

α -Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma

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Background: Oxidative stress may play an important role in the pathogenesis of bronchial asthma.

Objective: We evaluated the therapeutic effect of α -lipoic acid, a nonenzymatic antioxidant, in a mouse model of asthma.

Methods: BALB/c mice were immunized intraperitoneally with ovalbumin (OVA) on days 1 and 14 and challenged with inhaled OVA on days 28, 29, and 30. Mice were fed OVA-free standard mouse chow with 0%, 0.125%, 0.25%, 0.5%, and 1% (wt/wt) α -lipoic acid during the immunization and challenge periods.

On day 31, mice were challenged with inhaled methacholine, and enhanced pause was measured as an index of airway hyperresponsiveness. Severity of airway inflammation was determined by means of differential cell count of bronchoalveolar lavage (BAL) fluid and by means of histopathologic lung analysis. Levels of OVA-specific IgE in serum, IL-4 and IL-5 in BAL fluid, and intracellular reactive oxygen species in alveolar macrophages and lymphocytes obtained from regional perihilar lymph nodes were measured. Nuclear factor κ B DNA-binding activity in lung tissues was analyzed by means of electrophoretic gel mobility shift assay.

Results: Compared with untreated asthmatic mice, mice treated with α -lipoic acid had significantly reduced airway hyperresponsiveness, a lower proportion of eosinophils among BAL cells, and significantly improved pathologic lesion scores of the lungs. α -Lipoic acid also significantly reduced serum OVA-specific IgE concentrations, IL-4 and IL-5 concentrations in BAL fluid, and intracellular reactive oxygen species and nuclear factor κ B DNA-binding activity.

Conclusion: These results suggest that oxidative stress plays an important role in asthmatic airway inflammation and that α -lipoic acid may be useful as adjuvant therapy for bronchial asthma. (J Allergy Clin Immunol 2004;114:429-35.)

Key words: Allergic inflammation, airway hyperresponsiveness, antioxidant, α -lipoic acid

Bronchial asthma is a chronic airway disease characterized by eosinophilic inflammation and airway hyper-

Abbreviations used

AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
EMSA: Electrophoretic gel mobility shift assay
HED: Human equivalent dose
NF: Nuclear factor
OVA: Ovalbumin
Penh: Enhanced pause
ROS: Reactive oxygen species

responsiveness (AHR). Production of reactive oxygen species (ROS), including superoxide and hydroxyl radicals, hydrogen peroxide, and hypochlorous acid, is increased¹⁻³ and antioxidant reserves are decreased^{1,3-5} in asthmatic patients. Oxidative stress increases the peroxidation of lipids, proteins, and DNA and the production of chemoattractants, as well as enhancing AHR, airway secretion, and vascular permeability in asthmatic airways.⁶ ROS also promote the activities of proinflammatory redox-sensitive nuclear factors, including nuclear factor- κ B (NF- κ B), thus increasing the allergic inflammation that occurs in asthmatic patients.^{7,8}

Previous epidemiologic studies have suggested that high intake of food rich in antioxidants is related to higher FEV₁ and forced vital capacity values, less frequent and milder asthmatic symptoms, and a lower incidence of asthma.⁹⁻¹² Administration of dietary antioxidants, including vitamins C¹³⁻¹⁶ and E^{15,16} and selenium,¹⁷ however, has resulted in inconsistent therapeutic effects in patients with asthma. N-acetylcysteine,¹⁸⁻²⁰ superoxide dismutase,²¹ ambroxol,²² and a catalytic antioxidant²³ produced limited suppression of allergic airway inflammation and AHR in animal models of asthma.

α -Lipoic acid is a naturally occurring antioxidant that has been used clinically for treatment of oxidant-induced diseases, such as ischemia-reperfusion injury²⁴ and diabetic neuropathy.^{25,26} Suggested to be one of the ideal antioxidants, α -lipoic acid directly scavenges free radicals, recycles other antioxidants, accelerates glutathione synthesis, and modulates the activity of transcription factors, such as NF- κ B.²⁷ However, the therapeutic efficacy of α -lipoic acid in bronchial asthma had not previously been evaluated. Therefore we investigated the effects of α -lipoic acid on allergic airway inflammation and AHR in a mouse model of asthma. To further explore

