Lovastatin inhibits bronchial hyperresponsiveness by reducing RhoA signaling in rat allergic asthma

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Chiba Y, Arima J, Sakai H, Misawa M. Lovastatin inhibits bronchial hyperresponsiveness by reducing RhoA signaling in rat allergic asthma. Am J Physiol Lung Cell Mol Physiol 294: L705–L713, 2008. First published February 22, 2008; doi:10.1152/ajplung.00531.2007.—Recent studies revealed an importance of a monomeric GTP-binding protein, RhoA, in contraction of bronchial smooth muscle (BSM). RhoA and its downstream have been proposed as a new target for the treatment of airway hyperresponsiveness in asthma. Statins are known to inhibit the functional activation of RhoA via the depletion of geranylgeranylpyrophosphate. To determine the beneficial effects of statins on the airway hyperresponsiveness in allergic bronchial asthma, we investigated the effects of systemic treatment with lovastatin on the augmented BSM contraction and activation of RhoA in rats with allergic bronchial asthma. Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated Ascaris suum antigen. Animals were also treated with lovastatin (4 mg·kg⁻¹·day⁻¹·ip) once a day before and during the antigen inhalation period. Repeated antigen inhalation caused a marked BSM hyperresponsiveness to ACh with the increased expression and translocation of RhoA. Lovastatin treatments significantly attenuated both the augmented contraction and RhoA translocation to the plasma membrane. Lovastatin also reduced the increased cell number in bronchoalveolar lavage fluids and histological changes induced by antigen exposure, whereas the levels of immunoglobulin E in sera and interleukins-4, -6, and -13 in bronchoalveolar lavage fluids were not significantly changed. These findings suggest that lovastatin ameliorates antigen-induced BSM hyperresponsiveness, an important factor of airway hyperresponsiveness in allergic asthmatics, probably by reducing the RhoA-mediated signaling.

Lovastatin inhibits bronchial hyperresponsiveness; bronchial smooth muscle; airway inflammation

INCREASED AIRWAY NARROWING in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important symptom of the disease, although the pathophysiological variations leading to the hyperresponsiveness are unclear now. Several mechanisms have been suggested to explain the airway hyperresponsiveness (AHR), such as alterations in the neural control of airway smooth muscle (1), increased mucosal secretions (25), and mechanical factors related to remodeling of the airways (57). In addition, it has also been suggested that one of the factors that contribute to the exaggerated airway narrowing in asthmatics is an abnormality of the nature of airway smooth muscle (33, 46). Rapid relief from airway limitation in asthmatic patients by β-stimulant inhalation may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus it may be important for development of asthma therapy to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease.

Smooth muscle contraction is mainly regulated by an increase in cytosolic Ca²⁺ concentration in myocytes. Recently, an additional mechanism, termed Ca²⁺ sensitization, has also been suggested in the agonist-induced smooth muscle contraction including airways (6, 11, 12, 24, 26, 39, 60). Although the detailed mechanism is not fully understood, there is increasing evidence that a monomeric GTP-binding protein, RhoA, and its downstream target, Rho-kinase, are involved in the agonist-induced Ca²⁺ sensitization of airway smooth muscle contraction (11, 12, 24, 60). When the RhoA/Rho-kinase system is activated by contractile agonists, the activity of myosin light chain (MLC) phosphatase is reduced and the level of phosphorylated MLC is then increased, resulting in an augmentation of smooth muscle contraction. Recent studies revealed an augmentation of the RhoA/Rho-kinase-mediated Ca²⁺ sensitization in diseased smooth muscles such as coronary (27, 43, 49) and cerebral vasospasms (13, 41, 42) and hypertension (36, 45, 53). In addition, our previous studies demonstrated that the agonist-induced, RhoA-mediated Ca²⁺ sensitization of bronchial smooth muscle contraction is augmented in rats (11) and mice (12) with allergic bronchial asthma. It is thus possible that an augmentation of RhoA/Rho-kinase system might be one of the causes of the bronchial smooth muscle hyperresponsiveness. An importance of RhoA and its downstream Rho-kinases in contraction of human bronchial smooth muscle was also demonstrated (60), and the RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (19).

