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A novel thiol compound, *N*-acetylcysteine amide, attenuates allergic airway disease by regulating activation of NF-κB and hypoxia-inducible factor-1α

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Abbreviations: AD4, *N*-acetylcysteine amide; BAL, bronchoalveolar lavage; DCF, 2',7'-dichlorofluorescein; EBD, Evans blue dye; HIF, hypoxia-inducible factor; OVA, ovalbumin; PAS, periodic acid-Schiff; R_L, airway resistance; ROS, reactive oxygen species

Abstract

Reactive oxygen species (ROS) play an important role in the pathogenesis of airway inflammation and hyperresponsiveness. Recent studies have demonstrated that antioxidants are able to reduce airway inflammation and hyperreactivity in animal models of allergic airway disease. A newly developed antioxidant, small molecular weight thiol compound, *N*-acetylcysteine amide (AD4) has been shown to increase cellular levels of glutathione and to attenuate oxidative stress related disorders such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. However, the effects of AD4 on allergic airway disease such as asthma are unknown. We used ovalbumin (OVA)-inhaled mice to evaluate the role of AD4 in allergic airway disease. In this study with OVA-inhaled mice, the increased ROS generation, the increased levels of Th2 cytokines and VEGF, the increased vascular permeability, the increased mucus production, and the increased airway resistance in the lungs were significantly reduced by the administration of AD4. We also found that the administration of AD4 decreased the increases of the NF-κB and hypoxia-inducible factor-1α (HIF-1α) levels in nuclear protein extracts of lung tissues after OVA inhalation. These results suggest that AD4 attenuates airway inflammation and hyperresponsiveness by regulating activation of NF-κB and HIF-1α as well as reducing ROS generation in allergic airway disease.

Keywords: hypoxia-inducible factor-1, α subunit; lung inflammation; *N*-acetylcysteinamide; NF-κB; oxidative stress; respiratory hypersensitivity

Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by an associated increase in airway responsiveness (Bousquet *et al.,* 2000). Oxidative stress is caused by a large variety of free oxygen radicals known as reactive oxygen species (ROS). ROS play a crucial role in the pathogenesis of airway inflammation (Rahman *et al.,* 1996; Dworski, 2000). The inflammatory cells recruited to the asthmatic airways have a capability of producing ROS. Evidence for increase of oxidative stress in asthma is further provided by the finding of defective endogenous antioxidant capacity in asthmatic patients (Dworski, 2000). Recently, several studies have demonstrated that antioxidants are able to reduce airway inflammation and hyperreactivity in animal models of asthma (Cho *et al.,* 2004b; Lee *et al.,* 2004d). Therefore, antioxidant treatment of asthma has long been a subject of therapeutic strategy.

Glutathione (GSH) is synthesized from cysteine and is a vital intra- and extracellular protective antioxidant against oxidative stress. Alterations in alveolar and lung GSH metabolism are widely recognized as a central feature of many inflamma-

tory lung diseases such as asthma. Antioxidants which increase cellular cysteine levels, such as *N*-acetylcysteine (NAC), carbocysteine, and L-2-oxothiazolidine-4-carboxylic acid have been shown to exhibit anti-inflammatory effect and anti-hyperreactivity in animal models of asthma (Asti *et al.,* 1995; Dworski, 2000; Blesa *et al.,* 2002; Lee *et al.,* 2004d). Recently, a novel, low-molecular weight thiol antioxidant, *N*-acetylcysteine amide (AD4), has been developed (Offen *et al.,* 2004; Grinberg *et al.,* 2005; Penugonda *et al.,* 2005). In NAC, the carboxyl group is negatively charged at physiological pH, limiting the drug's ability to cross cell membranes. A newly designed amide form of NAC, AD4, in which the carboxyl group is neutralized, is expected to be more hydrophobic and membrane-permeable (Atlas *et al.,* 1999). In fact, AD4 has been shown to increase cellular levels of GSH either by providing the limiting substrate for GSH biosynthesis or reducing its oxidized form, glutathione disulfide (GSSG) in a thiol exchange reaction (Grinberg *et al.,* 2005; Bartov *et al.,* 2006). Moreover, studies have demonstrated that treatment with AD4 results in a remarkable restoration of intracellular thiols, a more effective protection against hemoglobin oxidation, and a substantial reduction of intracellular oxidation compared with NAC (Offen *et al.,* 2004; Grinberg *et al.,* 2005). However, there is no data on the influence and the molecular basis of AD4 on allergen-induced bronchial inflammation and airway hyperresponsiveness.

In the present study, we used a mouse model for allergic airway disease to evaluate the effect of a novel antioxidant, AD4 on hyperresponsiveness and inflammation of the airways and to investigate the related molecular mechanisms.

Materials and Methods

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 wk of age and free of murine specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, Korea), were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed (Institute of Laboratory Animal Research, 1996). Mice were sensitized on days 1 and 14 by i.p. injection of 20 μg ovalbumin (OVA) (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 with some modifications (Kwak *et al.,* 2003; Lee *et al.,* 2006a). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed at 48 h after the last challenge. At the time of lavage, the mice (8 mice in each group) were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg of body wt, administered i.p.). 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 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 (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. 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. Inter-investigator variation was $< 5\%$. The mean number from the two investigators was used to estimate the cell differentials.

