

Regulation of pro-inflammatory responses by lipoxygenases via intracellular reactive oxygen species *in vitro* and *in vivo*

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Abbreviations: 5-LOX, 5-lipoxygenase; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DCFH, 2',7'-dichlorodihydrofluorescein; EMSA, electrophoretic mobility shift assay; LTs, leukotrienes; MIF, macrophage migration inhibitory factor; NDGA, nordihydroguaiaretic acid; NOX, NADPH oxidase; OVA, ovalbumin; ROS, reactive oxygen species

Abstract

Reactive oxygen species (ROS) performs a pivotal function as a signaling mediator in receptor-mediated signaling. However, the sources of ROS in this signaling have yet to be determined, but may include lipoxygenases (LOXs) and NADPH oxidase. The stimulation of lymphoid cells with TNF- α , IL-1 β , and LPS resulted in significant ROS production and NF- κ B activation. Intriguingly, these responses were markedly abolished via treatment with the LOXs inhibitor nordihydroguaiaretic acid (NDGA). We further examined *in vivo* anti-inflammatory effects of NDGA in allergic airway inflammation. Both intraperitoneal and intravenous NDGA administration attenuated ovalbumin (OVA)-induced influx into the lungs of total leukocytes, as well as IL-4, IL-5, IL-13, and TNF- α levels. NDGA also significantly reduced serum levels of OVA-specific IgE and suppressed OVA-induced airway hyperresponsiveness to inhaled methacholine. The results of our histological studies and flow cytometric analyses showed that NDGA inhibits OVA-induced lung inflammation and the infiltration of CD11b⁺

macrophages into the lung. Collectively, our findings indicate that LOXs performs an essential function in pro-inflammatory signaling via the regulation of ROS regulation, and also that the inhibition of LOXs activity may have therapeutic potential with regard to the treatment of allergic airway inflammation.

Keywords: anti-inflammatory agents; asthma; lipoxygenase; macrophages; models, animal; NF- κ B; reactive oxygen species

Introduction

The physiological balance of reactive oxygen species (ROS) is ultimately determined by the rate of O₂⁻ production, the metabolism rate of O₂⁻ by endogenous superoxide dismutases (SODs), and the removal rate of H₂O₂ via antioxidant enzymes (catalase or glutathione peroxidase) and/or involvement in Haber-Weiss or Fenton chemistry (Adler *et al.*, 1999; Haddad *et al.*, 2002; Miller *et al.*, 2006). In mammalian cells, potential enzymatic O₂⁻ sources include the mitochondrial electron-transport chain, the arachidonic acid-metabolizing enzymes (cyclooxygenase and lipoxygenase), the cytochrome P450s, xanthine oxidase, NADPH-oxidases, and NO synthases (Babior *et al.*, 1999; Griendling *et al.*, 1999; Van Heerebeek *et al.*, 2002; Ogawa *et al.*, 2003; Genova *et al.*, 2004). Among these, the NADPH-oxidases (NOX) and cyclooxygenases / lipoxygenases are generally recognized as the principal physiological sources of O₂⁻, which is in turn dismutated into H₂O₂ (Babior *et al.*, 1999; Griendling *et al.*, 1999; Van Heerebeek *et al.*, 2002; Kuhn *et al.*, 1999; Rhee *et al.*, 2003). H₂O₂ is now generally believed to be one of the most important ROS molecules in the modulation of multiple cellular events, including receptor-mediated signaling, apoptosis, proinflammation, and metabolism (Davies *et al.*, 1999; Lee *et al.*, 1999; Aslan *et al.*, 2003; Tonks *et al.*, 2005; Saito *et al.*, 2006). Although the sources of ROS generated after stimulation with proinflammatory cytokines such as TNF- α and IL-1 β remain to be clearly elucidated, several reports have indicated that 5-lipoxygenase (5-LOX) activity may be critically related to intracellular ROS production (Los *et al.*, 1995; Lee *et al.*, 1997). The 5-LOX enzyme catalyzes the

production of leukotrienes and ROS from arachidonic acid (Lewis *et al.*, 1990; Harrison *et al.*, 1995). During *in vivo* inflammatory responses, the leukotrienes (LTs) B₄, C₄, D₄, and E₄ generated by the 5-LOX pathway of arachidonic acid metabolism have been experimentally determined to perform a function in each of these inflammatory mechanisms (Samuelsson *et al.*, 1983; O'Byrne *et al.*, 1997). Clinical and experimental studies have shown that selective LTD₄ receptor antagonists, including pranlukast, zafirlukast, MK-571, and MK-679 have potential effects with regard not only to the amelioration of asthma symptoms, but also in terms of the use of β 2-agonists and bronchoconstriction induced by exposure to allergens, exercise, aspirin, and cold air (Samuelsson *et al.*, 1983; O'Byrne *et al.*, 1997; Gaddi *et al.*, 2004; Leone *et al.*, 2007).

Asthma is a chronic airway disease characterized by eosinophilic inflammation and airway hyperresponsiveness (AHR) (Akdis *et al.*, 2006; Eder *et al.*, 2006; Umetsu *et al.*, 2006). Increased ROS generation, which results in an imbalance between oxidative forces and the antioxidant defense systems, has been implicated in the pathogenesis of asthma (Rahman *et al.*, 1999; Dworski *et al.*, 2000; Rahman *et al.*, 2000; Lee *et al.*, 2006, 2007; Kirkham *et al.*, 2006). ROS are capable of eliciting a variety of pathological changes, including the peroxidation of lipids, proteins, and DNA and the generation of chemoattractants, as well as enhancements of AHR, airway secretion, and vascular permeability in asthmatic airways (Barnes *et al.*, 1990; Henricks *et al.*, 2001; Andreadis *et al.*, 2003). These changes ultimately result in increased mediator release from the epithelium, which leads to the recruitment of immune effector cells. Inflammatory cells such as macrophages, lymphocytes, mast cells, B cells, and eosinophils have been proposed to perform critical functions in the initiation, development, and chronicity of this disease (Williams *et al.*, 2000; Elias *et al.*, 2003; Akdis *et al.*, 2006; Eder *et al.*, 2006; Umetsu *et al.*, 2006). These inflammatory cells contribute to the generation of Th2 cytokines (IL-4, IL-5, and IL-13), chemokines (eotaxin and RANTES), and TNF- α , which are detected at elevated levels in asthmatic lungs (Williams *et al.*, 2000; Elias *et al.*, 2003). Therefore, the regulation of intracellular ROS during inflammation may have potential advantages with regard to the treatment of inflammatory diseases.

In this study, we have attempted to determine the roles of lipoxygenases (LOXs) in both proinflammatory signaling *in vitro* and allergic airway inflammation *in vivo*. To that end, we utilized Nordihydroguaiaretic acid (NDGA), LOXs inhibitor. The results of previous studies have shown that NDGA

exerts pleiotropic effects capable of influencing a broad variety of cellular processes, including growth factor- and TNF-induced signal transduction, leukocyte chemotaxis, myoblast cell differentiation, cancer cell proliferation, and viral proliferation in infected cells (Goetzl *et al.*, 1980; Gnabre *et al.*, 1995; Lee *et al.*, 2003; West *et al.*, 2004; Ito *et al.*, 2005; Youngren *et al.*, 2005; Arasaki *et al.*, 2007). It can also induce the expression of NOS, regulate calcium channel activity, and inhibit the growth of β -amyloid protofibrils (Ramasamy *et al.*, 1999; Huang *et al.*, 2004; Moss *et al.*, 2004). Although many of the effects of NDGA on cellular events appear to be attributable to its function as LOXs inhibitor, the more detailed mechanism by which NDGA interferes as an antioxidant with the proinflammatory signaling pathway has yet to be clearly elucidated. Moreover, its therapeutic effects on allergic inflammation had not, prior to this study, been evaluated. Our findings indicate a specific role of LOXs in proinflammatory signaling via the regulation of ROS, and also point to the potential therapeutic utility inherent to the inhibition of LOXs activity in the treatment of allergic airway inflammation.

Materials and Methods

Intracellular 2',7'-dichlorodihydrofluorescein staining

The oxidation of 2',7'-dichlorodihydrofluorescein (DCFH) to fluorescent 2',7'-dichlorofluorescein (DCF) was evaluated in order to determine the intracellular levels of H₂O₂. RAW 264.7 (Mouse macrophage cell line), Jurkat (Human T lymphoma cell line), THP-1 (human acute monocytic leukemia cell line), and BEAS 2B (Human bronchial epithelial cell line) cells were pre-treated with 5 μ M NDGA (Plymouth Meeting, PA) or REV5901 for different times, as indicated in each experiment, in an atmosphere of 5% CO₂ at 37°C. In addition, the scavenger activity of REV5901 (Calbiochem, San Diego, CA) was evaluated in RAW 264.7 cells, following the protocol above. As an independent experiment, Jurkat cells were pre-treated with 5 μ M NDGA for different times (30 min, 45 min, and 60 min), then stimulated for 1 h with 1 ng/ml TNF- α , 10 ng/ml IL-1 β , and 1 μ g/ml LPS. For DCF staining, DCFH was added at a final concentration of 20 μ M and incubated for 30 min at 37°C. The cells were washed once in phosphate-buffered saline (PBS) and maintained in 1 ml of medium. Cellular fluorescence was determined via flow cytometry (FACSCalibur Becton- Dickinson, Franklin

Lakes, NJ). Measurements were conducted at 510–540 nm after the excitation of cells at 488 nm using an argon ion laser.

