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Antioxidant Down-Regulates Interleukin-18 Expression in Asthma

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ABSTRACT

An alteration in the balance between a T-helper type 2 cell (Th2) response and a Th1 response may predispose to the development of bronchial asthma. Interleukin-18 (IL-18) has an ability to promote both Th1 and Th2 responses, depending on the surrounding cytokine environment. Reactive oxygen species (ROS) play a crucial 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 asthma. In this study, we used a C57BL/6 mouse model of allergic asthma to examine the effects of antioxidants on the regulation of IL-18 expression. Our present study with ovalbumin-induced murine model of asthma revealed that ROS production in cells from bronchoalveolar lavage fluids was increased and that administration of L-2-oxothiazolidine-4-carboxylic acid or α-lipoic acid reduced the increased levels of ROS, the increased expression of IL-18 protein and mRNA, airway inflammation, and bronchial hyperresponsiveness. Our results also showed that antioxidants down-regulated a transcription factor, nuclear factor-κB (NF-κB), activity. These results indicate that antioxidants may reduce IL-18 expression in asthma by inhibiting the activity of NF-κB and suggest that ROS regulate the IL-18 expression.

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). ROS can lead to endothelial barrier dysfunction with subsequently increased permeability to fluids, macromolecules, and inflammatory cells (Henricks and Nijkamp, 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 patients with asthma (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., 2004; Lee et al., 2004). However, there are few data on the influence and the molecular basis of antioxidants on allergen-induced bronchial inflammation and airway hyperresponsiveness.

Asthma is a T-helper type 2 cell (Th2) cytokine-dominant disease with a particular profile of cytokine release (Robinson et al., 1992). However, other cytokines, which have been classically considered to belong to Th1 cell profiles, are also associated with the airway inflammatory response that characterizes bronchial asthma. An alteration in the balance between Th2 and Th1 cell response may predispose to the development of bronchial asthma (Umetsu et al., 2002).

IL-18 has been identified as a proinflammatory cytokine that induces interferon-γ in activated natural killer cells, Th1, and CD8+ cytotoxic T cells (Okamura et al., 1995, 1998; Dinarello, 1999). IL-18 plays important roles in the development of Th1 cell responses (Okamura et al., 1995) and Th2 cell responses, which are dependent on the surrounding cy-

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ABBREVIATIONS: ROS, reactive oxygen species; Th, T-helper cell; OTC, L-2-oxothiazolidine-4-carboxylic acid; BAL, bronchoalveolar lavage; R_L, Airway resistance; NF-κB, nuclear factor-κB; IL, interleukin; GSSG, glutathione disulfide; GSH, glutathione; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OVA, ovalbumin; BAY 11-7085, (*E*)-3[(4-*t*-butylphenyl)sulfonyl]-2-propenenitrile; buffer A, Tris-HCl, EDTA, glycerol, dithiothreitol, $MgCl_2$, and phenylmethylsulfonyl fluoride; buffer B, sucrose, $MgCl_2$, and potassium phosphate buffer.

tokine environment (Xu et al., 2000; Nakanishi et al., 2001). It is also able to induce the production of IgE (Hoshino et al., 2000; Yoshimoto et al., 2000), IL-4, IL-13, and histamine in basophils and mast cells in association with IL-3 (Yoshimoto et al., 1999), IL-4, and IL-13 secretion from natural killer cells and naive T lymphocytes in response to IL-2 (Hoshino et al., 1999). IL-18 is expressed in the human airway epithelium (Cameron et al., 1999) and is also produced by human alveolar macrophages (Shigehara et al., 2001). However, there are conflicting reports on the actions of IL-18 on the pathogenesis of asthma (Hofstra et al., 1998; Campbell et al., 2000; Kodama et al., 2000; Wild et al., 2000; Tanaka et al., 2001; Ho et al., 2002).

In the present study, we used a murine model of asthma to determine the effect of the antioxidants L-2-oxothiazolidine-4-carboxylic acid (OTC), which is a prodrug of cysteine, or α-lipoic acid on the regulation of IL-18 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 (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 of 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 described previously with some modifications (Fig. 1) (Kwak et al., 2003). 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, Tokyo, Japan). Bronchoalveolar lavage (BAL) was performed 72 h after the last challenge. At the time of lavage, the mice (8 mice per group) were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight, administered intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept 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 with a cytospin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico. Inc., Aguada, Puerto Rico) 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.

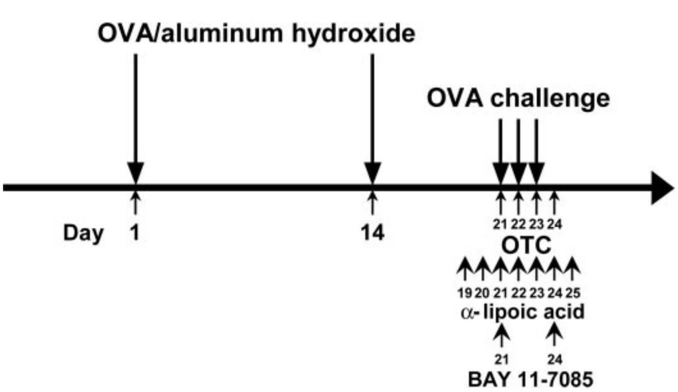

Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized on days 1 and 14 by intraperitoneal injection of ovalbumin emulsified in 1 mg of aluminum hydroxide. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) ovalbumin 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 a 24-h interval on days 21 to 24, beginning 1 h before the first challenge. α-Lipoic acid was administered 7 times by oral gavage at a 24-h interval on days 19 to 25, beginning 2 days before the first challenge. BAY 11-7085 was administered by intraperitoneal injection two times to each treated animal, once on day 21 and the second time on day 24.

Administration of Antioxidants, OTC or α-Lipoic Acid, and an Inhibitor of Nuclear Factor-κB Activation, BAY 11-7085. OTC solution (160 mg/kg body weight/day; Sigma-Aldrich) was freshly prepared as described elsewhere (Han et al., 2002) and administered intraperitoneally four times at a 24-h interval on days 21 to 24, beginning 1 h before the first challenge. α-Lipoic acid (100 mg/kg body weight/day; Sigma-Aldrich), which is a nonenzymatic antioxidant, was administered seven times by oral gavage at a 24-h interval on days 19 to 25, beginning 2 days before the first challenge. BAY 11-7085 (20 mg/kg body weight/day; BIOMOL International L.P., Plymouth Meeting, PA), dissolved in dimethyl sulfoxide and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each treated animal, once on day 21 (1 h before the first airway challenge with ovalbumin) and the second time on day 24 (1 day after the last airway challenge with ovalbumin) (Yang et al., 2004) (Fig. 1).

