

A Novel Dithiol Amide CB3 Attenuates Allergic Airway Disease through Negative Regulation of p38 Mitogen-activated Protein Kinase

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Rationale: Cellular redox homeostasis altered by excessive production of reactive oxygen species (ROS) and weakening of the antioxidant defense leads to oxidative stress. Oxidative stress is characterized as a decrease in glutathione/glutathione disulfide (GSH/GSSG) and the triggering of a number of the redox-sensitive signaling cascades. Recent studies have demonstrated that ROS play an important role in the pathogenesis of airway inflammation and hyperresponsiveness.

Objectives: Here we characterized for the first time the protective properties of a new hydrophobic thiol compound, *N*-acetyl cysteine proline cysteine amide (CB3), in allergic airway diseases.

Methods: We used ovalbumin (OVA)-inhaled mice to evaluate the role of CB3 as an antiinflammatory reagent and to determine its molecular signaling activity in allergic airways.

Measurements and Main Results: The administration of CB3 (1–50 mg/kg) to OVA-inhaled mice restored the decreased GSH levels, enhanced IL-10 expression, and significantly reduced the increase of Th2 cytokines and OVA-specific IgE. CB3 decreased the number of inflammatory cells and airway hyperresponsiveness in the lungs. We also found that the administration of CB3 dramatically decreased the nuclear translocation of the nuclear factor- κ B (NF- κ B) and the phosphorylation of p38 mitogen-activated protein kinases (MAPKs) in lungs after OVA inhalation. In addition, allergen-induced airway inflammation and hyperresponsiveness were substantially reduced by the administration of inhibitors of NF- κ B and p38 MAPK, BAY 11-7085, and SB 239063, respectively.

Conclusions: These results suggest that CB3 attenuates allergic airway disease by up-regulation of GSH levels as well as inhibition of NF- κ B and p38 MAPK activity.

Keywords: asthma; *N*-acetyl cysteine proline cysteine amide; glutathione; p38 mitogen-activated protein kinase; nuclear factor- κ B; oxidative stress

Exposure to airborne oxidant gases and particles leads to cellular damages and allergic airway diseases. These pathogeneses are mediated by a large variety of free oxygen radicals known as reactive oxygen species (ROS). Under physiological conditions, ROS participate in maintenance of cellular “redox homeostasis”

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Antioxidant treatment of asthma has long been a subject of therapeutic strategy. However, several human studies have yielded disappointing results with the effects of the existing antioxidant supplementation in asthma. In this study, a newly developed dithiol peptide, *N*-acetyl cysteine proline cysteine amide (CB3), has been used to study protective efficacy against the development of allergic airway diseases.

What This Study Adds to the Field

This study suggests that the regulation of p38 mitogen-activated protein kinase and/or nuclear factor- κ B signaling as well as up-regulation of glutathione levels could be the molecular mechanism of CB3 action in treating airway inflammatory disorders.

to protect cells against oxidative stress through various redox-regulatory mechanisms. The excess of ROS, which leads to oxidative stress, was shown to play an important role in the pathogenesis of airway inflammation and tissue injury observed in asthma, which comprises epithelial cell damage, cell shedding, and airway hyperresponsiveness (1–5). Further evidence for the involvement of oxidative stress in asthma is provided by the findings of a defective endogenous redox defense system in patients with asthma and animal models (1, 6–11). Recently, several studies have demonstrated that antioxidants are able to reduce airway inflammation and hyperresponsiveness in animal models of asthma (8, 12, 13). Thus, antioxidant treatment of asthma has long been a subject of therapeutic strategy.

There is considerable evidence that under pathological conditions, abnormally high concentrations of ROS in cells lead to the activation of signaling pathways involving nuclear factor- κ B (NF- κ B), p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, and Jun NH2-terminal kinase (JNK) (14–17). These signal transduction processes can induce various biological activities, such as muscle contraction, gene expression, cell growth, and nerve transmission (18). Therefore, these signaling pathways can be fundamentally critical targets for the action of various antioxidants.

Despite these findings, previous human studies have yielded disappointing results with the effects of antioxidant supplementation in asthma, such as vitamin C, vitamin E, and flavonoids. The ineffectiveness of these reducing reagents has been suggested to be due to their inability to restore glutathione (GSH) levels (5). Indeed, antioxidants that increase cellular cysteine levels, like *N*-acetylcysteine (NAC), carbocysteine, L-2-oxothiazolidine-4-carboxylic acid, and *N*-acetylcysteine amide

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(AD4), have been shown to exhibit antiinflammatory effects and anti-airway hyperresponsiveness in animal models of asthma (1, 8, 13, 19, 20). Several studies have shown that converting the carboxyl group of NAC to an amide increases hydrophobicity, thereby increasing membrane permeability (21–25). We have also demonstrated that treatment with AD4, an amide form of NAC, results in a remarkable restoration of intracellular thiols in airway inflammatory cells and a significant reduction of all pathophysiological symptoms of asthma through regulation of NF- κ B and hypoxia inducible factor-1 α signaling pathways (13).

In this study, we have characterized the protective properties of a novel dithiol peptide, *N*-acetyl cysteine proline cysteine amide (CB3), which is shown to be a more potent reducing agent than AD4 (24), using an ovalbumin (OVA)-inhaled murine model of asthma. We also evaluated the roles of CB3 in the airway hyperresponsiveness and inflammation and investigated the related molecular mechanisms, specifically, in the involvement of p38 MAPK signaling cascades. Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Additional detail on methods is provided in the online data supplement.

Animals and Experimental Protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, Korea), housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. Mice were sensitized and challenged as previously described, with some modifications (6, 27).

Administration of CB3, BAY 11-7085, and SB 239063

CB3 (Figure 1) was prepared by Atlas (24). CB3 (1, 10, or 50 mg/kg body weight/d) dissolved in phosphate buffered saline (PBS) was administered intraperitoneally to each animal at 24-hour intervals on days 21 to 24, beginning at 1 hour before the first challenge. A p38 MAPK inhibitor, SB 239063 (0.75 mg/kg body weight/d; Calbiochem-Novobiochem Corp., San Diego, CA), dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.9% NaCl, was administered by intravenous injection three times to each treated animal, once on Day 21 (1 h before the first challenge), the second time on Day 22 (24 h after the first challenge), and the third time on Day 24 (24 h after the last challenge). An inhibitor of NF- κ B activation, BAY 11-7085 (20 mg/kg body weight/d; BIOMOL International L.P., Plymouth Meeting, PA) dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each animal, once on Day 21 (1 h before the first challenge) and the second time on Day 23 (3 h after the last challenge).

