

Phosphoinositide 3-kinase- δ inhibitor reduces vascular permeability in a murine model of asthma

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Background: Bronchial asthma is characterized by inflammation of the airways, which is usually accompanied by increased vascular permeability, resulting in plasma exudation. Vascular endothelial growth factor (VEGF) has been implicated in contributing to asthmatic tissue edema through its effect on vascular permeability. Many cellular responses of VEGF are regulated by the lipid products of phosphoinositide 3-kinase (PI3K). However, the effect of PI3K catalytic subunit p110 δ on VEGF-mediated signaling is unknown. Recently, an isoform-specific small molecule inhibitor, IC87114, which is selective for p110 δ catalytic activity, has been identified.

Objective: We have sought to investigate the role of PI3K- δ , more specifically in the increase of vascular permeability.

Methods: Female BALB/c mice were sensitized and challenged with ovalbumin. We have investigated the effect of IC87114 on airway inflammation, T_H2 cytokines expression, airway hyperresponsiveness, plasma extravasation, hypoxia-inducible factor 1 α expression, and VEGF expression in a murine model of asthma.

Results: Our current study has revealed that IC87114 reduces antigen-induced airway infiltration of inflammatory cells, secretion of T_H2 cytokines in lungs, airway hyperresponsiveness, and vascular permeability. Moreover, we have found that inhibition of p110 δ reduces ovalbumin-induced upregulation of VEGF level.

Conclusion: These results suggest that PI3K- δ inhibitor attenuates antigen-induced airway inflammation and hyperresponsiveness by preventing vascular leakage in mice.

Clinical implications: These findings provide a crucial molecular mechanism for the potential role of PI3K- δ in

asthma and other airway inflammatory disorders. (J Allergy Clin Immunol 2006;118:403-9.)

Key words: Airway, PI3K- δ , vascular permeability, VEGF

Bronchial asthma is an increasingly prevalent and occasionally severe disease characterized by chronic inflammation of the airways, which is usually accompanied by increased vascular permeability, resulting in plasma exudation.¹ Changes in vascular permeability are crucial to the development and perpetuation of the inflammatory response. Increased vascular permeability causes secretion of intravascular components, which contribute to the airway obstruction and hyperresponsiveness.^{2,3}

Phosphoinositide 3-kinase (PI3K) is a signal transduction enzyme that catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate to form phosphatidylinositol (3,4,5)-trisphosphate (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.⁴⁻⁷ 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).⁸ Previous studies suggest that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells.^{9,10} PI3K plays a key role in induction of the T_H2 response.⁹⁻¹³ This enzyme is also essential for IL-5-induced eosinophil release from bone marrow¹¹ and migration of eosinophils caused by several chemoattractants.¹² Enhanced basal activity of PI3K has been reported in eosinophils derived from patients with allergic asthma.¹⁴ Some studies have reported that PI3K inhibition reduces T_H2 cytokine production, pulmonary eosinophilia, and airway inflammation and hyperresponsiveness in a mouse asthma model.¹⁵⁻¹⁷ 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.¹⁸

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis.¹⁹ VEGF also increases vascular permeability and leads to airway inflammation.^{2,20,21} VEGF is a mediator of vascular and extravascular remodeling and inflammation that enhances antigen sensitization and is crucial in adaptive T_H2-mediated inflammation.²¹ Studies have indicated that PI3K

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Abbreviations used

BAL: Bronchoalveolar lavage
 HIF: Hypoxia-inducible factor
 p-Akt: Phosphorylated Akt
 PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
 PI3K: Phosphoinositide 3-kinase
 R_L: Airway resistance
 VEGF: Vascular endothelial growth factor

plays a crucial role in VEGF-mediated signaling.²²⁻²⁴ Although PI3Ks are thought to contribute to the pathogenesis of asthma, the contribution of the individual PI3K isoforms in the increase of vascular permeability in asthma has not been examined.

Activation of hypoxia-responsive genes including VEGF is mediated by hypoxia-inducible factor (HIF)-1, a heterodimeric basic helix-loop-helix-Per-Arnt/AhR-Sim domain transcription factor.^{25,26} HIF-1 is composed of 2 subunits, HIF-1 α and HIF-1 β . Whereas the β -subunit is constitutively present, the stability of the α -subunit and its transcriptional activity are precisely controlled by the intracellular oxygen concentration.^{27,28}

On the basis of these observations, we hypothesized that PI3K- δ inhibitor attenuates antigen-induced airway inflammation and hyperresponsiveness by preventing vascular leakage via modulation of VEGF expression and HIF-1 α activation in a murine model of asthma. We found evidence that an isoform-specific small molecule inhibitor, IC87114, which is selective for p110 δ catalytic activity, inhibits increased vascular permeability, airway inflammation, and airway hyperresponsiveness. In addition, the specific inhibitor of p110 δ reduces VEGF expression.

METHODS

See the [Methods](#) section in the Online Repository at www.jacionline.org for additional material.

Animals and experimental protocol

Female BALB/c mice, 8 to 10 weeks of age and free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized and challenged, as previously described.¹⁵ Bronchoalveolar lavage (BAL) was performed at 6, 24, 48, and 72 hours after the last challenge.

