



Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma

Lan Xu ^{a,b,1}, Xing-wei Dong ^{c,1}, Liang-liang Shen ^b, Fen-fen Li ^c, Jun-xia Jiang ^c, Rui Cao ^c, Hong-yi Yao ^c, Hui-juan Shen ^c, Yun Sun ^{b,*}, Qiang-min Xie ^{c,**}

^a The First People's Hospital of Wujiang City 215200, China

^b Department of Pharmacology, Medical College of Yangzhou University, Yangzhou 225001, China

^c Zhejiang Respiratory Drugs Research Laboratory of the State Food and Drug Administration of China, Medical College of Zhejiang University, Hangzhou 310058, China

ARTICLE INFO

Article history:

Received 21 November 2011

Received in revised form 12 January 2012

Accepted 25 January 2012

Available online 8 February 2012

Keywords:

Airway inflammation

Simvastatin

Inhalation

Drug delivery

Pharmacokinetics

ABSTRACT

The dose–response of the pleiotropic effects of statins on airway inflammation has not yet been established and may differ from that of their cholesterol-lowering effects. High oral doses of statins may have adverse effects, and it may be possible to overcome the side effects and low clinical efficacy by administering statins via inhalation. In this study, we hypothesize that simvastatin is a potential anti-inflammatory drug with biological and pharmacokinetic properties suitable for delivery by the inhaled route. Mice were immunized with ovalbumin (OVA) and then challenged with aerosol OVA. Simvastatin was locally delivered by inhalation (i.h.) and intratracheal injection (i.t.) or systematically delivered by intraperitoneal injection (i.p.) and gavage (i.g.) during the OVA challenge. In a mouse model of asthma, i.h. simvastatin significantly and dose-dependently attenuated airway inflammation, remodeling and hyperresponsiveness in a RhoA-dependent pathway. Upon comparing the pharmacodynamics, i.h. simvastatin had a more potent effect than that of i.g. and i.p. simvastatin, and the i.h. or i.t. delivery routes led to a higher drug concentration in local lung tissue and a lower drug concentration in the plasma than that obtained by the i.g. These results suggest that simvastatin is a potential anti-inflammatory drug for airway inflammatory diseases with properties suitable for delivery by inhalation, which will probably reduce the side effects and increase clinical efficacy.

© 2012 Published by Elsevier B.V.

1. Introduction

Statins, which are inhibitors of 3-hydroxy-3-methyl-3-glutaryl coenzyme A reductase, not only reduce cholesterol but also have several other pharmacological actions that might be beneficial in airway inflammatory diseases, including anti-oxidant, anti-inflammatory, and immunomodulatory effects. Many of these pleiotropic effects of statins are mediated by the inhibition of the isoprenylation of small GTP-binding signaling molecules, such as Rho, Ras, and Rac [1]. McKay and colleagues showed for the first time that simvastatin treatment, either orally or by intraperitoneal injection (i.p.), effectively suppresses acute eosinophilic airway inflammation and Th2 cytokine secretion in a mouse asthmatic model [2]. Both oral and i.p. simvastatin treatment reduced the ovalbumin-specific IgE levels; the number of total inflammatory cells, macrophages, neutrophils, and eosinophils in the bronchoalveolar lavage fluid; the expression of CD40, CD40 L and VCAM-1; the mRNA and protein levels of interleukin (IL)-4, IL-13 and tumor necrosis factor (TNF)- α ; the number of goblet cells; and the activity of matrix

metalloproteinases (MMPs); and induced the activity of small G proteins, MAP kinases and NF-kappaB activities in bronchoalveolar lavage cells and lung tissues in ovalbumin-induced allergic asthma in mice [3]. Cotreatment with mevalonate and simvastatin reversed the anti-inflammatory effects observed with simvastatin alone. The mevalonate pathway appears to modulate allergic airway inflammation, whereas the beneficial effects of simvastatin on lung compliance and airway hyperreactivity may be independent of the mevalonate pathway [4]. In the clinic, the published data are controversial. Oral statin treatment is associated with reduced hospitalization from asthma attacks in patients with asthma [5] and the prevention of chronic rhinosinusitis and pulmonary exacerbation of obstructive airway diseases [6], suggesting possible applications of statins in patients with asthma and other airway inflammatory diseases. Furthermore, oral statin treatment enhances the anti-inflammatory effect of an inhaled corticosteroid in asthmatic patients, which was mediated by the reduction of sputum macrophage counts and the alteration of the activity of indoleamine 2, 3-dioxygenase (IDO), a tryptophan-degrading enzyme, in macrophages [7,8]. In asthma, there are theoretical reasons as to how statins might exert their therapeutic effects. In human tissue, statin actions include reducing both the proliferation and activation of T cells and the migration of leucocytes [9]; inhibiting the adhesion of eosinophils [10], the degranulation of mast cells [11], and the proliferation, synthesis and

* Corresponding author. Tel.: +86 514 87992152.

** Corresponding author. Tel.: +86 571 88208231.

E-mail addresses: jgz7718@sina.com (Y. Sun), xieqm@zju.edu.cn (Q. Xie).

¹ These authors contributed equally to this work.

secretion of extracellular matrix proteins by the human airway smooth muscle cells [12,13]; and enhancing inflammatory cell apoptosis [14]. However, there are three clinical studies of statins in asthma that have not demonstrated important differences between statin treatment and placebo for the spirometry or airway hyperresponsiveness (AHR) symptoms [15,16], although the sputum leukotriene B₄ and macrophages decreased significantly with statin treatment [17]. The administered dose in the three clinical studies was 40 mg oral atorvastatin or simvastatin daily, so we cannot exclude the possibility that the lack of clinical effects may have been because the dose of simvastatin was too low. Prospective, controlled trials are needed to establish if statins have a beneficial effect in patients with asthma and chronic obstructive pulmonary disease, especially those with systemic complications and comorbidities. The dose response for the pleiotropic effects of statins has not yet been established and may differ from the cholesterol-lowering effects. High oral doses of statins may have adverse effects, particularly on skeletal muscles [17], so it would be important for statins to be delivered by the inhaled route [18]. Inhaled aerosol drug delivery provides a unique opportunity for the topical treatment of the lungs. Ideally, this allows for high pulmonary concentrations of medication to be attained without high systemic levels, providing efficient and effective therapies that spare patients from the unwanted side effects. The object of this study was to determine if simvastatin delivered as an aerosol to the local airway of a murine model of asthma could reduce airway inflammation and AHR while maintaining a low systemic drug delivery by evaluating the concentrations in the blood and lung tissue after the aerosol and oral administration of simvastatin.

2. Materials and methods

2.1. Animals

Inbred, female, specific pathogen-free BALB/c mice (weighing 20 ± 2 g, 6 weeks old) were purchased from the Shanghai Slac Laboratory Animal Co. Ltd. (Certificate No. SCXK 2007–0005, Shanghai, China). The animals were housed in a room maintained at 23 ± 2 °C with 50 ± 10% humidity and a 12-h light/12-h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). The animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld for 8 h before the experiments. All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

To sensitize the mice, 100 µg ovalbumin (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed on a 2-mg aluminum hydroxide gel as an adjuvant was subcutaneously injected on days 0 and 14. On day 14, a booster of 50 µg ovalbumin and 2 mg aluminum hydroxide was intraperitoneally injected into mice as previously described [19,20].

Control mice were injected with aluminum hydroxide gel following the same protocol as in Fig. 1. All protocols described in this study were approved by the University of Zhejiang Committee for Animal Experimentation.

