

HIF-1 α inhibition ameliorates an allergic airway disease via VEGF suppression in bronchial epithelium

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Hypoxia-inducible factor-1 α (HIF-1 α) plays a critical role in immune and inflammatory responses. One of the HIF-1 α target genes is vascular endothelial growth factor (VEGF), which is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation in allergic airway diseases. Using OVA-treated mice and murine tracheal epithelial cells, the signaling networks involved in HIF-1 α activation and the role of HIF-1 α in the pathogenesis of allergic airway disease were investigated. Transfection of airway epithelial cells with HIF-1 α siRNA suppressed VEGF expression. In addition, the increased levels of HIF-1 α and VEGF in lung tissues after OVA inhalation were substantially decreased by an HIF-1 α inhibitor, 2-methoxyestradiol. Our data also show that the increased numbers of inflammatory cells, increased airway hyperresponsiveness, levels of IL-4, IL-5, IL-13, and vascular permeability in the lungs after OVA inhalation were significantly reduced by 2-methoxyestradiol or a VEGF inhibitor, CBO-P11. Moreover, we found that inhibition of the PI3K p110 δ isoform (PI3K- δ) or HIF-1 α reduced OVA-induced HIF-1 α activation in airway epithelial cells. These findings indicate that HIF-1 α inhibition may attenuate antigen-induced airway inflammation and hyperresponsiveness through the modulation of vascular leakage mediated by VEGF, and that PI3K- δ signaling may be involved in the allergen-induced HIF-1 α activation.

Key words: Allergic airway disease · Hypoxia-inducible factor-1 α · PI3K- δ · RNA interference · vascular endothelial growth factor



Supporting Information available online

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways that is characterized by airway remodeling with an increased vascular permeability that causes secretion of intravas-

cular components [1]. Exudation of plasma proteins into the airways contributes to airway obstruction and hyperresponsiveness [2, 3]. Studies have also revealed prominent increases in blood vessel numbers, size, vascular surface area, and vascular leakage, and shown a close correlation between such alterations and disease severity in asthma [3, 4].

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Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that mediates gene expression in response to cellular oxygen concentrations [5]. HIF-1 is composed of two subunits, HIF-1 α and HIF-1 β . While the β -subunit protein is constitutively expressed, the stability of the α -subunit and its transcriptional activity are controlled by the intracellular oxygen concentration [6]. 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 *via* oxygen independent pathways [7]. HIF-1 α has been reported to play an important role in inflammatory responses [8, 9]. Upon activation, HIF-1 α is known to stimulate the expression of genes that promote angiogenesis, vasodilation, vascular permeability, and glucose uptake [10]. In addition to HIF-1 α , three HIF- α isoforms have been identified to date with an obvious tissue-restricted expression pattern. Unlike HIF-1 α , which is ubiquitously expressed in organisms, HIF-2 α and HIF-3 α , which share pronounced sequence homology with HIF-1 α [11–13], are restricted to specific tissues [14, 15].

One of the genes whose expression is regulated by HIF-1 α is vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogenic peptide, which plays a key role in vasculogenesis and angiogenesis [16]. VEGF also increases vascular permeability and leads to airway inflammation [3, 17]. Recently, we have demonstrated that VEGF is one of the major inducers of asthma and thus the inhibition of VEGF may be a good therapeutic strategy [3]. In addition, Lee *et al.* have reported that VEGF is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and Th2 inflammation [17]. In addition, PI3K/Akt signaling has been shown to increase levels of HIF-1 α protein [18]. However, there are little data on the roles and molecular basis of HIF-1 α activation in allergic airway diseases.

In the current study, we investigated the signaling networks involved in HIF-1 α activation and the role of HIF-1 α in pathogenesis of allergic airway disease using primary mouse tracheal epithelial cells and a murine model of OVA-induced allergic airway disease. The results showed that HIF-1 α is activated in antigen-induced airway disease through PI3K- δ signaling.

Results

HIF-1 α RNA interference reduces the expression of HIF-1 α and VEGF in bronchial epithelium

Activation of HIF-1 α induces VEGF expression that is abnormally enhanced in asthma. Involvement of HIF-1 α activation in VEGF expression in bronchial epithelial cells from OVA-treated mice was evaluated using siRNA for HIF-1 α . The levels of nuclear HIF-1 α protein and VEGF protein in primary tracheal epithelial cells isolated from OVA-treated mice were increased compared with the levels in tracheal epithelial cells from the control mice (Fig. 1A). RNA interference using siRNA for HIF-1 α reduced the increased levels of HIF-1 α and VEGF in bronchial epithelial cells of OVA-treated mice. Additionally, RT-PCR and real-time RT-PCR

analyses revealed that the increased mRNA levels of HIF-1 α and VEGF were substantially decreased by the transfection of siRNA targeting HIF-1 α (Fig. 1B–D).

HIF-2 α RNA interference reduces the expression of HIF-2 α and VEGF in bronchial epithelium

Western blot analysis showed that levels of nuclear HIF-2 α protein and VEGF protein in primary tracheal epithelial cells isolated from OVA-treated mice were increased as compared with the levels in tracheal epithelial cells from the control mice (Supporting Information Fig. 1A). The RNA interference with siRNA for HIF-2 α reduced the increased levels of HIF-2 α and VEGF in bronchial epithelial cells isolated from OVA-treated mice. Consistent with the results, RT-PCR and real-time RT-PCR analyses revealed that the increased mRNA levels of HIF-2 α and VEGF were substantially decreased by the transfection of siRNA targeting HIF-2 α (Supporting Information Fig. 1B–D).

Effects of 2-methoxyestradiol (2ME2) on HIF-1 α protein levels in OVA-treated mice

The effects of 2ME2, an inhibitor of HIF-1 α translation, on HIF-1 α protein levels were evaluated in nuclear protein extracts of lung tissues and primary tracheal epithelial cells isolated from OVA-treated and control mice. HIF-1 α levels were increased in OVA-treated mice, as compared with the levels in the control mice (Fig. 2A, B, E, and F). The increased HIF-1 α levels in nuclear protein extracts were decreased by *in vitro* treatment with 2ME2 (Fig. 2A and B) as well as by oral administration of 2ME2 (Fig. 2E and F).

Effect of IC87114 on HIF-1 α protein levels in airway epithelial cells of OVA-treated mice

PI3K signaling has been shown to increase levels of HIF-1 α protein [18]. We examined whether activation of HIF-1 α is mediated through PI3K- δ signaling in allergic airway disease using primary tracheal epithelial cells isolated from OVA-treated and control mice. As expected, Western blot analysis showed that levels of HIF-1 α protein in nuclear protein extracts of tracheal epithelial cells from OVA-treated mice were increased significantly, as compared with the levels in tracheal epithelial cells from the control mice (Fig. 2C and D). Treatment with the PI3K- δ inhibitor IC87114 reduced significantly the increased HIF-1 α levels in tracheal epithelial cells from OVA-treated mice.