Measurement of Intracellular ROS in Bronchoalveolar Lavage Cells Isolated from OVA-inhaled Mice

ROS were measured by a method described previously (6). For measurement of intracellular ROS, bronchoalveolar lavage cells were washed

with PBS and then incubated with 3.3 μ mol/L 2',7'-dichlorofluorescein (DCF) diacetate (Molecular Probes, Eugene, OR) dissolved in PBS at room temperature for 10 minutes. FACSscan analysis was performed with DCF stained cells (1×10^4 cells) to measure ROS levels using a FACSCalibur instrument (BD Biosciences, San Jose, CA). The data were analyzed with a CellQuest Pro program (BD Biosciences).

Measurement of GSH and Glutathione Disulfide in Lung Tissues

Total GSH and glutathione disulfide (GSSG) levels were determined using a Glutathione Assay Kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

Western Blot Analysis

Protein expression levels were analyzed by means of Western blot analysis as described previously (27).

Cytosolic or Nuclear Protein Extractions for Analysis of NF- κ B p65

Cytosolic or nuclear extraction was performed as described previously (6).

Cell Culture and Treatment

Epidermal growth factor-transformed NIH-3T3 cells (DHER-14 cells) were pretreated with various concentrations of CB3 for 30 minutes and then incubated in the presence of cisplatin (CDDP; 100 μ mol/L) for 16 hours.

Murine tracheal epithelial cells were isolated from OVA- or saline-inhaled mice under sterile conditions as described previously (27). Isolated cells were seeded in culture dishes and grown until confluence. The medium was then replaced with a new medium containing vehicle (PBS) and/or CB3 (0.5 or 1.0 mmol/L), and then the cells were stressed with 1 μ g/ml of tumor necrosis factor- α . These primary cells were incubated for 16 hours at 37°C.

Measurement of Cytokines

Levels of IL-4, IL-5, IL-13, and IL-10 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (IL-4, IL-5, and IL-13: R&D Systems, Inc., Minneapolis, MN; IL-10: Invitrogen Corp. Carlsbad, CA). Levels of IL-10 in serum were also measured by an enzyme immunoassay (R&D Systems, Inc.).

Measurement of Serum OVA-Specific IgE

OVA-specific IgE levels were measured by ELISA according to the manufacturer's protocol, using a mouse OVA-IgE ELISA kit (MD Biosciences, Inc., St. Paul, MN).

Histology

For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (28).

Determination of Airway Responsiveness

Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere (29).

RESULTS

CB3 Lowers ROS Production Induced by CDDP or OVA

First, the ability of CB3 (Figure 1) to scavenge free radicals was tested by the DCF-DA assay. Preincubation of DHER-14 cells with NAC (0.1–100 mmol/L), GSH (0.1–1 mmol/L), and CB3 (0.5 μ mol/L to 50 mmol/L) for 1 hour suppressed CDDP (100 μ mol/L)-induced ROS accumulation during 4 hours (Table 1).

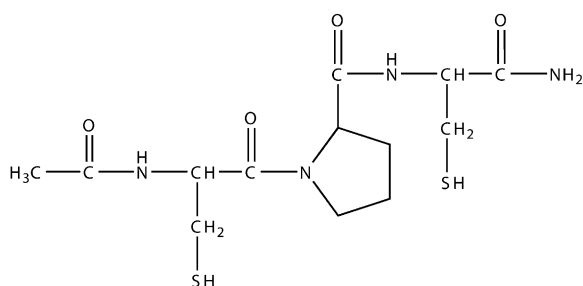


Figure 1. Structure of CB3, *N*-acetyl cysteine proline cysteine amide.

TABLE 1. THE REACTIVE OXYGEN SPECIES SCAVENGING EFFECT OF THIOL REAGENTS

Thiol Reagent	IC ₅₀ (μmol/L)	R ²
N-acetyl cysteine (NAC)	500	0.99
Glutathione (GSH)	140	0.99
N-acetyl cysteine proline cysteine amide (CB3)	1.1	0.99

Definition of abbreviations: IC₅₀ = concentration needed to inhibit 50% of the ROS produced; ROS = reactive oxygen species.

The apparent activity is presented by the concentration needed to inhibit 50% of the ROS produced (IC₅₀) by CDDP in DHER-14 cells.

In addition, we also examined the ROS scavenging ability of CB3 in BAL cells of OVA-inhaled mice. The ROS levels were significantly increased at 48 hours after OVA inhalation compared with the levels after saline inhalation (Figure 2). The increased ROS levels were substantially reduced by the administration of CB3.

CB3 Increases GSH Levels in Lung Tissues of OVA-inhaled Mice

The efficacy of CB3 in restoring GSH levels *in vivo* was evaluated using an animal model of asthma induced by OVA inhalation. GSH levels in lung tissues were significantly lowered (to 36% of control) at 48 hours after the last inhalation of OVA compared with the levels after saline inhalation (Figure 3A). The administration of 10 or 50 mg/kg of CB3 reversed the effect of OVA and restored GSH to near normal level. In contrast, GSSG levels in lung tissues were significantly increased at 48 hours after OVA inhalation compared with the levels after saline inhalation (Figure 3B). The increased GSSG levels were substantially reduced by the administration of CB3.

Effect of CB3 on Cellular Changes in BAL Fluids of OVA-inhaled Mice

Numbers of total cells, neutrophils, lymphocytes, and eosinophils in BAL fluids, were significantly increased at 48 hours after OVA inhalation. Administration of CB3 (1, 10, and 50 mg/kg)

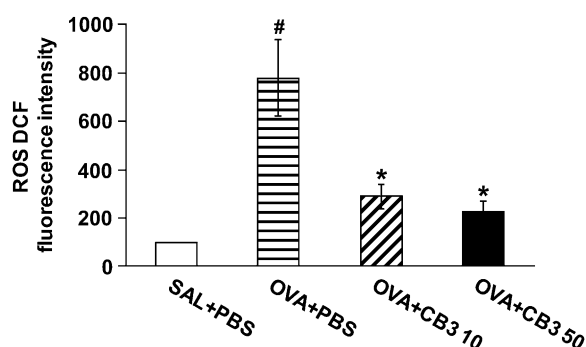


Figure 2. Dichlorofluorescein (DCF) fluorescence intensity in bronchoalveolar lavage (BAL) cells from ovalbumin (OVA)-inhaled mice. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered phosphate-buffered saline (PBS) (SAL+PBS), OVA-inhaled mice administered PBS (OVA+PBS), OVA-inhaled mice administered 10 mg/kg of N-acetyl cysteine proline cysteine amide (CB3) (OVA+CB3 10), and OVA-inhaled mice administered 50 mg/kg of CB3 (OVA+CB3 50). The reactive oxygen species (ROS) levels are presented as the relative ratio of values in OVA+PBS, OVA+CB3 10, or OVA+CB3 50 to those in SAL+PBS. The relative ratio of ROS levels in the BAL cells of SAL+PBS is arbitrarily presented as 100. Bars represent mean $\pm$ SEM from six mice per group. [#]*P* < 0.05 versus SAL+PBS; ^{*}*P* < 0.05 versus OVA+PBS.