2.2. Study design

2.2.1. Experiment 1

To estimate the inhibitory potency on airway inflammation produced by simvastatin administered by the different routes, the mice were divided into six groups. The treatment groups of mice were placed in a plastic box and administered simvastatin (Zhejiang Jingxin Pharmaceutical Co., Ltd., China; dissolved in 100% ethanol) by inhalation (i.h.) with 5 mg/mL (dissolved in 20% ethanol and 80% saline) by a jet nebulizer (BARI Co. Ltd., Germany), by intratracheal injection (i.t) with 20 µL/10 g body weight (2 mg/kg), by intragastric injection (i.g) with 40 mg/kg, and by intraperitoneal injection (i.p) with 40 mg/kg. The treatments were repeated daily from day 21 to day 28. One hour after the treatment, the mice were placed in a plastic box and challenged with aerosolized ovalbumin (10 mg/mL in saline) in the jet nebulizer for 30 min, which was also repeated daily from day 21 to day 28. An equal volume of inhaled ethanol was substituted for the simvastatin in the vehicle-OVA group (model) and the control group. Y-27632, a Rho-A kinase inhibitor, at 10 mg/kg by intranasal administration (i.n.) and 1 mg/kg i.g. dexamethasone were used as reference drugs for comparison and given once a day for 7 days [21].

2.2.2. Experiment 2

To investigate the dose–effect relationships in airway hyperresponsiveness, inflammation and remodeling, the treatment groups were administered simvastatin at 1, 5 and 20 mg/mL i.h. by a jet nebulizer (BARI Co. Ltd., Germany), and the treatment was repeated daily from day 21 to day 28. An equal volume of ethanol inhalation was substituted for the simvastatin in the vehicle-OVA group and control group, respectively. One hour after the treatment, the mice were placed in a plastic box and challenged daily from day 21 to day 28 with aerosolized ovalbumin (10 mg/mL in saline) in the jet nebulizer for 30 min.

2.2.3. Experiment 3

To determine the plasma and lung tissue drug concentrations of simvastatin administered by intratracheal injection (i.t.), inhalation (i.h.) and intragastric injection (i.g.), to compare the potential advantages and disadvantages of the different delivery routes and modes, and to discuss how to select a better delivery route and mode that meets the needs of asthma patients, the mice were placed in a plastic box and administered simvastatin as follows: a single dose of 5 mg/

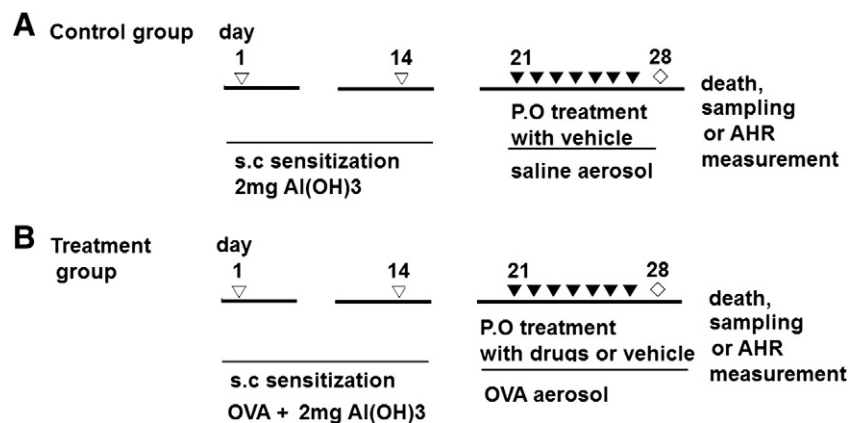


Fig. 1. Experimental procedure. Mice were subcutaneously (s.c.) sensitized twice with 100 µg ovalbumin allergen emulsified in 2 mg aluminum hydroxide adjuvant. The negative control mice (control group) were injected with 2 mg aluminum hydroxide adjuvant alone by the same protocol. After 21 days, all animals were placed in a plastic box and challenged with antigen via the airways with aerosolized OVA allergen (1% in saline) or an equal volume of saline (control group) in a jet nebulizer for 30 min, daily, for 7 consecutive days. The drug treatment was started from day 21 to 28.

mL via a jet nebulizer for 10 min for inhalation (i.h.), a single dose of 2 mg/kg (20 μ L/10 g body weight) by intratracheal injection (i.t), or a single dose of 100 mg/kg by intragastric injection (i.g).

2.3. The preparation of the bronchoalveolar lavage fluids

Seven days after the ovalbumin challenge, the mice were anesthetized with urethane (2 g/kg, i.p.). The bronchoalveolar lavage fluids were obtained by cannulating the trachea and lavaging with phosphate buffer solution (PBS) containing 1% bovine serum albumin and 5000 IU/L heparin. The bronchoalveolar lavage fluids were centrifuged at 500 $\times$ g for 10 min at 4 °C. The supernatant was stored at –80 °C. The pelleted bronchoalveolar lavage fluid cells were resuspended in PBS, and the total number of leucocytes was counted in a Neubauer chamber. Two hundred cells from the bronchoalveolar lavage fluids stained with Wright-Giemsa were identified under a light microscope. The total number of each cell type was determined by multiplying the percentage of each cell type by the total number of cells. The results were expressed as the number of each cell type in 1 L of bronchoalveolar lavage fluids.

2.4. The measurement of airway hyperresponsiveness

The changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were used to evaluate the changes in airway function after the intravenous administration of methacholine (Sigma, St. Louis, USA). The anesthetized and tracheostomized mice were assessed as previously described [20–22]. Each anesthetized mouse was placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fleisch tube connected to the airway in a pressure transducer. The changes in the lung volume were measured by detecting the pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure pleural pressure, a No. 12 needle with a multiholed tip was directly inserted into the pleural cavity (between the fourth and fifth rib at the left thorax) through a port in the connecting tube with a differential pressure transducer. The transpulmonary pressure was calculated as the difference between the mouth and pleural pressures. The signals of all the pressure transducers were continuously computed (MedLab, Nanjing Biotech Instruments, China) by fitting the flow, volume, and pressure values to an equation of motion. The cumulative dose–effect curves were established, and the provocation doses (PDs) of methacholine that produced a 100% increase in lung resistance (PD100) or a 25% decrease in lung dynamic compliance (PD25) were determined for each animal. These values were used as the indices of airway responsiveness. The cumulative doses of 0.0125–0.25 mg/kg methacholine were administered i.v. via the jugular vein cannula for a period of 5 min per dose with lung function measurements taken between each dose.

2.5. Reverse transcriptase polymerase chain reaction

The total RNA was extracted from lung homogenates in 1 mL TRIzol (Takara Bio, Dalian, China) as per the manufacturer's instructions. One-microgram aliquots of the total RNA were reverse transcribed to cDNA using moloney murine leukemia virus reverse transcriptase (M-MLV RTase, Takara Bio, Dalian, China) according to the manufacturer's instructions. The PCR amplification was performed with 1.25 U of LA Taq DNA polymerase (Takara, JP), 0.2 μ M sense and antisense primers, and 400 μ M each of dATP, dTTP, dGTP and dCTP in a total volume of 25 μ L. All PCR reactions were performed in a gradient thermal cycler PCR machine (Eppendorf, Germany). The mouse-specific sense and antisense primers used for the PCR amplifications are shown in Table 1. The PCR samples and markers were separated in 1.5% agarose gels containing 0.5 μ g/mL ethidium bromide. The photographs of the UV-transilluminated ethidium bromide-stained agarose gels were visualized with an imaging system (UVP Company Limited, England). The results were expressed as the ratio of the OD signal of CCL-11 and the cytokines to that of β -actin. All the primers were checked against GenBank for selectivity.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The frozen bronchoalveolar lavage fluids were thawed on ice and centrifuged at 2000 $\times$ g for 60 min at 4 °C. The concentrations of CCL-11, IL-4, IL-5 and IFN- γ in the bronchoalveolar lavage fluid supernatants were measured by sandwich ELISA using pair matched antibodies according to the manufacturer's instructions. The ELISA kits were obtained from eBioscience (San Diego, CA) and R&D (Minneapolis, MN).