Statins are a class of cholesterol-lowering drugs that decrease mortality from cardiovascular disease (48) and stroke (2). The beneficial effects of statins have been attributed to reduce cholesterol biosynthesis through competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate. Statins have pleiotropic effects beyond lowering cholesterol levels, such as anti-inflammatory actions and antioxidant activity (31, 34), and have also been proposed as a novel treatment of respiratory diseases (23). One of the mechanisms of the pleiotropic effects may result from the depletion of the downstream metabolites farnesylpyrophosphate and geranylgeranylpyrophosphate. These isoprenoid pyrophosphates are precursors required for the posttranscriptional prenylation and functional activation of certain proteins, including RhoA. It is thus likely that statins have an ability to reduce the Ca²⁺ sensitization of
smooth muscle contraction via an inhibition of RhoA function indirectly. Indeed, lovastatin can inhibit translocation of RhoA to plasma membrane, which is modulated by its geranylgeranylation, resulting in an inhibition of RhoA-mediated function in cultured tumor cell lines (32, 61).

In the present study, the effects of lovastatin on the augmented contraction and RhoA activation in bronchial smooth muscles of rats with experimental asthma were investigated to determine the beneficial effects of this agent on the AHR in allergic bronchial asthma. To our knowledge, this study is the first to show that lovastatin ameliorates antigen-induced bronchial smooth muscle hyperresponsiveness, probably by reducing the RhoA-mediated signaling.

METHODS

Animals and treatments. Male Wistar rats (6 wk of age, specific pathogen free, 170–190 g; Charles River Japan, Kanagawa, Japan) were used. All experiments were approved by the Animal Care Committee at the Hoshi University (Tokyo, Japan).

Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated Ascaris suum antigen (DNP-Asc) according to the method described previously (9, 11, 39). In brief, the rats were sensitized with DNP-Asc together with Bordetella pertussis on day 0 and were boosted 5 days later (day 5). Eight days after the first immunization (day 8), the rats were challenged by inhaling DNP-Asc for 40 min under conscious state. The animals were then subjected to a total of three repeated antigen challenges every 48 h with the same inhalational challenge method (days 8, 10, and 12). Animals also received intraperitoneal injection with lovastatin (4 mg·kg⁻¹·day⁻¹) dissolved in 50% DMSO in PBS; Toronto Research Chemicals, Ontario, Canada (37) or its vehicle once a day for 7 days from day 6 to day 12. The treatment was carried out 1 h before the antigen inhalation on the days of the antigen challenge (days 8, 10, and 12). The dosage of lovastatin was based on previous studies (15, 16, 18, 21, 37, 38) in which rats were used.

Functional study for bronchial smooth muscles. To determine the change in bronchial smooth muscle contractility, we measured the isometric contraction of the circular smooth muscle of the main bronchus as described previously (6, 9, 39). In brief, 24 h after the last antigen challenge (day 13), the rats were killed under chloral hydrate (400 mg/kg ip) anesthesia. After thoracotomy, blood samples were collected from the hearts to obtain sera, and bronchoalveolar lavage (BAL) was carried out as described previously (6). The airway tissues below the larynx to the lungs were then immediately removed. About a 4-mm length (3-mm diameter) of the left main bronchus was isolated (8–9 cartilages), and the resultant tissue ring preparation was then suspended in an organ bath at a resting tension of 1 g. The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): 118.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25.0 NaHCO₃, 1.2 KH₂PO₄, and 10.0 glucose (pH 7.4). The isometric contraction of the circular muscle was measured with a force-displacement transducer (TB-612T; Nihon Kohden, Tokyo, Japan). During an equilibration period, the tissues were washed three or four times at 15- to 20-min intervals and were equilibrated slowly to a baseline tension of 1 g. After the equilibration period, the concentration-response curve to acetylcholine (ACH; 10⁻⁷ to 10⁻³ M final concentration) was constructed cumulatively. A higher concentration of ACH was successively added after attainment of a plateau response to the previous concentration. In another series of experiments, isotonic K⁺ solution (10–90 mM final concentration) was cumulatively administered in the presence of atropine and indomethacin (both 10⁻⁶ M) to determine the bronchial smooth muscle responsiveness to high-K⁺ depolarization.