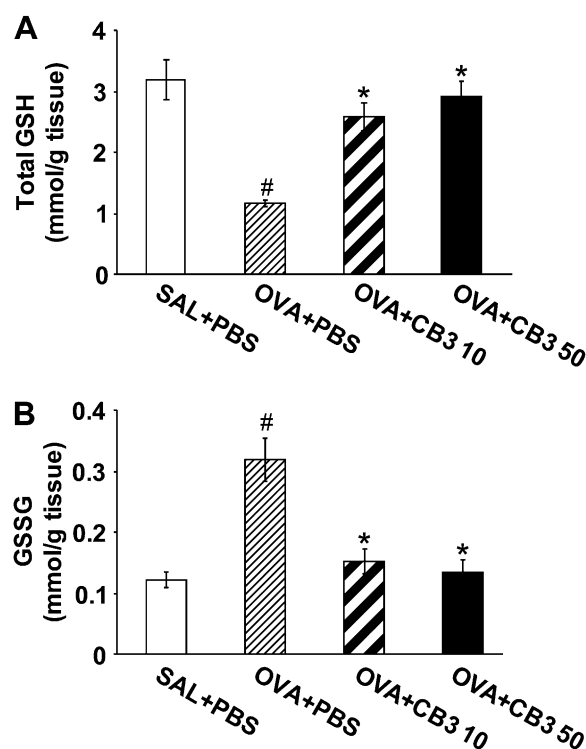


Figure 3. N-acetyl cysteine proline cysteine amide (CB3) increases glutathione (GSH) and decreases glutathione disulfide (GSSG) levels in lung tissues. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered phosphate-buffered saline (PBS) (SAL+PBS), OVA-inhaled mice administered PBS (OVA+PBS), OVA-inhaled mice administered 10 mg/kg of CB3 (OVA+CB3 10), and OVA-inhaled mice administered 50 mg/kg of CB3 (OVA+CB3 50). (A) GSH levels in lung tissues. (B) GSSG levels in lung tissues. Bars represent mean $\pm$ SEM from seven mice per group. [#]*P* < 0.05 versus SAL+PBS; ^{*}*P* < 0.05 versus OVA+PBS.

decreased the number of these cells in BAL fluids in a dose-dependent manner (Figure 4A).

Effect of CB3 on Pathologic Changes of OVA-inhaled Mice

Histological analysis showed typical features of an allergic airway disease in the OVA-inhaled mice. Numerous inflammatory cells infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Figure 4C) as compared with the control (Figure 4B). Mice treated with CB3 (Figures 4D and 4E) showed marked reduction in the thickening of airway epithelium. A considerable decrease was also observed in the infiltration of inflammatory cells in the peribronchiolar region, the number of inflammatory cells, and the amount of debris in the airway lumen. The scores of peribronchial, perivascular, and total lung inflammation were significantly increased at 48 hours after OVA inhalation compared with those after saline inhalation (Figure 4F). The increased peribronchial, perivascular, and total lung inflammation after OVA inhalation was significantly decreased by the administration of 10 and 50 mg/kg CB3.

CB3 Reduces Airway Hyperresponsiveness

Airway responsiveness was assessed as a percentage increase of respiratory system resistance (R_{rs}) in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curves of percent R_{rs} shifted to the left compared with that of control mice (Figure 4G). In addition, R_{rs} produced

