

Involvement of sirtuin 1 in airway inflammation and hyperresponsiveness of allergic airway disease

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Background: Bronchial asthma is a chronic inflammatory disorder of the airways characterized by increased expression of multiple inflammatory genes. Acetylation of histones by histone acetyltransferases is associated with increased gene transcription, whereas hypoacetylation induced by histone deacetylases is associated with suppression of gene expression. Sirtuin 1 (SIRT1) is a member of the silent information regulator 2 family that belongs to class III histone deacetylase. **Objective:** This study aimed to investigate the role of SIRT1 and the related molecular mechanisms in the pathogenesis of allergic airway disease.

Methods: By using a murine model of ovalbumin (OVA)-induced allergic airway disease and murine tracheal epithelial cells, this study investigated the involvement of SIRT1 and its signaling networks in allergic airway inflammation and hyperresponsiveness.

Results: In this study with mice after inhalation of OVA, the increased levels of SIRT1, hypoxia-inducible factor 1 α (HIF-1 α), and vascular endothelial growth factor protein in the lungs after OVA inhalation were decreased substantially by the administration of a SIRT1 inhibitor, sirtinol. We also showed that the administration of sirtinol reduced significantly the increased numbers of inflammatory cells of the airways; airway hyperresponsiveness; increased levels of IL-4, IL-5, and IL-13; and increased vascular permeability in the lungs after OVA inhalation. In addition, we have found that inhibition of SIRT1 reduced OVA-induced upregulation of HIF-1 α in airway epithelial cells.

Conclusions: These results indicate that inhibition of SIRT1 might attenuate antigen-induced airway inflammation and

hyperresponsiveness through the modulation of vascular endothelial growth factor expression mediated by HIF-1 α in mice. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergic airway disease, histone deacetylase, hypoxia-inducible factor 1 α , sirtuin 1, sirtinol, vascular endothelial growth factor

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by an associated increase in airway responsiveness.¹ T_H2 cells participate in asthma pathogenesis by stimulating B cells that produce allergen-specific IgE, as well as by inducing the infiltration of eosinophils and other inflammatory cells into the airways.^{2,3} Cytokines produced by T_H2 cells, including IL-4, IL-5, and IL-13, exacerbate airway inflammation.⁴

The status of histone acetylation is associated with the transcriptional regulation of genes and is controlled by the reversible covalent modifying enzymes histone acetyltransferase and histone deacetylase (HDAC).⁵ HDACs are divided into 2 classes. Class I members (HDACs 1, 2, 3, 8, and 11) are transcriptional corepressors homologous to yeast RPD3.⁶ Class II members (HDACs 4, 5, 6, 7, 9, and 10) have domains similar to yeast histone deacetylase 1.⁷ Class III HDACs are distinct from class I and II HDACs and are homologues of the yeast silent information regulator 2 (Sir2).⁸ Mammalian sirtuin 1 (SIRT1; Sir2 α) is a member of the Sir2 family. Sir2 has been implicated in caloric restriction, aging, and inflammation.⁹

SIRT1 deacetylates several transcription factors that govern metabolism, endocrine signaling, and inflammation, including peroxisome proliferator-activated receptor γ (PPAR γ), PPAR γ coactivator 1 α , forkhead box transcription factors, p53, and nuclear factor κ B (NF- κ B).^{9,10} It has been reported that HDACs might regulate hypoxia-inducible factor 1 α (HIF-1 α) activity.¹¹ Moreover, there are very recent studies in which SIRT1 is required for HIF-1 α activation and stabilization.^{12,13} HIF-1, a heterodimeric basic helix-loop-helix-Per-Arnt/AhR-Sim domain transcription factor, mediates gene expression in response to cellular oxygen concentrations.¹⁴ In addition to the oxygen-dependent regulation of HIF-1 α activity, several reports have demonstrated that HIF-1 α expression is regulated by a variety of cytokines and growth factors in oxygen-independent pathways¹⁵ and that HIF-1 α plays an important role in inflammatory responses.¹⁶⁻¹⁸ Recently, we have demonstrated that vascular endothelial growth factor (VEGF) is one of the major determinants of asthma and thus the inhibition of VEGF might be a good therapeutic strategy,^{19,20} and we have also revealed that VEGF expression is modulated through the regulation of HIF-1 α activity mediated by the phosphoinositide 3-kinase

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Abbreviations used

BAL: Bronchoalveolar lavage
 EBD: Evans blue dye
 HDAC: Histone deacetylase
 HIF-1 α : Hypoxia-inducible factor 1 α
 IC₅₀: Inhibitory concentration of 50
 2ME2: 2-Methoxyestradiol
 NF- κ B: Nuclear factor κ B
 OVA: Ovalbumin
 PAS: Periodic acid–Schiff
 p-Akt: Phosphorylated Akt
 PI3K: Phosphoinositide 3-kinase
 PPAR γ : Peroxisome proliferator-activated receptor γ
 R_{rs}: Respiratory system resistance
 Sir2: Silent information regulator 2
 SIRT1: Sirtuin 1
 VEGF: Vascular endothelial growth factor

(PI3K)/Akt pathway in an ovalbumin (OVA)–inhaled murine model of allergic airway disease.^{17,21–24} However, no data are available on the role of SIRT1 in allergic airway disease.

In the present study we used mice after OVA inhalation (OVA-inhaled mice) to examine the involvement of SIRT1 in the pathogenesis of allergic airway disease. In addition, we also evaluated the effect of an SIRT1 inhibitor, sirtinol, on bronchial hyperresponsiveness and airway inflammation in OVA-induced allergic airway disease of mice and investigated the role of SIRT1 in the signaling linked to HIF-1 α activation using murine tracheal epithelial cells.

METHODS

The details of the methods used in this study can be found in the Methods section of this article's Online Repository at www.jacionline.org.

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from Orientbio, Inc (Seoungnam, Korea); housed throughout the experiments in a laminar flow cabinet; and maintained on standard laboratory chow *ad libitum*. Mice were sensitized and challenged as previously described, with some modifications.²⁵

Administration of sirtinol, 2-methoxyestradiol, and CBO-P11

Sirtinol (0.1 or 0.5 mg/kg body weight per day; Alexis Corp, San Diego, Calif) or vehicle control (0.05% dimethyl sulfoxide) diluted with 0.9% NaCl was administered intraperitoneally 2 times to each animal, once on day 21 and the second time on day 23. An inhibitor of HIF-1 α , 2-methoxyestradiol (2ME2; 100 mg/kg body weight per day, Calbiochem, San Diego, Calif), was suspended in 0.5% carboxymethylcellulose (Calbiochem) and administered by means of oral gavage 7 times at 24-hour intervals on days 19 to 25.²⁶ The cyclopeptide vascular endothelial growth inhibitor CBO-P11 (Flt-1: inhibitory concentration of 50% [IC₅₀] = 700 nmol/L; Flk-1/KDR: IC₅₀ = 1.3 μ mol/L; D-Phe-Pro [79–93], Calbiochem) was used to inhibit VEGF activity. CBO-P11 (2 mg/kg body weight per day) was administered intraperitoneally 3 times at 24-hour intervals.²⁷

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis, as described previously.²⁵

Nuclear protein extractions for analysis of HIF-1 α , HIF-1 β , and SIRT1

Nuclear extraction was performed as described previously.²¹

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as described previously.²⁵

Sirtinol treatment on murine tracheal epithelial cells from OVA-inhaled mice

Cells were seeded in culture dishes and grown until 70% confluence. The medium was then replaced with a new medium containing vehicle (0.1% dimethyl sulfoxide) or sirtinol (2 or 10 μ mol/L) overnight at 37°C.

SIRT1 deacetylase assay

SIRT1 activity was determined by using a deacetylase fluorometric assay kit (Sir2 assay kit; CycLex Co, Ltd, Ina, Nagano, Japan).²⁸

Measurement of T_H2 cytokine and VEGF levels

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of bronchoalveolar lavage (BAL) fluids by means of enzyme immunoassays.

Measurement of plasma exudation

Evans blue dye (EBD) was used to assess lung permeability, as described previously.²¹

Histology

For histologic examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere.²⁹

Quantitation of airway mucus expression

The numbers of periodic acid–Schiff (PAS)–positive and PAS-negative epithelial cells in individual bronchioles were counted, as described previously, to quantitate the level of mucus expression in the airway.³⁰

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine through the airways, as described elsewhere.³¹

RESULTS**SIRT1 and HIF-1 α protein levels increase in lungs from OVA-inhaled mice**

To evaluate the activation of SIRT1 and HIF-1 α in OVA-induced allergic airway disease, we analyzed nuclear levels of SIRT1 and HIF-1 α using nuclear protein extracts of lung tissues. Western blot analysis revealed that nuclear SIRT1 protein levels in lung tissues were increased approximately 1.4-, 1.7-, 2.7-, 2.7-, and 3.0-fold at 1, 6, 24, 48, and 72 hours, respectively, after challenge with OVA compared with levels seen in the prechallenge period (Fig 1). In contrast, no significant changes in the

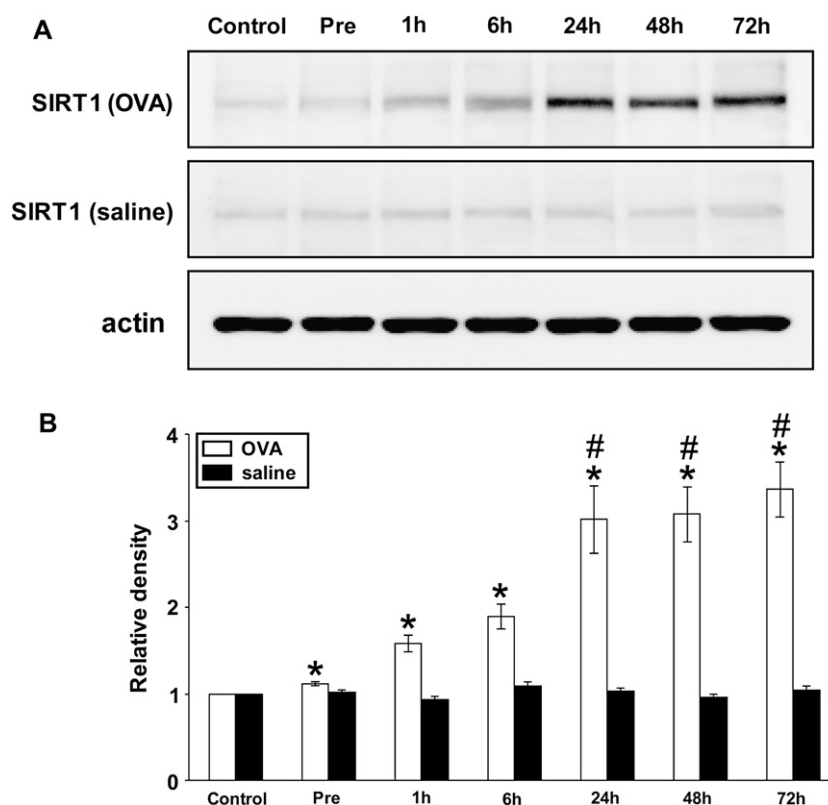


FIG 1. Levels of SIRT1 protein in lung tissues of OVA-sensitized and OVA-challenged mice. **A**, Western blot analyses of SIRT1. **B**, Densitometric analyses are presented as the relative ratio of SIRT1 to actin. The relative ratio of SIRT1 in the lung tissues of control mice is arbitrarily presented as 1. Data represent means $\pm$ SEMs from 8 mice per group. One, 6, 24, 48, and 72 hours are the time periods of the sampling after the last challenge in mice sensitized and challenged with OVA or saline. *Control*, No treatment (mice with no sensitization and no challenge); *Pre*, 1 hour before the first challenge (OVA-sensitized mice without OVA challenge or saline-sensitized mice without saline challenge). # $P < .05$ versus the Pre group. * $P < 0.05$ versus the saline group.

nuclear SIRT1 levels were observed after saline inhalation. Nuclear HIF-1 α protein levels in lung tissues were increased approximately 1.5-, 1.9-, 2.3-, 2.6-, and 2.7-fold at 1, 6, 24, 48, and 72 hours, respectively, after challenge with OVA compared with the levels seen in the prechallenge period (see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, no significant changes in the HIF-1 α protein level were observed after saline inhalation.

