

Simvastatin Improves Epithelial Dysfunction and Airway Hyperresponsiveness

From Asymmetric Dimethyl-Arginine to Asthma

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Altered arginine metabolism, the uncoupling of nitric oxide synthase (NOS) by asymmetric dimethyl-arginine (ADMA), increased oxo-nitrosative stress, and cellular injury were reported in airway epithelial cells in asthma. Statins improve vascular endothelial dysfunction by reducing ADMA and increasing endothelial NOS (eNOS), thereby reducing oxo-nitrosative stress in cardiovascular diseases. Whether statin therapy leads to similar beneficial effects in lung epithelium in asthma is unknown. The effects of simvastatin therapy after sensitization (40 mg/kg, intraperitoneally) on markers of arginine and NO metabolism and features of asthma were ascertained in a murine model of allergic asthma. The effects of simvastatin on the expression of NOS in A549 lung epithelial cells were studied *in vitro*. Simvastatin induced eNOS in lung epithelial cells *in vitro*. In acute and chronic models of asthma, simvastatin therapy was associated with significantly reduced airway inflammation, airway hyperresponsiveness, and airway remodeling. ADMA and inducible nitric oxide synthase were reduced by simvastatin, but eNOS was increased. A marked reduction of nitrotyrosine, a marker of oxo-nitrosative stress, was evident in airway epithelium. Cell injury markers such as cytosolic cytochrome c, caspases 3 and 9 and apoptotic protease activating factor 1 (Apaf-1) were also reduced. Simvastatin improves dysfunctional nitric oxide metabolism in allergically inflamed lungs. Important pleiotropic mechanisms may be responsible for the statin-induced reduction of airway inflammation, epithelial injury, and airway hyperresponsiveness.

Keywords: asthma; asymmetric dimethyl arginine; nitric oxide; endothelial nitric oxide synthase; epithelial cell injury

Asthma is a chronic inflammatory condition characterized by airway hyperresponsiveness and remodeling. The structural cells of the airway, such as bronchial epithelial cells, are considered active participants in the asthmatic process (1, 2). Increased oxo-nitrosative stress and epithelial injury are important features of asthma (1–4). We recently reported on altered arginine metabolism in allergically inflamed lungs leading to increased concentrations of asymmetric dimethyl-arginine (ADMA) and the relative depletion of L-arginine, which uncouples nitric oxide synthase (NOS) and leads to increased oxo-nitrosative stress (1). We also demonstrated that the administration of L-arginine to

CLINICAL RELEVANCE

Simvastatin improves nitric oxide metabolism in bronchial epithelium, similar to the known effects in vascular endothelium, and reduces asymmetric dimethyl-arginine, oxo-nitrosative stress, epithelial injury, and apoptosis. Simvastatin may have therapeutic potential in asthma, and further clinical investigations are warranted.

mice with asthma restores this metabolic dysfunction, reduces oxo-nitrosative stress, and reverses many of the features of asthma (5). Interestingly, exhaled nitric oxide (exhNO) was increased in L-arginine-treated mice compared with mice exhibiting allergic airway inflammation, that is, contrary to the usual concordance between exhNO and allergic inflammation. This finding highlights the controversial role of NO in asthma. Although eNOS was the predominant NOS isoform in L-arginine treated mice, inducible nitric oxide synthase (iNOS) predominated in mice with asthma. This asthma-related metabolic dysfunction and its reversal by L-arginine were prominent in bronchial epithelial cells, and are highly reminiscent of a similar pathology in the vascular endothelial dysfunction seen in obesity-related metabolic syndrome, namely, the higher expression of iNOS, the uncoupling of eNOS by increased ADMA, and increased oxo-nitrosative stress (6, 7). Interestingly, metabolic syndrome and obesity are known to increase the risk of asthma as well as its severity (8), suggesting that ADMA may represent an important therapeutic target. Under normal conditions, the low amounts of NO produced by eNOS play an important role in the regulation of metabolic homeostasis, whereas the induction of iNOS is associated with the production of larger amounts of NO, nitrates and nitrites that are proinflammatory. The exact effect of NO depends on its cellular source, stimulus, and local microenvironment. The iNOS-mediated synthesis of NO is more likely to cause the generation of reactive nitrogen species compared with eNOS. Thus, strategies to increase eNOS and inhibit iNOS induction may be beneficial in asthma.

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis, that was also shown to demonstrate pleiotropic actions independent of its cholesterol-lowering effect (9–11). In endothelial cells, statins were shown to upregulate the expression and activity of eNOS (12–14), thereby increasing the production of NO and reversing endothelial cell dysfunction. Statins may also reduce the induction of iNOS (15), and simvastatin was shown to attenuate the expression of cytokine-inducible NOS in embryonic cardiac myoblasts (16). Apart from their direct effects on NOS, statins were also shown to reduce concentrations of ADMA (17, 18). Statins were previously shown to inhibit allergic sensitization and attenuate the asthma phenotype, but important lacunae

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exist. First, most studies administered statins during allergic sensitization, limiting any inferences regarding their effects on asthma after the disease is established. Second, only one study investigated the effect of statins on airway hyperresponsiveness (AHR), the cardinal feature of asthma. Third, no study has investigated the effects of statins in chronic models of asthma that recapitulate clinically important, poorly reversible features, such as peribronchial fibrosis. Lastly, the mechanisms of action for statins in asthma are not fully explained by either the inhibition of cholesterol or the synthesis of mevalonate, suggesting that other important effects may be relevant (19).

We investigated whether simvastatin can modulate arginine and NO metabolism in lung epithelial cells, and whether this modulation is associated with an attenuation of the pathology in acute and chronic models of asthma. To exclude the effects of statins on the prevention of allergic sensitization, as reported earlier, we treated mice with simvastatin only after allergen sensitization. In this report, we demonstrate for the first time, to the best of our knowledge, that simvastatin alleviates asthmatic conditions by modulating NO metabolism in bronchial epithelium.

