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A prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid, regulates vascular permeability by reducing vascular endothelial growth factor expression in asthma

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Running title: L-2-oxothiazolidine-4-carboxylic acid in asthma

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ABBREVIATIONS: BAL, Bronchoalveolar lavage; HIF-1, hypoxia-inducible factor 1; OTC, L-2-oxothiazolidine-4-carboxylic acid; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidyl inositol-3,4,5-triphosphate; R_L, airway resistance; ROS, reactive oxygen species; TBST, Tris-buffered saline Tween 20; VEGF, vascular endothelial growth factor

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Abstract

Inflammation of the asthmatic airway is usually accompanied by increased vascular permeability and plasma exudation. Oxidative stress plays critical roles in airway inflammation. Although reactive oxygen species (ROS) are shown to cause vascular leakage, the mechanisms by which ROS induce increased vascular permeability are not clearly understood. We have used a murine model of asthma to evaluate the effect of L-2-oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine, which acts as an antioxidant, more specifically in the increase of vascular permeability. These mice develop the following typical pathophysiological features of asthma in the lungs: increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, increased vascular permeability, and increased levels of vascular endothelial growth factor (VEGF). Administration of OTC markedly reduced plasma extravasation and VEGF levels in allergen-induced asthmatic lungs. We also showed that the increased hypoxia-inducible factor-1α, transcriptional activator of VEGF, levels in nuclear protein extracts of lung tissues at 72 h after ovalbumin inhalation were decreased by the administration of OTC. These results indicate that OTC modulates vascular permeability by lowering VEGF expression.

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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). Reactive oxygen species (ROS) play a crucial role in the pathogenesis of airway inflammation (Rahman et al., 1996; Dworski, 2000; MacNee, 2001). The inflammatory cells recruited to the asthmatic airways have a capability of producing ROS. Evidence for increase oxidative stress in asthma is further provided by the finding of defective endogenous antioxidant capacity in asthmatic patients (Dworski, 2000). ROS can lead to endothelial barrier dysfunction with subsequent increased permeability to fluids, macromolecules, and inflammatory cells (Henricks and Nijkamp, 2001). However, the mechanisms by which ROS induce increased vascular permeability are not clearly understood.

ROS are shown to induce vascular endothelial growth factor (VEGF) expression in vitro and in vivo (Kuroki et al., 1996). 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 so that plasma proteins can leak into the extravascular space, which leads to edema and profound alterations in the extracellular matrix. 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.,

2002b). Activation of hypoxia-responsive genes including VEGF is mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric basic helix-loop-helix-PAS domain transcription factor (Wang and Semenza, 1995; Semenza, 1999). HIF-1 is composed of two subunits, HIF-1α and HIF-1ß. Whereas the ß-subunit protein is constitutively present, the stability of the α-subunit and its transcriptional activity are precisely controlled by the intracellular oxygen concentration (Maxwell et al., 1999; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Kaelin, 2002).

Recently, we have demonstrated that L-2-oxothiazolidine-4-carboxylic acid (OTC) is able to reduce inflammation and hyperreactivity in animal models of asthma (Lee et al., 2004b). In the present study, we used a murine model of asthma to evaluate the effect of a prodrug of cysteine, OTC, which is known as an antioxidant and a precursor of glutathione biosynthesis, more specifically on the increase of vascular permeability.

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Materials and Methods

Animals and Experimental Protocol. Female BALB/c mice, 8-10 weeks of age and free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejon, Korea), were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μg ovalbumin (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 μl, as previously described (Kwak et al., 2003) (Fig. 1). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed. At the time of lavage, the mice (6 mice in each group) were sacrificed with an overdose of pentobarbital sodium (100 mg/kg of 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

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into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. A part of each pool was then centrifuged and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Shadon Scientific Ltd., Cheshire, United Kingdom). 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. Interinvestigator variation was < 5%. The mean number from the two investigators was used to estimate the cell differentials.

Administration of OTC, α-Lipoic Acid, or VEGF Receptor Inhibitor. OTC solution (80 or 160 mg/kg body weight/day, Sigma-Aldrich) was freshly prepared as described elsewhere (Han et al., 2002) and administered intraperitoneally four times at 24 h intervals on days 21-24, beginning 1 h before the first challenge. α-Lipoic acid (50 or 100 mg/kg body weight/day, Sigma-Aldrich) which is a nonenzymatic antioxidant, was administered 7 times by oral gavage at 24 h intervals on days 19-25, beginning 2 days before the first challenge (Fig. 1). An inhibitor of VEGF receptor tyrosine kinase, SU5614 (Flk-1; IC_{50} = 1.2 μM, 5-Chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone, Calbiochem-Novabiochem Corp., San Diego, CA) was used to inhibit

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VEGF activity. SU5614 (2.5 mg/kg body weight/day) was dissolved in dimethyl sulfoxide, and administered intraperitoneally three times at 24 h intervals, beginning 1 h after the last airway challenge with ovalbumin (Fig. 1).

Measurement of Intracellular Reactive Oxygen Species. ROS were measured by a method previously described with modification (Sundaresan et al., 1995; Lee et al., 2002a). BAL fluids were washed with phosphate buffered saline (PBS). To measure intracellular ROS, cells were incubated for 10 min at room temperature with PBS containing 3.3 μM 2',7'-dichlorofluorescein diacetate (Molecular probes, Eugene, OR), to label intracellular ROS. The cells were then immediately observed under fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) and fluorescence-activated cell sorting analysis (Partec, Münster, Germany).

Measurement of Plasma Exudation. To assess lung permeability, Evans blue dye was dissolved in 0.9% saline 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 min, the animals were killed and their chests were opened. Normal saline containing 5 mM 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 h and the

absorption of light at 620 nm was measured in 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 is expressed as ng of dye/mg of wet lung.

Histology, Immunohistochemistry, and Immunocytochemistry. At 72 h 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% (v/v) 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). Inflammation score was graded by three independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (Tournoy et al., 2000). A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells, and

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a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. For immunohistochemistry and immunocytochemistry of VEGF or HIF-1α, 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 (Biomeda Corp., Foster City, CA) for 5 min and in pepsin solution for 4 min at 40°C. The slides were incubated in normal horse serum for 15 min at room temperature. The slides were then probed with antibody against VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) or HIF-1α (Novus Biologicals Inc., Littleton, CO) overnight at 4°C, and were incubated with prediluted biotinylated pan-specific IgG for 10 min. The slides were incubated in streptavidin/peroxidase complex reagent for 5 min, and then in 3-amino-9-ethylcarbazole substrate kit for 12 min. 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 min with Gill's hematoxylin in 20% ethylene glycol and then mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, CA) and photomicrographed (Vanox T; Olympus, Tokyo, Japan).

Measurements of VEGF in BAL Fluids. Levels of VEGF were quantified by an

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enzyme immunoassay according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN). The minimum detectable level of mouse VEGF is less than 3.0 pg/ml.

Western Blot Analysis. BAL fluids were obtained from the tracheas of ovalbumin challenged mice with 0.8 ml saline solution and were centrifuged at 4000 × *g* for 1 min. Each supernatant was recovered, and the remaining cell pellet was resuspended in PBS for cytospin. Each BAL fluid supernatant was quantified using the Bradford reagent (Bio-Rad, Hercules, CA) and 3 μg of BAL protein was loaded on a 12% SDS-polyacrylamide gel electrophoresis gel and separated at 120 V for 90 min. 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). Samples (30 μg 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 min). Nonspecific sites were blocked with 5% non-fat milk in Tris-buffered saline Tween 20 (TBST) (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 2 h, and the blots were then incubated with an anti-VEGF antibody (Santa Cruz Biotechnology), anti-Akt antibody (Cell Signaling Technology Inc., Beverly, MA), or

anti-phosphorylated Akt (p-Akt) antibody (Cell Signaling Technology Inc.) overnight at 4°C. Anti-rabbit 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).