Confocal microscopy

For confocal microscopy analysis, the RAW 264.7 cells were grown on 0.2% gelatin-coated coverslips, pre-treated for 1 h with or without 5 μ M NDGA, then stimulated for 1 h with 1 ng/ml TNF- α , 10 ng/ml IL-1 β , and 1 μ g/ml LPS. The cells were washed in PBS, fixed for 30 min with 4% paraformaldehyde at room temperature, and permeabilized with permeabilization buffer (0.05% saponin, 1% FBS, 10 mM HEPES, and 10 mM glycine in PBS, pH 7.5) for 30 min at room temperature. The cells were incubated with anti-5-LOX antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature, then incubated with goat anti-rabbit antibody conjugated with FITC. The cells were subsequently washed three times with PBS, mounted with GEL/MOUNT (Biomedex, Foster City, CA), and examined under an Olympus Fluoview 300 Confocal Laser Scanning head with an Olympus IX70 inverted microscope.

Luciferase reporter assay

RAW 264.7 cells (4×10^5) grown on 12-well plates were transiently transfected with the NF- κ B-dependent reporter construct, pBilx-luc, coupled with the *Renilla* luciferase vector, using FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN). The total DNA concentration in each of the experiments was maintained via the addition of the appropriate empty vector to the DNA mixture. 48 h after transfection, the cells were treated for 6 h with 1 μ g/ml of LPS and 1 ng/ml of TNF- α , after 1 h of pre-treatment with different concentrations of NDGA (1 μ M, 5 μ M, and 10 μ M). The cells were lysed, and the luciferase activity was determined using a dual luciferase assay kit (Promega Corp., Madison, WI). The κ B-specific luciferase activity was normalized on the basis of the renilla luciferase control.

Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells were plated at a density of 5×10^6 cells for 24 h in 6-well plates. The cells were then treated for 1 h with different concentrations of NDGA, and stimulated for 3 h with 1 μ g/ml of LPS or 1 ng/ml of TNF- α . The nuclear extracts were prepared using a specific extraction kit (Sigma, St. Louis, MO). NF- κ B activation was evaluated using consensus oligonucleotides of the transcription

factor. The consensus sequence was AGTTGA-GGGGACTTTCCCAGGC. The probes were end-labeled with T4 polynucleotide kinase in the presence of [γ - 32 P] ATP, and purified on a Nick column (Amersham Biosciences, Little Chalfont, UK). Nuclear protein (5 μ g) was incubated for 20 min with labeled probes at room temperature. The mixture was separated via 6% SDS-PAGE, and the resultant gel was dried and subjected to autoradiography.

Measurement of TNF- α production

RAW 264.7 cells were plated onto 24-well tissue culture plates. The cells (1×10^6 cells/ml) were then treated for 1 h with different concentrations of NDGA (1 μ M, 5 μ M, and 10 μ M), and stimulated for 5 h with 1 μ g/ml LPS. The quantity of TNF- α in the culture supernatant fractions was determined using an ELISA kit, in accordance with the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). The concentration of TNF- α in each sample was calculated from a standard curve which was prepared using known quantities of recombinant TNF- α .

A protocol for the animal model of asthma

Six-week-old male BALB/c mice were maintained in a specific, pathogen-free area and were provided with OVA-free feed and water. Mice were sensitized on days 1 and 14 via intraperitoneal injection of 20 μ g OVA emulsified in 1 mg Al(OH) $_3$ (alum), followed by an identical booster injection administered on day 14. On days 21, 22, and 23 after initial sensitization, the mice were challenged for 30 min with an aerosol of 5% OVA (wt/vol in PBS) using an ultrasonic nebulizer. For the inhalation challenge, the mice were placed in a 30 \times 30 \times 15 cm plastic chamber with small ventilation holes on one side, and the OVA solution was aerosolized using an ultrasonic nebulizer that was attached directly to the chamber opposite the ventilation holes. NDGA (10 mg/kg and 20 mg/kg) or vehicle control (0.05% DMSO) diluted with 0.9% NaCl was administered in a volume of 50 μ l either intraperitoneally or intravenously to each animal on days 21, 22, and 23. All experiments described herein were approved by the Animal Research Ethical Review Board of the Asan Institute for Life Sciences.

Determination of AHR

24 h after the final aerosol challenge, AHR was evaluated in conscious, unrestrained mice via whole-body plethysmography. Each mouse was

placed in a plastic chamber and exposed to aerosolized normal saline, followed by increasing concentrations of aerosolized methacholine solutions (2.5, 5, 10, and 20 mg/ml; Sigma) for 3 min. Beginning 10 s after each aerosol exposure and lasting for 3 min, enhanced pause (Penh) readings, calculated automatically on the basis of the mean pressure generated within the plethysmography chamber during inspiration and expiration combined with the time of each phase, were recorded and averaged. The values were expressed as the percentage change in Penh at each methacholine concentration, as compared with the baseline readings.

Collection of serum and BAL samples

24 h after the last challenge, the mice were sacrificed via overdose of pentobarbital-Na (100 mg/kg of body weight, administered via intraperitoneal injection). Blood was drawn via puncture of the vena cava, then centrifuged. Serum was shock-frozen in liquid nitrogen and stored at -70°C for measurements of IgE. In order to collect bronchoalveolar lavage (BAL) fluids, the chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated, and the catheter was secured with ligatures. Pre-warmed, 0.9% NaCl solution was then slowly infused into the lungs and withdrawn. The total BAL cells were counted with a hemocytometer. Differential cell counts were acquired from BAL cells spun down onto slides using a cytocentrifuge (Shannon Scientific Ltd., Cheshire, UK) and treated with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico). Two independent, blinded investigators counted the cells with a microscope. Approximately 400 cells in each of four different random locations were counted by two independent, blinded investigators. The inter-investigator variation was $< 7\%$. The mean number obtained from the reports of the two investigators was used to estimate the cell differentials.

Histologic assay

24 h after the final challenge, the mice were sacrificed and the lungs were removed and fixed with 10% (vol/vol) neutral-buffered formalin. The specimens were dehydrated and embedded in paraffin. For the histologic 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, de-paraffinized, and stained with hematoxylin 2, eosin-Y (Richard-Allan Scientific,

Kalamazoo, MI).

Measurement of BAL cytokines and the ELISPOT assay

Levels of IL-4, IL-5, IL-13, and TNF- α were quantified in the supernatants of the BAL fluids via enzyme immunoassays, all of which were conducted in accordance with the manufacturer's recommendations (R&D Systems, Inc., Minneapolis, MN). For the counting of IL-4, IL-5, IL-13, and TNF- α -producing cells, ELISPOT assay kits (R&D Systems, Inc.) were used, again in accordance with the manufacturer's instructions. 1.6×10^5 splenocytes were tested in a volume of 100 μl . The cells were stimulated with PMA (20 ng/ml; Sigma) and ionomycin (1 μM ; Sigma) for 12 h at 37°C in a humidified atmosphere containing 5% CO_2 . The spots were counted using Elispot leader 4.0 (AIDGmbH, Strassberg, Germany). Unstimulated cells were used as a negative control. No spots were detected in the control wells.

Flow cytometric analysis

Lung tissue was cut into small fragments, and washed thoroughly in RPMI culture medium. Each tissue sample was incubated for 1 h in RPMI containing 0.1% collagenase (Type IV; Sigma), 0.01% hyaluronidase (Sigma), and 0.002% DNase (Sigma). The samples were incubated for 1 h in RPMI containing 0.1% collagenase (Type IV; Sigma), 0.01% hyaluronidase (Sigma), and 0.002% DNase (Sigma). Cells were washed in medium containing 10% FBS. Viable cells were isolated via Ficoll-hypaque (Sigma) density centrifugation, and washed twice in RPMI medium without serum. The cells were incubated for 40 min on ice with FITC-conjugated anti-CD11b (M1/70) (BD Pharmingen, San Diego, CA). The cells were washed three times in PBS buffer, and analyzed using FACScalibur apparatus (Becton-Dickinson).

Measurement of BAL leukotriene C_4

Levels of LTC $_4$ were quantified in the supernatants of the BAL fluids via enzyme immunoassay, conducted in accordance with the manufacturer's protocols (Cayman Chemical Co., Ann Arbor, MI). The lower detection limit for LTC $_4$ in this assay was 10 pg/ml.

Statistical analysis

All data are expressed as the mean \pm SEM. For comparison between groups, the Mann-Whitney

and Kruskal-Wallis tests were used. Statistical significance was defined as $P < 0.05$.