MATERIALS AND METHODS

Animals

Male BALB/c mice (8–10-week-old; National Institute of Nutrition, Hyderabad, India) were acclimatized for 1 week before initiating the experiments. All animals were maintained according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (India), and protocols were approved by our Institutional Animal Ethics Committee.

Grouping of Mice

Mice were divided into two groups, that is, acute and chronic. Each group was further divided into three groups ($n = 6$), named according to the sensitization/challenge/treatment: SHAM/PBS/vehicle (VEH) (normal controls), chicken egg ovalbumin (OVA)/OVA/VEH (allergic

controls; OVA, Grade V, was from Sigma, St. Louis, MO), and OVA/OVA/STATIN (the statin was simvastatin; Sigma). Simvastatin (40 mg/kg in a 200- μ l volume) was administered by intraperitoneal injection for 7 consecutive days, 30 minutes before each local challenge. The dose of 40 mg/kg simvastatin was prepared by dissolving 4 mg of simvastatin in 100 μ l of ethanol and 150 μ l of 0.1 N NaOH, incubated at 50°C for 2 hours. The pH was adjusted to 7 with HCl, and the total volume consisted of 1 ml. The stock solution was diluted in sterile PBS (20). Because mice metabolize statins more rapidly than do humans, a dose of 40 mg/kg/day of simvastatin in mice is considered comparable to the maximum dose of 80 mg daily in humans, in terms of serum concentration (21).

Sensitization, Challenge, and Treatment of Mice

Mice were sensitized and challenged as shown in Figures 1A and 1B. Briefly, mice were injected intraperitoneally with 50 μ g OVA in 4 mg aluminum hydroxide or with only 4 mg aluminum hydroxide on days 0, 7, and 14, and challenged with 3% (acute) and 1.5% (chronic) OVA in PBS or PBS alone from Days 21–27 (acute) and from Days 21–50 on alternate days (chronic). Vehicle or statin was administered by intraperitoneal injections from Days 21–28 (acute) or from Days 36–51 (chronic).

Immunohistochemistry

Immunohistochemistry for iNOS, eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and nitrotyrosine (Sigma) was performed, and semi-quantitative analyses of eNOS-stained and iNOS-stained slides were performed as described previously (22).

Measurements of Nitrotyrosine and ADMA

Concentrations of nitrotyrosine (Cayman Chemical, Ann Arbor, MI) and ADMA (Diagnostika, GMBH, Hamburg, Germany) were measured using a competitive ELISA, as described earlier (1).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Apoptotic Assay and Measurement of 8-Hydroxy-2'-Deoxyguanosine

The TUNEL assay of apoptosis was performed (Dead End Calorimetric TUNEL System; Promega, Madison, WI), and 8-hydroxy-2'-deox-

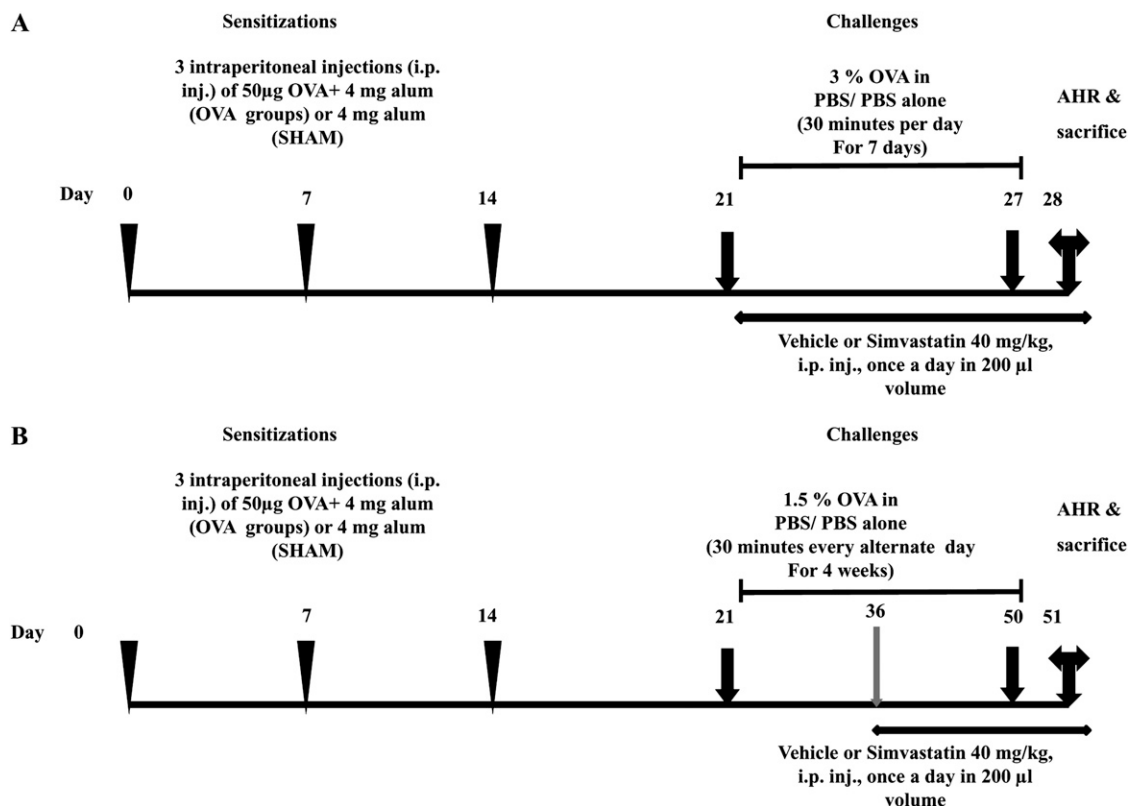


Figure 1. Protocol to induce allergic asthma in mice. Male BALB/c (8–10-week-old) mice were grouped, sensitized, and challenged. Simvastatin was administered via intraperitoneal injections (i.p. inj.) from Days 21–28 in an acute model (A), and from Days 36–51 in a chronic model (B). Measurements of lung function and sacrifice were performed on Days 28 and 51, respectively. AHR, airway hyperresponsiveness; OVA, chicken egg ovalbumin.

yguanosine (8-OHdG; Assay Designs, West Chester, PA) was measured according to the manufacturer's instructions.