2.7. Western blot analyses

The preparation of the protein samples from the lung tissues and the Western blot analyses were performed as described by Chiba et al. [23] with some improvements. The lung tissues were removed and immediately frozen with liquid nitrogen, and the tissue was crushed to powder with a mortar. The tissues were homogenized using a high-speed disperser in RIPA buffer (Biyuntian Biotechnology, Haimen, China) containing 1% PMSF (Haixin Biotechnology, Hangzhou, China). After homogenization and centrifugation at 12,000 rpm at 4 °C for 15 min, the resulting supernatant was collected. The protein samples (30 μ g) were subjected to 13% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis at 80–120 V for 2 h and then transferred to 0.45- μ m nitrocellulose membranes at 70 V for 1.5 h. The membranes were blocked by 5% fat-free milk and then incubated with the rabbit monoclonal antibody against RhoA (21 kDa) (Cell Signaling Technology; 1/1000) or the rabbit monoclonal antibody against β -actin (43 kDa) (Cell Signaling Technology; 1/1000) in Tris-buffered saline with Tween-20 overnight at 4 °C. After washing, the membranes were incubated with the goat anti-rabbit 800 antibodies (1:5000) for 2 h at room

Table 1
The gene primers (5'–3'), annealing temperature and cycle.

Subjects	Gene primers	Annealing temperature (°C)	Cycle
CCL-11	Forward-AGAGGCTGAGATCCAAGCAG Reverse-ACAGATCTCTTTGCCAACCT	61	32
IL-4	Forward-ACGGCACAGAGCTATTGATG Reverse-ATGGTGGCTCAGTACTACGA	55	35
IL-5	Forward-ATGACTGTGCTCTGTGCTGGAGC Reverse-CTGTTTTCTGGAGTAACTGGGG	61	35
IFN- γ	Forward-CCTCAGACTCTTTGAAGCTT Reverse-CAGCGACTCTTTTCTCTT	58	35
β -actin	Forward-CTGTCCCTGTATGCCTCTG Reverse-ATGTCACGCACGA TTTC	55	32

temperature. After the final washes, the membranes were scanned using the Odyssey™ Infrared Imager (169 μm resolution, 0.5 mm offset with medium quality). The integrated density values were analyzed with a computerized image analysis system (Quantity One 4.5, Bio-Rad). The RhoA expression levels were given as the ratio of the OD signal for RhoA to that for β -actin.

2.8. Tissue processing and histological analysis

The right lungs were taken from mice under terminal anesthesia, inflated with 10% formalin, and immersed in the same solution before processing in paraffin-embedded blocks. Five-micrometer sections were stained with H&E, periodic acid-Schiff (PAS) and Masson-trichrome to evaluate the general morphology. To determine the peribronchial cell counts and severity of the infiltration of the inflammatory cells, the assays for the mucus-secreting goblet cells and subepithelial fibrosis were performed based on the 5-point scoring system described in our previous papers [20,24]. The analyses were performed in a blind fashion, and the slides were presented in a random order for each examination.

2.9. The analysis of the simvastatin concentration in the plasma and lung tissues of mice

The method previously described by Otaegui et al. [25] and Koitabashi et al. [26] was used with improvements. Briefly, simvastatin was dissolved in a mixture of PEG, ethanol and water. The mice were grouped into 3 mice per treatment and administered a single dose of simvastatin at 40 mg/kg i.t., 40 mg/kg i.g. or 5 mg/ml i.h. for 10 min. At the predefined time points (0.5, 2 and 6 h), the mice were anesthetized by ether, and blood samples were collected by cardiac puncture. The blood samples were centrifuged for 10 min at 2000 rpm to separate the plasma, which was kept frozen at -80°C until analysis by high performance liquid chromatography (HPLC). In addition, the lungs were removed from the mice. Liquid-liquid extraction was used for the sample preparation. To a 150- μL aliquot of plasma sample, 150 μL acetonitrile was added. The samples were stirred for 10 min and centrifuged at 12,000 rpm for 10 min. After centrifugation, the upper organic layer was removed, and 50 μL of the obtained solution was injected into the HPLC system (Agilent 1100). The lung tissue samples were accurately weighed, and 500 μL acetonitrile was added per 100 mg tissue and homogenized. The tissue homogenates were processed in a similar manner as the plasma samples and analyzed by HPLC using a C₁₈ column (Agilent Hypersil ODS 5 μm , 4.0 $\times$ 250) as the stationary phase and an acetonitrile: water: acetic acid (70: 30: 0.1, V/V) mixture as the mobile phase. The flow rate was 1.0 mL/min. The simvastatin levels were quantified by UV at 238 nm with a limit of quantification in the range of 5–2000 ng/mL. The pharmacokinetic analyses were performed using the data of the plasma and lung tissue concentrations as described by Chapman [27].

2.10. Data analysis

The numerical data are presented as the means $\pm$ S.E.M. The statistical calculations were performed using SigmaStat software (SigmaStat 2.0, SPSS Inc., Chicago, IL, USA). ANOVA and the Student-Newman-Keuls multiple comparisons test were used for evaluating the differences in respiratory function, the number of inflammatory cells, the cytokine mRNA expression in the lungs and the cytokine levels in the bronchoalveolar lavage fluid in each group. The significance was assessed at a level of $P < 0.05$. The experiments were performed independently at least twice; the results were qualitatively identical.

3. Results

3.1. The effects of the different delivery routes of simvastatin on airway inflammatory cells

The total number of inflammatory cells in the bronchoalveolar lavage fluid (BALF) of the vehicle-ovalbumin-treated mice was 6.8-fold greater than that in control mice. Simvastatin delivered by different routes (5 mg/mL i.h. for 10 min, 2 mg/kg i.t., 40 mg/kg i.g., and 40 mg/kg i.p.), the reference drug Y-27632 (10 mg/kg i.n.) and dexamethasone (1 mg/kg i.p.) all significantly inhibited the accumulation of the inflammatory cells in the airways. The eosinophils in the BALF in the vehicle-OVA-treated mice increased 125-fold compared to that in saline-vehicle mice (Fig. 2). The pretreatment of the mice sensitized and challenged with ovalbumin with simvastatin by all routes and the reference drug for 7 days significantly decreased the number of eosinophils. However, simvastatin at 5 mg/mL i.h. and 2 mg/kg i.t. had a more potent inhibitory effect on the accumulation of the total number of inflammatory cells and eosinophils in the airways compared to that of simvastatin at 40 mg/kg i.g. and 40 mg/kg i.p. ($P < 0.05$). Simvastatin at 5 mg/mL i.h. and 2 mg/kg i.t. had similar potent effects as that of dexamethasone at 1 mg/kg i.p.

3.2. The dose-response of i.h. simvastatin on the inflammatory cells in the BALF

The total number of inflammatory cells in the BALF of vehicle-OVA mice was 7.2-fold greater than that in the control mice. The inhalation pretreatment with simvastatin at 1, 5 and 20 mg/mL significantly inhibited the infiltration of the inflammatory cells in a dose-dependent manner. The classification of these inflammatory cells indicated that in the vehicle-OVA treated mice, the number of eosinophils, lymphocytes, macrophagocytes and neutrophils in the BALF increased 273.6-, 3.2-, 2.1- and 17.1-fold, respectively, compared to the control mice (Fig. 3). The pretreatment of the mice sensitized and challenged with ovalbumin with varying doses of simvastatin for 7 days significantly decreased the number of eosinophils, lymphocytes, macrophagocytes and neutrophils in a dose-dependent manner, but not simvastatin at 1 mg/mL significantly

