Airway hyperresponsiveness (AHR) and airway inflammation are key pathophysiological features of many respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD). To evaluate the treatment responses of procaterol and CD38 inhibitors in an ozone-induced AHR mice model, we hypothesized that procaterol and two synthetic CD38 inhibitors (Compounds T and H) might have therapeutic effects on the ozone-induced AHR mice model, and the nuclear factor-kappaB (NF-κB) pathway and the CD38 enzymatic activity might be involved in the mechanisms. With the exception of the Control group, ozone exposure was used to establish an AHR model. Male Kunming mice in the Procaterol and CD38 inhibitors groups were treated with an emulsifier of procaterol hydrochloride, Compound T or H. Results indicated that (1) no drug showed severe toxicity in this study; (2) ozone exposure induced airway inflammation and AHR; (3) intragastric treatment with procaterol and Compound T achieved potent therapeutic effects, but Compound H did not show any therapeutic effect; (4) the NF-κB pathway was involved in both the pathogenic mechanisms of ozone and therapeutic mechanisms of procaterol and Compound T; (5) however, the in vivo effect of Compound T was not caused by its inhibitory activity on CD38. Taken together, procaterol and Compound T are potentially good drugs to treat asthma and COPD complicated with ozone exposure.

Key words  airway hyperresponsiveness; CD38 inhibitor; procaterol; ozone exposure; inflammation; nuclear factor-kappaB

Airway hyperresponsiveness (AHR), a common symptom exhibited in many respiratory diseases, is defined by an exaggerated response of the airways to nonspecific stimuli. Among such diseases, asthma is the most common, accounting for about 60–70% of all diseases with symptoms of AHR. Next are chronic obstructive pulmonary disease (COPD) (about 15%) and bronchiectasis (about 10%). Due to the influence of industry and automobile exhaust emissions, ozone is generated and aggregated near to the earth’s surface, to the extent that it has become a common urban environmental air pollutant. Ozone near the earth’s surface is harmful to humans, especially to the airways. Many studies on ozone-induced impairment have thus been performed in view of environmental protection. Ozone can exacerbate respiratory diseases, have a direct correlation with asthma attacks, asthma-related hospital visits and significantly contribute to hospitalizations for respiratory illness. Researchers have therefore been interested to explore the role of ozone on animal models of respiratory diseases. It was reported that ozone inhalation can exacerbate airway inflammation and AHR in allergen-sensitized animal models. Previous studies also found that ozone inhalation can solely induce a murine AHR model with lung injury and inflammation. The β2 selective agonists function as bronchodilators and are used as relievers and controllers of AHR symptoms. Procaterol is a selective β2 agonist, claimed to be more potent than salbutamol. Moreover, procaterol is known to inhibit inflammation and lung fibroblast migration. The expression and distribution of CD38 (a trans-membrane enzyme) is quite extensive, and it has been identified in the airway smooth muscle. Cyclic adenosine diphosphate ribose (ADPR), which is generated by the enzyme catalysis of CD38, can adjust cell contraction through modulating the release of intracellular Ca2+. The contractility of airway smooth muscle is related to the density of Ca2+ in smooth muscle cells. Given that CD38 could affect contraction of smooth muscle cells by adjusting intracellular Ca2+ density, it might play a very important role in the pathogenesis of AHR. Furthermore, CD38 contributes to the AHR symptom in allergy-induced AHR mice model, and the nuclear factor-kappaB (NF-κB) pathway is involved in the induction of CD38 expression; tumor necrosis factor-alpha (TNF-α) induces higher CD38 expression in the cultured airway smooth muscle cells from asthmatics donors than in cells from non-asthmatics donors; AHR is attenuated in CD38 knockout mice compared with wild-type controls. Based on the crystal structure of the CD38-oxidized form of nicotinamide adenine dinucleotide (NAD+) complex, we synthesized a series of simplified N-substituted nicotinamide derivatives and showed that some of them, including Compounds T and H, are good inhibitors of the enzymatic activity of CD38.