Effects of sirtinol on SIRT1 levels and enzyme activity in lung tissues and in primary airway epithelial cells of OVA-inhaled mice

Western blot analysis revealed that the increased SIRT1 levels in nuclear protein extracts after OVA inhalation were decreased by the administration of sirtinol (Fig 2, *A* and *B*). Consistent with our *in vivo* data, the increased SIRT1 protein levels in nuclear protein extracts of tracheal epithelial cells from OVA-inhaled mice were significantly reduced by the administration of sirtinol (Fig 2, *C* and *D*). In addition, the fluorometric assay showed that the deacetylase activity of SIRT1 substantially increased in lungs and airway epithelial cells from OVA-inhaled mice and that the treatment with sirtinol reduced significantly the increase

of SIRT1 enzyme activity (see Fig E2 in this article's Online Repository at www.jacionline.org).

Effects of sirtinol on HIF-1 α protein levels in lung tissues and in primary airway epithelial cells of OVA-inhaled mice

To investigate whether the activation of HIF-1 α is regulated by SIRT1 in allergic airway disease, we administered the SIRT1 inhibitor sirtinol *in vivo* and *in vitro*, measuring the HIF-1 α activation in the lung tissues and the primary cultured tracheal epithelial cells from OVA-inhaled mice. Western blot analysis showed that the administration of sirtinol reduced substantially the increased HIF-1 α levels in nuclear protein extracts of lung tissues (Fig 3, *A* and *B*) and primary tracheal epithelial cells (Fig 3, *C* and *D*) from OVA-inhaled mice.

Time course of inhibition for SIRT1 enzyme activity and protein levels of SIRT1 and HIF-1 α by sirtinol in primary tracheal epithelial cells from OVA-inhaled mice

Western blot analysis revealed that the SIRT1 protein level and enzyme activity started to decrease significantly at 8 hours of

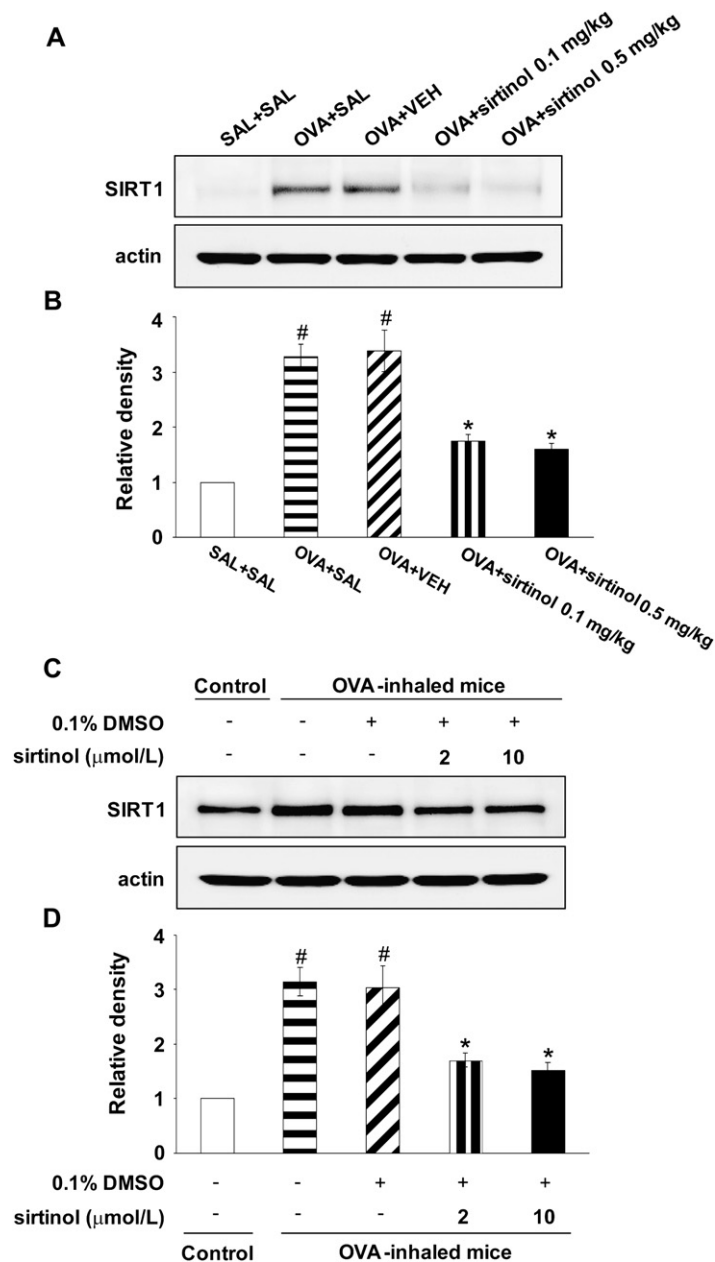


FIG 2. Effect of sirtinol on SIRT1 expression in nuclear protein extracts from lung tissues (**A** and **B**) and from primary cultured tracheal epithelial cells (**C** and **D**) of OVA-sensitized and OVA-challenged mice. **Fig 2, A**, Western blot analysis of SIRT1 in lung tissues. **Fig 2, B**, Densitometric analyses are presented as the relative ratio of SIRT1 to actin. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (*SAL*+*SAL*), OVA-inhaled mice administered saline (*OVA*+*SAL*), OVA-inhaled mice administered drug vehicle (*OVA*+*VEH*), OVA-inhaled mice administered 0.1 mg/kg sirtinol (*OVA*+*sirtinol* 0.1 mg/kg), and OVA-inhaled mice administered 0.5 mg/kg of sirtinol (*OVA*+*sirtinol* 0.5 mg/kg). The relative ratio of SIRT1 in the lung tissues of *SAL*+*SAL* mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 8 mice per group. #*P* < .05 versus the *SAL*+*SAL* group. **P* < .05 versus the *OVA*+*SAL* group. **Fig 2, C**, Western blot analysis of SIRT1 in airway epithelial cells. **Fig 2, D**, Densitometric analyses. The relative ratio of SIRT1 in the tracheal epithelial cells of control mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 6 independent experiments. Control, Epithelial cells isolated from saline-sensitized and saline-challenged mice; DMSO, dimethyl sulfoxide. #*P* < .05 versus control mice. **P* < .05 versus OVA-inhaled mice treated with drug vehicle only.

incubation with sirtinol in OVA-inhaled airway epithelial cells, whereas reduction of HIF-1 α activity started at 11 hours of incubation (see **Fig E3** in this article's Online Repository at www.jacionline.org).

Effects of sirtinol, 2ME2, and CBO-P11 on VEGF protein levels in lungs of OVA-inhaled mice

Western blot analysis revealed that the increased VEGF levels at 72 hours after OVA inhalation were decreased significantly by

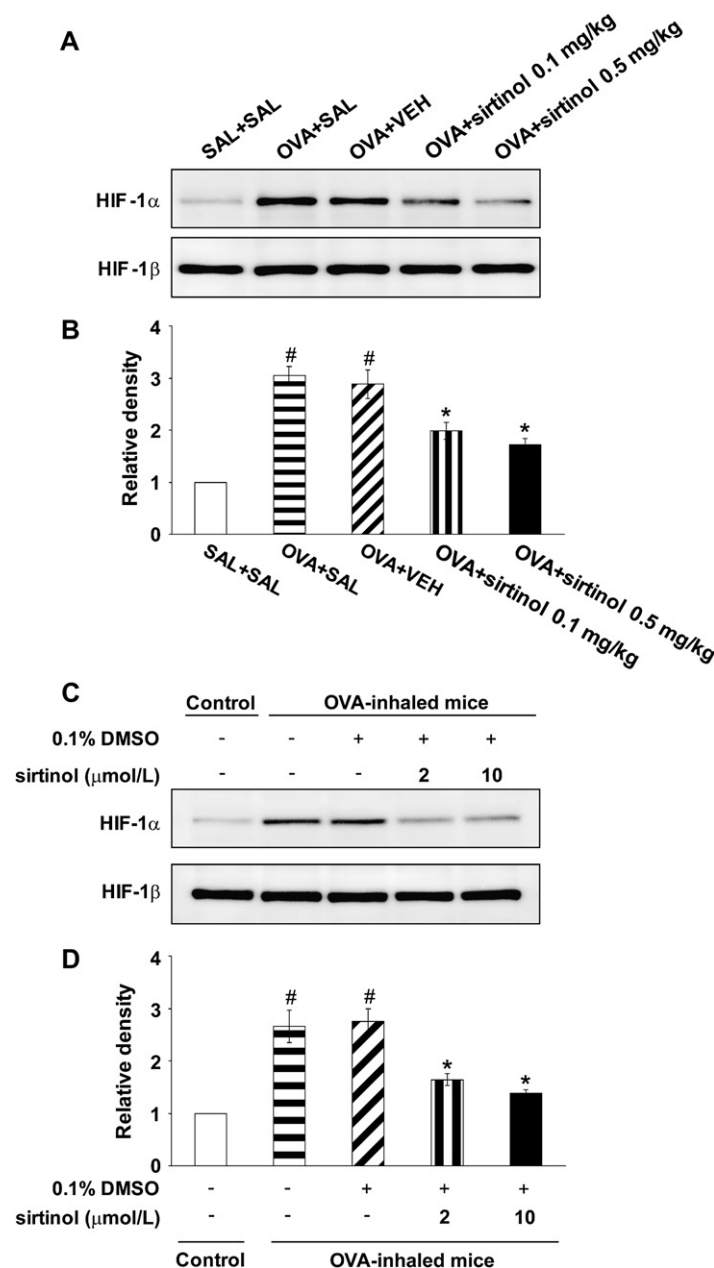


FIG 3. Effect of sirtinol on HIF-1 α and HIF-1 β levels in nuclear protein extracts from lung tissues (**A** and **B**) and from primary cultured tracheal epithelial cells (**C** and **D**) of OVA-sensitized and OVA-challenged mice. The groups are defined as in the legend for Fig 2. Fig 3, **A**, Western blot analysis of HIF-1 α and HIF-1 β protein in lung tissues. Fig 3, **B**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of HIF-1 α in the lung tissues of SAL+Sal mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 8 mice per group. # P < .05 versus the SAL+Sal group. * P < .05 versus the OVA+Sal group. Fig 3, **C**, Western blot analysis of HIF-1 α and HIF-1 β in airway epithelial cells. Fig 3, **D**, Densitometric analyses. The relative ratio of HIF-1 α in the tracheal epithelial cells of control mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 6 independent experiments. Control, Epithelial cells isolated from saline-sensitized and saline-challenged mice; DMSO, dimethyl sulfoxide. # P < 0.05 versus control mice. * P < 0.05 versus OVA-inhaled mice treated with drug vehicle only.

the administration of sirtinol (Fig 4, **A** and **B**). Consistent with these results, enzyme immunoassay showed that the increased VEGF levels in BAL fluids after OVA inhalation were decreased substantially by the administration of sirtinol (Fig 4, **C**). In addition, the administration of an HIF-1 α inhibitor, 2ME2, and a VEGF receptor inhibitor, CBO-P11, also reduced substantially the increase of VEGF levels in BAL fluids of OVA-inhaled mice (Fig 4, **C**).