Results

NDGA, LOXs inhibitor, evidences strong antioxidant activity in lymphoid cells

We first attempted to determine whether NDGA, LOXs inhibitor, manifests scavenger activity via the inhibition of LOXs in lymphoid cells. Raw 264.7 (Mouse macrophage cell line), THP1 (Human acute monocytic leukemia cell line), Jurkat (Human T lymphoma cell line), BEAS 2B (Human bronchial

epithelial cell) cells were treated with 5 μM NDGA, and its scavenger activity was analyzed using DCFH probes (Chang *et al.*, 2004). The NDGA treatments induced significant reductions in intracellular ROS in the tested cell lines, and these reductions occurred in a time-dependent manner (Figure 1A, Raw cells; Figure 1B, THP1 cells; Figure 1C, Jurkat cells; Figure 1D, BEAS 2B cells). With regard to mean fluorescence intensity, the elimination of ROS by NDGA occurred quickly and reached a plateau 30 min after the administration of NDGA treatment, as depicted in the left panels (Figure 1). Following treatment of REV5901, 5-LOX inhibitor, the significant reduction of intracellular

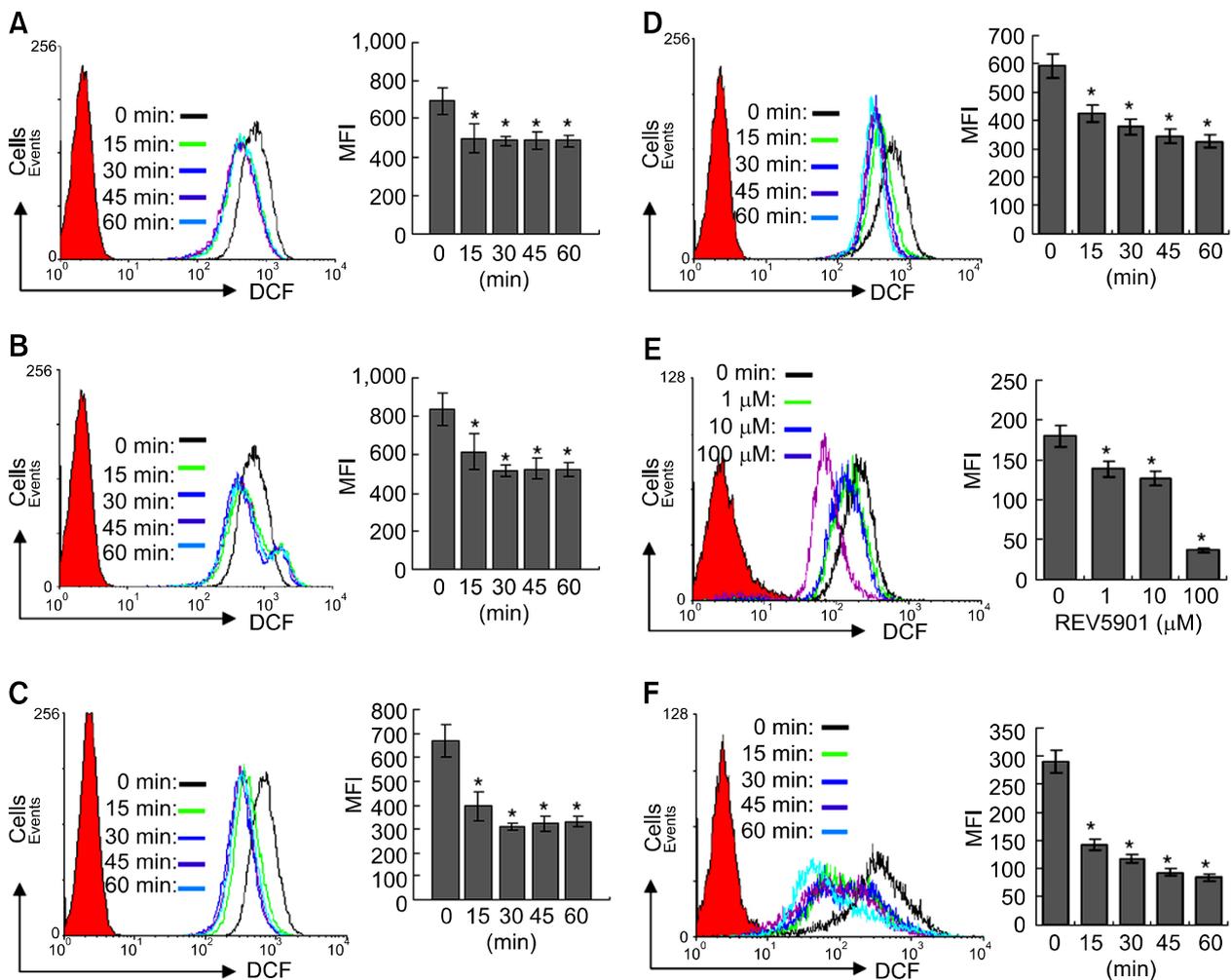


Figure 1. Scavenger activity of NDGA, LOXs inhibitor, in lymphoid cells. Raw 264.7 cells, THP-1 cells, Jurkat cells, BEAS 2B cells were treated with or without 5 μM NDGA for different times, as indicated. The cells were treated with 20 μM DCFH and incubated for 30 min at 37°C. The levels of ROS were determined via flow cytometry (A, RAW 264.7 cells; B, THP-1 cells; C, Jurkat cells; D, BEAS 2B cells). Raw 264.7 cells were treated with different concentrations of REV5901 for 60 min, as indicated (E), or 50 mM REV5901 for different times, as indicated (F). The cells were treated with 20 μM DCFH and incubated for 30 min at 37°C. The levels of ROS were determined via flow cytometry. Data are representative of three individual experiments. Decreases in mean fluorescence intensity were represented in the right panels. The bar represents the mean \pm SE from three individual experiments. * $P < 0.05$ vs control (0 min).

ROS could be detected with both a dose-dependent and a time-dependent manner in Raw 264.7 cells (Figure 1E and F). The results show that LOXs is essential for the generation of intracellular ROS in lymphoid cells. This observation is, in part, consistent with the results of previous reports (Los *et al.*, 1995; Lee *et al.*, 1997).

LOXs are essential for the production of intracellular ROS by pro-inflammatory stimuli such as TNF- α , IL-1 β , and LPS

We next attempted to determine whether pro-inflammatory stimuli including TNF- α , IL-1 β , and LPS can affect intracellular ROS levels. Jurkat cells

were stimulated for 1 h with TNF- α , IL-1 β , and LPS, and then ROS levels were evaluated using DCF dye. The levels of ROS were increased significantly after stimulation with TNF- α (Figure 2A, blue line), IL-1 β (Figure 2B, blue line), and LPS (Figure 2C, blue line). Intriguingly, when the cells were pre-treated with NDGA to inhibit LOXs activity, these stimuli were incapable of enhancing intracellular ROS levels; rather, ROS levels were markedly reduced, as is shown in Figure 2A, Figure 2B, and Figure 2C (purple lines). The results indicate that LOXs may prove to be essential for the generation of intracellular ROS by pro-inflammatory stimuli, including TNF- α , IL-1 β , and LPS. It is generally accepted that, upon stimulation,