Quantitation of total cholesterol and IgE levels in serum. Serum was obtained from the blood sample by centrifugation at 3,000 g for 10 min at 4°C. Total cholesterol in serum was measured using enzymatic methods with a Cholesterol E-Test (Wako, Osaka, Japan) according to the manufacturer’s instructions. Total IgE in serum was measured using enzyme-linked immunosorbent assay (ELISA) with a Rat IgE ELISA kit (AKRIE-011; Shibayagi, Gunma, Japan) according to the manufacturer’s instructions.

Quantitation of cytokine levels in BAL fluid. The BAL fluid obtained was centrifuged at 500 g for 5 min at room temperature, and the resultant supernatant was used for cytokine analyses. The levels of interleukin (IL)-4, IL-6, and IL-13 were determined using ELISA with Quantikine rat IL-4 immunoassay (R4000; R&D Systems, Minneapolis, MN), Endogen rat IL-6 ELISA kit (ER2IL6; Pierce Biotechnology, Rockford, IL), and BioSource rat IL-13 immunoassay kit (KRC0131; BioSource International, Camarillo, CA), respectively, according to the manufacturers’ instructions.

BAL cell counts. The resultant pellet of the centrifuged BAL fluid was resuspended in 500 µl of 10% formaldehyde and incubated for 10 min at room temperature. The cells were washed with PBS and resuspended in 500 µl of PBS. An aliquot of cell suspension was used for cell counts with a hemocytometer. Cell counts were determined in duplicate and expressed as total cell counts per milliliter of BAL fluids obtained.

Western blot analyses. Protein samples of bronchial tissues were prepared as described previously (6, 9, 11, 39). In brief, the airway tissues below the main bronchi to the lungs were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The airways were carefully cleaned of adhering connective tissues, blood vessels, and lung parenchyma under a stereomicroscope. The epithelium was removed as much as possible by gently rubbing with keen-edged tweezers; histological examinations revealed that almost all the cells other than bronchial smooth muscle cells and cartilages were removed by this process (6, 9, 11, 39). The bronchial tissue (containing the main and intrapulmonary bronchi) segments were then equilibrated in oxygenated Krebs-Henseleit solution (37°C) for 60 min with 10-min washout intervals. After the equilibration period, the tissue segments were stimulated with ACh (10⁻³ M) for 10 min. The reaction was stopped by quickly freezing with liquid nitrogen, and the tissue was crushed to pieces with a Cryopress (CP-100W; Microtec, Chiba, Japan; 15 × 3). The tissue powder was homogenized in ice-cold Tris (10 mM; pH 7.5) buffer containing 5 mM MgCl₂, 2 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 1% Triton X-100, and 1% sodium cholate. The tissue homogenate was centrifuged (3,000 g at 4°C for 15 min). An aliquot of the resultant supernatant was used as the total protein sample. The remainder of the resultant supernatant was then ultracentrifuged (105,000 g at 4°C for 30 min), and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 3 ml of the homogenization buffer and recentrifuged (105,000 g at 4°C for 30 min). The resultant pellet was resuspended in 2 ml of ice-cold homogenization buffer containing 1% Triton X-100 and 1% sodium cholate and used as the membrane fraction. These preparations were stored at −80°C until use.