Sirtinol reduces plasma extravasation in OVA-inhaled mice

EBD assay revealed that plasma extravasation was significantly increased at 72 hours after the last challenge of OVA (Fig 4, **D**). The increase of plasma extravasation after OVA inhalation was significantly reduced by the administration of sirtinol, 2ME2, or CBO-P11.

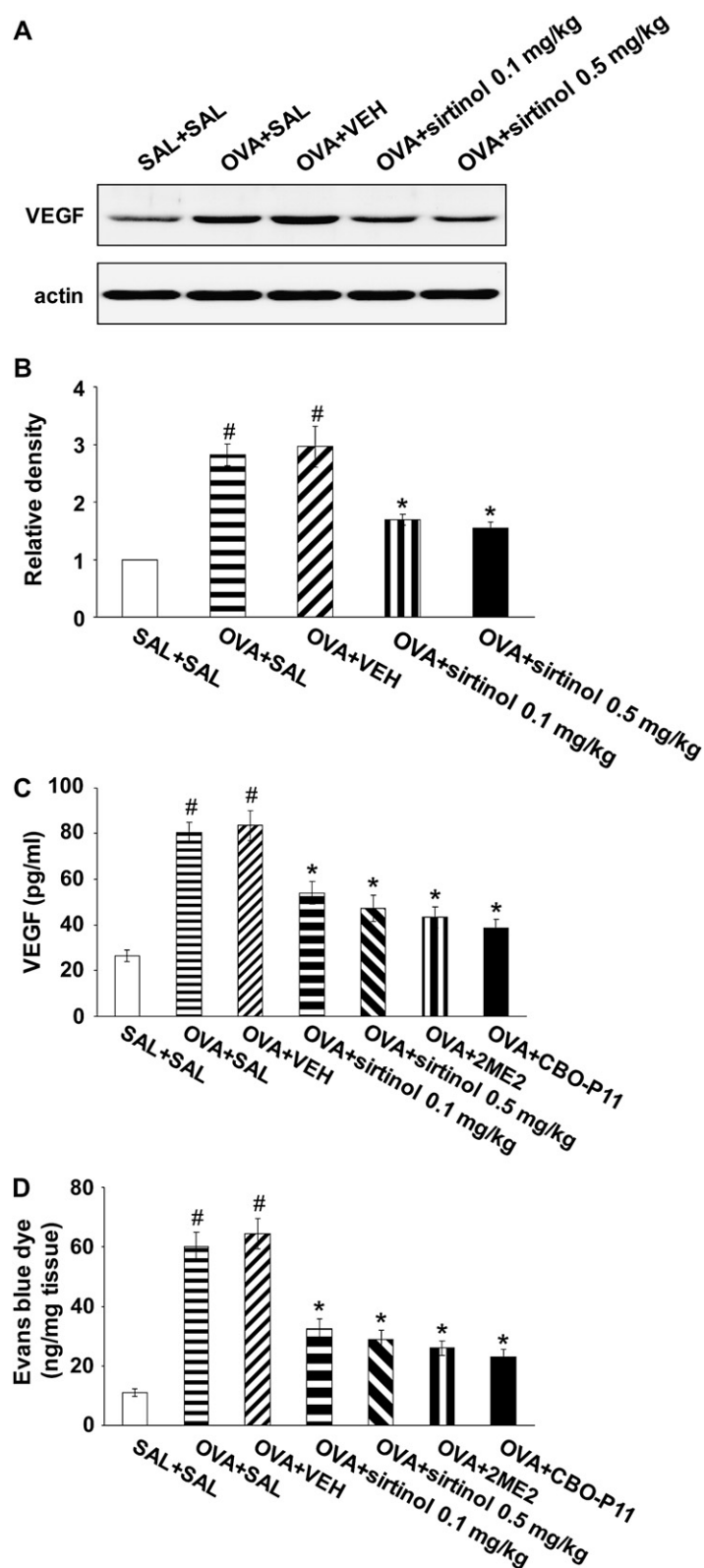


FIG 4. Effect of sirtinol, 2ME2, or CBO-P11 on VEGF expression and plasma exudation in OVA-sensitized and OVA-challenged mice. The groups are defined as in the legend for Fig 2. **A**, Western blot analysis of VEGF. **B**, Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of SAL+Sal mice is arbitrarily presented as 1. **C**, Enzyme immunoassay of VEGF in BAL fluids. **D**, Plasma exudation was quantified by means of EBD assay. Bars represent means $\pm$ SEMs from 8 mice per group. # $P < .05$ versus the SAL+Sal group. * $P < .05$ versus the OVA+Sal group.

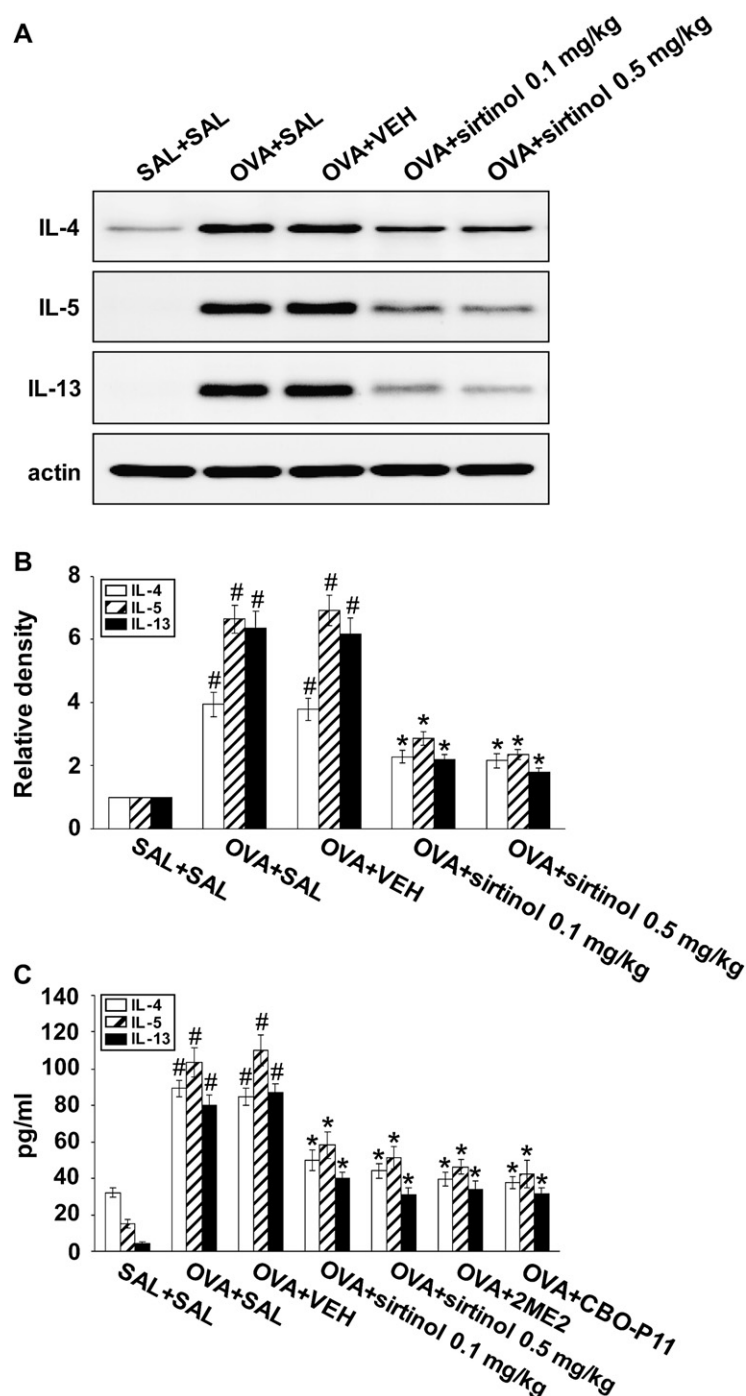


FIG 5. Effect of sirtinol, 2ME2, or CBO-P11 on IL-4, IL-5, and IL-13 levels in lungs of OVA-sensitized and OVA-challenged mice. The groups are defined as in the legend for Fig 2. **A**, Western blotting of IL-4, IL-5, and IL-13 in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissues of SAL+SAL mice is arbitrarily presented as 1. **C**, Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. Bars represent means $\pm$ SEMs from 8 mice per group. $\#P < .05$ versus SAL+SAL mice. $*P < 0.05$ versus OVA+SAL mice.

Effect of sirtinol, 2ME2, and CBO-P11 on IL-4, IL-5, and IL-13 levels in lungs of OVA-inhaled mice

Western blot analysis revealed that the increased IL-4, IL-5, and IL-13 levels at 72 hours after OVA inhalation were decreased significantly by the administration of sirtinol

(Fig 5, A and B). Consistent with these results, enzyme immunoassays also showed that the increased levels of IL-4, IL-5, and IL-13 in BAL fluids after OVA inhalation were significantly reduced by the administration of sirtinol, 2ME2, or CBO-P11 (Fig 5, C).

Effect of sirtinol on IFN- γ levels in lung tissues of OVA-inhaled mice

Western blot analysis showed that the levels of IFN- γ were significantly increased after OVA inhalation (see Fig E4 in this article's Online Repository at www.jacionline.org). Sirtinol did not show any significant effect on the IFN- γ protein levels.

Effect of sirtinol on NF- κ B p65 activity in lung tissues of OVA-inhaled mice

Western blot analysis revealed that levels of NF- κ B p65 in nuclear protein extracts of lung tissues were increased at 72 hours after OVA inhalation compared with levels seen in saline-sensitized and saline-challenged mice (see Fig E5, A, in this article's Online Repository at www.jacionline.org). The increased NF- κ B p65 levels were not affected significantly by the administration of sirtinol. In contrast, levels of NF- κ B p65 in cytosolic protein extracts of lung tissues were decreased after OVA inhalation. Consistent with these results, treatment with sirtinol did not affect the nuclear levels of NF- κ B p65 in airway epithelial cells isolated from OVA-inhaled mice (see Fig E5, B).