2ME2 and CBO-P11 reduce increased VEGF and plasma exudation of OVA-treated mice

Involvement of HIF-1 α in VEGF expression was evaluated using their respective inhibitors. Levels of VEGF protein in lung tissues

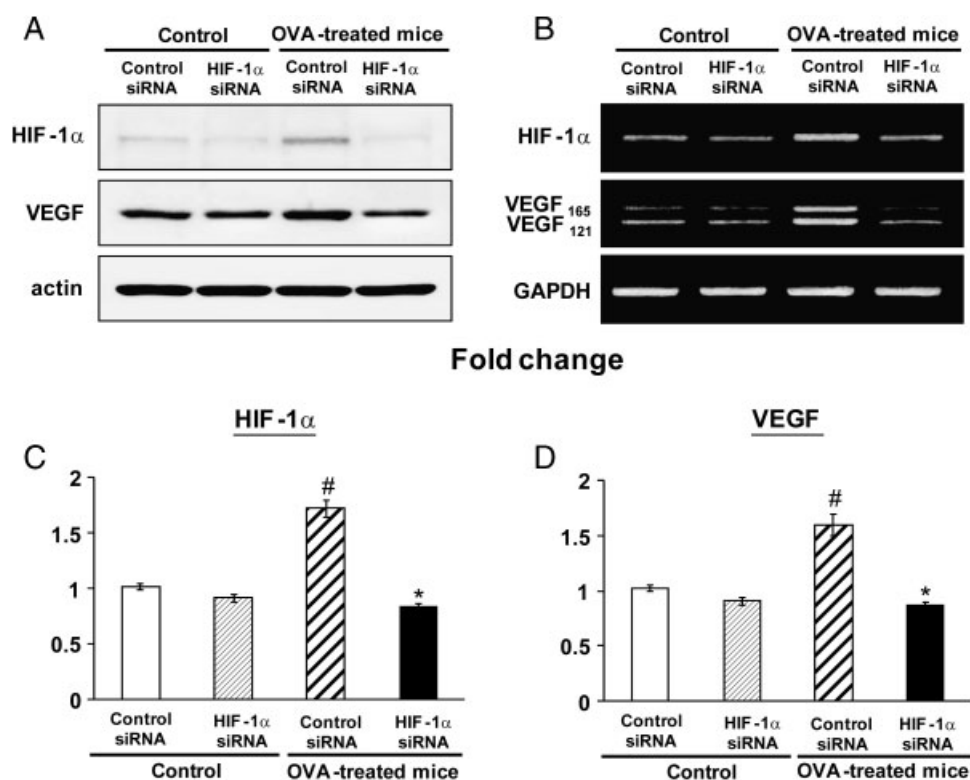


Figure 1. Increased expression of HIF-1 α and VEGF in bronchial epithelial cells of OVA-treated mice. Primary cultured tracheal epithelial cells isolated from OVA-treated and saline-treated mice were transfected with HIF-1 α or control siRNA. HIF-1 α and VEGF (A) protein and (B) mRNA levels were determined by Western blotting and RT-PCR, respectively. The data are representative of four independent experiments. Quantitative analysis of (C) HIF-1 α and (D) VEGF mRNA expression; mean $\pm$ SEM of four independent experiments (i.e. eight samples per group). Ten saline-treated mice and ten OVA-treated mice were used for each experiment. In each experiment, two samples per group were used for the quantitative analysis of mRNA expression. Statistical comparisons were performed using one-way ANOVA. # p < 0.05 versus both groups of control mice, * p < 0.05 versus cells from OVA-treated mice transfected with control siRNA.

and BALF were significantly increased 48 h after the last challenge of OVA, as compared with the levels in the control mice, and administration of 2ME2 (HIF-1 α translation inhibitor) or CBO-P11 (VEGF receptor inhibitor) substantially reduced the increased VEGF protein levels in lung tissues (Fig. 3A and B) and BALF (Fig. 3C).

In addition, Evans blue dye assay revealed that plasma extravasation was significantly increased 48 h after the last challenge of OVA (Fig. 3D). The increase in plasma extravasation was significantly reduced by administration of 2ME2 or CBO-P11.

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

To determine whether inhibition of HIF-1 α and VEGF suppresses Th2 inflammation in lungs of OVA-treated mice, we measured levels of Th2 cytokines. As shown in Fig. 4, the levels of IL-4, IL-5, and IL-13 in lung tissues and BALF were significantly increased 48 h after the last challenge of OVA, as compared with the levels in the control mice. The increased IL-4, IL-5, and IL-13 levels after the OVA inhalation were decreased significantly by administration of 2ME2 or CBO-P11.

Effects of 2ME2 or CBO-P11 on cellular changes in BALF of OVA-treated mice

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BALF were increased significantly 48 h after OVA inhalation, as compared with the numbers in BALF of the control mice (Fig. 5A). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by administration of 2ME2 or CBO-P11.

2ME2 or CBO-P11 reduces airway hyperresponsiveness of OVA-treated mice

Effects of the inhibitors of HIF-1 α and VEGF receptor on airway responsiveness were evaluated by measuring methacholine-mediated respiratory system resistance (R_{rs}). As presented in Fig. 5, at dose of 50 mg/mL of methacholine, percent R_{rs} increased significantly in the OVA-treated mice, as compared with the controls. Administration of 2ME2 or CBO-P11 to OVA-treated mice significantly reduced the levels of R_{rs} at 50 mg/mL of methacholine inhalation, as compared with the untreated mice. These results suggest that administration of 2ME2 or CBO-P11 reduces OVA-induced airway hyperresponsiveness.

Effect of 2ME2 or CBO-P11 on pathological changes of OVA-treated mice

Histologic analysis revealed that numerous inflammatory cells as well as eosinophils infiltrated tissue around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Fig. 5D and E), as compared to the control (Fig. 5C). Mice treated with 2ME2 (Fig. 5F) or CBO-P11 (Fig. 5G) showed marked reductions in the thickening of airway epithelium, infiltration of inflammatory cells in the peribronchial region, number of inflammatory cells, and amount of debris in the airway lumen. The results of inflammatory scoring showed that the increased peribronchial, perivascular, and total lung inflammation after OVA inhalation was significantly decreased by administration of 2ME2 or CBO-P11 (Fig. 5H).

Effect of 2ME2 or CBO-P11 on airway mucus expression of OVA-treated mice

Percentage of airway epithelium, which stained positively with periodic acid-Schiff (PAS) in OVA-treated mice (Fig. 5J, K, and N), was substantially greater than in the control mice (Fig. 5I and N). The increased levels of PAS-positive airway epithelium after OVA inhalation were decreased significantly by treatment of 2ME2 (Fig. 5L and N) or CBO-P11 (Fig. 5M and N).