To determine the levels of RhoA in each fraction, the samples (10 µg of total protein per lane) were subjected to 15% SDS-PAGE and the proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After being blocked with 3% gelatin, the PVDF membrane was incubated with polyclonal rabbit anti-RhoA antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2,500 dilution; Amersham Biosciences, Piscatway, NJ), detected using an enhanced chemiluminescent system (Amersham Biosciences), and analyzed with a densitometry system. Detection of housekeeping gene was also performed on the same membrane by using monoclonal mouse anti-β-actin antibody (1:3,000 dilution; Santa Cruz Biotechnology) to confirm the same amount of proteins loaded.

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Histological analyses. Airways below the main bronchi were fixed in 10% formaldehyde and embedded in Paraplast X-TRA paraffin (Fisher HealthCare, Houston, TX). Sections (4 μm) were obtained from blocks and mounted on silane-coated glass slides, deparaffinized with xylene and graded ethanol, and processed for hematoxylin and eosin staining.

Evaluation of in vivo airway responsiveness. In vivo airway responsiveness was measured 24 h after the last antigen challenge. Rats were anesthetized with chloral hydrate (400 mg/kg ip), and the trachea was dissected free of surrounding tissue and cannulated with an 18-gauge cannula. The rat was then connected to a small animal ventilator (flexiVent; SCIREQ, Montreal, PQ, Canada) and ventilated in a constant-flow inflation mode with a tidal volume of 10 ml/kg, an inspiratory-expiratory ratio of 2:3, a respiratory rate of 90 breaths/min, and maximum pressure of 30 cmH2O. Positive end-expiratory pressure was controlled by submerging the expiratory limb into a water trap. Each animal was paralyzed with pancuronium bromide (0.8 mg/kg ip) and allowed to equilibrate on the ventilator until spontaneous breathing ceased. By following a standard volume history, small-amplitude volume oscillations at random frequencies were applied at constant lung volume to the airway opening for 16 s, and Newtonian resistance (Rn), an index of the resistance of the central airways, was calculated. Airway responsiveness to ACh was determined by inhaling aerosolized ACh solution (0.001–0.1% in saline) using an ultrasonic nebulizer (Aeroneb; SCIREQ).

Statistical analyses. All the data are means ± SE. Statistical significance of differences was determined using unpaired Student’s t-test or two-way analysis of variance (ANOVA) with post hoc Bonferroni/Dunn (StatView for Macintosh version 5.0; SAS Institute, Cary, NC). A value of P < 0.05 was considered significant.

RESULTS

Effect of systemic treatments with lovastatin on total cholesterol level in serum. To determine the effectiveness of lovastatin treated under the condition described in METHODS, we measured total cholesterol levels in sera of nonsensitized normal rats that were treated with lovastatin (4 mg·kg−1·day−1) once a day for 7 days. Total cholesterol levels in sera of the lovastatin-treated rats (64.2 ± 4.5 mg/dl, n = 6) were significantly lower than those of the vehicle-treated animals (89.0 ± 7.7 mg/dl, n = 6; P < 0.01), indicating that lovastatin can reduce cholesterol biosynthesis by the treatment protocol used.

Bronchial smooth muscle hyperresponsiveness in repeatedly antigen-challenged rats. Figure 1, A and B, shows the ACh responsiveness of bronchial smooth muscles isolated from nonsensitized normal control and repeatedly antigen-challenged rats. The nonsensitized normal animals were used as controls, because our previous studies demonstrated that the sensitization procedure alone has no effect on the bronchial smooth muscle contractility and the expression and activation of RhoA (4, 5, 7, 9, 11). Application of ACh (10−7–10−3 M) to isolated bronchial smooth muscles elicited a concentration-dependent contraction in all animals used. The contractile response to ACh of bronchial smooth muscle from the repeatedly antigen-challenged rats was markedly augmented, i.e., bronchial smooth muscle hyperresponsiveness, compared with that from the control animals; the ACh concentration-response curve was significantly shifted upward by the antigen exposure (vehicle control group in Fig. 1A vs. vehicle-challenged group in Fig. 1B; P < 0.05). On the other hand, no significant change in the contractile response induced by high-K+ depolarization was observed between the groups (Fig. 1, C and D). These findings are consistent with our previous reports (5, 11, 39) that demonstrate an augmented agonist-induced Ca2+ sensitization of contraction in the repeatedly antigen-challenged rats.