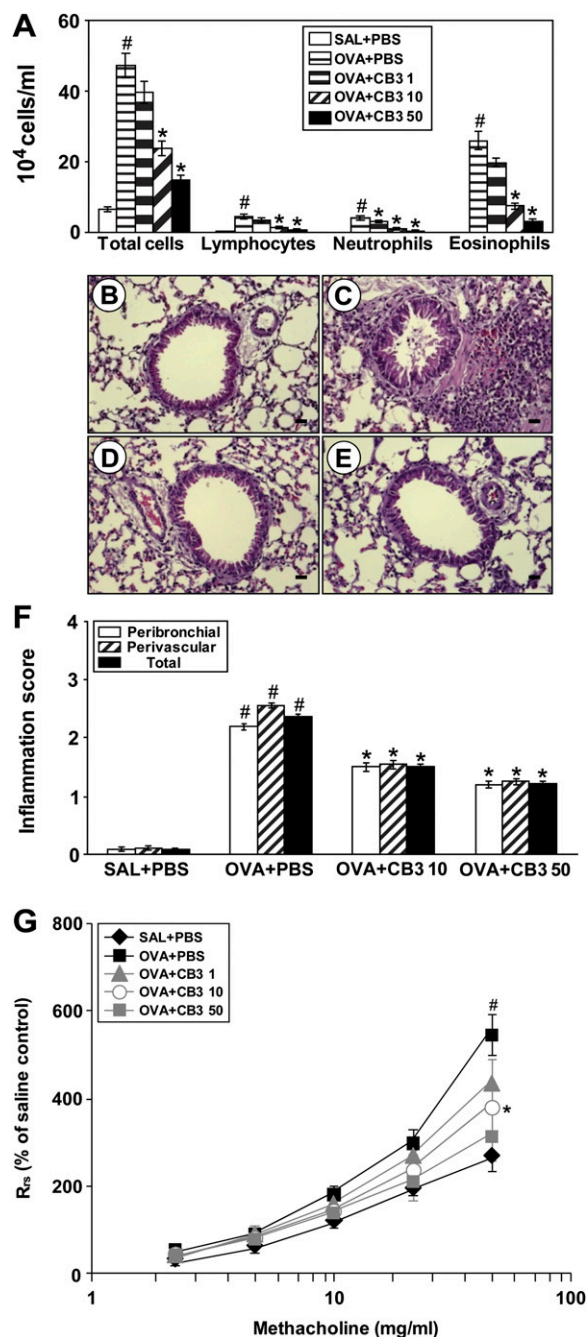


Figure 4. Effect of N-acetyl cysteine proline cysteine amide (CB3) on (A) changes in bronchoalveolar lavage (BAL) fluids, (B–F) pathologic changes in lung tissues, and (G) airway responsiveness of OVA-sensitized and -challenged mice. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered phosphate-buffered saline (PBS) (SAL+PBS), OVA-inhaled mice administered PBS (OVA+PBS), OVA-inhaled mice administered 1 mg/kg of CB3 (OVA+CB3 1), OVA-inhaled mice administered 10 mg/kg of CB3 (OVA+CB3 10), and OVA-inhaled mice administered 50 mg/kg of CB3 (OVA+CB3 50). (A) The numbers of total and differential cellular component of BAL fluids. (B–E) Representative hematoxylin and eosin-stained sections of the lungs from (B) saline-inhaled mice administered drug vehicle, (C) OVA-inhaled mice administered drug vehicle, (D) OVA-inhaled mice administered 10 mg/kg of CB3, and (E) OVA-inhaled mice administered 50 mg/kg of CB3. Bars indicate scale of 20 μm. (F) Peribronchovascular and perivascular lung inflammation was measured and total lung inflammation was defined as the average of the peribronchovascular and perivascular inflammation scores. (G) Airway responsiveness was presented by percent change of respiratory system resistance (R_{rs}). R_{rs} values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Bars represent mean ± SEM from seven mice per group. [#]P < 0.05 versus SAL+PBS; ^{*}P < 0.05 versus OVA+PBS.

tion of p38 MAPK. The increased level of phosphorylated p38 (phospho-p38) MAPK was decreased by the pretreatment with CB3 in a concentration-dependent manner.

We also evaluated the inhibitory effect of CB3 on p38 MAPK phosphorylation using primary tracheal epithelial cells isolated from OVA-inhaled mice. Consistent with the data from the study with DHER-14 cells, the levels of phospho-p38 MAPK in primary tracheal epithelial cells from OVA-inhaled mice were increased significantly compared with the levels in primary tracheal epithelial cells from the control mice (Figure 6). Treatment with CB3 reduced significantly the increased levels of phospho-p38 MAPK in primary tracheal epithelial cells from OVA-inhaled mice.

CB3 Decreases Phospho-p38 MAPK but Not Phospho-JNK or Phospho-ERK in Lung Tissues of OVA-inhaled Mice

Western blot analysis revealed that phospho-p38 MAPK levels in lung tissues were increased at 48 hours after challenge with OVA compared with the levels in the control group (Figures 7A and 7B). The increased phospho-p38 MAPK levels in lung tissues after OVA inhalation were significantly decreased by the administration of CB3. No significant changes in p38 MAPK levels were observed in lung tissues of any of the groups tested.

Phosphorylation of JNK and ERK in lung tissues was also substantially increased at 48 hours after challenge with OVA compared with the levels after saline inhalation (see Figure E1 in the online supplement). However, the increase of phosphorylated forms of JNK and ERK levels in lung tissues of OVA-inhaled mice was not affected by the treatment with CB3. JNK or ERK levels were not changed in lung tissues of any of the groups tested.

CB3 Prevents Nuclear Translocation of NF-κB p65 in Lung Tissues of OVA-inhaled Mice

Western blot analysis showed that levels of NF-κB p65 in nuclear protein extracts from lung tissues were increased at 48 hours after OVA inhalation compared with the levels in the control mice (Figures 7C and 7D). The increased NF-κB p65 levels in nuclear protein extracts after OVA inhalation were significantly decreased by the administration of 10 and 50 mg/kg CB3.

In contrast, levels of NF-κB p65 in cytosolic protein fractions from lung tissues were decreased after OVA inhalation compared with the levels in the control mice. The decreased NF-κB

by 50 mg/ml of methacholine was significantly increased in OVA-inhaled mice compared with the control mice. CB3 administered into the OVA-sensitized and -challenged mice reduced R_{rs} at 50 mg/ml of methacholine in a dose-dependent manner. These results indicate that the treatment with CB3 effectively decreased the OVA-induced airway hyperresponsiveness.

CB3 Lowers p38 MAPK Phosphorylation in Murine Fibroblasts and Tracheal Epithelial Cells

Previously, pretreatment of DHER-14 and NIH-3T3 cells with AD4 has been shown to suppress CDDP-induced ROS accumulation and phosphorylation of JNK, ERK1/2, and p38 MAPK (16, 23). We examined whether CB3 is able to reduce phosphorylation of p38 MAPK using DHER-14 cells. The cells were incubated with various concentrations of CB3 for 30 minutes before addition of CDDP. As shown in Figure 5, treatment with CDDP (100 μmol/L) induced the phosphoryla-

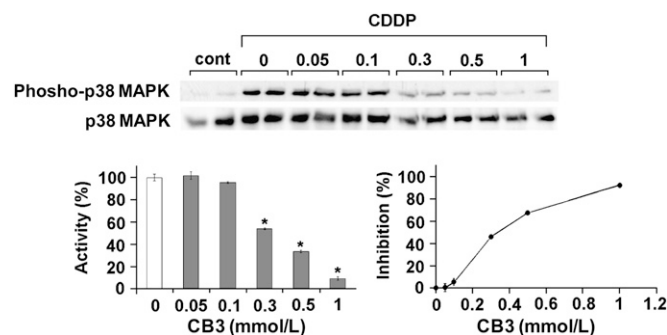


Figure 5. Effects of *N*-acetyl cysteine proline cysteine amide (CB3) on cisplatin (CDDP)-induced phosphorylation of p38 (phospho-p38) mitogen-activated protein kinases (MAPK) in epidermal growth factor-transformed NIH-3T3 cells (DHER)-14 cells. *Graphs* show percent activation. Results are representative of three independent experiments.

p65 levels in cytosolic protein fractions after OVA inhalation were substantially increased by the administration of CB3.