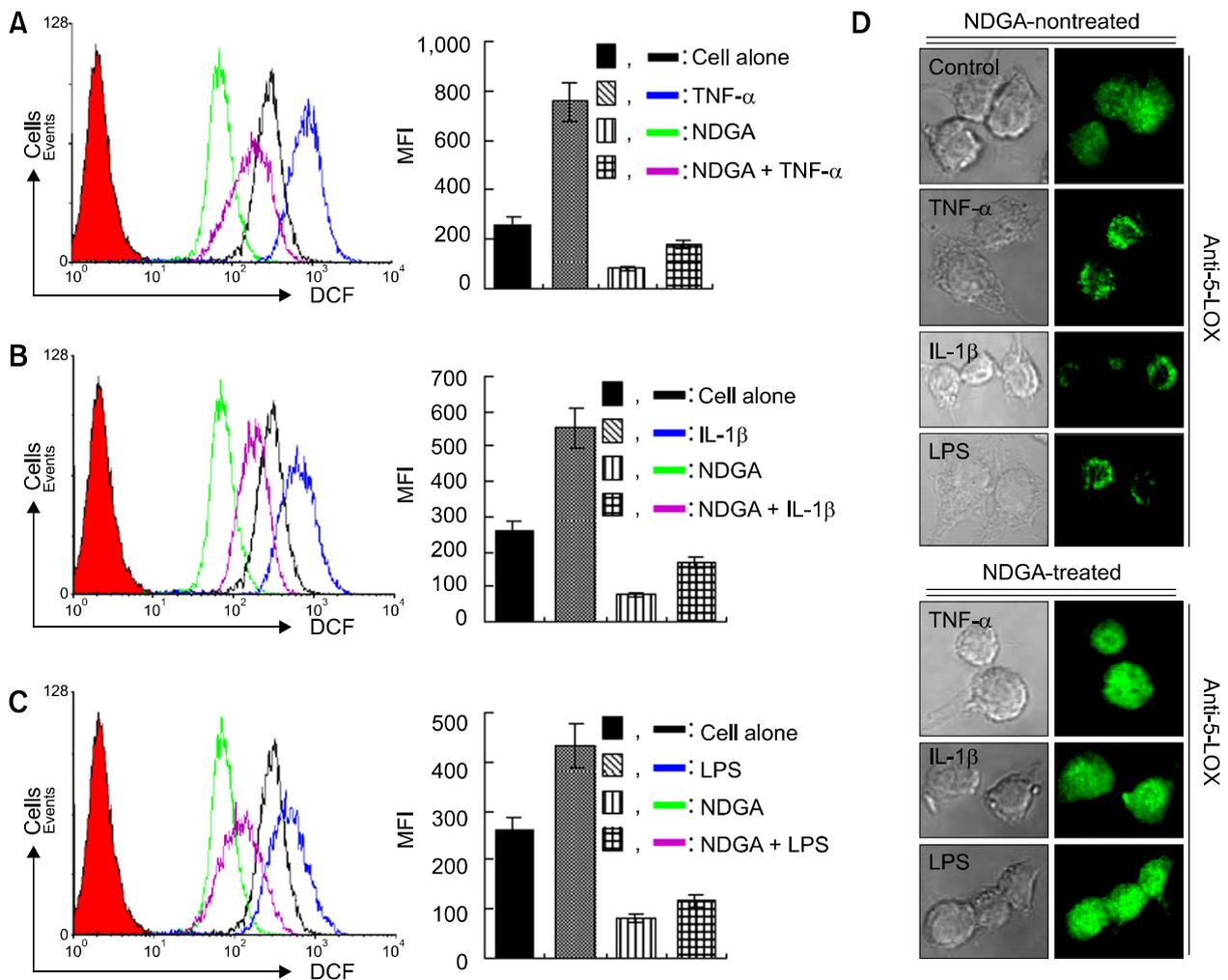


Figure 2. NDGA inhibits TNF- α , IL-1 β , and LPS-induced ROS production and translocation of 5-LOX into the nuclear membrane. Jurkat cells were treated for 1 h with or without 5 μ M NDGA and stimulated for 1 h with 1 ng/ml TNF- α , 10 ng/ml IL-1 β , and 1 μ g/ml LPS for 1 h. For DCF staining, DCFH was added at a final concentration of 20 μ M and incubated for 30 min at 37°C. ROS levels were analyzed via flow cytometry (A, TNF- α ; B, IL-1 β ; C, LPS). Data are representative of three individual experiments. Decreases in mean fluorescence intensity were represented in the right panels. (D) Raw264.7 cells were treated for 1 h with or without 5 μ M NDGA and stimulated for 1 h with 1 ng/ml TNF- α , 10 ng/ml IL-1 β , and 1 μ g/ml LPS. The localization of 5-LOX was analyzed via confocal microscopy, as described in the Materials and Methods section.

intranuclear or intracellular 5-LOX binds to the nuclear membrane for the activation of 5-LOX (Healy *et al.*, 1999). Interestingly, TNF- α , IL-1 β , and LPS stimulation induced a significant translocation of 5-LOX into the nuclear membrane (Figure 2D, upper panels). However, NDGA pre-treatment

markedly inhibited the localization of 5-LOX as a result of these stimuli (Figure 2D, down panels). These results strongly indicate that LOXs including 5-LOX may essentially involve intracellular ROS production in pro-inflammatory signaling such as

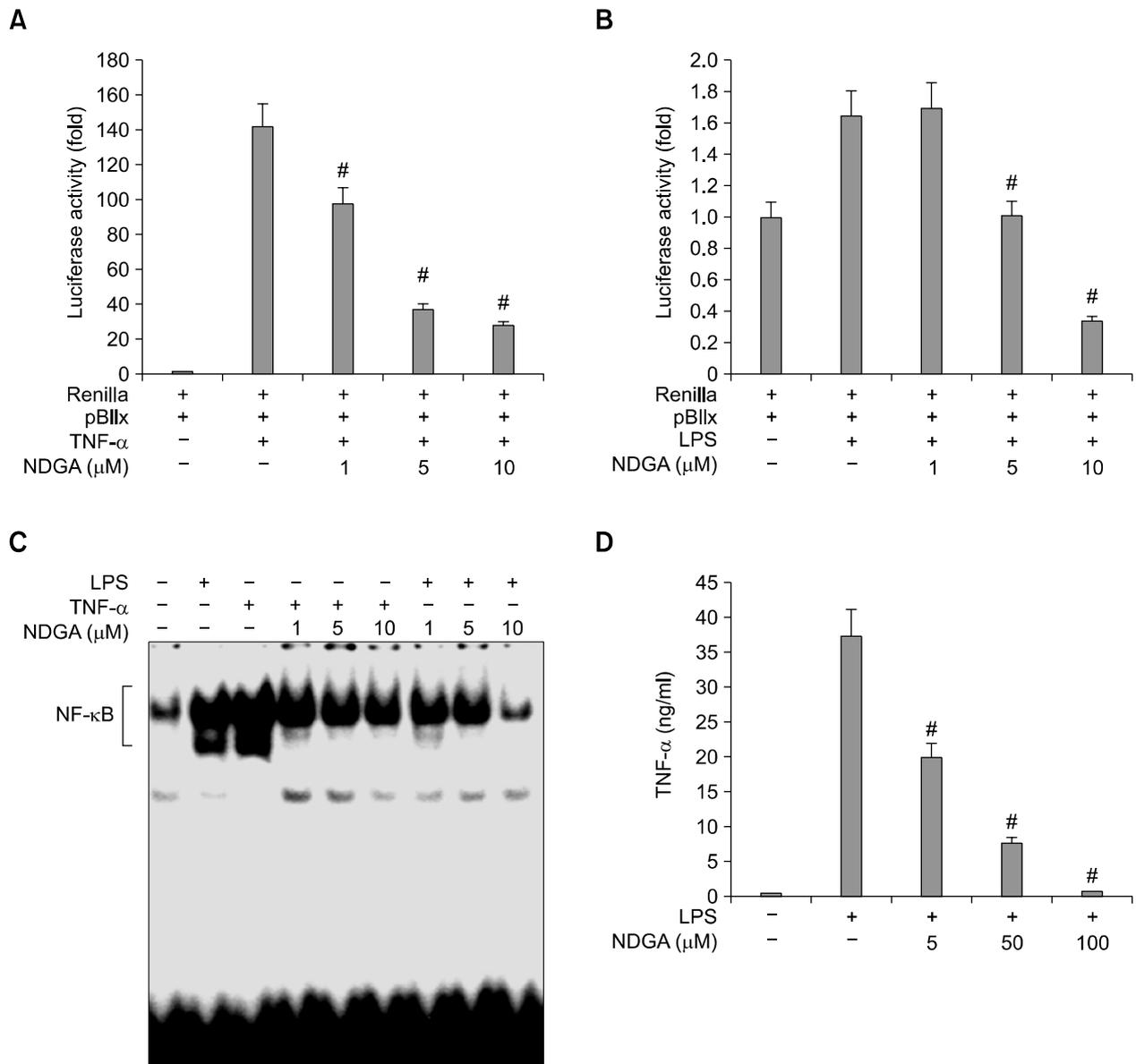


Figure 3. NDGA inhibits TNF- α and LPS-induced NF- κ B activation and LPS-mediated TNF- α production. (A) and (B) RAW 264.7 cells transfected (in 24-well plates) with a κ B-luciferase reporter (100 ng) and a control *Renilla* luciferase reporter. The cells were pre-treated with different NDGA concentrations as indicated for 1 h, then stimulated for 5 h with 1 ng/ml TNF- α (A) or 1 μ g/ml LPS (B). The luciferase assay was conducted as described in the Materials and Methods section. NF- κ B-specific luciferase activity was normalized on the basis of the *Renilla* luciferase control. The results are expressed as the fold induction in luciferase activity relative to that of untreated cells. Error bars indicate standard deviation. * P < 0.05 vs NDGA-no-treated control. (C) Raw 264.7 cells were treated with different NDGA concentrations as indicated for 1 h, stimulated for 5 h with 1 ng/ml TNF- α or 1 ng/ml LPS, and the nuclear extracts were prepared. NF- κ B DNA-binding activity was analyzed via EMSA as described in the Materials and Methods section. (D) Raw 264.7 cells were treated with different concentrations of NDGA as indicated for 1 h, and stimulated for 5 h with 1 μ g/ml LPS. The quantity of TNF- α in the culture supernatant fractions was determined via ELISA, as described in the Materials and Methods section. Error bars indicate standard deviation. * P < 0.05 vs NDGA-no-treated control.

that associated with TNF- α , IL-1 β , and LPS.

