

[Revised unmarked manuscript]

# **PTEN Reduces Vascular Endothelial Growth Factor Expression in Allergen-induced Airway Inflammation**

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**The Running title:** PTEN and VEGF in Asthma

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**Number of text pages: 40**

**Number of figures: 12**

**Number of references: 40**

**Number of words in *Abstract, Introduction, and Discussion*: 202, 568, and 1,340**

**Abbreviations used in this paper:**

VEGF, vascular endothelial growth factor; T<sub>H</sub>2, T-helper type 2 cell; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; PIP3, phosphatidylinositol 3,4,5-triphosphate; AdPTEN, adenovirus gene transfer vector expressing a PTEN cDNA; BAL, bronchoalveolar lavage; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ ; R<sub>L</sub>, airway resistance

## ABSTRACT

Vascular endothelial growth factor (VEGF) plays a pivotal role in the pathogenesis of bronchial asthma. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) has been implicated in regulating cell survival signaling through the phosphoinositide 3-kinase (PI3K)/Akt pathway. The key role of PI3K in VEGF-mediated signal transduction is established. However, the effects of PTEN on VEGF-mediated signaling in asthma are unknown. This study was aimed to determine the effect of PI3K inhibitors and PTEN on VEGF expression in allergen-induced airway inflammation. We have used a female C57BL/6 mouse model for asthma to determine the role of PTEN in allergen-induced airway inflammation, specifically in the expression of VEGF. Allergen-induced airway inflammation leads to increased activity of PI3K in lung tissue. These mice develop the following typical pathophysiological features of asthma in the lungs: increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, increased expression of IL-4, IL-5, IL-13, ICAM-1, VCAM-1, RANTES, and eotaxin, increased vascular permeability, and increased levels of VEGF. Administration of PI3K inhibitors or adenoviruses carrying PTEN cDNA reduced the symptoms of asthma and decreased the increased levels of plasma extravasation and VEGF in allergen-induced asthmatic lungs. These results indicate that PTEN reduces VEGF expression in allergen-induced airway inflammation.

## Introduction

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 (Bousquet et al., 2000). Several studies have revealed prominent increases in vessel number, vessel size, vascular surface area, and vascular leakage, including important correlations between these alterations and disease severity in asthma (Hoshino et al., 2001; Lee and Lee, 2001; Orsida et al., 2001; Salvato, 2001; Lee et al., 2002; Lee KS et al., 2004). Increased vascular permeability causes secretion of intravascular components. Exudation of plasma proteins into the airways contributes to the airway obstruction and hyperresponsiveness (Van de Graaf et al., 1991; Lee et al., 2002).

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis (Dvorak et al., 1995). VEGF also increases vascular permeability and leads to airway inflammation (Lee et al., 2002; Lee CG et al., 2004; Lee KS et al., 2004). Recently, we have demonstrated that VEGF is one of the major determinants of asthma and thus the inhibition of VEGF receptor may be a good therapeutic strategy (Lee and Lee, 2001; Lee et al., 2002; Lee KS et al., 2004). In addition, VEGF is a mediator of vascular and extravascular remodeling and inflammation that enhances antigen sensitization and is crucial in adaptive T-helper type 2 cell (T<sub>H</sub>2)-mediated inflammation (Lee CG et al., 2004).

Phosphoinositide 3-kinase (PI3K) is a signal transduction enzyme, which phosphorylates the D3 position of the inositol ring of phosphoinositide and its phosphorylated derivatives (Whitman et al., 1988). Recent studies suggest that PI3K contributes to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells (Fukao et al., 2002a; Fukao et al., 2002b). PI3K plays a key role in induction of the T<sub>H</sub>2 response (Dunzendorfer et al., 1998; Palframan et al., 1998; Fukao et al., 2002a; Fukao et al., 2002b).

This enzyme is also essential for IL-5-induced eosinophil release from bone marrow (Palframan et al., 1998) and migration of eosinophils caused by a number of chemoattractants (Dunzendorfer et al., 1998). Enhanced basal activity of PI3K has been reported in eosinophils derived from allergic asthmatics (Bracke et al., 2000). In addition, the key role of PI3K in VEGF-mediated signal transduction is established (Fujio and Walsh, 1999; Thakker et al., 1999). Phosphatase and tensin homologue deleted on chromosome ten (PTEN) functions primarily as a lipid phosphatase to regulate crucial signal transduction pathways (Yamada and Araki, 2001). PTEN has been implicated in regulating cell survival signaling through the PI3K/Akt pathway. PTEN blocks the action of PI3K by dephosphorylating the signal lipid phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3, produced by PI3K following activation by receptor tyrosine kinases, activated Ras, or G proteins, leads to the stimulation of several downstream targets, including the serine/threonine protein kinase Akt (Cantley and Neel, 1999). PTEN plays a pivotal role in  $T_H2$ -mediated inflammation and airway hyperresponsiveness (Kwak et al., 2003). However, the effect of PTEN on VEGF-mediated signaling in asthma has not been defined.

In the present study, we used a murine model of allergen-induced airway inflammation to determine the role of the PTEN, more specifically in the expression of VEGF. We found evidence that specific inhibitors of PI3K or adenovirus gene transfer vector expressing a PTEN cDNA (AdPTEN) inhibits increased vascular permeability, airway inflammation, and airway hyperresponsiveness. In addition, specific inhibitors of PI3K or AdPTEN reduces VEGF expression.

## Materials and Methods

**Animals and Experimental Protocol.** Female C57BL/6 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). The mice 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 intraperitoneally injection of 20  $\mu$ g ovalbumin (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200  $\mu$ l. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (weight/volume) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). Bronchoalveolar lavage (BAL) was performed 72 hours after the last challenge. At the time of lavage, the mice (six mice in each group) were sacrificed 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, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico) in order to examine the cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Variation of results between investigators

was less than 5%. The mean of the values from the two investigators was used for each cell count.

**Vectors.** The E1/E3-deleted replication-deficient recombinant adenovirus was made using the AdEasy system (Quantum Biotechnologies, Montreal, Quebec, Canada) described by He et al (He et al., 1998). KpnI-XhoI restriction fragments from pcDNA3/wild-type PTEN cDNA were ligated into KpnI-XhoI-digested pShuttleCMV, as previously described (Hwang et al., 2001). To create AdLacZ, a Sall-NotI restriction fragment from pcDNA3.1/LacZ (Invitrogen Corp., San Diego, CA) was ligated to Sall-NotI-digested pShuttleCMV. Recombination into the pAdEasy viral backbone was accomplished in bacteria (*E. coli* strain BJ5183, which is recombination deficient) according to the manufacturer's instructions. The recombination was verified and the adenoviral recombinant DNA was transferred to a regular strain of *E. coli* (DH5 $\alpha$ ), which generates far greater yields of DNA. Recombinant pAdEasy plasmids containing CMV-cDNA inserts were purified over QIAGEN columns (QIAGEN Inc., Valencia, CA), and 5  $\mu$ g of PacI-digested DNA was used to transfect QBI-293A cells using the calcium phosphate method (Promega Corp., Madison, WI). Cells were seeded at  $2 \times 10^6$  cells per 150-mm culture dish 24 hours prior to transfection. Lysis of transfected cells, indicating adenoviral growth, occurred within 4 days. Following amplification, lysates containing clonal recombinant adenovirus were prepared from 150-mm culture dishes and purified by CsCl gradient centrifugation. Recovered virus was aliquoted and stored at  $-20^{\circ}\text{C}$  in 5 mmol/L Tris (pH 8.0) buffer containing 50 mmol/L NaCl, 0.05% bovine serum albumin (BSA), and 25% glycerol. Virus was titrated by serial dilution infection of QBI-293A cells and plaques were counted under an overlay of 0.3% agarose, 10% fetal bovine serum (FBS), and 1x Dulbecco's modified Eagle's medium (DMEM).

**Administration of Wortmannin, LY-294002, Adenoviral Vectors, or VEGF Receptor Inhibitor.** Wortmannin (100  $\mu\text{g}/\text{kg}$  body weight/day; Calbiochem-Novabiochem Corp., San Diego, CA) or LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (1.5 mg/kg body weight/day; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.9% NaCl was administered in a volume of 50  $\mu\text{l}$ , as described previously (Tigani et al., 2001; Kwak et al., 2003). Wortmannin or LY-294002 was administered intratracheally two times to each treated 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). The vehicle was 0.9% NaCl containing DMSO. Adenoviral vectors ( $10^9$  plaque-forming units) were administered intratracheally two times to each animal under the same administration schedule described above. An inhibitor of VEGF receptor tyrosine kinase, SU5614 (Flk-1;  $\text{IC}_{50} = 1.2 \mu\text{mol}/\text{L}$ , 5-Chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone, Calbiochem-Novabiochem Corp.) was used to inhibit VEGF activity. SU5614 (2.5 mg/kg body weight/day) was dissolved in DMSO, and administered intraperitoneally three times at 24 hour-interval, beginning 1 hour after the last airway challenge with ovalbumin.