SB 239063 Reduces Degradation of Inhibitory κ B α Protein and Nuclear Translocation of NF- κ B p65 in Lung Tissues of OVA-inhaled Mice

Western blot analysis revealed that inhibitory κ B α (I κ B α) protein levels in lung tissues were significantly decreased at 48

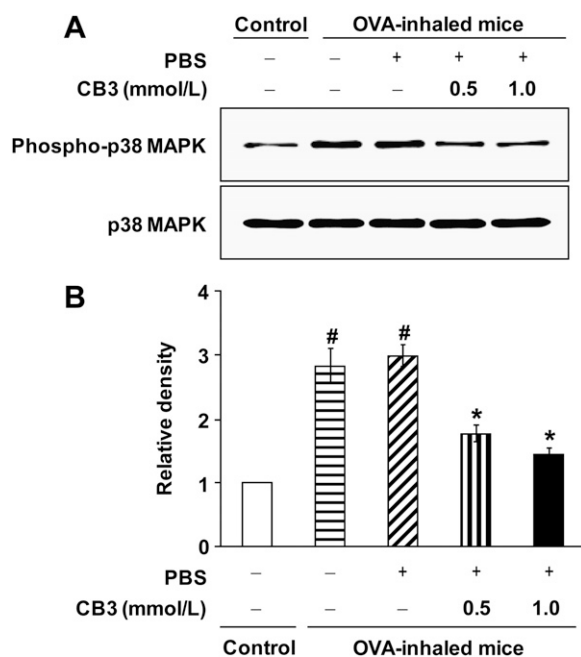


Figure 6. *N*-acetyl cysteine proline cysteine amide (CB3) decreases phosphorylation of p38 mitogen-activated protein kinase (MAPK) levels in primary tracheal epithelial cells isolated from ovalbumin (OVA)-inhaled mice. (A) Western blotting of p38 MAPK and phosphorylated p38 (phospho-p38) MAPK in primary tracheal epithelial cells of OVA-inhaled mice. To evaluate the effect of CB3 on p38 MAPK phosphorylation in airway epithelial cells, the primary tracheal epithelial cells from OVA-inhaled mice were treated with CB3 (0.5 mmol/L or 1.0 mmol/L). The primary tracheal epithelial cells isolated from saline-inhaled mice were used as a control. (B) Densitometric analyses are presented as the relative ratio of phospho-p38 MAPK to p38 MAPK. The relative ratio of phospho-p38 MAPK in tracheal epithelial cells of control mice is arbitrarily presented as 1. *Bars* represent mean $\pm$ SEM from five independent experiments. #*P* < 0.05 versus control mice; **P* < 0.05 versus OVA-inhaled mice without treatment of CB3.

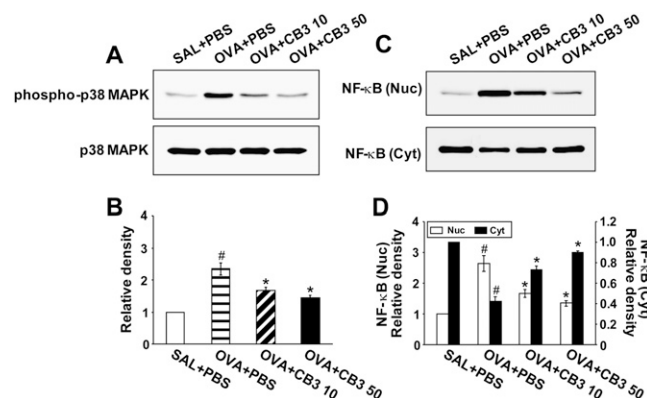


Figure 7. *N*-acetyl cysteine proline cysteine amide (CB3) decreases phosphorylated p38 (phospho-p38) mitogen-activated protein kinases (MAPK) levels and nuclear factor (NF)- κ B p65 activity. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered phosphate-buffered saline (PBS) (SAL+PBS), ovalbumin (OVA)-inhaled mice administered PBS (OVA+PBS), OVA-inhaled mice administered 10 mg/kg of CB3 (OVA+CB3 10), and OVA-inhaled mice administered 50 mg/kg of CB3 (OVA+CB3 50). (A) Western blot analyses of phospho-p38 MAPK. (B) Densitometric analyses are presented as the relative ratio of phospho-p38 MAPK to p38 MAPK. (C) Western blot analyses of NF- κ B p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. (D) Densitometric analyses are presented as the relative ratio of NF- κ B p65 levels in OVA+PBS, OVA+CB3 10, or OVA+CB3 50 to those in SAL+PBS. The relative ratio of phospho-p38 MAPK in the lung tissues or NF- κ B in nuclear protein extracts from the lung tissues of SAL+PBS is arbitrarily presented as 1. *Bars* represent mean $\pm$ SEM from seven mice per group. #*P* < 0.05 versus SAL+PBS; **P* < 0.05 versus OVA+PBS.

hours after OVA inhalation compared with those in the control mice (Figures 8A and 8B). The decrease in I κ B α protein levels after OVA inhalation was significantly blocked by the administration of SB 239063.

In addition, SB 239063 significantly reduced the increase of nuclear NF- κ B p65 levels in lung tissues of OVA-inhaled mice (Figures 8C and 8D), whereas the decrease in NF- κ B p65 levels in the cytosolic fractions after OVA inhalation was substantially inhibited by the administration of SB 239063.

Effect of BAY 11-7085 and SB 239063 on Cellular Changes and on Airway Hyperresponsiveness of OVA-inhaled Mice

Numbers of total cells, neutrophils, lymphocytes, and eosinophils in BAL fluids were increased significantly at 48 hours after OVA inhalation compared with the numbers after saline inhalation (Figure 9A). The elevated number of these cells after OVA inhalation was substantially reduced by the administration of a NF- κ B inhibitor, BAY 11-7085, or a p38 MAPK inhibitor, SB 239063.

In OVA-sensitized and -challenged mice, the dose-response curves of percent R_{rs} shifted to the left compared with that of control mice (Figure 9B). In addition, the percent R_{rs} produced by administration of methacholine of 50 mg/ml increased significantly in OVA-inhaled mice compared with the control mice. The administration of BAY 11-7085 or SB 239063 decreased the levels of percent R_{rs} substantially at the 50 mg/ml of methacholine compared with that in untreated mice (Figure 9B). These results indicate that inhibition of NF- κ B or p38 MAPK activity reduces OVA-induced airway hyperresponsiveness.