Bronchoalveolar Lavage

On day 28, each mouse was killed, bronchoalveolar lavage (BAL) was performed, and an absolute cell count for each cell type was calculated after determining the total cell count and differential cell count, as described previously (23).

Lung Histopathology

Formalin-fixed, paraffin-embedded lung-tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, and Masson's trichrome, as described earlier (23).

Measurements of IL-4, IL-5, IL-10, IL-13, and Transforming Growth Factor- β 1 Concentrations in the Lung

The ELISA of IL-4, IL-5, IL-10, TGF- β 1 (BD Biosciences, Rockville, MD), and IL-13 (R&D Systems, Minneapolis, MN) was performed according to the manufacturer's protocols.

Measurement of AHR

AHR to methacholine (MCh; Sigma) was determined by double-chamber, whole-body plethysmography, (PLY 3351; Buxco Electronics, Winchester, UK) and Flexivent (SCIREQ, Montreal, Canada), respectively, as described earlier, with some modifications (5).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical significance was set at $P \leq 0.05$. ANOVA with *post hoc* correction was used to compare groupwise data.

RESULTS

Simvastatin Treatment Induces eNOS Expression in Lung Epithelial Cells and Improves Nitric Oxide Metabolism during Allergic Airway Inflammation

To determine whether simvastatin could modulate NO metabolism in allergically inflamed lungs, we measured the expression of NOS in mice with allergic airway inflammation treated with simvastatin or the control. Figure 2 shows the immunohistochemistry and semiquantitative morphometry of eNOS and iNOS expression in control and simvastatin-treated mice. Simvastatin induces the expression of eNOS in bronchial epithelium, while simultaneously downregulating iNOS in both bronchial epithelia and inflammatory cells. Because the induction of eNOS in bronchial epithelium by simvastatin was previously unreported, it was also independently confirmed by fluorescently labeled immunohistochemistry (Figure E1 in the online supplement). Further, exhNO had not diminished in mice receiving simvastatin, despite a previously reported direct correlation between inflammation and exhNO (Figure E2a), confirming that the increased eNOS was functional. Because the downstream effects of increased NO production by eNOS would include guanyl cyclase activation in local tissues, we measured cyclic guanosine monophosphate (cGMP) in lung homogenate, and found it to be increased in simvastatin-treated mice (Figure E2b). To confirm whether simvastatin could directly induce NOS in lung epithelial cells, we measured the expression of NOS in A549 cells cultured with varying doses of simvastatin. We found that simvastatin potently induced the expression of eNOS in A549 cells, as measured by immunocytochemistry (Figure E3a) and flow cytometry (Figure E3b).

Simvastatin Treatment Inhibits ADMA, Reduces Oxo-Nitrosative Stress, and Diminishes Cellular Injury

Because increased ADMA in allergically inflamed bronchial epithelium causes oxo-nitrosative stress, we investigated whether

simvastatin inhibits ADMA and the generation of peroxynitrite in lungs. We found that simvastatin treatment was associated with significant reductions in ADMA and nitrotyrosine, a marker for peroxynitrite, in lungs (Figure 3). Immunohistochemistry showed that oxo-nitrosative stress was substantially increased in lungs of allergic controls and was reduced, to near normal in treated mice. This was seen most prominently in the airway epithelium. Because we had found improved NO metabolism and oxo-nitrosative stress in lungs after simvastatin treatment, we also measured whether any effects on cellular injury and apoptosis were evident. We found that simvastatin treatment diminished epithelial injury and apoptosis, as indicated by the reduced TUNEL staining of nuclei (Figure 3). This effect was most prominent in the airways, but was also evident in other regions, as confirmed by other markers of cellular injury in lung lysates (Figure E4).

Simvastatin Treatment Reduces BAL Cell Count

Table 1 summarizes the analysis of BAL fluid from normal control mice (SHAM/PBS/VEH), allergic control mice (OVA/OVA/VEH), and simvastatin-treated mice (OVA/OVA/STATIN). BAL cell counts were dramatically reduced in simvastatin-treated mice.

Simvastatin Treatment Prevents Airway Inflammation, Mucus Hypersecretion, and Airway Remodeling in a Model of Chronic Asthma

Because airway inflammation, mucus hypersecretion, and airway remodeling are important features of asthma, we stained lung sections from control and simvastatin-treated mice with hematoxylin and eosin, periodic acid-Schiff, and Masson's trichrome, seeking alterations in inflammatory cell infiltrates, mucous metaplasia, and collagen deposition, respectively (Figure 4). We found that simvastatin treatment was associated with a reduction of cell infiltrates, mucous metaplasia, and the deposition of peribronchial collagen. An analysis of the lung cytokine profile showed a reduction in the Th2 cytokines IL-4, IL-5, and IL-13, and an increase in the immunomodulatory cytokine IL-10 (Figure 5). Similarly, simvastatin treatment reduced airway inflammation in a model of acute asthma (Figure E5).