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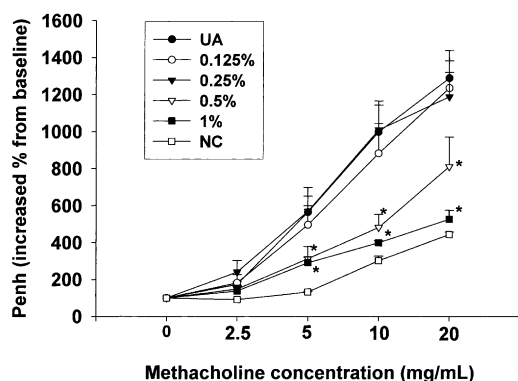


FIG 1. Effect of α -lipoic acid on airway responsiveness to inhaled methacholine. UA, Untreated asthmatic mice; NC, normal control mice; 0.125%, 0.25%, 0.5%, and 1%, asthmatic mice treated with mouse chow containing 0.125%, 0.25%, 0.5%, and 1% α -lipoic acid, respectively. * $P < .01$ compared with untreated asthmatic group.

the mechanism, we also assessed the effects of α -lipoic acid on cytokine levels in bronchoalveolar lavage (BAL) fluid and intracellular ROS in inflammatory cells and assessed the degree of NF- κ B DNA-binding activity in lung tissues.

METHODS

Allergen sensitization and challenge

Six-week-old male BALB/c mice were maintained in a specific pathogen-free area and allowed ovalbumin (OVA)—free feed and water. The mice were divided into 6 groups, and asthma was induced in 5 groups. On day 1, each mouse was immunized through intraperitoneal injection with 20 μ g of chicken OVA (Grade III; Sigma Chemical Co, St Louis, Mo) and 2 mg of Al(OH)₃ (alum), followed by an identical booster injection on day 14. Throughout the immunization and challenge periods, mice were fed standard mouse chow with 0%, 0.125%, 0.25%, 0.5%, and 1% (wt/wt) α -lipoic acid. On days 28, 29, and 30, each mouse underwent aerosolized antigen challenge with 5% OVA (wt/vol in PBS) for 30 minutes per day. During 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 through an ultrasonic nebulizer attached directly to the chamber opposite the ventilation holes. The negative control group of mice not administered α -lipoic acid was sensitized and challenged with PBS. All the experiments described in this article were approved by the animal research ethical review board of the institution.

Determination of AHR

Twenty-four hours after the final aerosol challenge, AHR was assessed in conscious and unrestrained mice by means of 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 minutes. Beginning 10 seconds after each aerosol exposure and lasting for 3 minutes, enhanced pause (Penh) readings, calculated automatically on the basis of the mean pressure generated in the plethysmography chamber during inspiration and expiration combined with the time of each phase, were recorded and averaged. Values were represented as the percentage change in Penh at each methacholine concentration compared with baseline readings.

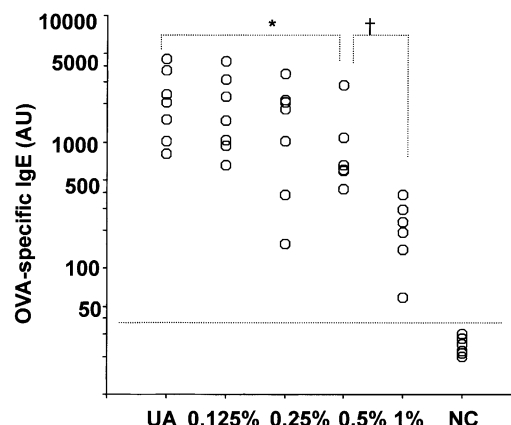


FIG 2. Effect of α -lipoic acid on serum levels of OVA-specific IgE. UA, Untreated asthmatic mice; NC, normal control mice; 0.125%, 0.25%, 0.5%, and 1%, asthmatic mice treated with mouse chow containing 0.125%, 0.25%, 0.5%, and 1% α -lipoic acid, respectively. * $P < .05$, † $P < .01$.