Effects of IC87114 on Akt phosphorylation and PI3K enzyme activity in OVA-treated mice

To ascertain the inhibitory effect of IC87114 on PI3K- δ , we determined levels of Akt phosphorylation by Western blotting and PI3K activity by phosphatidyl inositol-3,4,5-triphosphate (PIP3) competition enzyme immunoassay. Levels of phosphorylated Akt (p-Akt) protein in lung tissues were significantly increased 48 h after OVA inhalation, as compared with the levels in the control mice (Fig. 6A and B). However, no significant changes in Akt protein levels were observed in any of the group tested. The increased p-Akt, but not Akt protein, levels in lung tissues after OVA inhalation were significantly reduced by administration of IC87114. Supporting the results, the increased PIP3 levels in lung tissues after OVA inhalation were significantly decreased by administration of IC87114 (Fig. 6C).

Discussion

HIF-1 α plays an important role in immune and inflammatory responses [8, 9]. In fact, HIF-1 α is activated oxygen dependently or independently by various mediators including hypoxia, nitric oxide, reactive oxygen species, cytokines, apoptotic cell debris, and infectious pathogens in inflammatory tissues [19–27]. Studies have shown that HIF-1 α activation during inflammation enhances its target gene expression such as VEGF, glucose transporter 1, and metalloproteinase [20, 28]. In addition, a close interaction between HIF-induced glycolytic energy production

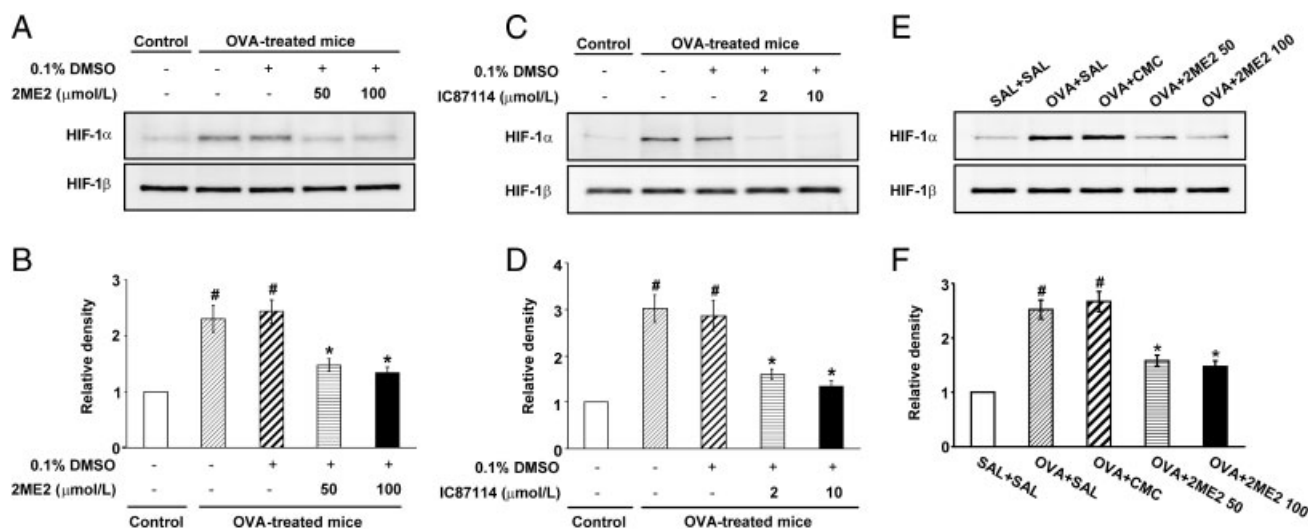


Figure 2. 2ME2 or IC87114 decreases HIF-1 α levels in OVA-treated mice. Primary airway epithelial cells of OVA-treated mice were cultured in the presence/absence of (A, B) 2ME2 for 24 h or (C, D) IC87114 for 2 h and HIF-1 α and HIF-1 β protein levels in the nuclear extracts determined. (A, C) Representative Western blot and (B, D) densitometric analyses (mean $\pm$ SEM) of 6 independent experiments (i.e. six samples per group). Ten saline-treated mice and ten OVA-treated mice were used for each experiment, in which one sample per group was quantified. (E, F) The levels of HIF-1 α and HIF-1 β in nuclear protein extracts from lung tissues were measured 48 h after the last challenge in saline/OVA-treated mice administered with either saline, or 0.5% carboxymethylcellulose, or 50 or 100 mg/kg of 2ME2. (E) Representative Western blot and (F) densitometric analysis (mean $\pm$ SEM) of eight mice per group. All densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β with the ratio in control mice being set as 1. Statistical comparisons were performed using one-way ANOVA. # p <0.05 versus control/SAL+Sal mice, * p <0.05 versus OVA-treated mice without 2ME2 or IC87114 (B, D) or versus OVA+Sal and OVA+CMC (F).