Simvastatin Treatment Reduces AHR

To elucidate the effects of simvastatin on airway function, we challenged asthmatic control and simvastatin-treated mice with MCh. In a model of acute asthma, simvastatin treatment was associated with an attenuation of AHR, measured using double-chamber plethysmography (Figure 6A). To determine whether the beneficial effects of statins were related mostly to anti-inflammatory effects or to alterations in airway remodeling, we further tested the effects of statins in a model of chronic asthma characterized by low concentrations of inflammatory cell infiltrate but pronounced mucus metaplasia and peribronchial collagen deposition. In this model, simvastatin treatment was associated with a marked improvement of invasively determined lung-resistance, airway-resistance, and tissue-resistance parameters to near-normal concentrations (Figures 6B–D).

Chronic Allergic Airway Inflammation Is Associated with Increased Serum ADMA

To determine whether chronic asthma was associated with systemic metabolic stress, we determined the serum concentrations of ADMA in a model of chronic asthma. We found that ADMA had increased more than twofold in such mice, compared with naive control mice (Figure 7; mean $\pm$ SEM, 1.64 ± 0.23 μ mol/ml versus 0.72 ± 0.05 μ mol/ml; $P < 0.01$). The

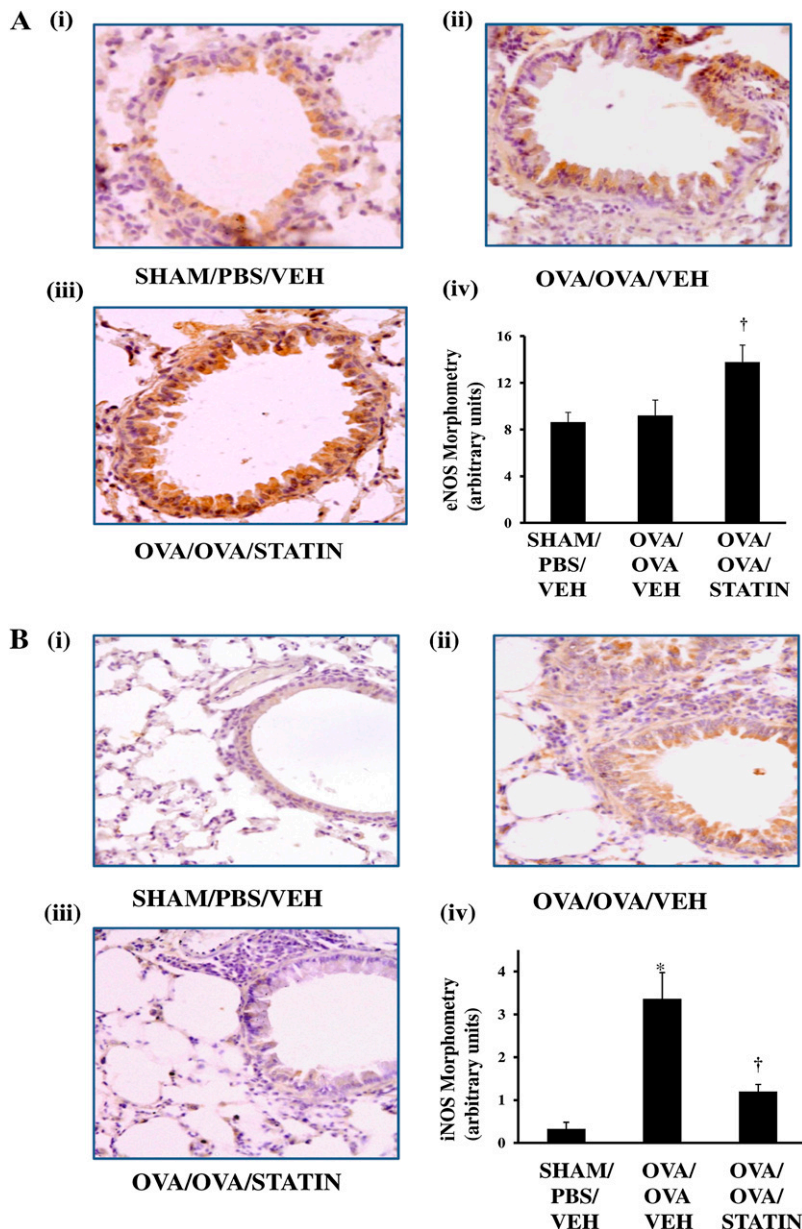


Figure 2. Simvastatin enhances the expression of endothelial nitric oxide synthase (*eNOS*) and reduces inducible nitric oxide synthase (*iNOS*). Representative photomicrographs of immunohistochemical staining of *eNOS* (A), sham (i), OVA (ii), and statin (iii) and for *iNOS* (B), sham (i), OVA (ii), and statin (iii) in lung sections, and semi-quantitative analyses of staining are shown for *eNOS* (A, iv) and *iNOS* (B, iv). Brown indicates a positive stain. All photographs were taken at $\times 20$ magnification. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ versus sham/PBS/vehicle (SHAM/PBS/VEH). † $P < 0.05$ versus OVA/OVA/VEH.

administration of statin was associated with a significant reduction of ADMA to concentrations similar to those of naive control mice ($0.9 \pm 0.2 \mu\text{mol/ml}$).