Analysis of serum OVA-specific IgE antibody

After measurement of AHR, the mice were anesthetized and killed by means of cardiac puncture, and the blood was centrifuged at 13,000 rpm for 20 minutes. The levels of serum OVA-specific IgE antibody were measured with an ELISA. Briefly, each well of a 96-well plate was coated with 100 μ L of 5 μ g/mL OVA in PBS and incubated overnight at 4°C. Plates were washed 3 times with 0.1% Tween 20 in PBS and incubated with 10% FBS in PBS for 1 hour. To each well, 100 μ L of a serum sample diluted 1:50 in PBS was added, and the plates were incubated for 2 hours at room temperature. The plates were washed 5 times, 100 μ L of a 1:2000 dilution of rat anti-mouse IgE (Pharmingen, San Diego, Calif) was added to each well, and the plates were incubated for 1 hour at 37°C. The plates were washed 7 times, 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate (Pharmingen) was added to each well, and the plates were incubated for 30 minutes in the dark at room temperature. Finally, stop solution (2N H₂SO₄) was added, and the plates were read at 450 nm on a microplate reader. Values were converted to arbitrary units by using pooled sera from immunized mice as a standard.

Analysis of BAL fluids and histologic examination

After drawing blood by means of cardiac puncture, the trachea was cannulated, and BAL was performed with 1 mL of normal saline at 37°C. The recovered fluid was centrifuged at 400g to separate cells from supernatant. The concentrations of IL-4, IL-5, IFN- γ , IL-12, IL-1 β , and TNF- α in each supernatant were determined by using commercial ELISA kits (R&D Systems, Minneapolis, Minn). Each BAL cell pellet was resuspended in RPMI 1640 medium, and the total number of cells was determined. Slides for differential cell counts were prepared with cytopsin and stained with Diff-Quik. For each sample, 2 investigators blinded to the treatment group counted 300 cells in each sample, and the values were averaged.

For histopathologic assessment, the lungs were fixed overnight in formalin, embedded in paraffin, sectioned to 4 μ m, and stained with hematoxylin and eosin. The degree of lung inflammation was evaluated by using the method previously described.²⁸ Briefly, lung lesions, including alveolar septal infiltrates, perivascular infiltrates, and peribronchial infiltrates, were subjectively graded on a numeric scale of 1 to 4, corresponding to minimal, mild, moderate, and marked severity by 2 independent pathologists blinded to the treatments.

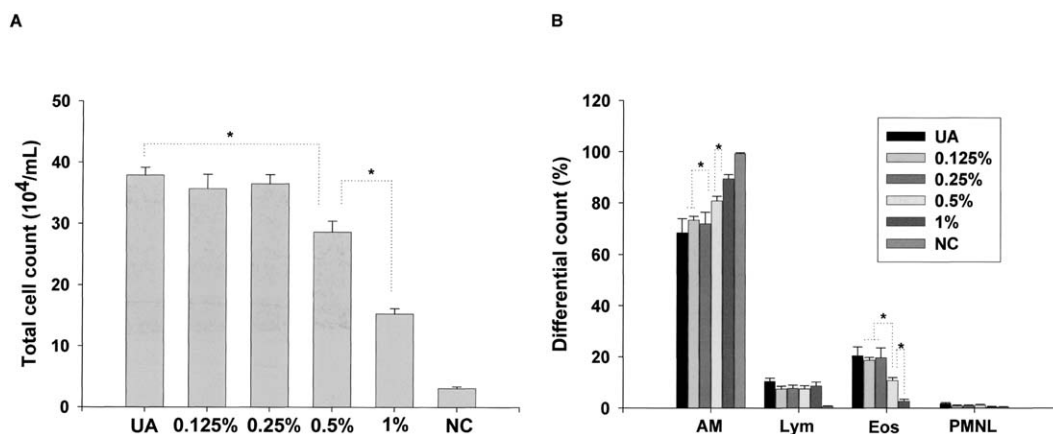


FIG 3. Effect of α -lipoic acid on BAL cells of OVA-induced asthmatic mice. **A**, Total number of BAL cells in each study group. **B**, Differential count of BAL cells in each study group. UA, Untreated asthmatic mice; NC, normal control mice; 0.125%, 0.25%, 0.5%, and 1%, asthmatic mice treated with mouse chow containing 0.125%, 0.25%, 0.5%, and 1% α -lipoic acid, respectively. * $P < .05$.

