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Effect of oral administration with pravastatin and atorvastatin on airway hyperresponsiveness and allergic reactions in asthmatic mice

Ching-Feng Huang, MD, PhD^{*}; Ho-Jen Peng, MD, PhD[†]; Chia-Chao Wu, MD, PhD[‡]; Wen-Tsung Lo, MD, PhD^{*}; Yu-Lueng Shih, MD, PhD[§]; and Tzee-Chung Wu, MD^{||}

^{*} Department of Pediatrics, Tri-Service General Hospital and School of Medicine, National Defense Medical Center, Taipei, Taiwan

[†] Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

[‡] Division of Nephrology, Department of Medicine, Tri-Service General Hospital, Taipei, Taiwan

[§] Division of Gastroenterology, Department of Medicine, Tri-Service General Hospital, Taipei, Taiwan

^{||} Department of Pediatrics, Taipei Veterans General Hospital and School of Medicine, National Yang-Ming University, Taipei, Taiwan

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ABSTRACT

Background: Asthma is characterized by airway hyperresponsiveness and remodeling. Pravastatin and atorvastatin are used clinically as cholesterol-lowering agents but also exhibit anti-inflammatory and immunomodulating properties.

Objective: To investigate the therapeutic effect of oral statins on airway hyperresponsiveness and allergic reaction.

Methods: BALB/c mice received intraperitoneal sensitization and aerosol inhalation with ovalbumin consequently. One week after ovalbumin aerosol challenge, pravastatin, atorvastatin, or phosphate-buffered saline were given by intragastric gavage daily for 2 weeks. Airway hyperresponsiveness, serum allergen specific antibody levels, cytokine production by splenocytes, and bronchoalveolar lavage fluid were examined.

Results: Both pravastatin and atorvastatin effectively reduced airway hyperresponsiveness. Pravastatin effectively suppressed both T_H1- and T_H2-mediated antibody responses, reducing serum specific IgE, IgG, IgG1, and IgG2a levels. Pravastatin also effectively reduced interleukin (IL) 4, IL-5, and interferon γ production but significantly enhanced IL-10 levels in splenocytes and BALF. Similarly, atorvastatin effectively attenuated production of specific IgE, IgG1, and IgG2a antibodies. It also significantly attenuated IL-4, interferon γ , and increased IL-10 concentration in bronchoalveolar lavage fluid and splenocytes.

Conclusion: Oral administration of pravastatin or atorvastatin not only was able to inhibit T_H1 inflammatory responses but also had therapeutic effects on airway hyperresponsiveness and T_H2 allergic responses. These results seem to suggest that these drugs have potential as a nonimmunosuppressive therapy for asthma and allergic diseases.

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Introduction

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase and reduce cholesterol biosynthesis. They have been widely prescribed as cholesterol-lowering agents and have been shown to reduce morbidity and mortality from coronary artery disease.^{1,2} Statins have been recently recognized as anti-inflammatory

Reprints: Ching-Feng Huang, MD, PhD, Department of Pediatrics, Tri-Service General Hospital and School of Medicine, National Defense Medical Center, No. 325, Sec.2, Cheng-Gong Road, Neihs District, Taipei 114, Taiwan; E-mail: h-cf@yahoo.com.tw.

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agents and shown to possess immunomodulating effects.^{3,4} However, airway inflammation does not always induce coordinate airway hyperresponsiveness.⁵ Statins also have a potential therapeutic role in respiratory disease. They effectively reduced endotracheal intubation rates in obstructive airway disease.⁶ They have also been considered as potential candidates for asthma.⁷ Bronchial smooth muscle hyperresponsiveness induced by ovalbumin stimulation has been found to be significantly attenuated by an intraperitoneal injection or inhalation of statins.^{8,9} In a nationwide, population-based study, statin use was found to be associated with reduced hospitalization for asthma attacks in patients with asthma.¹⁰

In contrast to these findings, Menzies et al¹¹ found that simvastatin does not improve inflammatory outcome, lung capacity, or airway resistance, and their results do not support a therapeutic

role for statins when treating asthma patients. Ostroukhova et al.¹² retrospectively reviewed medical records and demonstrated that patients with asthma who received statins had a worse clinical course, including a worse forced expiratory volume, than controls. Simvastatin has also been shown to be without steroid-sparing effects in patients with eosinophilic asthma.¹³ Therefore, the effects of statins on airway hyperresponsiveness are still under debate and need further evaluation.

