory potential of S100A8 [16–18]. Although the role of S100A8 in AHR and ASM contraction is unknown, a previous study showed that LPS induced S100A8 overexpression inhibiting RAGE-mediated cardiomyocyte contractility [19]. Thus, we reasoned that given its role in Ca^{2+} signaling and cell contractility [11], S100A8 may be a promising candidate to regulate ASM-contraction in asthma. Here, we analyze the potential of recombinant S100A8 to control allergen-induced AHR and ASM contraction.

2. Materials and methods

2.1. Preparation of recombinant rat S100A8 protein

The PET-22b-rS100A8 plasmid used in this study was previously described [15]. Briefly, PET-22b-rS100A8 was cloned using Nde I and Xho I restriction sites and transformed into BL21 (DE3) cells by calcium chloride transformation. A positive *Escherichia coli* strain containing the recombinant pET-22b-rS100A8 plasmid was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). Recombinant rat S100A8 protein (rS100A8) was then purified by affinity chromatography purification using Ni Sepharose 6 Fast Flow beads (GE Healthcare, Uppsala, Sweden) and by ion-exchange chromatography purification using Q Sepharose Fast Flow beads (GE Healthcare, Uppsala, Sweden) by gravity flow according to the manufacturer's instructions. The purity of the recombinant S100A8 protein was higher than 95% without loss in antigenic activity as determined by immunoblotting assay.

2.2. Ovalbumin sensitization and challenge model

Sprague-Dawley rats (4 weeks old, weighing 100–120 g) were purchased from the Shanghai Laboratory Animal Centre (Shanghai, China), housed in a pathogen-free animal facility maintained at a controlled temperature of 20–22 °C and air humidity of 45–55% with a regular light-dark schedule. The animal experiments were reviewed and approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine and performed according to the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' as well as the guidelines of the Animal Welfare Act. The rats were sensitized and challenged with ovalbumin (OVA, grade V, Sigma, Taufkirchen, Germany) as previously described [14]. Briefly, on day 0, the rats were intraperitoneally injected with 1 mg of OVA precipitated with 10 mg of aluminum hydroxide gel and dissolved in 1 ml saline (0.9% NaCl). On day 14, allergic rats were anesthetized with 1% sodium pentobarbitone (w/v) at a dose of 50 mg/kg by intraperitoneal injection, followed by pretreatment with rS100A8 protein (10 ng/kg, 100 ng/kg and 1000 ng/kg), 55.0 $\mu\text{g/kg}$ terbutaline (TB) or saline solution (vehicle control) injection into the external jugular vein. Finally, the allergic rats were challenged with 1 ml/kg of 5% OVA in saline by injection into the external jugular vein over a 10-s period. Normal control rats received a saline solution injection on the same schedule as the rats receiving OVA.

2.3. Pulmonary resistance (RL) measurements

Rats were placed in a supine position and warmed with an incandescent lamp after anesthesia. A T-shaped incision was made in the upper part of the trachea, and a T-shaped cannula, which was directly attached to a heater-controlled pneumotachograph (Godart 17212, Bilthoven, Holland), was gently inserted into the trachea.

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). To measure the transpulmonary pressure, a water-filled PE-90 tube was inserted into the esophagus to the mid-thorax level (lower one-third of the esophagus) and coupled to a pressure transducer (PT14MX, Jialong Teaching Equipment; Shanghai, China). The pneumotachograph tidal flow signal was integrated with respect to time to obtain the tidal volume. The RL was calculated over a complete respiratory cycle using an integration method in which flows, volumes, and pressures were continuously recorded with software (Shanghai Medical College, Fudan University) designed for physiology experiments.

2.4. Tracheal ring contraction assay

Animals were anesthetized, and then the trachea was excised and surrounding connective tissues were removed, taking care not to damage the epithelium. Each trachea was cut transversally into a ring of 3–4 segments in length in ice-cold Krebs solution (pH 7.4, composition in mM: NaCl 119, NaHCO_3 25, MgSO_4 1.2, KH_2PO_4 1.2, KCl 4.7, CaCl_2 2.5, and D-glucose 11). The tracheal rings were incubated with or without rS100A8 (1 $\mu\text{g/ml}$) for 12 h before measuring the force of contraction in response to acetylcholine (ACh). To begin an experiment, the tracheal rings were suspended in organ bath chambers (Panlab Harvard Apparatus, Holliston, Massachusetts, USA) containing oxygenated (95% O_2 ; 5% CO_2) Krebs solution maintained at 37 °C. Following equilibration for 30 min under a resting tension of 1 g, the tracheal rings were contracted with ACh (10^{-9} to 10^{-4} M) in a cumulative manner every 15 min. Changes in isometric tension were measured by attaching the upper end of the ring to an isometric tension transducer (MLT0420, AD Instruments, Australia) and recorded on AD Instruments PowerLab Chart software. For the positive control, atropine (0.1 μM) was added 15 min before eliciting ACh response.