**Western Blot Analysis.** Lung tissues were homogenized in the presence of protease inhibitors and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA), as described previously (Kwak et al., 2003). Samples (30  $\mu\text{g}$  of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, 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% non-fat dry milk in Tris-Buffered Saline Tween-20 buffer (TBST; 25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) for 1



hour, and the blots were then incubated with an anti-PTEN antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-4 antibody (Serotec Ltd., Oxford, UK), anti-IL-5 antibody (Santa Cruz Biotechnology), anti-IL-13 antibody (R&D Systems, Inc. Minneapolis, MN), anti-IL-1 $\beta$  antibody (R&D Systems, Inc.), anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) antibody (R&D Systems, Inc.), anti-VEGF antibody (Santa Cruz Biotechnology), anti-intercellular adhesion molecule-1 (ICAM-1) antibody (Santa Cruz Biotechnology), anti-vascular cell adhesion molecule-1 (VCAM-1) antibody (Santa Cruz Biotechnology), anti-RANTES antibody (Abcam Ltd., Cambridge, UK), anti-eotaxin antibody (Abcam Ltd.), anti-Akt antibody (Cell Signaling Technology, Beverly, MA), or anti-phosphorylated Akt antibody (Cell Signaling Technology), overnight at 4°C. Anti-rabbit or anti-mouse horseradish peroxidase conjugated-IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was visualized by exposing to a photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

**Measurement of PI3K Enzyme Activity in Lung Tissues.** Lung tissues were homogenized in the presence of protease inhibitors. 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, UT). The enzyme activity was expressed as amounts of PIP3 (pmol/ml) produced by 1 ml of lung tissue extracts containing equal amounts of protein.

**Measurement of PTEN Activity.** PTEN activities were measured using the PTEN malachite green assay kit according to the protocol provided by the manufacturer (Upstate

Biotechnology Inc., Lake Placid, NY).

**Measurement of Plasma Exudation.** To assess lung permeability, Evans blue dye was dissolved in 0.9% NaCl at a final concentration of 5 mg/ml. Animals were weighed and injected with 20 mg/kg Evans blue dye in the tail vein. After 30 minutes, the animals were killed and their chests were opened. Saline containing 5 mM ethylene diamine tetraacetic acid (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. Evans blue dye 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 using a spectrophotometer (Spectra Max Plus Microplate Spectrophotometer, Sunnyvale, CA). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01-10 µg/ml and the results were expressed as ng of dye/mg of wet lung.

**Histology, Immunohistochemistry, and Immunocytochemistry.** At 72 hours after the last challenge, 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% (volume/volume) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4-µm 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, MI). For immunohistochemistry or immunocytochemistry of VEGF, the deparaffinized 4-µm sections or the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the instructions of the R. T. U. Vectastain Universal Quick kit

from Vector Laboratories Inc. (Burlingame, CA). Briefly, the slides were incubated in Endo/Blocker (Biomedica Corp., Foster City, CA) 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) overnight at 4°C, and were incubated with prediluted biotinylated pan-specific IgG for 10 minutes. The slides were incubated in streptavidin/oxidase complex reagent for 5 minutes, and then in 3-amino-9-ethylcarbazole substrate kit for 12 minutes. Controls consisted of sections of lung tissue or BAL cells from mice were incubated without the primary antibody. After immunostaining, the slides were counterstained for 1 minute with Gill's hematoxylin in 20% ethylene glycol and then mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, CA) and photomicrographed (VENOX-T, Olympus, Tokyo, Japan).

**Measurements of T<sub>H</sub>2 Cytokines and VEGF in BAL Fluids.** Levels of IL-4, IL-5, IL-13, and VEGF were quantified by an enzyme immunoassay according to the manufacturer's protocol (IL-4; Endogen, Inc., Woburn, MA, IL-5; BioSource International, Inc. Camarillo, CA, IL-13; R&D Systems, Inc., VEGF; R&D Systems Inc.). Sensitivities for IL-4, IL-5, IL-13, and VEGF assays were 5, 3, 1.5, and less than 3.0 pg/ml, respectively.

**Nuclear Protein Extractions for Analysis of Hypoxia-Inducible Factor (HIF)-1 $\alpha$ .** Lungs were removed and homogenized in 8 volumes of a lysis buffer containing 1.3 mol/L sucrose, 1.0 mmol/L MgCl<sub>2</sub>, and 10 mmol/L potassium phosphate buffer, pH 7.2. The homogenate was filtered through four layers of gauze and centrifuged at 1,000  $\times$  g 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 MgCl<sub>2</sub> to maintain a

final 2.2 mol/L sucrose concentration and centrifuged at  $100,000 \times g$  for 1 hour. The resulting nuclear pellets were washed once with a solution containing 0.25 mol/L sucrose, 0.5 mmol/L  $MgCl_2$ , and 20 mmol/L Tris-HCl, pH 7.2 and centrifuged at  $1,000 \times g$  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 (PMSF), and protein inhibitor cocktails. The mixture was kept on ice for 2 hours with gentle stirring and centrifuged at  $12,000 \times g$  for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for detection of HIF-1 $\alpha$ . For Western analysis, samples (30  $\mu$ g of protein per lane) were loaded on an 8% SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) by the wet transfer method (250 mA, 90 minutes). Nonspecific sites were blocked with 5% non-fat dry milk in TBST (25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) for 1 hour, and the blots were incubated with antibody against HIF-1 $\alpha$  (Novus Biologicals Inc., Littleton, CO) overnight at 4°C. Anti-rabbit horseradish peroxidase conjugated-IgG was used to detect binding of antibody. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

**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 (Takeda et al., 1997). 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 quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H<sub>2</sub>O 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 in-line 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 to 50 mg/ml in saline). After each methacholine challenge, the data of calculate airway resistance ( $R_L$ ) was 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 mean  $\pm$  SD. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at  $p < 0.05$ .

## Results

**Allergen-Induced Airway Inflammation Leads to Increased Levels of VEGF and Increased Plasma Exudation.** In a first set of experiments the changes of VEGF levels and plasma exudation at 6, 12, 24, 48, and 72 hours after a single ovalbumin inhalation using an enzyme immunoassay and the Evans blue dye assay were analyzed. The levels of VEGF protein in BAL fluids were increased significantly at 6, 12, 24, 48, and 72 hours after a single ovalbumin inhalation compared with the levels of control mice and the levels before ovalbumin inhalation (Fig. 1A). Consistent with the increased VEGF protein levels, the Evans blue dye assay revealed that plasma exudation was significantly increased at 6, 12, 24, 48, and 72 hours after a single ovalbumin inhalation compared with the levels of control mice and the levels before ovalbumin inhalation (Fig. 1B).

**LY-294002, Wortmannin, AdPTEN, or VEGF Receptor Inhibitor Decreased VEGF Protein Levels in BAL Fluids and Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice.** To investigate the effects of PI3K inhibitors, AdPTEN, or VEGF receptor inhibitor on VEGF expression in BAL fluids and the lung tissues at 72 hours after the last challenge, enzyme immunoassay, Western blot analysis, and immunohistochemical analysis were performed. Administration of LY-294002, wortmannin, AdPTEN, or SU5614 dramatically reduced the increased levels of VEGF in BAL fluids at 72 hours after the last challenge in three times ovalbumin-inhaled mice (Fig. 2). Consistent with the results obtained from the enzyme immunoassay, Western blot analysis revealed that the intratracheal administration of LY-294002, wortmannin, or AdPTEN reduced significantly the increased levels of VEGF in the lung tissues at 72 hours after ovalbumin inhalation (Fig. 3, A and B).

Immunohistochemical analysis showed the localization of immunoreactive VEGF in inflammatory cells and epithelial layers around the bronchioles of mice with ovalbumin-

induced asthma (Fig. 4B) whereas in the control mice VEGF was hardly detected in inflammatory cells around the bronchioles (Fig. 4A). Ovalbumin-sensitized and -challenged mice treated with AdPTEN resulted in a decrease of immunoreactive VEGF localized in inflammatory cells except in epithelial layers around the bronchioles (Fig. 4C), but AdLacZ did not (Fig. 4D). Immunocytologic analysis of BAL fluids showed the localization of immunoreactive VEGF in the precipitated cells from the ovalbumin-sensitized and -challenged mice (Fig. 4F). However, immunoreactive VEGF was reduced in BAL cells from control mice (Fig. 4E). Ovalbumin-sensitized and -challenged mice treated with AdPTEN decreased immunoreactive VEGF expression in BAL cells from allergen-induced asthmatic mouse lungs (Fig. 4G), but AdLacZ did not (Fig. 4H).

**Effect of LY-294002, Wortmannin, or AdPTEN on HIF-1 $\alpha$  Protein Levels in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice.** To evaluate the effects of PI3K inhibitors and AdPTEN on HIF-1 $\alpha$  levels at 72 hours after the last challenge, the Western blot analysis was employed. Western blot analysis revealed that levels of HIF-1 $\alpha$  protein in nuclear protein extracts from lung tissues were increased significantly at 72 hours after ovalbumin inhalation compared with the levels in the control mice (Fig. 3, C and D). The increased HIF-1 $\alpha$  levels in nuclear protein extracts from lung tissues at 72 hours after ovalbumin inhalation were decreased significantly by the administration of LY-294002, wortmannin, or AdPTEN.

**LY-294002, Wortmannin, AdPTEN, or VEGF Receptor Inhibitor Reduced Plasma Extravasation in Ovalbumin-Sensitized and -Challenged Mice.** To determine the effects of PI3K inhibitors, AdPTEN, and VEGF receptor inhibitor on plasma extravasation at 72 hours after the last challenge, the Evans blue dye assay was performed. The Evans blue dye

assay revealed that plasma extravasation was significantly increased at 72 hours after the last challenge (Fig. 5). The increase in plasma extravasation at 72 hours after ovalbumin inhalation was significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN. The administration of a VEGF receptor blocker, SU5614, also reduced plasma extravasation.