Administration of AD4

AD4 was prepared as previously described (Adam *et al.,* 1999; Bahat-Stroomza *et al.,* 2005; Bartov *et al.,* 2006). AD4 (60 or 120 mg/kg body wt/day), dissolved in PBS, was administered i.p. four times to each animal at 24-h intervals on days 21-24, beginning 1 h before the first challenge. For the evaluation of the effect of AD4 on OVA-induced ongoing allergic airway inflammation, AD4 (120 mg/kg body wt/day by i.p.) was administered two times to each animal at 24-h intervals on days 24-25, beginning 3 h after the last OVA challenge.

Measurement of intracellular ROS

ROS were measured by a method previously described (Lee *et al.,* 2006a). BAL cells were washed with PBS. To measure intracellular ROS, cells were incubated for 10 min at room temperature with PBS containing 3.3 μM 2',7'-dichlorofluorescein (DCF) diacetate (Molecular probes, Eugene, OR),

to label intracellular ROS. We performed FACScan analysis with DCF stained cells (1×10^4 cells) in BAL fluids to measure ROS levels using a FACSCalibur instrument (BD Biosciences, San Jose, CA). The data were analyzed with a CellQuest Pro program (BD Biosciences).

Measurement of GSH and GSSG in lung tissues

Lung tissues were homogenized with 10 ml of ice-cold buffer (50 mM phosphate buffer containing 1 mM EDTA) per gram tissue. After centrifugation at 10,000 $\times$ *g* for 15 min at 4°C, the supernatants were removed, deproteinated, and then stored at −20°C until the samples were assayed. Total GSH and GSSG levels were determined using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/ml. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 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. Lungs were removed and weighed wet. EBD 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 using a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). 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.

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). Samples were loaded on SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to PVDF membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST; 25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the blots were then incubated overnight at 4°C with an anti-IL-4 Ab (Serotec Ltd., Oxford, United Kingdom), anti- IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-13 Ab (R&D Systems, Inc., Minneapolis, MN), or anti-VEGF Ab (Santa Cruz Biotechnology). Anti-rabbit or -mouse HRP-conjugated-IgG was used to detect binding of Ab. The membranes were stripped and reblotted with an anti-actin Ab (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific Ab was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

Cytosolic or nuclear protein extractions for analysis of hypoxia-inducible factor (HIF)-1α, HIF-1β, and NF-κB p65

Lungs were removed and homogenized in 2 vol of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 5 mM $MgCl_2$, and 1 mM PMSF) containing protease inhibitor cocktails. The homogenates were centrifuged at 1,000 $\times$ *g* for 15 min at 4°C. The supernatants collected were incubated on ice for 10 min and centrifuged at 100,000 $\times$ *g* for 1 h at 4°C to obtain cytosolic proteins for analysis of NF-κB p65. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 mM sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 6.8) and pelleted at 1,000 $\times$ *g* for 15 min. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 M and centrifuged at 100,000 $\times$ *g* for 1 h. The resulting 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 $\times$ *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 1 h with gentle stirring and centrifuged at 12,000 $\times$ *g* for 30 min. The resulting supernatants were used as soluble nuclear proteins for analysis of HIF-1α, HIF-1β, and NF-κB p65. The levels of these proteins were analysed by Western blotting using Ab against HIF-1α (Novus Biologicals Inc., Littleton, CO), HIF-1β (Novus Biologicals Inc.), or NF-κB p65 (Upstate Biotech, Lake Placid, NY) as described above.

Measurement of Th2 cytokines and VEGF

Levels of IL-4, IL-5, IL-13, and VEGF were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's

protocol (IL-4; BioSource International, Inc. Camarillo, CA; IL-5; Endogen, Inc., Woburn, MA; IL-13 and VEGF; R&D Systems, Inc.). Sensitivities for IL-4, IL-5, IL-13, and VEGF assays were 5, 5, 1.5, and 3.0 pg/ml, respectively.

Determination of airway responsiveness

Airway responsiveness was also 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 i.p. 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 vol 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 vol 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 calculated airway resistance (R_L) were continuously collected. Maximum values of R_L were selected to express changes in airway function which was represented as a percentage change from baseline after saline aerosol.

Processing of lungs for histologic and image analysis

At 48 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. The lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. Specimens were dehydrated and embedded in paraffin. After section of the specimens, they were placed on slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI) or periodic acid-Schiff (PAS). Stained slides were all quantified under identical light microscope conditions, including magnification ($\times$ 20), gain, camera position, and background illumination (Cho *et al.,* 2004a).

Histology

For histological examination, 4-μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). 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 a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

Quantitation of airway mucus expression

To quantitate the level of mucus expression in the airway, the number of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as described previously (Ikeda *et al.,* 2003; Cho *et al.,* 2004a). Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells of each bronchiole.