NF-κB is a key transcriptional factor that coordinates the expression of various inflammatory genes. Ozone-induced lung inflammation and injury are mediated via NF-κB pathways according to many studies. Phosphorylation of NF-κB p65 (serine 536) plays an important role in regulating activation of NF-κB following exposure to inflammatory stimuli. NF-κB complexes retain in the cytosol through binding to the inhibitory protein, IκB-α. Upon stimulation, IκB-α is phosphorylated, which results in proteasome-mediated degradation of IκB-α. IκB-α degradation plays a pivotal role on NF-κB activation.
To evaluate the treatment responses of procaterol and CD38 inhibitors in an ozone-induced AHR mice model, we hypothesized that procaterol, Compounds T and H might have therapeutic effects on the ozone-induced AHR mice model, and the NF-κB pathway and the CD38 enzymatic activity might be involved in the mechanisms. In this study, we made an ozone-induced AHR mice model, evaluated the therapeutic effects of the three drugs and studied the mechanisms for the involvement of the NF-κB pathway and the CD38 enzymatic activity.

MATERIALS AND METHODS

Animal Groupings and Treatment Fifty adult male Kunming mice weighing 30–35g were purchased from the Department of Laboratory Animal Science of the Peking University Health Science Center (certificate No. SCXK (Jing) 2006–0008). Features of the Kunming mice (the most commonly used outbred mouse strain in China) are white color, low cytoimmunity sensitivity, comparatively large habitus, good adaptive capacity, high breeding coefficient and good survival rate. Mice were housed under controlled temperature (22±2°C), humidity (50±19%) and lighting (6:30 a.m.–6:30 p.m.) in solid bottom cages with food and water available ad libitum. All experiments were performed under the guidelines of the Experimental Laboratory Animal Committee of Peking University Health Science Center. Animal experiments were in strict accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mice were acclimatized for 1 week prior to experimentation, and then randomly divided into the following five groups (10 mice per group): Control group, Model group, Procaterol group, Compound T group and Compound H group. All groups were exposed to ozone with the exception of the Control group. Due to the insolubility of drugs, an emulsifier with 0.5% carboxymethylcellulose sodium and 6% vegetable oil was used as a drug carrier. To reduce the probability of infection, the intragastric route was chosen for administration. Mice in the Control and Model groups were intragastrically administered with 0.2 mL drug carrier every morning (8:30–10:30 a.m.) and night (8:30–10:30 p.m.) over 5 consecutive days (synchronizing with ozone exposure) and on the 6th morning. During the same period, mice in the Procaterol group were intragastrically administered with 0.2 mL×7.5 μg/mL emulsifier of procaterol hydrochloride (Otsuka Co., Ltd., Zhejiang, China); mice in the Compound T and Compound H groups were intragastrically administered with 0.2 mL×5 mg/mL emulsifier of Compound T (Fig. 1A) and 0.2 mL×6 mg/mL emulsifier of Compound H (Fig. 1B), respectively.

Ozone Exposure Protocol After repeated modification, an AHR mouse model was established by way of ozone exposure. The details are listed as follows: mice were placed in plastic boxes (40×30×25 cm³), which were then infused with mixed gas containing 4.0 ppm (ppm) of ozone and fresh air. During 5 consecutive days of intragastric administration, 10 min every afternoon (2:00–4:00 p.m.) was needed to expose the whole body of the mice in the mixed gas. Ozone was generated by directing an air source through an ozone generator (JC-006, Ruda shiji Co., Ltd., Beijing, China). Ozone concentration was continuously monitored with a UV light photometer (UV-100, Tayasaf Co., Ltd., Beijing, China).

Body Weight Changes and Evaluation of Airway Responsiveness All mice were weighed the night before intragastric administration and on the 5th night of intragastric administration. The body weight changes during the 5d were calculated for comparison and analysis.