Nuclear Protein Extractions for Analysis of HIF-1α. Lungs were removed and homogenized in 8 volumes of a lysis buffer containing 1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 7.2. The homogenate was filtered through four layers of gauze and centrifuged at 1,000 × *g* for 15 min. The resulting pellets were carefully harvested and resuspended in 10 mM potassium phosphate buffer (pH 7.2) containing 2.4 M sucrose and 1.0 mM $MgCl_2$ to maintain a final 2.2 M sucrose concentration and centrifuged at 100,000 × *g* for 1 h. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.2 and centrifuged at 1,000 × *g* for 10 min. The pellets were solubilized with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM PMSF, and protease inhibitor cocktails. The mixture was kept on ice for 2 h with gentle stirring and centrifuged at 12,000 × *g* for 30 min. The resulting supernatant was used as soluble nuclear proteins

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for detection of HIF-1α. For Western analysis, samples (30 μg of protein per lane) were loaded on an 8% SDS-polyacrylamide gel electrophoresis gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% non-fat dry milk in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the blots were incubated with antibody against HIF-1α (Novus Biologicals Inc.) overnight at 4°C. Anti-rabbit horseradish peroxidase conjugated IgG was used to detect binding of antibodies. The binding of the specific antibodies was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

Measurement of Phosphatidylinositol 3-kinase (PI3K) Enzyme Activity in Lung Tissue. . 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). The amount of phosphatidyl inositol-3,4,5-triphosphate (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 pmol PIP3 produced by 1 ml of lung tissue extracts containing equal

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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 (Takeda et al., 1997; Eum et al., 2003). Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, and inserted with an 18-gauge metal needle. 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/min and a positive end-expiratory pressure of 2 cm H_2O 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 (2.5-50 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

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from baseline after saline aerosol.

Densitometric Analyses and Statistics. The immunoreactive signals of HIF-1α were analyzed by densitometric scanning (Gel Doc XR, Bio-Rad Laboratories Inc., Hercules, CA). Data were expressed as mean ± S.E.M. Statistical comparisons were performed using one-way analysis of variance followed by the Fisher's test. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at $p < 0.05$.

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Results

Effect of OTC on ROS Generation in BAL Fluids. ROS generation in BAL fluids was increased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 2). The increased ROS generation was significantly reduced by the administration of OTC.

OTC, α-Lipoic Acid, and VEGF Receptor Inhibitor Decreased VEGF Levels in BAL Fluids of Ovalbumin-sensitized and -challenged Mice. Administration of OTC, α-lipoic acid, or SU5614 dramatically reduced the increased levels of VEGF in BAL fluids at 72 h after the last challenge (Fig. 3A). Consistent with the results obtained from the enzyme immunoassay, Western blot analysis revealed that OTC reduced the increased levels of VEGF in the BAL fluid at 72 h after ovalbumin inhalation (Fig. 3B).

Localization of Immunoreactive VEGF in Lung Tissues and in BAL Fluids of Ovalbumin-induced Asthma. Immunohistochemical analyses showed the localization of immunoreactive VEGF in inflammatory cells around the bronchioles of mice with ovalbumin-induced asthma (Fig. 4B). In control mice and in ovalbumin-sensitized and -challenged mice treated with OTC, VEGF positive cells were hardly detected (Fig. 4, A, C and D). Immunocytologic analyses of BAL fluids showed the localization of immunoreactive VEGF in the precipitated cells from the ovalbumin-sensitized and -

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challenged mice (Fig. 4F). However, immunoreactive VEGF was significantly reduced in BAL cells from control mice and from ovalbumin-sensitized and -challenged mice treated with OTC (Fig. 4, E, G and H).

OTC, α-Lipoic Acid, and VEGF Receptor Inhibitor Reduced Plasma Extravasation in Ovalbumin-sensitized and challenged Mice. The Evans blue dye assay revealed that plasma extravasation was significantly increased at 72 h after the last challenge (Fig. 5). The increase in plasma extravasation at 72 h after ovalbumin inhalation was significantly reduced by the administration of the OTC, α-lipoic acid, or VEGF receptor inhibitor.

Effect of OTC on HIF-1α Protein Levels in Lung Tissues of Ovalbumin-sensitized and -challenged Mice. Western blot analysis revealed that levels of HIF-1α protein in nuclear protein extracts from lung tissues were increased at 72 h after ovalbumin inhalation compared with the levels in the control mice (Fig. 6, A and B). The increased HIF-1α levels in nuclear protein extracts from lung tissues at 72 h after ovalbumin inhalation were decreased by the administration of OTC.

Localization of Immunoreactive HIF-1α in Lung Tissues of Ovalbumin-induced Asthma. Immunohistochemical analyses showed the localization of immunoreactive HIF-1α in inflammatory cells around the bronchioles of mice with ovalbumin-induced

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asthma (Fig. 6D). In control mice and in ovalbumin-sensitized and -challenged mice treated with OTC, almost no HIF-1α positive cells were detected (Fig. 6, C, E and F).

Effect of OTC on PI3K Enzyme Activity and Akt Phosphorylation in Lung Tissues of Ovalbumin-sensitized and -challenged Mice. We used Western blotting to determine the activation of Akt which can activate the HIF pathway. Levels of p-Akt protein in the lung tissues were increased at 72 h after ovalbumin inhalation compared with the levels in the control mice (Fig. 7A). However, no significant changes in Akt protein levels were observed in any of the groups tested. The increased p-Akt but not Akt protein levels in lung tissues at 72 h after ovalbumin inhalation were significantly reduced by the administration of OTC. In addition, we have also measured PI3K enzyme activity using the PIP3 competition enzyme immunoassay. PI3K activitiy in the lung tissues was increased at 72 h after ovalbumin inhalation compared with the control mice (Fig. 7B). The increased PI3K activity in lung tissues at 72 h after ovalbumin inhalation was significantly reduced by the administration of OTC.

Effect of OTC, α-Lipoic Acid, and VEGF Receptor Inhibitor on Cellular Changes in BAL Fluids. Numbers of total cells, eosinophils, lymphocytes, and neutrophils in BAL fluids were increased significantly at 72 h after ovalbumin inhalation compared with the numbers after saline inhalation (Fig. 8). The increased

numbers of these cells were significantly reduced by the administration of OTC**,** α-lipoic acid, or SU5614.

Effect of OTC on Pathological Changes of Ovalbumin-induced Asthma. Histological analyses revealed typical pathologic features of asthma in the ovalbumin-exposed mice. Numerous inflammatory cells including eosinophils infiltrated around the bronchioles (Fig. 9B) as compared to the control (Fig. 9A). Mice treated with OTC (Fig. 9, C and D) showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after ovalbumin inhalation compared with the scores after saline inhalation (Fig. 9E). The increased peribronchial, perivascular, and total lung inflammation was significantly reduced by the administration of OTC. These results suggest that OTC inhibits antigen-induced inflammation in the lungs, including the influx of eosinophils.

Effect of OTC, α-Lipoic Acid, and VEGF Receptor Inhibitor on Airway Hyperresponsiveness. Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways using invasive assessment technique. 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. 10). In

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addition, the percent R_L produced by methacholine administration (at doses from 2.5 mg/ml to 50 mg/ml) increased significantly in the ovalbumin-sensitized and -challenged mice compared with the controls. Ovalbumin-sensitized and -challenged mice treated with OTC**,** α-lipoic acid, or 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 OTC, α-lipoic acid, or SU5614 treatment reduces ovalbumin-induced airway hyperresponsiveness.