Effects of lovastatin on antigen-induced bronchial smooth muscle hyperresponsiveness. To determine the effect of systemic treatment with lovastatin on the augmented bronchial smooth muscle responsiveness observed in the repeatedly antigen-challenged rats, animals also received intraperitoneal injection with lovastatin (4 mg·kg−1·day−1) or its vehicle (50% DMSO in PBS) before and during the period of repeated antigen exposure. The lovastatin treatments had no effect on the contractility of bronchial smooth muscle to ACh (Fig. 1A) or high-K+ depolarization (Fig. 1C) in the nonsensitized nor-
mal control animals. However, the augmented ACh-induced contraction, i.e., bronchial smooth muscle hyperresponsiveness, observed in the repeatedly antigen-challenged rats was significantly inhibited by the systemic treatments with lovastatin (Fig. 1B; P < 0.05). The bronchial smooth muscle responsiveness to ACh of the antigen-challenged animals completely returned to the control level: no significant difference was observed between the lovastatin-challenged (Fig. 1B) and the vehicle control (Fig. 1A) groups.

Effects of lovastatin on the expression of RhoA protein in bronchial smooth muscle. The levels of total RhoA protein expression were assessed in the total protein samples of the main and intrapulmonary bronchial smooth muscles without epithelia and lung parenchyma (6, 7, 9, 11, 39). As reported previously, the expression of RhoA protein in bronchial smooth muscle of the repeatedly antigen-challenged rats was markedly and significantly increased compared with that of the nonsensitized normal control group (Fig. 2, vehicle control vs. vehicle challenged; P < 0.05). The systemic treatments with lovastatin had no effect on the RhoA protein expression in the control animals (Fig. 2, vehicle control vs. lovastatin control). Moreover, lovastatin also had no effect on the upregulation of RhoA induced by repeated antigen exposure (Fig. 2, vehicle challenged vs. lovastatin challenged).

Effects of lovastatin on the acetylcholine-induced translocation of RhoA protein in bronchial smooth muscle. ACh (10^-3 M) stimulation elicited an approximately twofold increase in membrane RhoA protein, i.e., ACh-induced translocation of RhoA from cytosol to membrane, in bronchial smooth muscle of nonsensitized normal rats (7). In the present study, the effect of systemic treatment with lovastatin on the ACh-induced translocation of RhoA in bronchial smooth muscle was determined. As shown in Fig. 3, membrane-associated RhoA in the ACh-stimulated bronchial smooth muscle of the repeatedly antigen-challenged rats (vehicle-challenged group) was significantly increased compared with that of nonsensitized normal control animals (vehicle control group; P < 0.01). In the ACh-stimulated muscles of the control animals, lovastatin had no effect on the level of membrane-associated RhoA (vehicle control vs. lovastatin control groups). On the other hand, in the ACh-stimulated muscles of the antigen-challenged rats, the increased level of membrane-associated RhoA was significantly inhibited by the systemic treatments with lovastatin (vehicle challenged vs. lovastatin challenged groups; P < 0.01): the relative membrane-associated RhoA to total RhoA returned to the control level (Fig. 3B), whereas the absolute amount of membrane-associated RhoA was still higher (Fig. 3A).