Effects of sirtinol, 2ME2, and CBO-P11 on cellular changes in BAL fluids

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with the numbers after saline inhalation (Fig 6, A). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of sirtinol, 2ME2, or CBO-P11.

Sirtinol reduces lung inflammation of OVA-inhaled mice

The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 hours after OVA inhalation compared with scores after saline inhalation (Fig 6, B). The increased peribronchial, perivascular, and total lung inflammation after OVA inhalation was significantly decreased by the administration of sirtinol.

Effect of sirtinol on airway mucus expression of OVA-inhaled mice

The percentage of airway epithelium that stained positively with PAS in OVA-inhaled mice was substantially greater (Fig 6, C and E) than in saline-inhaled mice (Fig 6, C and D). The increased levels of PAS-positive airway epithelium after OVA inhalation were decreased significantly by the treatment of sirtinol (Fig 6, C and F).

Sirtinol reduces airway hyperresponsiveness of OVA-inhaled mice

Airway responsiveness was assessed as a percentage increase of respiratory system resistance (R_{rs}) in response to increasing doses of methacholine. In OVA-sensitized and OVA-challenged mice the dose-response curve of R_{rs} shifted to the left compared with that of control mice (Fig 6, G). In addition, the percentage of R_{rs} produced by administration of 50 mg/mL methacholine increased significantly in the OVA-inhaled mice compared with

that seen in the control animals. OVA-sensitized and OVA-challenged mice treated with sirtinol, 2ME2, or CBO-P11 showed a significant reduction of percentage R_{rs} at 50 mg/mL methacholine compared with that seen in untreated mice. These results suggest that administration of sirtinol, 2ME2, or CBO-P11 reduces OVA-induced airway hyperresponsiveness.

Effects of sirtinol on Akt phosphorylation in OVA-inhaled mice

We performed Western blotting to determine Akt phosphorylation, which can activate the HIF pathway. Levels of phosphorylated Akt (p-Akt) protein in the lung tissues were significantly increased at 72 hours after OVA inhalation compared with levels seen in control mice (see Fig E6 in this article's Online Repository at www.jacionline.org). However, no significant changes in Akt protein levels were observed in any of the groups tested. The increased p-Akt, but not Akt protein, levels in the lung tissues after OVA inhalation were significantly reduced by the administration of sirtinol.

PI3K inhibitors decrease the HIF-1 α activity and VEGF expression in lung tissues of OVA-inhaled mice

Western blot analysis showed that the increased HIF-1 α levels in nuclear protein extracts of lung tissues after OVA inhalation were significantly decreased by the administration of LY294002 or wortmannin (see Fig E7 in this article's Online Repository at www.jacionline.org). In addition, the administration of the PI3K inhibitors substantially reduced the increase of VEGF levels in lung tissues after OVA inhalation (see Fig E8 in this article's Online Repository at www.jacionline.org).

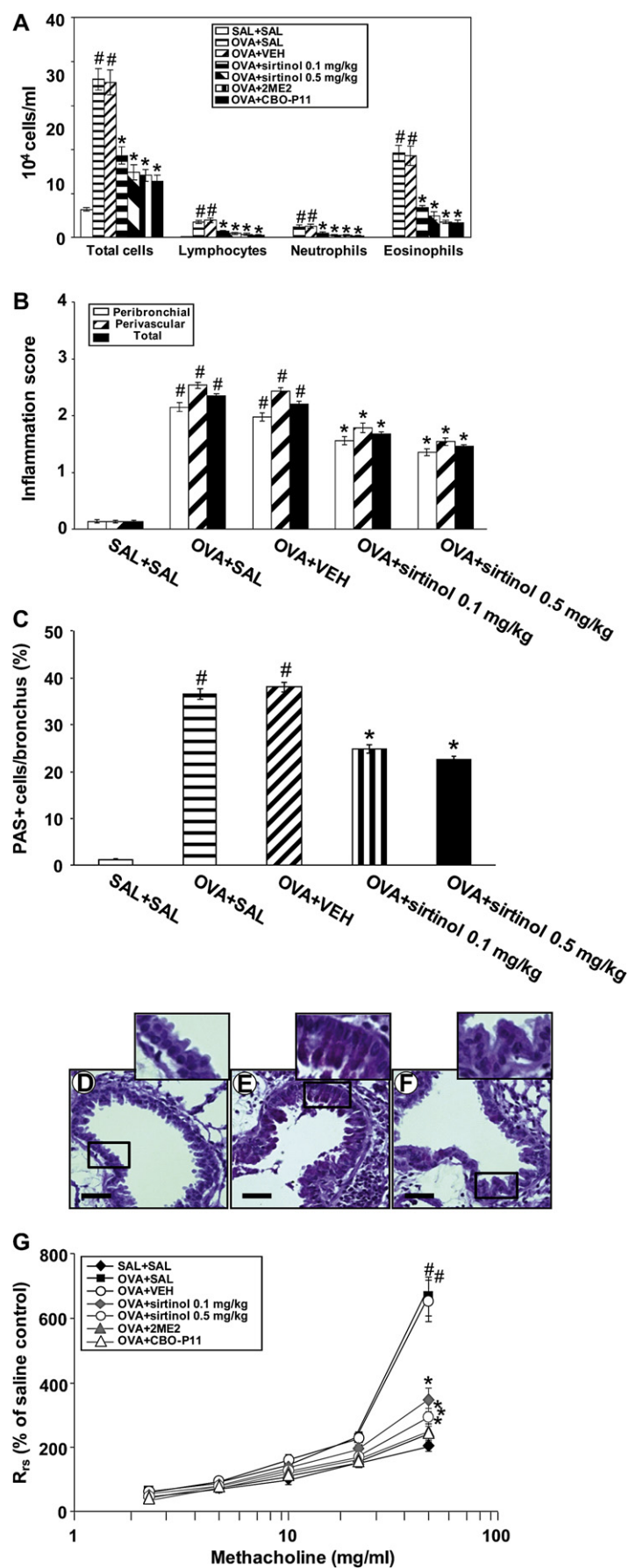
Effect of LY294002 or wortmannin on SIRT1 in nuclear extracts of lung tissues from OVA-inhaled mice

Western blot analysis revealed that the nuclear levels of SIRT1 protein increased in lungs after OVA inhalation were significantly decreased by treatment with the PI3K inhibitor LY294002 or wortmannin (see Fig E9 in this article's Online Repository at www.jacionline.org). Additional results (Fig E10) can be found in the Results section of this article's Online Repository at www.jacionline.org.

DISCUSSION

To our knowledge, this report is the first to describe the involvement of SIRT1 in airway hyperresponsiveness and inflammation in a murine model of allergic airway disease. Our present study with a murine model of allergen-induced airway disease suggests that sirtinol attenuates antigen-induced airway inflammation and hyperresponsiveness at least in part by means of modulation of VEGF expression through inhibition of HIF-1 α activation in allergic airway disease.

Bronchial asthma is a chronic inflammatory disease of the airways characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyperresponsiveness to inhaled allergens and nonspecific stimuli.³² The inflammatory response involves the recruitment and activation of inflammatory



cells and changes in the structural cells of the airway.^{33,34} The increased expression of these inflammatory proteins results from enhanced gene transcription in a cell-specific manner.³⁵ Acetylation of nuclear histones is crucial to gene expression because transcriptionally activated genes have been found to be highly associated with acetylation.³⁶⁻³⁸ Histone acetyltransferases and HDACs are identified as a transcriptional coactivator and corepressor, respectively.³⁹⁻⁴¹ Mammalian HDACs can be divided into 3 classes based on their similarity to yeast HDACs.⁶ The third class of HDACs (class III) is homologous to the Sir2 protein family, including SIRT1. Sir2 protein is widely distributed in all the phyla of life and is involved in aging, cell-cycle regulation, apoptosis, metabolism, and inflammation.⁴²⁻⁴⁵ More recently, SIRT1 has been shown to regulate proinflammatory mediator release in sustained inflammation and aging of the lungs.⁹ Our present study with a murine model of allergen-induced airway inflammation has revealed that SIRT1 protein expression and enzyme activity were increased and that sirtinol reduced the increased level of SIRT1 protein and activity after OVA inhalation.

Although the yeast Sir2 protein has been extensively characterized as an HDAC, SIRT1 deacetylates nonhistone substrates, including various transcription factors, such as PPAR γ , PPAR γ coactivator 1 α , forkhead box transcription factors, p53, and NF- κ B, which regulate a wide range of cellular processes.^{9,10} HIF-1 is a key transcriptional activator responsible for cellular responses to various stimuli by a variety of inflammatory cytokines and growth factors, as well as cellular oxygen concentrations.¹⁵ HIF-1 α is known to play an important role in inflammatory responses.^{10,11,14} It has been shown that HIF-1 α activation stimulates the expression of genes that promote angiogenesis, vasodilation, vascular permeability, glucose uptake, and glycolysis in inflammatory tissues.⁴⁶ In addition, the modulation of HIF-1 α stability and its activation involves several posttranslational modifications, such as hydroxylation, ubiquitination, phosphorylation, and acetylation. The hypoxia-regulated subunit of HIF-1, HIF-1 α , is an acetylated protein.⁴⁷ Acetylation of HIF-1 α decreases its protein stability through enhancement of ubiquitination.⁴⁸ HDAC regulates HIF-1 α activity.⁴⁹ Lee et al¹¹ have also reported that an HDAC inhibitor can downregulate hypoxia-responsive angiogenesis through suppression of HIF-1 α activity. Furthermore, there are very recent studies in which SIRT1 is required for HIF-1 α activation and stabilization.^{12,13} In the present study determination of HIF-1 α protein levels in nuclear extracts has revealed that the protein levels were substantially increased in our current OVA-induced model of allergic airway disease and OVA-treated tracheal epithelial cells, suggesting that HIF-1 α is activated. The increased levels of HIF-1 α were significantly reduced after the administration of sirtinol. Therefore these findings indicate that SIRT1 might upregulate HIF-1 α activity through deacetylation in allergic airway disease. Supporting

this contention, our results have also shown that the decrease in SIRT1 expression, activity, or both caused by sirtinol administration occurs before that of HIF-1 α *in vitro*, suggesting that SIRT1 is upstream of HIF-1 α .

Expression of VEGF, one of the crucial mediators in asthma, is regulated through HIF-1 α .^{46,50} Very recently, we have shown that increased expression of VEGF is decreased by the inhibition of HIF-1 α activity in OVA-inhaled mice.¹⁷ In this study we have found that the increased levels of VEGF after OVA inhalation were significantly reduced by the administration of sirtinol or 2ME2. Taken together, these findings suggest that sirtinol inhibits the increased VEGF expression through the downregulation of HIF-1 α activity in allergic airway disease.