LOXs are required for the activation of NF- κ B in pro-inflammatory signaling

We then attempted to determine whether or not LOXs are required for the activation of NF- κ B in pro-inflammatory signaling. In order to address this question, we initially ascertained whether NDGA

inhibits TNF- α -induced NF- κ B reporter activity. Raw 264.7 cells were pre-treated with different NDGA concentrations, as is indicated in Figure 3A, for 1 h, and then stimulated for 5 h with TNF- α . The luciferase assay showed that NDGA pretreatment resulted in a marked inhibition of TNF- α -induced NF- κ B reporter activity in a dose-dependent manner (Figure 3A). In parallel with those results, upon pretreatment with NDGA, NF- κ B reporter

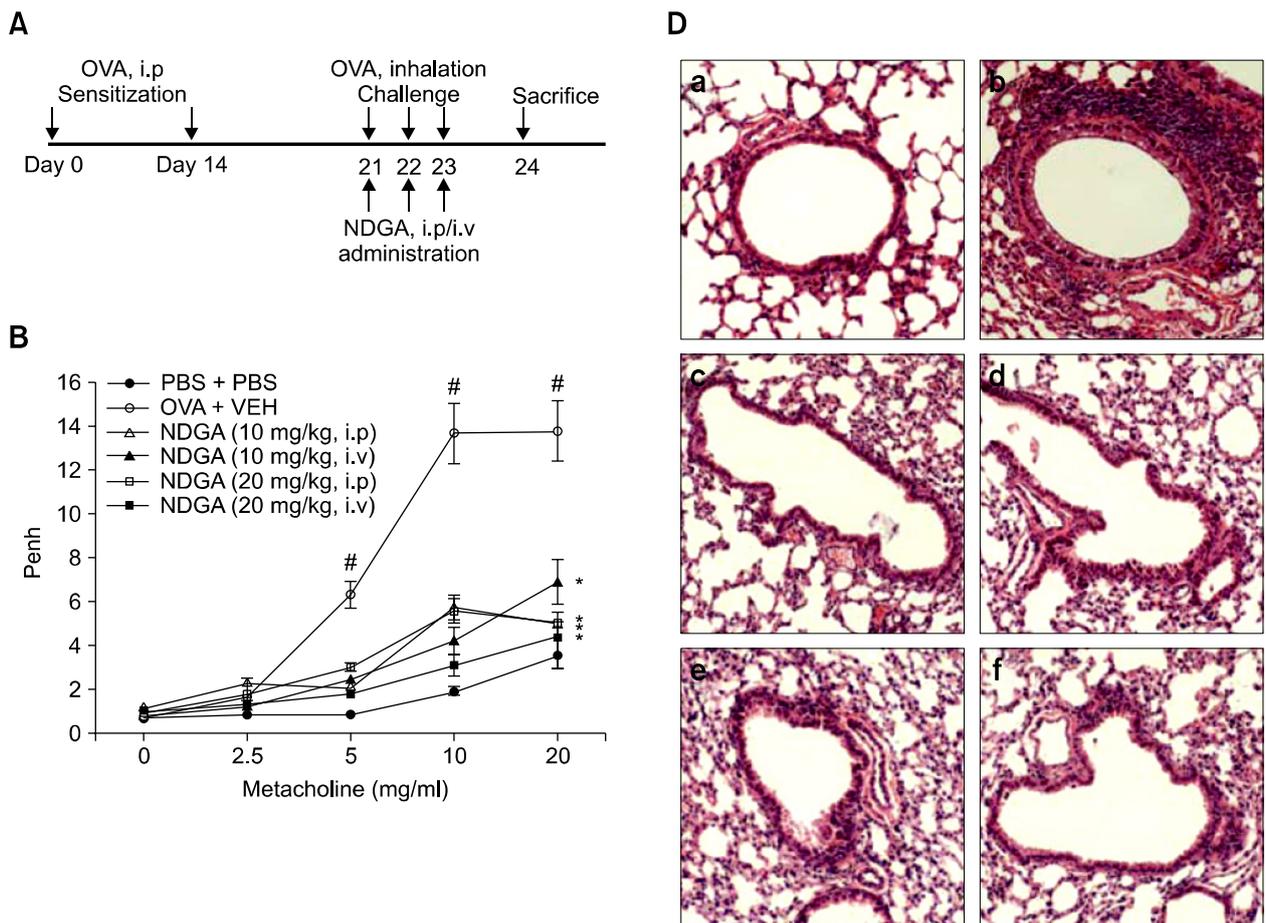


Figure 4. NDGA attenuates airway hyperresponsiveness and inflammation in a murine asthma model. (A) Six-week-old male BALB/c mice were sensitized on days 1 and 14 via intraperitoneal injection of 20 μ g OVA emulsified in 1 mg Al(OH)₃ (alum), followed by an identical booster injection on day 14. On days 21, 22, and 23 after initial sensitization, the mice were challenged for 30 min with an aerosol of 5% OVA (wt/vol in PBS) using an ultrasonic nebulizer. NDGA (10 mg/kg and 20mg/kg) or vehicle control (0.05% DMSO) diluted with 0.9% NaCl, was administered either intraperitoneally or intravenously in a volume of 50 μ l in triplicate to each animal on days 21, 22, and 23. (B) Airway hyperresponsiveness was measured 24 h after the last challenge in saline-inhaled mice to which PBS was administered (PBS + PBS), OVA-inhaled mice to which drug vehicle was intraperitoneally administered (OVA + VEH), OVA-inhaled mice to which 10 mg/kg NDGA was intraperitoneally administered (NDGA 10 mg/kg *i.p.*), OVA-inhaled mice to which 10 mg/kg NDGA was intravenously administered (NDGA 10 mg/kg *i.v.*), OVA-inhaled mice to which 20 mg/kg NDGA was intraperitoneally administered (NDGA 20 mg/kg *i.p.*), and OVA-inhaled mice to which 20 mg/kg NDGA were intravenously administered (NDGA 20 mg/kg *i.v.*), as described in the Materials and Methods. Data represent mean \pm SE from 6 independent experiments. #*P* < 0.05 vs PBS+PBS mice and **P* < 0.05 vs OVA + VEH mice. (C) The number of total cells and differential cellular component of BAL fluids from each of the mice described above were counted 24 h after the last challenge. Differential cell counts were conducted on a minimum of 400 cells in each of four different random locations to identify macrophages (Mac), eosinophils (Eos), neutrophils (Neu), and lymphocyte (LyM). Bars represent mean \pm SE from 6 independent experiments. #*P* < 0.05 vs PBS + PBS mice and **P* < 0.05 vs OVA + VEH mice. (D) Lung tissue was fixed, sectioned to a thickness of 4 μ m and stained as described in the Materials and Methods section (a, PBS + PBS; b, OVA + VEH, c, NDGA 10 mg/kg *i.p.*; d, NDGA 10 mg/kg *i.v.*; e, NDGA 20 mg/kg *i.p.*; f, NDGA 20 mg/kg *i.v.*). (E) Mouse serum samples were collected 24 h after the last challenge. The levels of OVA-specific IgE were analyzed using ELISA. Bars represent mean \pm SE from 6 independent experiments. **P* < 0.05 vs OVA + VEH mice.

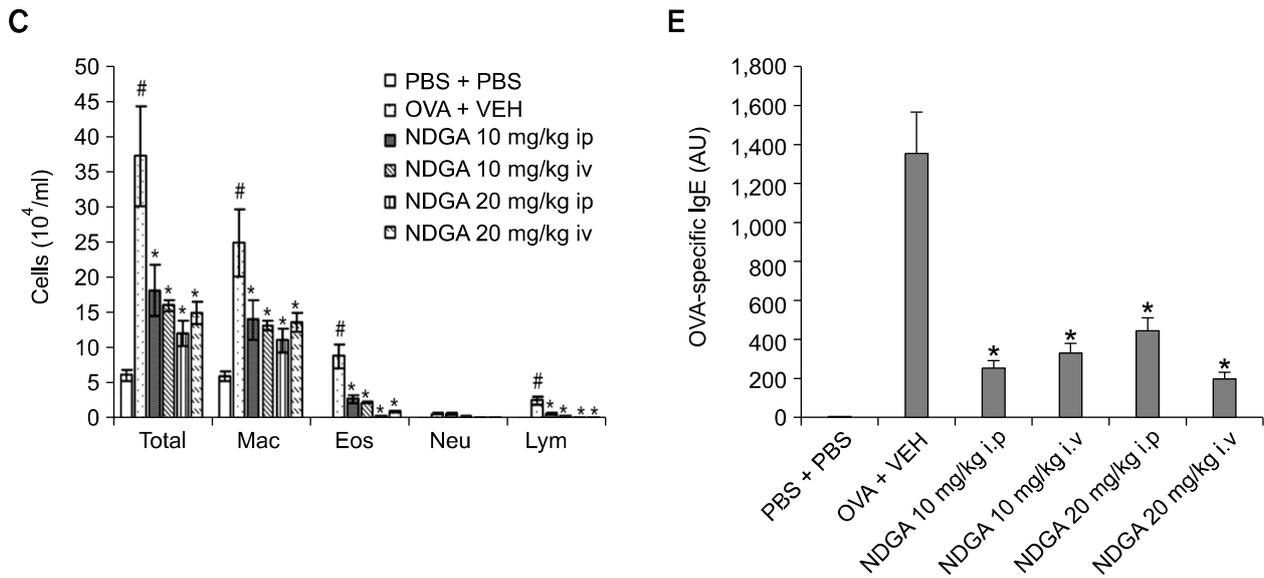


Figure 4. Continued.

activity after LPS stimulation was inhibited significantly (Figure 3B). In the electrophoretic gel mobility shift (EMSA) assay, moreover, NF- κ B DNA-binding activities were substantially reduced in NDGA-pre-treated Raw cells, followed by TNF- α and LPS stimulation (Figure 3C). Moreover, NDGA markedly attenuated LPS stimulation-induced TNF- α production (Figure 3D). Collectively, these results strongly indicate that LOXs are required for the activation of NF- κ B in pro-inflammatory signaling, such as that associated with TNF- α and LPS.