2.5. Collagen gel contraction assay

Primary rat ASM cells were dissected, purified, and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Labs, Logan, UT) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (HyClone Labs, Logan, UT) as described previously [15]. Confluent cells at passage 4, 6 and 8 were harvested and resuspended at a density of $1 \times 10^5/\text{ml}$ in a collagen solution that was prepared using Collagen Type I (BD Biosciences, San Diego, CA) at a final concentration of 2 mg/ml. Then, 0.5 ml of the collagen solution with or without rS100A8 (1 $\mu\text{g/ml}$) was added to each well of a 24-well plate and incubated at 37 °C for 30 min to form the gels. The embedded ASM cells were cultured for 24 h in DMEM at 37 °C in 5% CO_2 to allow for cell growth in the formed gel matrix. In the stimulated collagen contraction assay, 1 mM ACh was first added to the well. The gels were then detached from the culture dish using a 27-gauge needle. Gel surface images were captured with a GelDoc XR system (Bio-Rad, Hercules, CA) 24 h later, and the surface areas were quantified using NIH Image software.

2.6. Western blots

ASM cells were seeded into 6-well plates with 10% FBS-DMEM and allowed to grow to 80% confluence. Then, the cells were treated with rS100A8 protein (1 $\mu\text{g/ml}$) for 1 h, followed by stimulation with ACh (1 mM) for 2 h. Total cell protein was extracted and separated by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with antibodies against myosin light chain (MLC; Abcam, 1:2000 dilution) and Phospho-Myosin Light Chain 2

(Ser19) (p-MLC; Cell Signaling Technology, 1:1000 dilution). Immunocomplexes were visualized using chemiluminescence and imaged on an Amersham Imager 600 (GE Healthcare). Determination of band densities was performed using NIH Image software and is presented as p-MLC/MLC and normalized to basal levels.

2.7. Statistical analysis

Data were expressed as the means $\pm$ SD. One-way analysis of variance (ANOVA) was performed to determine the differences among the groups. Post hoc comparisons, if applicable, were performed using the least significant difference (LSD) test, with $P < 0.05$ considered statistically significant.

3. Results

3.1. Recombinant S100A8 reduces ovalbumin-induced AHR

Acute airway hyperresponsiveness to OVA challenge, mainly manifested as exaggerated pulmonary resistance (RL), appears in OVA-sensitized mice at 2 min after intravenous challenge with OVA. Pre-treatment with terbutaline, a well-known β -adrenergic receptor agonist, suppresses the pulmonary resistance for 2–8 min after the OVA challenge. Pre-treatment with recombinant S100A8 (0.01–1 μ g/kg rS100A8) suppresses the pulmonary resistance for 3–9 min after the OVA challenge. Of note, the inhibitory effect of rS100A8 (1 μ g/kg) is more efficient than that of terbutaline (55.0 μ g/kg) at 6–8 min (Fig. 1).

3.2. Recombinant S100A8 inhibits the hypercontractility of tracheal rings induced by acetylcholine

AHR in asthma is mainly due to ASM contractility. Thus, we analyzed the potential of rS100A8 to control AHR in tracheal ring