Inflammation of the asthmatic airway is usually accompanied by increased vascular permeability and plasma exudation.¹ Although other inflammatory mediators, including platelet-activating factor, can promote microvascular leakage,^{51,52} one of the major roles of VEGF in asthma appears to be the enhancement of vascular permeability.^{20,53} The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesiculovacuolar organelles.⁵³ Previous studies have shown that overproduction of VEGF causes an increase in vascular permeability, which results in leakage of plasma proteins, including inflammatory mediators and inflammatory cells, into the extravascular space, thereby allowing migration of inflammatory cells into the airways.²⁰ In addition, VEGF also plays a pivotal role in adaptive T_H2-mediated inflammation.⁵⁴ Consistent with these observations, we have found that VEGF expression was upregulated and that the levels of IL-4, IL-5, and IL-13 and vascular permeability were increased in the present murine model of allergic airway disease. The increase of vascular permeability, bronchial inflammation, airway hyperresponsiveness, and VEGF, IL-4, IL-5, and IL-13 levels was significantly reduced by the administration of the SIRT1 inhibitor sirtinol or a VEGF inhibitor, CBO-P11. However, sirtinol did not affect the increased levels of a T_H1 cytokine, IFN- γ , in lung tissues of OVA-inhaled mice. Additionally, when we measured the activity of NF- κ B, a well-known player in immune and inflammatory responses, including asthma, the OVA-induced increase in nuclear NF- κ B p65 levels in lung tissues and primary airway epithelial cells was not affected significantly by the administration of sirtinol. Therefore these findings indicate that SIRT1 might have an important role in inducing and maintaining allergic airway disease, especially T_H2-mediated inflammation, through the regulation of HIF-1 α and VEGF expression, even though an involvement of the SIRT1–NF- κ B signaling pathway in the disease could not be excluded.

Some studies have reported that PI3K inhibition reduces T_H2 cytokine production, pulmonary eosinophilia, airway inflammation, and bronchial hyperresponsiveness in a murine model of asthma.^{25,55} In addition, PI3K is shown to be involved in

FIG 6. Total cells and differential cellular components in BAL fluids, inflammation in lung tissues, airway mucus expression, and airway responsiveness of OVA-sensitized and OVA-challenged mice. The groups are defined as in the legend for Fig 2. **A**, The numbers of total and differential cellular components of BAL fluids. **B**, Inflammation scores. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. **C**, Quantitation of airway mucus expression. **D–F**, Representative PAS-stained sections of the lungs. Sampling was performed in saline-inhaled mice administered saline (Fig 6, D), OVA-inhaled mice administered saline (Fig 6, E), and OVA-inhaled mice administered 0.5 mg/kg sirtinol (Fig 6, F). The violet color indicates PAS-positive mucus expression. Bars indicate 50 μ m. **G**, Airway responsiveness in OVA-sensitized and OVA-challenged mice. Bars represent means $\pm$ SEMs from 8 mice per group. #*P* < .05 versus SAL+OVA mice. **P* < .05 versus OVA+SAL mice.

HIF-1 α activation induced by oxygen-dependent or oxygen-independent pathways.⁵⁶⁻⁵⁸ Moreover, we have recently demonstrated that HIF-1 α activity is regulated through modulation of PI3K/Akt signaling in OVA-induced airway inflammation.²¹ Consistent with these observations, in the present study levels of p-Akt protein in the lung tissues were increased after OVA inhalation. The increased levels of p-Akt were significantly reduced after administration of sirtinol. Furthermore, we have also shown that the increased levels of HIF-1 α were significantly reduced after administration of sirtinol in primary murine tracheal epithelial cells isolated from OVA-sensitized and OVA-challenged mice. The increased protein levels of HIF-1 α and VEGF in lung tissues after OVA inhalation were significantly decreased by the administration of the PI3K inhibitor LY294002 or wortmannin. Together, these findings suggest that SIRT1 regulates HIF-1 α activation, therewith inducing VEGF expression through the PI3K/Akt pathway, as well as HIF-1 α deacetylation, in a murine model of allergic airway disease.

Interestingly, in this study we have found that sirtinol, a SIRT1 enzyme inhibitor, suppressed the SIRT1 protein expression in lung tissues and primary tracheal epithelial cells of OVA-inhaled mice. SIRT1 is known to regulate its own transcription through various positive- and negative-feedback loop systems.⁵⁹ Recent studies have also revealed that the PI3K signaling pathway has an active role in SIRT1 nuclear retention.^{60,61} Supporting this contention, our data have shown that the levels of p-Akt increased by means of OVA inhalation were significantly reduced by sirtinol and that the increase of SIRT1 protein levels in lung tissues after OVA inhalation was significantly decreased by PI3K inhibition. These findings suggest that SIRT1 regulates its expression through its own transcriptional regulation system and the modulation of the nuclear localization involving the PI3K/Akt pathway.

In summary, we have shown that the administration of sirtinol was effective in reversing all pathophysiologic symptoms of allergic airway disease examined. Our data have also revealed that sirtinol substantially reduced the increase in VEGF expression, including the activity of VEGF through inhibition of HIF-1 α activation mediated by PI3K signaling in our murine model of allergic airway disease. In support of these findings, the administration of sirtinol reduced the increase in HIF-1 α activity in OVA-treated airway epithelial cells. Therefore one likely mechanism for the effectiveness of sirtinol is the reduction of VEGF expression to physiologic levels through inhibition of HIF-1 α activity stimulated in part by the PI3K/Akt signaling pathway, as well as HIF-1 α deacetylation, even though the roles of other related signaling molecules could not be excluded (see Fig E11 in this article's Online Repository at www.jacionline.org). Thus these findings provide a crucial molecular mechanism for the potential of SIRT1 inhibition in preventing or treating allergic airway disease and other airway inflammatory disorders.

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Clinical implications: These findings provide a crucial molecular mechanism for the potential of an SIRT1 inhibitor, sirtinol, in preventing or treating asthma and other airway inflammatory disorders.

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METHODS

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from Orientbio, Inc (Seoungnam, Korea); housed throughout the experiments in a laminar flow cabinet; and maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University. Mice were sensitized on days 1 and 14 by means of intraperitoneal injection of 20 μ g of OVA (Sigma-Aldrich, St Louis, Mo) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co, Rockford, Ill) in a total volume of 200 μ L, as previously described with some modifications.^{E1} On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) by using an ultrasonic nebulizer (NE-U12; Omron, Kyoto, Japan). BAL was performed at 72 hours after the last challenge. At the time of lavage, the mice (8 mice in each group) were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight administered intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Prewarmed 0.9% NaCl solution (800 μ L) was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. Part of each pool was then centrifuged, and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, Mass). The smears were stained with Diff-Quik solution (Dade Diagnostics of P.R., Inc, Aguada, Puerto Rico) to examine the cell differentials. Two independent blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of 4 different random locations. Interinvestigator variation was less than 5%. The mean number from the 2 investigators was used to estimate the cell differentials.

Administration of sirtinol, 2ME2, CBO-P11, LY294002, and wortmannin

Sirtinol (0.05 or 0.5 mg/kg body weight per day; Alexis Corp) or vehicle control (0.05% dimethyl sulfoxide) diluted with 0.9% NaCl was administered intraperitoneally 2 times to each animal, once on day 21 (1 hour before the first airway challenge with OVA) and the second time on day 23 (3 hours after the last airway challenge with OVA). An inhibitor of HIF-1 α , 2ME2 (100 mg/kg body weight per day, Calbiochem), was suspended in 0.5% carboxymethylcellulose (Calbiochem) and administered by means of oral gavage 7 times at 24-hour intervals on days 19 to 25, beginning 2 days before the first challenge.^{E2} The cyclopeptidic vascular endothelial growth inhibitor CBO-P11 (Flt-1: IC₅₀ = 700 nmol/L; Flk-1/KDR: IC₅₀ = 1.3 μ mol/L; D-Phe-Pro [79-93], Calbiochem) was used to inhibit VEGF activity. CBO-P11 (2 mg/kg body weight per day) was administered intraperitoneally 3 times at 24-hour intervals, beginning at 1 hour before the last inhalation.^{E3} LY-294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one; 1.5 mg/kg body weight per day; BIOMOL Research Laboratories, Inc, Plymouth Meeting, Pa) or wortmannin (100 μ g/kg body weight per day, Calbiochem) dissolved in dimethyl sulfoxide and diluted with 0.9% NaCl was administered intratracheally 2 times to each animal, once on day 21 (1 hour before the first airway challenge with OVA) and the second time on day 23 (3 hours after the last airway challenge with OVA).

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors, and protein concentrations were determined by using the Bradford reagent (Bio-Rad Laboratories, Inc, Hercules, Calif). Samples were loaded onto an SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by means of the wet transfer method (250 mA for 90 minutes). Nonspecific sites were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20) for 1 hour, and the blots were then incubated overnight at 4°C with an anti-IL-4 antibody (Serotec Ltd, Oxford,

United Kingdom), anti-IL-5 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-IL-13 antibody (R&D Systems, Inc, Minneapolis, Minn), anti-Akt antibody (Cell Signaling Technology, Inc, Beverly, Mass), anti-p-Akt antibody (Cell Signaling Technology, Inc), anti-IFN- γ antibody (Santa Cruz Biotechnology), or anti-VEGF antibody (Santa Cruz Biotechnology). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with an anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was visualized by means of exposure to photographic film after treatment with enhanced chemiluminescence system reagents (GE Healthcare).

Nuclear protein extractions for analysis of HIF-1 α , HIF-1 β , and SIRT1

Lung tissues or primary airway epithelial cells were homogenized in 8 volumes of a lysis buffer containing 1.3 mol/L sucrose, 1.0 mmol/L MgCl₂, and 10 mmol/L potassium phosphate buffer (pH 7.2). The homogenate was filtered through 4 layers of gauze and centrifuged at 1,000g for 15 minutes. The resulting pellets were carefully harvested and resuspended in 10 mmol/L potassium phosphate buffer (pH 7.2) containing 2.4 mol/L sucrose and 1.0 mmol/L MgCl₂ to maintain a final 2.2 mol/L sucrose concentration and centrifuged at 100,000g for 1 hour. The resulting nuclear pellets were washed once with a buffer (pH 7.2) containing 0.25 mol/L sucrose, 0.5 mmol/L MgCl₂, and 20 mmol/L Tris-HCl and centrifuged at 1,000g for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150 mmol/L NaCl, 2 mmol/L EDTA, 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor cocktails. The mixture was kept on ice for 2 hours with gentle stirring and centrifuged at 12,000g for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for detection of HIF-1 α , HIF-1 β , or SIRT1. The levels of these proteins were analyzed by means of Western blotting with antibodies against HIF-1 α (Novus Biologicals, Inc, Littleton, Colo), HIF-1 β (Novus Biologicals, Inc), SIRT1 (Abcam, Cambridge, Mass), and actin (Sigma-Aldrich), as described above.