NDGA attenuates airway hyperresponsiveness and airway inflammation in an experimental asthma model

As it has been well established that, in cases of allergic inflammation, the oxidative stress-induced production of ROS promotes the activities of pro-inflammatory redox-sensitive nuclear factors, including NF- κ B (Rahman *et al.*, 1999; Dworski *et al.*, 2000; Rahman *et al.*, 2000; Kirkham *et al.*, 2006) and leukotriene release into the airspace as a result of the 5-LOX-mediated arachidonic acid pathway contributes to the release of airway mucus and the infiltration of inflammatory cells, therapy with both antioxidants and the inhibition of 5-LOX activity may have potential advantages. We recently reported that α -lipoic acid, a naturally-occurring antioxidant, may exert suppressive effects on allergic airway inflammation and AHR in an experimental asthma model (Cho *et al.*, 2004). Therefore, we attempted to determine whether the administration of NDGA is capable of suppressing the development of the

bronchial asthma. To that end, we generated a mouse model of ovalbumin (OVA)-induced asthma, as is depicted in Figure 4A. On days 21, 22, and 23, different NDGA concentrations were either intraperitoneally or intravenously administered. NDGA treatment markedly attenuated OVA-induced hyper-responsiveness to methacholine (Figure 4B). Moreover, both intraperitoneal and intravenous NDGA administration profoundly inhibited the infiltration of inflammatory cells such as macrophages, eosinophils, and lymphocytes into the BAL fluid (Figure 4C). In parallel with this result, NDGA treatment prevented lung inflammation, as was shown by the observed reduction of eosinophil-rich leukocyte infiltration in the peribronchiolar regions, whereas typical pathologic features of asthmatic inflammation were observed in OVA-challenged mice not treated with NDGA (Figure 4D). In addition, enzyme immunoassays revealed that the level of OVA-specific IgE was decreased markedly in NDGA-treated mice as compared with the untreated mice (Figure 4E).

NDGA inhibits the production of Th2 cytokines and proinflammatory cytokine TNF- α

Considering the essential role of Th2 cytokines and TNF- α with regard to the elicitation of allergic inflammatory responses, we measured the concentrations of IL-4, IL-5, IL-13, and TNF- α in BAL fluid, as well as the cells that produce them in the peripheral system. OVA-challenge elicited substantial increases in the concentration of all four cytokines in the BAL fluid, as well as in the IL-4, IL-5, IL-13,

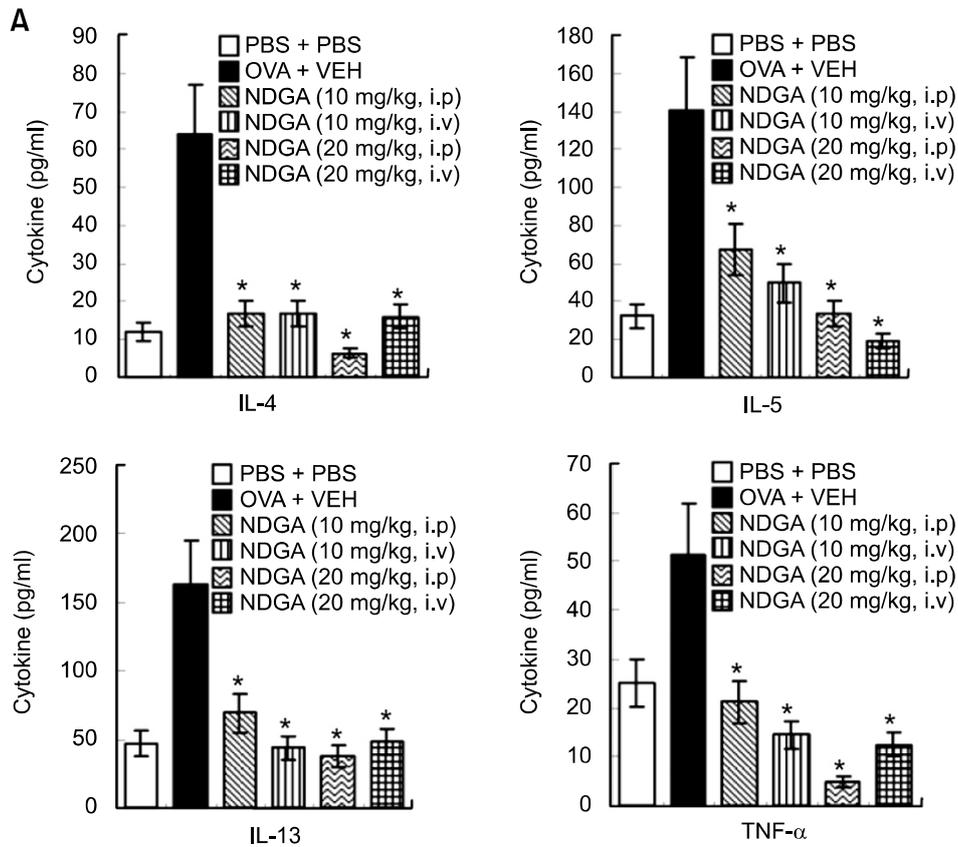


Figure 5. NDGA reduces inflammatory cytokines and the number of cytokine-producing cells. (A) Mouse BAL fluid samples from mice treated as described in the Figure 3 legend were collected 24 h after the last challenge. Enzyme immunoassays for IL-4, IL-5, IL-13, and TNF- α were conducted as described in the Materials and Methods section. Bars represent mean \pm SE from 6 independent experiments. * P < 0.05 vs OVA + VEH mice. (B) Mouse splenocytes from mice treated as described in the Figure 3 legend were harvested 24 h after the last challenge. For the detection of numbers of IL-4, IL-5, IL-13, and TNF- α -producing cells, ELISPOT assays were conducted as described in the Materials and Methods section. Spots were counted using Elispot leader 4.0. As a negative control, unstimulated cells were used. No spots were detected in the control wells. Bars represent mean \pm SE from 6 independent experiments. * P < 0.05 vs OVA + VEH mice.

and TNF- α -producing cells in splenocytes (Figure 5A and Figure 5B, closed bars), as compared with the levels and numbers of cells observed after PBS challenge (Figure 5A and B, open bars). The increased levels of these cytokines were markedly reduced by NDGA in a dose-dependent manner, as was the number of the cells that produce them in the splenocytes (Figure 5A and B).

NDGA inhibits OVA-induced production of TNF- α and the infiltration of macrophages into lung tissue and eosinophils into BAL fluid

Recent reports have shown that TNF- α , along with the Th2 cytokines, may critically contribute to several features of the response, antigen-induced inflammation and AHR, thus promoting allergic responses (Rudmann *et al.*, 2000; Williams *et al.*, 2000; Elias *et al.*, 2003; Akdis *et al.*, 2006; Eder *et al.*, 2006; Umetsu *et al.*, 2006). Furthermore, the

release of leukotriene into the airspace as a consequence of the 5-LOX-mediated arachidonic acid pathway contributes to the release of airway mucus and the infiltration of inflammatory cells (Barnes *et al.*, 1998). We finally attempted to determine whether NDGA affects the production of TNF- α to be processed in an allergen-dependent manner. When co-cultured with OVA allergen, a significant upregulation of TNF- α was observed in splenocytes derived from OVA-sensitized and challenged mice, whereas these levels were reduced markedly in NDGA-co-cultured splenocytes derived from OVA-sensitized and challenged mice. This result indicates that NDGA can inhibit the allergen-induced generation of the inflammatory cytokine, TNF- α , and thus its effects may contribute, in part, to an amelioration of allergic inflammation, coupled with reductions of Th2 cytokines *in vivo*, as is shown in Figure 5 (Figure 6A). Intriguingly, when we measured secreted leukotriene C₄ (LTC₄), which