Interestingly, the mice previously used to evaluate the relationship between statins and asthma have almost received statins by intraperitoneal injection and not by oral administration.^{8,14,15} Statins can be easily taken orally and are never injected in humans. Many factors can influence drug absorption, including food and drug interactions. It is more reasonable therefore to evaluate the effects of statins on asthma using intestinal absorption even in an animal model. Oral simvastatin has been used to examine its anti-inflammatory role in a murine model of allergic asthma.¹⁶ However, the mice received simvastatin before allergen inhalation. In addition, they received allergen challenge from nostril instillation. It may be difficult to say that asthma is successfully induced from nasal challenge. Their effects were also not confirmed by any pulmonary function test.

In addition, mice in previous studies usually received the statin injection before allergen inhalation,^{9,14,16} which is also out of keeping with the clinical situation. Most asthma patients have become unwittingly sensitized and inhaled to an aeroallergen in childhood, and as a result they would take statins after airway hyperresponsiveness. Thus, previous animal studies may therefore, at least partially, represent asthma prevention rather than treatment. Atorvastatin had been examined for its therapeutic effect in asthma mice. However, it was given by intraperitoneal injection, and its effect was not tested by pulmonary function test.¹⁵ The effect of oral statin treatment on airway hyperresponsiveness has never been elucidated. In the present study, we used intragastric gavage with pravastatin or atorvastatin after systemic and airway sensitization. We also evaluated the therapeutic effect of statins on airway hyperresponsiveness and allergic reactions in ovalbumin-sensitized mice. We have demonstrated for the first time to our knowledge that either pravastatin or atorvastatin, when given orally, offers a feasible method of effectively suppressing not only airway hyperresponsiveness but also systemic T_H1 inflammation, T_H2 allergic responses, and ongoing IgE antibody production.

Methods

Animals

Female (6 to 8 weeks old) BALB/c mice were purchased from the National Animal Center of Taiwan. They were bred on a standard diet (LabDiet; PMI Feeds, St. Louis, Missouri) in the Animal House of National Defense Medical Center. Sprague-Dawley rats (300–450 g) were obtained from the Animal Center, National Yang-Ming University (Taipei, Taiwan). All the experiments were repeated at least 4 times. All protocols of animal experiments were approved by the Institutional Animal Care and Use Committee of National Defense Medical Center.

Experimental Protocol

Mice were sensitized intraperitoneally with 10 μ g of ovalbumin (Sigma, St. Louis, Missouri) adsorbed onto 4 mg of alum and suspended in 0.5 mL of phosphate-buffered saline (PBS) twice with a 2-week interval. Two weeks after the last sensitization, the mice were allowed to inhale 3% ovalbumin in a PBS (10 mL of a 30-mg/mL ovalbumin solution) aerosol for 10 minutes. Ovalbumin aerosol exposure was repeated 3 times within 1 week. One

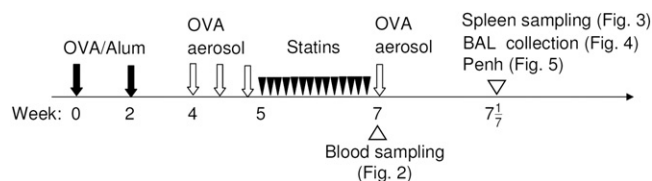


Figure 1. Experimental protocol. BALB/c mice were sensitized intraperitoneally with ovalbumin (OVA) adsorbed onto alum twice within a 2-week interval. Two weeks after the last sensitization, the mice were exposed to 3 courses of OVA aerosol within 1 week. One week after the first OVA inhalation, the mice were treated with intragastric pravastatin, atorvastatin, or phosphate-buffered saline once daily for 2 weeks. After blood sampling, all the mice were inhaled with OVA aerosol again 1 day after the final statin gavage. Spleen sampling, bronchoalveolar lavage (BAL), and airway responsiveness detection were performed 1 day after the final OVA inhalation.

week after the first ovalbumin inhalation, the mice were treated with pravastatin (Sigma) or atorvastatin (Lipitor; Pfizer, New York) dissolved in 0.5 mL of PBS (4 mg/kg/d) once daily for 2 weeks using intragastric gavage. Control mice received PBS only. After blood sampling, mice were exposed to ovalbumin aerosol again 1 day after the final statin gavage. Spleen sampling, bronchoalveolar lavage, and airway hyperresponsiveness detection were performed 1 day after the final ovalbumin inhalation. These protocols are summarized in Figure 1.