Densitometric analysis and statistics

All immunoreactive signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc.). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Significant differences between two groups were determined using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

Effect of AD4 on ROS levels in BAL cells and GSH and GSSG levels in lung tissues of OVA-sensitized and -challenged mice

ROS generation in BAL cells and GSSG levels in lung tissues were significantly increased at 48 h after OVA inhalation compared with the levels after saline inhalation (Figure 1A and C). The increased ROS generation and GSSG levels were sub-

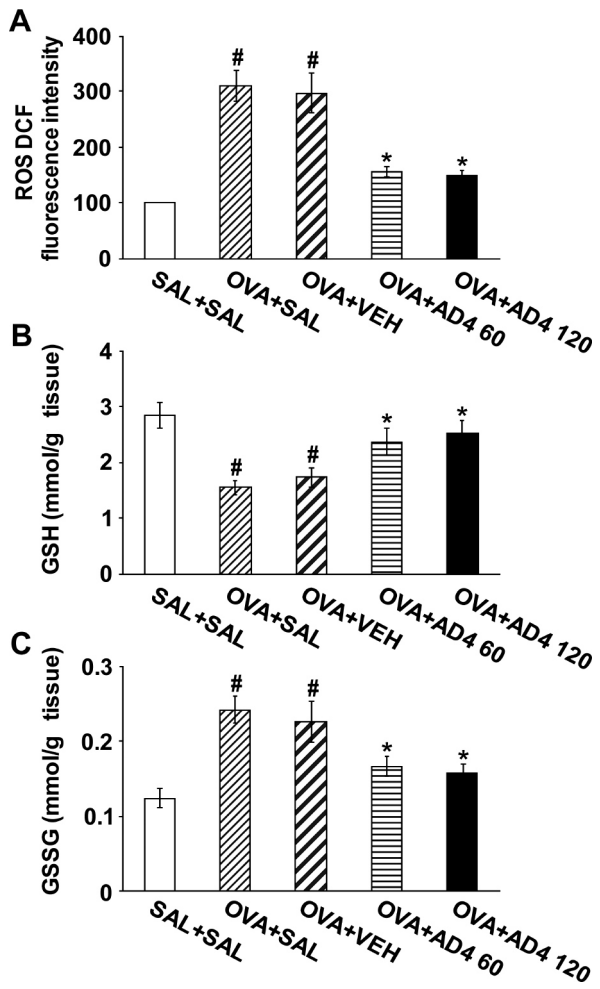

Figure 1. Effect of AD4 on ROS levels in BAL cells and GSH and GSSG levels in lung tissues. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) DCF fluorescence intensity. The ROS levels are presented as the relative ratio of values in OVA + SAL, OVA + VEH, OVA + AD4 60, or OVA + AD4 120 to those in SAL + SAL. The relative ratio of ROS levels in the BAL cells of SAL + SAL is arbitrarily presented as 100. (B) GSH levels in lung tissues. (C) GSSG levels in lung tissues. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

stantially reduced by the administration of AD4. In contrast, GSH assay revealed that GSH levels in lung tissues were significantly decreased at 48 h after the last inhalation of OVA compared with the levels after saline inhalation (Figure 1B). The decreased OVA-induced GSH levels were significantly increased by the administration of AD4.

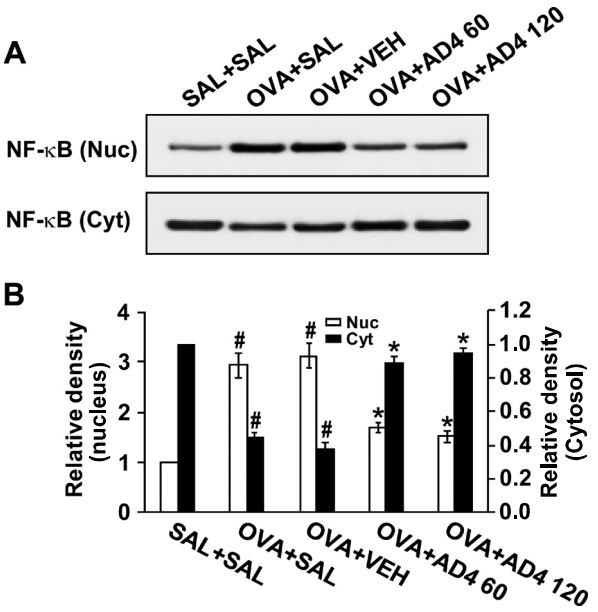

Figure 2. Effect of AD4 on levels of NF-κB p65 in lung tissues of OVA-sensitized and -challenged mice. Levels of NF-κB p65 were measured at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) Western blot analyses of NF-κB p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. (B) Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in OVA + SAL, OVA + VEH, OVA + AD4 60, or OVA + AD4 120 to those in SAL + SAL. The relative ratio of NF-κB in nuclear protein extracts from the lung tissues of SAL + SAL is arbitrarily presented as 1. Bars represent mean ±SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

Effect of AD4 on NF-κB p65 protein levels in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis showed that levels of NF-κB p65 in nuclear protein extracts from lung tissues were increased at 48 h after OVA inhalation compared with the levels in the control mice (Figure 2). The increased NF-κB p65 levels in nuclear protein extracts after OVA inhalation were significantly decreased by the administration of AD4. In contrast, levels of NF-κB p65 in cytosolic protein fractions from lung tissues were decreased after OVA inhalation compared with the levels in the control mice. The decreased NF-κB p65 levels in cytosolic protein fractions after OVA inhalation were substantially increased by the administration of AD4.