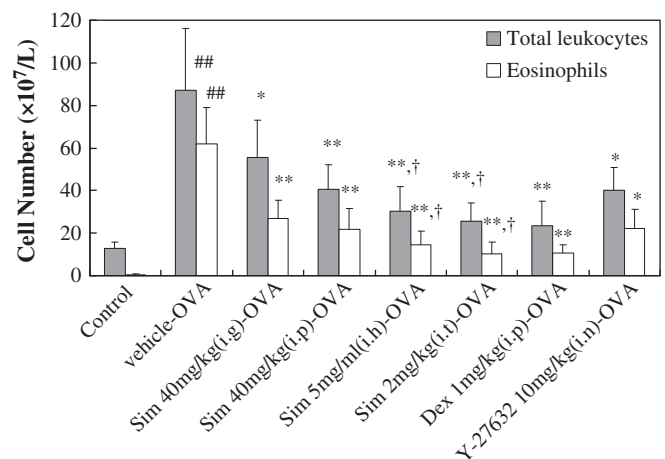


Fig. 2. The effects of simvastatin delivered via the different routes on the airway inflammatory cells in the BALF. The mice were treated as described in Fig. 1, and the bronchoalveolar lavage fluid was harvested 24 h after the final ovalbumin challenge. The data are expressed as the mean $\pm$ S.E.M. $n = 9-10$ for each group. $^{##}P < 0.01$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus the OVA-vehicle group; $^{\dagger}P < 0.05$ versus the Sim 40 mg/kg (i.g.)-OVA group. The treatments were as follows: simvastatin at 5 mg/mL via inhalation (i.h.) for 10 min, 2 mg/kg by intratracheal injection (i.t.), 40 mg/kg by intragastric injection (i.g.), or 40 mg/kg by intraperitoneal injection (i.p.); dexamethasone (Dex) at 1 mg/kg by intragastric injection (i.g.) and Y-27632 at 10 mg/kg by intranasal administration (i.n.), daily, for 7 consecutive days.

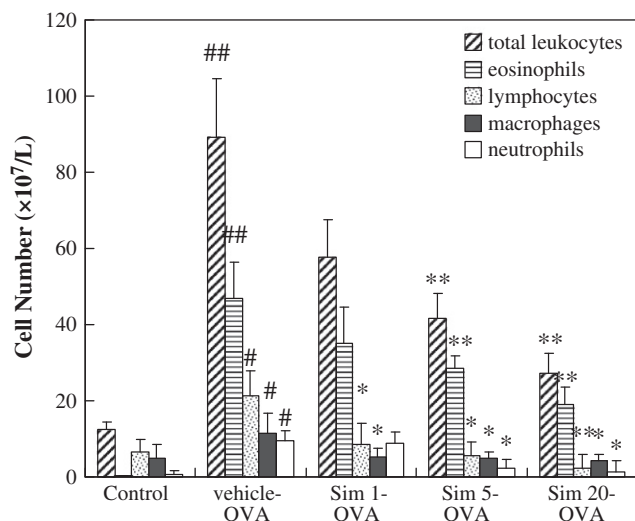


Fig. 3. The dose-response of simvastatin delivered via inhalation on the inflammatory cells in the BALF. The mice were treated as described in Fig. 1, and the bronchoalveolar lavage fluid was harvested 24 h after the final ovalbumin challenge. The data are expressed as the mean $\pm$ S.E.M. $n = 10$ for each group. ## $P < 0.01$ versus the control group; * $P < 0.05$, ** $P < 0.01$ versus the OVA-vehicle group.

inhibited the accumulation of the total number of inflammatory cells, eosinophils and neutrophils in airways.

3.3. The allergen-induced airway hyperresponsiveness to methacholine

The airway responsiveness to intravenous methacholine treatment at the doses of 0, 0.05, 0.1, 0.15, 0.2 and 0.25 mg/kg was assessed 24 h after the final challenge. The responsiveness of the control mice to methacholine was observed as a weak change in airway resistance (R_L) and lung dynamic compliance (C_{dyn}). However, the vehicle-OVA-treated mice had obvious airway responsiveness with a significant and dose-dependent increase in R_L and decrease in C_{dyn} compared to the control mice (Fig. 4 and Table 2). The aerosol pretreatment with simvastatin at the doses of 1, 5 and 20 mg/ml significantly and dose-dependently reduced the airway responsiveness provoked by methacholine. Additionally, the reference drug Y-27632 at 10 mg/kg i.n. had a significant inhibitory effect on the airway responsiveness. The inhibitory effect of simvastatin at the dose of 5 mg/ml i.h. was comparable to that of Y-27632 at 10 mg/kg i.n.

3.4. CCL-11, IL-4, IL-5 and IFN- γ mRNA expression in the lung tissues

To measure the CCL-11, IL-4, IL-5 and IFN- γ mRNA expression in vivo, the lung tissues were harvested and tested 24 h after the last ovalbumin challenge. As shown in Fig. 5, the vehicle-OVA-treated

Table 2

Effects of simvastatin and Y-27632 on methacholine-induced increase of airway resistance (PD_{100}) and reduction of lung dynamic compliance (PD_{25}) in actively sensitized mice. Sensitized mice react at smaller doses of intravenous methacholine, indicating airway hyperresponsiveness. Data are given as mean (95% confidence limits).

Groups	n	R_L PD_{100} (95% CI) mg/kg, i.v.	C_{dyn} PD_{25} (95% CI) mg/kg, i.v.
Control (vehicle i.h.)	10	0.148(0.112–0.196)	0.182(0.126–0.246)
vehicle-OVA (i.h.)	10	0.079(0.059–0.1120) #	0.072 (0.051–0.101) #
Sim 1 mg/ml-OVA (i.h.)	9	0.127(0.114–0.143)	0.089(0.062–0.105)
Sim 5 mg/ml-OVA (i.h.)	10	0.103(0.096–0.134)*	0.125(0.098–0.168)*
Sim 20 mg/ml-OVA (i.h.)	9	0.121(0.107–0.156)**	0.162(0.127–0.211)**
Y-27632 10 mg/kg-OVA (i.n.)	9	0.098(0.078–0.129)*	0.118(0.096–0.158)*

$P < 0.05$, versus control group.

* $P < 0.05$.

** $P < 0.01$, versus vehicle-OVA.

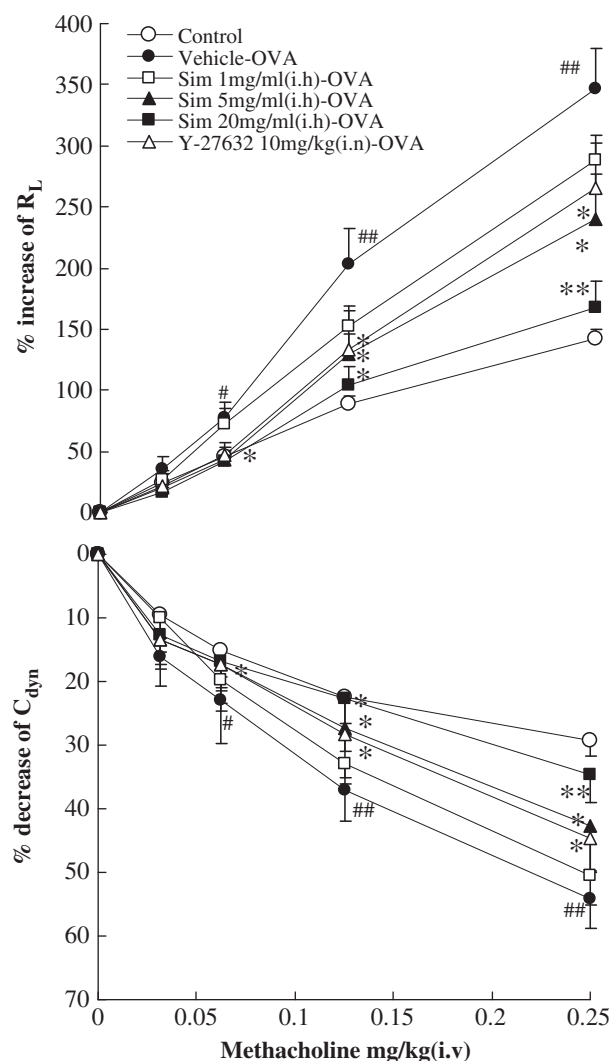


Fig. 4. The dose-dependent response of R_L and C_{dyn} to methacholine. The ovalbumin-sensitized mice were treated with 1, 5 and 20 mg/ml simvastatin (Sim) via inhalation, 10 mg/kg Y-27632 or vehicle once a day for 7 days. Fifteen minutes after the aerosol administration of Sim and vehicle or Y-27632 i.n., the mice were challenged with aerosolized antigen or normal saline (NS) for 30 min daily. Twenty-four hours after the final ovalbumin challenge, the R_L and C_{dyn} against the intravenous application of methacholine was determined as described in the Materials and methods. The data are expressed as the mean $\pm$ S.E.M. $n = 9$ –10 for each group. # $P < 0.05$, ## $P < 0.01$ versus the control mice; * $P < 0.05$, ** $P < 0.01$ versus the OVA-vehicle group.