Overall histopathologic scores were generated from the sum of the individual lesion scores for each mouse. In addition, periodic acid–Schiff staining was also performed to evaluate mucus secretion of the lung sections.

Determination of intracellular ROS in alveolar macrophages and lymphocytes from regional perihilar lymph nodes

Mice from each group ($n = 8-10$) were subjected to 4 sequential BAL procedures as described above, and the BAL cells from each mouse were pooled. The regional draining lymph nodes from each mouse were obtained by dissecting out the perihilar region, which was placed in RPMI 1640 medium (Sigma) at 4°C. Single-cell suspensions were prepared by gently pressing the lymph nodes through a steel mesh (stainless steel 60 mesh screen) with a rubber plunger, followed by centrifugation at 1800 rpm for 5 minutes at 4°C.

Cells obtained from BAL and perihilar lymph nodes were washed once with PBS at 1800 rpm for 10 minutes. Each cell preparation was resuspended in 300 μ L of PBS and incubated with 20 μ M of 2',7'-dichlorofluorescein diacetate (Molecular Probes, Eugene, Ore) for 20 minutes at 37°C. The cells were washed with PBS and incubated for 1 hour at 37°C, followed by flow cytometry gated for DCF emission. Data were collected from at least 10,000 events.

Electrophoretic gel mobility shift assay for evaluation of NF- κ B DNA binding

Mice from each group ($n = 8-10$) were killed, and the lungs were removed and homogenized for electrophoretic gel mobility shift assay (EMSA). Nuclear proteins were extracted from each lung tissue sample, as previously described.¹⁹

EMSA was performed by using double-stranded oligonucleotides encoding the consensus target sequence of NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') that had been labeled with γ -³²P-ATP and T4 polynucleotide kinases. Nuclear extracts (5 μ g per sample) were incubated at room temperature for 20 minutes in binding reaction mixture (10 mmol/L HEPES [pH 7.8], 60 mmol/L KCl, 1 mmol/L EDTA, 7% glycerol, and 1 mmol/L dithiothreitol) and 1 μ g of poly(dI-dC). Unlabeled oligonucleotide was added and incubated on ice for 30 minutes for the negative controls. For the supershift analysis, antibodies to NF- κ B p50 and p65 were added and incubated on ice for 1 hour. The samples were subsequently electrophoresed on

a 6% nondenaturing polyacrylamide gel, and the bands were visualized by means of autoradiography and semiquantified by means of densitometry.

Statistical analysis

All data are expressed as means $\pm$ SEM. For comparison between groups, Mann-Whitney and Kruskal-Wallis tests were used. Statistical significance was defined as a P value of less than .05.

RESULTS

Airway responsiveness in the methacholine provocation test

Airway responsiveness, as determined on the basis of Penh, was increased in the asthmatic mice sensitized by and challenged with OVA and then challenged with methacholine. Compared with untreated asthmatic mice, mice given 0.5% and 1% α -lipoic acid had significantly decreased AHR (Fig 1).

Serum allergen-specific IgE levels, analysis of BAL samples, and histopathologic examination of the lungs

The OVA-specific IgE levels were higher in asthmatic mice than in control mice. OVA-specific serum IgE levels were significantly decreased in a dose-dependent fashion by α -lipoic acid compared with those seen in untreated asthmatic mice (Fig 2).

Examination of inflammatory cells obtained from BAL fluids revealed that, compared with control mice, asthmatic mice had significantly increased total cell numbers and proportions of eosinophils, lymphocytes, and neutrophils, with a concomitantly decreased proportion of alveolar macrophages. Treatment of mice with 0.5% and 1% α -lipoic acid induced a significant and dose-dependent decrease in total cell numbers and the percentage of eosinophils compared with those seen in untreated asthmatic mice (Fig 3). The relative proportion of macrophages in BAL cells was increased in the α -lipoic acid–treated groups because of the decreased proportion