Effect of AD4 on levels of HIF-1α and HIF-1β in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that HIF-1α levels in nuclear protein extracts from lung tissues were

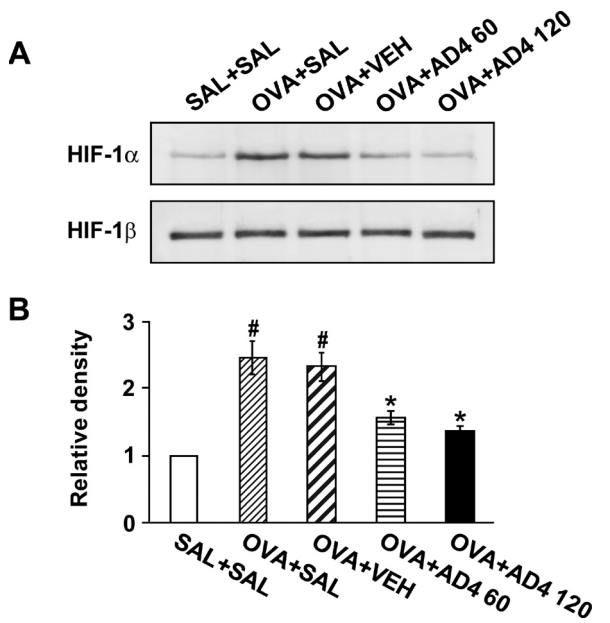

Figure 3. Effect of AD4 on HIF-1α and HIF-1β levels in nuclear protein extracts from lung tissues of OVA-sensitized and -challenged mice. HIF-1α and HIF-1β levels were measured at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) Western blot analyses of HIF-1α and HIF-1β protein. (B) Densitometric analyses are presented as the relative ratio of HIF-1α to HIF-1β. The relative ratio of HIF-1α in nuclear protein extracts from the lung tissues of SAL + SAL is arbitrarily presented as 1. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

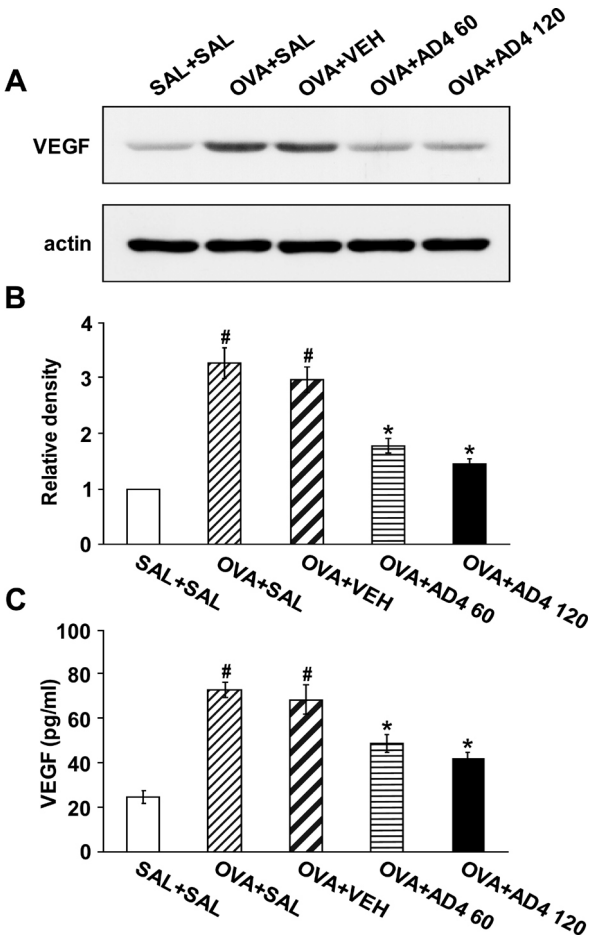

Figure 4. Effect of AD4 on VEGF levels in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) Western blot analyses of VEGF. (B) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of SAL + SAL is arbitrarily presented as 1. (C) Enzyme immunoassay of VEGF in BAL fluids. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

increased at 48 h after challenge with OVA compared with the levels in the control group (Figure 3). The increased HIF-1α levels in nuclear protein extracts after OVA inhalation were significantly decreased by the administration of AD4. In contrast, there were no significant changes in HIF-1β levels in nuclear protein extracts from lung tissues of any of the groups tested.

Effect of AD4 on VEGF protein levels in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice

Western blot analysis revealed that VEGF levels in lung tissues were increased at 48 h after OVA inhalation compared with the levels in the control mice (Figure 4A and B). The increased VEGF levels in lung tissues after OVA inhalation were significantly decreased by the administration of AD4. Consistent with the results obtained from the Western blot analysis, enzyme immunoassay revealed that levels of VEGF protein in BAL fluids were increased at 48 h after OVA inhalation compared with the levels in the control mice (Figure 4C). The increased VEGF levels after last OVA inhalation were significantly decreased by the administration of AD4.

Effect of AD4 on plasma exudation in OVA-sensitized and -challenged mice

The EBD assay revealed that plasma extravasation was significantly increased at 48 h after OVA inhalation (Figure 5). The increase in plasma extravasation was decreased significantly by the ad-

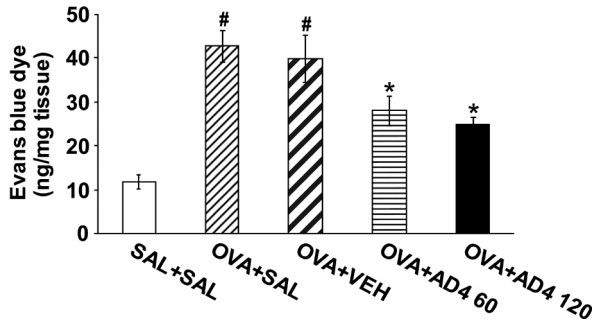

Figure 5. Effect of AD4 on plasma exudation in OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). The plasma exudation was quantified by EBD assay. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

ministration of AD4.