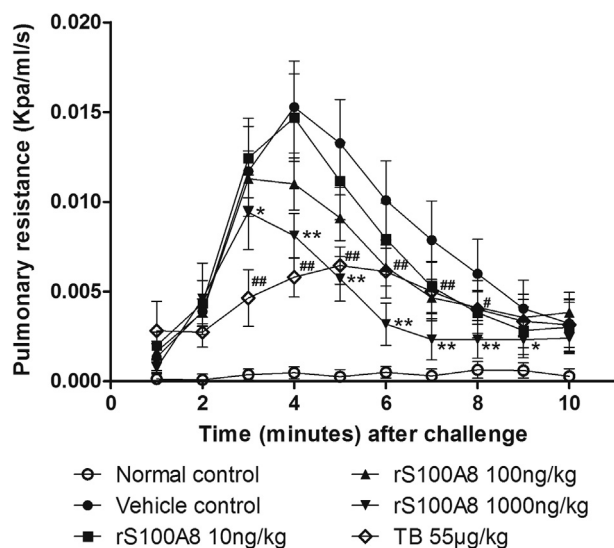


Fig. 1. rS100A8 attenuates exaggerated AHR after OVA sensitization and challenge. Rats were sensitized with an OVA protocol as described in Materials and Methods. rS100A8 (10 ng/kg, 100 ng/kg, 1000 ng/kg), terbutaline (TB, 55.0 μ g/kg) or saline solution (vehicle control) was administered intravenously before OVA challenge. The normal control group was sensitized and challenged with saline solution without OVA. Pulmonary resistance (RL) was recorded for 10 min and was expressed as the differential value subtracted from the corresponding baseline value. The data are presented as the means $\pm$ SD ($n = 8$ in each experimental group, $*P < 0.05$, $**P < 0.01$ for the rS100A8 1000 ng/kg-treated group compared to the vehicle-control group; $\#P < 0.05$, $\#\#P < 0.01$ for the TB-treated group compared to the vehicle-control group).

contraction *ex vivo* assay. Ach is a muscarinic agonists that induces a dose-dependent (10^{-9} to 10^{-3} M) contraction in the isolated rat tracheal rings. Atropine is one of the most characterized cholinergic antagonists for muscarinic receptors. As expected, atropine (0.1 μ M) completely abrogates the Ach-induced contraction of tracheal rings. Addition of rS100A8 (1 μ g/ml) does not affect basal tension in the rat tracheal rings (data not shown), but pre-incubation of the rings with rS100A8 (1 μ g/ml) significantly inhibits the Ach-induced hypercontractility of the tracheal rings by over 60–75% (Fig. 2).

3.3. Recombinant S100A8 inhibits hypercontractility in primary ASM cells

We next examined the potential of rS100A8 to control AMS contractility. Ach (1 mM) induces around 80% contraction of the ASM cell-embedded collagen gel, resulting in around 20% of the initial surface area after 6 h (Fig. 3). rS100A8 does not affect the contraction of normal dormant ASM cells. However, pre-treatment with rS100A8 (1 μ g/ml) significantly inhibits the Ach-induced hypercontractility of primary ASM cells resulting in around 50% of the initial area as compared to the final 21% of the initial surface area reported in the absence of rS100A8 (Fig. 3). These results indicate that Ach-induced 80% contraction, and rS100A8 inhibits this effect by 40% resulting in a final 50% contraction of the initial area. Thus, rS100A8 treatment inhibits the Ach-induced contraction of both tracheal rings and ASM cells.

3.4. Recombinant S100A8 inhibits Ach-induced phosphorylation of MLC in ASM cells

Given that ASM contraction is regulated by the phosphorylation of the myosin light chain (MLC), we analyzed whether rS100A8 affects MLC-phosphorylation. Ach stimulation significantly induces MLC-phosphorylation (0.90 ± 0.10 p-MLC/Total MLC) when compared to the untreated control samples (0.14 ± 0.05 p-MLC/Total MLC, $P < 0.01$). Pre-treatment with rS100A8 followed by Ach stimulation significantly inhibits MLC-phosphorylation as

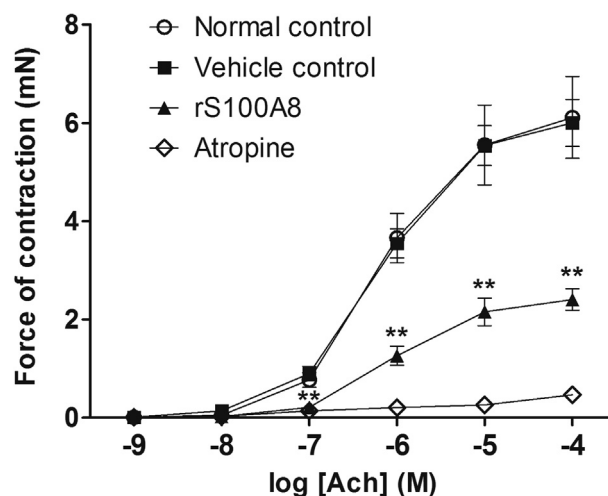


Fig. 2. rS100A8 suppresses Ach-induced hypercontractility in tracheal rings. Tracheal rings were incubated with or without rS100A8 (1 μ g/ml) for 12 h and then stimulated with Ach (10^{-9} to 10^{-4} M) in a cumulative manner every 15 min. Changes in isometric tension were recorded and measured using AD Instruments PowerLab Chart software. For the positive control, atropine (0.1 μ M) was added 15 min before eliciting Ach response. The data are presented as the means $\pm$ SD ($n = 6$ in each experimental group; $**P < 0.01$ for the rS100A8-treated group compared to the normal-control and vehicle-control groups).