Administration of a selective p110 δ inhibitor, IC87114, and VEGF receptor inhibitors

IC87114 (0.1 or 1 mg/kg body weight/d) or vehicle control (0.05% dimethyl sulfoxide) diluted with 0.9% NaCl was administered in a volume of 50 μ L by intratracheal instillation 2 times to each animal, once on day 21 (1 hour before the first airway challenge with ovalbumin) and the second time on day 23 (3 hours after the last airway challenge with ovalbumin). An inhibitor of VEGF receptor tyrosine kinases, SU5614 (2.5 mg/kg body weight/d; Calbiochem,

San Diego, Calif) or SU1498 (9 mg/kg body weight/d; Calbiochem) was dissolved in dimethyl sulfoxide and administered intraperitoneally 3 times at 24-hour intervals, beginning 1 hour after the last airway challenge with ovalbumin.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye was used as described previously.²⁰

Histology and immunocytochemistry

For histologic examination, 4- μ m sections of fixed embedded tissues were cut, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, Mich). For immunocytochemistry of VEGF, the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the instructions of the RTU Vectastain Universal Quick kit from Vector Laboratories Inc (Burlingame, Calif). The slides were then probed with an antibody against VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif).

Measurements of VEGF and T_H2 cytokines in BAL fluids

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by an enzyme immunoassay (IL-4, Endogen, Inc, Woburn, Mass; IL-5, BioSource International, Inc, Camarillo, Calif; IL-13 and VEGF, R&D Systems, Inc, Minneapolis, Minn).

Western blot analysis

Western blot analysis was performed as described previously.²⁰ The blots were incubated with an anti-VEGF Ab (Santa Cruz Biotechnology), anti-IL-4 Ab (Serotec Ltd, Oxford, United Kingdom), anti-IL-5 antibody (Santa Cruz Biotechnology), anti-IL-13 Ab (R&D Systems, Inc.), anti-Akt Ab (Cell Signaling Technology Inc, Beverly, Mass), or antiphosphorylated Akt (p-Akt) Ab (Cell Signaling Technology Inc).

Cytosolic or nuclear protein extractions for analysis of HIF-1 α and HIF-1 β

Cytosolic or nuclear extractions were performed as described previously.²⁰

Measurement of PI3K enzyme activity in lung tissues

The measurement of PI3K enzyme activity in lung tissues was performed as described previously.^{29,30} The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays (Echelon, Inc, Salt Lake City, Utah).

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere.^{31,32}

Densitometric analyses and statistics

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc, Hercules, Calif). Data are expressed as means $\pm$ SEMs. Statistical comparisons were performed by using 1-way ANOVA followed by the Scheffé test. Significant differences between groups were determined by using the unpaired Student *t* test. Statistical significance was set at *P* < .05.

RESULTS

Effect of PI3K- δ inhibitor, IC87114 and VEGF receptor inhibitors on VEGF protein expression in BAL fluids and lung tissues of ovalbumin-sensitized and ovalbumin-challenged mice

Western blot analysis revealed that administration of IC87114, SU5614, or SU1498 dramatically reduced the increased levels of VEGF in lung tissues at 72 hours after the last challenge (Fig 1, A and B). Consistent with the results obtained from the Western blot analysis, enzyme immunoassay revealed that the administration of IC87114, SU5614, or SU1498 reduced significantly the increased levels of VEGF in BAL fluids at 72 hours after ovalbumin inhalation (Fig 1, C).

Immunocytologic analysis of BAL fluids showed the localization of immunoreactive VEGF in the precipitated cells. Level of immunoreactive VEGF in BAL cells from the ovalbumin-sensitized and ovalbumin-challenged mice (Fig 2, B) was higher than the level in BAL cells from control mice (Fig 2, A). Ovalbumin-sensitized and ovalbumin-challenged mice treated with IC87114 decreased immunoreactive VEGF expression in BAL cells from allergen-induced asthmatic mouse lungs (Fig 2, C and D).

Plasma extravasation in ovalbumin-sensitized and ovalbumin-challenged mice was reduced by administration of PI3K- δ inhibitor, IC87114, or VEGF receptor inhibitors

The Evans blue dye assay revealed that plasma extravasation was significantly increased at 72 hours after the last challenge (Fig 3). The increase in plasma extravasation at 72 hours after ovalbumin inhalation was significantly reduced by the administration of IC87114, SU5614, or SU1498.

Effect of IC87114 on HIF-1 α expression in nuclear protein extracts from lung tissues

Western blot analysis showed that levels of HIF-1 α protein in nuclear protein extracts from lung tissues were increased significantly at 72 hours after the last challenge compared with levels in the control animals (Fig 4). The increased HIF-1 α levels were significantly reduced by the administration of IC87114.

Effect of IC87114 on p-Akt and Akt protein expression and PI3K enzyme activity in lung tissues of ovalbumin-sensitized and ovalbumin-challenged mice

We used Western blotting to determine the activation of Akt that can activate the HIF pathway. Levels of p-Akt protein in the lung tissues were increased significantly at 72 hours after ovalbumin inhalation compared with levels in the control mice (see this article's Fig E1, A and B, in the Online Repository at www.jacionline.org). However, no significant changes in total Akt protein levels were observed in any of the groups tested. The increased p-Akt