Airway responsiveness to methacholine (Mch, Sigma, St. Louis, MO, U.S.A.) was evaluated using an AniRes 2005 animal lung function analysis system (Beijing Bestlab High-Tech Co., Ltd., China) 2 h after the last intragastric administration on the 6th day. Mice were anesthetized with pentobarbital sodium (150 mg/kg, Sigma, St. Louis, MO, U.S.A.) by intraperitoneal injection. The mice were then intratracheally intubated, placed in a rodent plethysmograph and mechanically ventilated with a constant pressure of 10 cmH₂O at a respiratory rate of 90 breaths per min. After reaching a stable tracing, lung resistance (RL) and dynamic pulmonary compliance (Cdyn) were measured at baseline and following intravenous administration of Mch. Mch was injected via the jugular vein through a micro-infusion pump in progressive doses (120 μg/mL×0.1 mL, 240 μg/mL×0.1 mL, 480 μg/mL×0.1 mL, 960 μg/mL×0.1 mL, 1.92 mg/mL×0.1 mL) about every other 5 min. The RL and Cdyn signals were collected continuously from 5 s to 1 min for each dose, and the mean RL and Cdyn were recorded to reflect the change in airway responsiveness.

Collection of Blood, Bronchoalveolar Lavage Fluid (BALF) and Lung Tissue After completion of the airway responsiveness evaluation, anesthetized mice were bled from the orbits, and 1 V blood was kept in 19 V anticoagulant buffer (1.5 mM citric acid, 13.5 mM sodium citrate, 90% phosphate buffered saline (PBS)) at 4°C for counting of erythrocytes and leukocytes. The collecting of blood was followed by exsanguination via the abdominal artery. For BALF collection, the right lung was separated by ligating at the right mainstem bronchus; BALF was collected from the left lung. The left lung was lavaged three times with 0.5 mL PBS. The total collected BALF was centrifuged at 206×g for 5 min at 4°C. The supernatant was extracted, and then the malondialdehyde (MDA) concentration in the supernatant was assayed by MDA detecting kits (Jiancheng Bioengineering Institute, Nanjing, China). The pellet was resuspended with 500 μL leukocytes diluent (Jiancheng Bioengineering Institute, Nanjing, China), and the leukocytes in BALF were counted by the help of a counting slide. After BALF collection, right lung tissues were removed from the chest cavity. Half of the right lung tissues (5 in each group) were fixed with 4% paraformaldehyde at room temperature for next histopathological analysis. The other half...
were stored at −80°C for Western blotting and HPLC detection.

Histopathological Analysis After fixation, right lung tissues were embedded in paraffin. The lower lobes were sectioned at 4 μm, stained with hematoxylin & eosin (H&E) or periodic acid schiff (PAS), analyzed using an Olympus IX73 microscope for 10× objectives. Pictures were taken using an Olympus DP72 charge-coupled device (Olympus Co., Ltd., Tokyo, Japan). Histological changes were scored in terms of the extent of lung edema, infiltration of inflammatory cells into lung tissues and mucus secretion, as follows: (0) none; (1) slight; (2) mild; (3) moderate; and (4) severe. The three individual scores were added together to obtain a total lung pathological score. The grading data are presented in histograms.

Western Blotting Western blotting was performed as described previously.25 Briefly, lobe of lung tissues were lysated by tissue lysis buffer (50 mM Tris–HCl, PH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1 mM Na3VO4, 1% Triton X-100, 0.25% Sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 mM dithiothreitol (DTT) along with the protease inhibitor cocktail). Total protein concentration of the lung tissues was determined using a bicinchoninic acid protein assay reagent kit (Beijing CellChip Biotechnology Co., Ltd., China). Equal amounts (20 μg) of protein extracts were subjected to a 12.5% SDS–polyacrylamide electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Millipore, MA, U.S.A.) for 35 min at 250 mA. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline solution with Tween (TBST; 20 mM Tris–HCl (pH 7.4), 0.15M NaCl, and 0.05% Tween-20) for 2 h at room temperature under agitation and processed for immunodetection. P-NF-κB p65 Antibody (Ser 536) (1:160, Santa Cruz Biotechnology, Inc., Texas, U.S.A.), IκB-α Antibody (C-21) (1:700, Santa Cruz Biotechnology, Inc., Texas, U.S.A.) and Actin Antibody (I-19) (1:800, Santa Cruz Biotechnology, Inc., Texas, U.S.A.) were used as the primary antibodies, and horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (1:2000, M&C Gene Technology, Beijing, China) was used as the secondary antibody. Actin was used as an internal standard. An enhanced chemiluminescence detection system was applied to detect the target proteins. The optical density of each band was quantified by using ImageJ software (NIH, Maryland, U.S.A.). Levels of p-NF-κB p65 and IκB-α were normalized against actin.