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Discussion

Oxidative stress has been shown to play critical roles in airway inflammation, including vascular leakage (Rahman et al., 1996; Dworski, 2000; MacNee, 2001). Alterations in alveolar and lung glutathione metabolism are widely recognized as a central feature of many inflammatory lung diseases such as asthma. Glutathione is synthesized from cysteine and is a vital intra- and extracellular protective antioxidant against oxidative stress (Asti et al., 1995; MacNee, 2001; Blesa et al., 2002). In this study, we have used OTC, which is a precursor of glutathione biosynthesis and thus functioning as an antioxidant, to examine roles of ROS and the molecular mechanism in vascular permeability of ovalbumin-induced asthma murine model. The results have revealed that ROS generation, VEGF expression, and HIF-1α protein level as well as vascular permeability are increased in induction of asthma. The increased levels of these molecules are significantly reduced by administration of OTC. These observations suggest that ROS generation in asthma up-regulates VEGF expression due to the increased HIF-1α activity.

Several studies have demonstrated that OTC is more effective than N-acetylcysteine in replenishing intracellular glutathione stores (Williamson and Meister, 1981; Mesina

et al., 1989) and blocks airway hyperresponsiveness and inflammation in an asthmatic animal model (Lee et al., 2004b). A thiazolidine derivative, OTC is a prodrug of cysteine that raises the plasma concentrations of cysteine and glutathione (Porta et al., 1991; Vita et al., 1998; Oiry et al., 1999). This compound is readily transportable into cells and converted to L-cysteine by an ubiquitous intracellular enzyme, 5-oxoprolinase (Williamson and Meister, 1981). Cysteine derivatives, N-acetylcysteine and carbocysteine, have been used to block inflammation and hyperreactivity in animal models of asthma (Asti et al., 1995; Blesa et al., 2002). Recently, α-lipoic acid which is a naturally occurring antioxidant has been used clinically for the treatment of oxidant-induced diseases (Cao and Phillis , 1995; Ametov et al., 2003). Cho et al. have shown that α-lipoic acid reduces airway inflammation and hyperresponsiveness in a murine model of asthma (Cho et al., 2004). Our results indicate that plasma extravasation caused by increased vascular permeability is elevated after inducing asthma and that administration of OTC or α-lipoic acid significantly reduces the increased plasma extravasation at 72 h after ovalbumin inhalation. Consistent with previous observations, administration of OTC or α-lipoic acid also decreases eosinophilic inflammation and airway hyperresponsiveness. 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 (Foster et al., 1982; Persson, 1996).

Recently, we have reported that over-production of VEGF is associated with increased vascular permeability and plasma exudation in a murine model of asthma (Lee et al., 2002b). Consistent with these observations, we have found that VEGF expression is up-regulated in ovalbumin-induced asthma. Interestingly, administration of the antioxidant, OTC or α-lipoic acid, reduces the increased VEGF expression. These results suggest that oxidative stress is associated with the regulation of VEGF expression and that treatment of the antioxidant may decrease the vascular permeability by inhibiting up-regulation of VEGF expression. It is now well established that VEGF plays a critical role in asthma (Hoshino et al., 2001; Lee and Lee, 2001; Lee et al., 2004a). The major role of VEGF in asthma appears to be the enhancement of vascular permeability, resulting in leakage of plasma proteins into the extravascular space (Dvorak et al., 1995; Lee et al., 2002b). This may cause edema and profound alterations

in the extracellular matrix. 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). To distinguish direct effects of antioxidant on the asthma phenotype from those of VEGF, VEGF receptor inhibitor, SU5614 was administered. We have found that SU5614 inhibits the increased vascular permeability, the airway inflammation, and the airway hyperresponsiveness. These results suggest that VEGF may affect vascular permeability without affecting ROS production. VEGF expression is regulated through HIF-1α expression (Wang and Semenza, 1995; Semenza, 1999). HIF-1 is a transcriptional activator that mediates changes in gene expression in response to changes in cellular oxygen concentrations (Semenza, 2001). Previous reports have demonstrated that HIF-1α plays a critical role in immune and inflammatory responses (Lukashev et al., 2001; Jung et al., 2003). Determination of HIF-1α protein level in nuclear extracts has revealed that this protein level is substantially increased in our present ovalbumin-induced model of asthma, suggesting that HIF-1α is activated. The administration of OTC results in significant reduction of nuclear HIF-1α level as well as expression of VEGF. Previous reports have shown that increase of PI3K/Akt activity can activate the HIF pathway (Zhong et al., 2000; Laughner et al., 2001; Treins et al., 2002; Mottet et al., 2003). Li et al. have also

reported that activation of Akt turns on HIF-1α independently of hypoxia (Li et al., 2005). In addition, ROS have been shown to stabilize HIF-1α during hypoxia and/or non-hypoxia (Chandel et al., 2000; Haddad and Land, 2000). In the present study, levels of p-Akt protein in the lung tissues were increased after ovalbumin inhalation. The increased p-Akt but not Akt protein levels were significantly reduced by the administration of OTC. In addition, PI3K activitiy in the lung tissues was also increased after ovalbumin inhalation. The increased PI3K activity in lung tissues was significantly reduced by the administration of OTC. Taken together, we suggest that OTC regulates HIF-1α action through a PI3K/Akt pathway, resulting in decreased VEGF expression in a murine model of asthma.

In summary, we have examined the role of the ROS in a murine model of allergic asthma, more specifically in the increase of vascular permeability. By using OTC, an antioxidant agent, we have shown the important role for ROS in ovalbumin-induced airway hyperresponsiveness and eosinophilic inflammation. By examining the effects of administration of OTC on plasma exudation and VEGF expression, we conclude that antioxidant treatments such as administration of OTC modulate vascular permeability by reducing VEGF expression.

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Footnotes

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Figure legends

Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized on days 1 and 14 by intraperitoneal injection of ovalbumin emulsified in 1 mg of aluminum hydroxide. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer. In the case of treatment with OTC, it was administered intraperitoneally four times at 24 h intervals on days 21-24, beginning 1 h before the first challenge. In the case of treatment with SU5614, SU5614 was administered intraperitoneally three times at 24 h intervals, beginning 1 h after the last airway challenge with ovalbumin. α-Lipoic acid was administered 7 times by oral gavage at 24 h intervals on days 19-25, beginning 2 days before the first challenge.

Fig. 2. Effect of OTC on ROS levels in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), and ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160). A-D, representative

fluorescence microscopy shows ROS-positive cells in the BAL fluids. E, dichlorofluorescein fluorescence intensity is presented as the relative ratio of ROS levels in ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered OTC 80 mg/kg (OVA+OTC 80), and ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160). The relative ratio of ROS levels in the BAL fluids of SAL+SAL is arbitrarily presented as 100. Bars represent mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC treatment groups versus OVA+SAL.

Fig. 3. Effect of OTC, α-lipoic acid, or SU5614 on VEGF secretion in BAL fluids. A, enzyme immunoassay of VEGF. B, Western blotting of VEGF. Sampling was performed at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614), ovalbumin-inhaled mice administered α-lipoic acid 50 mg/kg (OVA+α-lipoic acid 50),

and ovalbumin-inhaled mice administered α-lipoic acid 100 mg/kg (OVA+α-lipoic acid 100). Bars represent the mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC, α-lipoic acid, or SU5614 treatment groups versus OVA+SAL.

Fig. 4. Localization of immunoreactive VEGF in lung tissues and BAL fluids of ovalbumin-sensitized and -challenged mice. A and E, sampling was performed at 72 h after the last challenge in lung tissues and BAL fluids from sensitized mice challenged with saline, from sensitized mice challenged with ovalbumin (B and F), from ovalbumin-inhaled mice administered OTC 80 mg/kg (C and G), and from ovalbumin-inhaled mice administered OTC 160 mg/kg (D and H). A-D, representative light microscopy of VEGF-positive cells in the bronchioles. The brown color indicates VEGF-positive cells. E-H, representative light microscopy of VEGF-positive cells in the BAL fluids. The brown color indicates VEGF-positive cells. Scale bars: 50 μm (A-D) or 10 μm (E-H).