Measurement of Intracellular ROS. ROS were measured by a method described previously with modifications (Sundaresan et al., 1995; Lee et al., 2002). BAL fluids were washed with phosphate-buffered saline. To measure intracellular ROS, cells were incubated for 10 min at room temperature with phosphate-buffered saline 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). The numbers of ROS-positive cells stained by dichlorofluorescein were counted. Data were presented as the number of ROS-positive cells divided by number of total cells in each group.

Measurement of Glutathione and Glutathione Disulfide in Lung Tissues. Lung tissues were homogenized with 10 ml of ice-cold buffer (50 mM phosphate buffer containing 1 mM EDTA) per gram of tissue. After centrifugation at 10,000*g* for 15 min at 4°C, the supernatant was removed, deproteinated, and then stored at −20°C until the sample was assayed. Total glutathione (GSH) and glutathione disulfide (GSSG) levels were determined using a Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol.

Western Blot Analysis. Lung tissues were homogenized in the presence of protease inhibitors, and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA) as described previously (Kwak et al., 2003). Samples (30 μg of protein per lane) were loaded on a 12% SDS-polyacrylamide gel electrophoresis gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by the wet-transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween 20 (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-IL-4 antibody (Serotec Ltd., Oxford, UK), anti-IL-5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-13 antibody (R&D Systems, Inc., Minneapolis, MN), or anti-IL-18 antibody (Santa Cruz Biotechnology) overnight at 4°C. Anti-rabbit horseradish peroxide-conjugated IgG was used to detect binding of antibody. The membranes were stripped and reblotted with antiactin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

Measurement of Th2 Cytokines. Levels of IL-4, IL-5, and IL-13 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (IL-4: Endogen, Inc., Woburn, MA; IL-5: BioSource International, Inc. Camarillo, CA; IL-13: R&D Systems). Sensitivities for IL-4, IL-5, and IL-13 assays were 5, 3, and 1.5 pg/ml, respectively.

RNA Isolation and RT-PCR. Total RNA from lung tissues was isolated using a rapid-extraction method (TRI-Reagent) as described previously (Chomczynski and Sacchi, 1987). RNA was quantified by measuring absorption at 260 nm and stored at −80°C until use. Total RNA (4 μg) was reverse-transcribed to cDNA in a buffer containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 μg of random hexanucleotide primers, 2.5 mM dNTP, 40 units of RNase inhibitor, and 50 units/μl SuperScript II RT (Invitrogen, Carlsbad, CA), in a final volume of 20 μl. This mixture was incubated for 50 min at 42°C and then digested with 2 U/μl *Escherichia coli* RNase H for 20 min at 37°C. The first-strand cDNAs were used for PCR amplification of IL-18 or the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed by mixing 3 μl of the reverse-transcription reaction mixture with 47 μl of buffer containing 2.5 units of TaqDNA polymerase (Promega, Madison, WI) and 30 pmol of specific primer pairs for mouse cDNA of IL-18 or GAPDH, designed from published mouse gene sequences (Faust et al., 2002). The primers used were as follows: IL-18 (predicted length, 320 base pairs) sense, 5′-ACTGTACAACCGCAGTAATAC-3′; antisense, 5′-AGTGAACATTACAGATTTATCCC-3′; and GAPDH (predicted length, 609 base pairs) sense, 5′-GCCATCAACGACCCCTTCATTGAC-3′; antisense, 5′-ACGGAAGGCCATGCCAGTGAGCTT-3′. PCR reactions were performed in a thermocycler (GeneAmp PCR System 2400; Applied Biosystems, Foster City, CA) using the following reaction conditions: after an initial incubation for 2 min at 95°C, samples were subjected to 35 cycles of 1 min at 94°C, 2 min at 54°C (GAPDH), or 58°C (IL-18) and 1 min at 72°C. A final extension step at 72°C for 10 min was performed. The RT-PCR products were electrophoretically fractioned on 2% agarose gels stained with ethidium bromide. DNA bands were visualized under UV light.

Quantitative Real-Time PCR. Quantitative real-time PCR analysis was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The sequences of primers used were as follows: IL-18 sense, 5′-ACTGTACAACCGCAGTAATAC-3′; antisense, 5′-AGTGAACATTACAGATTTATCCC-3′; and β-actin sense, 5′-CAGATCATGTTTGAGACCTTC-3′; antisense, 5′-ACTTCATGATGGAATTGAATG-3′. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol. The data have been normalized to the expression of β-actin.

Cytosolic or Nuclear Protein Extractions for Analysis of Nuclear Factor-κB p65. Lungs were removed and homogenized in 2 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. The homogenates were centrifuged at 1000*g* for 15 min at 4°C. The supernatant fractions were incubated on ice for 10 min and centrifuged at 100,000*g* for 1 h at 4°C to obtain cytosolic proteins for analysis of nuclear factor-κB (NF-κB) p65. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 6.8) and pelleted at 1000*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*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 1000*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 phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 h with gentle stirring and centrifuged at 12,000*g* for 30 min. The resulting supernatant was used as soluble nuclear proteins for the determination of NF-κB p65 levels. The levels of these proteins were analyzed by Western blotting using antibody against NF-κB p65 (Upstate Biotechnology, Lake Placid, NY) as described above.