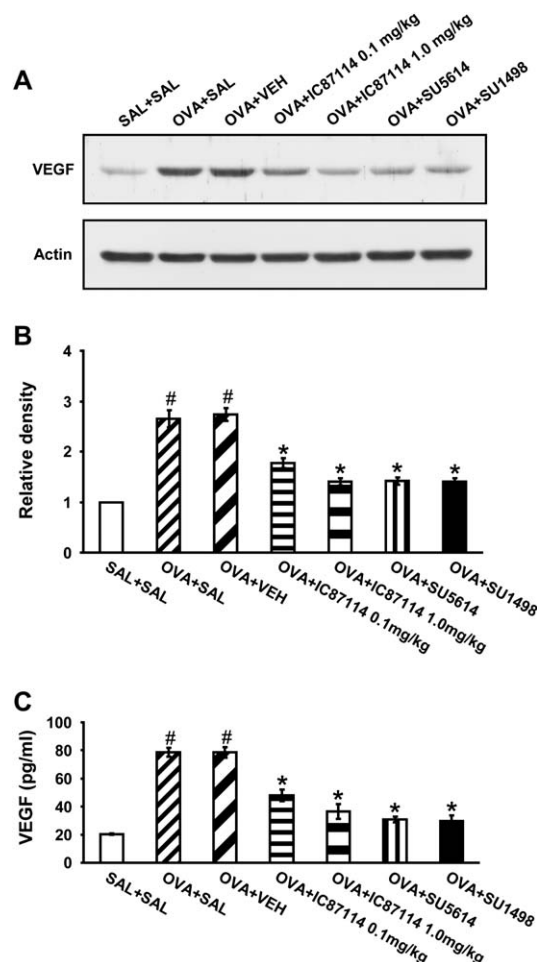


FIG 1. Effect of IC87114, SU5614, or SU1498 on VEGF protein expression. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+ SAL), ovalbumin (OVA)-inhaled mice administered saline (OVA+ SAL), OVA-inhaled mice administered drug vehicle (OVA+ VEH), OVA-inhaled mice administered 0.1 mg/kg of IC87114 (OVA+IC87114 0.1 mg/kg), OVA-inhaled mice administered 1 mg/kg of IC87114 (OVA+IC87114 1.0 mg/kg), OVA-inhaled mice administered SU5614 (OVA+SU5614), or OVA-inhaled mice administered SU1498 (OVA+SU1498). **A**, Western blotting of VEGF in lung tissues. **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 is arbitrarily presented as 1. **C**, Enzyme immunoassay of VEGF in BAL fluids. Bars represent means $\pm$ SEMs from 8 mice per group. # P < .05 vs SAL+ SAL; * P < .05 vs OVA+ SAL.

but not Akt protein levels in lung tissues at 72 hours after ovalbumin inhalation were significantly reduced by the administration of IC87114. In addition, we have also measured PI3K enzyme activity using the PIP3 competition enzyme immunoassay. PI3K activity in the lung tissues was increased significantly at 72 hours after ovalbumin inhalation compared with that in the control mice (see this article's Fig E1, C, in the Online Repository at www.jacionline.org). The increased PI3K activity in lung tissues at 72 hours after ovalbumin inhalation was significantly reduced by the administration of IC87114.

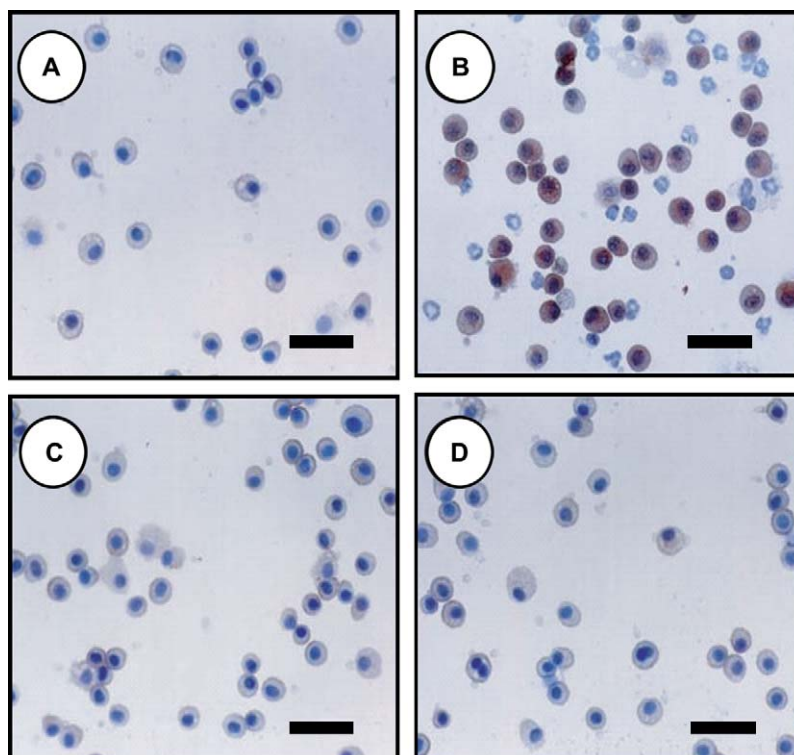


FIG 2. Localization of immunoreactive VEGF. Sampling was performed at 72 hours after the last challenge in BAL fluids from sensitized mice challenged with saline (**A**), from sensitized mice challenged with ovalbumin (**B**), from ovalbumin-inhaled mice administered 0.1 mg/kg of IC87114 (**C**), and from ovalbumin-inhaled mice administered 1 mg/kg of IC87114 (**D**). Brown indicates VEGF⁺ cells. Bars indicate scale of 50 μ m.