HPLC Analysis of cADPR and ADPR Concentration in Lung Tissues Frozen lung tissues were washed three times with PBS, added with 15 μL cold 0.6 M perchloric acid (PCA) per 1 mg lungs, and fully ground with a glass homogenizer on ice. PCA was removed by mixing the aqueous sample with an organic phase containing 3 vol. of chloroform to 1 V of tri-n-octylamine. Then, 1 part sample was added to 4 parts organic phase and vortexed for 1 min. Following a centrifugation step of 1500×g 10 min at 4°C, the upper aqueous layer containing the cADPR and ADPR was removed and adjusted to pH 8 with an equal amount of 200 mM Tris base. Before HPLC detection, the residual protein and other high-molecular-weight Compounds were then removed from samples by centrifugation for 8100×g 30 min at 4°C via ultrafiltration centrifuge tubes (10kDa MW cut-off; Millipore, MA, U.S.A.).

Reversed Phase-HPLC analysis of nucleotides was performed on a 250×4.6 mm hypersil BDS C18-5 μm column (Thermo, MA, U.S.A.). The separation was performed at a flow rate of 1 mL/min with RP-HPLC buffer (20 mM KH2PO4, 5 mM tetrabutylammonium dihydrogen phosphate, pH 6) containing gradient amounts of methanol. Column temperature was 30°C, whereas the autosampler temperature was 4°C. The gradient used for separation was (% methanol) 0 min (6.5), 3.5 min (7.5), 5.5 min (16), 8 min (25), 16 min (25), 18 min (6.5) and 27 min (6.5). ADPR and cADPR were detected using an UV detector (Waters 2998, MA, U.S.A.) at 270 nm. Integration of peaks was performed with the data-acquisition system from Waters Instruments (Waters Corporation, MA, U.S.A.).

Statistical Analysis Values are expressed as mean ± S.D. The SPSS 16.0 software was used for statistical analysis. Mann–Whitney test was used to compare the total lung pathological score between groups. Other statistical comparisons between groups were evaluated by ANOVA. Values of p<0.05 were considered statistically significant.

RESULTS

Body Weight Changes and Peripheral Blood Cell Analysis As can be seen from Fig. 2A, the body weight of mice in the Control group increased, but mice in the other groups exposed to ozone lost weight to varying extents. The body weight changes between the Model group and the Control group were significant. Compared with the Model group, treatment with procaterol, Compound T or H did not significantly change the body weight.

![Fig. 2](image-url)
Next, the number of erythrocytes and leukocytes in the blood were examined to evaluate toxic effects of procaterol, Compounds T and H preliminarily. Compared with the number of erythrocytes and leukocytes in the Model group, no drug treatment affected the number of erythrocytes and leukocytes (data not shown).

**BALF Assay**

To study the level of inflammation and oxidative damage, the number of leukocytes and MDA concentration in BALF were examined. As shown in Fig. 2B, the numbers of leukocytes in the Model group were more than that in the Control group ($p < 0.05$). Compared with the number of leukocytes in the Model group, treatment with procaterol and Compound T showed a tendency to decrease ($p = 0.068, 0.090$). The MDA concentration in BALF was significantly elevated by ozone exposure (Fig. 2C). Treatment with procaterol and Compound T significantly reduced the MDA concentration. However, treatment with Compound H did not show such pharmacological effects.