DISCUSSION

In this study, we report on the potential beneficial effects of simvastatin in AHR and epithelial cell injury in asthma. Mice metabolize statins much more quickly than humans do, and our 40 mg/kg/day dose of simvastatin is comparable to the upper limit of clinically indicated dosage for cholesterol lowering (80 mg/day), and well within the range of doses used in cancer trials (21). Although the anti-inflammatory effects of high-dose statin therapy in murine models of allergic airway inflammation were previously documented, key differences are evident. First, we are not aware of any previous reports of the effects of statin therapy on NO and ADMA metabolism in the lungs, or of the effects of simvastatin on airway epithelium. Second, we treated mice with simvastatin after allergen sensitization and during both acute and chronic allergen exposure. Because most

patients with atopic asthma are already allergen-sensitized, the effects of statins after sensitization may be more relevant, especially in chronic models. Third, this is the first report on the effect of statins in AHR in acute and chronic models. Importantly, we used invasive methods that measured mechanical properties of the respiratory system and split them into airway and parenchymal components, and we also used the more physiologic and noninvasive double-chamber plethysmography, which directly estimates specific airway conductance (SGaw). SGaw, unlike enhanced pause (Penh), is a valid non-invasive index of airway obstruction with the advantage of being relatively volume-independent and therefore resistant to the hyperinflation-associated reduction in airway resistance. We found similar benefits for L-arginine supplementation in asthma, when administered at the high doses used to treat cardio-metabolic syndrome. Thus we provide a novel theoretical framework for understanding and treating airway epithelial dysfunction in asthma, where the well-understood arginine and NO metabolic disturbances in vascular endothelium are at least partly recapitulated in bronchial epithelium.

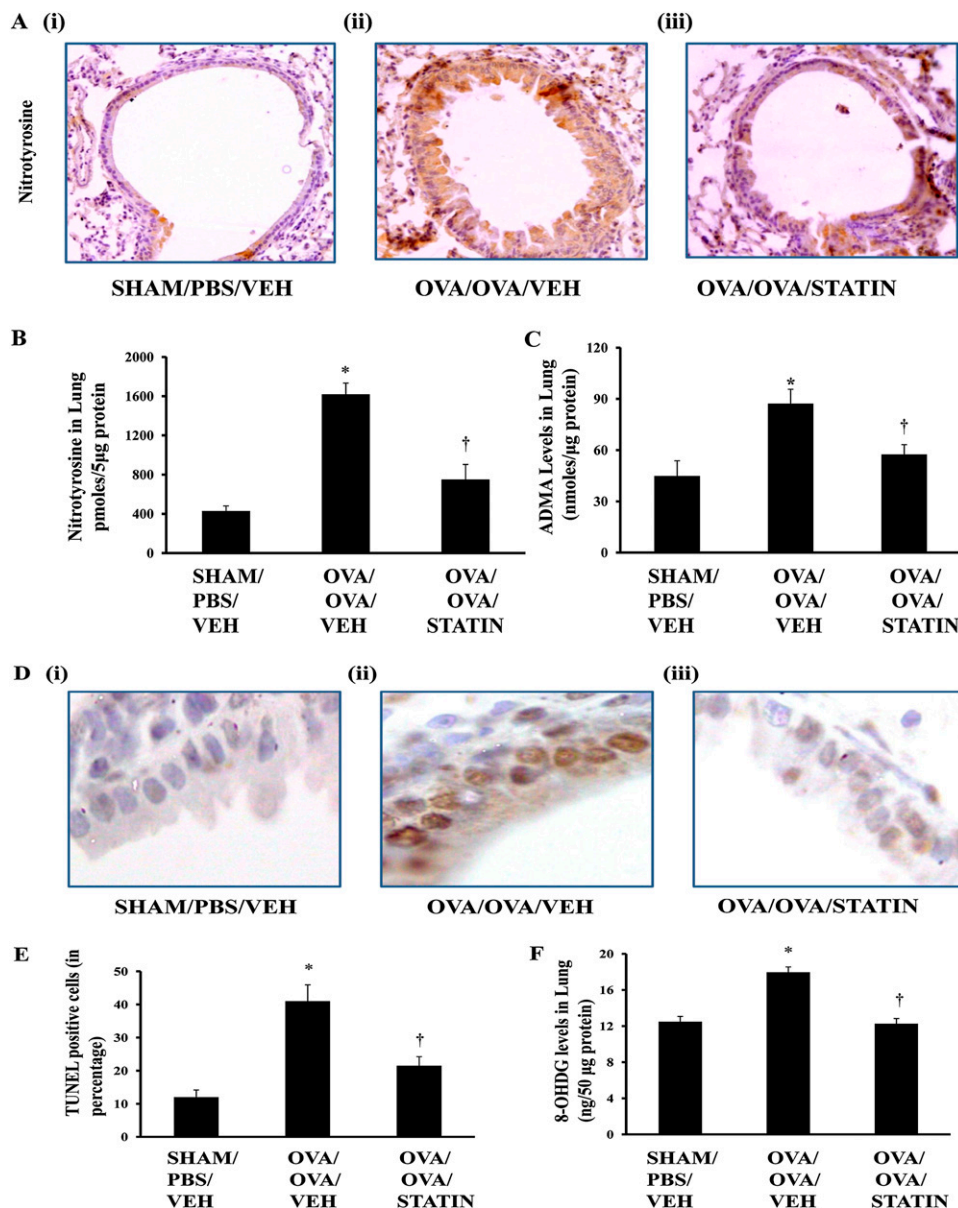


Figure 3. Simvastatin inhibits concentrations of nitrotyrosine and asymmetric dimethyl-arginine (ADMA) in lungs. Results of immunohistochemistry for nitrotyrosine (A): SHAM (i), OVA (ii) and STATIN (iii); ELISA for nitrotyrosine (B); ADMA concentrations (C); terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay (D): SHAM (i), OVA (ii), and STATIN (iii); apoptotic index (E); and 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations (F) in lungs. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ versus SHAM/PBS/VEH. † $P < 0.05$ versus OVA/OVA/VEH.