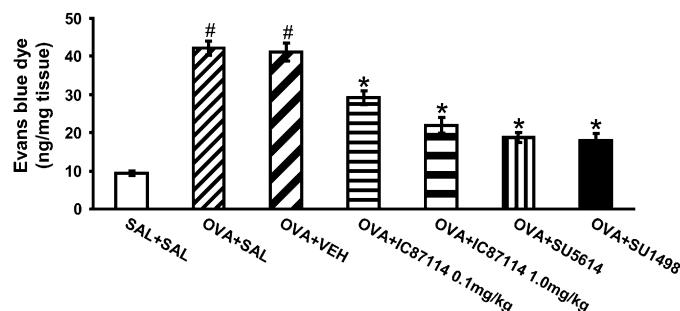


FIG 3. Effect of IC87114, SU5614, or SU1498 on plasma exudation. Bars represent means $\pm$ SEMs from 8 mice per group. [#] $P < .05$ vs saline (SAL) + SAL; ^{*} $P < .05$ vs ovalbumin (OVA) + SAL.

Effect of PI3K- δ inhibitor, IC87114, and VEGF receptor inhibitors on IL-4, IL-5, and IL-13 expression

Western blot analysis revealed that IL-4, IL-5, and IL-13 protein levels in lung tissues were increased significantly at 72 hours after ovalbumin inhalation compared with the levels after saline inhalation (see this article's Fig E2, A and B, in the Online Repository at www.jacionline.org). The increased levels of these cytokines at 72 hours after ovalbumin inhalation were significantly reduced by the administration of IC87114, SU5614, or SU1498. Consistent with the results, enzyme immunoassays also showed the significant increase in levels of

these cytokines in BAL fluids at 72 hours after ovalbumin inhalation compared with the levels after saline inhalation. The increased levels of these cytokines at 72 hours after ovalbumin inhalation were significantly reduced by the administration of IC87114, SU5614, or SU1498 (see this article's Fig E2, C, in the Online Repository at www.jacionline.org).

Effect of PI3K- δ inhibitor, IC87114, and VEGF receptor inhibitors on cellular changes in BAL fluids

Numbers of total cells, eosinophils, lymphocytes, and neutrophils in BAL fluids were increased significantly at

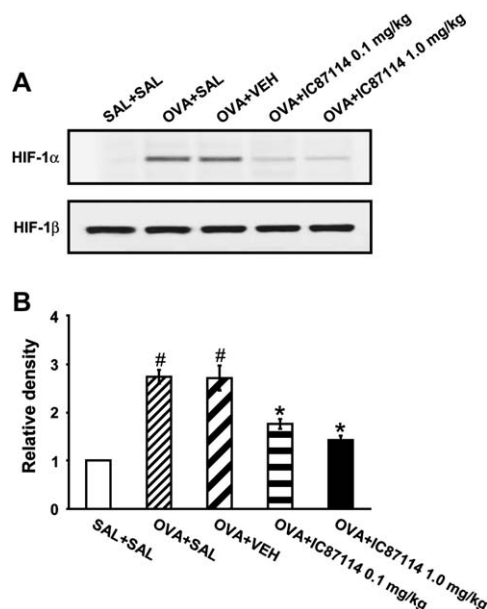


FIG 4. Effect of IC87114 on HIF-1 α and HIF-1 β expression in nuclear protein extracts from lung tissues. **A**, HIF-1 α and HIF-1 β expression in nuclear protein extracts from lung tissues. **B**, Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of HIF-1 α in the lung tissues of saline (SAL) + SAL is arbitrarily presented as 1. Bars represent means $\pm$ SEMs from 8 mice per group. $^{\#}P < .05$ vs SAL+ α SAL; $^{*}P < .05$ vs ovalbumin (OVA) + α SAL. VEH, Vehicle.

72 hours after ovalbumin inhalation compared with the numbers after saline inhalation (Fig 5, A). The increased numbers of these cells were significantly reduced by the administration of IC87114, SU5614, or SU1498.

Effect of PI3K- δ inhibitor, IC87114, and VEGF receptor inhibitors on airway hyperresponsiveness

In ovalbumin-sensitized and ovalbumin-challenged mice, dose-response curve of percent airway resistance (R_L) shifted to the left compared with that of control mice (Fig 5, B). In ovalbumin-sensitized and ovalbumin-challenged mice treated with IC87114, SU5614, or SU1498, the dose-response curve of percent R_L was shifted to the right compared with that of untreated mice. These results indicate that treatment with IC87114 or VEGF receptor inhibitors reduces ovalbumin-induced airway hyperresponsiveness.