Cytosolic or nuclear protein extractions for analysis of NF- κ B p65

Lung tissues or primary tracheal epithelial cells were homogenized in 2 volumes of buffer A (50 mmol/L Tris-HCl [pH 7.5], 1 mmol/L ethylene diamine tetraacetic acid, 10% glycerol, 0.5 mmol/L dithiothreitol, 5 mmol/L MgCl₂, and 1 mmol/L phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. The homogenates were centrifuged at 1,000g for 15 minutes at 4°C. For analysis of cytosolic NF- κ B p65, supernatants were incubated on ice for 10 minutes and centrifuged at 100,000g for 1 hour at 4°C, and the supernatant was used to determine the level of NF- κ B p65. For determination of nuclear NF- κ B levels, the resulting pellets were washed twice in buffer A and resuspended in buffer B (1.3 mol/L sucrose, 1.0 mmol/L MgCl₂, and 10 mmol/L potassium phosphate buffer [pH 6.8]) and placed in pellets at 1,000g for 15 minutes. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 mol/L and centrifuged at 100,000g for 1 hour. The resulting pellets were washed once with a solution containing 0.25 mol/L sucrose, 0.5 mmol/L MgCl₂, and 20 mmol/L Tris-HCl (pH 7.2) and centrifuged at 1,000g for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150 mmol/L NaCl, 2 mmol/L EDTA, 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 hour with gentle stirring and centrifuged at 12,000g for 30 minutes. The resulting supernatant was used for detection of nuclear NF- κ B p65. The levels of NF- κ B p65 were analyzed by means of Western blotting with antibody against NF- κ B p65 (Upstate Biotech, Lake Placid, NY), as described above.

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as previously described.^{E1} The trachea proximal to the bronchial bifurcation was

excised, and adherent adipose tissue was removed. The trachea was opened longitudinally and cut into 3 pieces. The isolated tracheas were incubated in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) containing 0.1% protease overnight at 4°C. After tissue digestion, FBS (10% final concentration) was added to the medium to deactivate enzymes, undigested fragments of tissue were removed, and tracheal epithelial cells were harvested by means of centrifugation at 100g for 5 minutes. The epithelial cells were seeded onto 35-mm collagen-coated dishes for submerged culture. The growth medium Dulbecco modified Eagle medium containing 10% FBS, penicillin, streptomycin, and amphotericin B was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The cells were maintained in a humidified 5% CO₂ incubator at 37°C until they adhered.

Sirtinol treatment on murine tracheal epithelial cells from OVA-inhaled mice

Cells were seeded in culture dishes and grown until 70% confluence. The medium was then replaced with a new medium containing vehicle (0.1% dimethyl sulfoxide) or sirtinol (2 or 10 μ mol/L) overnight at 37°C. The levels of SIRT1, HIF-1 α , or HIF-1 β in cells were analyzed by means of Western blotting with respective antibodies against them, as described above.

SIRT1 deacetylase assay

SIRT1 deacetylase activity was evaluated in crude nuclear extracts of lung tissues and primary tracheal epithelial cells. We measured SIRT1 using a deacetylase fluorometric assay kit (Sir2 assay kit; CycLex Co, Ltd), as described elsewhere.^{E4} All experiments were performed in duplicate, and the results are presented as arbitrary fluorescence units.

Measurement of T_H2 cytokine and VEGF levels

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by enzyme immunoassays, according to the manufacturer's protocol (IL-4: BioSource International, Inc, Camarillo, Calif; IL-5: Endogen, Inc, Woburn, Mass; IL-13 and VEGF: R&D Systems, Inc). Sensitivities for IL-4, IL-5, IL-13, and VEGF assays were 5, 5, 1.5, and 3.0 pg/mL, respectively.

Measurement of plasma exudation

EBD was dissolved in 0.9% saline at a final concentration of 5 mg/mL to assess lung permeability. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 minutes, the animals were killed, and their chests were opened. Normal saline containing 5 mmol/L EDTA was perfused through the aorta until all venous fluid returning to the opened right atrium was clear. Lungs were removed and weighed wet. EBD was extracted in 2 mL of formamide kept in a water bath at 60°C for 3 hours, and the absorption of light at 620 nm was measured with a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). The dye extracted was quantified by means of interpolation against a standard curve of dye concentration in the range of 0.01 to 10 μ g/mL and is expressed as nanograms of dye per milligram of wet lung.

Processing of lungs for histologic and image analysis

At 72 hours after the last challenge, lungs were removed from the mice after death. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin and 4% acetic acid) by using a ligature around the trachea. Lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. After section of the specimens, they were placed on slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, Mich) or PAS. Stained slides were all quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination.^{E5}

Histology

For histologic examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The inflammation score was graded by 3 independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere.^{E6} A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by a thin layer (1-5 cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells.

Quantitation of airway mucus expression

The numbers of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as described previously to quantitate the level of mucus expression in the airway.^{E5} Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells from each bronchiole.

Determination of airway responsiveness

Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine through the airways, as described elsewhere.^{E7} Anesthesia was achieved with 80 mg/kg pentobarbital sodium injected intraperitoneally. The trachea was then exposed through a midcervical incision and tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small-animal ventilator (flexiVent; SCIREQ, Montreal, Canada). The mouse was quasisinusoidally ventilated with a nominal tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that seen during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to a water column. Methacholine aerosol was generated with an inline nebulizer and administered directly through the ventilator. Each mouse was challenged with methacholine aerosol in increasing concentrations (2.5-50 mg/mL in saline) to determine the differences in airway response to methacholine. After each methacholine challenge, the data of calculated R_{rs} were continuously collected. Maximum values of R_{rs} were selected to express changes in airway function.

Densitometric analysis and statistics

All immunoreactive and phosphorylative signals were analyzed by using densitometric scanning (Gel Doc XR; Bio-Rad Laboratories, Inc). Data were expressed as means $\pm$ SEMs. Statistical comparisons were performed by using 1-way ANOVA, followed by the Scheffe test. Significant differences between 2 groups were determined by using the unpaired Student *t* test. Statistical significance was set at a *P* value of less than .05.

RESULTS

Levels of SIRT1 and HIF-1 α in the mice challenged with OVA without prior sensitization

The effect of challenge with OVA without prior sensitization on the SIRT1 and HIF-1 α levels in nuclear extracts was examined. Western blot analysis showed that in OVA-challenged mice with or without prior sensitization, levels of SIRT1 and HIF-1 α in nuclear protein extracts from lung tissues were increased at 72 hours after OVA challenge compared with the levels of saline-sensitized and saline-challenged mice (Fig E10). However, the increase in SIRT1 and HIF-1 α levels in nuclear protein extracts after OVA challenge without prior sensitization was significantly less than the levels seen in OVA-sensitized and OVA-challenged mice.

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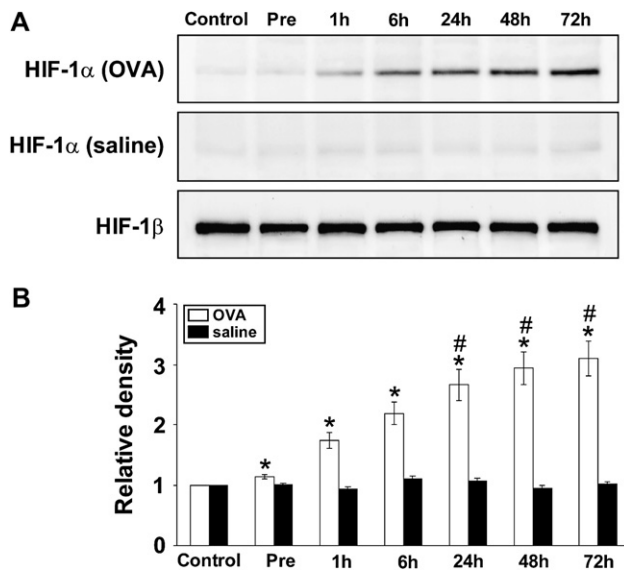


FIG E1. Levels of HIF-1 α protein in lung tissues of OVA-sensitized and OVA-challenged mice. **A**, Western blot analyses of HIF-1 α protein. **B**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of HIF-1 α in the lung tissues of control mice is arbitrarily presented as 1. Data represent means $\pm$ SEMs from 8 mice per group. One, 6, 24, 48, and 72 hours are the time periods of the sampling after the last challenge in mice sensitized and challenged with OVA or saline. *Control*, No treatment (mice with no sensitization and no challenge); *Pre*, 1 hour before the first challenge (OVA-sensitized mice without OVA challenge or saline-sensitized mice without saline challenge). # $P < .05$ versus the Pre group. * $P < .05$ versus the saline group.

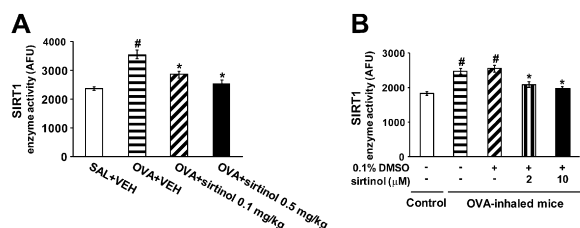


FIG E2. Effect of sirtinol on SIRT1 enzyme activity in OVA-sensitized and OVA-challenged mice. **A**, The fluorometric assay for SIRT1 deacetylase in lung tissues. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered drug vehicle (*SAL+VEH*), OVA-inhaled mice administered drug vehicle (*OVA+VEH*), OVA-inhaled mice administered 0.1 mg/kg sirtinol (*OVA+sirtinol 0.1 mg/kg*), and OVA-inhaled mice administered 0.5 mg/kg sirtinol (*OVA+sirtinol 0.5 mg/kg*). Bars represent means $\pm$ SEMs from 6 mice per group. [#] $P < .05$ versus *SAL+VEH* mice. ^{*} $P < .05$ versus *OVA+VEH* mice. **B**, Fluorometric assay for SIRT1 deacetylase in primary tracheal epithelial cells. Bars represent means $\pm$ SEMs from 6 independent experiments. *Control*, Epithelial cells isolated from saline-sensitized and saline-challenged mice; *DMSO*, dimethyl sulfoxide; *AFU*, arbitrary fluorescence units. [#] $P < .05$ versus control mice. ^{*} $P < .05$ versus OVA-inhaled mice treated with drug vehicle only.