Effect of lovastatin on antigen-induced increase in total IgE level in serum. Figure 4A shows the levels of total IgE in sera of rats. In the nonsensitized normal control rats that were also treated with vehicle for lovastatin (vehicle control group, Fig. 4A), a detectable total IgE level that is not significantly different from that in naive animals (data not shown) was found. Total IgE level was markedly and significantly increased when animals were sensitized and repeatedly challenged with DNP-Asc antigen (vehicle-challenged group: P < 0.01 vs. vehicle control group; Fig. 4A). Neither the basal level nor the antigen-induced increment of total IgE was affected by the systemic treatment with lovastatin: no significant difference was observed between the vehicle control and the lovastatin control groups or between the vehicle-challenged and the lovastatin-challenged groups (Fig. 4A).

Effects of lovastatin on antigen-induced increase in IL-4, IL-6, and IL-13 levels in BAL fluid. As shown in Fig. 4, the levels of IL-4 (B), IL-6 (C), and IL-13 (D) in BAL fluids of repeatedly antigen-challenged rats (vehicle-challenged groups) were significantly increased compared with those of nonsensitized normal control animals (vehicle control groups). In the nonsensitized control animals, lovastatin treatments had no effect on the levels of these parameters (vehicle control vs. lovastatin control, respectively). The levels of IL-4 and IL-6 tended to be decreased by the systemic treatments with lovastatin in the antigen-challenged rats but were still higher than in the control groups (Fig. 4, B and C). No significant difference was observed between the vehicle-challenged and lovastatin-challenged groups in any parameter measured.
Effects of systemic treatments with lovastatin on antigen-induced airway inflammation. To determine the effects of lovastatin on the changes in airway biology induced by repeated antigen exposure, we recorded total cell counts in BAL fluids and performed histological examinations. Total cell counts in BAL fluids of the repeatedly antigen-challenged rats were significantly increased compared with those of the control group (Fig. 5, vehicle control vs. vehicle challenged group; \( P < 0.001 \)). Diff-Quik staining of the cells revealed that most of the increased cells were eosinophils (data not shown). The increase in BAL cells induced by antigen challenge was significantly reduced by the systemic treatments with lovastatin (Fig. 5A). Histological examinations also revealed a marked inflammation of the lungs in the antigen-challenged rats: many inflammatory cells were infiltrated into the subepithelial and smooth muscle layers of intrapulmonary bronchi by the repeated antigen exposure (Fig. 5D, vehicle-challenged group). Lovastatin treatments reduced the cell infiltration, especially between the epithelial and smooth muscle layers, induced by antigen challenge (Fig. 5E, lovastatin-challenged group).

Effects of systemic treatments with lovastatin on antigen-induced airway hyperresponsiveness in vivo. Finally, airway responsiveness to inhaled ACh was measured in anesthetized animals. The baselines of \( R_n \), a parameter of the resistance of the central airways, among vehicle-treated control, lovastatin-treated control, vehicle-treated antigen-challenged, and lovastatin-treated antigen-challenged groups were not statistically different (0.077 ± 0.010, 0.065 ± 0.012, 0.079 ± 0.016, and 0.062 ± 0.011 cmH\(_2\)O·s·ml\(^{-1}\), respectively). In all of the animals used, inhalation of aerosolized ACh caused an increase in \( R_n \) in a nearly concentration-dependent manner. The in vivo airway responsiveness to ACh of the repeatedly antigen-challenged rats was augmented, i.e., airway hyperresponsiveness,
compared with that of the control animals: the ACh concentration-response curve was significantly shifted upward by the antigen exposure (vehicle control group in Fig. 6A vs. vehicle challenged group in Fig. 6B; \( P < 0.05 \)). The augmented ACh responsiveness induced by antigen challenge was significantly inhibited by pretreatment with lovastatin (Fig. 6B; \( P < 0.05 \) by 2-way ANOVA).

**DISCUSSION**

Statins have pleiotropic effects beyond lowering cholesterol levels, and its potential benefits on inflammatory airway diseases such as asthma have been suggested. In experimental allergic asthma, simvastatin caused a reduction of inflammatory cell infiltration into airways and a decrease in the levels of proinflammatory cytokines in BAL fluids (34). Similar anti-inflammatory effects of pravastatin have also been reported (59). We have shown presently for the first time that lovastatin can improve the augmented bronchial smooth muscle contractility, an important factor of AHR in allergic bronchial asthma (33, 46).