Fig. 5. Effect of OTC, α-lipoic acid, or SU5614 on plasma exudation. Sampling was performed at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614), ovalbumin-inhaled mice administered α-lipoic acid 50 mg/kg (OVA+α-lipoic acid 50), and ovalbumin-inhaled mice administered α-lipoic acid 100 mg/kg (OVA+α-lipoic acid 100). Data represent the means ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC, α-lipoic acid, or SU5614 treatment groups versus OVA+SAL.

Fig. 6. Effect of OTC on HIF-1α expression in nuclear protein extracts from lung tissues and localization of immunoreactive HIF-1α in lung tissues of ovalbumin-sensitized and -challenged mice. A, HIF-1α expression in nuclear protein extracts from lung tissues. HIF-1α expression was measured at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), and ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160). B, densitometric analyses

are presented as the relative ratio of HIF-1α to actin. The relative ratio of HIF-1α in the lung tissues of control mice is arbitrarily presented as 1. Results were similar in six independent experiments. C-F, localization of immunoreactive HIF-1α in lung tissues of ovalbumin-sensitized and -challenged mice. Representative light microscopy of HIF-1α-positive cells in the bronchioles. Sampling was performed at 72 h after the last challenge in lung tissues and BAL fluids from sensitized mice challenged with saline **(**C**),** from sensitized mice challenged with ovalbumin (D), from ovalbumin-inhaled mice administered OTC 80 mg/kg (E), and from ovalbumin-inhaled mice administered OTC 160 mg/kg (F). The brown color indicates HIF-1α-positive cells. Scale bars: 50 μm.

Fig. 7. Effect of OTC on p-Akt and Akt protein expression and PI3K enzyme activity in lung tissues of ovalbumin-sensitized and -challenged mice. A, p-Akt and Akt protein expression in lung tissues was measured at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160). Results were similar in six independent experiments. B, PIP3 generation by PI3Ks in lung tissue extracts. Data

represent mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC treatment groups versus OVA+SAL.

Fig. 8. Effect of OTC, α-lipoic acid, or SU5614 on total and differential cellular components of BAL of ovalbumin-sensitized and -challenged mice. The numbers of each cellular component of BAL from 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 OTC 80 mg/kg (OVA+OTC 80), ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614), ovalbumin-inhaled mice administered α-lipoic acid 50 mg/kg (OVA+α-lipoic acid 50), and ovalbumin-inhaled mice administered α-lipoic acid 100 mg/kg (OVA+α-lipoic acid 100) were counted at 72 h after the last challenge. Bars represent mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC, α-lipoic acid, or SU5614 treatment groups versus OVA+SAL.

Fig. 9. Effect of OTC on pathologic changes in lung tissues of ovalbumin-sensitized and

-challenged mice. A-D, representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (A), ovalbumin-inhaled mice administered saline (B), ovalbumin-inhaled mice administered OTC 80 mg/kg (C), and ovalbumin-inhaled mice administered OTC 160 mg/kg (D). Bars indicate scale of 50 μm. E, peribronchial and perivascular lung inflammation were measured at 72 h after the last challenge in SAL+SAL, OVA+SAL, OVA+VEH, OVA+OTC 80, and OVA+OTC 160. Bars represent mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC treatment groups versus OVA+SAL.

Fig. 10. Effect of OTC, α-lipoic acid, or SU5614 on airway responsiveness in ovalbumin-sensitized and -challenged mice. Airway responsiveness was measured at 72 h 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 OTC 80 mg/kg (OVA+OTC 80), ovalbumin-inhaled mice administered OTC 160 mg/kg (OVA+OTC 160), and ovalbumin-inhaled mice administered SU5614 (OVA+SU5614), ovalbumin-inhaled mice administered α-lipoic acid 50 mg/kg (OVA+α-lipoic acid 50),

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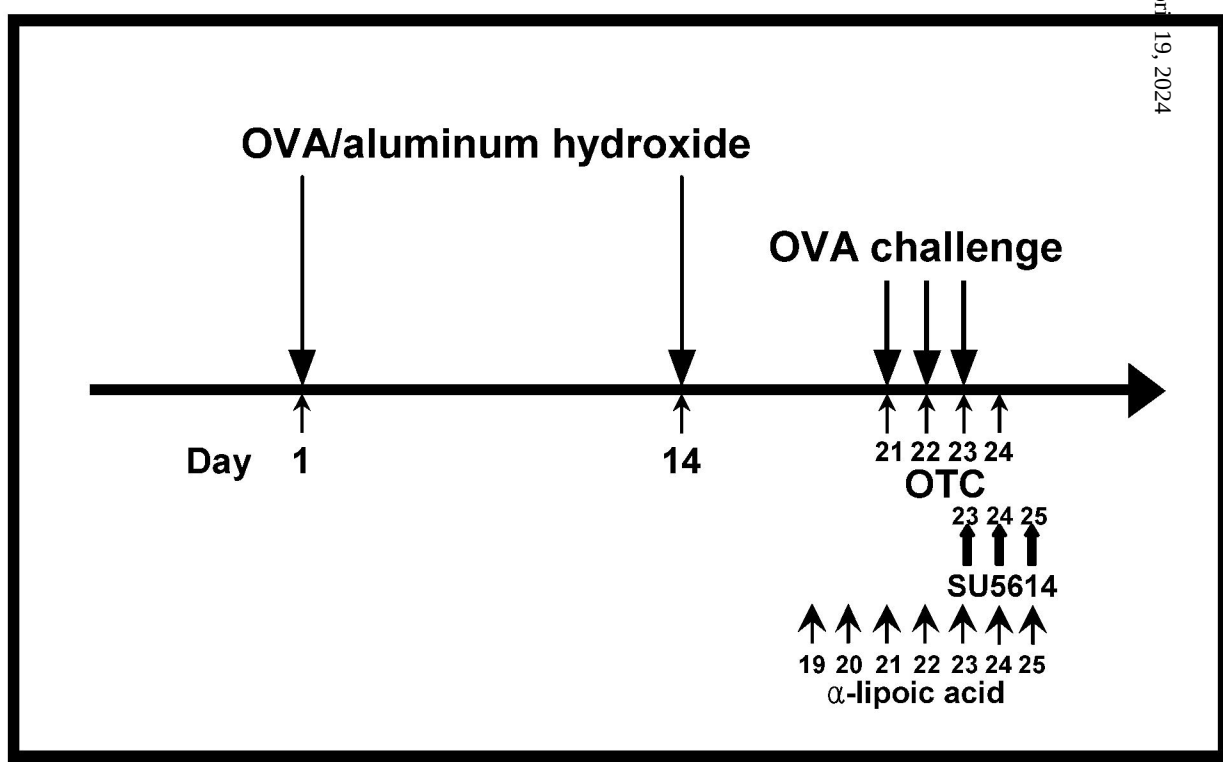
and ovalbumin-inhaled mice administered α-lipoic acid 100 mg/kg (OVA+α-lipoic acid 100). Airway responsiveness was measured at 72 h after the last challenge as described in materials and methods. Data represent mean ± S.E.M. from 6 independent experiments. *, $p < 0.05$. OVA+SAL and OVA+VEH versus SAL+SAL; †, $p < 0.05$. OTC, α-lipoic acid, or SU5614 treatment groups versus OVA+SAL.

Fig. 1.