Assessment of antibody responses

Antibody levels were examined by enzyme-linked immunosorbent assays (ELISAs) as previously described.¹⁷ In brief, wells of plates (96 well; Nunc, Kamstrup, Roskilde, Denmark) were coated with 100 μ L of ovalbumin (100 μ g/mL) in 0.05M carbonate buffer, pH 9.6, overnight at 4°C. Free from ovalbumin-binding sites were blocked with 3% skimmed milk in PBS-Tween 20 for 1 hour. Serum samples (1/30–1/1,000) and standards (pooled hyperimmune sera after monthly treatment with ovalbumin emulsified in the complete Freund adjuvant) were added in duplicate after incubation for 5 hours at room temperature. After washing, 100 μ L of horseradish peroxidase-conjugated goat anti-mouse IgG (1/4,000; Jackson, West Grove, Pennsylvania), IgG2a, or IgG1 (1/4,000 for both; SBA, Birmingham, Alabama) were added, and the plates were incubated overnight at 4°C. After washing, color was developed by adding orthophenyldiamine (0.5 mg/mL; Sigma) in citrate-carbonate buffer containing 0.015% hydrogen peroxide, incubated in the dark at room temperature. The color reaction was stopped by 4N sulfuric acid and read at 492 nm using a reader (SPECTRAMax 250, Molecular Devices, Sunnyvale, California), and unknowns were interpolated.

Bronchoalveolar lavage fluid

Mice were killed by intraperitoneal injection of 20 mg of pentobarbitone 1 day after final aerosol challenge. The trachea were cannulated and then lavaged twice with 1 mL of PBS. The recovered fluid, bronchoalveolar lavage fluid (BALF), was centrifuged at 600g for 10 minutes at 4°C, and then the supernatant was stored at –70°C until examination.

Cytokine Assays

One day after the final aerosol challenge, spleen cells from 6 BALB/c mice in each group were sampled and cultured (4×10^6 cells per well) with ovalbumin in duplicate in complete RPMI-1640 medium (1 mg/mL), 10% fetal calf serum, and antibiotics in 24-well flat-bottomed microtiter plates (1 mL per well; Costar, Cambridge, Massachusetts). The supernatants were harvested after 1 to 3 days of culture. The levels of the cytokines interleukin (IL) 4, IL-5, IL-10, and interferon γ (IFN- γ) from the harvested supernatants and