It has been suggested that one of the factors that contribute to the AHR in asthmatics is an abnormality of the nature of airway smooth muscle (33, 46). The airway smooth muscle is the main structure of airway walls and plays a major role in the contraction of the airways. The excessive contraction of airway smooth muscles may be one of the crucial factors that directly cause the asthma symptoms such as the AHR. In the present study, an increased responsiveness of the isolated bronchial smooth muscle to ACh was observed in rats that were sensitized and repeatedly challenged with antigen (Fig. 1, vehicle-challenged group). This observation is consistent with our previous reports (4, 5, 7, 11, 35, 39), indicating that reproducible bronchial smooth muscle hyperresponsiveness has occurred with the antigen sensitization and challenge procedure used. In the rats with allergic bronchial asthma, an augmented agonist-induced, RhoA-mediated Ca\(^{2+}\) sensitization of bronchial smooth muscle contraction accompanied with an upregulation of RhoA protein has been demonstrated (11). Moreover, an in vivo AHR to inhaled contractile agonists such as ACh (Refs. 4 and 35 and the present study) and neurokinin A (35) was also demonstrated. Similar observations, including an
in vivo AHR (28), bronchial smooth muscle hyperresponsiveness (12), and augmented RhoA-mediated Ca\(^{2+}\) sensitization of contraction (12), have also been reported in a murine model of bronchial asthma induced by ovalbumin antigen. An importance of RhoA and its downstream Rho-kinases in contraction of human bronchial smooth muscle was also demonstrated (60), and the RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (19).

It is known that geranylgeranylation is required for the activation and translocation of monomeric GTP-binding proteins including RhoA (50, 51). In the resting state, GDP-bound inactive form of RhoA exists in the cell cytosol with GDP dissociation inhibitor, called RhoGDI, which buries the geranylgeranylated, hydrophobic tail of RhoA. Activation of RhoA is initiated by guanine nucleotide exchange factors (RhoGEFs) through activation of plasma membrane receptors coupled to certain heterotrimeric GTP-binding proteins. The active RhoGEFs exchange GDP for GTP on RhoA and the RhoGDI dissociates from RhoA, resulting in the association of GTP-bound RhoA with the plasma membrane (50). Indeed, receptors stimulation by contractile agonists such as ACh (7) and endothelin-1 (8) caused translocation of RhoA correlated with induction of contraction in rat bronchial smooth muscle. Therefore, HMG-CoA reductase inhibitors that block synthesis of isoprenoids and prevent translocation of RhoA to the membrane could modulate intracellular signaling including smooth muscles. In the present study, systemic treatments with lovastatin significantly inhibited the bronchial smooth muscle hyperresponsiveness induced by antigen exposure (Fig. 1B). The augmented ACh-induced translocation of RhoA to the plasma membrane observed in the repeatedly antigen-challenged rats was also significantly reduced by the lovastatin treatments (Fig. 3), whereas the increased expression of RhoA protein was not changed (Fig. 2). On the other hand, the ACh-induced contraction (Fig. 1A) and translocation of RhoA (Fig. 3) were not affected by the lovastatin treatments in bronchial smooth muscles of the control animals. It is thus possible that lovastatin treatments might inhibit geranylgeranylation of RhoA proteins newly synthesized by the upregulated mRNAs (10) in the repeatedly antigen-challenged rats, resulting in an inhibition of the augmented bronchial smooth muscle contraction. Alternatively, the lack of effect of lovastatin in the nonsensitized control animals may be explained by its indirect effects, i.e., inhibition of the release of inflammatory mediators (such as eicosanoids, histamine, ATP, and serotonin) induced by antigen challenge: statins could inhibit the release of these mediators (30, 38, 44, 47, 52).