Histology, Immunohistochemistry, and Immunocytochemistry. At 72 h after the last challenge, mice were killed, and the lungs and trachea were filled intratracheally with a fixative (0.8% formalin and 4% acetic acid) using a ligature around the trachea. Lungs were removed, and 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 Microsystems Nussloch GmbH, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). For immunohistochemistry and immunocytochemistry of IL-18, the deparaffinized 4-μm sections or the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with instructions for the RTU Vectastain Universal Quick Kit from Vector Laboratories Inc. (Burlingame, CA). In brief, 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. After incubation in normal horse serum for 15 min at room temperature, the slides were probed with an affinity-purified rabbit polyclonal IL-18 IgG (Santa Cruz Biotechnology) overnight at 4°C and then incubated with prediluted biotinylated panspecific IgG for 10 min. The slides were incubated with horseradish peroxide-conjugated streptavidin for 5 min and then in 3-amino-9-ethylcarbazole substrate mixtures for peroxidase for 12 min. For the control, sections of lung tissue or BAL cells prepared from mice were treated without the primary antibody under the same conditions. After immunostaining, the slides were counterstained for 1 min with Gill's hematoxylin in 20% ethylene glycol and then mounted with Aqueous Mounting Medium from InnoGenex (San Ramon, CA) and photomicrographed (Vanox T; Olympus Optical Co., Tokyo, Japan).

Determination of Airway Responsiveness. 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 pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montreal, Canada). The mouse was quasisinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm of 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 the water column. Methacholine aerosol was generated with an inline nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/ml in saline). After each methacholine challenge, the data of 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.

Densitometric Analysis and Statistics. All immunoreactive signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad). Data were expressed as mean ± S.E.M. Statistical comparisons were performed using one-way analysis of variance followed by 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 OTC or α-Lipoic Acid on ROS Levels in BAL Fluids of Ovalbumin-Sensitized and -Challenged Mice. ROS generation in BAL cells was increased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 2). The increased ROS generation was substantially reduced by the administration of OTC or α-lipoic acid.

Effect of OTC or α-Lipoic Acid on GSH and GSSG Levels in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice. GSH assay revealed that the levels of total GSH in lung tissues were decreased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation. The decreased GSH levels after ovalbumin inhalation were significantly increased by the administration of OTC or α-lipoic acid (Fig. 3A). However, levels of GSSG in lung tissues were increased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 3B). The increased GSSG levels after ovalbumin inhalation were significantly reduced by the administration of OTC or α-lipoic acid.

IL-18 Protein Levels and mRNA Expression Increased in Ovalbumin-Sensitized and -Challenged Mice. Western blot analysis revealed that IL-18 protein levels in lung tissues were increased approximately 2.7-, 2.8-, 4.0-, 5.4-, 5.4-, and 6.1-fold at 6, 12, 24, 36, 48, and 72 h, respectively, after challenge with ovalbumin compared with the levels in the control group (Fig. 4, A and B). In contrast, no significant changes in the IL-18 protein level were observed after saline inhalation. Real-time PCR analysis revealed that IL-18 mRNA expression had increased approximately 1.6-, 1.7-, 2.1-, 2.6-, 2.9-, and 2.9-fold at 6, 12, 24, 36, 48, and 72 h, respectively, after ovalbumin inhalation compared with the expression in the control group (Fig. 4, C and D). In contrast, no significant changes in the IL-18 mRNA expression were observed after saline inhalation.

Effect of OTC or α-Lipoic Acid on IL-18 Protein Levels and mRNA Expression in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice. Western blot analysis revealed that levels of IL-18 protein in lung tissues were increased at 72 h after ovalbumin inhalation compared with the levels in the control mice (Fig. 5, A and B). The increased IL-18 levels at 72 h after ovalbumin inhalation were decreased significantly by the administration of OTC or α-lipoic acid. Real-time PCR analyses showed that IL-18 mRNA expression in lung tissues was increased 72 h after ovalbumin inhalation compared with the expression after saline inhalation (Fig. 5, C and D). The increased IL-18 mRNA expression 72 h after ovalbumin inhalation was reduced by the administration of OTC or α-lipoic acid.

Localization of Immunoreactive IL-18 in Lung Tissues and in BAL Fluids of Ovalbumin-Induced Asthma. Immunohistochemical analyses showed the localization of immunoreactive IL-18 in epithelial cells around the bronchioles of mice with ovalbumin-induced asthma (Fig. 6B). Treatment of ovalbumin-sensitized and -challenged mice with OTC or α-lipoic acid resulted in a marked reduction of the immunoreactive IL-18 level (Fig. 6, C and D), similar to that in the control (Fig. 6A).

Immunocytological analysis of BAL fluids showed localization of immunoreactive IL-18 in the BAL cells from ovalbumin-exposed mice (Fig. 6, F, I, and J). However, treatment of ovalbumin-sensitized and -challenged mice with OTC or α-lipoic acid markedly reduced immunoreactive IL-18 level (Fig. 6, G and H) similar to that in precipitated cells from control mice (Fig. 6E). To examine the cell differentials present in BAL cells, the slides used for the detection of IL-18 were

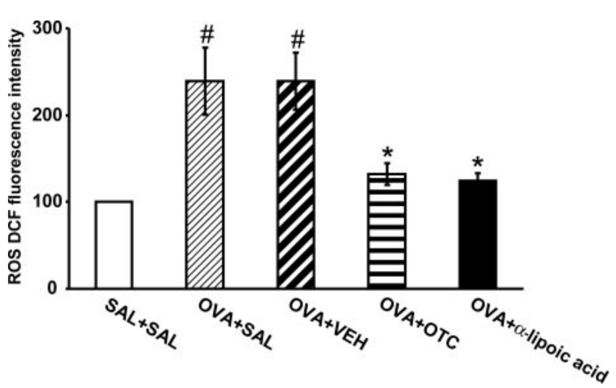

Fig. 2. Effect of OTC or α-lipoic acid on ROS levels in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered OTC (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). Dichlorofluorescein fluorescence intensity is presented as the relative ratio of ROS levels. 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 eight mice per group. #, $p < 0.05$ versus SAL+SAL;*, $p < 0.05$ versus OVA+SAL.