DISCUSSION

Bronchial asthma is a chronic inflammatory disease of the airways that is characterized by airway remodeling, which is usually accompanied by increased vascular permeability, resulting in plasma exudation.^{1,33} Several studies have revealed prominent increases in vessel number, vessel size, vascular surface area, and vascular leakage, including a significant correlation between these alterations and disease severity in asthma.^{2,20,34-40} VEGF has been

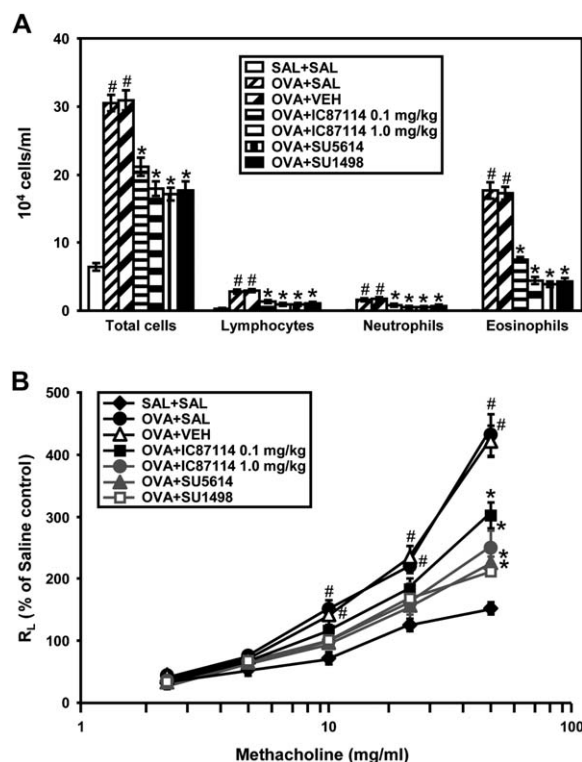


FIG 5. Effect of IC87114, SU5614, or SU1498 on total cells and differential cellular components of BAL fluids (**A**) and airway responsiveness (**B**) of ovalbumin (OVA)-sensitized and ovalbumin-challenged mice. **A**, The numbers of each cellular component of BAL were counted at 72 hours after the last challenge. **B**, Airway responsiveness was measured at 72 hours after the last challenge as described in Methods. Bars represent means $\pm$ SEMs from 8 mice per group. $^{\#}P < .05$ vs saline (SAL)+ α SAL; $^{*}P < .05$ vs OVA+ α SAL. VEH, Vehicle.

implicated to contribute to edema of asthmatic tissue through its effect on vascular permeability.⁴⁰⁻⁴² Many cellular responses of VEGF are regulated by the lipid products of PI3K.²²⁻²⁴ However, the effect of PI3K- δ on VEGF-mediated signaling is unknown. Very recently, an isoform-specific small molecule inhibitor, IC87114, which is selective for PI3K- δ catalytic activity, has been identified.⁴³⁻⁴⁵ Our current study with the ovalbumin-induced model of asthma has revealed that IC87114 reduces antigen-induced airway infiltration of inflammatory cells, increased secretion of T_H2 cytokines in lungs, airway hyperresponsiveness, and increased vascular permeability. Moreover, we have found that inhibition of PI3K- δ reduces ovalbumin-induced upregulation of VEGF level. These findings suggest that IC87114 attenuates antigen-induced airway inflammation and hyperresponsiveness by preventing vascular leakage in mice.

Phosphoinositide 3-kinase activity is stimulated after antigen challenge in a murine model of allergic asthma and administration of wortmannin or LY294002, 2 broad-spectrum inhibitors of PI3Ks, attenuates inflammation and airway hyperresponsiveness.¹⁵⁻¹⁷ The results of the current study indicate that plasma extravasation caused by increased vascular permeability is elevated after inducing

asthma and that administration of PI3K- δ inhibitor significantly reduces the plasma extravasation increased after ovalbumin inhalation. Although the pathogenesis of asthma induced by plasma extravasation is not clearly defined, plasma protein leakage has been implicated to play a role in the induction of a thickened, engorged, and edematous airway wall, resulting in the airway lumen narrowing.³ It has been also suggested that the plasma exudate may readily pass the inflamed mucosa and reach the airway lumen through leaky epithelium, thus compromising epithelial integrity and reducing ciliary function and mucus clearance.^{46,47} Recently, several investigators have demonstrated that PI3K- δ plays a prominent role in leukocyte trafficking in animal models of inflammation.⁴³ Therefore, these observations suggest that inhibition of PI3K- δ reduces antigen-induced airway inflammation by preventing vascular leakage as well as blocking leukocyte trafficking.

Vascular endothelial growth factor is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and T_H2 inflammation.²¹ Although other inflammatory mediators, including platelet-activating factor, can promote microvascular leakage,^{48,49} one of the major roles of VEGF in asthma appears to be the enhancement of vascular permeability.^{2,19,20} The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesiculo-vacuolar organelles.^{19,50} VEGF can be produced by a wide variety of cells, including macrophages, neutrophils, eosinophils, and lymphocytes.^{2,20,21,51,52} Several 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, allowing migration of inflammatory cells into the airways.^{2,20,41} Consistent with these observations, we have found that VEGF expression is upregulated and vascular permeability is increased in ovalbumin-induced asthma. Interestingly, administration of a PI3K- δ inhibitor, IC87114, significantly reduces the VEGF expression induced by ovalbumin inhalation, suggesting that PI3K- δ is involved in the regulation of VEGF expression. To distinguish direct effects of PI3K- δ on the asthma phenotype from those of VEGF, VEGF receptor inhibitor, SU5614 or SU1498 was administered. We have found that SU5614 or SU1498 inhibits the increased vascular permeability, the airway inflammation, and the airway hyperresponsiveness. These results suggest that VEGF may affect vascular permeability without affecting PI3K- δ signaling pathway.