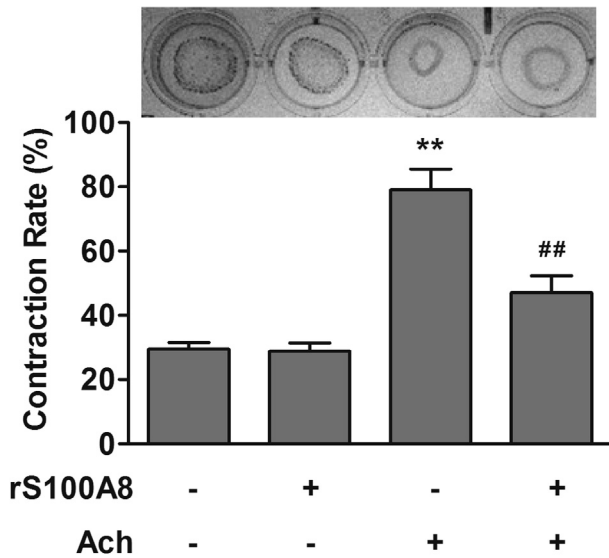


Fig. 3. rS100A8 suppresses Ach-induced hypercontraction in ASM cells. Collagen gels containing ASM cells and rS100A8 (1 μ g/ml) were cultured for 24 h and then stimulated with Ach (1 mM). After being detached from the culture dish, the surface areas of the gels were recorded for 24 h to estimate the contractile response of ASM cells. There were 4 replicate wells per group, and 3 parallel experiments were performed using ASMCs at passage 4, 6 and 8. Data are presented as the means $\pm$ SD. ** P < 0.01 for the Ach-treated control compared to the normal-control group; ## P < 0.01 for the rS100A8 + Ach group compared to the Ach-treated control group.

compared to the Ach group (rS100A8+ACh = 0.40 ± 0.10 p-MLC/Total MLC vs Ach (alone) = 0.90 ± 0.10 p-MLC/Total MLC; P < 0.01, n = 3; Fig. 4). As a control, we also confirmed that rS100A8 alone does not significantly affect MLC-phosphorylation in resting ASM cells (0.13 ± 0.05 p-MLC/Total MLC).

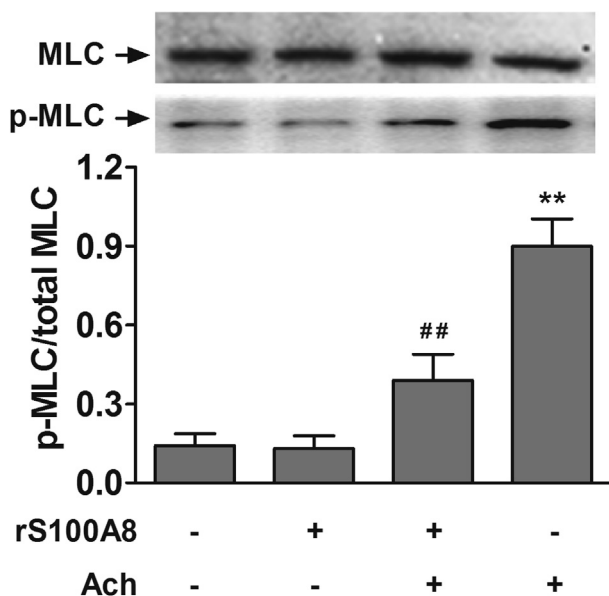


Fig. 4. rS100A8 inhibits the Ach-induced phosphorylation of MLC in ASM cells. Phosphorylation levels of MLC in Ach-stimulated ASM cells that were pretreated with or without rS100A8 (1 μ g/ml) were detected by Western blotting. A representative immunoblot (top) shows unphosphorylated MLC and phosphorylated MLC (p-MLC) induced by Ach. Data are presented as the means $\pm$ SD of three separate experiments. ** P < 0.01 for the Ach-treated control compared to the normal-control group, ## P < 0.01 for the rS100A8 + Ach group compared to the Ach-treated control group.