**Effect on Airway Responsiveness**

**RL and Cdyn graphs** produced by the AniRes 2005 animal lung function analysis system are shown in Fig. S1. At first, RL and Cdyn values of each mouse were recorded for six different conditions (baseline, five gradient doses of Mch challenge), and then 1 was set as the baseline value of RL and Cdyn of each mouse. To minimize the baseline value variation of mice and reflect the true airway responsiveness to Mch challenge, values on conditions of Mch challenge were normalized against the absolute baseline values. Values were expressed as fold change relative to the baseline values, and the outcomes were called RL ratio and Cdyn ratio, respectively. The RL ratio and Cdyn ratio were used to evaluate airway responsiveness.

As shown in Fig. 3, mice in the Model group exhibited a significantly elevated RL ratio and reduced Cdyn ratio compared with mice in the Control group. This illustrates that ozone exposure by this method successfully caused AHR. It should be noted that treatment with procaterol and Compound T could significantly ameliorate AHR symptoms. However, treatment with Compound H did not show such pharmacological effects.

**Effect on Pathological Changes**

Compared with the Control group, ozone exposure induced lung edema, infiltration of inflammatory cells and mucus hyper-secretion (Fig. 4). Treatment with procaterol and Compound T showed a marked reduction of pathological changes (Figs. 4A, B) and total lung pathological scores (Fig. 4C). But treatment with Compound H did not reduce the inflammatory reaction ($p > 0.1$).

**Activation of NF-κB**

Our results showed that ozone exposure significantly induced phosphorylation of p65 (Fig. 5). Treatment with procaterol and Compound T strikingly suppressed the NF-κB p65 phosphorylation levels ($p < 0.05$), but they were not affected by Compound H treatment. Ozone exposure also significantly induced IκB-α degradation (Fig. 6). Treatment with procaterol and Compound T suppressed the ozone-induced degradation of IκB-α ($p < 0.05$). But treatment with Compound H did not increased the IκB-α level ($p > 0.1$).

**Influence on CD38 Catalytic Activity**

To study the CD38 catalytic activity of lungs of mice in all groups, both cADPR and ADPR levels in lungs were detected by HPLC. From a comparison of the peak areas of the unspiked samples (Fig. 7c) with those of the spiked samples (Fig. 7b), it was found that cADPR concentration in unspiked samples was under the detection limit of this method; but ADPR in unspiked samples was one single baseline-resolved peak with the same retention time as that of ADPR standards. Integration of peaks, linear correlation analysis and a series of mathematical conversions could convert the ADPR peak area to the ADPR content of lungs (data not shown). According to a comparison of the ADPR levels in lungs among six groups (Figs. 7d–i), we got following results: ozone exposure did not result in any obvious changes to the ADPR levels.
in mice lungs; treatment with Compound H or T could significantly reduce ADPR levels in lungs, but procaterol did not show such activity.

**DISCUSSION**

The results of the present study indicate that no drug showed severe toxicity in this study. The ozone exposure protocol induced airway inflammation and AHR. Intragastric treatment with procaterol and Compound T achieved potent therapeutic effects, but Compound H did not show a protective role against the AHR mice model. The NF-κB pathway was involved in both the pathogenic mechanisms of ozone and therapeutic mechanisms of procaterol and Compound T. However, the *in vivo* effect of Compound T was not caused by its inhibitory activity on CD38.

As far as drug toxicology is concerned, body weight changes and peripheral blood cell counting were used as toxicological indicators. Our results showed that no drug treatment resulted in changes to these toxicological indicators.

AHR and airway inflammation are key pathophysiological features of asthma and COPD.\(^{26,27}\) In this study, compared with the Control group, the ozone-induced AHR mice model exhibited higher MDA levels in BALF, more leukocytes in BALF, pathological features of airway inflammation, and a significantly elevated RL ratio and reduced Cdyn ratio. MDA, as a by-product of lipid peroxidation, has been identified in the past as a marker of oxidative damage.\(^{28,29}\) Accumulated oxida-
secretion, airway smooth muscle hypertrophy and infiltration are characterized by goblet cell hyperplasia, mucus hyper-secretion. Lung pathological changes in the AHR murine model control group.

