



## Protective effects of pyrrolidine dithiocarbamate against airway inflammation in the ovalbumin-induced mouse model

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### ABSTRACT

Pyrrolidine dithiocarbamate (PDTC) is known to exert anti-tumor and anti-inflammatory effects. However, the effects of PDTC against airway inflammation and its underlying mechanisms have not been reported. In the present study, we examined the protective effects of PDTC in a murine model of asthma induced by ovalbumin. PDTC reduced the number of infiltrating inflammatory cells in concert with reduced eosinophil peroxidase (EPO) activity in bronchoalveolar lavage fluid. In parallel, PDTC decreased airway hyperresponsiveness in a dose dependent manner. All these effects were correlated with heme oxygenase-1 (HO-1) mRNA and protein induction, and reversed by ZnPP, a HO-1 inhibitor. In addition, PDTC reduced the secretion of Th<sub>2</sub> cytokines such as IL-4 and IL-5, whereas ZnPP blocked the inhibitory effects of PDTC on Th<sub>2</sub> cytokine secretion. These results suggest that PDTC protects against airway inflammation at least in part via HO-1 induction, and that inhibitory action on Th<sub>2</sub> cytokines may be associated with the protective mechanism of PDTC.

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### 1. Introduction

Asthma is a complex disease characterized by chronic airway inflammation, hyperresponsiveness of airway smooth muscle to various spasmogens, and periodic airway obstruction resulting in decreased lung function (Bochner et al., 1994; Barnes, 1996). In these conditions, the airway wall is infiltrated by a variety of inflammatory cells, including eosinophils, mast cells, macrophages, Th<sub>2</sub> lymphocytes and neutrophils, and is damaged by the mediators produced by the cells (Bousquet et al., 1990; Barnes et al., 1998). For example, IL-4 produced by Th<sub>2</sub> cells induces the growth and development of Th<sub>2</sub> cells, B cells and mast cells with concurrent IgE secretion while IL-5 induces eosinophil generation in bone marrow and lung eosinophilia (Foster et al., 2002; Zimmermann et al., 2003).

PDTC was initially regarded to be a potent inhibitor of nuclear factor-κB (NF-κB). It has since been used as an anti-oxidant compound to counteract the toxic effects of proinflammatory cytokines (Li et al., 2001; Muller et al., 2000; Liu et al., 1999), and has been advocated for use in the treatment of immune deficiency syndrome and neurode-

generative diseases (Schreck et al., 1992; Reisinger et al., 1990). Besides the inhibitory actions on NF-κB, PDTC also has the potential to activate gene expression of endogenous anti-oxidants, such as superoxide dismutase via an NF-κB independent pathway (Borrello and Demple, 1997). Recently, PDTC has been reported to induce HO-1 in cultured cells (Hartsfield et al., 1998) and in *in vivo* models (Hata et al., 2003; Muhl et al., 2004) independent of NF-κB, which provides protection against oxidative stress.

HO catalyzes the oxidation of heme to carbon monoxide (CO), iron and biliverdin, which is then converted to bilirubin by biliverdin reductase. Three isoforms of HO have been identified; inducible HO-1 and constitutively expressed HO-2 and HO-3 (Maines, 1988; Montellano, 2000). In contrast to HO-2 and HO-3, the HO-1 isoform has long been implicated as a cytoprotective protein against oxidative stress and inflammatory response. For example, prior induction of HO-1 reduced tissue injury in various experimental models of acute and chronic inflammation such as hyperoxic lung injury, ischemia/reperfusion injury, uveitis, hepatitis and colitis; in contrast enzymatic inhibition of HO-1 activity exacerbated the injuries (Fujita et al., 2001; Juan et al., 2001; Otterbein et al., 1999; Nakahira et al., 2003; Wang et al., 2001). In addition, HO-1 was shown to attenuate airway inflammation and hyperreactivity in guinea pig (Almolki et al., 2004).