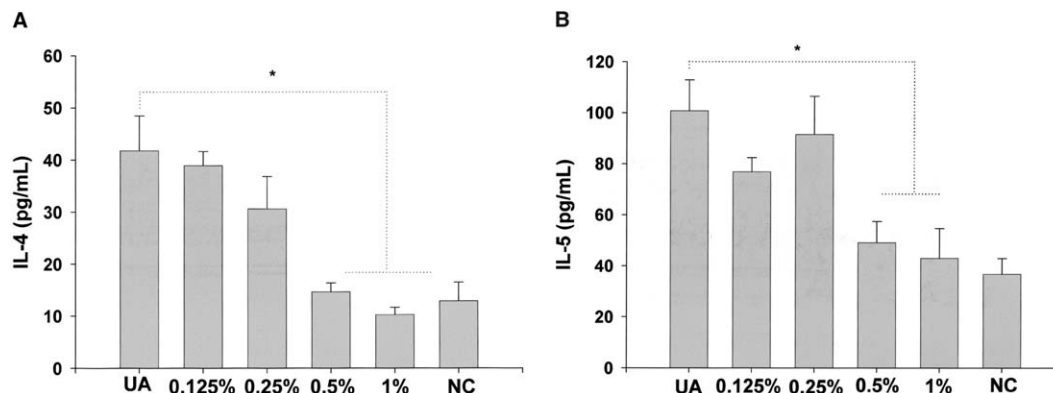


FIG 4. Effect of α -lipoic acid on IL-4 and IL-5 concentrations in BAL fluid of OVA-induced asthmatic mice. **A,** Levels of IL-4. **B,** Levels of IL-5. UA, Untreated asthmatic mice; NC, normal control mice; 0.125%, 0.25%, 0.5%, and 1%, asthmatic mice treated with mouse chow containing 0.125%, 0.25%, 0.5%, and 1% α -lipoic acid, respectively. * $P < .03$.

of eosinophils. Because the total number of BAL cells was significantly decreased in the α -lipoic acid-treated mice, the total number of macrophages in BAL cells was also decreased.

The levels of IL-4 and IL-5 in BAL fluids were significantly higher in asthmatic mice than in control mice. In contrast, asthmatic mice treated with 0.5% and 1% α -lipoic acid had significantly lower concentrations of IL-4 and IL-5 in BAL fluid than untreated asthmatic mice (Fig 4). The levels of other cytokines, such as IFN- γ , IL-1 β , and TNF- α in BAL fluid tended to be decreased in the α -lipoic acid-treated groups, although not significantly. The levels of IL-12 in BAL fluid were not affected by α -lipoic acid treatment in asthmatic mice (data not shown).

The extent and anatomic distribution of allergic inflammation was further examined by means of histologic analysis of lung tissue. Intense inflammation was evident in the lungs of asthmatic mice, with infiltration of an admixture of eosinophils and mononuclear cells surrounding the bronchi and some epithelial damage. Some perivascular inflammation and infiltration of the air spaces were also evident. Asthmatic mice given 0.5% and 1% α -lipoic acid showed a significant improvement in lesion scores compared with untreated asthmatic mice (summed lesion score, 7.6 ± 0.49 in untreated asthmatic mice and 6.33 ± 0.33 and 4.50 ± 0.43 in 0.5% and 1% α -lipoic acid-treated asthmatic mice, respectively; $P < .01$; Fig 5). The number of periodic acid–Schiff–positive cells in bronchi was also considerably increased in untreated asthmatic mice, whereas a significant reduction was observed in asthmatic mice fed mouse chow with 0.5% and 1% α -lipoic acid (data not shown).

Intracellular oxidative stress and NF- κ B DNA-binding activities

The amounts of intracellular ROS were considerably higher in the alveolar macrophages of BAL fluids and lymphocytes obtained from the perihilar lymph nodes of asthmatic mice compared with control mice. After treatment with α -lipoic acid, intracellular ROS levels

were decreased significantly in the lymphocytes of the perihilar lymph nodes. Intracellular ROS levels in alveolar macrophages were also reduced after α -lipoic acid therapy, although not significantly (Fig 6).

EMSA showed that specific NF- κ B DNA-binding activity was greatly increased in the lungs of asthmatic mice compared with control mice. The values of relative density represented as a percentage of the p65-treated sample were $146\% \pm 7.1\%$ in control mice and $268\% \pm 22.1\%$ in untreated asthmatic mice ($P < .03$). This increased activity was dose dependently inhibited by α -lipoic acid. The values were decreased to $203\% \pm 18.2\%$ and $156.8\% \pm 4.3\%$ in each asthmatic mouse group treated with 0.5% and 1% α -lipoic acid, respectively ($P < .05$).