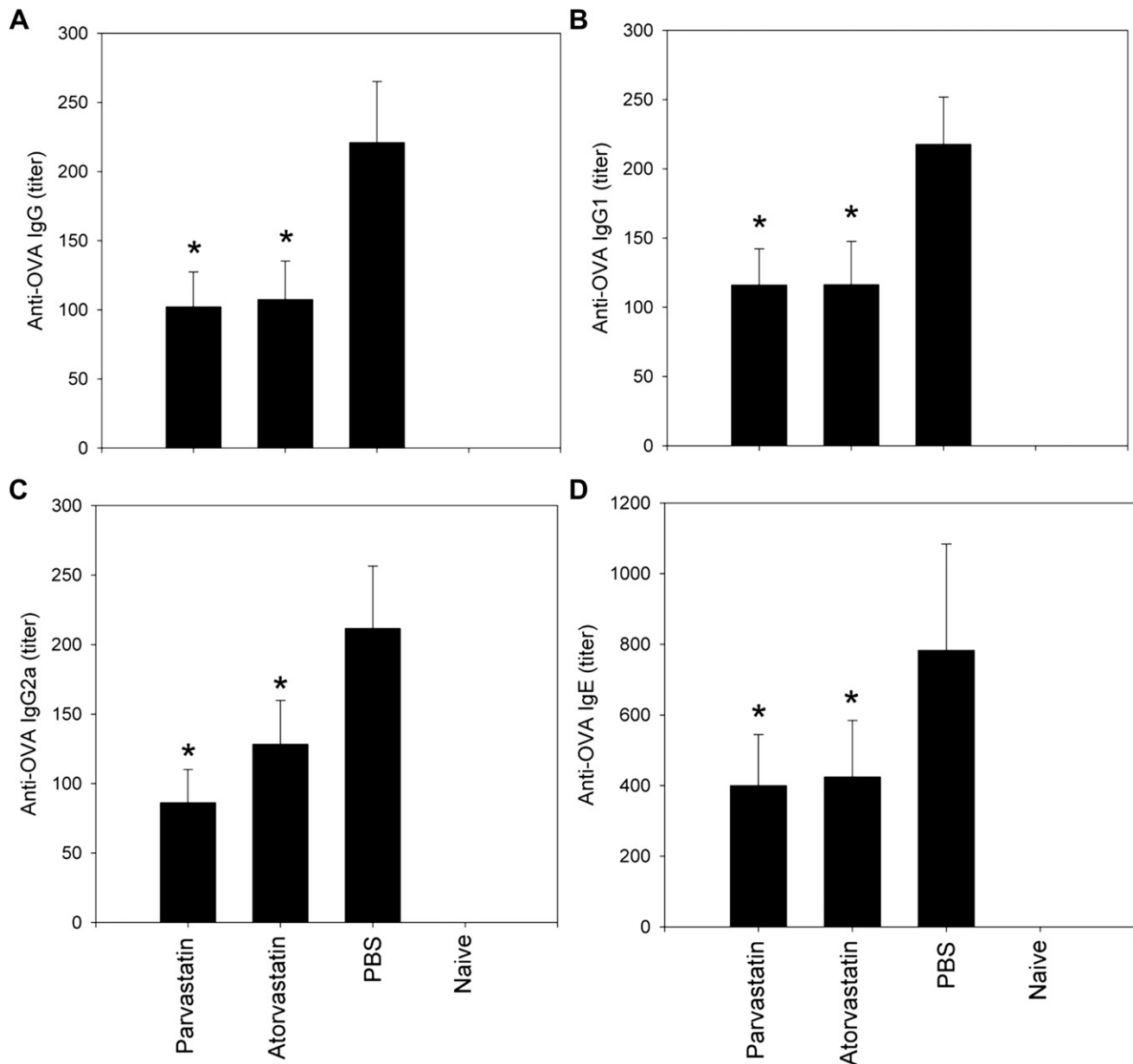


Figure 2. Effect of oral pravastatin and oral atorvastatin treatment on specific systemic antibody responses. Specific antibody responses after treatment with pravastatin or atorvastatin in ovalbumin (OVA)-sensitized mice. IgG (A), IgG1 (B), and IgG2a (C) were examined by enzyme-linked immunosorbent assay and IgE (D) by in vivo passive cutaneous anaphylaxis test. Results are expressed as mean (SD) (n=24). Statistical significant was determined with 1-way analysis of variance with a post hoc test. * $P < .05$ compared with the phosphate-buffered saline (PBS) group.

rewarmed BALF were measured using appropriate sandwich ELISA kits (e-bioscience, San Diego, California).

Passive cutaneous anaphylaxis

Serum specific IgE antibody responses were assessed in in vivo animal models as described previously.¹⁸ Briefly, passive cutaneous anaphylaxis tests were examined in Sprague-Dawley rats. Aliquots of 100 μ L of 2-fold dilutions of mouse serum samples (1/10–1/1,600) were intradermally injected into the rats. They were then challenged 24 hours later by an intravenous injection of 2 mg of ovalbumin and 5 mg of Evans Blue in 1 mL of PBS. The reaction was read 30 minutes later. The passive cutaneous anaphylaxis titer was taken as the highest dilution giving a positive reaction of at least 5 mm in diameter, and the results were expressed as mean (SD).

Airway Hyperresponsiveness

The airway hyperresponsiveness of mice was measured 24 hours after the final ovalbumin challenge using whole-body plethysmography (Buxco, Troy, New York). Mice were acclimatized in the main chamber for 10 minutes. After measuring baseline values for 5 minutes, the mice were subsequently challenged with aerosolized PBS or increasing concentration of methacholine (6.25, 12.5, and 25 mg/mL; Sigma) for 3 minutes. The response in terms of enhanced pause (Penh) was recorded for 6 minutes after each dose. Penh reflects airway resistance and is used to monitor pulmonary dysfunction.

Statistical analyses

Antibody, cytokine titers, and Penh were expressed as mean (SD), and all group comparisons were analyzed using 1-way analysis of variance with post hoc Scheffe or Duncan test.