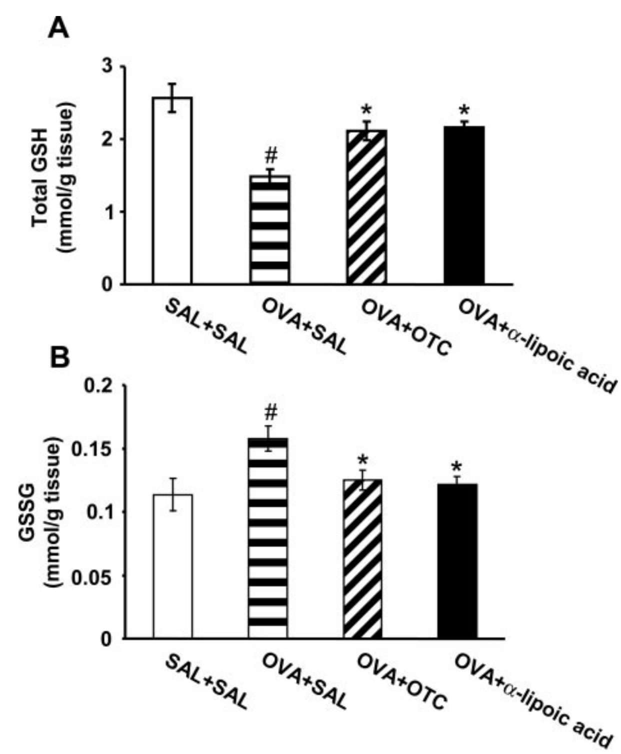

Fig. 3. Effect of OTC or α-lipoic acid on GSH (A) and GSSG (B) levels in lung tissues of ovalbumin-sensitized and -challenged mice. Sampling was performed 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered 160 mg/kg OTC (OVA+OTC), and ovalbumin-inhaled mice administered 100 mg/kg α-lipoic acid (OVA+α-lipoic acid). Bars represent mean ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; *, $p < 0.05$ versus OVA+SAL.

destained with 70% ethyl alcohol. The smears of BAL cells were stained with Diff-Quik solution. Immunoreactive IL-18 was localized on macrophages (Fig. 6, I and J).

Effect of OTC or α-Lipoic Acid on NF-κB p65 Protein Levels in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice. Western blot analysis revealed that levels of NF-κB p65 in nuclear protein extracts from lung tissues were increased at 72 h after ovalbumin inhalation compared with the levels in the control mice (Fig. 7A). The increased NF-κB p65 levels at 72 h after ovalbumin inhalation were decreased by the administration of OTC or α-lipoic acid. In contrast, levels of NF-κB p65 in cytosolic protein extracts

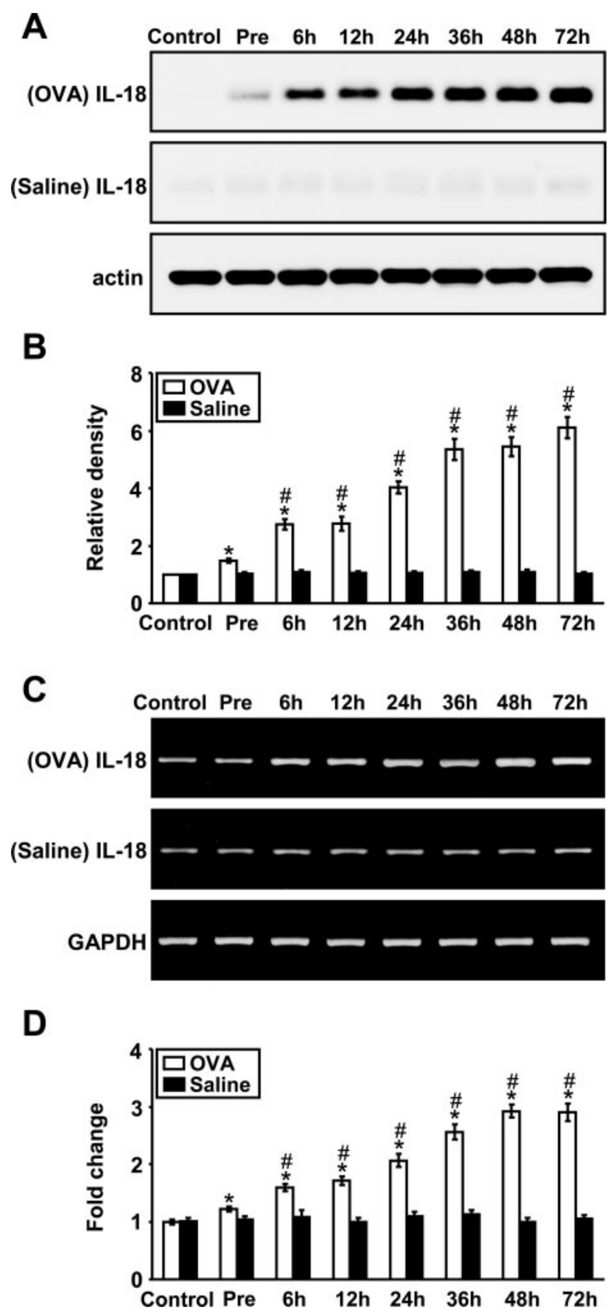

Fig. 4. Levels of IL-18 protein and mRNA in lung tissues of ovalbumin-sensitized and -challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with ovalbumin or saline. A, Western blot analyses of IL-18 protein. The Western blot was probed with an anti-IL-18 antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. B, densitometric analyses are presented as the relative ratio of IL-18 to actin. The relative ratio of IL-18 in lung tissues of the control group is arbitrarily presented as 1. C, representative RT-PCR of IL-18 mRNA expression. D, quantitative analysis of IL-18 mRNA expression by means of real-time PCR. Data represent mean ± S.E.M. from eight mice per group; 6, 12, 24, 36, 48, and 72 h are time periods after the last challenge; Control, no treatment; Pre, 1 h before the first challenge; #, $p < 0.05$ versus Pre; ∗, $p < 0.05$ versus saline inhalation.