**Effect of LY-294002, Wortmannin, or AdPTEN on IL-4, IL-5, and IL-13 Levels in Lung.**

To show the effects of PI3K inhibitors and AdPTEN on T<sub>H</sub>2 cytokines at 72 hours after the last challenge, the Western blot analysis and enzyme immunoassay were employed. Western blot analysis revealed that IL-4, IL-5, and IL-13 protein levels in lung tissues were increased significantly at 72 hours after OVA inhalation compared with the levels after saline inhalation (Fig. 6, A and B). The increased levels of them at 72 hours after ovalbumin inhalation were significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays also showed the significant increase in levels of them in BAL fluids at 72 hours after ovalbumin inhalation as compared to the levels after saline inhalation. The increased levels of them at 72 hours after ovalbumin inhalation were significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN (Fig. 6C).

**Effect of LY-294002, Wortmannin, or AdPTEN on Levels of Adhesion Molecules and Chemokines.**

For the evaluation of the effects of PI3K inhibitors and AdPTEN on expression of adhesion molecules and chemokines at 72 hours after the last challenge, we measured VCAM-1, ICAM-1, eotaxin, and RANTES protein levels in lung tissues. Western blot analysis showed that VCAM-1, ICAM-1, eotaxin, and RANTES protein levels in the lung tissues were increased significantly at 72 hours after ovalbumin inhalation compared with the



levels after saline inhalation (Fig. 7, A and B). The increased levels of the proteins at 72 hours after ovalbumin inhalation were significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN.

**Effect of LY-294002, Wortmannin, or AdPTEN on TNF- $\alpha$  and IL-1 $\beta$  Levels.** For the examination of the effects of PI3K inhibitors and AdPTEN on levels of TNF- $\alpha$  and IL-1 $\beta$  at 72 hours after the last challenge, the Western blot analysis was performed. Western blot analysis showed that TNF- $\alpha$  and IL-1 $\beta$  protein levels in lung tissues were increased significantly at 72 hours after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 7, C and D). The increased levels of the proteins at 72 hours after ovalbumin inhalation were significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN.

**Effect of LY-294002, Wortmannin, AdPTEN, or VEGF Receptor Inhibitor on Cellular Changes in BAL Fluids.** To investigate the cellular changes in BAL fluids after administration of PI3K inhibitors, AdPTEN, and a VEGF receptor inhibitor at 72 hours after ovalbumin inhalation, total cells and eosinophils in BAL fluids were counted. Numbers of total cells and eosinophils in BAL fluids were increased significantly at 72 hours after ovalbumin inhalation compared with the numbers after saline inhalation (Fig. 8A). The increased numbers of eosinophils were significantly reduced by the administration of LY-294002, wortmannin, AdPTEN, or SU5614 (Fig. 8A).

**Effect of LY-294002, Wortmannin, or AdPTEN on Pathological Changes of Ovalbumin-Induced Asthma.** To assess the pathological changes of ovalbumin-inhaled mice by administration of PI3K inhibitors and AdPTEN at 72 hours after ovalbumin inhalation,

histologic analyses were performed. Histologic analyses revealed typical pathologic features of asthma in the ovalbumin-exposed mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Fig. 8C) as compared to the control (Fig. 8B). Mice treated with LY-294002 (Fig. 8D), wortmannin (Fig. 8E), or AdPTEN (Fig. 8F) showed marked reductions in the thickening of airway epithelium, in the infiltration of inflammatory cells in the peribronchiolar region, in the number of inflammatory cells, and in the amount of debris in the airway lumen. These results indicate that LY-294002, wortmannin, and AdPTEN inhibit antigen induced inflammation in the lungs, including the influx of eosinophils.

**Effect of LY-294002, Wortmannin, AdPTEN, or VEGF Receptor Inhibitor on Airway Hyperresponsiveness.** To examine the effect of PI3K inhibitors, AdPTEN, or VEGF receptor inhibitor on airway hyperresponsiveness at 72 hours after ovalbumin inhalation, we assessed airway responsiveness as a change in airway function after challenge with aerosolized methacholine via airways. In ovalbumin-sensitized and -challenged mice, the dose-response curve of percent  $R_L$  shifted to the left compared with that of control mice (Fig. 9). ovalbumin-sensitized and -challenged mice treated with LY-294002, wortmannin, AdPTEN, or VEGF receptor inhibitor, SU5614 showed a dose-response curve of percent  $R_L$  that shifted to the right compared with that of untreated mice. These results indicate that LY-294002, wortmannin, AdPTEN, or VEGF receptor inhibitor treatment reduces ovalbumin-induced airway hyperresponsiveness.

**Determination of Akt Phosphorylation and PI3K Enzyme Activity in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice.** To support the contention that effects of

wortmannin, LY-294002 or AdPTEN on allergen-induced airway inflammation were specifically directed through the PI3K pathway, we performed Western blotting to determine Akt phosphorylation and PIP3 competition enzyme immunoassay to measure PI3K enzyme activity. Levels of phosphorylated Akt protein in the lung tissues were significantly increased at 72 hours after ovalbumin inhalation compared with the levels in the control mice (Fig. 10, A and B). However, no significant changes in Akt protein levels were observed in any of the group tested. The increased phosphorylated Akt, but not Akt protein, levels in the lung tissues after ovalbumin inhalation were significantly reduced by the administration of LY-294002, wortmannin, or AdPTEN. The PI3K activity in the lung tissues was increased at 72 hours after ovalbumin inhalation compared with the control mice (Fig. 10C). The increased PI3K activity in the lung tissues after ovalbumin inhalation was significantly decreased by the administration of LY-294002, wortmannin, or AdPTEN.

**PTEN Protein Levels and PTEN Activities in Lung Tissues of OVA-Sensitized and -Challenged Mice.** Western blot analysis revealed that PTEN protein levels were decreased significantly at 72 hours after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 11, A and B). The decreased PTEN levels after ovalbumin inhalation was increased by the administration of AdPTEN. Consistent with these results, PTEN enzyme assays revealed that PTEN activity was decreased significantly at 72 hours after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 11C). The decreased PTEN activity after ovalbumin inhalation was increased by the administration of AdPTEN.

## Discussion

Bronchial asthma is characterized by inflammation of the airways which is usually accompanied by increased vascular permeability, resulting in plasma exudation (Bousquet et al., 2000). VEGF has been implicated to contribute to asthmatic tissue edema through its effect on vascular permeability (Thurston et al., 2000; Antony et al., 2002). Many cellular responses of VEGF are regulated by the lipid products of PI3K (Fujio and Walsh, 1999; Thakker et al., 1999). The PTEN has been shown to down-regulate PI3K signaling, yet the effect of PTEN on VEGF-mediated signaling is unknown. Our present study with the murine model of allergen-induced airway inflammation has revealed that PTEN, which blocks the action of PI3K, reduces antigen-induced airway infiltration of inflammatory cells, increased secretion of  $T_H2$  cytokines, adhesion molecules, and chemokines in lungs, airway hyperresponsiveness, and increased vascular permeability. Moreover, we have found that inhibition of PI3K or increase of PTEN expression reduces ovalbumin-induced up-regulation of VEGF level. These findings suggest that PTEN attenuates antigen-induced airway inflammation and hyperresponsiveness by modulation of VEGF expression in mice.

Many inflammatory mediators attract and activate eosinophils via signal transduction pathways involving the enzyme PI3K (Dunzendorfer et al., 1998; Palframan et al., 1998; Zhu et al., 2000; Ezeamuzie et al., 2001). Recently, we have demonstrated that administration of either PI3K inhibitors or AdPTEN reduces eosinophilic inflammation and airway hyperresponsiveness in a murine model of asthma (Kwak et al., 2003). Duan et al. have also shown that inhibition of PI3K signaling pathway may suppress  $T_H2$  cytokine production, eosinophil infiltration, mucus production, and airway hyperresponsiveness (Duan et al., 2005). Consistent with these observations, our present study has shown that PI3K inhibitors or AdPTEN attenuated antigen-induced airway inflammation and hyperresponsiveness. In addition, plasma extravasation caused by increased vascular permeability was elevated after

ovalbumin inhalation and that administration of PI3K inhibitors or AdPTEN significantly reduced the increased plasma extravasation. 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. Exudation of plasma proteins into the airways correlates with bronchial hyperreactivity (Van de Graaf et al., 1991). It is also possible 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 (Persson, 1996).

Recently, Lee et al. have reported that VEGF is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and T<sub>H</sub>2 inflammation (Lee CG et al., 2004). Several studies have also demonstrated that VEGF exhibits chemotactic effects on monocytes, neutrophils, and eosinophils via PI3K pathway (Clauss et al., 1990; Berleon et al., 1996; Feistritzer et al., 2004; Jia et al., 2004). One of the major roles of VEGF in asthma appears to be the enhancement of vascular permeability (Dvorak et al., 1995; Lee et al., 2002; Lee KS et al., 2004). The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesicular-vacuolar organelles (Dvorak et al., 1994; Dvorak et al., 1995). VEGF can be produced by a wide variety of cells including macrophages, neutrophils, eosinophils, and lymphocytes (Horiuchi and Weller, 1997; Hoshino et al., 2001; Lee et al., 2002; Lee CG et al., 2004; Lee KS et al., 2004). 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 and allowing the migration of inflammatory cells into the airways (Thurston et al., 2000; Lee et al., 2002; Lee KS et al., 2004). Consistent with these observations, we have found that VEGF expression

was up-regulated in ovalbumin-induced airway inflammation. Interestingly, administration of the PI3K inhibitors or AdPTEN reduced the increased VEGF expression. In addition, we have found that VEGF receptor inhibitor, SU5614, inhibited the increased vascular permeability, the airway inflammation, and the increased VEGF expression. These results suggest that PI3K signaling pathway is associated with the regulation of VEGF expression and that treatment of the PI3K inhibitors and AdPTEN may decrease the vascular permeability by inhibiting up-regulation of VEGF expression which leads to airway inflammation in asthma. Recent studies have reported that migration of cells into sites of inflammation are blocked directly by PI3K inhibition (Sasaki et al., 2000; Funamoto et al., 2002; Hannigan et al., 2002; Wang et al., 2002). Moreover, class IA PI3K plays a key role in induction of the T<sub>H</sub>2 response (Palframan et al., 1998; Fukao et al., 2002a; Fukao et al., 2002b), and PI3K inhibitors are known to reduce the levels of chemokines and adhesion molecules as well as T<sub>H</sub>2 cytokines (Morel et al., 2001; Kwak et al., 2003; Duan et al., 2005; Amin et al., 2005). In the present study, the results have revealed that the increased levels of T<sub>H</sub>2 cytokines (IL-4, IL-5, and IL-13), adhesion molecules (ICAM-1 and VCAM-1), and chemokines (RANTES and eotaxin) in lungs after ovalbumin inhalation were significantly decreased by treatment of PI3K inhibitors, LY-294002 or wortmannin. Taken together, these observations suggest that PI3K inhibitors can decrease the migration of inflammatory cells by inhibiting VEGF-induced vascular permeability and by blocking chemotactic effects of VEGF on leukocytes. In addition, the direct effects of the PI3K inhibitors on leukocytes and/or effects of PI3K on the release of chemokines, which subsequently leads to a reduced number of leukocytes, may have an effect on the migration of inflammatory cells.