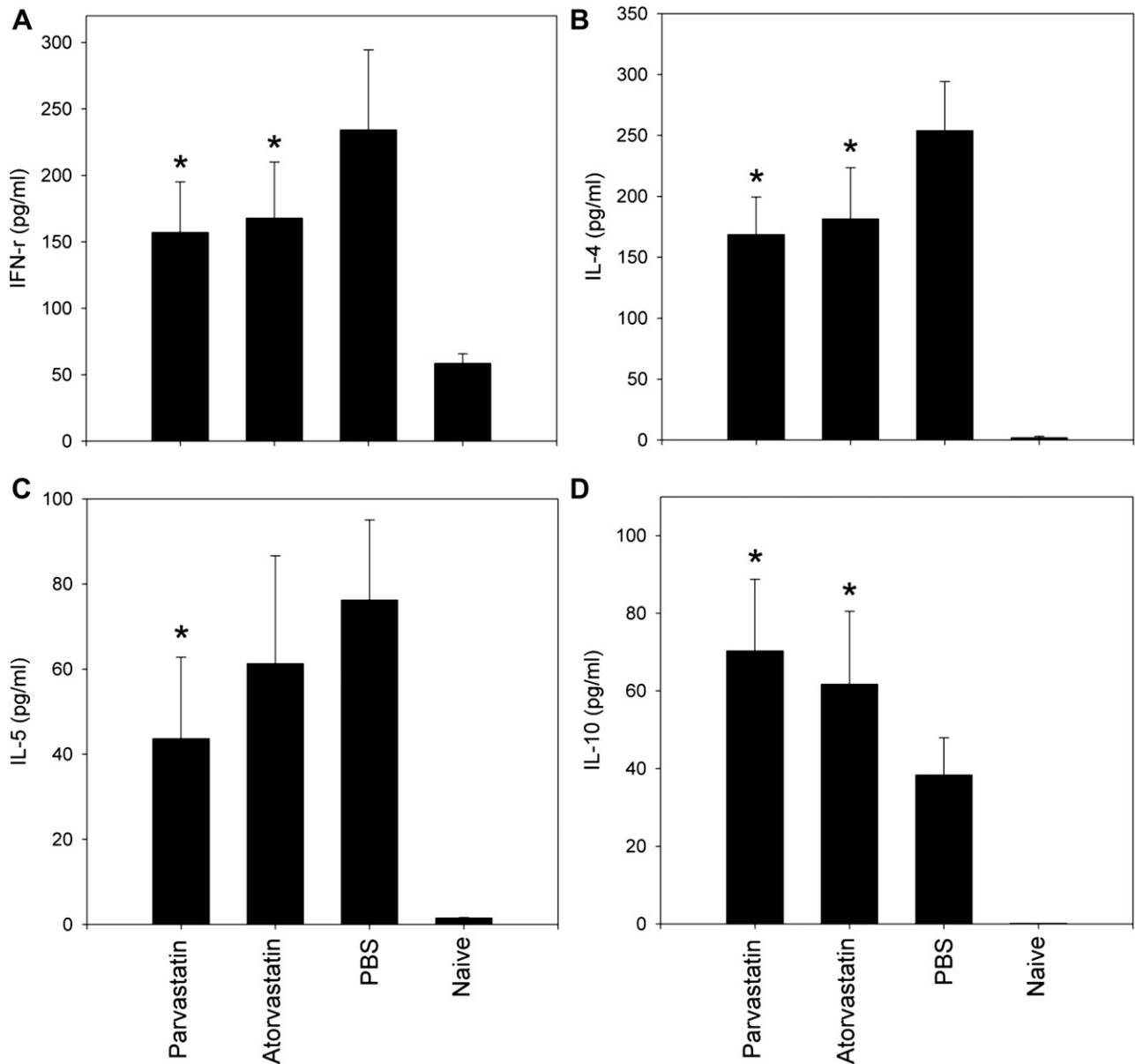


Figure 3. Effect of pravastatin and atorvastatin treatment on cytokine secretion of spleen cells. Cytokine levels of ovalbumin cocultured spleen cells from ovalbumin-sensitized mice after treatment with pravastatin or atorvastatin. Interferon γ (IFN- γ) (A), interleukin 4 (IL-4) (B), IL-5 (C), and IL-10 (D) were detected by sandwich enzyme-linked immunosorbent assay kits. Results are expressed as mean (SD) (n=24). * P <.05 compared with the phosphate-buffered saline (PBS) group.

Results

Effect of oral pravastatin and atorvastatin treatment on specific antibody responses in the ovalbumin-sensitized mice

The therapeutic effect of statin on the ovalbumin-specific antibody response was studied first. Ovalbumin-sensitized mice were treated with oral pravastatin, atorvastatin, or PBS. Their systemic antibody responses were examined and are shown in Figure 2. The mice that had received either pravastatin or atorvastatin had significantly reduced specific IgG, IgG1, and IgG2a antibody responses compared with the controls (P <.05; Fig 2A-C). Pravastatin produced a similar therapeutic effect to atorvastatin in terms of reducing the specific antibody responses. In addition, the mice receiving either pravastatin or atorvastatin also had a significantly lower allergen specific IgE antibody level than the control mice (P <.05; Fig 2D). The mice receiving pravastatin or atorvastatin without previous ovalbumin sensitization had no ovalbumin-specific antibody responses (data not shown).

Effect of pravastatin and atorvastatin treatment on the cytokine levels of splenocytes and BALF

To further investigate the effect of oral pravastatin and atorvastatin treatment in sensitized mice, cytokines were measured from cultured splenocytes and in BALF. Their results were shown in Figure 3 and Figure 4, respectively. Compared with the mice receiving PBS, those receiving pravastatin had effectively reduced cytokine production levels, including IFN- γ , IL-4, and IL-5 (P <.05; Fig 3A-C), but also had a significant increase in IL-10 secretion by the splenocytes (P <.05; Fig 3D). The mice treated with atorvastatin also had diminished IFN- γ and IL-4 levels (P <.05; Fig 3A and B) and a higher IL-10 concentration in the cultured splenocytes than the controls (P <.05; Fig 3D).