Vascular endothelial growth factor expression is regulated through HIF-1 α .^{25,26} Previous reports have demonstrated that HIF-1 α plays a critical role in immune and inflammatory responses.^{16,53} Determination of HIF-1 α protein level in nuclear extracts has revealed that this protein level is substantially increased in our current ovalbumin-induced model of asthma, suggesting that HIF-1 α is activated. However, the increased levels of HIF-1 α are significantly reduced after administration of PI3K- δ inhibitor. Previous reports have shown that increase of

PI3K/Akt activity activates the HIF signaling pathway.⁵⁴⁻⁵⁷ Li et al⁵⁸ have also reported that activation of Akt turns on HIF-1 α independently of hypoxia. In the current study, levels of p-Akt protein and PI3K activity in the lung tissues are increased after ovalbumin inhalation. The levels of p-Akt and PI3K activity increased by ovalbumin inhalation are significantly reduced after administration of IC87114. Taken together, these findings suggest that PI3K- δ regulates HIF-1 α action through a PI3K/Akt signaling pathway, resulting in increased VEGF expression in a murine model of asthma.

In summary, we have examined the role of PI3K- δ in a murine model of allergic asthma, more specifically in the increase of vascular permeability. Our data demonstrate that administration of a selective PI3K- δ inhibitor, IC87114, substantially reduces expression of VEGF, including the activity of VEGF. The activity includes plasma leakage and migration of inflammatory cells. Therefore, 1 likely mechanism for the effectiveness of PI3K- δ is the reduction of VEGF expression to a physiological level. In addition, these findings also provide a crucial molecular mechanism for the potential role of PI3K- δ in asthma and other airway inflammatory disorders.

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METHODS

Animals and experimental protocol

Female BALB/c mice, 8 to 10 weeks of age and free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, 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 treated according to guidelines approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μ g ovalbumin (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.²¹ On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (wt/vol) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron Corp, Tokyo, Japan). BAL was performed at 6, 24, 48, and 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 secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and stored 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 by cytopspin (Thermo Electron, Waltham, Mass). The smears were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc, Aguada, Puerto Rico) to examine the cell differentials. Two independent, blind investigators counted the cells using a microscope. Approximately 400 cells were counted in each of 4 different random locations. Variation of results between investigators was less than 5%. The mean of the values from the 2 investigators was used for each cell count.

Administration of a selective p110 δ inhibitor, IC87114, and VEGF inhibitors

IC87114 (0.1 or 1 mg/kg body weight/d) or vehicle control (0.05% dimethyl sulfoxide) diluted with 0.9% NaCl was administered in a volume of 50 μ L by intratracheal instillation 2 times to each animal, once on day 21 (1 hour before the first airway challenge with ovalbumin) and the second time on day 23 (3 hours after the last airway challenge with ovalbumin). Inhibitors of VEGF receptor tyrosine kinase, SU5614 (Flk-1; IC₅₀ = 1.2 μ mol/L, 5-chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone; Calbiochem, San Diego, Calif) and SU1498 ([E]-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-n-propyl)amino-carbonyl]acrylonitrile; Calbiochem) were used to inhibit VEGF activity. SU5614 (2.5 mg/kg body weight/d) or SU1498 (9 mg/kg body weight/d) was dissolved in dimethyl sulfoxide and administered intraperitoneally 3 times at 24-hour intervals, beginning 1 hour after the last airway challenge with ovalbumin.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/mL. 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. The lungs were removed and weighed wet. EBD was extracted

in 2 mL formamide kept in a water bath at 60°C for 3 hours, and the absorption of light at 620 nm was measured in a spectrophotometer (Spectra Max Plus Microplate Spectrophotometer; Molecular Devices, Sunnyvale, Calif). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01 to 10 μ g/mL and is expressed as ng dye/mg wet lung.

Histology and immunocytochemistry

At 72 hours after the last inhalation, lungs were removed from the mice after sacrifice. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) 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. For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, Mich). For immunocytochemistry of VEGF, the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the instructions of the RTU Vectastain Universal Quick kit from Vector Laboratories Inc (Burlingame, Calif). Briefly, the slides were incubated in Endo/Blocker (Biomedex Corp, Foster City, Calif) for 5 minutes and in pepsin solution for 4 minutes at 40°C. The slides were incubated in normal horse serum for 15 minutes at room temperature. The slides were then probed with antibody against VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C, and then incubated with prediluted biotinylated pan-specific IgG for 10 minutes. The slides were incubated with streptavidin/peroxidase complex reagent for 5 minutes, and then with 3-amino-9-ethyl-carbazole substrate for 12 minutes. Controls consisted of BAL cells from mice were incubated without the primary antibody. After immunostaining, the slides were counterstained with Gill's hematoxylin in 20% ethylene glycol for 1 minute and then mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, Calif) and photomicrographed (Vanox T; Olympus, Tokyo, Japan).

Measurements of VEGF and T_H2 cytokines in BAL fluids

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by an enzyme immunoassay according to the manufacturer's protocol (IL-4, Endogen, Inc, Woburn, Mass; IL-5, BioSource International, Inc, Camarillo, Calif; IL-13 and VEGF, R&D Systems, Inc, Minneapolis, Minn). Sensitivities for IL-4, IL-5, IL-13, and VEGF assays were 5, 3, 1.5, and less than 3.0 pg/mL, respectively.