Cytokine-inducible leukocyte-endothelial adhesion molecules are important in the recruitment and migration of leukocytes to the sites of inflammation (Montefort and Holgate, 1991). Expression of ICAM-1 or VCAM-1 is modulated by cytokines such as IL-1 $\beta$ , IL-4,

and TNF- $\alpha$  (Dustin et al., 1986; Osborn et al., 1989; Hirata et al., 1998). We have revealed that PI3K inhibitors or AdPTEN reduced the increased numbers of inflammatory cells in the airways which are the source of IL-1 $\beta$ , IL-4, and TNF- $\alpha$ . Consistent with these observations, the levels of IL-1 $\beta$ , IL-4, and TNF- $\alpha$  in BAL fluids of ovalbumin-sensitized and -challenged mice were decreased by the administration of these agents. Decrease in the expression of ICAM-1 and VCAM-1 proteins may be due to the reduced IL-1 $\beta$ , IL-4, and TNF- $\alpha$  levels in the lungs. Hence, these results strongly indicate that PI3K inhibitors or AdPTEN regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression and possibly also by suppressing IL-1 $\beta$ , IL-4, and TNF- $\alpha$  expression.

VEGF expression is regulated through HIF-1 $\alpha$  expression (Semenza, 1999). It has known that regulation of HIF-1 $\alpha$  expression and activity is based upon the cellular O<sub>2</sub> concentration and that HIF-1 $\alpha$  expression and activity also are regulated by major signal transduction pathways, including those involving PI3K and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) (Berra et al., 2000; Semenza, 2002). Previous reports have demonstrated that HIF-1 $\alpha$  plays a critical role in immune and inflammatory responses (Lukashev et al., 2001; Jung et al., 2003). Consistent with previous reports, determination of HIF-1 $\alpha$  protein level in nuclear extracts has revealed that this protein level was substantially increased in our present ovalbumin-induced model of airway inflammation. The administration of PI3K inhibitor or AdPTEN, which blocks the action of PI3K, resulted in significant reduction of nuclear HIF-1 $\alpha$  level as well as expression of VEGF. These results suggest that AdPTEN inhibits VEGF expression through the regulation of HIF-1 $\alpha$  expression by inhibition of PI3K/Akt pathway.

In summary, we have examined the role of the PTEN in a murine model of allergen-induced airway inflammation, more specifically in the expression of VEGF. The administration of AdPTEN was effective in reversing all pathophysiological symptoms examined. Our data

also suggest that administration of AdPTEN substantially reduces expression of VEGF including the activity of VEGF. The activity includes plasma leakage and migration of inflammatory cells (Fig. 12). Therefore, one likely mechanism for the effectiveness of PTEN is the reduction of VEGF expression to physiological level thereby pathological actions of VEGF cease. Thus, these findings provide a crucial molecular mechanism for the potential role of PTEN in preventing asthma and other airway inflammatory disorders.



## **Acknowledgments**

We thank professor Mie-Jae Im for critical readings of the manuscript.

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**Footnotes**

This work was supported by grants from the National Research Laboratory Program of the Korea Science and Engineering Foundation and by Korea Research Foundation Grants funded by Korea Government (MOEHRD, Basic Research Promotion Fund) (KRF-2005-201-E00014), Republic of Korea.

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### Figure legends

**Fig. 1.** The kinetics of VEGF expression and plasma exudation after single ovalbumin-challenge. (A) Enzyme immunoassay of VEGF in BAL fluids. (B) Plasma exudation measured using Evans blue dye assay. Data represent mean  $\pm$  SD from 6 mice per group. 6, 12, 24, 48, and 72 hours are time periods after single challenge. Control, no treatment; Pre, 1 hour before the first challenge; #,  $p < 0.05$  versus Pre; \*,  $p < 0.05$  versus saline inhalation.

**Fig. 2.** Effect of LY-294002, wortmannin, AdPTEN, or SU5614 on VEGF protein levels in BAL fluids. BAL was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), saline-inhaled mice administered LY-294002 (SAL+LY294002), saline-inhaled mice administered wortmannin (SAL+wortmannin), saline-inhaled mice administered AdLacZ (SAL+AdLacZ), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614). VEGF protein levels in BAL fluids were quantified using an enzyme immunoassay. Bars represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 3.** Effect of LY-294002, wortmannin, or AdPTEN on VEGF protein and HIF-1 $\alpha$  protein levels in lung tissues. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice

administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), and ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ). (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) HIF-1 $\alpha$  levels in nuclear protein extracts from lung tissues. (D) Densitometric analyses are presented as the relative ratio of HIF-1 $\alpha$  to HIF-1 $\beta$ . The relative ratio of HIF-1 $\alpha$  in the lung tissues of SAL+SAL is arbitrarily presented as 1. Bars represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 4.** Effect of LY-294002, wortmannin, or AdPTEN on VEGF expression in lung tissues of ovalbumin-sensitized and -challenged mice. (A-D) Localization of immunoreactive VEGF in lung tissues of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 hours after the last challenge in lung tissues from sensitized mice challenged with saline (A), from sensitized mice challenged with ovalbumin (B), from ovalbumin-inhaled mice administered AdPTEN (C), and from OVA-inhaled mice administered AdLacZ (D). Representative light microscopy of VEGF-positive cells in the bronchioles. The brown color indicates VEGF-positive cells. Bars indicate scale of 50  $\mu$ m. (E-F) Localization of immunoreactive VEGF in BAL cells of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 hours after the last challenge from sensitized mice challenged with saline (E), from sensitized mice challenged with ovalbumin (F), from ovalbumin-inhaled mice administered AdPTEN (G), and from ovalbumin-inhaled mice administered AdLacZ (H). Representative light microscopy of VEGF-positive cells in BAL fluids. The brown color indicates VEGF-positive cells. Bars indicate scale of 50  $\mu$ m.

**Fig. 5.** Effect of LY-294002, wortmannin, AdPTEN, or SU5614 on plasma exudation. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), saline-inhaled mice administered LY-294002 (SAL+LY294002), saline-inhaled mice administered wortmannin (SAL+wortmannin), saline-inhaled mice administered AdLacZ (SAL+AdLacZ), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614). The plasma exudation was quantified by Evans blue dye assay. Bars represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 6.** Effect of LY-294002, wortmannin, or AdPTEN on IL-4, IL-5, and IL-13 levels in lung tissues and in BAL fluids. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), saline-inhaled mice administered LY-294002 (SAL+LY294002), saline-inhaled mice administered wortmannin (SAL+wortmannin), saline-inhaled mice administered AdLacZ (SAL+AdLacZ), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), and ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ). (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 mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 7.** Effect of LY-294002, wortmannin, or AdPTEN on VCAM-1, ICAM-1, eotaxin, RANTES, TNF- $\alpha$ , and IL-1 $\beta$  in lung tissues of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), and ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ). (A) Western blotting of VCAM-1, ICAM-1, eotaxin, and RANTES 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) Western blotting of TNF- $\alpha$  and IL-1 $\beta$  in lung tissues. (D) 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. Bars represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL and OVA+AdLacZ.