Compared with the control mice, those receiving pravastatin had an effective reduction in cytokine levels from BALF, including IFN- γ , IL-4, and IL-5 (P <.05; Fig 4A-C), but a significant increase in IL-10 secretion (P <.05; Fig 4D). The mice receiving atorvastatin

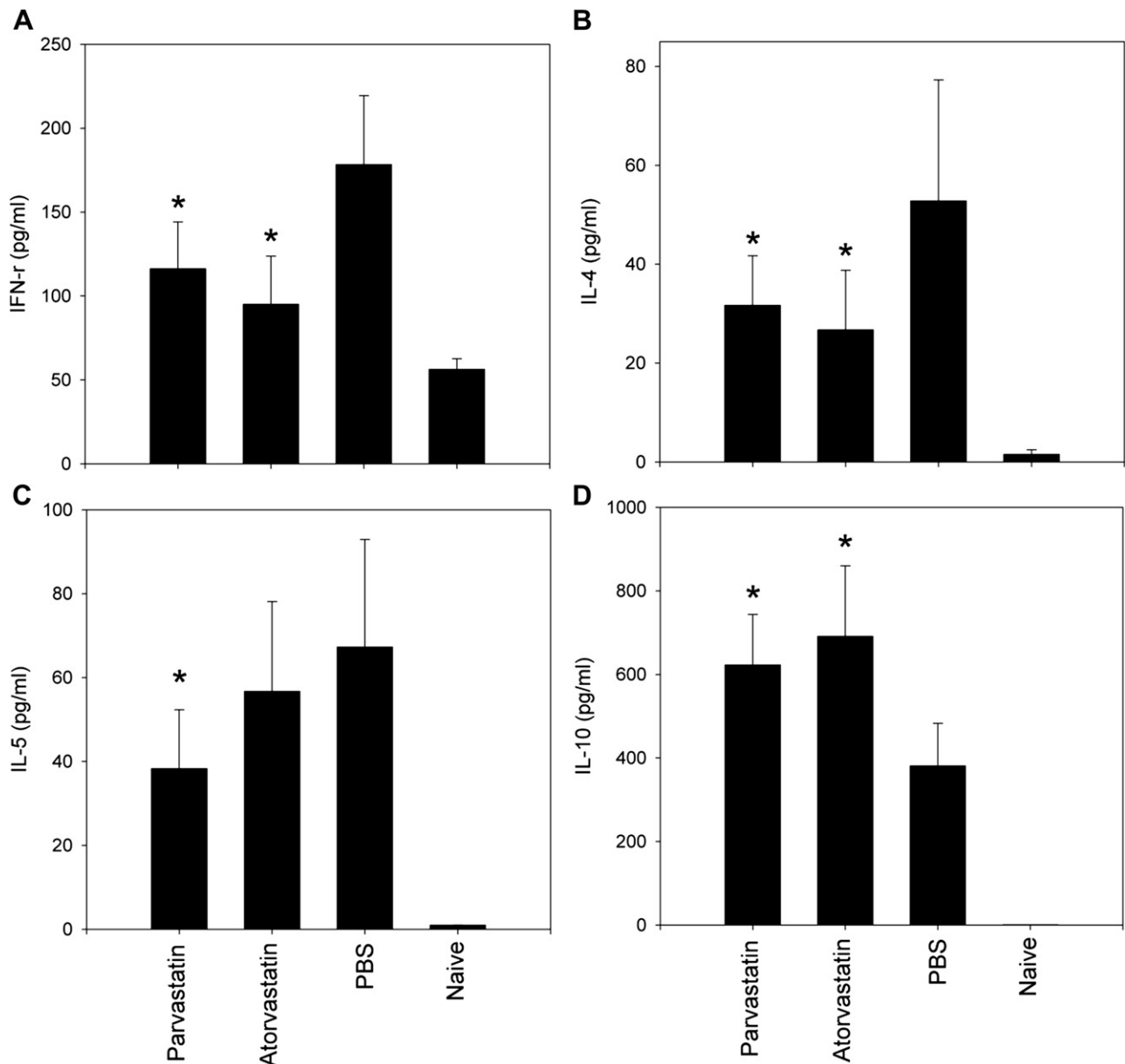


Figure 4. Effect of pravastatin and atorvastatin treatment on the cytokine levels of bronchoalveolar lavage fluid (BALF). Cytokine levels of BALF from ovalbumin-sensitized mice after treatment with pravastatin or atorvastatin. Interferon γ (IFN- γ) (A), interleukin 4 (IL-4) (B), IL-5 (C), and IL-10 (D). Results are expressed as mean (SD) (n=24). * $P < .05$ compared with the phosphate-buffered saline (PBS) group.

treatment also had significantly attenuated IFN- γ and IL-4 levels ($P < .05$; Fig 4A and B) but a significant enhancement of IL-10 secretion in the BALF ($P < .05$; Fig 4D). The cytokine levels of spleen cells and BALF from the mice without ovalbumin sensitization but receiving oral pravastatin or atorvastatin treatment were similar to those from naive mice (data not shown).