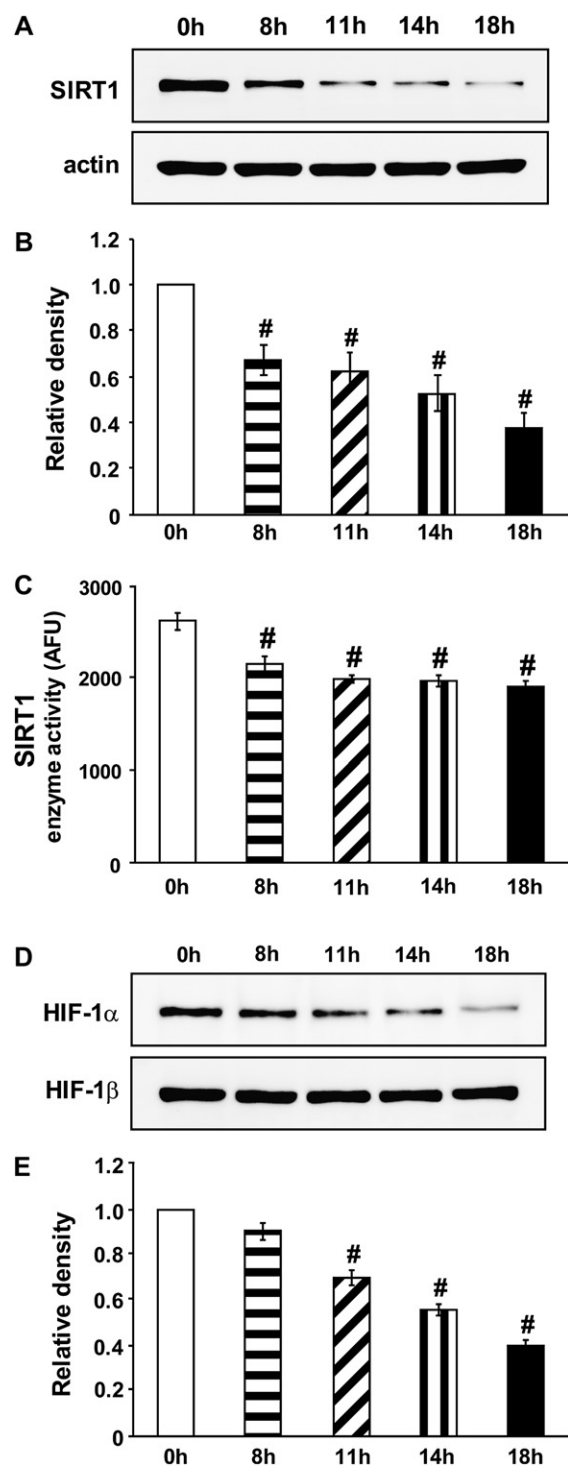


FIG E3. Kinetics of SIRT1 enzyme activity and SIRT1 and HIF-1 α protein expression after treatment with sirtinol in nuclear extracts of primary tracheal epithelial cells of OVA-sensitized and OVA-challenged mice. **A**, Western blot analyses of SIRT1. **B**, Densitometric analyses are presented as the relative ratio of SIRT1 to actin. **C**, The fluorometric assay for SIRT1 deacetylase. **AFU**, Arbitrary fluorescence units. **D**, Western blot analyses of HIF-1 α and HIF-1 β protein. **E**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of SIRT1 or HIF-1 α in airway epithelial cells of the mice before treatment with sirtinol (0h) is arbitrarily presented as 1. Each sample was incubated with sirtinol (10 μ mol/L) for 0, 8, 11, 14, and 18 hours, respectively. Bars represent means $\pm$ SEMs from 6 independent experiments. # P < .05 versus 0h.

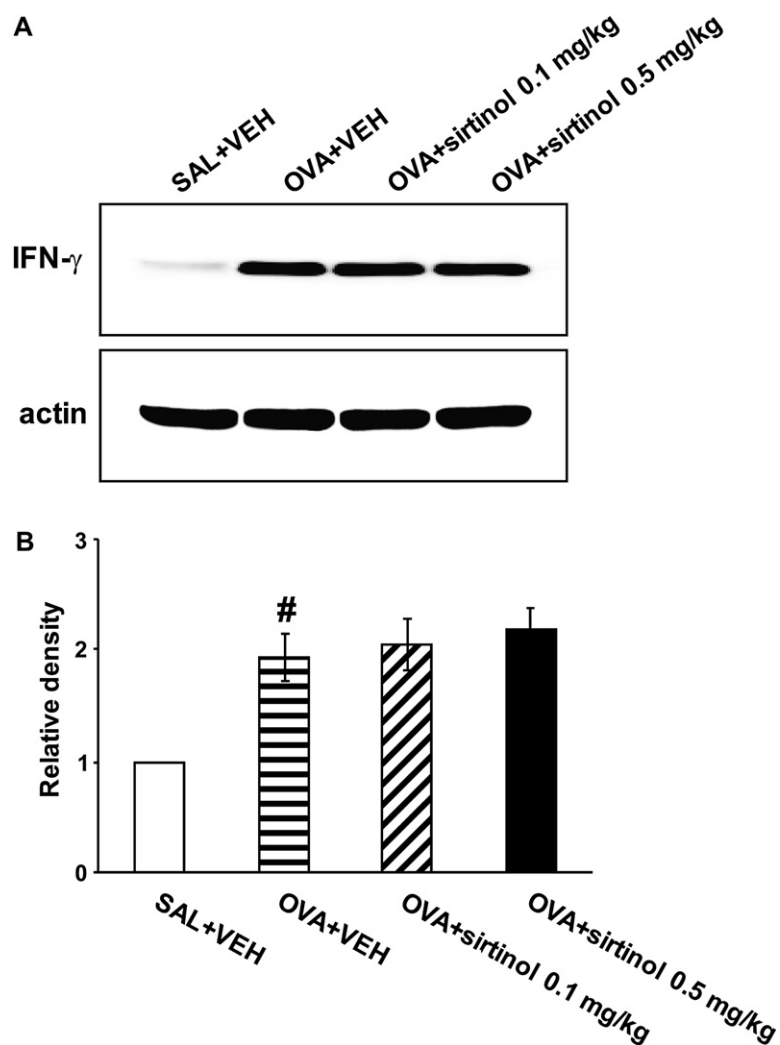
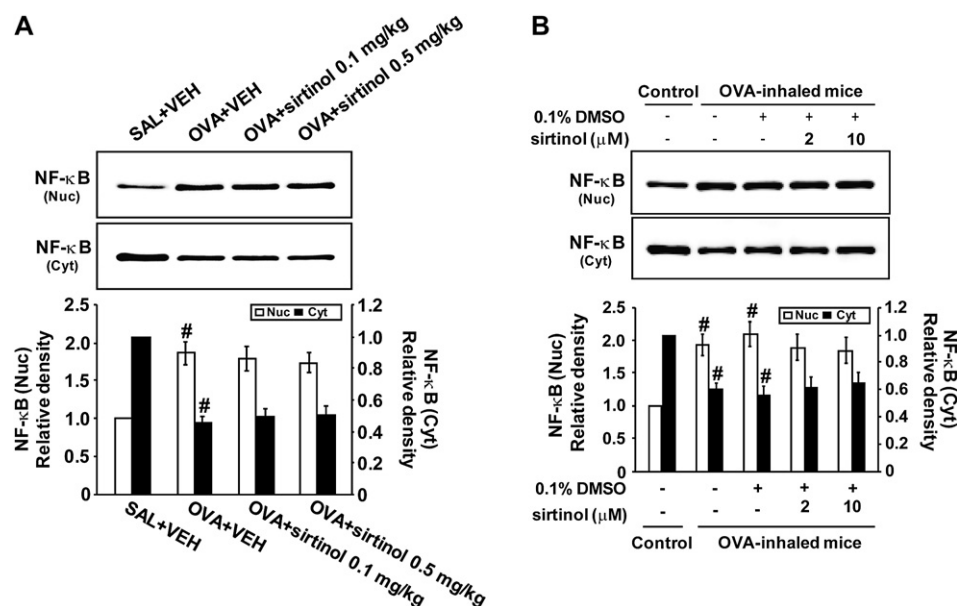


FIG E4. Effect of sirtinol on IFN- γ protein levels in OVA-sensitized and OVA-challenged mice. Groups are defined as in the legend for Fig 2. **A**, Western blotting of IFN- γ protein in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of IFN- γ to actin. The relative ratio of IFN- γ in the lung tissues of SAL+VEH mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 6 mice per group. [#] $P < 0.05$ versus SAL+VEH mice.



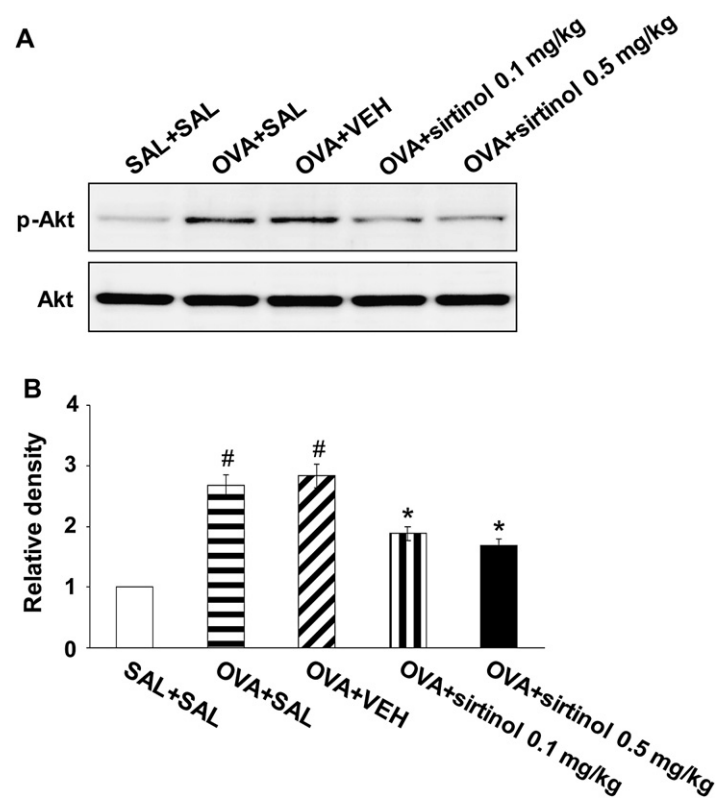


FIG E6. Effect of sirtinol on p-Akt and Akt protein levels in OVA-sensitized and OVA-challenged mice. Groups are defined as in the legend for Fig 2. **A**, Western blotting of p-Akt and Akt in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of p-Akt to Akt. The relative ratio of p-Akt in the lung tissues of SAL+SAL mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 8 mice per group. # $P < 0.05$ versus SAL+SAL mice. * $P < 0.05$ versus OVA+SAL mice.

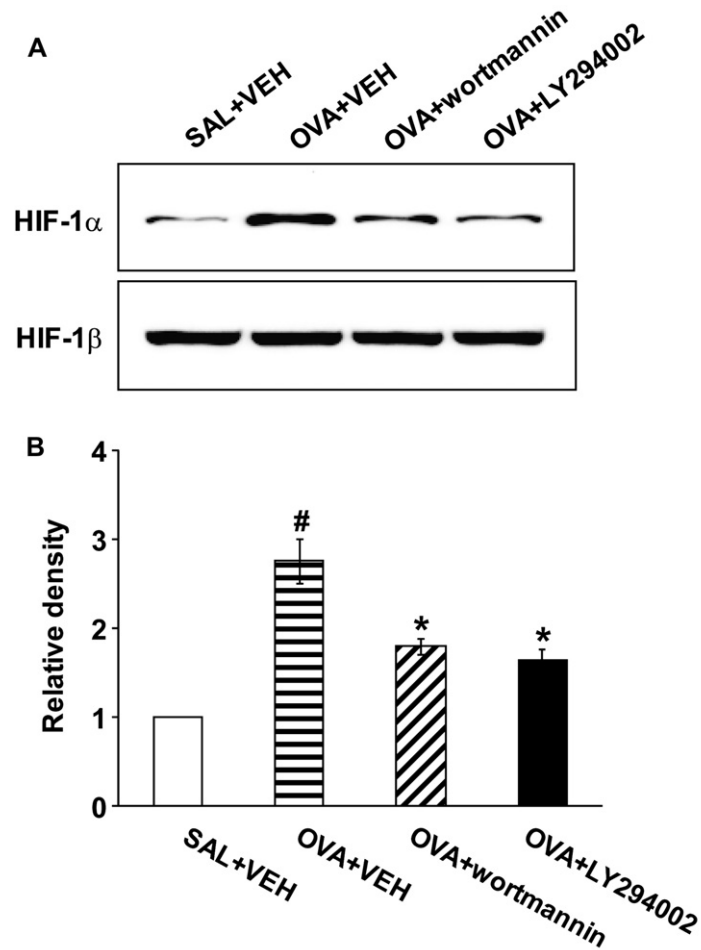


FIG E7. Effect of LY294002 or wortmannin on HIF-1 α levels in nuclear protein extracts of lung tissues from OVA-sensitized and OVA-challenged mice. Groups are defined as in the legend for Fig 2. **A**, Representative Western blot of HIF-1 α and HIF-1 β in lung tissues administered LY294002 or wortmannin. **B**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of HIF-1 α in nuclear protein extracts of the lung tissues of SAL+VEH mice is arbitrarily presented as 1. Data represent means $\pm$ SEMs from 6 mice per group. # P < .05 versus SAL+VEH mice. * P < 0.05 versus OVA+VEH mice.