**Fig. 8.** Effects of LY-294002, wortmannin, AdPTEN, or SU5614 on cellular changes in BAL fluids and pathologic changes in lung tissues. The BAL cells was measured at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), saline-inhaled mice administered LY-294002 (SAL+LY294002), saline-inhaled mice administered wortmannin (SAL+wortmannin), saline-inhaled mice administered AdLacZ (SAL+AdLacZ), ovalbumin-

inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614). (A) The numbers of total cells and eosinophils of BAL fluids. (B-F) Representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed at 72 hours after the last challenge in saline-inhaled mice administered saline (B), ovalbumin-inhaled mice administered saline (C), ovalbumin-inhaled mice administered LY-294002 (D), ovalbumin-inhaled mice administered wortmannin (E), and ovalbumin-inhaled mice administered AdPTEN (F). Bars indicate scale of 50  $\mu$ m. Data represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 9.** Effects of LY-294002, wortmannin, AdPTEN, or SU5614 on airway responsiveness. Airway responsiveness was measured at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), saline-inhaled mice administered LY-294002 (SAL+LY294002), saline-inhaled mice administered wortmannin (SAL+wortmannin), saline-inhaled mice administered AdLacZ (SAL+AdLacZ), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614). (A)  $R_L$  on methacholine challenge in OVA-inhaled mice.  $R_L$  values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine as described in Materials and Methods. (B)  $R_L$  on

methacholine challenge in saline-inhaled mice. Data represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 10.** Effect of LY-294002, wortmannin, or AdPTEN on phosphorylated Akt and Akt protein levels and PI3K enzyme activity in lung tissues of ovalbumin-sensitized and -challenged mice. Phosphorylated Akt and Akt protein levels were measured at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered LY-294002 (OVA+LY294002), ovalbumin-inhaled mice administered wortmannin (OVA+wortmannin), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), and ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ). (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 is arbitrarily presented as 1. (C) Enzyme immunoassay of PIP3 generation by PI3Ks in lung tissue extracts. Bars represent mean  $\pm$  SD from 6 mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

**Fig. 11.** Effect of AdPTEN on PTEN protein and PTEN activity in lung tissues of ovalbumin-sensitized and -challenged mice. (A) Western blotting of PTEN. PTEN protein expression was measured at 72 hours after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered AdPTEN (OVA+AdPTEN), and ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ). (B) Densitometric analyses are presented as the relative ratio of PTEN to actin. The relative ratio of PTEN in the lung tissues of SAL+SAL is arbitrarily presented as 1. (C) PTEN activity was measured in lung tissues from sensitized mice

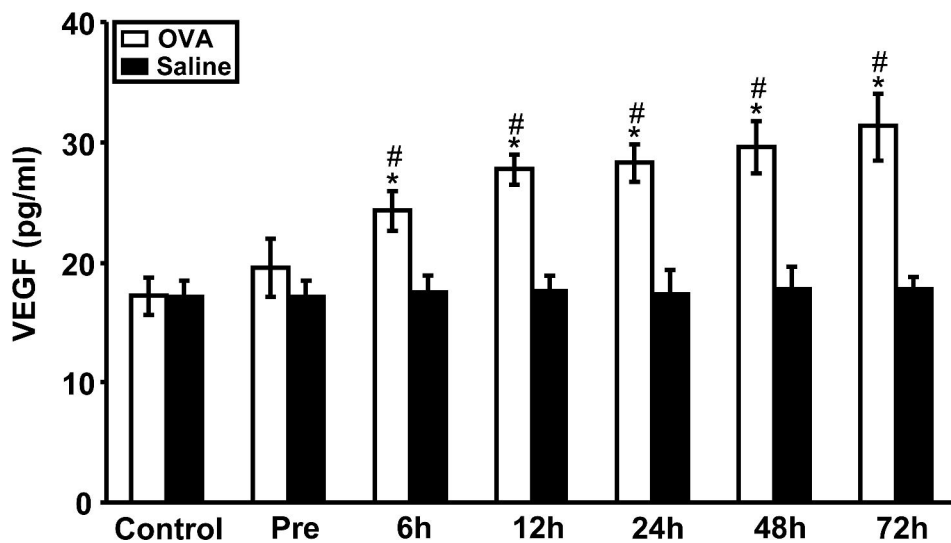
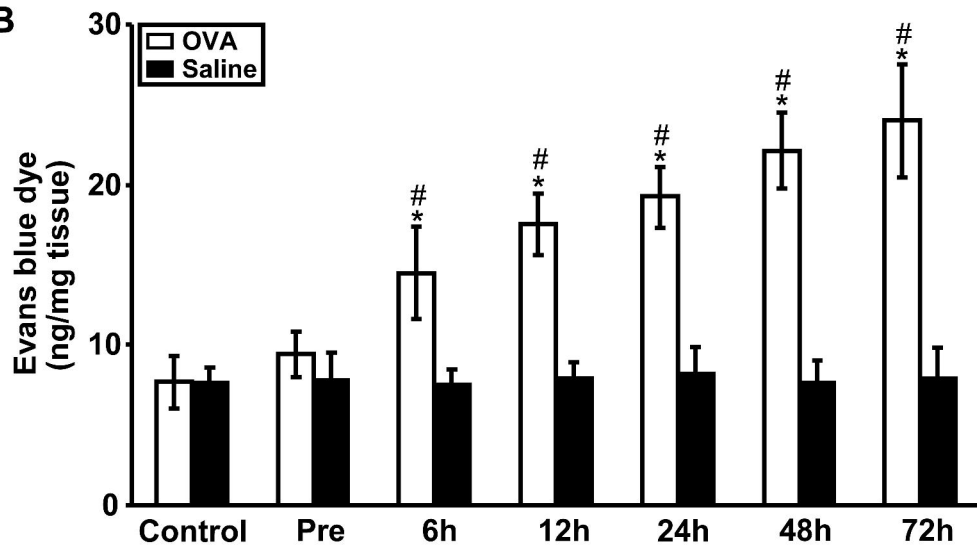
challenged with ovalbumin or with saline. Data represent mean  $\pm$  SD from 6 mice per group.

#,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.

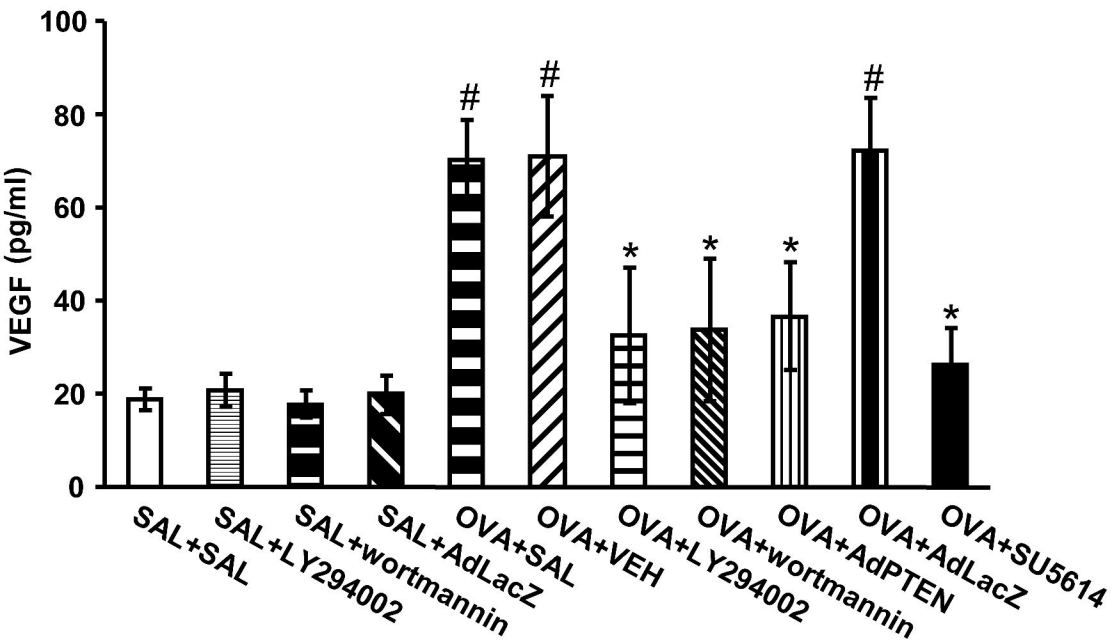
**Fig. 12.** A proposed mechanism of PTEN signaling pathway. It suggests that PTEN inhibits VEGF expression through the regulation of HIF-1 $\alpha$  activation by inhibition of PI3K/Akt pathway.