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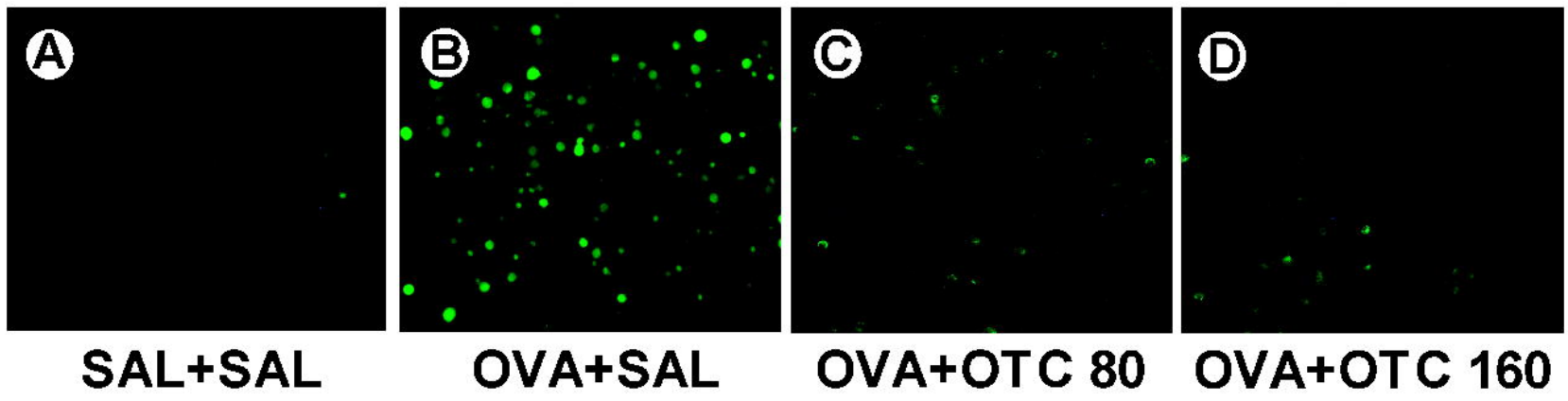
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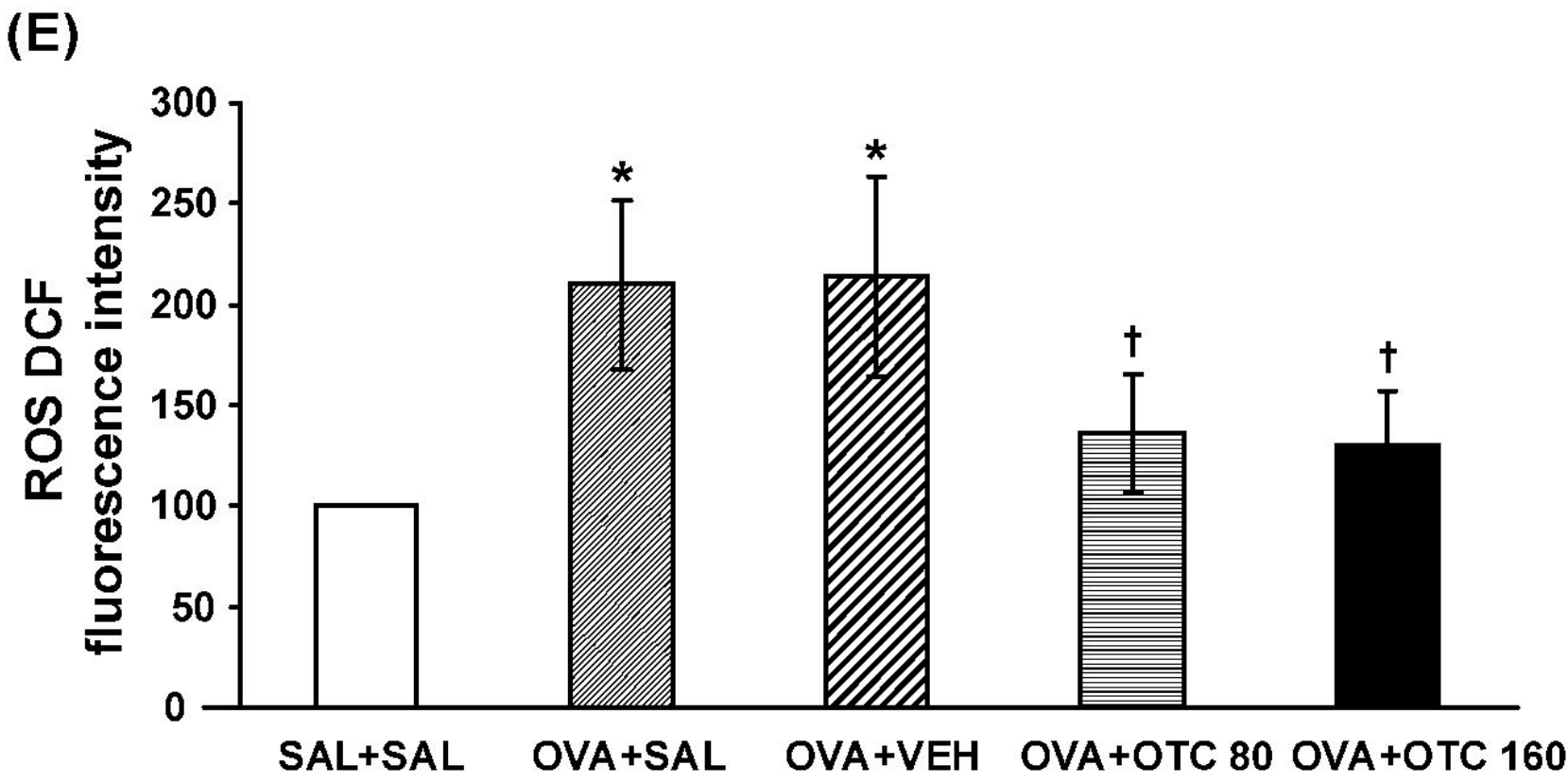
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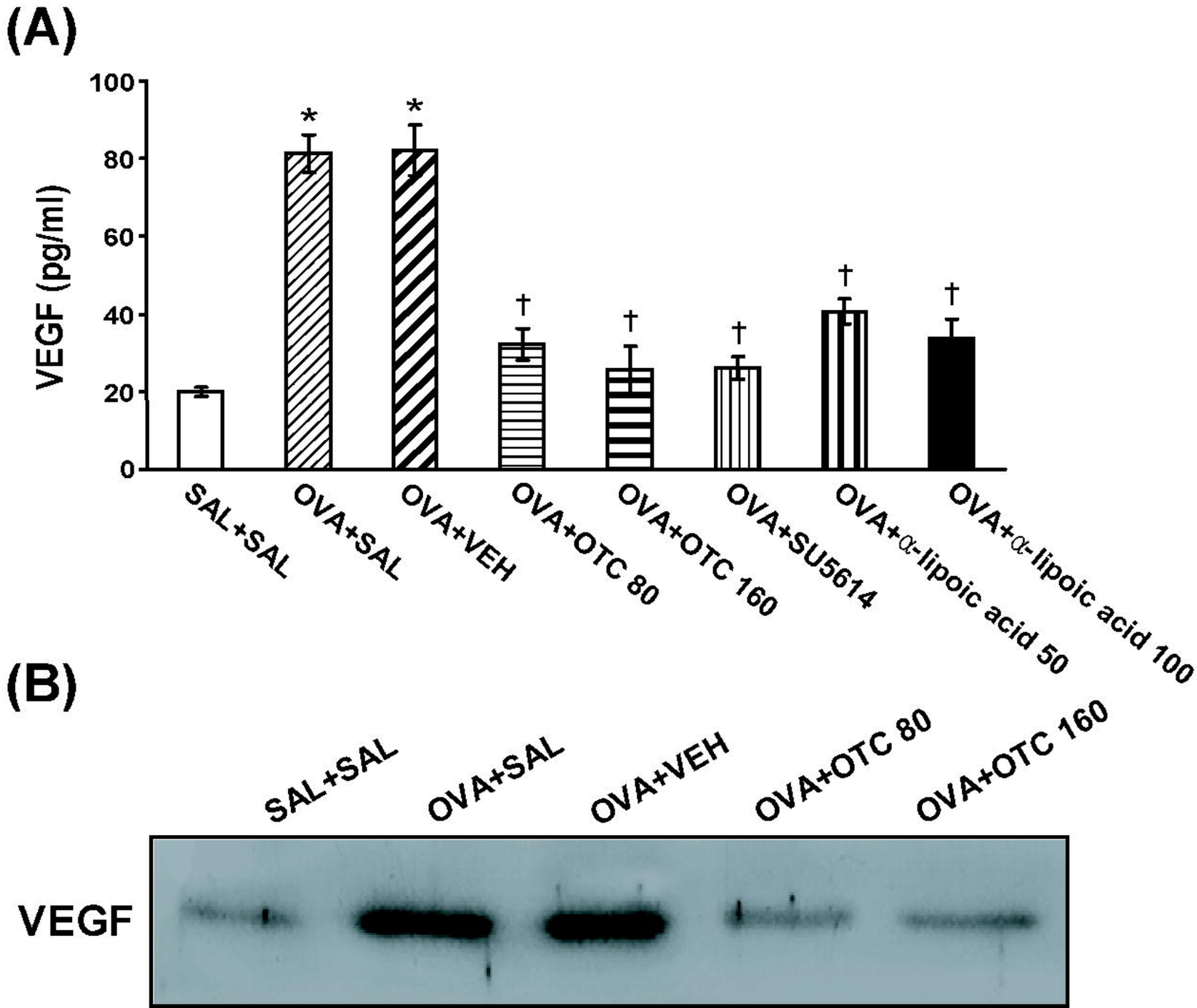