DISCUSSION

We have shown here that α -lipoic acid, a nonenzymatic antioxidant, effectively suppressed allergic inflammation and AHR in a mouse model of asthma. We also found that these effects of α -lipoic acid were related to decreased activity of the redox-sensitive transcription factor NF- κ B. These results support the hypothesis that oxidative stress is important in asthmatic airway inflammation and that α -lipoic acid may be used as adjuvant therapy for patients with bronchial asthma.

Asthmatic subjects have higher plasma concentrations of isoprostanes²⁹; enhanced production of ROS in blood monocytes, neutrophils, and eosinophils,^{1,29-31}; increased levels of oxidized glutathione in BAL fluids³; and increased nitric oxide concentrations in exhaled air.³² Furthermore, concentrations of natural antioxidants, including glutathione peroxidase, superoxide dismutase, glutathione, and vitamins C and E, are reduced in the blood cells, plasma, or BAL fluids of asthmatic patients.^{1-3,5,33-36}

Both α -lipoic acid and its reduced form, dihydrolipoic acid, act as a redox couple to carry electrons from the substrate of the dehydrogenase to NAD⁺.²⁷ Notably,

α -lipoic acid is considered an ideal antioxidant because it possesses many beneficial characteristics, including specific free radical quenching, metal chelating activity, interaction with other antioxidants, and suppressive effects on redox-sensitive gene expression,²⁷ all of which are important for efficacy. In addition, α -lipoic acid is easily absorbed and has a good bioavailability. Furthermore, α -lipoic acid, currently used in the management of diabetic neuropathy, has not caused significant adverse reactions.²⁶ In this regard clinical use of α -lipoic acid as adjunctive therapy for asthma may be more beneficial than use of other compounds or biologic agents.

In this murine model of asthma, significant therapeutic effects on allergic inflammation were observed in the groups fed mouse chow containing 0.5% and 1% α -lipoic acid, which was well tolerated. Because we administered the drug mixed in the food, doses varied with food intake. The average drug doses were 412 and 794 mg $\cdot$ kg⁻¹ $\cdot$ d⁻¹ for 0.5% and 1% α -lipoic acid, respectively. The human equivalent doses (HEDs), calculated by the FDA formula (HED = Animal dose in mg/kg $\times$ [Animal weight in kg/human weight in kg]^{0.33}), of the murine doses of 0.5% and 1% α -lipoic acid are 33.5 and 60.8 mg $\cdot$ kg⁻¹ $\cdot$ d⁻¹. These doses are slightly higher than the clinical doses currently administered for other diseases (10-30 mg $\cdot$ kg⁻¹ $\cdot$ d⁻¹; 600-1800 mg/d). A well-designed clinical study is necessary to determine the safety and efficacy of α -lipoic acid in human asthmatic subjects.

Chronic airway inflammation is a major factor in the pathogenesis of asthma, and the eosinophil has long been recognized as the central effector cell in the inflamed asthmatic airway. ROS released by eosinophils and other infiltrating leukocytes, including neutrophils, are important in the allergic inflammation and AHR observed in asthma. Increased ROS production leads directly to the oxidation of proteins, DNA, and lipids, all of which directly injure the airways and may induce pathophysiologic events in the lungs. Many antioxidant compounds have anti-inflammatory properties, possibly because of effects on inflammatory cellular messenger systems rather than direct prevention of free radical-mediated damage to biomolecules. However, the exact mechanisms by which increased oxidative stress leads to the molecular hallmarks of acute or chronic asthma have not been ascertained.

We have shown here that α -lipoic acid effectively suppressed the activity of NF- κ B, a redox-sensitive transcription factor, in the lung tissues through reduced oxidative stress. NF- κ B activity is increased in asthmatic airways,³⁷ and many stimuli involved in allergic inflammation have been shown to activate NF- κ B. In addition, many inflammatory proteins expressed in asthmatic airways are regulated by NF- κ B, including the cytokines IL-4, IL-5, and TNF- α ; the chemokines RANTES and eotaxin; and the adhesion molecules intercellular adhesion molecule 1 and vascular cell adhesion molecule 1,³⁸⁻⁴⁰ all of which are closely involved in the pathogenesis of asthma. Thus the development of an oxidant-antioxidant imbalance in the lungs of asthmatic patients may be the stimulus that activates redox-sensitive