# Figure 1

**A****B**

# Revised figure 2



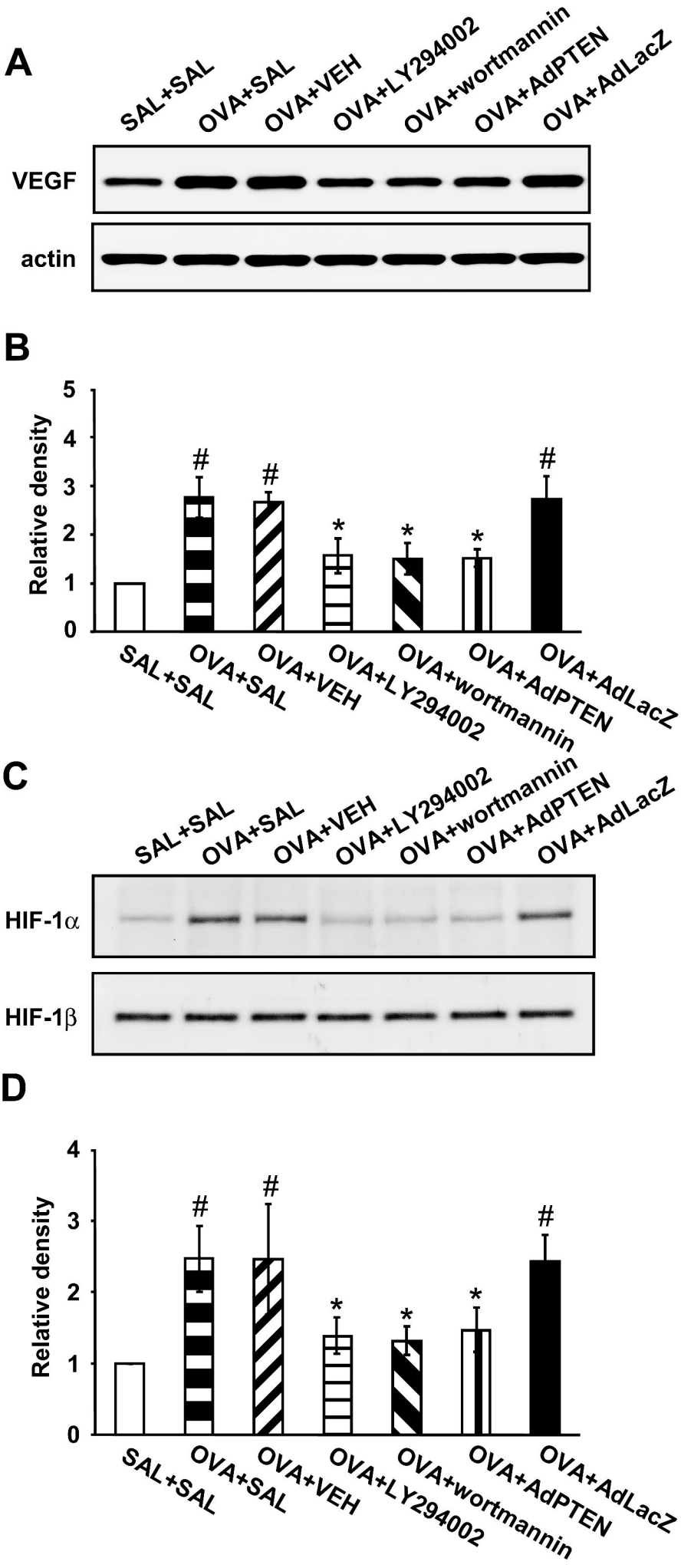
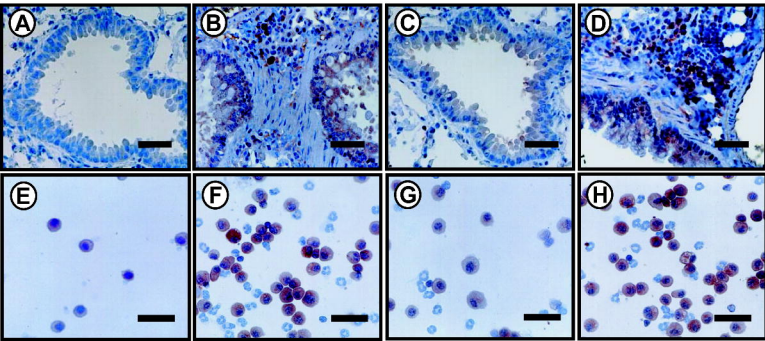
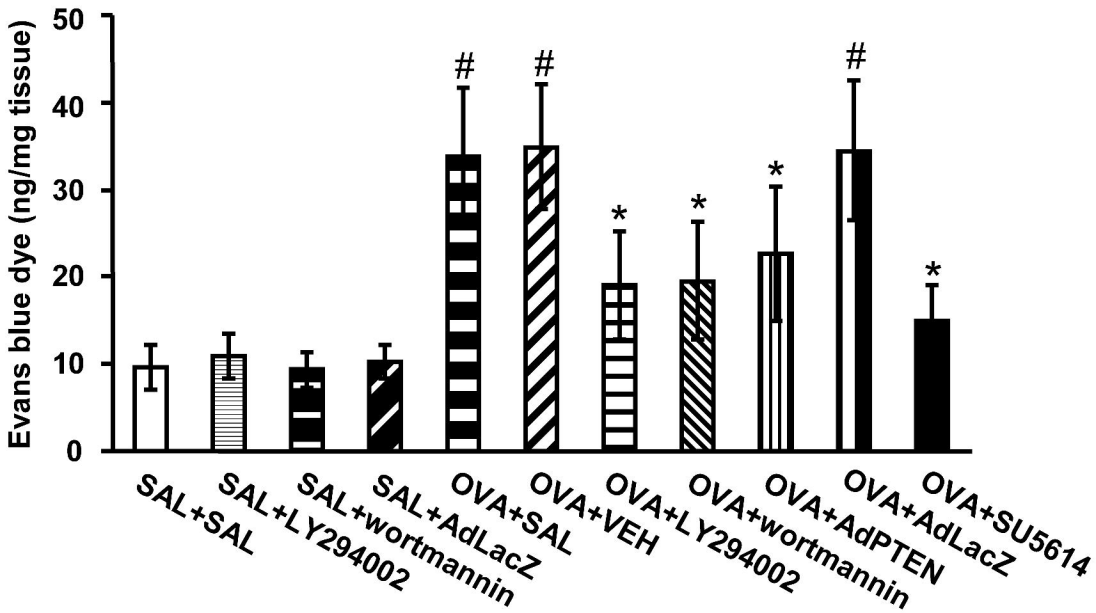