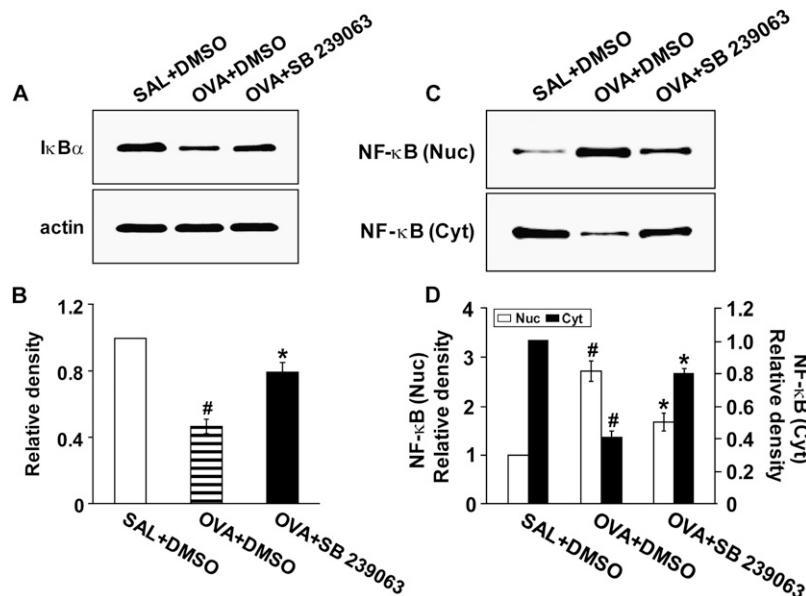


Figure 8. Effects of SB 239063 on inhibitory $\kappa B\alpha$ ($I\kappa B\alpha$) protein levels and nuclear factor (NF)- κB p65 activity in lung tissues of ovalbumin (OVA)-inhaled mice. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered dimethyl sulfoxide (DMSO) (SAL+DMSO), OVA-inhaled mice administered DMSO (OVA+DMSO), and OVA-inhaled mice administered SB 239063 (OVA+SB 239063). (A) Western blotting of $I\kappa B\alpha$ protein. (B) Densitometric analyses are presented as the relative ratio of $I\kappa B\alpha$ to actin. The relative ratio of $I\kappa B\alpha$ in the lung tissues of SAL+DMSO is arbitrarily presented as 1. (C) Western blot analyses of NF- κB p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. (D) Densitometric analyses are presented as the relative ratio of NF- κB p65 levels in OVA+DMSO, or OVA+SB 239063 to those in SAL+DMSO. The relative ratio of NF- κB in nuclear protein extracts from the lung tissues of SAL+DMSO is arbitrarily presented as 1. Bars represent mean $\pm$ SEM from six mice per group. [#] $P < 0.05$ versus SAL+DMSO; ^{*} $P < 0.05$ versus OVA+DMSO.

CB3 Increases IL-10 Protein Levels in OVA-inhaled Mice

Western blot analysis revealed that IL-10 levels in lung tissues were increased at 48 hours after OVA inhalation compared with the levels in the control mice (Figures 10A and 10B). The increased IL-10 levels in lung tissues after OVA inhalation were further increased by the administration of CB3. Consistent with the results, the levels of IL-10 protein in serum (Figure 10C) and BAL fluids (Figure E2) were increased at 48 hours after OVA inhalation compared with the levels in the control mice. The increased IL-10 levels after OVA inhalation were further increased by the administration of CB3.

CB3 Lowers Th2 Cytokines in OVA-inhaled Mice

Enzyme immunoassays revealed that the increased levels of Th2 cytokines in BAL fluids after OVA inhalation were significantly decreased by CB3 in a dose-dependent manner (Figure 10D).

CB3 Lowers OVA-Specific IgE Levels in OVA-inhaled Mice

OVA-specific IgE levels were increased in OVA-sensitized and -challenged mice. A significant decrease in OVA-specific IgE levels in the serum of OVA-inhaled mice was observed with 10 mg/kg CB3 (59.6% reduction) and 50 mg/kg CB3 (73.5% reduction) compared with the levels in OVA-inhaled mice administered with drug vehicle (Figure 10E).

Effect of CB3 Administered after Completion of OVA Inhalation on Cellular Changes in BAL Fluids and on Airway Hyperresponsiveness of OVA-inhaled Mice

To investigate the effect of CB3 on the ongoing or established asthmatic symptoms, CB3 was administered after OVA challenge was completed, and then the changes in BAL cells and airway hyperresponsiveness were determined. Numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly increased in the BAL fluid at 48 hours after the last OVA inhalation compared with the numbers after saline inhalation (Figure 11A). The increased numbers of these cells after OVA inhalation were substantially reduced by the administration of CB3.

In OVA-sensitized and -challenged mice, the dose-response curves of percent R_{rs} shifted to the left compared with that of saline-inhaled mice (Figure 11B). In addition, the percent R_{rs}

produced by administration of 50 mg/ml methacholine increased significantly in OVA-inhaled mice compared with the saline-inhaled mice. OVA-sensitized and -challenged mice treated with CB3 showed a substantial reduction of R_{rs} at