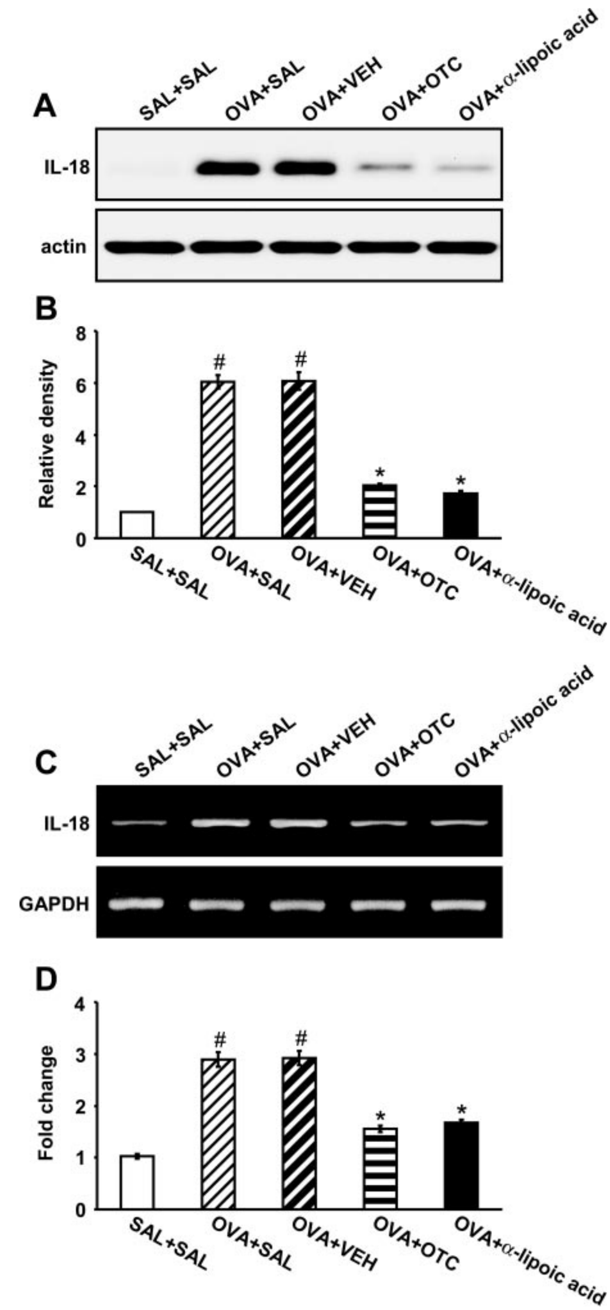

Fig. 5. Effect of OTC or α-lipoic acid on IL-18 protein and mRNA in lung tissues of ovalbumin-sensitized and -challenged mice. A, Western blot analysis of IL-18. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered OTC (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). B, densitometric analyses are presented as the relative ratio of IL-18 to actin. The relative ratio of IL-18 in lung tissues of SAL+SAL is arbitrarily presented as 1. C, representative RT-PCR analysis of IL-18 mRNA expression. D, quantitative analysis of IL-18 mRNA by means of real-time PCR. Data represent mean ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; ∗, $p < 0.05$ versus OVA+SAL.

from lung tissues were decreased at 72 h after ovalbumin inhalation compared with the levels in the control mice (Fig. 7B). The decreased NF-κB p65 levels in cytosol preparations were increased by the administration of OTC and α-lipoic acid. These results indicate that OTC and α-lipoic acid inhibit NF-κB activity by preventing translocation of this transcription factor into the nucleus.

Effect of BAY 11-7085 on IL-18 Levels in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice. Western blot analysis showed that IL-18 protein levels in lung tissues were increased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 8, A and B). The increased IL-18 levels were significantly reduced by the administration of BAY 11-7085.

Effect of OTC or α-Lipoic Acid on Cellular Changes in BAL Fluids. Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 72 h after ovalbumin inhalation compared with the numbers after saline inhalation (Fig. 9A). The increased numbers of total cells, lymphocytes, neutrophils, and eosin-

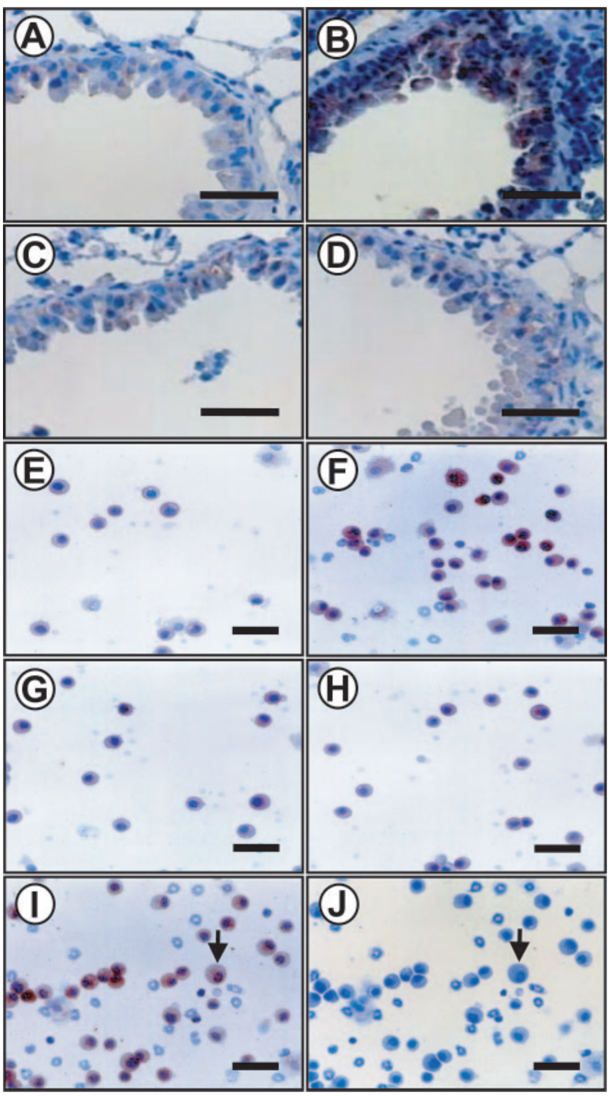

Fig. 6. Localization of immunoreactive IL-18 in lung tissues and in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed 72 h after the last challenge in lung tissues from sensitized mice challenged with saline (A), from sensitized mice challenged with ovalbumin (B), from ovalbumin-inhaled mice administered OTC (C), and from ovalbumin-inhaled mice administered α-lipoic acid (D). Sampling was also performed in BAL fluids from sensitized mice challenged with saline (E), sensitized mice challenged with ovalbumin (F, I, and J), ovalbumin-inhaled mice administered OTC (G), and from ovalbumin-inhaled mice administered α-lipoic acid (H). A–I, representative light microscopy showing IL-18-positive cells in the BAL fluids; the brown color indicates IL-18-positive cells. J, to examine the cell differentials in BAL cells prepared from the control mice, the slides used for the detection of IL-18 (I) were destained with 70% ethyl alcohol. The smears of BAL cells were stained with Diff-Quik solution and were viewed under a light microscope. The arrow indicates a macrophage. Bars, 50 μm.