Effect of AD4 on IL-4, IL-5, and IL-13 protein levels in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice

Western blot analysis revealed that IL-4, IL-5, and IL-13 protein levels in lung tissues were increased significantly at 48 h after OVA inhalation compared with the levels after saline inhalation (Figure 6A and B). The increased levels of these cytokines after OVA inhalation were dramatically decreased by the administration of AD4. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays revealed that levels of these cytokines in BAL fluids were increased substantially at 48 h after OVA inhalation compared with the levels in the control mice (Figure 6C). The increased levels of these cytokines were significantly decreased by the administration of AD4.

Effect of AD4 on cellular changes in BAL fluids and lung inflammation of OVA-sensitized and -challenged mice

Numbers of total cells, eosinophils, lymphocytes, and neutrophils in BAL fluids were increased significantly at 48 h after OVA inhalation compared with the numbers after saline inhalation (Figure 7A). The increased numbers of these cells after OVA inhalation were substantially reduced by the administration of AD4.

The scores of peribronchial, perivascular, and total lung inflammation were increased significantly

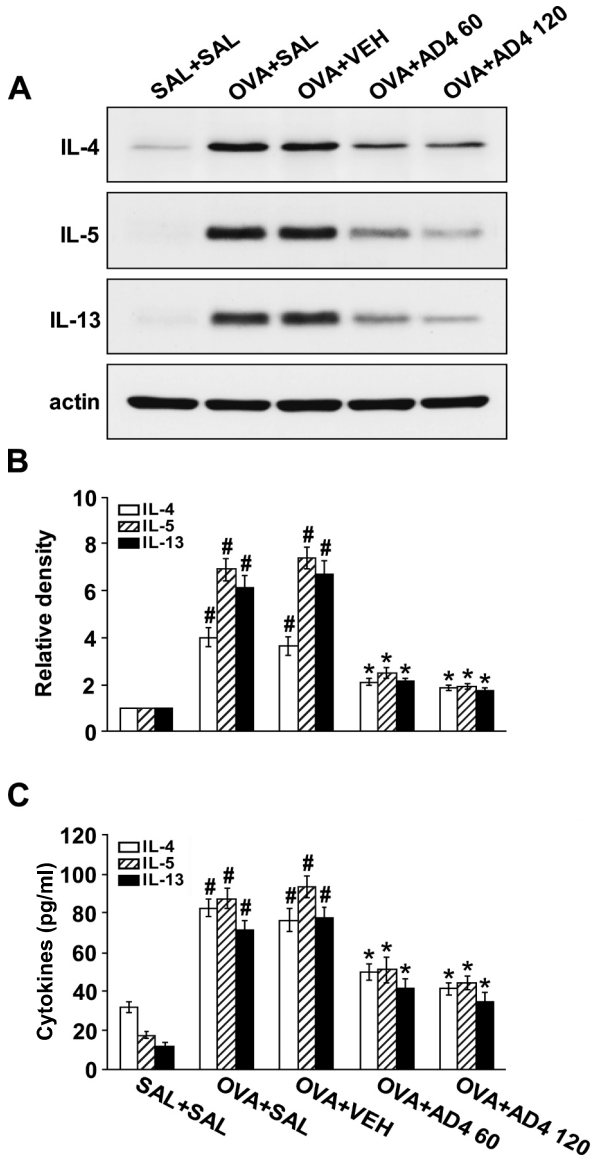

Figure 6. Effect of AD4 on IL-4, IL-5, and IL-13 protein levels in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) Western blot analyses 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 ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

at 48 h after OVA inhalation compared with scores after saline inhalation (Figure 7B). The increased peribronchial, perivascular, and total lung inflammation after OVA inhalation was significantly de-

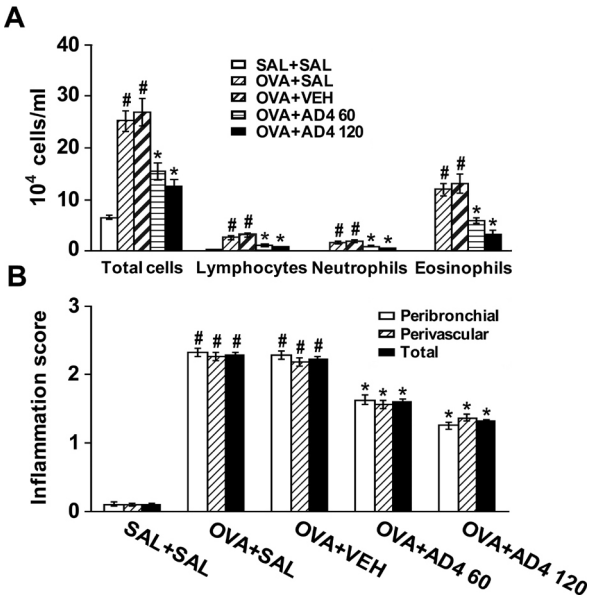

Figure 7. Effect of AD4 on bronchial inflammation of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). (A) The numbers of total and differential cellular component of BAL fluids. (B) Peribronchial and perivascular lung inflammation were measured and total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