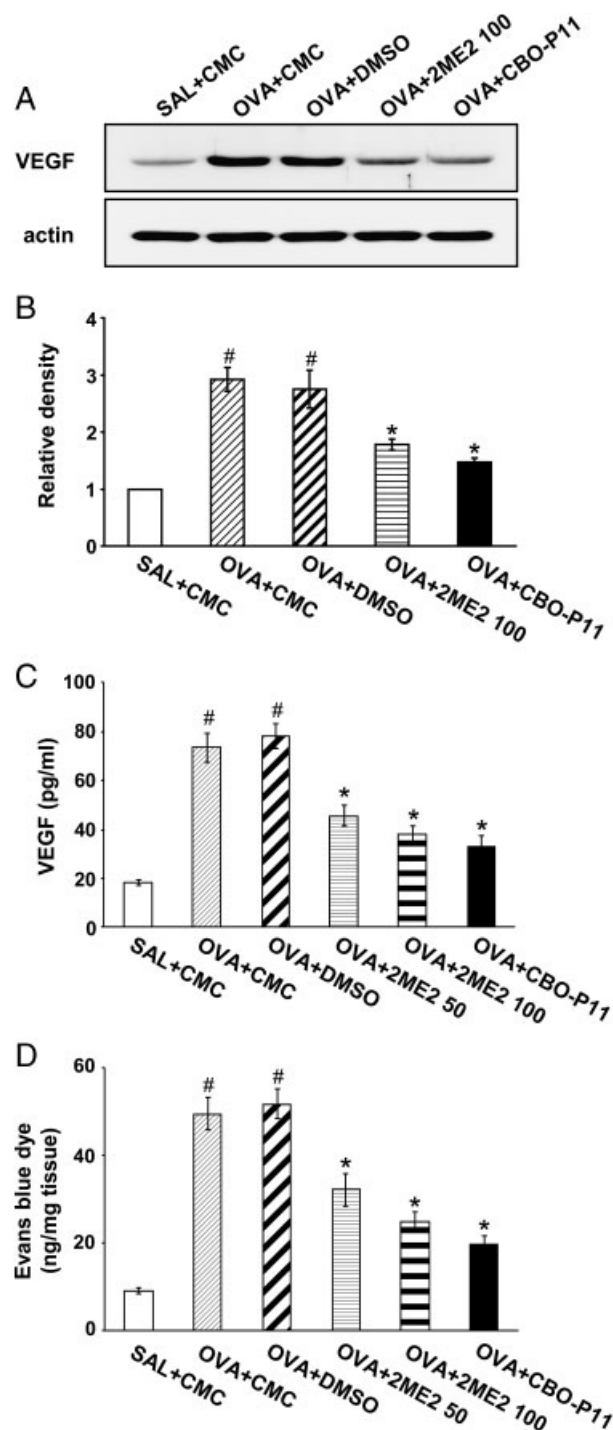


Figure 3. Effect of 2ME2 or CBO-P11 on VEGF expression and plasma exudation in OVA-treated mice. Sampling was performed 48 h after the last challenge in saline/OVA-treated mice administered with either 0.5% carboxymethylcellulose, or 0.05% DMSO, or 50 or 100 mg/kg of 2ME2, or CBO-P11. (A) Representative Western blot analysis of VEGF. (B) Densitometric analysis (mean $\pm$ SEM) of eight mice per group. All densitometric analyses are presented as the relative ratio of VEGF to actin with the ratio in control mice being set as 1. (C) Enzyme immunoassay of VEGF in BALF (mean $\pm$ SEM) of eight mice per group. (D) The plasma exudation was quantified by Evans blue dye assay (mean $\pm$ SEM) of eight mice per group. Statistical comparisons were performed using one-way ANOVA. # p < 0.05 versus SAL+CMC, * p < 0.05 versus OVA+CMC.

and immune cell function has been reported [29]. HIF-1 α activation promotes motility, invasiveness, and bacterial killing of neutrophils and macrophages in bacterial induced inflammation [29]. Furthermore, knockdown of HIF-1 α gene in dendritic

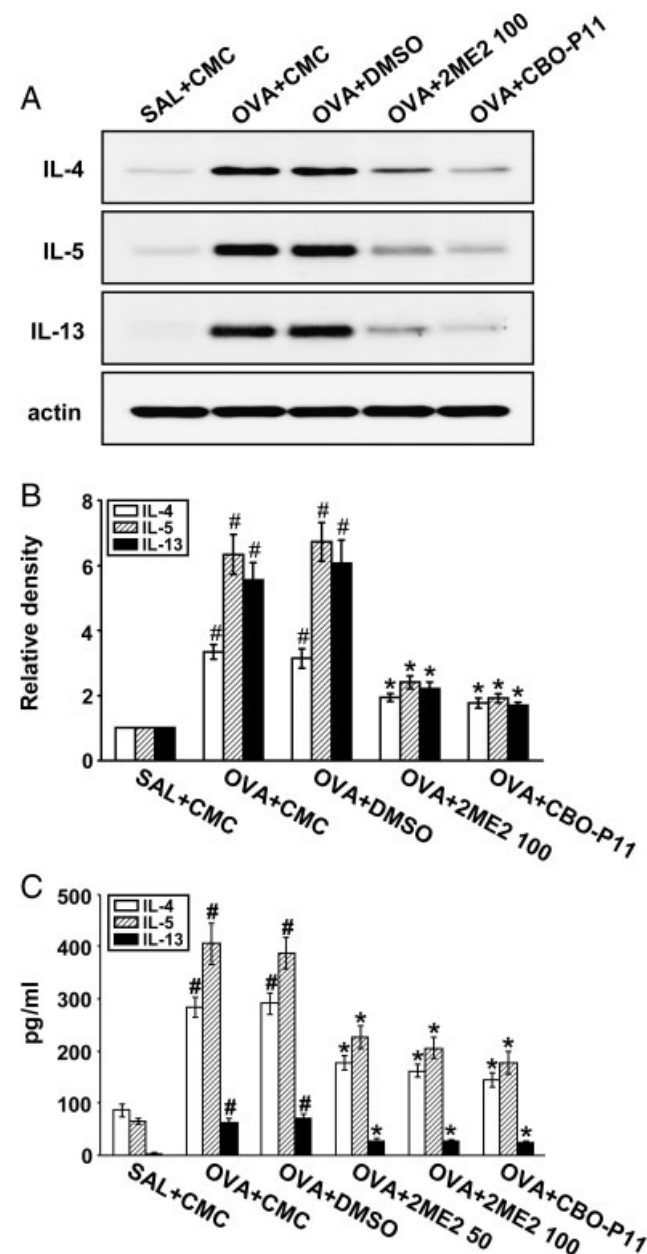


Figure 4. Effect of 2ME2 or CBO-P11 on IL-4, IL-5, and IL-13 levels in lung tissues and in BALF of OVA-treated mice. Sampling was performed 48 h after the last challenge in saline/OVA-treated mice administered with either 0.5% carboxymethylcellulose, or 0.05% DMSO, or 50 or 100 mg/kg of 2ME2, or CBO-P11. (A) Representative Western blotting of IL-4, IL-5, and IL-13 in lung tissues. (B) Densitometric analysis (mean $\pm$ SEM) of eight mice per group. All densitometric analyses are presented as the relative ratio of each molecule to actin with the ratio in control mice being set as 1. (C) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BALF (mean $\pm$ SEM) of eight mice per group. Statistical comparisons were performed using one-way ANOVA. * p < 0.05 versus OVA+CMC.