Fig. 3.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OVA+OTC 80
OVA+OTC 160
OVA+SU5614
OVA+α-lipoic acid 50
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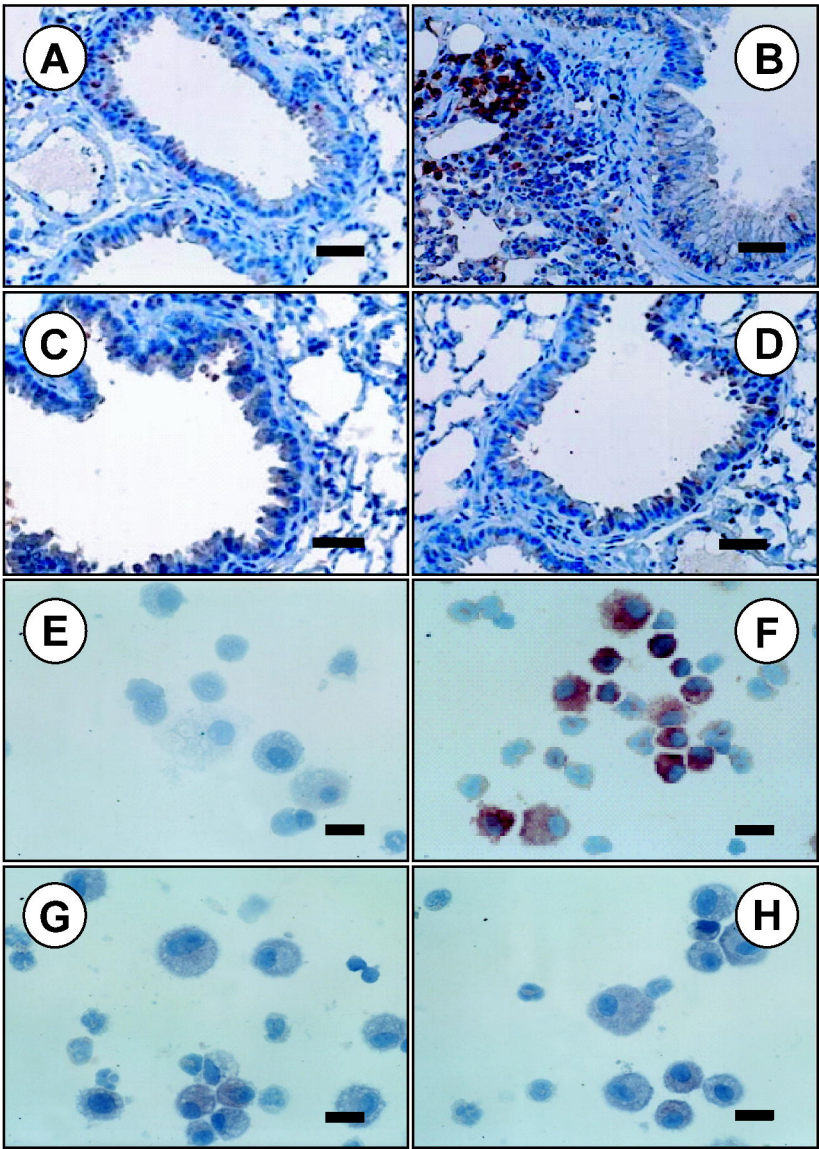
OVA+OTC 80
OVA+OTC 160
VEGF