mice showed an obvious up-regulation in the CCL-11, IL-4 and IL-5 mRNA expression compared to the control mice, and the aerosol pretreatment with simvastatin at the doses of 1, 5 and 20 mg/ml significantly inhibited this up-regulation, with the inhibition of CCL-11 and IL-4 up-regulation occurring in a dose-dependent manner. The inhibitory effect of simvastatin at 5 mg/ml i.h. was comparable to that of Y-27632 at 10 mg/kg i.n.

3.5. CCL-11, IL-4, IL-5 and IFN- γ levels in the BALF

The levels of the CCL-11, IL-4, IL-5 and IFN- γ proteins were measured by ELISA. As shown in Fig. 6, in the vehicle-OVA-treated mice, the levels of CCL-11, IL-4 and IL-5 in the BALF increased 1.2-, 3.3- and 3.9-fold, respectively, and the IFN- γ level did not decrease, compared to the control mice. The aerosol pretreatment with simvastatin significantly and dose-dependently inhibited the up-regulation of the CCL-11, IL-4 and IL-5 levels in the BALF. The effect of simvastatin at 5 mg/ml i.h. was comparable to that of Y-27632 at 10 mg/kg i.n. Simvastatin

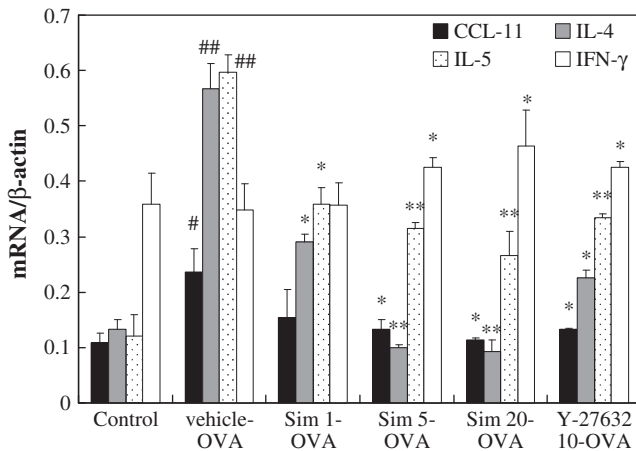


Fig. 5. The effects of simvastatin (Sim) and Y-27632 on the mRNA expression of CCL-11, IL-4, IL-5 and IFN- γ in the lungs of asthmatic mice. The mice were processed and treated as described in Fig. 1. Twenty-four hours after the final ovalbumin challenge, 100 mg of lung tissue from each mouse was harvested for the isolation of total RNA, and 1- μ g aliquots of the total RNA sample were used for reverse transcriptional synthesis of first-strand that was used as template for the PCR amplification. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, and specific bands were digitized using an imaging system. The PCR reactions were performed in triplicates, and the relative expression of the mRNA from the independent triplicate experiments was normalized by dividing the CCL-11 and cytokine values by the β -actin values, and the mean levels of the mRNA expression from the control mice were defined as 1. The results are shown as the mean $\pm$ S.E.M. $n=9$ for each group. * $P<0.05$, ** $P<0.01$ versus the control group; * $P<0.05$, ** $P<0.01$ versus the OVA-vehicle group.

at 5 and 20 mg/ml i.h. and Y-27632 at 10 mg/kg i.n. increased the IFN- γ level compared to the control or vehicle-OVA mice.

3.6. Eosinophil infiltration, mucus production and collagen deposition in lung tissues

The lung tissue was harvested 24 h after the final ovalbumin challenge. The vehicle-OVA-treated mice showed an obvious infiltration of the inflammatory cells into the peribronchiolar and perivascular connective tissues. Goblet cell hyperplasia and mucus hypersecretion within the bronchi of the lungs were also observed. There was a dramatic increase in both the extent of collagen deposition and the intensity of staining compared with control mice. Simvastatin at 5 or 20 mg/ml i.h. and Y-27632 at 10 mg/kg i.n. markedly attenuated

the ovalbumin-induced eosinophil infiltration, mucus production and collagen deposition in the lung tissues compared with the vehicle-OVA group (Figs. 7A and B). However, simvastatin at 1 mg/ml i.h. did not have a significant effect on eosinophil infiltration, mucus production or collagen deposition in the lung tissues.

3.7. RhoA protein expression

The lung tissue was harvested 24 h after the final ovalbumin challenge. The vehicle-OVA-treated mice showed a significant up-regulation of RhoA protein expression compared to the control mice, and aerosol pretreatment with simvastatin at 5 and 20 mg/ml and Y-27632 at 10 mg/kg i.n. significantly inhibited the up-regulation of RhoA protein expression (Fig. 8).

3.8. The lung and plasma concentrations of the simvastatin delivered via different routes

To estimate the relative contribution of the systemically absorbed drug to the biological activity when simvastatin was delivered topically to the lungs, a series of pharmacokinetic and biological studies were performed. Table 3 shows the drug concentrations in the lung tissue and plasma after i.h., i.t. and i.g. simvastatin administration. To assay the drug concentration of the lung tissue and plasma after simvastatin was delivered by the i.g. route, the mice were treated with a high dose (100 mg/kg) of simvastatin. The results show a lower concentration of drug in the lung tissue following the high-dose treatment compared to that following simvastatin treatment at 5 mg/ml i.h. for 10 min. The plasma concentration 0.5, 2 and 6 h after the i.h. administration was only 5-, 1.9- and 2.7-fold, respectively. However, the lung concentration was 25.8-, 5.5- and 4.8-fold of the levels following the i.g. treatment, respectively. After 0.5, 2 and 6 h of simvastatin treatment at 40 mg/ml i.t., the plasma levels were only 25-, 5.5- and 5.0-fold of the levels following the i.g. treatment, respectively, and were increased more than twenty-thousand-fold in the lung tissues.

4. Discussion

This study describes the pharmacology of simvastatin, which has a biological and pharmacokinetic profile ideal for delivery as an inhaled drug. Simvastatin is a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and has been widely prescribed to treat hyperlipidemia. Statins have several other pharmacological actions that might be beneficial in airway inflammation disease, including anti-inflammatory, anti-oxidant, and immunomodulatory effects. Many of these pleiotropic effects of statins are mediated by the inhibition of the isoprenylation of small GTP-binding signaling molecules. However, the dose response of the pleiotropic effects of statins has not yet been established and may differ from that of the cholesterol-lowering effects. High doses of oral statins may have adverse effects, particularly on skeletal muscles, so it is important for statins to be delivered by inhalation [18]. In this study, i.h. simvastatin attenuated airway inflammation, remodeling and hyperresponsiveness by inhibiting the RhoA pathway in a dose-dependent manner in the mouse model of asthma. Simvastatin is active when administered by inhalation but is weakly active when administered orally. These results are consistent with the pharmacokinetic properties of simvastatin, which show low plasma levels after oral administration but dose-dependent increases in efficacy after inhalation.