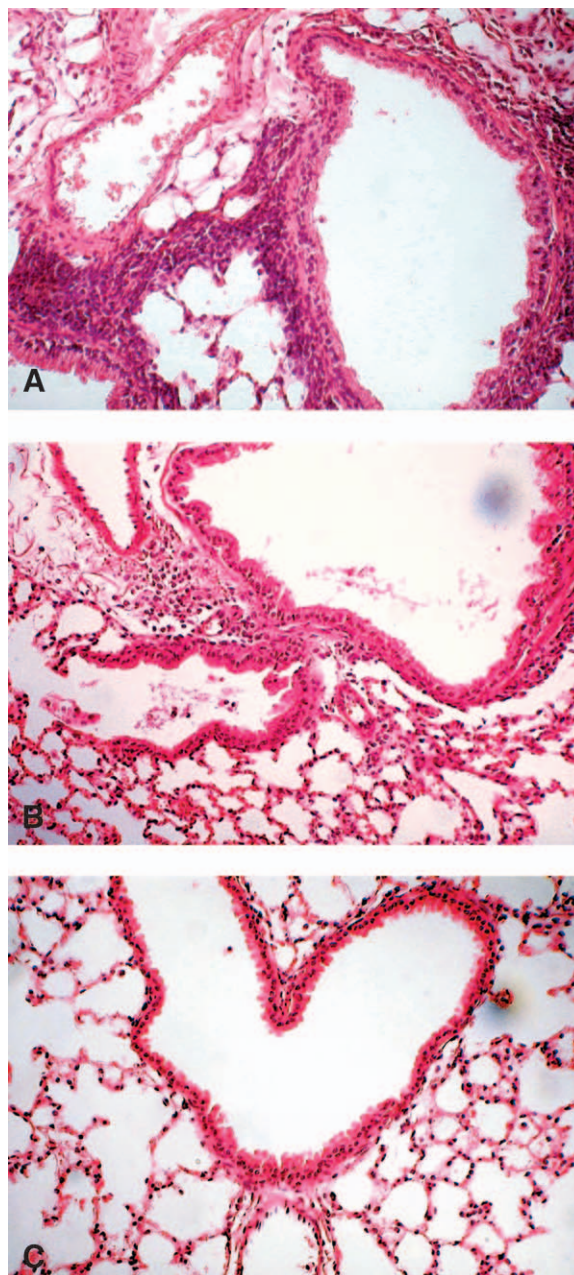


FIG 5. Effect of α -lipoic acid treatment on lung histopathology in a mouse model of OVA-induced asthma. **A**, Microscopic examination of lung tissue from an untreated asthmatic mouse. **B**, Microscopic examination of lung tissue from an asthmatic mouse fed mouse chow containing 1% α -lipoic acid. **C**, Microscopic examination of lung tissue from a normal control mouse. All tissue samples were stained with hematoxylin and eosin and viewed at 200 $\times$ magnification.

transcription factors, such as NF- κ B and activator protein 1. In agreement with this hypothesis, a small molecular inhibitor of redox-regulated NF- κ B and activator protein 1 transcription significantly blocked allergic airway inflammation in a mouse model of asthma, suggesting that redox-sensitive transcription factors could be a useful therapeutic target in asthma.⁸