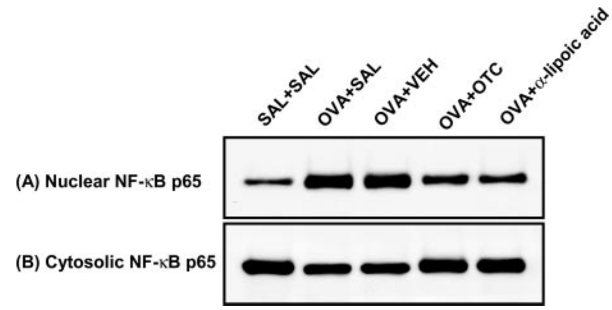

Fig. 7. Effect of OTC or α-lipoic acid on NF-κB p65 protein expression in nuclear and cytosolic protein extracts from lung tissues. NF-κB p65 protein expression in nuclear protein extracts (A) and in cytosolic protein extracts (B) from lung tissues. NF-κB protein expression was measured 72 h after the 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 (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). Results were similar in eight mice per group.

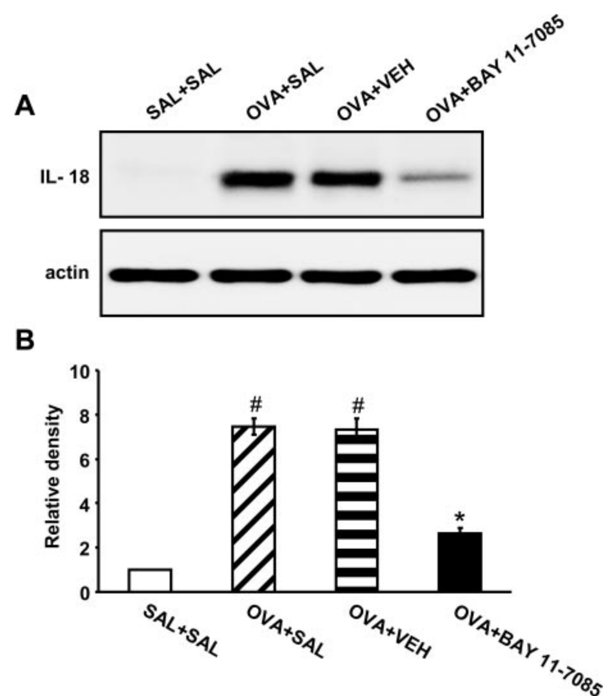

Fig. 8. Effects of BAY 11-7085 on IL-18 expression in lung tissues of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), and ovalbumin-inhaled mice administered BAY 11-7085 (OVA+BAY 11-7085). A, Western blotting of IL-18 in lung tissues. B, densitometric analyses are presented as the relative ratio of IL-18 to actin. The relative ratio of IL-18 in the lung tissues of SAL+SAL is arbitrarily presented as 1. Bars represent mean ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; *, $p < 0.05$ versus OVA+SAL.

ophils at 72 h after ovalbumin inhalation were significantly reduced by the administration of OTC or α-lipoic acid.

Antioxidants Reduced Ovalbumin-Induced Airway Hyperresponsiveness. Airway responsiveness was assessed as a percentage of increase of R_L in response to increasing doses of methacholine. In ovalbumin-sensitized and -challenged mice, the dose-response curve of R_L shifted to the left compared with that of control mice (Fig. 9B). In addition, the percentage of R_L produced by methacholine administration (at doses from 10 to 50 mg/ml) increased significantly in the ovalbumin-sensitized and -challenged mice compared with the controls. kovalbumin-sensitized and -challenged mice treated with OTC or α-lipoic acid showed a dose-response curve of the percentage of R_L that shifted to the right compared with that of untreated mice. These results indicate that OTC and α-lipoic acid treatment reduce ovalbumin-induced airway hyperresponsiveness.

Effect of OTC or α-Lipoic Acid on IL-4, IL-5, and IL-13 Protein Levels in Lung Tissues and in BAL Fluids of Ovalbumin-Sensitized and -Challenged Mice. Western blot analysis showed that IL-4, IL-5, and IL-13 protein levels in lung tissues were increased significantly at 72 h after ovalbumin inhalation compared with the levels after saline inhalation. The increased IL-4, IL-5, and IL-13 levels were significantly reduced by the administration of OTC or α-lipoic acid (Fig. 10, A and B). Consistent with these results, enzyme immunoassays revealed that levels of IL-4, IL-5, and IL-13 in BAL fluids were also increased significantly 72 h after ovalbumin inhalation compared with the levels after saline inhalation. The increased IL-4, IL-5, and IL-13 levels were significantly reduced by the administration of OTC or α-lipoic acid (Fig. 10C).

Effect of OTC or α-Lipoic Acid on Total IgE or OVA-Specific IgE Levels in Serum of Ovalbumin-Sensitized and -Challenged Mice. Levels of total IgE or ovalbumin-

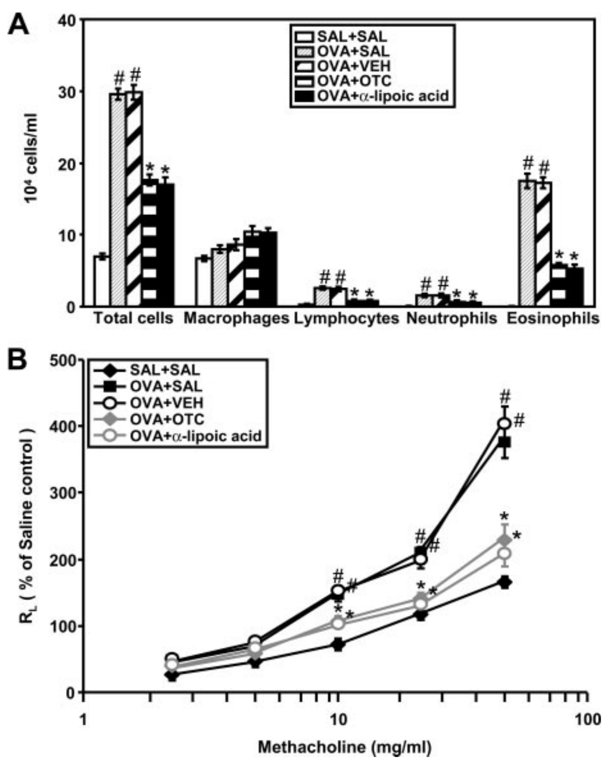

Fig. 9. Effects of OTC or α-lipoic acid on total and differential cellular components in BAL fluids and on airway responsiveness of ovalbumin-sensitized and -challenged mice. The numbers of total and differential cellular components of BAL fluid (A) and airway responsiveness (B) were measured at 72 h 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 (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). R_L values were obtained in response to increasing doses (2.5–50 mg/ml) of methacholine as described under *Materials and Methods*. Bars represent the mean ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; ∗, $p < 0.05$ versus OVA+SAL.