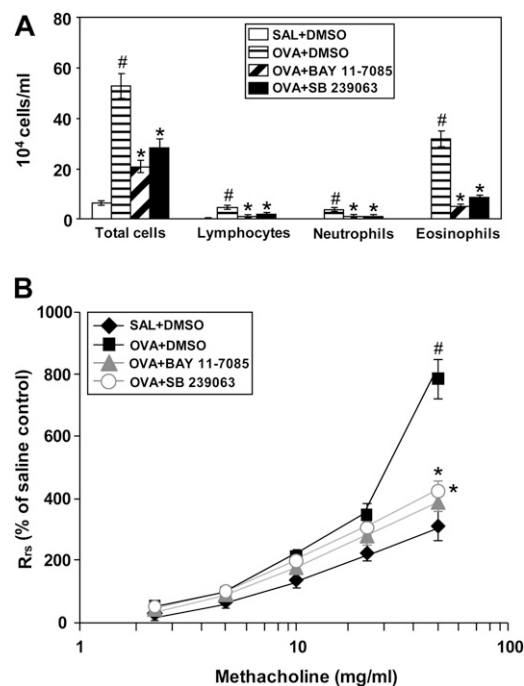
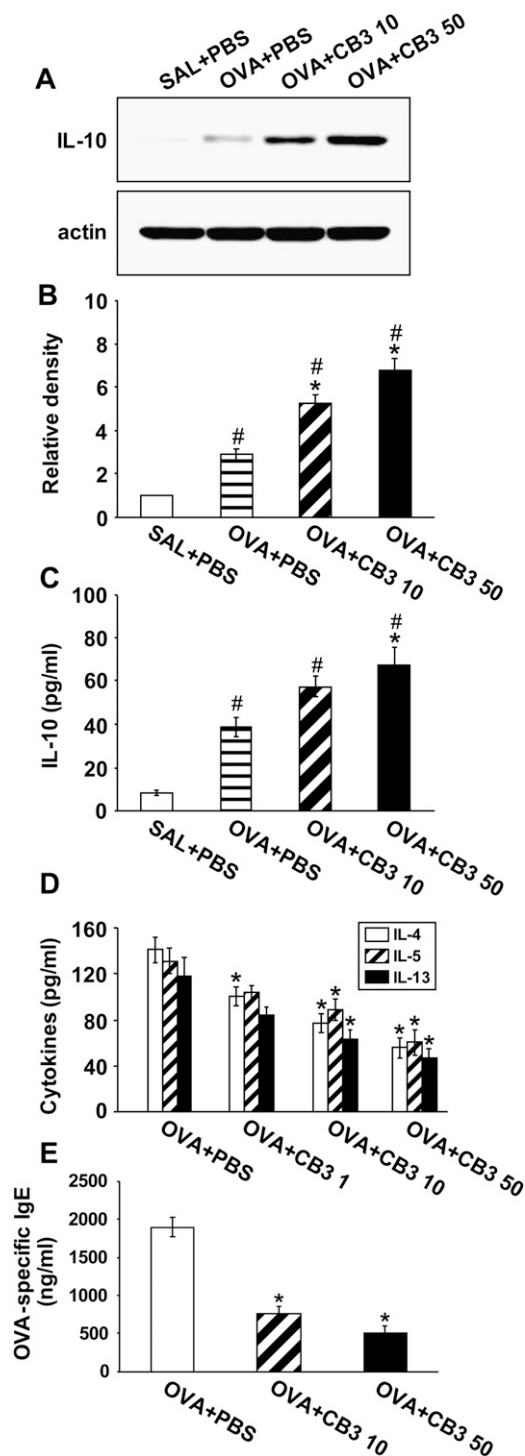


Figure 9. Effect of BAY 11-7085 and SB 239063 on (A) the numbers of total and differential cellular component of bronchoalveolar lavage fluids and (B) airway responsiveness in ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered dimethyl sulfoxide (DMSO) (SAL+DMSO), OVA-inhaled mice administered DMSO (OVA+DMSO), OVA-inhaled mice administered BAY 11-7085 (OVA+BAY 11-7085), and OVA-inhaled mice administered SB 239063 (OVA+SB 239063). Bars represent mean $\pm$ SEM from seven mice per group. [#] $P < 0.05$ versus SAL+DMSO; ^{*} $P < 0.05$ versus OVA+DMSO.

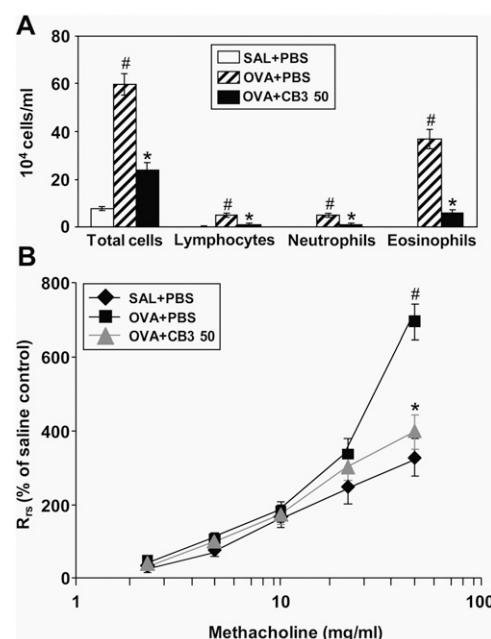


50 mg/ml of methacholine compared with that of mice treated with drug vehicle only. These results indicate that CB3 attenuates OVA-induced airway hyperresponsiveness that is a typical asthmatic symptom.

DISCUSSION

In the present study, we demonstrate for the first time the protective molecular bases of a novel dithiol peptide, *N*-acetyl cysteine proline cysteine amide (CB3), in the development of asthma using an OVA-induced model of allergic airway disease. Our results showed that CB3 treatment restores the OVA-

induced decrease of GSH levels in lungs. Moreover, CB3 treatment resulted in a reduction of Th2 cytokine expression (IL-4, IL-5, and IL-13) and an increase of IL-10 in lung tissues, BAL fluids, and serum. Furthermore, CB3 reduced OVA-specific IgE in serum, airway inflammation, and airway hyperresponsiveness in our animal model of asthma. In addition, CB3 treatment decreased OVA-induced phosphorylation of p38 MAPK, degradation of I κ B α , and nuclear translocation of NF- κ B, suggesting that CB3 modulates p38 MAPK signaling



Effect of *N*-acetyl cysteine proline cysteine amide (CB3) administered after ovalbumin (OVA) inhalation on total cells and differential cellular components in bronchoalveolar lavage (BAL) fluids and on airway responsiveness of OVA-sensitized and -challenged mice. (A) The numbers of total and differential cellular component of BAL fluids. (B) Airway responsiveness. Sampling was performed at 48 hours after the last challenge in saline-inhaled mice administered phosphate-buffered saline (PBS) (SAL+PBS), OVA-inhaled mice administered PBS (OVA+PBS), and OVA-inhaled mice administered 50 mg/kg of CB3 (OVA+CB3 50). Respiratory system resistance (R_s) values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine. Bars represent mean $\pm$ SEM from seven mice per group. # P < 0.05 versus SAL+PBS; * P < 0.05 versus OVA+PBS.

pathway involving I κ B and NF- κ B, thereby attenuating asthmatic pathological features in lungs of OVA-inhaled mice.

One of the causative mechanisms of airway inflammation and airway obstruction is oxidative stress or tilt in the delicate balance of the cellular redox state. In fact, reducing reagents that scavenge ROS and elevate cellular levels of GSH, such as NAC and NAC amides (e.g., AD4) (22, 30, 31), has been shown to attenuate asthmatic pathophysiological characteristics (13, 20). More recently, CB3 has been shown to increase GSH and lower GSSG levels *in vitro* (24). In the present *in vivo* study, our results showed that OVA inhalation reduces GSH levels in lung tissues while increasing GSSG levels and that CB3 restores GSH levels to near normal level and decreases GSSG levels. These observations indicate that CB3 is capable of replenishing GSH level *in vivo*.