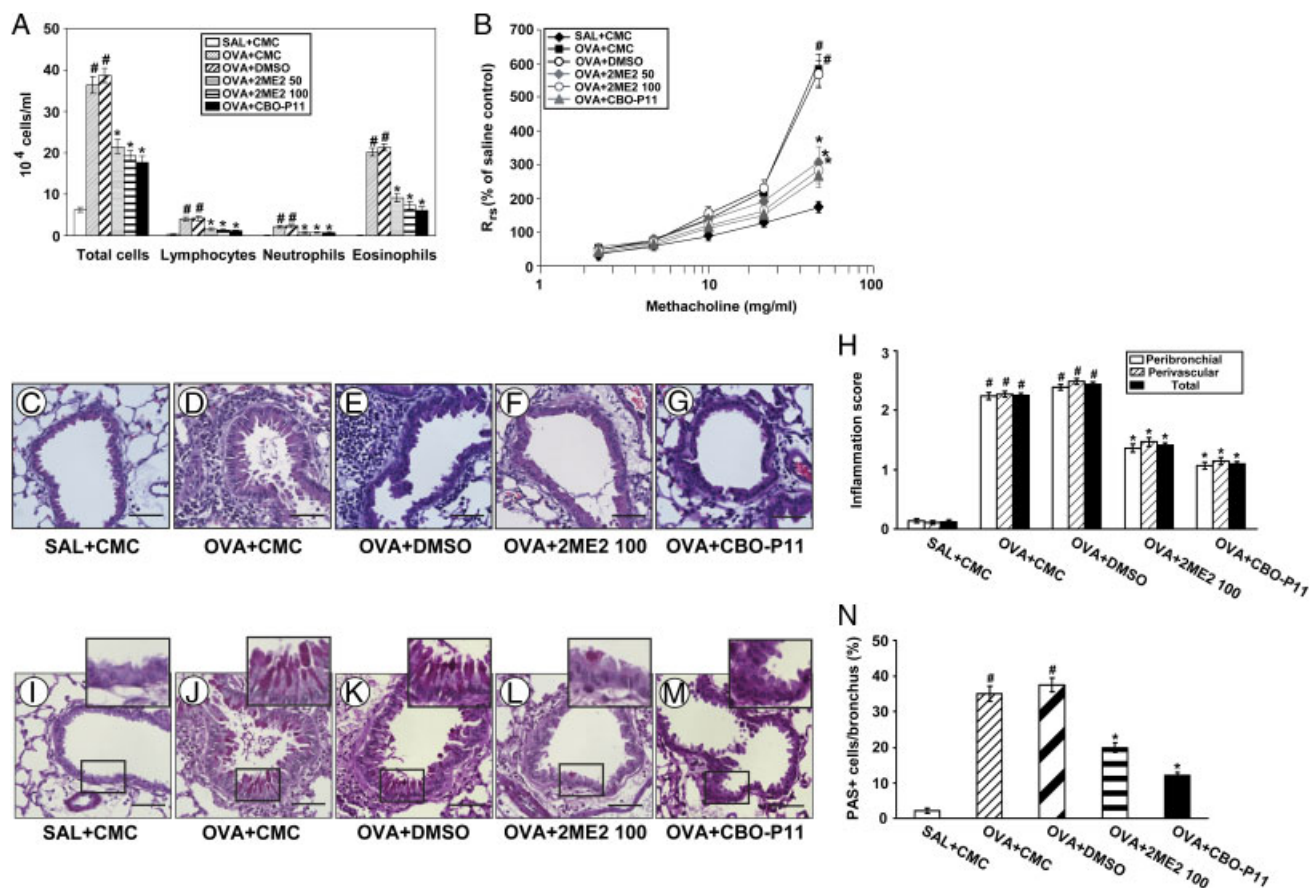


Figure 5. 2ME2 or CBO-P11 reduces airway inflammation and airway hyperresponsiveness of OVA-treated mice. Sampling was performed 48 h after the last challenge in saline/OVA-treated mice administered with either 0.5% carboxymethylcellulose, or 0.05% DMSO, or 50 or 100 mg/kg of 2ME2, or CBO-P11. (A) Cell numbers in BALF (mean $\pm$ SEM) of eight mice per group. (B) Airway responsiveness was assessed as a percent increase of R_{rs} in response to increasing doses of methacholine (mean $\pm$ SEM) of eight mice per group. (C–G) Representative hematoxylin and eosin-stained sections of the lungs from the indicated mice (C) SAL+CMC, (D) OVA+CMC, (E) OVA+DMSO, (F) OVA+2ME2 100, and (G) OVA+CBO-P11 (magnification, $\times 200$). Bars indicate 50 μ m. (H) Peribronchial and perivascular lung inflammation was measured and total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. (I–N) Representative PAS-stained sections of the lungs of the indicated mice (I) SAL+CMC, (J) OVA+CMC, (K) OVA+DMSO, (L) OVA+2ME2 100, and (M) OVA+CBO-P11, (magnification, $\times 200$). The violet color indicates PAS-positive mucus expression. Each inset highlights mucus producing lesions and enlarges the indicated area 4 times on screen of the indicated area in each digital image. Bars indicate 50 μ m. (N) Results are expressed as the percentage of PAS-positive cells per bronchiole (mean $\pm$ SEM) of eight mice per group. Statistical comparisons were performed using one-way ANOVA. # $p < 0.05$ versus SAL+CMC, * $p < 0.05$ versus OVA+CMC.

cells reduces glucose utilization and impairs the capability to stimulate T cells [30].

One of the target genes of HIF-1 α is VEGF, which is known as an important mediator of airway inflammatory diseases [31]. However, the roles of HIF-1 α and its activation mechanism in allergic airway diseases remain unknown. Our present study with a murine model of OVA-induced airway inflammation and primary bronchial epithelial cells has indicated that HIF-1 α is involved in the antigen-induced airway inflammation and hyperresponsiveness through regulation of VEGF expression and that PI3K- δ signaling activates HIF-1 α 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 [32], VEGF

appears to be the critical mediator of vascular permeability in asthma [3, 16, 33, 34]. The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesiculo-vacuolar organelles [17, 33]. VEGF can be produced by a wide variety of cells such as macrophages, neutrophils, eosinophils, and lymphocytes [3, 17, 33–35]. Several studies have shown that overproduction of VEGF causes an increase in vascular permeability, which results in leakage of plasma proteins, inflammatory mediators, and inflammatory cells into the extravascular space thereby allowing migration of inflammatory cells into the airway [3, 33, 36]. In addition, VEGF also plays a crucial role in adaptive Th2-mediated inflammation [17].

Consistent with these observations, we have found that allergic airway disease of mice induced by OVA inhalation

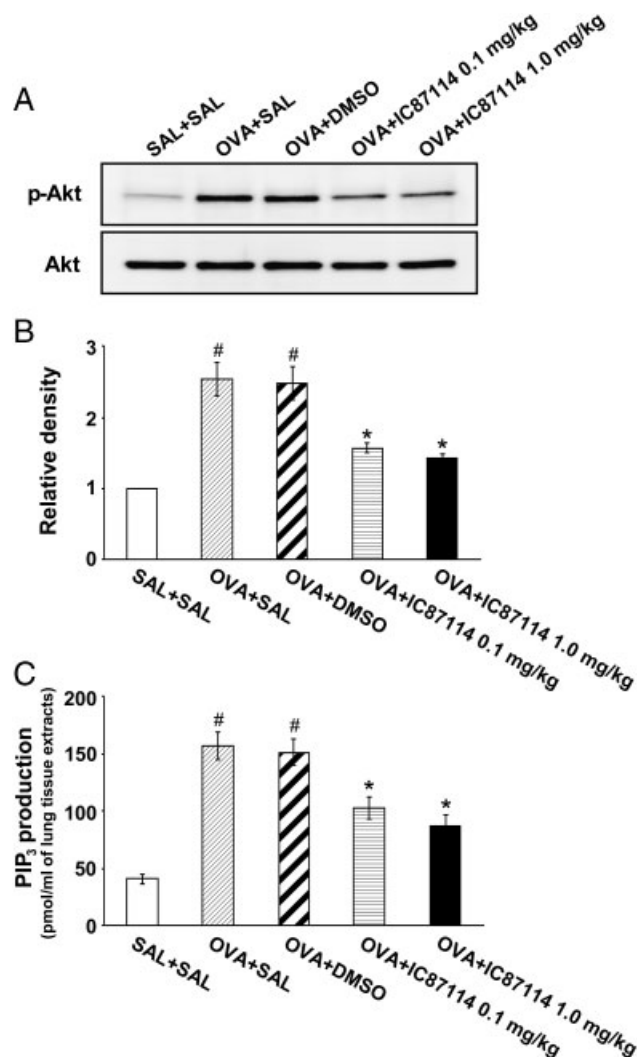


Figure 6. IC87114 suppresses p-Akt protein levels and PI3K enzyme activity in OVA-inhaled mice. Sampling was performed 48 h after the last challenge in saline/OVA-treated mice administered with either saline, or 0.05% DMSO, or 0.1 or 1.0 mg/kg of IC87114. (A) Representative Western blotting of p-Akt and Akt in lung tissues. (B) Densitometric analysis of Western blots (mean $\pm$ SEM) of eight mice per group. All densitometric analyses are presented as the relative ratio of p-Akt to Akt with the ratio in control mice being set as 1. (C) Enzyme immunoassay of PIP₃ generation by PI3K in lung tissue extracts (mean $\pm$ SEM) of eight mice per group. Statistical comparisons were performed using one-way ANOVA. [#] $p < 0.05$ versus SAL+Sal, ^{*} $p < 0.05$ versus OVA+Sal.