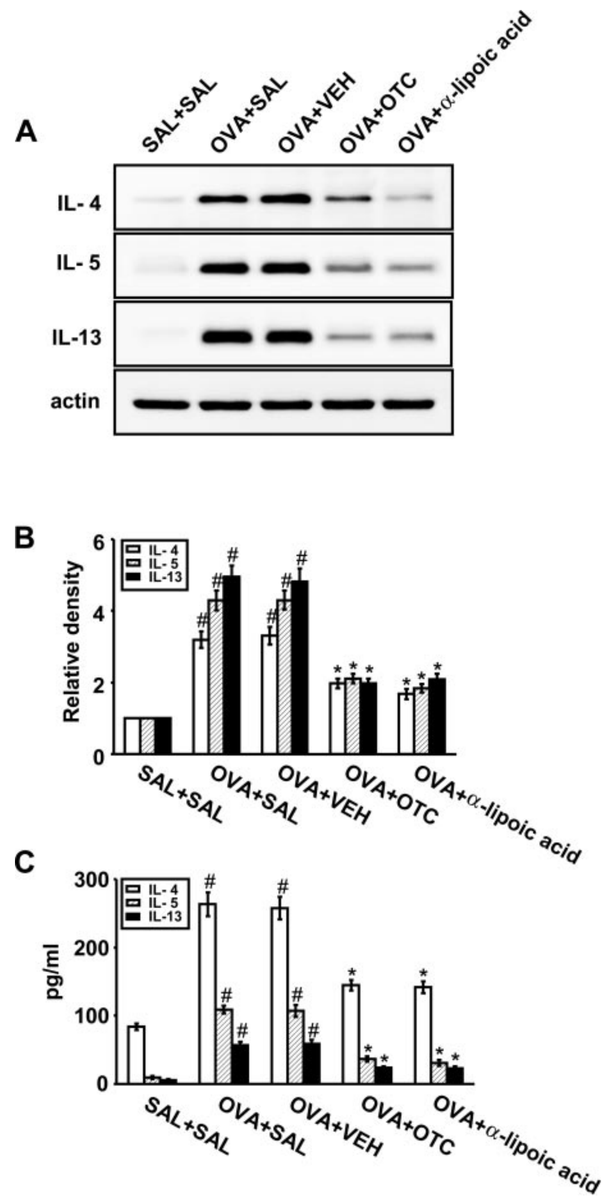

Fig. 10. Effect of OTC or α-lipoic acid on IL-4, IL-5, and IL-13 protein levels in lung tissues and in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered OTC (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). 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 ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; ∗, $p < 0.05$ versus OVA+SAL.

specific IgE levels in serum were increased significantly at 72 h after ovalbumin inhalation compared with levels after saline inhalation (Fig. 11). The increased total IgE or ovalbumin-specific IgE levels at 72 h after ovalbumin inhalation were significantly reduced by the administration of OTC or α-lipoic acid.

Discussion

Bronchial asthma is a long-term inflammatory disease of the airways characterized by airway eosinophilia and hyperresponsiveness to inhaled allergens and nonspecific stimuli (Bousquet et al., 2000). Airway inflammation and hyperresponsiveness are connected by complex signaling networks. Therefore, the molecular mechanisms of this disorder remain to be poorly understood. In this study, we have examined the effects of ROS on the regulation of IL-18 expression using antioxidant, OTC, or α-lipoic acid. Our present study with ovalbumin-induced murine model of asthma has revealed that ROS production in cells from BAL fluids was increased, and that administration of OTC or α-lipoic acid reduced the increased ROS production, the increased expression of IL-18 protein and mRNA, airway inflammation, and bronchial hyperresponsiveness. Our results also have indicated that antioxidants down-regulated a transcription factor, NF-κB activity. These findings suggest that antioxidants inhibit NF-κB signal transduction pathway by decreasing NF-κB binding activity to the promoter region of IL-18 gene involved in airway inflammation and remodeling in asthma.

IL-18 is produced by many cell types, including activated monocytes/macrophages and airway epithelial cells (Cameron et al., 1999; Shigehara et al., 2001) and has an ability to promote both Th1 and Th2 responses, depending on the surrounding cytokine environment (Xu et al., 2000; Nakanishi et al., 2001). Several reports have shown that IL-18 has a proinflammatory action in asthma. IL-18 can increase serum IgE levels and promote allergen-induced eosinophil influx into the airways in murine models of asthma (Campbell et al., 2000; Hoshino et al., 2000; Wild et al., 2000). Moreover, level of IL-18 is found to be raised in patients with asthma during acute exacerbations and in patients with stable asthma (Tanaka et al., 2001). In contrast, IL-18 exhibits antiallergic properties when administered with IL-12 in a murine asthma model (Hofstra et al., 1998), and in IL-18-deficient mice, an increase in airway eosinophilia has been observed (Kodama et al., 2000). In this study, we found that IL-18 expression was up-regulated in ovalbumin-induced asthma, and immunoreactive IL-18 was observed in BAL fluid macrophages and in airway epithelial cells. It is interesting that administration of the antioxidant OTC or α-lipoic acid reduced the increased IL-18 expression, the increased Th2 cytokines (IL-4, IL-5, and IL-13), and the increased ovalbumin-specific IgE. These results suggest that ROS signaling is associated with the regulation of IL-18 expression and that treatment of the antioxidants may improve the asthmatic features via regulation of IL-18 expression. Consistent with this present study, several studies have demonstrated that antioxidants are able to reduce airway inflammation and hyperresponsiveness through the regulation of NF-κB activity and modulate vascular permeability by lowering vascular endothelial growth factor expression in animal models of asthma (Cho et al., 2004; Lee et al., 2004, 2005).