How could these effects of statins be mediated? Important differences are evident between the cholesterol-lowering effects of statins and the anti-inflammatory effects of statins. The "classic" cholesterol pathway also modulates intracellular signaling pathways through farnesyl pyrophosphate and isoprenylation. This modulation can be inhibited by statins and reversed by mevalonate. In a recent study, the intraperitoneal adminis-

tration of simvastatin (40 mg/kg) had beneficial effects on asthma that were not reversible by mevalonate, indicating that other novel mechanisms may be important (19). Statins were also shown to activate important kinases directly, such as adenosine monophosphate activated protein kinase (AMPK), phosphoinositide 3-kinase (PI3K)/Akt (protein kinase B), and more controversially, mitogen-activated protein kinase, which

TABLE 1. EFFECTS OF SIMVASTATIN ON TOTAL, DIFFERENTIAL, AND ABSOLUTE CELL COUNTS IN BRONCHOALVEOLAR LAVAGE FLUID (DIFFERENTIAL COUNTS IN PERCENT AND ABSOLUTE CELLS IN $\times 10^4$)

	TCC ($\times 10^4$)	Macro	Mono	Neutro	Eosino	Macro	Mono	Neutro	Eosino
Sham	7.9 $\pm$ 0.9	80.0 $\pm$ 4.3	7.0 $\pm$ 2.3	10.1 $\pm$ 2.8	2.6 $\pm$ 0.9	6.3 $\pm$ 0.03	0.5 $\pm$ 0.01	0.8 $\pm$ 0.02	0.2 $\pm$ 0.008
OVA	41.2 $\pm$ 3.7*	29.3 $\pm$ 4.9	11.1 $\pm$ 1.1	20.8 $\pm$ 2.6	46.9 $\pm$ 5.1	12.0 $\pm$ 0.18*	4.5 $\pm$ 0.02	8.5 $\pm$ 0.46	19.3 $\pm$ 0.19*
Statin	13.0 $\pm$ 1.4†	63.2 $\pm$ 3.1	10.4 $\pm$ 1.1	12.1 $\pm$ 2.4	14.1 $\pm$ 2.1	8.1 $\pm$ 0.04†	1.3 $\pm$ 0.02	1.5 $\pm$ 0.03	1.5 $\pm$ 0.03†

Definition of abbreviations: OVA, chicken egg ovalbumin; TCC, total cell count; Macro, macrophages; Mono, mononuclear leukocytes; Neutro, neutrophils; Eosino, eosinophils; VEH, vehicle.

Data are given as mean $\pm$ SEM.

* $P < 0.05$ versus SHAM/PBS/VEH group.

† $P < 0.05$ versus OVA/OVA/VEH group.

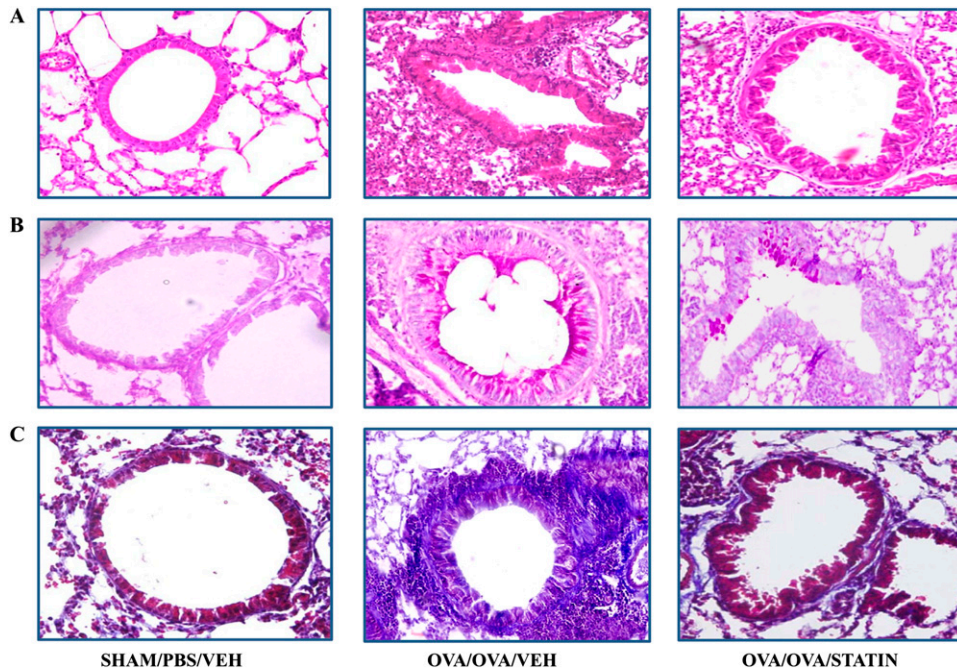


Figure 4. Effects of simvastatin on airway inflammation, mucus hypersecretion, and airway remodeling. Murine groups are named according to their sensitization/challenge/treatment. Photomicrographs are representative of hematoxylin and eosin stain (A), periodic acid-Schiff stain (B), and Masson's trichrome stain (C).

may be activated or inhibited. Our study design does not permit us to distinguish which, if any, of these mechanisms may be important in the observed effects on arginine and NO metabolism. However, the activation of AMPK or PI3K was found to cause a statin-induced direct upregulation of eNOS (24). We speculate that these results may represent the mevalonate-independent effects of statins in asthma found by Zeki and colleagues (19). Further study of statins in murine models of asthma with the selective suppression or deletion of these genes will be required.