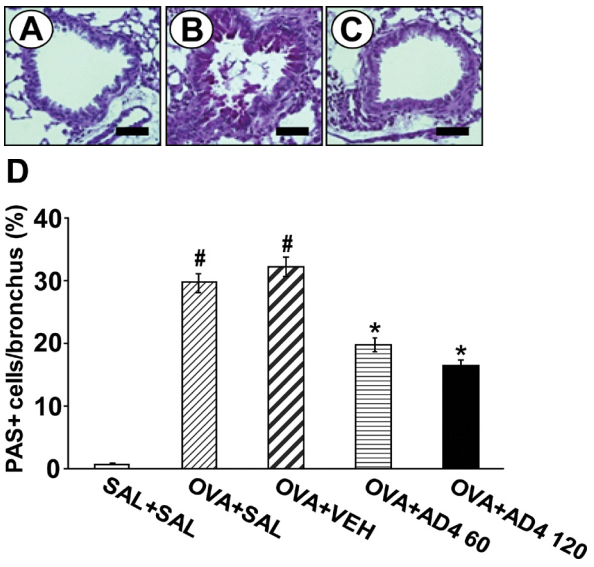

Figure 8. Effect of AD4 on airway mucus expression in OVA-sensitized and -challenged mice. (A-C) Representative PAS-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered saline (A), OVA-inhaled mice administered saline (B), and OVA-inhaled mice administered 120 mg/kg of AD4 (C). The violet color indicates PAS-positive mucus expression. Bars represent 50 μm. (D) Quantification of airway mucus expression. The percentage of PAS-positive cells per bronchiole was measured at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

creased by the administration of AD4.

Effect of AD4 on airway mucus expression of OVA-sensitized and -challenged mice

The percentage of airway epithelium, which stained positively with PAS in OVA-inhaled mice (Figure 8B and D), was substantially greater than in saline-inhaled mice (Figure 8A and D). The increased levels of PAS-positive airway epithelium were decreased significantly by the treatment of AD4 compared with the level of untreated mice after OVA inhalation (Figure 8C and D).

Effect of AD4 on airway hyperresponsiveness

Airway responsiveness was assessed as a percentage increase of R_L in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curves of percent R_L shifted to the left compared with that of control mice (Figure 9). In addition, the percent R_L produced by administration of methacholine of 50 mg/ml increased significantly in OVA-inhaled mice

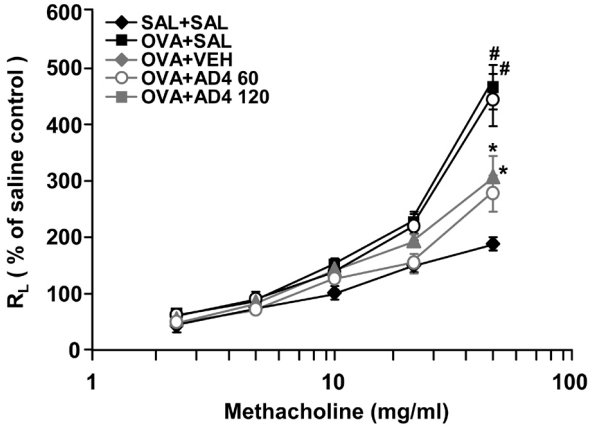

Figure 9. Effect of AD4 on airway responsiveness in OVA-sensitized and -challenged mice. Airway hyperresponsiveness was measured at 48 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered 60 mg/kg of AD4 (OVA + AD4 60), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). RL values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Bars represent mean (SEM. from 8 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

compared with the controls. OVA-sensitized and -challenged mice treated with AD4 showed a substantial reduction of R_L at dose of 50 mg/ml of methacholine compared with that of untreated mice after OVA inhalation. These results indicate that AD4 treatment reduces OVA-induced airway hyperresponsiveness.

Effect of AD4 administered after OVA inhalation on cellular changes in BAL fluids and on airway hyperresponsiveness of OVA-sensitized and -challenged mice

Numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly increased in the BAL fluid at 48 h after the last OVA inhalation compared with the numbers after saline inhalation (Figure 10A). The increased numbers of these cells after OVA inhalation were substantially reduced by the administration of AD4.