The present study also suggested that lovastatin treatments could reduce the inflammatory cell infiltration: both the increase in BAL cells and the histological changes in lungs induced by antigen exposure were attenuated (Fig. 5). It is unlikely that the inhibitory effects ofLovastatin on inflammatory cell infiltration are due to the inhibition of proinflammatory cytokines release or IgE production, because Lovastatin treatments did not significantly alter the increased expression of IL-4, IL-6, and IL-13 in BAL fluids and total IgE in sera induced by antigen (Fig. 4). Although the mechanism(s) of action of Lovastatin is not known here, its direct suppressive effect on inflammatory cells such as eosinophils and macrophages might be involved. Migration of leukocytes is believed to be involved in a coordinated remodeling of the actin cytoskeleton. The monomeric GTP-binding proteins RhoA, Rac1, and Cdc42 are critical regulators of actin reorganization in fibroblasts and other nonhemopoietic cells (20, 55). These Rho family GTPases are modulated by their geranylgeranylation. An involvement of Rho family GTPases in the transmigration across endothelial cell layers has also been demonstrated in leukocytes (22, 56, 58). It is thus likely that Lovastatin inhibits geranylgeranylation of Rho family GTPases in leukocytes, resulting in an inhibition of infiltration of these cells. Alternatively, Lovastatin might also act on endothelial and/or epithelial cells directly. It has been suggested that endothelial cells actively participate in the migration of leukocytes by the activation of signaling pathways (14) including Rho family GTPases (3).

In contrast to the current results of rats, McKay et al. (34) reported that in vivo treatments with simvastatin inhibited inflammatory cell infiltration in association with a reduction of cytokines in BAL fluids in a murine model of allergic asthma. They also suggested that the reduction of BAL cytokine levels is due to a reduction of antigen-induced cytokines production in lymphocytes (34). In addition, an in vitro treatment with fluvastatin inhibited the production of cytokines and chemokines in peripheral blood mononuclear cells obtained from patients with allergic asthma (40). It has recently become apparent that the different statins may have different biochemical functions (23). For example, lipophilic statins such as atorvastatin and simvastatin have a much greater effect on inflammatory responses than the hydrophilic pravastatin in human isolated monocytes and mouse inflamed peritoneal cavity (29). Some statins have differing effects on protein expression: in monocytes stimulated by lipopolysaccharide, pravastatin and fluvastatin may induce the production of tumor necrosis factor-α (TNF-α), whereas atorvastatin and simvastatin inhibit the production of TNF-α (23). A clinical study also found significant differences between the ex vivo responses after atorvastatin and simvastatin treatments in T lymphocytes from healthy volunteers (17). It is therefore important to recognize that all statins might not have the same therapeutic potential. Some species difference may also be involved in the action of statins.

Because the prevalence of asthma is recently rising (54), there is an increased need for the development of new drugs for its treatment, especially for patients who respond poorly to conventional therapy such as glucocorticoids. In the present study, it has been shown that Lovastatin has an effective action on bronchial smooth muscle hyperresponsiveness in rats with allergic bronchial asthma. The increased contractility of bronchial smooth muscle is considered an important factor of AHR in allergic bronchial asthma (33, 46). Statins also have an anti-inflammatory action in rat (present study) and murine (34) models of allergic airway inflammation, although the mechanism of action seems to be different. Thus statins and similar agents may have potential as therapeutic agents in allergic asthmatics. Clinical studies are necessary to assess whether statins have therapeutic potential in allergic asthma.

In conclusion, the present study demonstrates that Lovastatin ameliorates the antigen-induced bronchial smooth muscle hyperresponsiveness in association with a reduction of RhoA-mediated signaling. Lovastatin also reduces the infiltration of inflammatory cells into airways induced by antigen exposure.
These inhibitory effects of lovastatin may be beneficial for the treatment of allergic bronchial asthma.

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