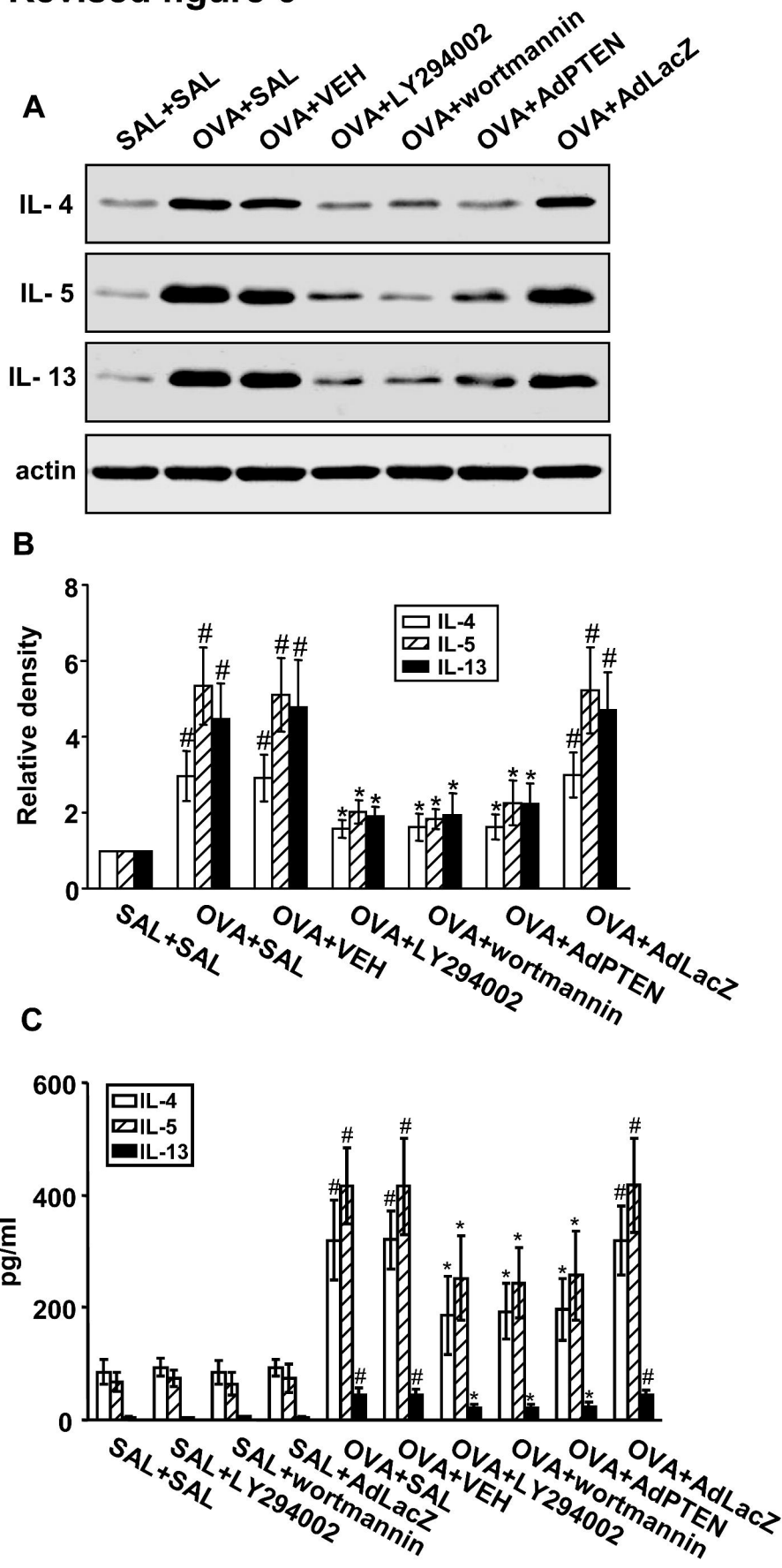
Figure 4



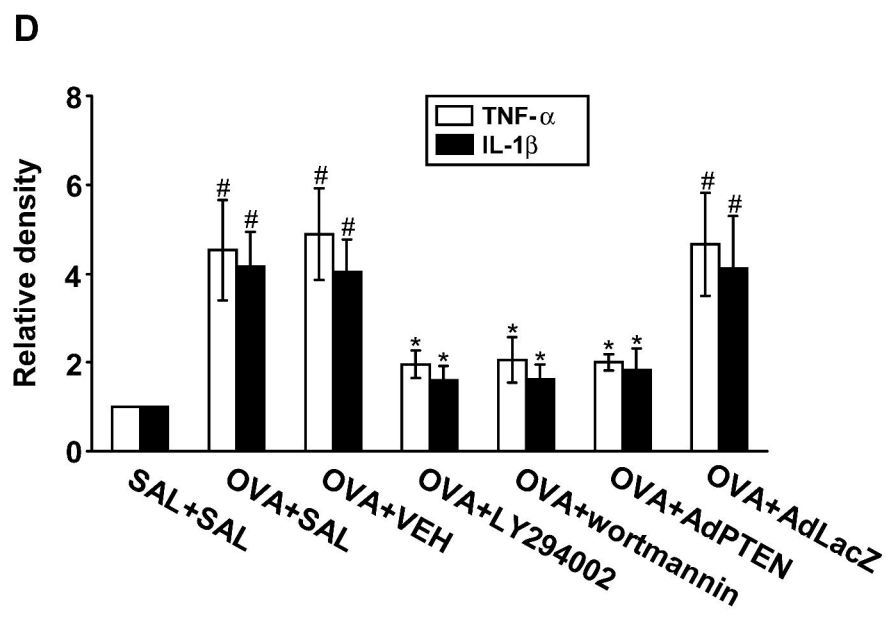
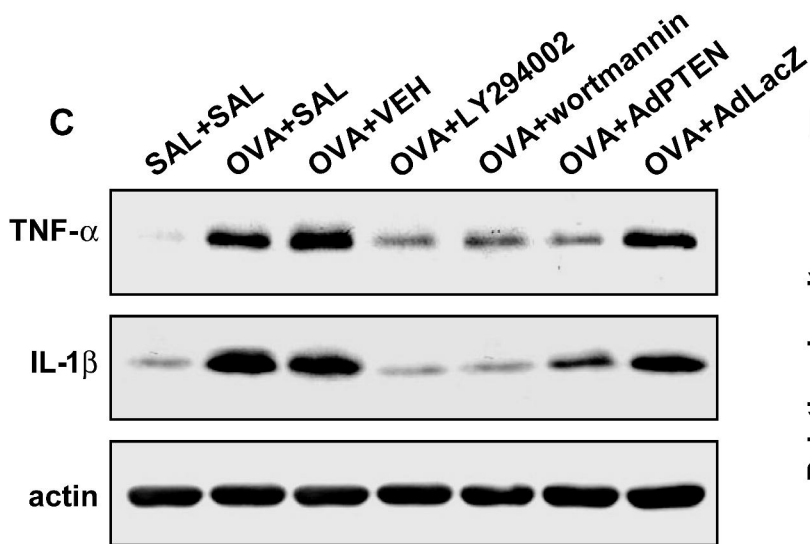
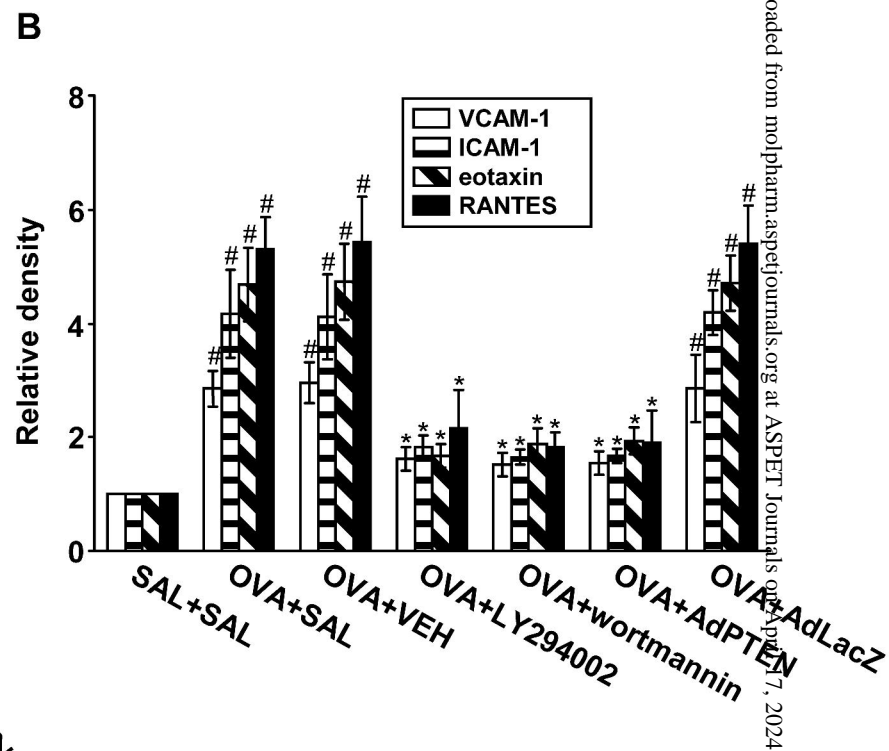
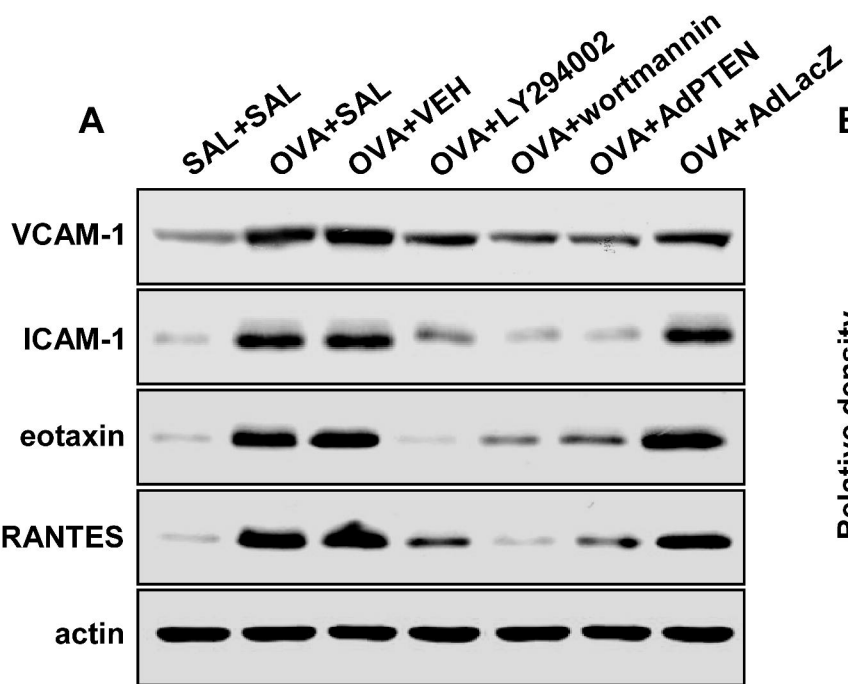
# Revised figure 5



# Revised figure 6

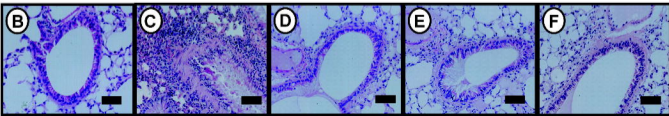
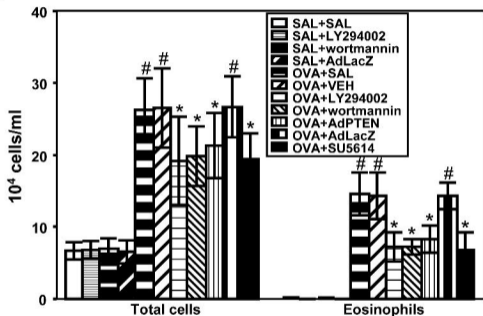