The selection of simvastatin as an appropriate compound for inhalation was based upon several important pharmacokinetic factors. First, simvastatin has low oral bioavailability. In pharmacokinetic studies with simvastatin at 40 mg/kg p.o., the calculated oral bioavailability was 5%. Low oral bioavailability is a critical factor for inhaled drugs that have the potential for adverse side effects when absorbed systemically [28]. This rationale has been successfully used for the inhaled

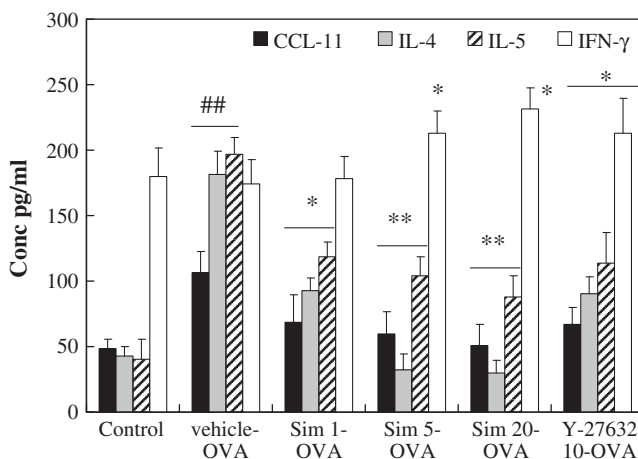


Fig. 6. The effects of simvastatin (Sim) and Y-27632 on the protein level of CCL-11, IL-4, IL-5 and IFN- γ in the BALF of asthmatic mice. The mice were processed and treated as described in Fig. 1, and the bronchoalveolar lavage fluid was harvested 24 h after the final ovalbumin challenge. The levels of CCL-11, IL-4, IL-5 and IFN- γ in the BALF were analyzed by ELISA. $n=8$ for each group. The results are shown as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ versus the control group; * $P<0.05$, ** $P<0.01$ versus the OVA-vehicle group.

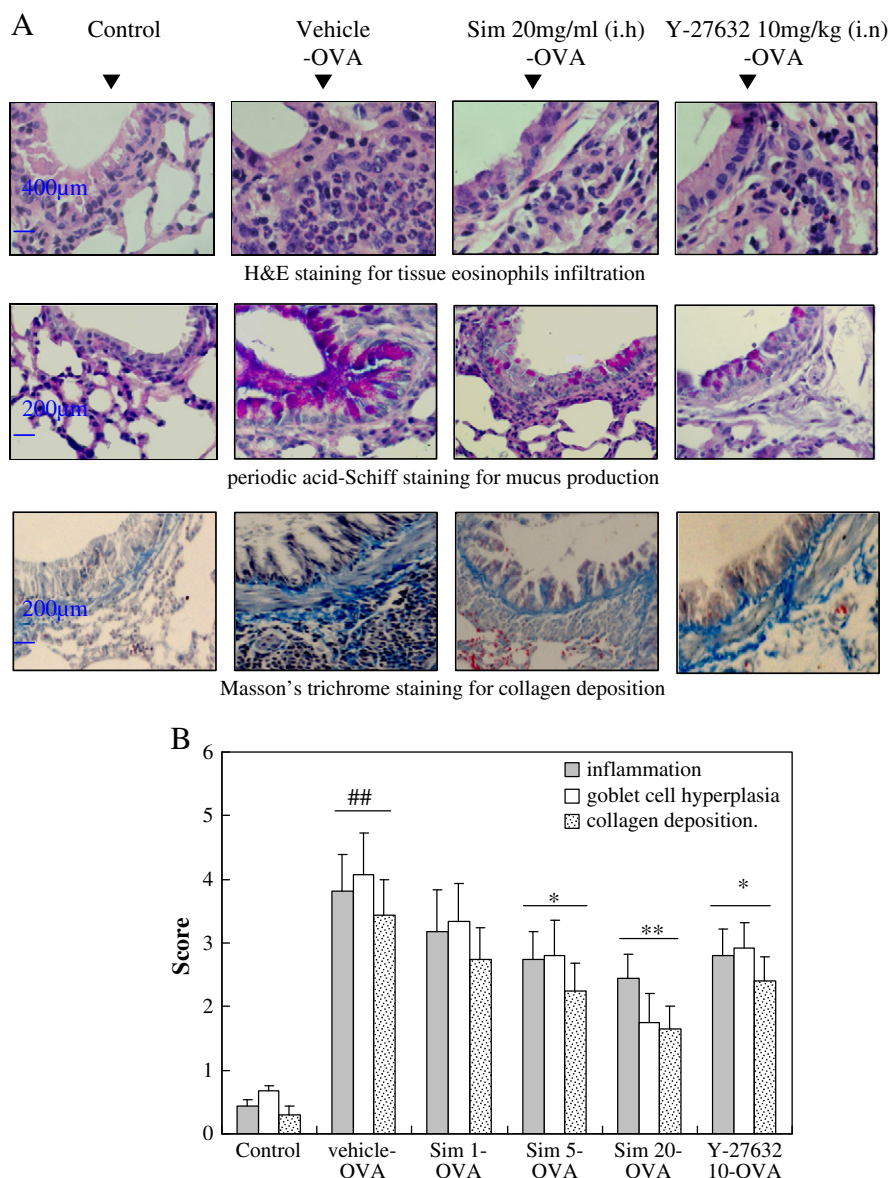


Fig. 7. The effects of simvastatin (Sim) and Y-27632 on inflammatory infiltration, mucus production and collagen deposition in lungs. The histological examinations of lung tissue eosinophil infiltration, mucus secretion and collagen deposition were performed 24 h after the final allergen challenge as described in the [Materials and methods](#). The lung tissues were fixed, sectioned to 5 µm, and stained with H&E to measure tissue eosinophil infiltration, periodic acid-Schiff to measure mucus production (red are positive for mucus) or Masson's trichrome to measure collagen deposition (blue are positive for collagen deposition). The data are expressed as the mean ± S.E.M. $n = 10$ for each group. * $P < 0.05$, ** $P < 0.01$ versus the control mice; * $P < 0.05$, ** $P < 0.01$ versus the OVA-vehicle group.

corticosteroids and long-acting muscarinic antagonists [28,29]. The very low bioavailability of inhaled or oral simvastatin is probably due to its poor absorption, which significantly limits its systemic exposure to cause adverse side effects. Second, simvastatin is highly protein-bound (94%–98% plasma protein binding in human blood), which further limits the systemic exposure to the free drug following inhalation [30]. Finally, simvastatin has a good lipophilicity, which is suitable for inhaled delivery as a dry powder system or a pressure metered-dose inhaler; this rationale has also been successfully used for inhaled corticosteroids [31]. The selection of simvastatin for the development as an inhaled compound was also based upon important biological information. In the present study, i.h. or i.t. simvastatin have an extremely potent anti-inflammatory effect *in vivo*, which is between 20- and 100-fold greater than the effect observed by the i.p. or i.g. routes. The potency of simvastatin was reduced in the presence of human serum. This probably reflects the binding of simvastatin to the plasma proteins in addition to its poor absorption. In the present study, we compared the pharmacokinetic parameters of the

lung tissues and plasma following the i.h. and i.t. administration of simvastatin in mice, which aimed to determine how much i.h. simvastatin is absorbed as compared to the 100% absorption by the i.t. administration. The results showed that the lung tissue and plasma concentration following i.h. delivery was only 3% to 4% compared to that following i.t. administration. However, in rat, these values were 8% to 10%, and a healthy volunteer study showed a 55% to 60% increase in the pattern of deposition within the lung due to treatment with an HFA-beclomethasone metered-dose inhaler [32]. As discussed above, these results may suggest that simvastatin is a potential anti-inflammatory drug that has pharmacological and pharmacodynamic properties suitable for delivery via inhalation.