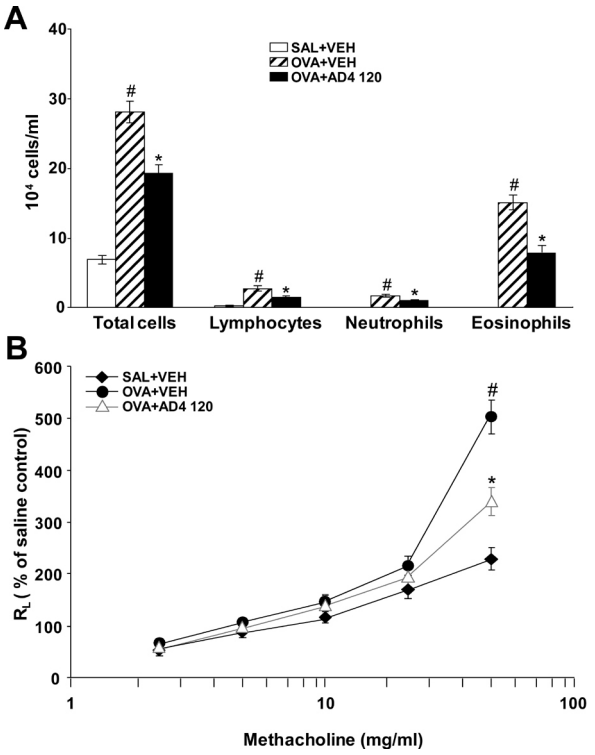

Figure 10. Effect of AD4 administered after OVA inhalation on total cells and differential cellular components in BAL fluids and on airway responsiveness of OVA-sensitized and -challenged mice. (A) The numbers of total and differential cellular component of BAL fluids. (B) Airway responsiveness. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL + VEH), OVA-inhaled mice administered drug vehicle (OVA + VEH), and OVA-inhaled mice administered 120 mg/kg of AD4 (OVA + AD4 120). R_L values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Bars represent mean ± SEM from 8 mice per group. $^{\#}P < 0.05$ versus SAL + VEH; $^{*}P < 0.05$ versus OVA + VEH.

In OVA-sensitized and -challenged mice, the dose-response curves of percent R_L shifted to the left compared with that of saline-inhaled mice (Figure 10B). In addition, the percent R_L produced by administration of methacholine of 50 mg/ml increased significantly in OVA-inhaled mice compared with the saline-inhaled mice. OVA-sensitized and -challenged mice treated with AD4 showed a substantial reduction of R_L at dose of 50 mg/ml of methacholine compared with that of mice treated with drug vehicle only after OVA inhalation. These results indicate that AD4 treatment after OVA challenge reduces OVA-induced airway hyperresponsiveness.

Discussion

Bronchial asthma is a chronic inflammatory disease of the airways characterized by airway eosinophilia and hyperresponsiveness to inhaled allergens and nonspecific stimuli (Bousquet *et al.,* 2000). Oxidative stress plays an important role in the pathogenesis of bronchial asthma (Dworski, 2000). Our present study with the OVA-induced model of allergic airway disease has revealed that ROS production in cells from BAL fluids was increased and that administration of a novel thiol compound AD4, which is the amide form of *N*-acetylcysteine, reduced significantly the increased ROS levels, the increased expression of Th2 cytokines and VEGF, plasma exudation, bronchial inflammation, and airway hyperresponsiveness. We have also found that the increased NF-κB and HIF-1α levels in nuclear protein extracts of lung tissues after OVA inhalation were decreased by the administration of AD4. These results suggest that AD4 attenuates airway inflammation and hyperresponsiveness through regulation of activation of NF-κB and HIF-1α as well as modulation of ROS generation in allergic airway disease of mice.

Level of ROS has been shown to be increased in the airways of OVA-challenged animals (Nishida *et al.,* 2002; Zhang *et al.,* 2002; Lee *et al.,* 2004d, 2005, 2006a). ROS released by inflammatory cells infiltrating the airways play an important role in the airway tissue injury observed in asthma (Dworski, 2000). Although the lung has a well-developed antioxidant system (Rahman *et al.,* 1996, 1997), overproduction of ROS or depression of the protective system results in epithelial cell damage, cell shedding, and bronchial hyperreactivity (Hulsmann *et al.,* 1994; Cortijo *et al.,* 1999). Studies with animal models have indicated that ROS contribute to airway hyperresponsiveness by increasing vagal

tone due to damage of oxidant sensitive β-adrenergic receptors as well as decreasing mucociliary clearance (Owen *et al.,* 1991; Adam *et al.,* 1999). Consistent with these observations, our data have shown that generation of ROS in BAL cells was significantly increased after allergen challenge in OVA-inhaled mice and that administration of AD4 reduced the ROS generation and inhibited airway inflammation and hyperresponsiveness. On the basis of these findings, we suggest that AD4 attenuates airway inflammation and hyperresponsiveness through the reduction of ROS levels in lungs of OVA-inhaled mice.

NF-κB is present in most cell types and is known to play a pivotal role in immune and inflammatory responses, including asthma (Siebenlist *et al.,* 1994; Baeuerle and Baltimore, 1996; Baldwin, 1996; Barnes and Adcock, 1997; Barnes and Karin, 1997; Gilmore, 1999). Development of oxidant/antioxidant imbalance in asthma leads to activation of redox-sensitive transcription factor NF-κB (Henderson *et al.,* 2002). ROS have also been directly implicated as second messengers in the activation of NF-κB, based upon the ability of oxidants to activate NF-κB by the oxidation of its cysteine-SH group or by ubiquitination and proteolysis of IκB (Ginn-Pease and Whisler, 1996; Shang *et al.,* 1997; Rahman and MacNee, 1998). Consistent with these observations, NF-κB levels in nuclear protein extracts from lung tissues were substantially increased in the OVA-induced model of allergic airway disease used for the present study. It is known that activation of this transcription factor induces a variety of inflammatory genes that are abnormally expressed in asthma (Barnes and Adcock, 1997; Mori *et al.,* 1999; Stutz and Woisetschlager, 1999). We have also assessed whether these inflammatory cytokines are up-regulated in the OVA-induced model of allergic airway disease. As expected, expression of IL-4, IL-5, and IL-13 was increased significantly after allergen challenge in OVA-inhaled mice. The administration of AD4 resulted in significant reduction of NF-κB translocation into nucleus and of expression of these cytokines after OVA inhalation. These results indicate that AD4 may inhibit NF-κB activity, preventing translocation of this transcription factor into nucleus induced by the increased ROS after allergen inhalation and that AD4 consequently reduces the expression of inflammatory cytokines mediated by NF-κB activation.