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of lung proteins. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories Inc, Hercules, Calif). Samples (30 μ g protein per lane) were loaded on a 10% SDS-PAGE gel. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by the wet transfer method (250 mA, 90 minutes). Nonspecific sites were blocked with 5% nonfat milk in TRIS-buffered saline Tween 20 (25 mmol/L TRIS pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) for 2 hours, and the blots were then incubated with an anti-VEGF Ab (Santa Cruz Biotechnology), anti-IL-4 Ab (Serotec Ltd, Oxford, United Kingdom), anti-IL-5 Ab (Santa Cruz Biotechnology), anti-IL-13 antibody (R&D Systems, Inc), anti-Akt Ab (Cell Signaling Technology Inc, Beverly, Mass), or p-Akt Ab (Cell Signaling Technology Inc) overnight at 4°C. Antirabbit or antimouse horseradish

peroxidase-conjugated IgG was used to detect binding of the antibodies. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

Cytosolic or nuclear protein extractions for analysis of HIF-1 α and HIF-1 β

Lungs were removed and homogenized in 2 volumes of buffer A (50 mmol/L TRIS-HCl, pH 7.5, 1 mmol/L EDTA, 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 1000g for 15 minutes at 4°C. The supernatant fraction was incubated on ice for 10 minutes and centrifuged at 100,000g for 1 hour at 4°C to obtain cytosolic proteins for analysis of HIF-1 α and HIF-1 β . The 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 pelleted at 1000g 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 1000g 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 as soluble nuclear proteins for analysis of HIF-1 α and HIF-1 β . The levels of these proteins were analyzed by Western blotting using antibody against HIF-1 α (Novus Biologicals Inc, Littleton, Colo) or HIF-1 β (Novus Biologicals Inc) as described.

Measurement of PI3K enzyme activity in lung tissues

The measurement of PI3K enzyme activity in lung tissues was performed as described previously.^{E2,E3} Lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of lung proteins. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories Inc). The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays according to the manufacturer's protocol (Echelon, Inc, Salt Lake City, Utah). The enzyme activity was expressed as pmol PIP3 produced by 1 mL of lung tissue extracts containing equal amounts of total protein.

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere.^{E4,E5} Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, 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 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 during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an inline nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each

mouse was challenged with methacholine aerosol in increasing concentrations (2.5-50 mg/mL in saline). After each methacholine challenge, the data of R_L were continuously collected. Maximum values of R_L were selected to express changes in airway function, which was represented as a percentage change from baseline after saline aerosol.

Densitometric analyses and statistics

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

RESULTS

Time-course analysis of cellular changes in BAL fluids

Total cell numbers in BAL fluids were significantly greater from 6 hours to 72 hours after ovalbumin inhalation compared with cell numbers before ovalbumin inhalation (Fig E3). The number of eosinophils in the BAL fluids was increased in a time-dependant manner, reached maximum level at 48 hours, and was sustained up to 72 hours after ovalbumin inhalation. The numbers of lymphocytes and neutrophils were also increased significantly from 24 to 72 hours after ovalbumin inhalation compared with cell numbers before ovalbumin inhalation.

Time-course analysis of airway hyperresponsiveness

Airway responsiveness produced by methacholine administration at dose of 25 mg/mL was increased significantly at 24, 48, and 72 hours after ovalbumin inhalation compared with the prechallenge period (Fig E4). At dose of 50 mg/mL, airway responsiveness was increased significantly at 6, 24, 48, and 72 hours after ovalbumin inhalation.

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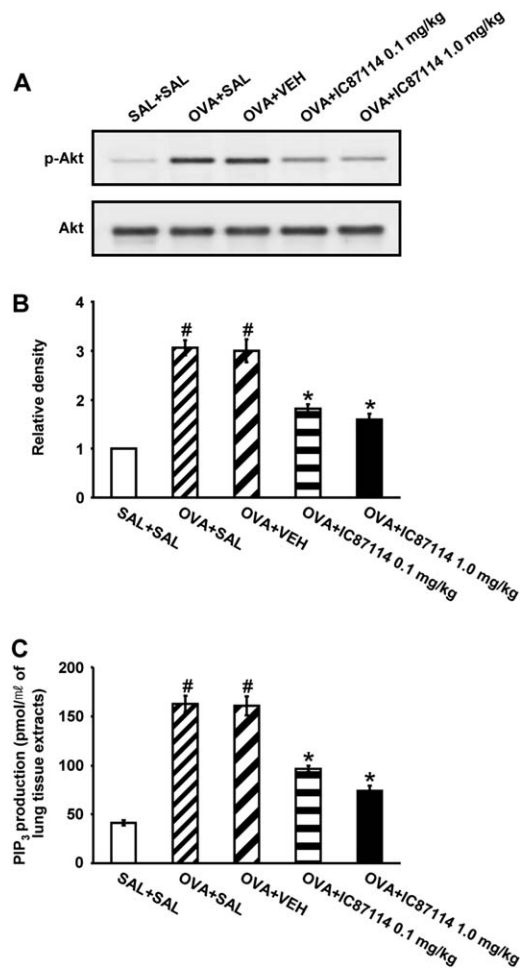


FIG E1. Effect of IC87114 on p-Akt and Akt protein expression and PI3K enzyme activity in lung tissues of ovalbumin (OVA)-sensitized and OVA-challenged mice. **A**, p-Akt and Akt protein expression were measured 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), OVA-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice administered 0.1 mg/kg IC87114 (OVA+IC87114 0.1 mg/kg), or OVA-inhaled mice administered 1 mg/kg IC87114 (OVA+IC87114 1.0 mg/kg). **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 is arbitrarily presented as 1. **C**, PIP₃ generation by PI3Ks in lung tissue extracts. Bars represent means $\pm$ SEMs from 8 mice per group. [#] $P < .05$ vs SAL+SAL; ^{*} $P < .05$ vs OVA+SAL. VEH, Vehicle.