resulted in up-regulation of VEGF expression, increases in IL-4, IL-5, and IL-13 levels, and enhancement of vascular permeability. The increased VEGF, IL-4, IL-5, and IL-13 levels, vascular permeability, bronchial inflammation, and airway hyperresponsiveness were significantly reduced after administration of a VEGF receptor inhibitor, CBO-P11. This inhibitor is a cyclic peptide of 17 amino acids derived from VEGF residue 79–93 and thus blocks binding of VEGF to its receptor, thereby VEGF signaling is obstructed [37]. In addition, our previous studies with a murine model of asthma have revealed that the VEGF receptor tyrosine kinase inhibitors SU5614 and SU1498 reduce

asthmatic features such as the increase in Th2 cytokines, VEGF, vascular permeability, inflammatory cells in airways, and airway hyperresponsiveness [3, 38, 39]. Together, these findings suggest that VEGF is a key player in inducing and maintaining allergic airway disease.

HIF-1 α regulates VEGF expression, and activation of HIF-1 α is controlled by a variety of inflammatory cytokines and growth factors as well as by cellular oxygen concentrations [7]. Very recently, we have shown that increased expression of VEGF after OVA inhalation is decreased by administration of an HIF-1 α inhibitor [9]. In keeping with these observations, determination of HIF-1 α protein levels in nuclear extracts in this study revealed that this protein is substantially increased in our current mouse model of OVA-induced allergic airway disease and tracheal epithelial cells isolated from OVA-treated mice, suggesting that HIF-1 α is activated. The increased levels of HIF-1 α were significantly reduced after administration of 2ME2 or transfection of siRNA targeting HIF-1 α . In addition, the increased levels of VEGF in lungs after OVA inhalation were significantly reduced by administration of 2ME2 *in vivo*, and the increased mRNA and protein levels of VEGF in tracheal epithelial cells isolated from OVA-treated mice were dramatically decreased by siRNA targeting HIF-1 α *in vitro*. Taken together, these findings suggest that HIF-1 α inhibition suppresses the VEGF expression in lungs, specifically in tracheal epithelial cells, of allergic airway disease.

2ME2 was initially introduced as a direct angiogenic inhibitor having antiproliferative and proapoptotic effects on endothelial cells. Recently, 2ME2 has been shown to inhibit activation of HIF-1 α by suppressing HIF-1 α translation and its nuclear translocation [40]. Therefore, on the basis of our present observations, we suggest that 2ME2 could reduce the levels of HIF-1 α protein in the nuclear fractions from lung tissues and airway epithelial cells of OVA-treated mice through the inhibition of HIF-1 α translation and its nuclear translocation, thereby suppresses the VEGF expression. However, the effects through other mechanisms of 2ME2 cannot be overlooked.

In addition, our results have also revealed a dramatic reduction in allergen-induced goblet cell hyperplasia in 2ME2-treated mice. Since Th2 cytokines, VEGF, T cells, and eosinophils are required to produce airway mucus accumulation and goblet cell degranulation [17, 41, 42], the decrease in allergen-induced goblet cell hyperplasia by 2ME2 may be attributed to a substantial drop in the levels of Th2 cytokines and VEGF as well as reduction in eosinophilia in OVA-treated mice.

Meanwhile, VEGF also represents one of the most important targets preferentially regulated by HIF-2 α [43]. HIF-2 α , one isoform of HIF- α subunits, is also referred to as endothelial PAS domain protein-1 or HIF-1 α -like factor and bears functional resemblance to HIF-1 α regarding hypoxic stabilization and binding to HIF-1 β , although it has also different roles in tumorigenesis [14, 44]. In fact, HIF-2 α can directly activate expression of genes encoding a number of pro-angiogenic factors, including VEGF, erythropoietin, angiopoietin, and Tie-2 receptors [11]. In this study, we have found that HIF-2 α protein and mRNA

expression was substantially increased in primary tracheal epithelial cells isolated from OVA-treated mice and that transfection with siRNA for HIF-2 α into the cells reduced significantly the increase of HIF-2 α and VEGF expression in primary tracheal epithelial cells (see the Supporting Information). These findings suggest that HIF-2 α inhibition also suppresses OVA-induced VEGF expression in bronchial epithelial cells.

PI3K catalyzes phosphorylation of phosphatidylinositol (4,5)-bisphosphate to form PIP3 in response to activation of either receptor tyrosine kinase, G-protein coupled receptors, or cytokine receptors, which ultimately regulate cell growth, differentiation, survival, proliferation, migration, and cytokine production [33, 34, 45]. The class IA PI3K consists of a heterodimer composed of a 110-kD (p110 α , β , δ) catalytic subunit and an adaptor protein (p85 α , p85 β , p55 α , p55 γ , p50 α) [46]. Previous studies have suggested that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells [47]. Some studies have reported that PI3K inhibition reduces Th2 cytokine production, pulmonary eosinophilia, airway inflammation, and bronchial hyperresponsiveness in a mouse asthma model [48, 49]. In addition, PI3K is shown to be involved in HIF-1 α activation induced by oxygen-dependent or oxygen-independent pathways [50, 51]. Recently, p110 δ and p110 γ isoforms have sparked a great deal of interest, as there is increasing evidence that these isoforms play key roles in immunity [52]. We have also demonstrated that OVA-induced HIF-1 α activation is significantly reduced by administration of a PI3K- δ inhibitor and suggested that inhibition of the p110 δ signaling pathway has therapeutic potential for allergic airway inflammation [33]. Consistent with these observations, in the present study, levels of p-Akt protein and PI3K activity in lung tissues were increased after OVA inhalation. The increased levels of p-Akt and PI3K activity were significantly reduced after administration of IC87114, a PI3K- δ inhibitor. Moreover, the increased levels of HIF-1 α after OVA inhalation were significantly reduced by IC87114 in primary tracheal epithelial cells isolated from OVA-treated mice. These findings suggest that PI3K- δ regulates HIF-1 α activation therewith inducing VEGF expression in a murine model of allergic airway disease.