Effect of pravastatin and atorvastatin on the airway hyperresponsiveness to methacholine aerosol

To determine whether statin administration has a therapeutic effect on the development of airway hyperresponsiveness, airway reactivity to methacholine was assessed after aerosolized ovalbumin challenge. A significant reduction was found in the mice's Penh values in response to the methacholine aerosol among the mice receiving either pravastatin or atorvastatin treatment at concentrations of 12.5 and 25 mg/mL ($P < .005$) compared with the PBS-treated mice (Fig 5). Pravastatin had a better therapeutic effect

in reducing Penh values than atorvastatin, but no statistical difference was found between them.

Discussion

We have demonstrated for the first time to our knowledge the therapeutic potential of oral pravastatin and atorvastatin administration as treatments for bronchial asthma and allergic reaction. Our results indicate that the airway hypersensitivity of ovalbumin-sensitized mice was markedly improved after treatment with either of these 2 statins. When compared with the mice receiving PBS only, the present study also revealed that treatment with either oral pravastatin or atorvastatin has efficacy in terms of inhibiting a systemic ongoing T_H2 allergic reaction, including a reduction in the levels of specific IgE antibodies in serum, specific IgG1 antibodies in serum, and IL-4 in BALF and spleen. These effects may be related to regulatory cytokines because there was a higher IL-10 concentration present in statin-treated mice compared with

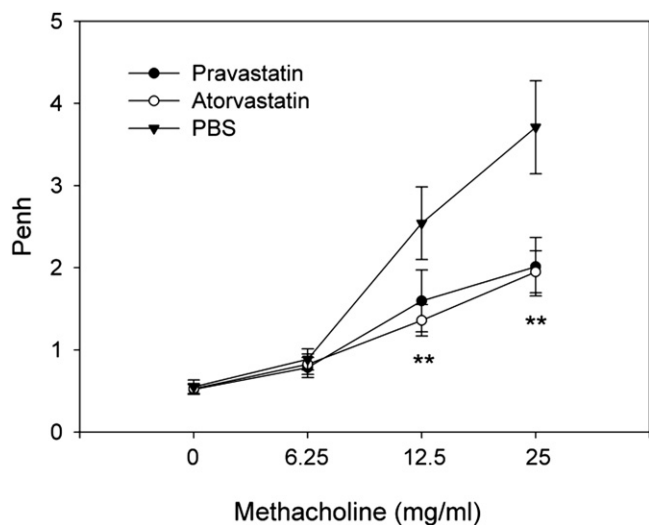


Figure 5. Effect of pravastatin and atorvastatin treatment on airway responsiveness to methacholine aerosol. Airway responsiveness in ovalbumin-sensitized mice after treatment with pravastatin or atorvastatin and challenge with ovalbumin aerosol. The responses were measured after aerosolized methacholine challenge using whole-body plethysmography. Results are expressed as mean (SD) ($n=24$). * $P<.05$ and ** $P<.005$, both statin-treated groups compared with the phosphate-buffered saline PBS group. Penh indicates enhanced pause.

those that received PBS only. This is the first study to our knowledge to prove that treatment with oral pravastatin or atorvastatin in sensitized mice seems to suppress both the T_H1 and T_H2 dominant immune reaction and reduce airway asthma.

The presence of anti-inflammatory effects is considered to be an important determinant that supports the therapeutic effect of statins on airway inflammation. Simvastatin attenuated inflammatory cell infiltration into airways and caused a decrease in the levels of proinflammatory cytokines in BALFs.¹⁶ Inflammatory intermediate substances, such as isoprenoids, have been found to be inhibited by statins.¹⁹

Fluvastatin and lovastatin have been found to effectively inhibit eosinophil migration and adhesion by inhibiting granulocyte-macrophage colony-stimulating factor stimulated human eosinophil adhesion to the intercellular adhesion molecule 1 in an in vitro model, and this is believed to demonstrate their potential anti-inflammatory effects.²⁰ Simvastatin has been found to reduce effectively various allergic responses via the regulation of small G proteins, mitogen-activated protein kinases, and nuclear factor- κB ²¹ and to attenuate bronchoalveolar lavage total cellularity, eosinophilia, and IL-4 and IL-5 levels in an asthma murine model.¹⁶ Injected pravastatin also regulates pulmonary dendritic cells and attenuates allergen presentation and sensitization with suppression of IL-17 production in a murine model.²²