Inflammation of the asthmatic airway usually accompanies an increase in vascular permeability and plasma exudation (Stutz and Woisetschlager, 1999; Bousquet *et al.,* 2000). Although other inflammatory mediators, including platelet-activating factor, can promote microvascular leakage (Kirsch *et al.,* 1992; Tamaoki *et al.,* 1999), 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*,* 2004c). The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesiculo-vacuolar organelles (Dvorak *et al.,* 1994*,* 1995). VEGF is produced by a wide variety of cells, including macrophages, neutrophils, eosinophils, and lymphocytes (Horiuchi and Weller, 1997; Taichman *et al.,* 1997; Lee *et al.,* 2002*,* 2004a*,* c). Several studies have shown that overproduction of VEGF causes the increase in vascular permeability, which results in leakage of plasma proteins including inflammatory mediators and inflammatory cells into the extravascular space thereby allowing migration of inflammatory cells into the airways (Thurston *et al.,* 2000; Lee *et al.,* 2002*,* 2004c). Although the pathogenesis of allergic airway disease induced by plasma extravasation is not clearly defined, plasma protein leakage has been implicated to induce 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). In addition, VEGF also plays a crucial role in adaptive Th2-mediated inflammation (Lee *et al.,* 2004a). Consistent with these observations, we have found that VEGF expression was up-regulated and that vascular permeability was increased in allergic airway disease of mice. The increased levels of VEGF, increased vascular permeability, bronchial inflammation, and airway hyperresponsiveness were significantly reduced by the administration of AD4. Taken together, these findings suggest that VEGF have an important role in inducing and maintaining allergic airway disease and that one likely mechanism for effects of AD4 on airway inflammation and bronchial hyperresponsiveness could be the modulation of vascular permeability mediated by VEGF.

VEGF is one of genes whose expression is regulated through HIF-1α activation (Wang and Semenza, 1995; Semenza, 1999). HIF-1 is a transcriptional activator that mediates gene expression in response to cellular oxygen concentrations (Semenza, 2001). HIF-1 is composed of 2 subunits, HIF-1α and HIF-1β. Whereas the β-subunit protein is constitutively expressed, the stability of the α-subunit and its transcriptional activity are controlled by the intracellular oxygen concentration (Ivan *et al.,* 2001; Kaelin, 2002). In addition to the oxygen-dependent regulation of HIF-1α activity, several reports have demonstrated that HIF-1α

expression is modulated by a variety of cytokines and growth factors in oxygen-independent pathway (Feldser *et al.,* 1999). Previous reports have demonstrated that HIF-1α also plays an important role in inflammatory responses and that ROS stabilize HIF-1α during hypoxia and/or nonhypoxia (Chandel *et al.,* 2000; Haddad and Land, 2001; Jung *et al.,* 2003; Lee *et al.,* 2006a, b). Very recently, we have shown that increased expression of VEGF in OVA-inhaled mice is decreased by the inhibition of HIF-1α activation (Lee *et al.,* 2006a). In keeping with these observations, determination of HIF-1α protein level in nuclear extracts has revealed that this protein levels were increased substantially in our current OVA-induced model of allergic airway disease suggesting that HIF-1α is activated. The increased levels of HIF-1α were reduced significantly after the administration of AD4. Moreover, the increased levels of VEGF after OVA inhalation were significantly reduced by the administration of AD4. Taken together, these findings suggest that AD4 inhibits the increased VEGF expression through the down-regulation of HIF-1α activity in allergic airway disease.

We have found a dramatic reduction in allergen-induced goblet cell hyperplasia in AD4-treated mice. Th2 cytokines, VEGF, T cells, and eosinophils are required to produce airway mucus accumulation and goblet cell degranulation (Zhu *et al.,* 1999; Justice *et al.,* 2002; Lee *et al.,* 2004a, b). Although a direct role of ROS in these cells can not be ruled out, the observed decrease in mucus production in AD4-treated lung tissue may be attributed to an indirect effect on goblet cells resulting from the combination of a substantial drop in Th2 cytokine and VEGF levels as well as reduction in eosinophilia in OVA-sensitized and -challenged mice.

In summary, we have examined the effect of AD4 on allergen-induced airway inflammation and bronchial hyperresponsiveness in mice. A significant amount of data showing an increase of oxidative stress in allergic airway disease and indicating a potential role of ROS in pathogenesis of the disease has been accumulated over years. Based on these observations, we have administered a newly developed antioxidant agent AD4 to allergen-inhaled mice to unearth a potential therapeutic strategy treating allergic airway disease. Administration of AD4 results in a significant reduction of all pathophysiological symptoms examined. Our data have also shown that AD4 modulates ROS generation in BAL cells, VEGF expression through regulation of HIF-1α, and activation of NF-κB. These findings suggest that oxidative stress is one of the important determinants of allergic airway disease and that antioxidant treatment such as administration of AD4 may be a recommendable therapeutic strategy. Thus, this study provides a crucial molecular mechanism for the potential of AD4 in preventing or treating allergic airway disease and other airway inflammatory disorders.

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