In summary, we have examined the roles of HIF-1 α in allergen-induced airway inflammation and bronchial hyperresponsiveness using an HIF-1 α inhibitor, 2ME2, and siRNA targeting HIF-1 α and evaluated the role of PI3K- δ signaling in HIF-1 α activation in allergic airway disease. The administration of 2ME2 was effective in reversing all pathophysiological symptoms examined. Our data have also revealed that HIF-1 α inhibition substantially reduces the increase in VEGF expression as well as the activity of VEGF in lungs, especially in bronchial epithelial cells, of our murine model of allergic airway disease. In addition, administration of IC87114 reduced the increase in HIF-1 α activity in OVA-treated airway epithelial cells. Therefore, one likely mechanism for the roles of HIF-1 α in pathobiology of allergen-induced airway inflammation and hyperresponsiveness is induction of vascular leakage via up-regulation of VEGF expression in lungs as well as bronchial epithelium. Thus, these findings provide a crucial molecular mechanism for the potential of

HIF-1 α inhibition in preventing and/or treating asthma and other airway inflammatory disorders.

Materials and methods

Animals and experimental protocol

Female C57BL/6 mice, aged 8–10 wk and free of murine specific pathogens, were obtained from the Orientbio (Seoungnam, Korea) were housed throughout the experiments in a laminar flow cabinet and were 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 (Jeonju, Korea). Mice were sensitized on days 1 and 14 by i.p. injection of 20 μ g OVA (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical, Rockford, IL, USA) in a total volume of 200 μ L, as previously described with some modifications [9, 48]. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (weight/volume) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). OVA-treated mice are defined throughout the manuscript as OVA-sensitized and OVA-challenged mice. BAL was performed 48 h after the last challenge as described previously [9]. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA, USA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R., Aguada, Puerto Rico) in order to examine the cell differentials.

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions as described previously [48]. The epithelial cells were seeded onto 35-mm collagen-coated dishes for submerged culture. The growth medium, DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA), containing 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and bovine serum albumin. However, DMEM without antibiotics was used as the growth medium for the transfections of siRNA. The cells were maintained in a humidified 5% CO₂ incubator at 37°C until they adhered.

Transfections with siRNA

RNA interference was performed with Stealth RNA interference (Invitrogen Life Technologies). We transfected primary cultured tracheal epithelial cells in third passage with siRNAs in six-well

plates, but not coated with collagen. Stealth siRNA targeting HIF-1 α or negative control siRNA was transfected to the cells grown until 30–50% confluence. After the transfections, the cells were incubated for 72 h and then harvested. For transfections, siRNA duplexes were incubated with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instruction. The sequences of Stealth siRNA were as follows: mouse HIF-1 α , 5'-AAGCAUUU-CUCUCAUUUCCUCAUGG-3' (sense); corresponding negative control, 5'-AAGACCUUUUACUCUUACUCCUUGG-3' (sense); mouse HIF-2 α , 5'-GUCACCAGAACUUGUGCAC-3' (sense); corresponding negative control, 5'-UAGCGACUAAACACAUCAA-3' (sense).

2ME2 or IC87114 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% DMSO), 2ME2 (50 or 100 μ mol/L, Calbiochem-Novobiochem, San Diego, CA, USA) for 24 h at 37°C, or IC87114 (2 or 10 μ mol/L) for 2 h at 37°C, respectively [40].

RNA isolation and RT-PCR

Levels of mRNA expression were analyzed by RT-PCR assay using total RNA isolated from lung tissues and epithelial cells by a rapid extraction method (TRI-Reagent) as previously described [53, 54]. The primers used were as follows: HIF-1 α (predicted length 343 bp) sense: 5'-TGCTCATCAGTTGCCACTT-3', antisense: 5'-TGGGCCATTTCTGTGTGTA-3'; HIF-2 α used were sense: 5'-GACGGTGACATGATCTTTCTGTC-3', antisense: 5'-CACTTCATCCTCATGAAGAAGTCAC-3'; VEGF (predicted length; VEGF₁₆₅: 535 bp and VEGF₁₂₁: 403 bp) sense: 5'-CCAAGTGGTCCCAGGCTGCACC-3', antisense: 5'-GGTTAATCGGTCTTTCCGGTGAG-3', and GAPDH (predicted length 609 bp) sense: 5'-GCCATCAACGACCCCTTCATTGAC-3', antisense: 5'-ACGGAAGGCCATGCCAGTG AGCTT-3'. PCR reactions were performed in a thermocycler (GeneAmp[®] PCR System 2400, Applied Biosystems, Foster City, CA, USA).

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed using the LightCycler[®] FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The ΔC_T -method was used for the calculation of relative changes of mRNA by LightCycler 480[®] Multiple Plate Analysis Software (Roche Diagnostics) [55]. The data were normalized to the expression of β -actin and was confirmed by quantitative real-time RT-PCR to be ubiquitously and consistently expressed gene among all groups analyzed. The sequences of primers used were as follows: HIF-1 α sense: 5'-TGCTCATCAGTTGCCACTT-3', antisense: 5'-TGGGCCATT-

TCTGTGTGTA-3'; HIF-2 α used were sense: 5'-GACGGTGACATGATCTTTCTGTC-3', antisense: 5'-CACTTCATCCTCATGAAGAAGTCAC-3'; VEGF sense: 5'-CCAAGTGGTCCCAGGCTGCACC-3', antisense: 5'-GGTTAATCGGTCTTTCCGGTGAG-3', and β -actin sense: 5'-CAGATCATGTTTGAGAC CTTC-3' and antisense: 5'-ACTTCATGATGGAATTGAATG-3'.

Measurement of PI3K enzyme activity in lung tissues

PI3K enzyme activity was measured as described previously [33]. The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays according to the manufacturer's protocol (Echelon, Salt Lake City, UT, USA).

Administration of 2ME2, CBO-P11, and IC87114

An inhibitor of HIF-1 α , 2ME2 (50 or 100 mg/kg body weight/day), was suspended in 0.5% carboxymethylcellulose (Sigma-Aldrich) and administered by oral gavage six times at 24-h interval on days 19–24, beginning 2 days before the first challenge [56]. 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-Novobiochem) was used to inhibit VEGF activity. CBO-P11 (2 mg/kg body weight/day) was administered i.p. three times at 24-h interval, beginning at 1 h before the first inhalation. IC87114 (0.1 or 1.0 mg/kg body weight/day) or vehicle control (0.05% DMSO) diluted with 0.9% NaCl was administered in a volume of 50 μ L by intratracheal instillation two times to each animal, once on day 21 (1 h before the first airway challenge with OVA) and the second time on day 23 (3 h after the last airway challenge with OVA) [33].