On the basis of a rat allergic asthma model, intraperitoneal injection with lovastatin effectively inhibits bronchial hyper-responsiveness by reducing the presence of the monomeric guanosine triphosphate-binding protein RhoA during contractions of the bronchial smooth muscle.¹⁴ It also reduces the expression of geranylgeranyltransferase I in bronchial smooth muscle, which is upregulated in the allergen-induced mice.²³ Simvastatin has been proven to improve impaired nitric oxide metabolism by reducing asymmetric dimethyl-arginine and increasing endothelial nitric oxide synthase in allergic lungs.²⁴ In contrast, some other studies have claimed that statins promote T_H2 bias immune reaction. Furthermore, an in vitro study has found that statins enhance the T_H2 cell-mediated immune reaction but suppress the T_H1 cell-mediated immune reaction.^{25–27}

Recently, the relationship between statins and regulatory cytokine has been elucidated. In rats with induced anti-glomerular basement membrane glomerulonephritis, statins have been found to augment macrophages by the upregulation of IL-10.²⁸ Serum IL-10 levels have been shown to increase significantly in patients with acute coronary syndrome after treatment with fluvastatin.²⁹ In the present study, sensitized mice treated with pravastatin had a significantly higher IL-10 level in their cultured splenocytes and BALF compared with the controls. BALF IL-10 was also effectively enhanced in mice after treatment with atorvastatin. This regulatory cytokine may execute a suppressive effect on both inflammation and allergy via attenuation of the T_H1 and T_H2 cell-mediated immune reactions. However, further studies are necessary to check their interaction.

According to radiolabeled studies, the oral absorption of pravastatin is approximately 34%, whereas the oral bioavailability is only 18%.³⁰ The low oral absorption and bioavailability may be caused by first-pass metabolism. Nonetheless, pravastatin is used clinically in the oral form, and therefore it is more reasonable to evaluate its therapeutic effects after enteral administration even in animal studies. Intraperitoneal or intravenous injection with pravastatin may have unexpected serum concentration results that are different from when the administration is oral. Such differences may influence experimental interpretation and help to explain the diverse findings across human and animal studies.^{10–13}

Most human studies discussing statins and respiratory diseases have been analyzed using a retrospective review of medical records,^{6,12} and the conclusions have usually involved a statistical analysis. However, any prospective human studies may be limited by ethical issues of volunteers having the asthma trait only and no individuals with exacerbated asthma being included. Furthermore, enrolled patients usually need to have their asthma controlled by the use of a concomitant anti-inflammatory medicine, such as a steroid, and under these circumstances the multiple confounding factors present may interfere with the interpretation of such prospective human studies.

In patients with allergic asthma, symptom control medication, including steroids and β_2 -agonists, are usually feasible.³¹ However, long-term use of steroids is associated with severe adverse effects, and lethal infection may manifest in various organs.³² Overuse of inhaled β_2 -agonists has been associated with possible increased risk of death from asthma.³³ Many new classes of drugs have been tested for asthma control, including anti-IgE antibody, cytokine antagonists, transcription factor antagonists, immunostimulatory DNA therapy, cytokine therapy, and anti-T-cell strategies.³⁴ All the medications mentioned have not only attenuated the allergic reaction but also changed the host's immune reaction. These possible effects have limited their clinical application. However, statins are not clinically used as immunosuppressive drugs. On the contrary, patients with sepsis may benefit from their anti-inflammatory effects.³⁵ Furthermore, obese children have a higher incidence of asthma.³⁶ Statins are not only useful as cholesterol-lowering agents in these children but should also help to attenuate asthma attacks. There were no abnormal findings from the serum of statin-treated mice, including liver, renal function, and glucose and lipid concentrations (data not shown). However, the efficacy and safety of long-term statin administration on asthma patients need further evaluation.

Penh has been previously used to detect change of airway reactivity. Recently, the use of Penh to evaluate pulmonary function has been challenged. Zhang et al³⁷ suggest that lower airway resistance must be confirmed by a direct invasive measurement. However, they still agree that Penh detection can be used as a screening procedure in conscious mice.³⁷ Frazer et al³⁸ also conclude that Penh measurements can be adopted to detect changes in specific airway resistance.

The present study explores the therapeutic effects of pravastatin and atorvastatin and provides evidence that these drugs improve the physiologic condition and allergic immune reactions of sensitized mice with the asthma trait. Our results also support the idea that either pravastatin or atorvastatin is able to reduce the airway hyperresponsiveness and T_H2 -dominant immune reaction after oral treatment. The potential application of oral statin administration should be considered for application to the treatment of allergy and asthma in the future.

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