Although the mechanisms remain unclear, we provide the first evidence that simvastatin increases eNOS in lung epithelial

cells *in vitro* and *in vivo*, beyond normal baseline concentrations. This increase is associated with a reduced expression of iNOS and diminished concentration of ADMA. Most of what we know about statins and NOS is derived from cardiovascular studies. Although statins potentially activate eNOS via AMPK or PI3K/Akt-dependent pathways (24), they were variably shown to increase (25) or decrease (15) the expression of iNOS. Although both eNOS and iNOS catalyze the production of NO from L-arginine, important differences are evident. Inducible NOS, as suggested by its name, is induced by stress and inflammation (26, 27), and because it occurs in an activated form, it is a much larger source of NO than eNOS. Various

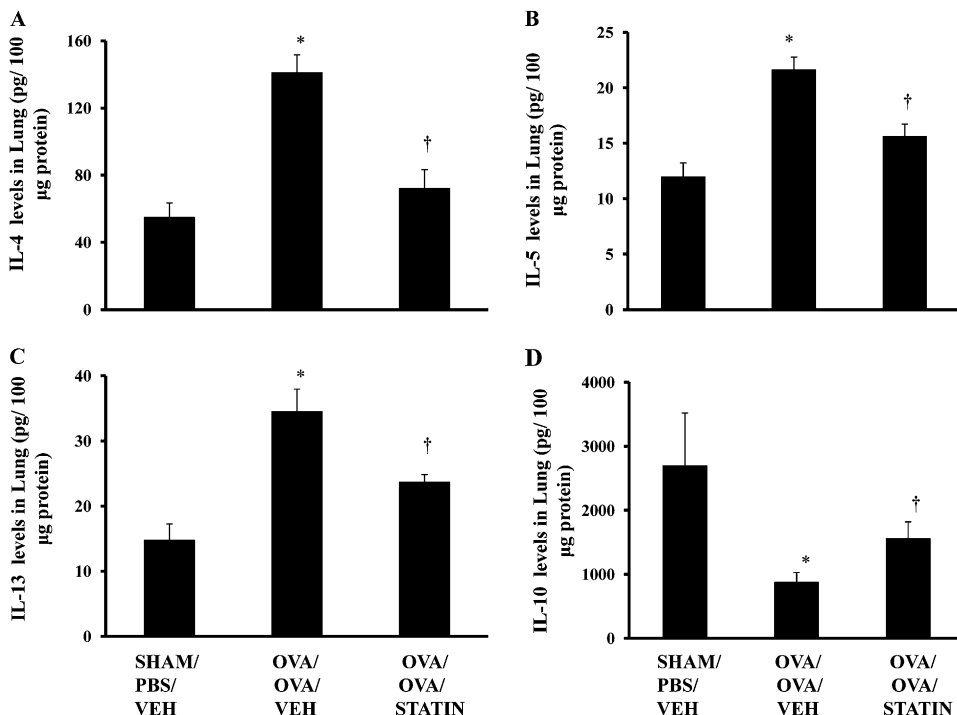


Figure 5. Effects of simvastatin on lung cytokines. Concentrations of IL-4 (A), IL-5 (B), IL-13 (C), and IL-10 (D) in lung homogenates are shown as mean $\pm$ SEM. * $P < 0.05$ versus SHAM/PBS/VEH. † $P < 0.05$ versus OVA/OVA/VEH.

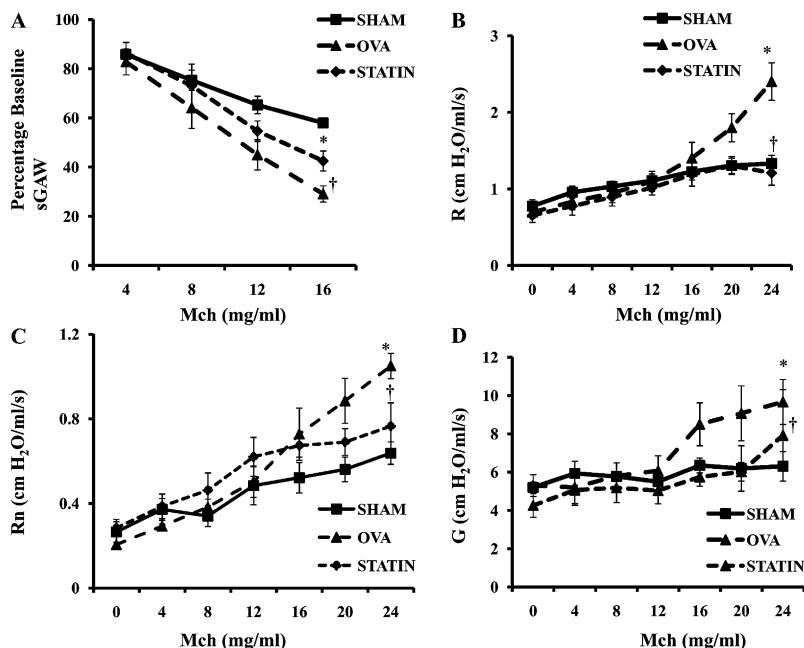


Figure 6. Effects of simvastatin on airway hyperresponsiveness. Specific airway conductance (sGAW) was measured in a model of acute asthma (A), whereas airway resistance (R), Newtonian resistance (Rn), and tissue damping (G) was measured by Flexivent in a model of chronic asthma (B–D). Mch, methacholine.

studies showed that the expression of iNOS was linked to oxo-nitrosative stress and potentiation of the inflammatory state (27), whereas the expression of eNOS was linked to the beneficial effects of regulated NO production via the activation of guanyl cyclase (28, 29). Our data clearly indicate that in acute allergic inflammation of the lungs, ADMA, iNOS, and oxo-nitrosative stress are increased and the activation of guanyl cyclase is diminished, despite a normal amount of eNOS (according to immunohistochemistry). Simvastatin treatment restores ADMA and iNOS to near-normal concentrations, and increases eNOS beyond baseline concentrations. Because ADMA is known to uncouple eNOS but not iNOS, the combination of increased eNOS, reduced iNOS, and reduced ADMA is likely to preserve NO synthesis at a much lower cost in terms of oxo-nitrosative stress. Indeed, exhNO had not decreased much with simvastatin treatment, but a striking reduction in oxo-nitrosative stress and cellular injury occurred, whereas the activation of guanyl cyclase, a downstream effect of NO, was potentiated. We speculate that physiologic NO production from eNOS is beneficial in airway epithelial cells, and that diminished eNOS activity because of physiologic uncouplers such as ADMA and leptin, both of which are increased in obesity, may be deleterious. The reduction of iNOS may not be the major beneficial effect of simvastatin in this model, because the treatment of OVA-induced mice with 1400 W, a potent iNOS inhibitor, reduced neither AHR nor airway inflammation, although exhNO was reduced (Figure E7). Because a new clinical phenotype of obesity-related asthma associated with low concentrations of exhNO was recently described, an investigation of these mechanisms in such patients may be useful.