Fig. 4.



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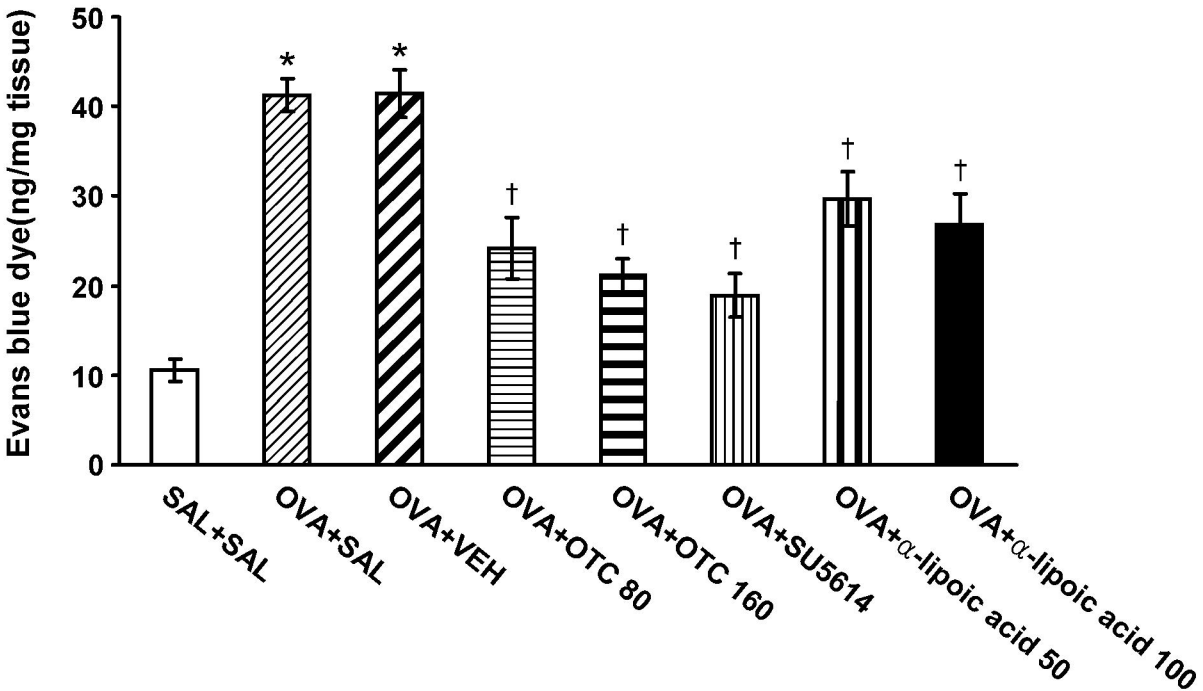
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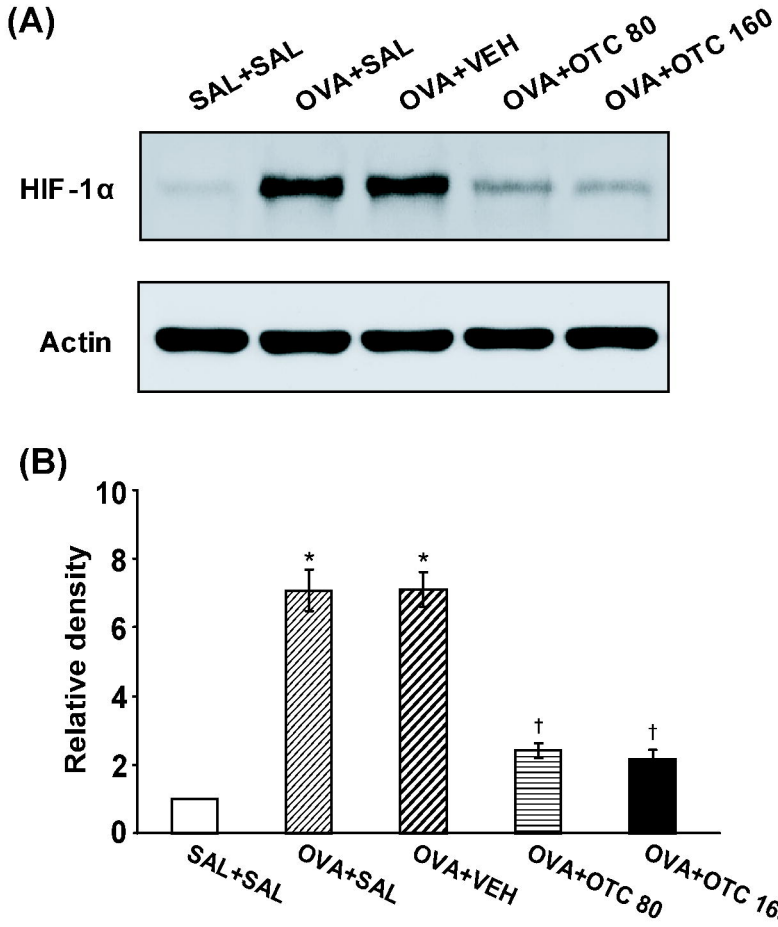
OVA+OTC 160
OVA+SU5614
OVA+α-lipoic acid 50
OVA+α-lipoic acid 100

Fig. 6.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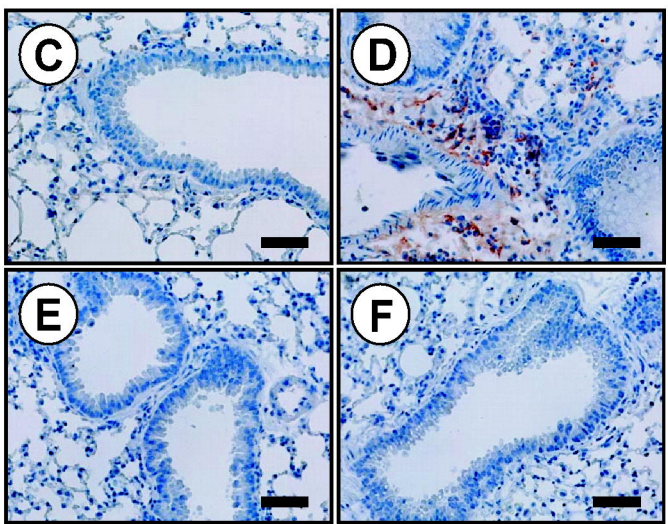
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D
E
F

Fig. 7.

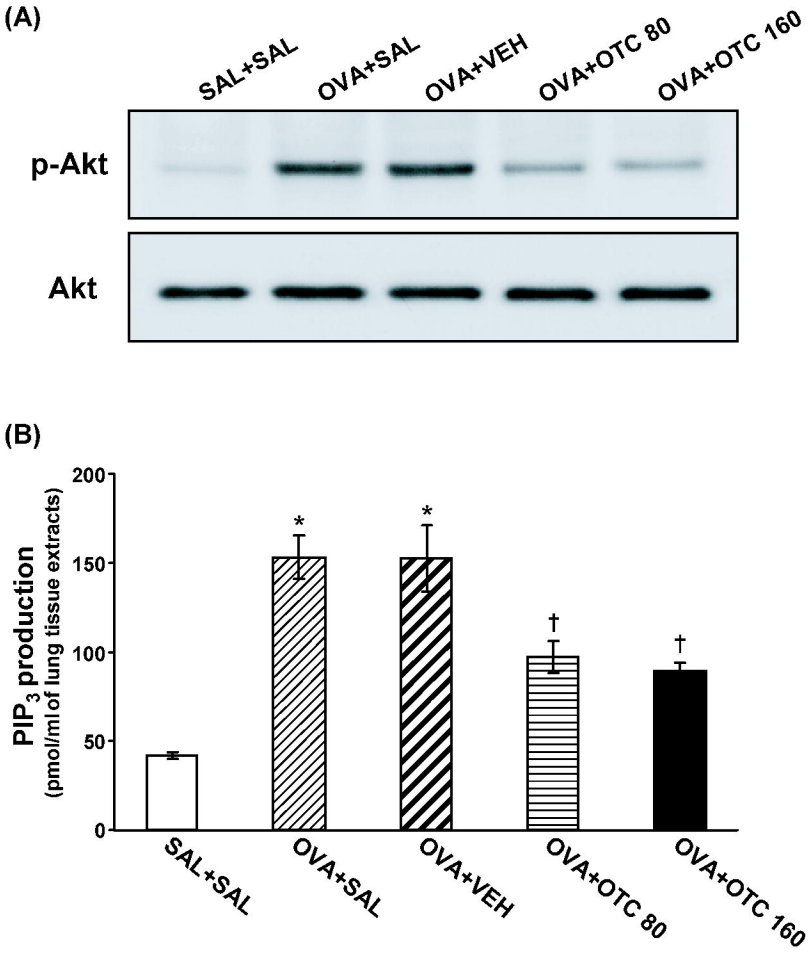

(A)
SAL+SAL
OVA+SAL
OVA+VEH
OVA+OTC 80
OVA+OTC 160
p-Akt
Akt
(B)
PIP3 production
(pmol/ml of lung tissue extracts)
200
150
100
50
0
*
*
†
†
SAL+SAL
OVA+SAL
OVA+VEH
OVA+OTC 80
OVA+OTC 160

Fig. 8.