# Revised figure 7



# Revised figure 8

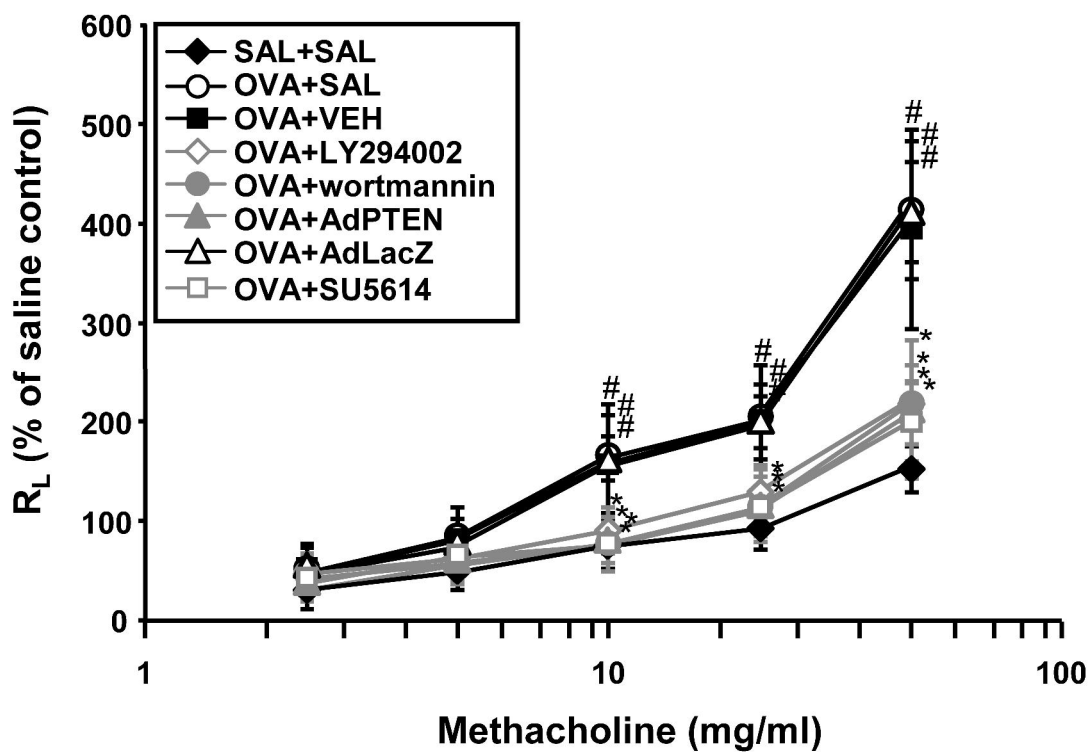
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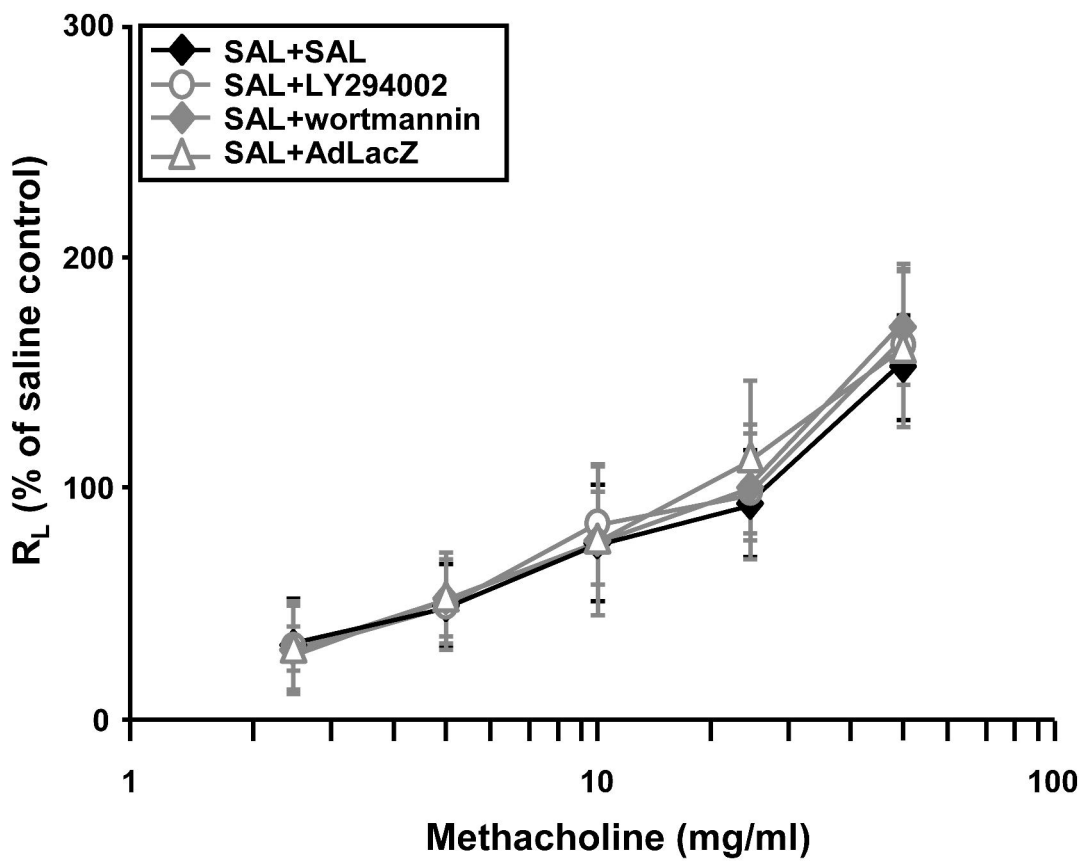


# Revised figure 9

## A

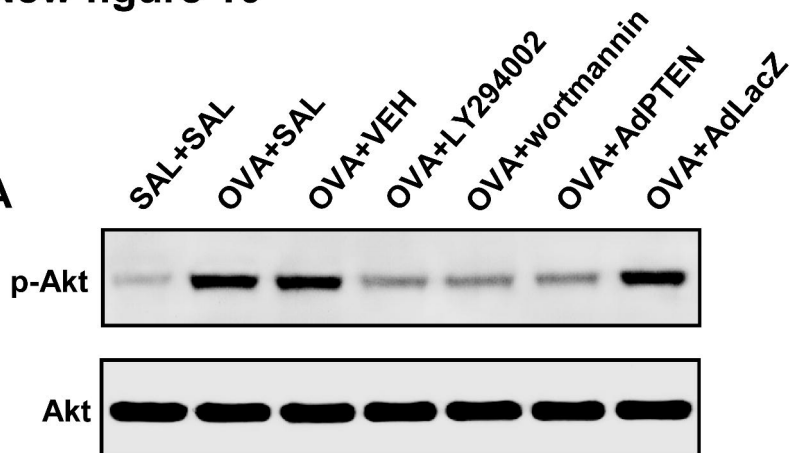


## B

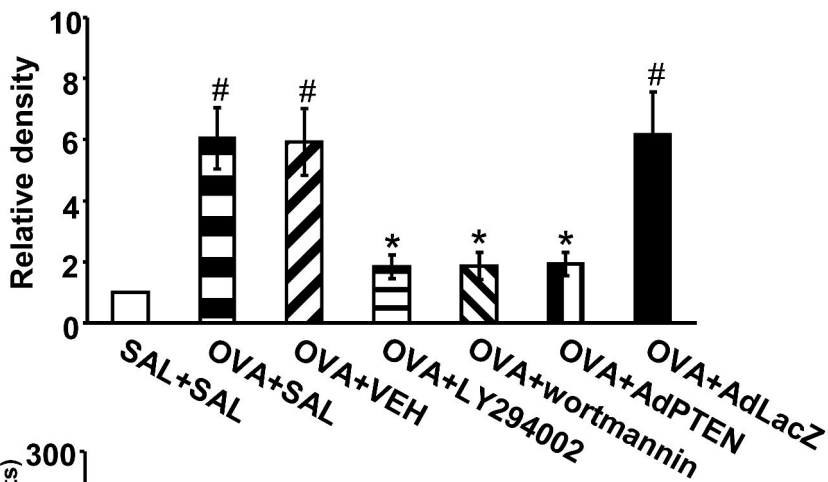


# New figure 10

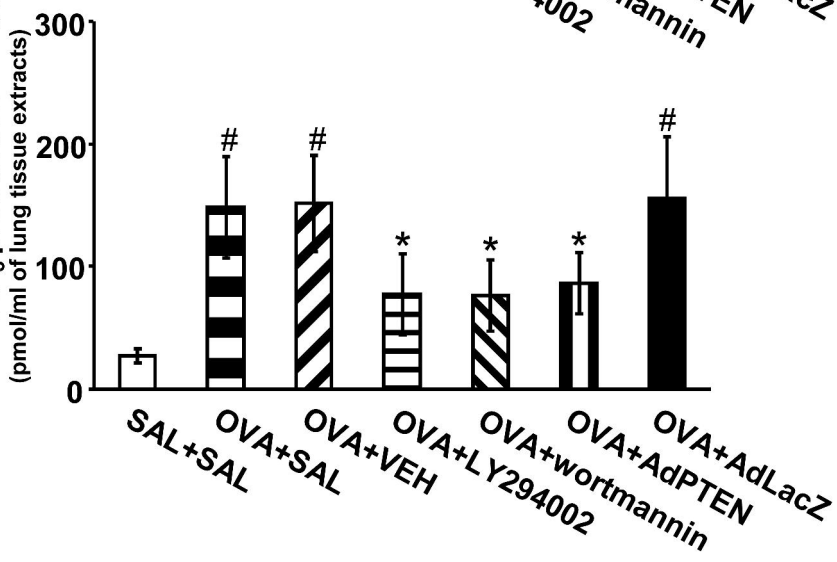
**A**



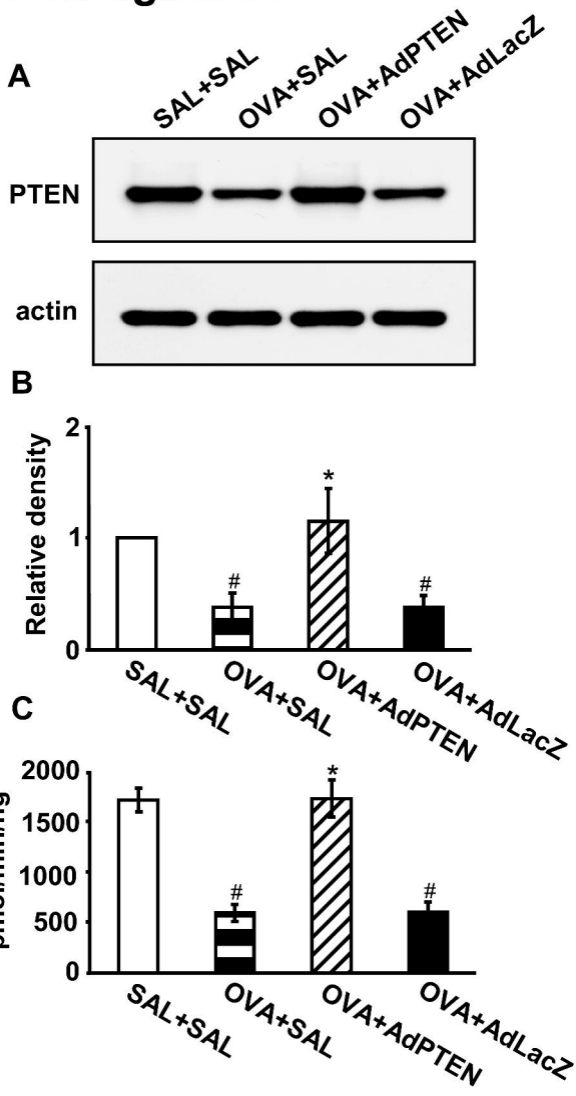
**B**



**C**



# New figure 11



# New figure 12

**PTEN/PI3K inhibitor**

```
graph TD; A([PTEN/PI3K inhibitor]) --> B[Inhibition of PI3K/Akt pathway]; B --> C[Inhibition of HIF-1α activation]; C --> D[Inhibition of VEGF expression]; D --> E[Inhibition of vascular leakage and inflammation];
```

Inhibition of PI3K/Akt pathway

Inhibition of HIF-1 $\alpha$  activation

Inhibition of VEGF expression

Inhibition of vascular leakage  
and inflammation