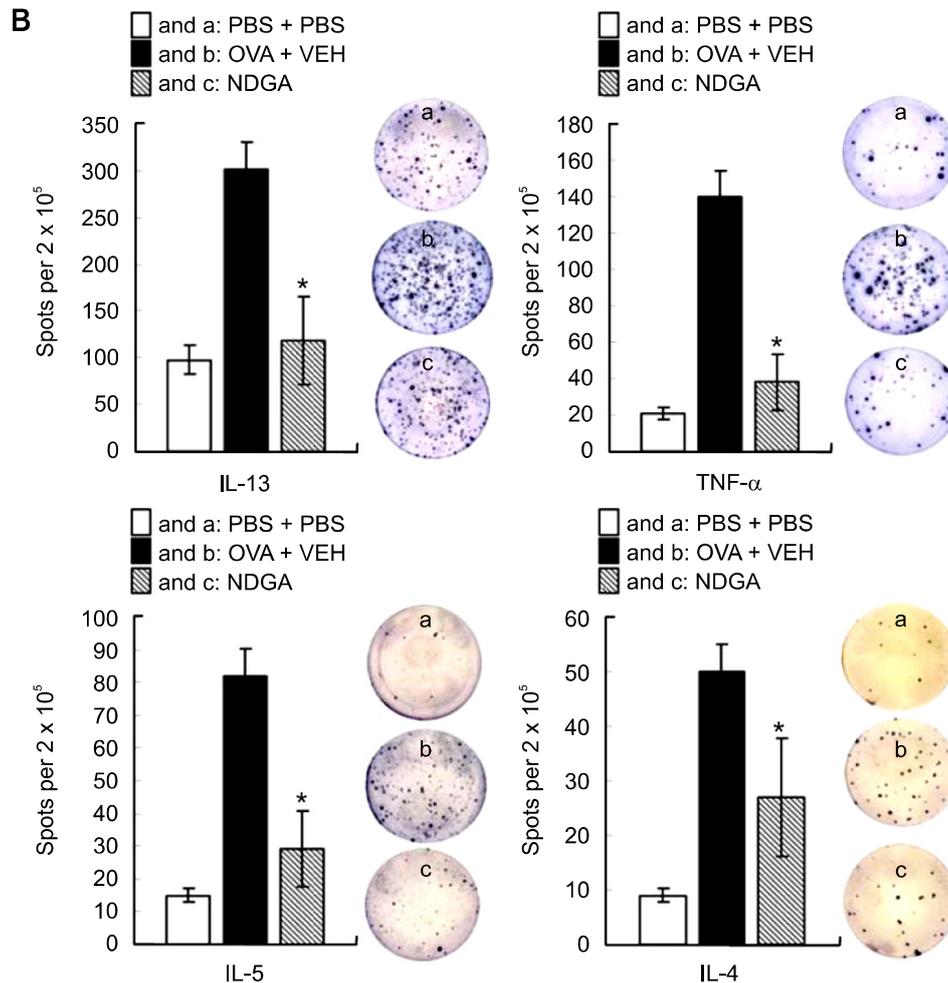


Figure 5. Continued.

is a critical mediator capable of increasing the infiltration of inflammatory cells into the lung, NDGA effected a significant inhibition of LTC_4 levels by more than 50% (Figure 6B). Furthermore, the reduced levels of LTC_4 appeared to exert a critical effect on the infiltration of macrophages into the lung. With regard to the relative proportion of macrophages, no significant differences could be detected in the PBS- or OVA-treated mice, whereas in the NDGA-treated mice, significant reductions were detected (Figure 6C). When the absolute numbers of cells were compared, marked increases were detected in the OVA-treated mice, whereas a dramatic reduction was observed in the NDGA-treated mice (Figure 6D). Additionally, the reduction of LTC_4 markedly affected the infiltration of eosinophils into BAL fluid as well as macrophages (Figure 6E). These results strongly suggest that NDGA may also function in the arachidonic acid pathway to produce LTC_4 as well as the inhibition

of pro-inflammatory signaling via the regulation of LOXs, and may result in an attenuation of airway inflammation.

Discussion

A great deal of emerging evidence suggests that ROS are crucial mediators in intracellular signaling, including the Toll-like receptor signaling pathway, and ROS scavenging or NOX inhibition suppresses LPS-induced cytokine production (Bonizzi *et al.*, 1999; Davies *et al.*, 1999; Lee *et al.*, 1999; Aslan *et al.*, 2003; Tonks *et al.*, 2005; Nakahira *et al.*, 2006; Saito *et al.*, 2006). However, the sources of ROS after the administration of these stimuli remain largely unknown. Primary cellular sources of ROS may be associated with NOX, 5-LOX, and the mitochondria (Babior *et al.*, 1999; Griendling *et al.*, 1999; Kuhn *et al.*, 1999; Van Heerebeek *et al.*,

2002; Rhee *et al.*, 2003). These ROSs are involved in pro-inflammatory signaling pathway (Babior *et al.*, 1999; Griendling *et al.*, 1999; Van Heerebeek *et al.*, 2002; Ogawa *et al.*, 2003; Genova *et al.*, 2004). The data presented in this study indicate a crucial role for lipoxygenases in pro-inflammatory signaling. NDGA, as LOXs inhibitor, strongly elicited the scavenging activity of intracellular ROS, inhibits TNF- α -induced ROS accumulation, and blocks

TNF- α -induced NF- κ B activation. NDGA has also been consistently shown to inhibit LPS-induced TNF- α production and NF- κ B activation, thereby indicating that those signalings are critically linked to LOXs activation. The results of previous studies have also shown that IL-1 β stimulation resulted in ROS generation via 5-LOX activity (Bonizzi *et al.*, 1999). Those results strongly indicate that 5-LOX activity is required for pro-inflammatory signaling,