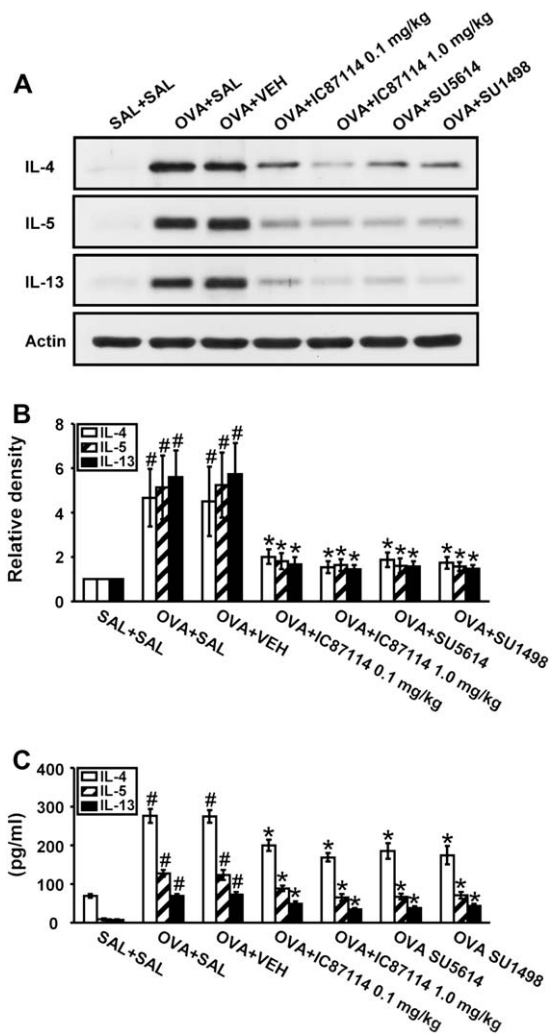


FIG E2. Effect of IC87114 on IL-4, IL-5, and IL-13 in lung tissues and in BAL fluids of ovalbumin (OVA)-sensitized and OVA-challenged mice. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (*SAL+ SAL*), OVA-inhaled mice administered saline (*OVA+ SAL*), OVA-inhaled mice administered drug vehicle (*OVA+ VEH*), OVA-inhaled mice administered 0.1 mg/kg IC87114 (*OVA+ IC87114* 0.1 mg/kg), OVA-inhaled mice administered 1 mg/kg IC87114 (*OVA+ IC87114* 1.0 mg/kg), OVA-inhaled mice administered SU5614 (*OVA+ SU5614*), or OVA-inhaled mice administered SU1498 (*OVA+ SU1498*). **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* 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 vs *SAL+ SAL*; **P* < .05 vs *OVA+ SAL*.

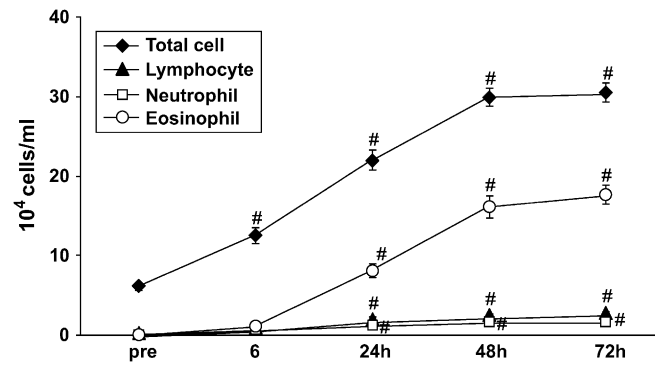


FIG E3. Time-course analysis of total cells and differential cellular components of BAL fluids of ovalbumin-sensitized and ovalbumin-challenged mice. BAL was performed at different times after the last ovalbumin inhalation. Six, 24, 48, and 72 hours are periods after the last ovalbumin challenge. *Pre*, 1 hour before the first challenge. Bars represent means $\pm$ SEMs from 8 mice per group. #*P* < .05 vs *Pre*.

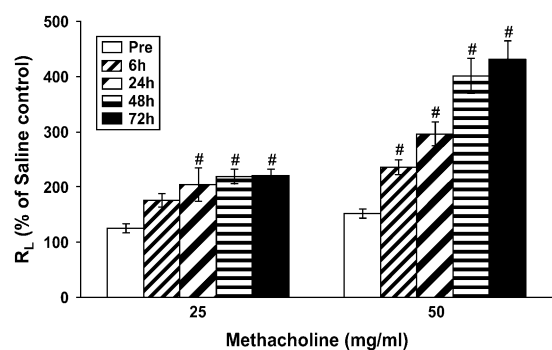


FIG E4. Time-course analysis of airway responsiveness in ovalbumin-sensitized and ovalbumin-challenged mice. Airway responsiveness produced by methacholine administration (at doses of 25 and 50 mg/mL) was performed at different times after the last ovalbumin inhalation. Six, 24, 48, and 72 hours are periods after the last ovalbumin challenge. *Pre*, 1 hour before the first challenge. Bars represent means $\pm$ SEMs from 8 mice per group. #*P* < .05 vs *Pre*.