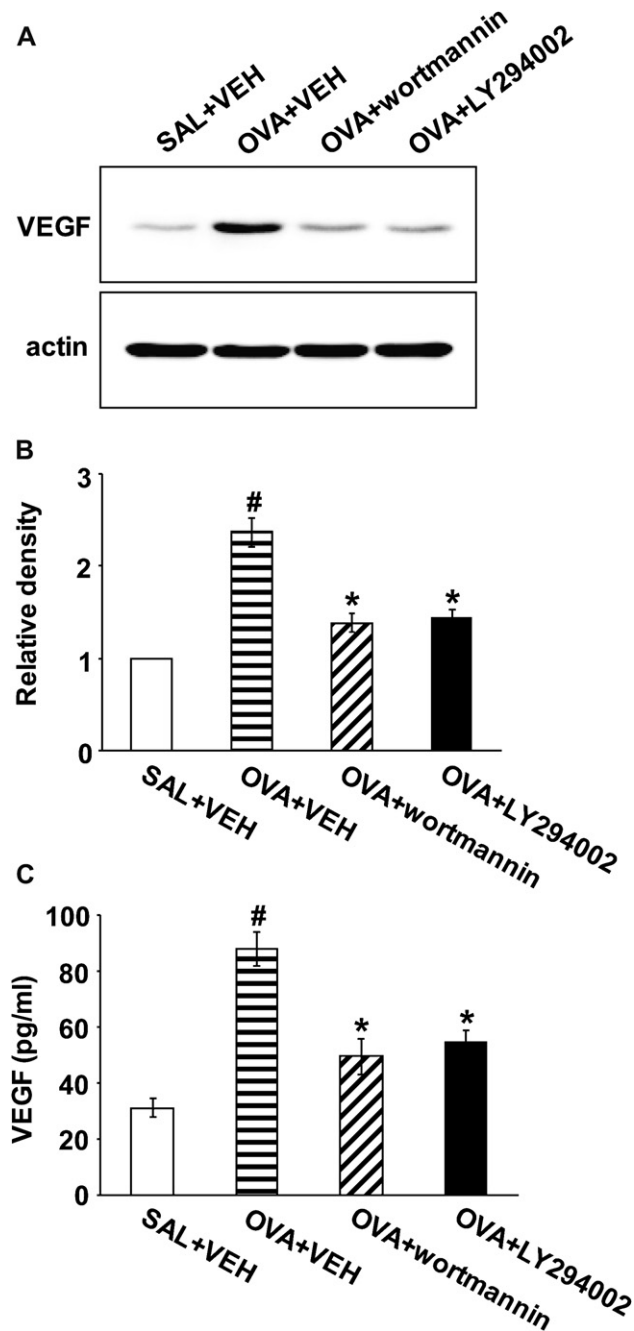


FIG E8. Effect of LY294002 or wortmannin on VEGF protein levels in lungs of OVA-sensitized and OVA-challenged mice. Groups are defined as in the legend for Fig 2. **A**, Western blot analyses of VEGF levels in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in lung tissues of SAL+VEH mice is arbitrarily presented as 1. **C**, Enzyme immunoassay of VEGF in BAL fluids. Data represent means $\pm$ SEMs from 6 mice per group. [#] $P < .05$ versus SAL+VEH mice. ^{*} $P < .05$ versus OVA+VEH mice.

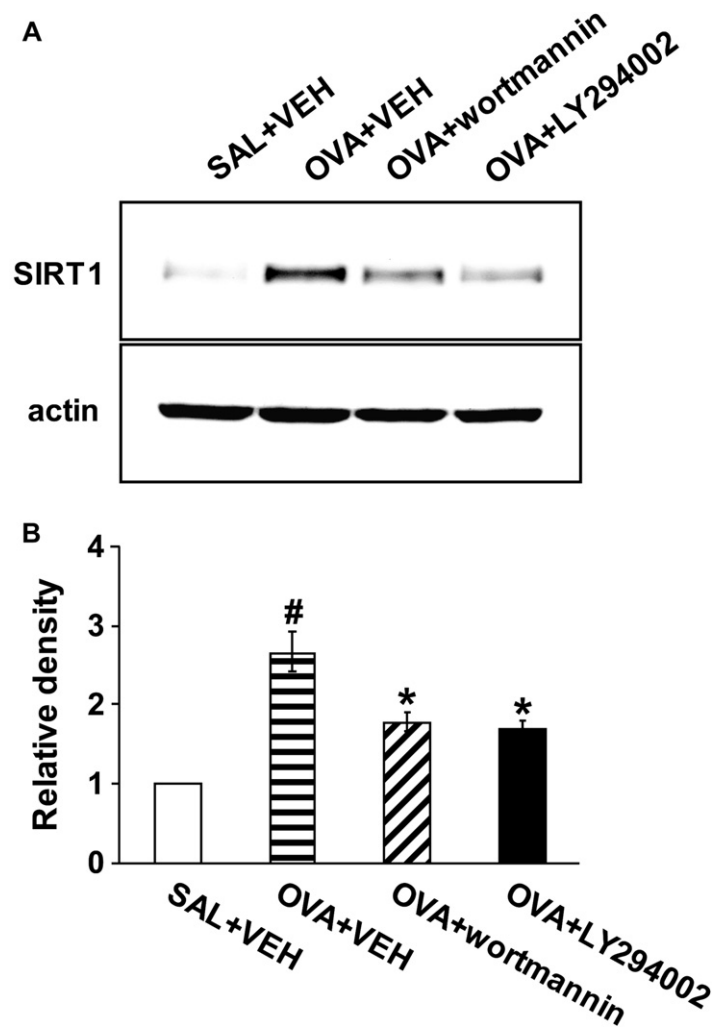


FIG E9. Effect of LY294002 or wortmannin on SIRT1 in nuclear extracts of lung tissues from OVA-sensitized and OVA-challenged mice. Groups are defined as in the legend for Fig 2. **A**, Western blot analyses of nuclear SIRT1 levels in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of SIRT1 to actin. The relative ratio of SIRT1 in lung tissues of SAL+VEH mice is arbitrarily presented as 1. Data represent means $\pm$ SEMs from 6 mice per group. # $P < 0.05$ versus SAL+VEH mice. * $P < 0.05$ versus OVA+VEH mice.

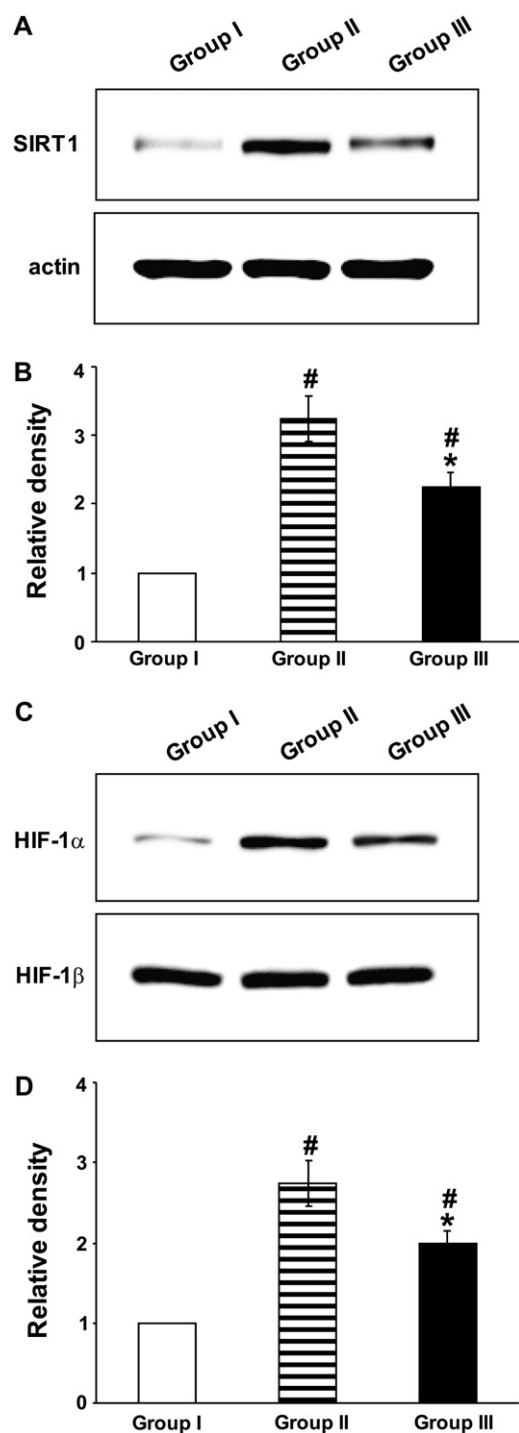


FIG E10. SIRT1 and HIF-1 α levels in nuclear extracts of lung tissues of mice challenged with OVA or saline. Sampling was performed at 72 hours after the last challenge in saline-sensitized and saline-challenged mice (group I), OVA-sensitized and OVA-challenged mice (group II), and OVA-challenged mice without prior sensitization (group III). **A**, Western blotting of SIRT1 in nuclear extracts of lung tissues. **B**, Densitometric analyses are presented as the relative ratio of SIRT1 to actin. **C**, Western blotting of HIF-1 α and HIF-1 β in nuclear extracts of lung tissues. **D**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of SIRT1 or HIF-1 α in the lung tissues of saline-sensitized and saline-challenged mice is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 6 mice per group. [#] P < .05 versus group I. ^{*} P < 0.05 versus group II.

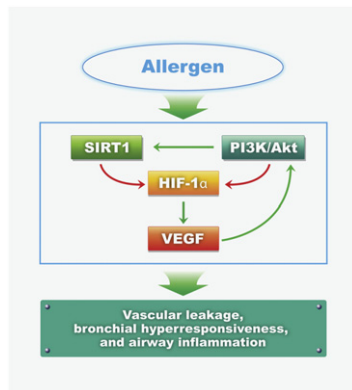


FIG E11. A proposed mechanism for the involvement of SIRT1 in allergic airway disease.