Although i.g. and i.p. simvastatin have shown good efficacy in pre-clinical models [2–4,6,33,34], it is not clear if i.h. simvastatin influences the proinflammatory factors and remodeling. In the present study, i.h. simvastatin significantly decreased the levels of CCL-11, IL-4 and IL-5 mRNA in lung tissue and their protein concentrations in the BALF and

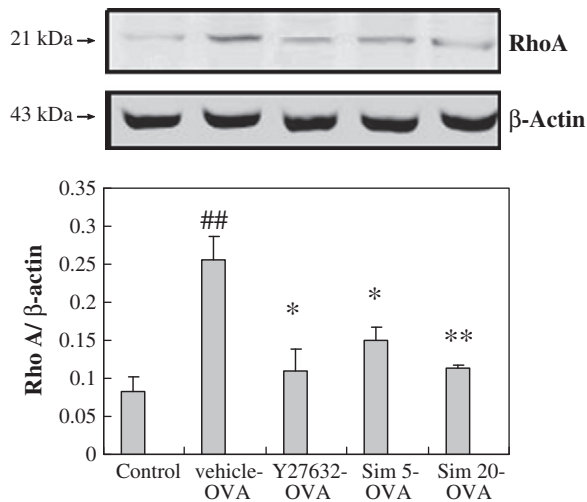


Fig. 8. The inhibitory effects of simvastatin on the antigen-induced up-regulation of RhoA in the bronchial tissues of sensitized mice. The mice were actively sensitized and repeatedly challenged with the OVA antigen as described in [Materials and methods](#). The animals also received 5 and 20 mg/ml simvastatin and vehicle via inhalation or Y-27632 by intranasal administration 1 h before each OVA challenge. Twenty-four hours after the final OVA challenge, the RhoA protein expression levels in the lung tissues were determined by Western blot. Representative blots for RhoA and β -actin are shown in the upper panels. The bands were analyzed by a densitometer, and the data are summarized in the lower panel. The data are expressed as the mean $\pm$ S.E.M. $n = 5$ for each group in three to four independent experiments. ## $P < 0.01$ versus the control group; * $P < 0.05$, ** $P < 0.01$ versus the vehicle-OVA group.

increased IFN- γ mRNA in lung tissue and its protein concentrations in the BALF; these cytokines play a central role in Th1 and Th2 allergic inflammation and AHR (Figs. 5 and 6). CCL-11, IL-4 and IL-5 are known to enhance AHR, whereas IL-4 maintains the allergic response via its effects on T and B lymphocytes and IgE. CCL-11 and IL-5 play a critical role in the expansion of eosinophil pools in both the bone marrow and blood in response to allergen provocation of the airways [34]. IL-4 probably acts by activating eosinophil-specific adhesion pathways and by regulating the production of IL-5 and CCL-11 in the lung compartment. IL-5 and CCL-11 co-operate locally in pulmonary tissues to selectively and synergistically promote eosinophilia. Thus, IL-5 acts systemically to induce eosinophilia and within tissues to promote local chemotactic signals. The regulation of the IL-5 and CCL-11 levels within the lung by IL-4 and IL-13 allows the Th2 cells to elegantly coordinate tissue and peripheral eosinophilia. Whereas the inhibition of either the IL-4/IL-13 or IL-5/eotaxin pathways resulted in the abolition of tissue eosinophils and AHR, only the depletion of IL-5 and CCL-11 concurrently results in the marked attenuation of pulmonary inflammation, AHR [35] and airway remodeling [36].

The beneficial effects of i.h. simvastatin on AHR may be due to the inhibition of these key cytokines. Chiba and colleagues showed that

IL-4 and IL-13 up-regulate the small G protein RhoA, thereby resulting in increased bronchial smooth muscle cell hyperresponsiveness in a mouse model of allergic inflammation [23,37]. Moreover, the CCL11-induced migration is also fully dependent on both RhoA and ROCK [38]. Simvastatin may work through multiple mechanisms: first, by reducing CCL11, IL-4 and IL-13 production (or by inhibiting the cells that make these cytokines); and second, by depleting mevalonate and geranylgeranyl pyrophosphate (GGPP) [4], thereby preventing the activation of RhoA signaling. In the present study, we studied the functional relevance of RhoA expression in lung tissues using the ROCK inhibitor Y-27632 by i.n. and simvastatin by i.h. Allergen exposure activates RhoA in lungs, and i.n. Y-27632 and i.h. simvastatin significantly inhibited inflammation and RhoA expression. Similarly, Schaafsma and colleagues showed that allergen challenge increases the role of Rho-kinase in airway smooth muscle (ASM) contraction. The basal tone in the preparations from both the control and OVA-sensitized animals was strongly decreased in the presence of the Rho-kinase inhibitor Y-27632. The expression of RhoA, an upstream activator of Rho-kinase, was significantly increased (2.6-fold) in the lung homogenates of the OVA-sensitized animals compared to the control animals, as determined by Western analysis [39]. The contribution of Rho-kinase to the contractile airway responsiveness, both in vivo and ex vivo, is augmented after active allergic sensitization, presumably as a consequence of the increased expression of RhoA. Henry and colleagues showed that Y-27632 inhibits pulmonary eosinophilia, bronchoconstriction and airway hyperresponsiveness in allergic mice [21]. Therefore, the inhibition of the RhoA/Rho-kinase pathway may be considered to be a useful pharmacotherapeutic target for the treatment of allergies and asthma.

In conclusion, our study demonstrates that i.h. simvastatin attenuates the allergic airway inflammation in a RhoA-dependent manner, inhibits the mRNA expression or protein release of Th2 cytokines and chemokines, and improves airway remodeling and lung function. These data demonstrate for the first time that the inhalation of simvastatin caused a higher drug concentration in the local lung tissue, a more potent anti-inflammatory action, and a lower drug concentration in the plasma and therefore fewer potential side effects than that of oral simvastatin. These results suggest that simvastatin is a potential anti-inflammatory drug for airway inflammatory diseases with properties suitable for delivery by inhalation, which will probably overcome the side effects and low clinical efficacy of large oral doses of simvastatin.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Scientific Foundation of China (No. 81172880 and 30973542).

References

- [1] Liao JK, Laufs U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* 2005;45:89–118.
- [2] McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol* 2004;172:2903–8.
- [3] Kim DY, Ryu SY, Lim JE, Lee YS, Ro JY. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur J Pharmacol* 2007;557:76–86.
- [4] Zeki AA, Franzi L, Last J, Kenyon NJ. Simvastatin inhibits airway hyperreactivity: implications for the mevalonate pathway and beyond. *Am J Respir Crit Care Med* 2009;180:731–40.
- [5] Huang CC, Chan WL, Chen YC, Chen TJ, Chou KT, Lin SJ, et al. Statin use in patients with asthma: a nationwide population-based study. *Eur J Clin Invest* 2011;41:507–12.
- [6] Wang W, Le W, Ahuja R, Cho DY, Hwang PH, Upadhyay D. Inhibition of inflammatory mediators: role of statins in airway inflammation. *Otolaryngol Head Neck Surg* 2011;144:982–7.

Table 3

Comparison of plasma and lung concentration of simvastatin following doses administered by inhalation, intragastric injection or intratracheal injection. Data are expressed as mean $\pm$ S.E.M. $n = 5$ (each group).