4. Discussion

Our results show for the first time the protective role of exogenous S100A8 protein in allergen-induced acute AHR. Here, we show that intravenously injected rS100A8 attenuates AHR in an allergen-induced rat model. rS100A8 markedly reduces the ACh-induced contraction in isolated tracheal rings and primary ASM cells, as well as MLC-phosphorylation. These data indicate that the effects of S100A8 on AHR are likely due to the suppression of ASM-contraction. Although previous studies showed that OVA challenge induces airway inflammation and hyperresponsiveness in sensitized animals, the link between airway inflammation and AHR is still controversial [20]. We previously reported the induction of S100A8 protein expression in the lung of OVA-induced asthmatic animals [14]. S100A8 is a small EF-hand protein capable of exerting both auto and paracrine cell regulation. The ambivalent role of S100A8 in cellular inflammation has been documented in a number of studies [18,21]. However, there is little information about the functional role of S100A8 in allergen-induced AHR. Our study is the first evidence of the protective role of exogenous S100A8 protein in allergen-induced acute AHR. Of note, the inhibitory effects of rS100A8 on AHR are more efficient than that of terbutaline for 6–8 min after the OVA challenge. Terbutaline is a well characterized β_2 -adrenoceptor agonist conventionally used as a “reliever” inhaler in the management of asthma symptoms. However, the inhibitory effect of terbutaline was stronger than that of S100A8 at 2–4 min after the OVA challenge. These results suggest that terbutaline and S100A8 may induce different mechanisms in acute AHR. Indeed, AHR can be either transient or persistent [22]. Transient acute increases in responsiveness are associated with the release of inflammatory mediators. By contrast, the persistent component of AHR appears to be related to the effects of chronic airway inflammation and thereby leading to a structural changes in the airway named airway remodeling (including ASM hyperplasia and hypertrophy) [20]. Airway remodeling in asthma is mainly attributable to ASM hypertrophy and hyperplasia caused by ASM cell proliferation and migration. We reported that S100A8 significantly inhibits ASM cell proliferation (data not published) and migration *in vitro* [15]. Thus, it is possible that S100A8 plays a beneficial role regulating persistent AHR as a promising hypothesis to be explored.

Although the pathophysiology of AHR remains uncertain, the acute airway narrowing and airflow obstruction is mostly due to the ASM contraction, as evidenced by the rapid reversal of AHR after treatment with inhaled β_2 -adrenergic agonists [1]. Given its role in Ca^{2+} signaling and cell contractility [11], we reasoned that S100A8 can be a promising candidate to regulate muscular contraction. Indeed, one previous study showed that LPS induced S100A8 overexpression inhibiting RAGE-mediated cardiomyocyte contractility [19]. Thus, we reasoned that S100A8 may also inhibit the contraction of ASM in asthma. Given that muscarinic receptors are the common agents used in bronchial provocation, we showed the potential of rS100A8 to inhibit ACh-induced tracheal rings and ASM cell contraction.

Several lines of evidence suggested that ASM-contraction is regulated by the phosphorylation of the myosin light chain (MLC) leading to the activation of actin and myosin crossbridging resulting in ASM shortening and contraction [23–25]. Therefore, we analyzed the effects of rS100A8 on ACh-induced MLC-phosphorylation at Ser19. rS100A8 inhibits ASM-contraction by preventing ACh-induced MLC-phosphorylation. It is now possible to propose that a similar mechanism may inhibit RAGE-mediated cardiomyocyte contractility after LPS stimulation as discussed above [19]. This MLC-phosphorylation can be mediated by the RhoA/Rho kinase signaling pathway. Activation of the RhoA/Rho kinase leads

to the inhibition of myosin light chain phosphatase (MLCP), resulting in increased p-MLC and subsequently increased ASM contractility [26]. Our previous proteomic study showed that increased S100A8 expression in asthmatic lung tissues correlates with higher expression of Rho-GDP dissociation inhibitor [14]. Rho-GDP dissociation inhibitor forms a complex with the GDP-bound forms of the Rho family GTPases and inhibits their activation [27]. It is therefore tempting to propose that S100A8 controls ASM-contraction by regulating the RhoA/Rho kinase signaling pathway, however the role of S100A8 on Ca^{2+} signaling may also suggest other mechanisms. Further studies are needed to determine the precise mechanism of S100A8 regulating MLC-phosphorylation.

In conclusion, the present study demonstrated that rS100A8 attenuates allergen-induced AHR, and this functional role of S100A8 was associated with its suppression of ASM hyper-contraction. Giving the tolerance and resistance to β_2 -adrenoceptor agonists, our findings might offer novel potential therapeutic targets for treating asthma by targeting ASM.

Conflict of interest

The authors declare that they have no competing interests.

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