Although the role of ADMA in asthma is still relatively new, strong evidence exists that ADMA importantly modulates oxo-nitrosative stress, epithelial injury (30), apoptosis (31), airway remodeling, and AHR (32) in models of asthma. Concentrations of ADMA are increased in allergically inflamed lungs, and correlate with oxo-nitrosative stress and mitochondrial dysfunction (1). Intracellular and plasma concentrations of ADMA reflect its production in the course of normal protein turnover by protein arginine N-methyltransferases, renal clearance from

plasma, and intracellular degradation by dimethylarginine dimethylaminohydrolase (DDAH). Studies examining the effects of statin *in vivo* did not examine tissue concentrations of ADMA, and focused only on plasma ADMA. Thus, the reported failure of statin therapy to decrease plasma concentrations of ADMA in some studies (18) does not exclude important effects elsewhere. We previously reported that lung and bronchial epithelial DDAH2 concentrations are diminished in asthma, which leads to an increase in tissue concentrations of ADMA. Although the mechanisms are not fully understood, membrane cholesterol depletion by statins was recently shown to activate the transcription factor sterol regulatory element binding proteins (SREBP), which increases the expression of DDAH. Lungs are an important source of circulating ADMA, and increased serum concentrations of ADMA are evident in lung fibrosis and pulmonary

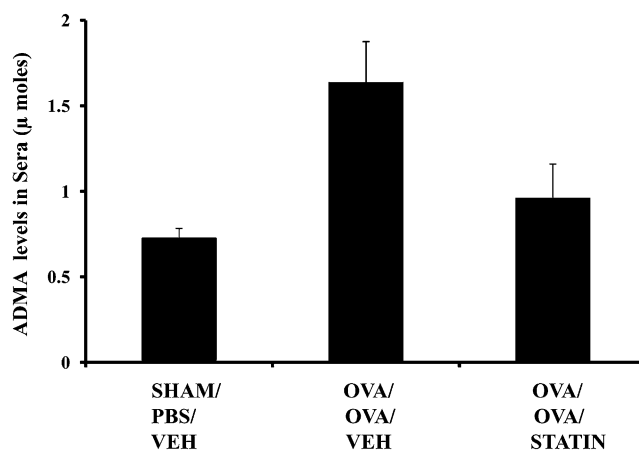


Figure 7. Concentrations of ADMA were increased in sera samples of mice with chronic allergic airway inflammation (OVA/OVA/VEH) compared with normal control mice (SHAM/PBS/VEH). Simvastatin treatment significantly reduced the increased concentrations of ADMA (OVA/OVA/STATIN). Data are shown as mean $\pm$ SEM. * $P < 0.05$ versus SHAM/PBS/VEH. † $P < 0.05$ versus OVA/OVA/VEH.

hypertension (33). We show for the first time, to the best of our knowledge, that chronic allergic airway inflammation can also increase serum concentrations of ADMA. Whether this increase would lead to systemic metabolic stress is unknown, but the association between inflammatory lung disease and metabolic syndrome appears to be bidirectional (34). Simvastatin was previously reported to be potentially beneficial in the treatment of both lung fibrosis (35) and pulmonary hypertension (36), and was also found to exert anti-asthma effects. Although these effects may be coincidental, ADMA is unlikely to be simply an innocent biomarker. The infusion of ADMA leads to the peribronchial deposition of collagen, fibrosis, and AHR in mice. We speculate that ADMA is an important therapeutic target, and that statin therapies should be investigated further in diseases associated with increased concentrations of ADMA.

Our study of the effects of simvastatin in asthma contains some limitations. First, although our work focuses on NO metabolism and the nonclassic effects of statins, this focus does not diminish the importance of classic mevalonate pathway-related effects (Figure E8). Indeed, we found that extracellular signal-regulated kinases (ERK1 and ERK2) phosphorylation was reduced in the lungs of simvastatin-treated mice (Figure E6), confirming the downregulation of the ras homolog/rat sarcoma pathways observed by others (37, 38). Second, although statin therapy seems to improve allergic airway inflammation in mice, as shown here and elsewhere, this improvement occurs at doses equivalent to the highest dose approved for cholesterol lowering in humans. Such doses are uncommon in usual clinical practice. Lastly, the benefits of statin therapy in asthma and other obstructive pulmonary diseases must ultimately be proven at the clinical level. Although multiple reports of the benefits of statins in chronic obstructive pulmonary disease outcomes were published (39), two small, prospective studies of human asthma found no effects of medium-dose statin therapy, or else marginal effects that lasted for short durations (less than 2 months) (40, 41). Although a recent retrospective study reported potentially worse outcomes of statin therapy (42), such retrospective comparisons may be biased by the increased prevalence of metabolic syndrome or obesity-related asthma in statin-treated patients. Patients with such comorbidities are known to exhibit poorer outcomes (8, 34). The success of prospective statin therapy in airway disease may relate to one or more such variables as appropriate patient selection, dosage, duration, and endpoints. Given the strong evidence for statin-mediated beneficial effects in models of allergic inflammation, scientifically designed, adequately powered, and targeted prospective clinical investigations are warranted.

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