The numbers of leukocytes in BALF and histological analysis group (Fig. 2C) are caused by the oxidative damage of ozone. It is apparent that the elevated BALF MDA levels in the Model group (Fig. 4) are caused by the oxidative damage of ozone. The numbers of leukocytes in BALF and histological analysis of lung tissue are widely used as indicators of airway inflammation. Lung pathological changes in the AHR murine model are characterized by goblet cell hyperplasia, mucus hyper-secretion, airway smooth muscle hypertrophy and infiltration of inflammatory cells. Due to the lack of bronchia or bronchus in the PAS stained lung tissues and the rare distribution of globlet cells in the bronchiole and terminal bronchiole, the number of globlet cells were hard to calculate in this study. And the airway smooth muscle hypertrophy could not be recorded. So lung edema, inflammatory cells infiltration and mucus hyper-secretion were taken into consideration. RL and Cdyn response to Mch challenge is regarded as the classic method to evaluate airway response in both experimental and clinical studies. In short, it is likely that the ozone exposure protocol induces airway inflammation and AHR, and that they may be the chain reaction of ozone-induced oxidative damage.

In the present study, treatment with procaterol and Compound T decreased the number of leukocytes in BALF to a certain extent, reduced the MDA concentration in BALF, and ameliorated the AHR symptoms (Fig. 3) and lung pathological changes (Fig. 4) clearly. In contrast, Compound H did not show any obvious therapeutic effect. Although controversies of anti-inflammatory effects remain on other β2 selective agonists, numerous pharmacological studies have shown the positive anti-inflammation and bronchodilatory effects of procaterol in murine AHR models. A randomized, double-blind, parallel group study in 140 patients with moderate acute asthma also demonstrated that nebulized procaterol is effective in improving peak expiratory flow rate and decreasing the asthma score.

Procaterol can inhibit the activation of NF-κB pathway in the previous studies. In this study, ozone exposure resulted in elevated phosphorylation levels of NF-κB p65 and degradation of IκB-α. Treatment with procaterol and Compound T decreased the phosphorylation levels of NF-κB p65. Consistent with this observation, procaterol and Compound T suppressed the ozone-induced degradation of IκB-α. But molecules in the NF-κB pathway were not affected by Compound H treatment. It suggests that ozone exposure may induce oxidative damage of lungs, leading to lung inflammation and AHR via the NF-κB pathway. Procaterol and Compound T may exert its therapeutic role via this pathway, although the exact mechanisms by which this occurs are potentially important and yet to be investigated.

CD38 is a multifunctional enzyme with both ADP-ribosyl cyclase and cADPR hydrolase activities, being capable of cleaving NAD⁺ to cADPR and hydrolyzing cADPR to ADPR. Both cADPR and ADPR levels in lungs, therefore, provide an accurate reflection of CD38 catalytic activity. However, cADPR contributes little to the products of CD38-catalysed metabolism of NAD⁺, and the major (96%) product is ADPR. Moreover, cADPR is unstable at high temperatures and acidic conditions, whereby it transforms to ADPR. The above reasons indicate that ADPR is a good indicator of CD38 catalytic activity. In our study, treatment with Compound T or H could significantly reduce ADPR levels in lung, showing a similar efficacy for inhibiting CD38 catalytic activity. Compound T and procaterol (which did not show any activity of inhibiting CD38 catalytic activity), however, were able to treat oxidative stress induced AHR and lung inflammatory pathological changes. We thus conclude that the in vivo effect of Compound T is not caused by its inhibitory activity on CD38.

Taken together, procaterol and Compound T are potentially good drugs to treat asthma and COPD complicated with ozone exposure. Further studies should be performed for the overall evaluation of Compound T’s potency for the therapy of AHR diseases such as asthma, COPD, bronchiectasis and so on.

Acknowledgments We thank Dr. Mark Bartlam for his help with language. This work was supported by the Research Fund of the State Key Laboratory of Natural and Biomimetic Drugs, Peking University, People’s Republic of China. The content is solely the responsibility of the authors and does not necessarily represent the official views of the State Key Laboratory of Natural and Biomimetic Drugs, Peking University,
People’s Republic of China. This work was also supported by the National Natural Science Foundation of China (NSFC 91213302).

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