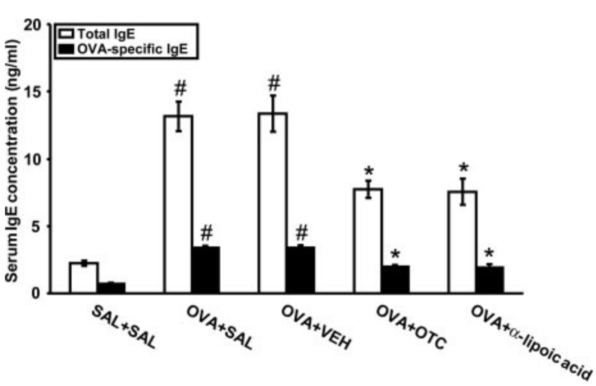

Fig. 11. Effect of OTC or α-lipoic acid on total IgE or ovalbumin-specific IgE levels in serum of ovalbumin-sensitized and -challenged mice. Sampling was performed 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered OTC (OVA+OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA+α-lipoic acid). Bars represent mean ± S.E.M. from eight mice per group. #, $p < 0.05$ versus SAL+SAL; ∗, $p < 0.05$ versus OVA+SAL.

Although OTC or α-lipoic acid has been studied clinically for the treatment of oxidant-induced diseases, such as ischemia-reperfusion injury (Cao and Phillis, 1995), diabetic neuropathy (van Dam, 2002), and coronary artery disease (Vita et al., 1998), there are no clinical studies using OTC or α-lipoic acid for airway diseases. The calculated human equivalent dose of the murine oral dosage of α-lipoic acid in this study is within the reported range of the therapeutic dose of α-lipoic acid in human diseases. Moreover, we revealed previously that the even lower concentration of OTC or α-lipoic acid than the concentration used in present study also reduced airway inflammation and bronchial hyperresponsiveness (Lee et al., 2004, 2005). However, a well-designed clinical study is necessary to determine the safety and efficacy of OTC or α-lipoic acid in human subjects with asthma.

The inflammatory cells recruited to the asthmatic airways have an exceptional capability of producing ROS. Activated eosinophils, neutrophils, monocytes, and macrophages by many causes, including allergen inhalation, can generate O_2^- via the membrane-associated NADPH-dependent complex. Thereafter, dismutation of O_2^- gives hydrogen peroxide (H_2O_2). O_2^- and H_2O_2 per se are moderate oxidants. However, both species are critical for the formation of potent cytotoxic radicals in biological systems through their interaction with other molecules (Dworski, 2000). Therefore, these stimulated cells, such as neutrophils, eosinophils, lymphocytes, and macrophages, produce a large amount of ROS (Conner and Grisham, 1996; Leusen et al., 1996; Babior, 1999). Consistent with these findings, our present results showed that ROS generation in BAL cells, which mainly consist of recruited inflammatory cells, was increased significantly in ovalbumin-sensitized and -challenged mice. The increased ROS generation was substantially reduced by the administration of OTC or α-lipoic acid.

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 inflammatory lung diseases such as asthma. OTC is a prodrug of cysteine that increases the plasma concentrations of cysteine and GSH

(Porta et al., 1991; Vita et al., 1998; Oiry et al., 1999). Several studies have also demonstrated that OTC repletes cellular GSH stores when GSH is acutely depleted (Hazelton et al., 1986; Moslen et al., 1989) and is more effective than *N*-acetylcysteine in repleting intracellular GSH stores (Williamson et al., 1982; Mesina et al., 1989). In addition, α-lipoic acid is reduced intracellularly to dihydrolipoic acid by lipoamide dehydrogenase. Dihydrolipoic acid is a highly reactive thiol capable of reducing GSSG to GSH and of affecting the oxidation state of thioredoxin and other thiol-containing proteins (Packer et al., 1995). Consistent with these observations, our data have shown that the decreased GSH levels in lung tissues were significantly increased by the administration of OTC or α-lipoic acid in ovalbumin-sensitized and -challenged mice.

Previous studies have shown that several proteins, including various transcriptional factors, are related to ROS-based regulation of signal transduction and gene expression in various immune and inflammatory processes (Sen, 1998; Lee et al., 2005). However, the molecular basis of ROS-mediated induction of asthma has not yet been clearly defined. A recent study has indicated that the development of oxidant/antioxidant imbalance in asthma leads to the activation of redox-sensitive transcription factor NF-κB (Henderson et al., 2002). NF-κB is present in most cell types and is known to play a critical role in immune and inflammatory responses, including asthma (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Baldwin, 1996; Barnes and Karin, 1997). As expected, the NF-κB protein level was substantially increased in the ovalbumin-induced model of asthma 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. These genes include cytokines (e.g., IL-4, IL-5, IL-9, IL-15, and TNF-α), chemokines (e.g., regulated on activation normal T cell expressed and secreted, eotaxin, and monocyte chemotactic protein-3), and adhesion molecules (e.g., intercellular adhesion molecule-1 and vascular cell adhesion molecule-1). IL-18 expression is also shown to be regulated through NF-κB activation (Chandrasekar et al., 2003, 2004). In this study, expression of IL-18 was increased significantly after allergen challenge in our murine model of asthma. Administration of OTC or α-lipoic acid resulted in significant reduction of NF-κB activity and expression of IL-18. In addition, the increased IL-18 protein levels after OVA inhalation were decreased by administration of an inhibitor of NF-κB activation, BAY 11-7085. Therefore, these results strongly suggest that antioxidants inhibit IL-18 expression in asthma by inhibiting the activity of NF-κB.

In summary, we examined the effect of ROS on the regulation of IL-18 expression in a murine model of allergic asthma. By using OTC and α-lipoic acid, antioxidants, we have shown the important role of ROS in ovalbumin-induced airway hyperresponsiveness and inflammation. Moreover, our results have revealed that administration of OTC or α-lipoic acid reduced IL-18 expression at protein and mRNA levels, including NF-κB activation. On the basis of these observations, we have concluded that antioxidants inhibit IL-18 expression in asthma by inhibiting activity of NF-κB. Thus, these findings provide an important molecular mechanism for the potential use of antioxidants to prevent and/or treat asthma and other airway inflammatory disorders.

Acknowledgments

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