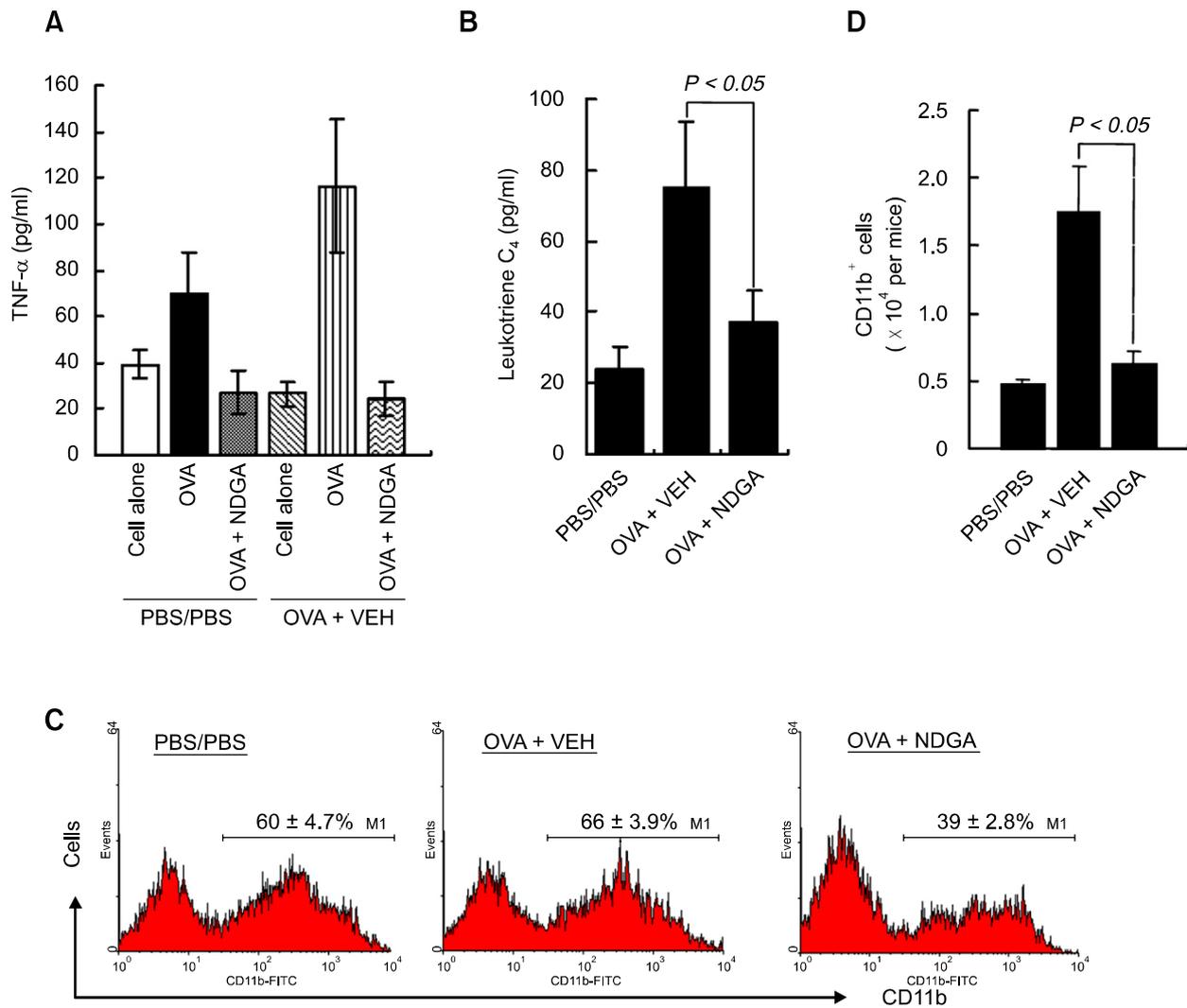


Figure 6. Effects of NDGA on the OVA-specific production of TNF- α , infiltration of macrophages into the lung, and leukotriene C4 levels in BAL fluids. (A) Splenocytes were harvested from saline-inhaled mice treated with PBS (PBS + PBS) and OVA-inhaled mice treated with drug vehicle (OVA + VEH). 5×10^5 cells per well were plated in 96-well plates, co-cultured with or without 20 μ g/ml OVA for 48 h in the presence or absence of 5 μ M NDGA. The quantity of TNF- α in the culture supernatant fractions was measured via ELISA, as described in the Materials and Methods section. Bars represent mean \pm SE from 6 independent experiments. (B) Leukotriene C4 levels in BAL fluids were analyzed via ELISA, as described in the Materials and Methods section. Bars represent mean \pm SE from 6 independent experiments. (C) and (D) Lungs were isolated from saline-inhaled mice treated with PBS (PBS+PBS), OVA-inhaled mice treated with drug vehicle (OVA + VEH), and OVA-inhaled mice intraperitoneally treated with 20 mg/kg NDGA (OVA + 20 mg/kg *i.p.*). Lung tissue was cut into small fragments, and the infiltrated cells were isolated as described in the Materials and Methods section. After the cells were counted (D), 10^6 per tube were incubated for 40 min on ice with FITC-conjugated anti-CD11b antibody and analyzed using a FACScalibur apparatus (C). \pm SE indicate standard deviations obtained from 6 different mice. (E) BAL fluids were harvested as described in the Materials and Methods and stained with Diff-Quik solution. The cells were visualized and identified by a microscope.

E

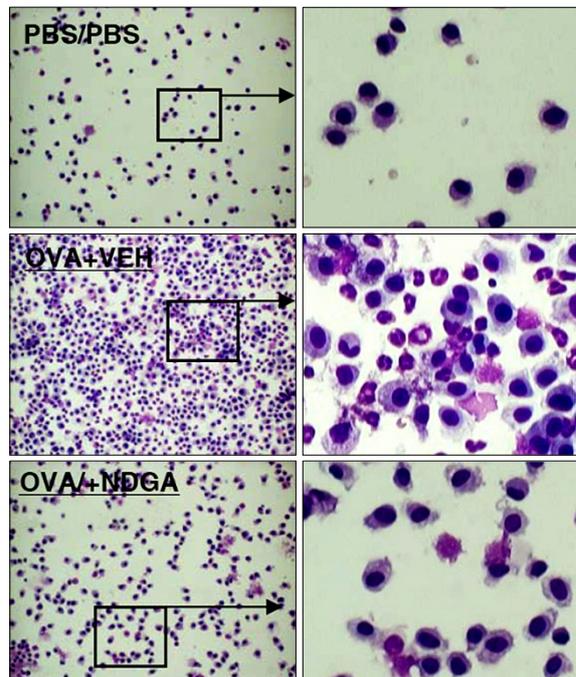


Figure 6. Continued.

such as that associated with TNF- α , IL-1 β , and LPS.

This study, for the first time, evaluated the contribution of LOXs to the pathogenesis of OVA-induced bronchial asthma in mice. The results of numerous studies have revealed that the production of ROS, including superoxide and hydroxyl radicals, hydrogen peroxide, and hypochlorous acid, is augmented, and antioxidant reserves are reduced in asthmatic patients (Rahman *et al.*, 1999; Dworski *et al.*, 2000; Rahman *et al.*, 2000; Kirkham *et al.*, 2006). Moreover, oxidative stress induces an increase in the peroxidation of lipids, proteins, and DNA, as well as the production of chemoattractants, as well as enhancing AHR, airway secretion, and vascular permeability in asthmatic airways (Barnes *et al.*, 1990; Henricks *et al.*, 2001; Andreadis *et al.*, 2003). ROS also promotes the activities of pro-inflammatory redox-sensitive nuclear factors, including NF- κ B, thus increasing the allergic inflammation observed in asthmatic patients (Henderson *et al.*, 2002; Rahman *et al.*, 2003). These results show that ROS may perform essential functions for both the induction of airway inflammation and for the pathogenesis of asthmatic diseases. Previous studies have revealed that NDGA exerts pleiotropic effects capable of influencing a wide variety of cellular processes, including TGF- β -induced signal transduction, leukocyte chemotaxis, myoblast cell differentiation, cancer cell proliferation, and viral

proliferation in infected cells (Goetzl *et al.*, 1980; Gnabre *et al.*, 1995; Lee *et al.*, 2003; West *et al.*, 2004; Ito *et al.*, 2005; Youngren *et al.*, 2005; Arasaki *et al.*, 2007). However, its anti-inflammatory effects in allergic inflammation have been investigated to a lesser degree. Our data indicated that treatment with NDGA markedly attenuates the development of airway hyperresponsiveness, OVA-induced bronchiolar inflammation, the infiltration of inflammatory cells into the airway, the release of pro-inflammatory cytokines including Th2, and the number of cytokine-generating cells in the peripheral system in OVA-sensitized and challenged mice.

Several cytokines, including IL-4, IL-5, IL-13, IL-1 β , and TNF- α , have been detected at elevated levels in the asthmatic lung, and may perform critical functions in the development and maintenance of asthmatic inflammation (Williams *et al.*, 2000; Elias *et al.*, 2003). IL-4, IL-5, and IL-13 regulate allergic inflammation via the promotion of Th2 cell differentiation, eosinophilic inflammation, and B cell differentiation, respectively. A number of studies have previously suggested a role for TNF- α in the development of AHR (Gosset *et al.*, 1991, 1992; Williams *et al.*, 2000; Coward *et al.*, 2002; Aggarwal *et al.*, 2003). In murine asthma models, a deficiency in TNF- α receptors has been determined to result in a marked attenuation of antigen-induced airway inflammation and AHR (Rudmann *et al.*, 2000). Additionally, recent studies have

shown that anti-TNF- α treatment administered to asthma patients resulted in a marked improvement in asthma symptoms, lung function, and AHR. The most striking of these improvements was that of AHR (Howarth *et al.*, 2005), which further points to the pivotal function of TNF- α in the development of AHR. Additionally, several studies have demonstrated that macrophage migration inhibitory factor (MIF), which is known to be elevated in asthmatic patients and also is known to play a critical function in the pathogenesis of asthma, is a pleiotropic molecule. MIF evidences several pro-inflammatory functions, including the induction of TNF- α , the release of IL-1 and NO from the macrophages, and the generation of arachidonic acid and eicosanoids via the induction of phospholipase A2 and cyclooxygenase (Bernhagen *et al.*, 1994; Mitchell *et al.*, 1999). The release of leukotriene into the airspace in response to allergens also critically contributes to the release of airway mucus and infiltration by inflammatory cells (Lewis *et al.*, 1990; Harrison *et al.*, 1995). Our *in vivo* data show that treatment with NDGA results in a marked reduction in the levels of pro-inflammatory cytokines, and suppresses the infiltration of inflammatory cells into the airway. A possible cellular mechanism by which NDGA may act on these responses involves the activity of LOXs. The LTC₄ levels in BAL fluid detected 24 h after the last challenge were 3-fold higher in the OVA-sensitized and challenged mice than in the mice receiving PBS alone. Moreover, infiltrations of inflammatory cells into BAL fluid and lung tissue, in addition to the levels of pro-inflammatory cytokines, were determined to have been markedly increased in the OVA-sensitized and challenged mice. These results show that the release of LTC₄ may induce the recruitment or migration of inflammatory cells into the lung, and then the secretion of pro-inflammatory cytokines, including Th2 and TNF- α . Intriguingly, treatment with NDGA resulted in a significant inhibition of LTC₄ levels, pro-inflammatory cytokine levels, and the infiltration of inflammatory cells in OVA-sensitized and challenged mice. This result indicates the essential role of LOXs during airway inflammation. In addition, NDGA also inhibited OVA-induced TNF- α secretion in the peripheral system. Although other functions of LOXs in airway inflammation cannot yet be ruled out, the inhibitory effects of NDGA as a antioxidant *in vitro* via the regulation of ROS, as well as its effects as an anti-inflammatory agent *in vivo* may contribute to either direct or indirect effects on the *in vivo* attenuation of airway inflammation.

In conclusion, our data show the pivotal role of LOXs in pro-inflammatory signaling via intracellular ROS regulation, which may be critical for the

activation of NF- κ B by that signaling, and the important role of LOXs in the pathogenesis of allergic inflammation.

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