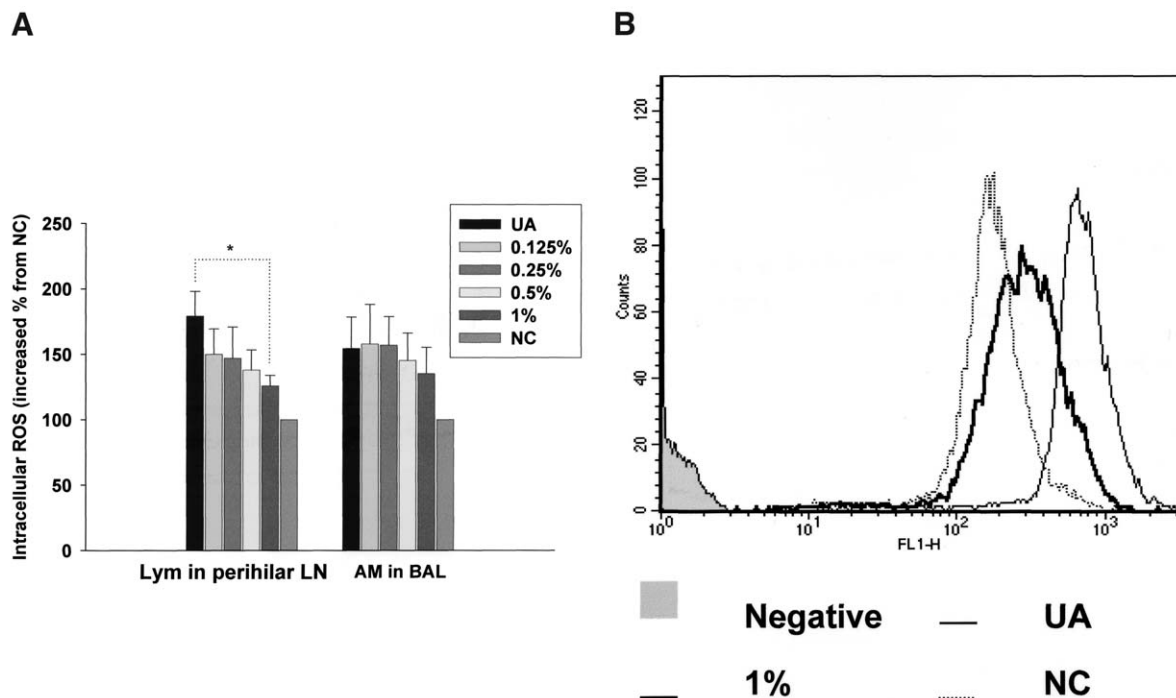


FIG 6. Effect of α -lipoic acid treatment on intracellular ROS of alveolar macrophages of BAL fluids and lymphocytes from perihilar lymph nodes in a mouse model of OVA-induced asthma. **A**, Amount of intracellular ROS in each study group. **B**, Result of FACS analysis for lymphocytes from perihilar lymph nodes. Lym, Lymphocyte; LN, lymph node; AM, alveolar macrophage; FL1-H, 2',7'-dichlorofluorescein diacetate; UA, Untreated asthmatic mice; NC, normal control mice; 0.125%, 0.25%, 0.5%, and 1%, asthmatic mice treated with mouse chow containing 0.125%, 0.25%, 0.5%, and 1% α -lipoic acid, respectively. * $P < .05$.

In our study decreased NF- κ B activity was accompanied by not only a reduction in the levels of important inflammatory mediators of allergic asthma, such as IL-4 and IL-5 in BAL fluids and serum OVA-specific IgE, but also by improvements in eosinophilic airway inflammation and AHR. These findings are in agreement with previous results from an animal asthma model showing that a catalytic antioxidant and N-acetylcysteine, which act as a free radical scavenger and a precursor of reduced glutathione, could suppress NF- κ B DNA-binding activity, reduce expression of adhesion molecules and mucin-specific mRNA in airways, and inhibit production of cytokines, such as IL-4, IL-5, and TNF- α .^{19,23} In addition, vitamin E, one of the most important natural antioxidants, was shown to inhibit *IL4* gene expression in peripheral blood T cells.⁴¹ Taken together, the findings of our study suggest that the functional mechanism underlying the therapeutic efficacy of α -lipoic acid as an antioxidant in allergic asthma may be related to inhibition of NF- κ B activation through decreased oxidative stress and subsequent reduction in proinflammatory chemical mediators. However, because the amount of ROS in alveolar macrophages of α -lipoic acid-treated asthmatic mice was not significantly different from the amount in untreated asthmatic mice, α -lipoic acid may act by other mechanisms, such as direct intervention in intracellular inflammatory signaling pathways without reduction of

ROS. α -Lipoic acid may induce antiasthmatic effects through several different anti-inflammatory mechanisms, which must be elucidated.

In conclusion, we have shown that α -lipoic acid effectively suppresses allergic inflammation and AHR in a mouse model of asthma. These results suggest that oxidative stress plays an important role in the asthmatic airway and that α -lipoic acid may be useful as adjunctive therapy for asthmatic patients.

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