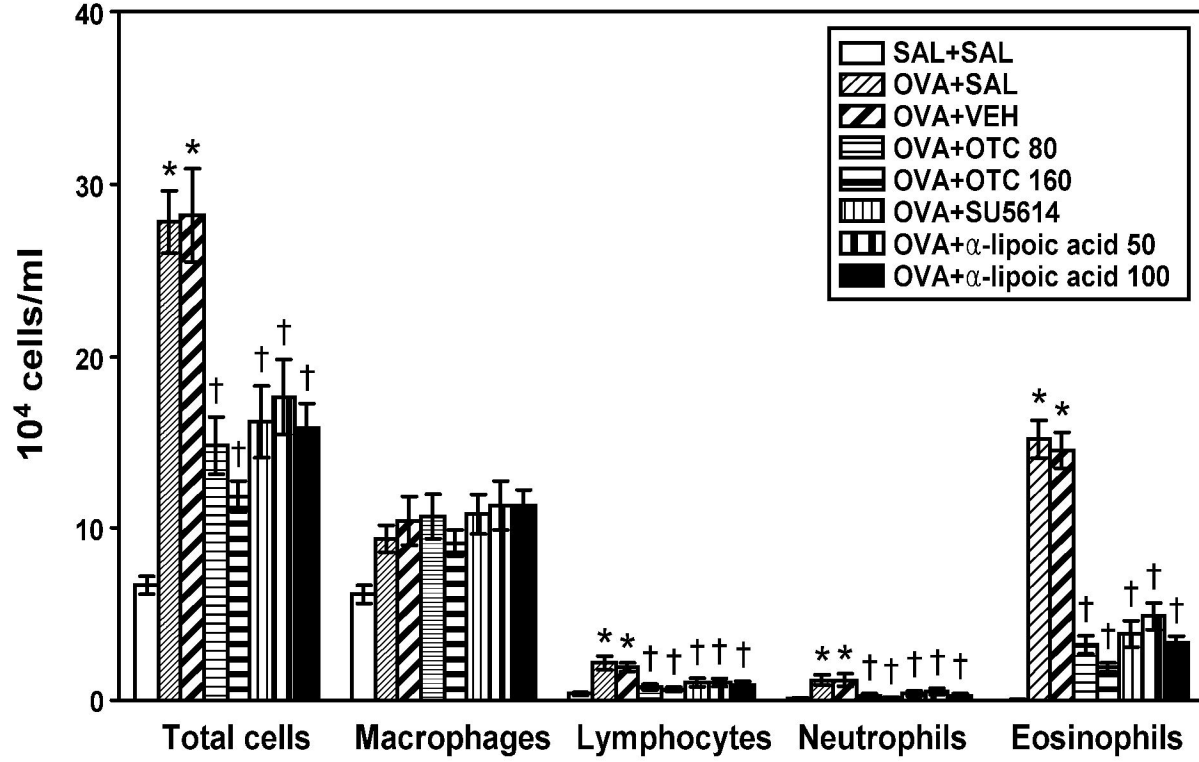
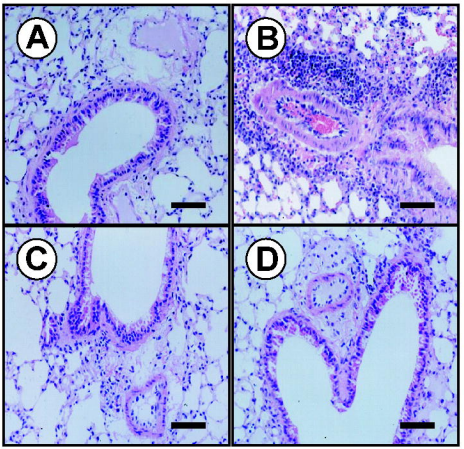

40
30
20
10
0
10^4 cells/ml
SAL+SAL
OVA+SAL
OVA+VEH
OVA+OTC 80
OVA+OTC 160
OVA+SU5614
OVA+α-lipoic acid 50
OVA+α-lipoic acid 100
Total cells
Macrophages
Lymphocytes
Neutrophils
Eosinophils

Fig. 9.


A
B
C
D

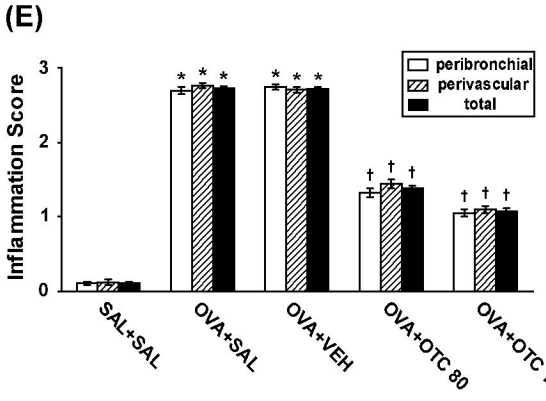
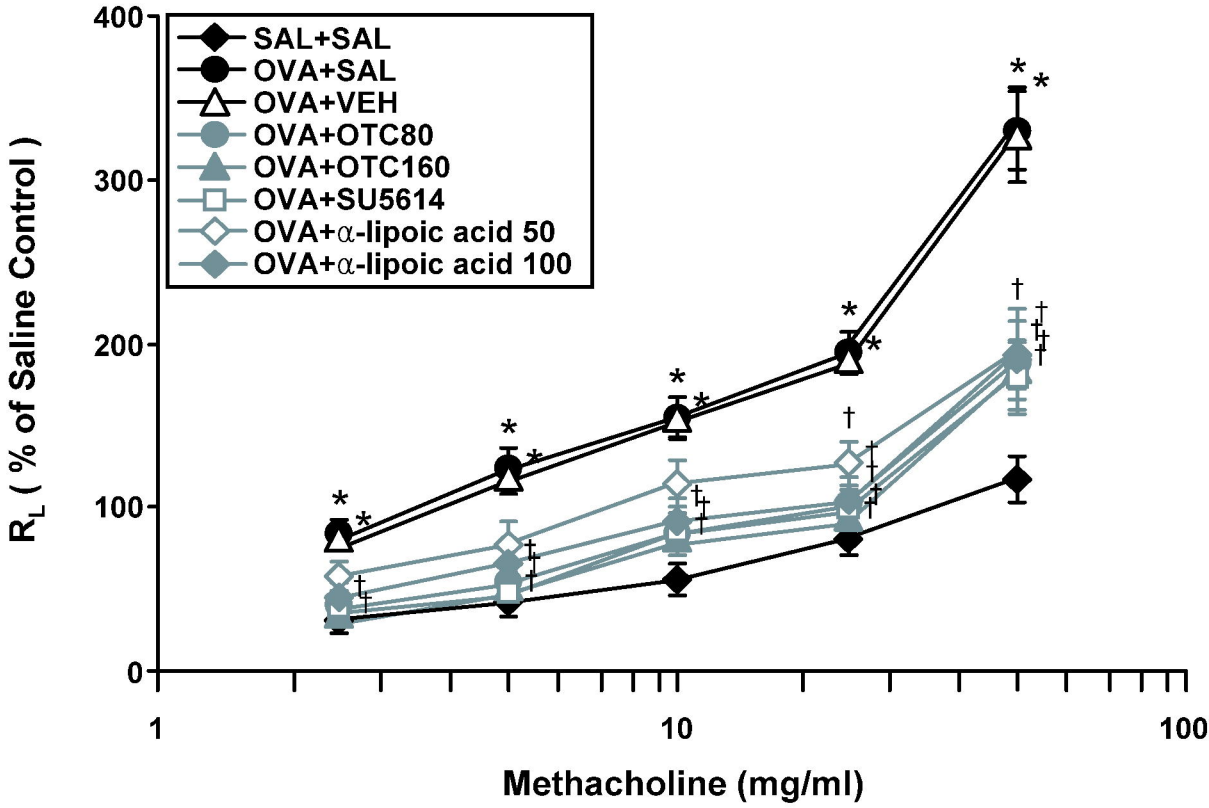

(E)
Inflammation Score
0
1
2
3
peribronchial
perivascular
total
SAL+SAL
OVA+SAL
OVA+VEH
OVA+OTC 80
OVA+OTC 160

Fig. 10.


SAL+SAL
OVA+SAL
OVA+VEH
OVA+OTC80
OVA+OTC160
OVA+SU5614
OVA+α-lipoic acid 50
OVA+α-lipoic acid 100
R_L (% of Saline Control)
400
300
200
100
0
1
10
100
Methacholine (mg/ml)