Routes (doses)	Time (h)	Conc. of plasma (μ g/ml)	Conc. of lung (μ g/g tissue)	Ratio of Lung/plasma
i.h (5 mg/ml, 10 min)	0.5	0.282 $\pm$ 0.055	6.850 $\pm$ 0.244	24.3
	2	0.261 $\pm$ 0.031	1.838 $\pm$ 0.121	7.1
	6	0.260 $\pm$ 0.059	1.258 $\pm$ 0.099	4.8
i.g (100 mg/kg)	0.5	0.057 $\pm$ 0.006	0.270 $\pm$ 0.043	10.0
	2	0.139 $\pm$ 0.013	0.335 $\pm$ 0.067	2.4
	6	0.094 $\pm$ 0.028	0.262 $\pm$ 0.053	2.7
i.t (2 mg/kg)	0.5	0.267 $\pm$ 0.042	186.3 $\pm$ 21.28	697.8
	2	0.578 $\pm$ 0.137	170.4 $\pm$ 26.74	294.8
	6	0.467 $\pm$ 0.034	139.5 $\pm$ 15.31	298.7

- [7] Hothersall EJ, Chaudhuri R, McSharry C, Donnelly I, Lafferty J, McMahon AD, et al. Effects of atorvastatin added to inhaled corticosteroids on lung function and sputum cell counts in atopic asthma. *Thorax* 2008;63:1070–5.
- [8] Maneechotesuwan K, Ekjiratrakul W, Kasetsinsombat K, Wongkajornsilp A, Barnes PJ. Statins enhance the anti-inflammatory effects of inhaled corticosteroids in asthmatic patients through increased induction of indoleamine 2, 3-dioxygenase. *J Allergy Clin Immunol* 2010;126:754–62 e1.
- [9] Samson KT, Minoguchi K, Tanaka A, Oda N, Yokoe T, Yamamoto Y, et al. Inhibitory effects of fluvastatin on cytokine and chemokine production by peripheral blood mononuclear cells in patients with allergic asthma. *Clin Exp Allergy* 2006;36:475–82.
- [10] Robinson AJ, Kashanin D, O'Dowd F, Fitzgerald K, Williams V, Walsh GM. Fluvastatin and lovastatin inhibit granulocyte macrophage-colony stimulating factor-stimulated human eosinophil adhesion to inter-cellular adhesion molecule-1 under flow conditions. *Clin Exp Allergy* 2009;39:1866–74.
- [11] Krauth MT, Majlesi Y, Sonneck K, Samorapoompichit P, Ghannadan M, Hauswirth AW, et al. Effects of various statins on cytokine-dependent growth and IgE-dependent release of histamine in human mast cells. *Allergy* 2006;61:281–8.
- [12] Schaafsma D, Dueck G, Ghavami S, Kroeker A, Mutawe MM, Hauff K, et al. The mevalonate cascade as a target to suppress extracellular matrix synthesis by human airway smooth muscle. *Am J Respir Cell Mol Biol* 2011;44:394–403.
- [13] Takeda N, Kondo M, Ito S, Ito Y, Shimokata K, Kume H. Role of RhoA inactivation in reduced cell proliferation of human airway smooth muscle by simvastatin. *Am J Respir Cell Mol Biol* 2006;35:722–9.
- [14] Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, et al. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol* 2006;176:7657–65.
- [15] Menzies D, Nair A, Meldrum KT, Fleming D, Barnes M, Lipworth BJ. Simvastatin does not exhibit therapeutic anti-inflammatory effects in asthma. *J Allergy Clin Immunol* 2007;119:328–35.
- [16] Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Simvastatin in the treatment of asthma: lack of steroid-sparing effect. *Thorax* 2010;65:891–6.
- [17] Di Stasi SL, MacLeod TD, Winters JD, Binder-Macleod SA. Effects of statins on skeletal muscle: a perspective for physical therapists. *Phys Ther* 2010;90:1530–42.
- [18] Barnes PJ. Future treatments for chronic obstructive pulmonary disease and its comorbidities. *Proc Am Thorac Soc* 2008;5:857–64.
- [19] Deng YM, Xie QM, Tang HF, Sun JG, Deng JF, Chen JQ, et al. Effects of ciclamilast, a new PDE 4 PDE4 inhibitor, on airway hyperresponsiveness, PDE4D expression and airway inflammation in a murine model of asthma. *Eur J Pharmacol* 2006;547:125–35.
- [20] Cao R, Dong XW, Jiang JX, Yan XF, He JS, Deng YM, et al. M(3) muscarinic receptor antagonist bencycloquidinium bromide attenuates allergic airway inflammation, hyperresponsiveness and remodeling in mice. *Eur J Pharmacol* 2011;655:83–90.
- [21] Henry PJ, Mann TS, Goldie RG. A rho kinase inhibitor, Y-27632 inhibits pulmonary eosinophilia, bronchoconstriction and airways hyperresponsiveness in allergic mice. *Pulm Pharmacol Ther* 2005;18:67–74.
- [22] Bao ZS, Hong L, Guan Y, Dong XW, Zheng HS, Tan GL, et al. Inhibition of airway inflammation, hyperresponsiveness and remodeling by soy isoflavone in a murine model of allergic asthma. *Int Immunopharmacol* 2011;11:899–906.
- [23] Chiba Y, Sato S, Hanazaki M, Sakai H, Misawa M. Inhibition of geranylgeranyl-transferase inhibits bronchial smooth muscle hyperresponsiveness in mice. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L984–91.
- [24] Xie QM, Wu X, Wu HM, Deng YM, Zhang SJ, Zhu JP, et al. Oral administration of allergen extracts from *Dermatophagoides farinae* desensitizes specific allergen-induced inflammation and airway hyperresponsiveness in rats. *Int Immunopharmacol* 2008;8:1639–45.
- [25] Otaegui D, Rodriguez-Gascon A, Zubia A, Cossio FP, Pedraz JL. Development and validation of a liquid chromatography-tandem mass spectrometry for the determination of Kendine 91, a novel histone deacetylase inhibitor, in mice plasma and tissues: application to a pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008;870:109–16.
- [26] Koitabashi Y, Kumai T, Matsumoto N, Watanabe M, Sekine S, Yanagida Y, et al. Orange juice increased the bioavailability of pravastatin, 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, in rats and healthy human subjects. *Life Sci* 2006;78:2852–9.
- [27] Chapman RW, House A, Richard J, Prelusky D, Lam J, Wang P, et al. Pharmacology of a potent and selective inhibitor of PDE4 for inhaled administration. *Eur J Pharmacol* 2010;643:274–81.
- [28] Cerasoli Jr F. Developing the ideal inhaled corticosteroid. *Chest* 2006;130:545–64S.
- [29] Disse B, Speck GA, Rominger KL, Witek Jr TJ, Hammer R. Tiotropium (Spiriva): mechanistical considerations and clinical profile in obstructive lung disease. *Life Sci* 1999;64:457–64.
- [30] Corsini A, Bellosa S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol Ther* 1999;84:413–28.
- [31] Crim C, Pierre LN, Daley-Yates PT. A review of the pharmacology and pharmacokinetics of inhaled fluticasone propionate and mometasone furoate. *Clin Ther* 2001;23:1339–54.
- [32] Leach CL, Davidson PJ, Boudreau RJ. Improved airway targeting with the CFC-free HFA-beclomethasone metered-dose inhaler compared with CFC-beclomethasone. *Eur Respir J* 1998;12:1346–53.
- [33] Zeki AA, Bratt JM, Rabowsky M, Last JA, Kenyon NJ. Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling? *Transl Res* 2010;156:335–49.
- [34] Elsner J, Escher SE, Forssmann U. Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. *Allergy* 2004;59:1243–58.
- [35] Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP, et al. Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* 2001;179:173–81.
- [36] Halwani R, Al-Abri J, Beland M, Al-Jahdali H, Halayko AJ, Lee TH, et al. CC and CXC chemokines induce airway smooth muscle proliferation and survival. *J Immunol* 2011;186:4156–63.
- [37] Chiba Y, Todoroki M, Misawa M. Interleukin-4 upregulates RhoA protein via an activation of STAT6 in cultured human bronchial smooth muscle cells. *Pharmacol Res* 2010;61:188–92.
- [38] Muesel MJ, Scott KS, Friedl P, Bradding P, Wardlaw AJ. CCL11 and GM-CSF differentially use the Rho GTPase pathway to regulate motility of human eosinophils in a three-dimensional microenvironment. *J Immunol* 2008;180:8354–60.
- [39] Schaafsma D, Gosens R, Bos IS, Meurs H, Zaagsma J, Nelemans SA. Allergic sensitization enhances the contribution of Rho-kinase to airway smooth muscle contraction. *Br J Pharmacol* 2004;143:477–84.