Western blot analysis

Protein expression levels were analyzed by Western blot analysis as described previously [48]. The blots were incubated with an anti-IL-4 Ab (Serotec, Oxford, UK), anti-IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IL-13 Ab (R&D Systems, Minneapolis, MN, USA), anti-p-Akt Ab (Cell Signaling Technology, Beverly, MA, USA), anti-Akt Ab (Cell Signaling Technology), or anti-VEGF Ab (Santa Cruz Biotechnology), overnight at 4°C. The binding of the specific Ab was visualized by exposing to photographic film after treating with ECL system reagents (GE Healthcare). The film was scanned and quantified using the quantification software (Gel Doc XR, Bio-Rad Laboratories). For the quantification of specific bands, the same size square was drawn around each band to measure the density and then the value was adjusted by the density of the background near that band. The results of densitometric analyses were expressed as the relative ratio of the target protein to reference protein. The relative ratio of the target protein of control group is arbitrarily presented as 1.

Nuclear protein extractions for analysis of HIF-1 α , HIF-2 α , and HIF-1 β

Nuclear extraction for lung tissues or primary airway epithelial cells was performed as described previously [33]. For Western analysis, samples were loaded on SDS-PAGE gel. The blots were incubated with Ab against HIF-1 α (Novus Biologicals, Littleton, CO, USA), HIF-1 β (Novus Biologicals), or HIF-2 α (Novus Biologicals) overnight at 4°C.

Measurement of Th2 cytokines and VEGF

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BALF by enzyme immunoassays according to the manufacturer's protocol (IL-4 and IL-5; Endogen, Woburn, MA, USA; IL-13 and VEGF; R&D Systems). 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

To assess lung permeability, Evans blue dye was used as described previously [33].

Processing of lungs for histologic and image analysis

At 48 h after the last challenge, lungs were removed from the mice after sacrifice. The specimens were dehydrated and embedded in paraffin. After section of the specimens, they were placed on slides, deparaffinized, and stained sequentially with H&E (Richard-Allan Scientific, Kalamazoo, MN, USA) or PAS. Stained slides were quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination [42, 57].

Histology

For histological 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 0–3, as previously described [42, 48, 58].

Determination of airway responsiveness

Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine *via* airways, as previously described [42, 59]. Each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/mL in saline). After each methacholine chal-

lenge, the data of calculated R_{rs} were continuously collected. Maximum values of R_{rs} were selected to express changes in airway function.

Quantitation of airway mucus expression

To quantitate the level of mucus expression in the airway, the number of PAS-positive and PAS-negative epithelial cells in individual bronchioles was counted as described previously [42, 57].

Statistical analysis

We used SPSS statistical software (version 16.0, SPSS, Chicago, IL, USA). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Statistical significance was set at $p < 0.05$.

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Conflict of interest: Kamal D. Puri is employed by Calistoga Pharmaceuticals, Inc. The other authors declare no financial or commercial conflict of interest.

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Abbreviations: HIF-1 α : Hypoxia-inducible factor-1 α · 2ME2: 2-methoxyestradiol · PAS: Periodic acid-Schiff · PIP3: phosphatidyl inositol-3,4,5-triphosphate · R_{rs}: respiratory system resistance · VEGF: Vascular endothelial growth factor

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[Supporting Information]

HIF-1 α inhibition ameliorates an allergic airway disease via VEGF suppression in bronchial epithelium

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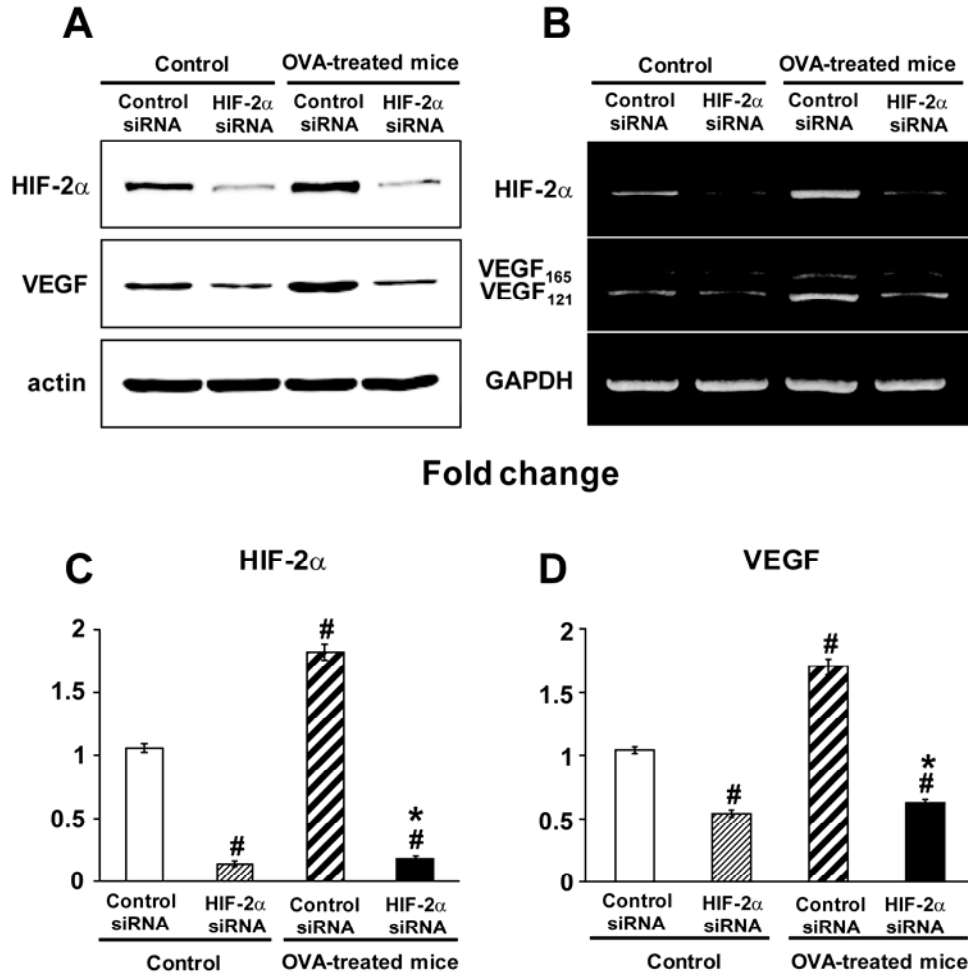


Figure S1. Increased expression of HIF-2α and VEGF in bronchial epithelial cells of OVA-treated mice. Primary cultured tracheal epithelial cells isolated from OVA-treated and saline-treated mice were transfected with HIF-2α or control siRNA. HIF-2α and VEGF (A) protein and (B) mRNA levels were determined by Western blotting and RT-PCR, respectively. The data are representative of four independent experiments. Quantitative analysis of (C) HIF-2α and (D) VEGF mRNA expression; mean ± SEM of 4 independent experiments (i.e. 8 samples per group). Ten saline-treated mice and ten OVA-treated mice were used for each experiment. In each experiment, two samples per group were used for the quantitative analysis of mRNA expression. Statistical comparisons were performed using one-way ANOVA. #p<0.05 versus both groups of control mice, *p<0.05 versus cells from OVA-treated mice transfected with control siRNA.