A persistent increase in ROS and nitric oxide (NO) in asthma leads to formation of reactive nitrogen species (RNS) and subsequent oxidation and nitration of proteins, which may cause alterations in protein function that are biologically relevant to airway injury/inflammation (32). On the other hand, S-nitrosothiol (SNO), the bioactive endogenous form of NO, including S-nitrosoglutathione (GSNO), has been shown to exert relatively potent bronchodilator activity (33, 34). Studies have also observed a paradoxical drop in GSNO concentration, which occurs despite the presence of elevated nitrite concentrations in asthma (35). Therefore, it is possible that CB3 may ameliorate the asthmatic features through reducing the RNS formation and/or restoring the GSNO level. However, further studies are required to understand the effect of CB3 on these cellular events.

The inflammatory responses in the OVA-inhaled mice, similar to the responses observed in asthma (36), are characterized by (1) up-regulation of several transcription factors, which act on expression of genes that encode inflammatory cytokines, chemokines, and other proinflammatory proteins; (2) elevated levels of Th2 cytokines; and (3) increased level of the OVA-specific IgE in the serum. Among the transcription factors, NF- κ B, a multiprotein complex, is involved in the early cellular defense reactions in higher organisms and plays a pivotal role in immune and inflammatory responses (37–39). Development of oxidant/antioxidant imbalance in asthma leads to the activation of this redox-sensitive transcription factor, NF- κ B (40). ROS have also been directly implicated as second messengers in the activation of NF- κ B, based on the ability to activate NF- κ B by the oxidation of a cysteine-SH group or by the ubiquitination and proteolysis of the I κ B (41, 42). Consistent with these observations, our results showed that NF- κ B levels in nuclear protein extracts from lung tissues are substantially increased in the OVA-induced model of allergic airway disease. In addition, the increase of the number of airway inflammatory cells and airway hyperresponsiveness in our OVA-induced model of allergic airway disease was dramatically reduced by the administration of BAY 11-7085, an NF- κ B inhibitor. The administration of CB3 resulted in a significant reduction of NF- κ B translocation into the nucleus, accompanying reduction in the expression of Th2 cytokines, IL-4, IL-5, and IL-13 in lungs, and OVA-specific IgE levels in serum induced by OVA sensitization and challenge. These results indicate that CB3 reduces the traits of asthmatic symptoms through the modulation of NF- κ B activation.

MAPKs are serine/threonine kinases that include ERKs, JNKs, and p38 MAPK (43–45). The group of proteins termed MAPKs relays signals generated by exogenous and endogenous stimuli to intracellular space via phosphorylation of proteins (43–45). The phosphorylation states and/or activities of all three MAPK members are up-regulated in animal models of asthma

(46, 47). In addition, recent studies have shown the potential antiinflammatory effects of p38 MAPK inhibition in animal models of asthma: SB 239063, a potent and selective p38 MAPK inhibitor, significantly inhibited antigen-induced eosinophilia and promoted apoptosis of eosinophils from BAL fluid (48), and p38 MAPK inhibition reduced antigen-induced airway inflammatory cell infiltration, levels of IL-4, IL-5 and IL-13, mucus hypersecretion, and airway hyperresponsiveness (49). Consistent with these data, our present results showed that phosphorylation of ERK, JNK, and p38 MAPK is markedly increased after OVA inhalation and that CB3 decreases dramatically the levels of phospho-p38 MAPK, but not the levels of phospho-ERK and phospho-JNK, in lung tissues of OVA-inhaled mice. In addition, CB3 inhibited CDDP-induced phosphorylation of p38 MAPK in DHER-14 cells. Supporting the observations, other cysteine amides, such as AD4, have been reported to inhibit CDDP-induced p38 MAPK phosphorylation in neuron cells (16). We have also found that SB 239063, a p38 MAPK inhibitor, not only reduces allergen-induced airway inflammation and airway hyperresponsiveness but also degradation of I κ B α and nuclear translocation of NF- κ B. These findings suggest that modulation of p38 MAPK signaling pathway involving I κ B and NF- κ B can be one of the molecular bases for the beneficial effects of CB3 on allergic airway disease.

IL-10 recently emerged as an antiinflammatory cytokine that inhibits the secretion of proinflammatory cytokines by monocytes and/or macrophages and the release of free oxygen radicals (50). In addition, IL-10 is a representative regulatory cytokine produced by several cell types, including regulatory T cells (Tregs) (51). Tregs can suppress established airway inflammation and airway hyperresponsiveness (51). The role of IL-10 as an antiinflammatory cytokine in allergic inflammation, including asthma, has been reported (52, 53). IL-10 has also been shown to abrogate allergen-induced airway inflammation and hyperresponsiveness and to down-regulate IL-4, IL-5, and IL-13 expression by Th2 lymphocytes (53–55). Additionally, a recent study has demonstrated that antioxidants quench the release of proinflammatory mediators through the up-regulation of antiinflammatory responses mediated by IL-10 (56). Consistent with these findings, Chang and colleagues have reported that NAC treatment further enhances Tregs induced by OVA administration (57). In this current study, the results revealed that levels of IL-10 in lung tissues, BAL fluids, and serum are substantially increased after OVA inhalation and that the enhanced levels of IL-10 protein are further increased by the administration of CB3, suggesting that induction of Tregs is one of the molecular bases of CB3 in ameliorating allergic airway disease.

In summary, we have examined the effects of a newly developed antioxidant dithiol amide, CB3, on allergen-induced airway inflammation and hyperresponsiveness and its relationship with p38 MAPK as well as NF- κ B in mice. Treatment of OVA-inhaled mice with CB3 results in restoration of GSH level, reduction of p38 MAPK phosphorylation and NF- κ B activation, induction of IL-10, and reduction in the levels of Th2 cytokine, OVA-specific IgE, airway inflammation, and airway hyperresponsiveness. Moreover, the inhibition of p38 MAPK or NF- κ B activation with respective inhibitor attenuates the allergen-induced airway inflammation and hyperresponsiveness. The extent of reduction in the airway inflammatory phenotype by the selective p38 MAPK or NF- κ B inhibitor is similar to CB3, strongly suggesting that the regulation of p38 MAPK/NF- κ B pathway could be the molecular mechanism of CB3 action. Taken together, this study provides a crucial molecular basis for the preventive and/or therapeutic capability of CB3, a GSH precursor, for allergic airway diseases.

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