Until now, no study has been reported to show the effects of PDTC on airway inflammation and its underlying mechanisms. Therefore,

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the aim of the present study was to examine whether *in vivo* administration of PDTC protects against ovalbumin-challenged murine airway inflammation, and if so, whether HO-1 is involved in its action. Our data suggest that PDTC has beneficial effects on airway inflammation at least in part via HO-1 induction, and the protective effects of PDTC may be associated with the inhibition of IL-4 and IL-5 production.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6J (8–9 wks) mice, weighing 20–25 g, were produced and maintained in specific pathogen-free conditions at our animal breeding facility. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (National Academy Press, 1996), and approved by the animal care and use committee of the KRICT in Korea.

### 2.2. Reagents

Zinc protoporphyrin IX (ZnPP) and cobalt protoporphyrin IX (CoPP) were obtained from Porphyrin Products (Logan, UT). Aluminum hydroxide [Al(OH)<sub>3</sub>] was purchased from Pierce Biotechnology (Rockford, IL). Pyrrolidine dithiocarbamate (PDTC), ovalbumin, *o*-phenylenediamine (OPD) and total RNA extraction kit were obtained from Sigma Chemical (St. Louis, MO). Reverse transcription system for RT-PCR was obtained from Promega (Madison, WI). All other reagents were purchased from Sigma Chemical unless indicated otherwise.

### 2.3. Induction of asthma and chemical treatment

C57BL/6J mice were immunized intraperitoneally (i.p.) with 20 µg ovalbumin (grade V) in 0.2 ml of 2.3 mg/ml suspension of [Al(OH)<sub>3</sub>]. A second injection was given 10 days later with 10 µg ovalbumin with the same concentration of [Al(OH)<sub>3</sub>]. On day 6th after the secondary immunization, mice were challenged with an aerosol of 5% ovalbumin for 20 min with ultrasonic nebulizer and the challenge was repeated for 3 days. In all experiments, negative control groups were treated identically with saline instead of ovalbumin in both the sensitization and challenge stages of the protocol. An injection of PDTC (50, 100, 200 mg/kg, i.p.), CoPP (5, 10, 30 mg/kg, i.p.), an inducer of HO-1, and ZnPP (10 mg/kg, i.p.), a HO-1 inhibitor was given at 12 h before challenge with aerosolized ovalbumin. On day 20th after the first immunization, the lung function was monitored by measuring airway responsiveness to methacholine (Mch, 25 and 50 mg/ml). Positive control mice were treated with saline only instead of chemicals. For all protocols, 8–10 animals were used for each group, and no visible side effects including lethality, sluggishness, sleeping and ruffled hair were observed in any of the experimental groups studied.

### 2.4. Analysis of airway hyperresponsiveness

Airway responsiveness to methacholine was measured on day 20th after the first immunization in conscious and unrestrained mice as described previously (Hamelmann et al., 1997). Mice were placed in a barometric plethysmographic chamber (Allmedicus Co, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (0, 25 and 50 mg/ml) was then nebulized through an inlet of the main chamber for 1 min, and the readings were taken and averaged for 3 min after each nebulization. Airway responsiveness was expressed by enhanced pause (Penh), a calculated value that correlates with airway resistance.

$$\text{Penh} = (\text{Te}/\text{Tr} - 1) \times (\text{pef}/\text{pif})$$
 (Te, expiration time; Tr, relaxation time; pef, peak expiratory flow; pif, peak inspiratory flow). Results are expressed as the percentage increase of PenH following the challenge with each concentration of methacholine where the baseline PenH (negative control group) is expressed as 100% as previously described (Lee et al., 2004).

### 2.5. Extraction of bronchoalveolar lavage fluid and assay of EPO activity

On day 21st (after measurement of airway hyperresponsive-ness), mice were anesthetized by i.p. injection of 0.25 ml of sodium pentobarbitone (60 mg/ml). After the chest was opened, the left lungs were ligated at the left main branch of the trachea by using surgical forceps, and the right lungs were slowly lavaged twice with 0.8 ml of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline (PBS). The remaining lobe of the left lung was stored for RNA extraction (upper lobe of the left lung) and histological analyses (bottom lobe of the left lung). The recovered fluid (80 to 90% of the injected volume) was centrifuged at 100 ×g for 5 min at 4 °C. The supernatant was removed for the analysis of EPO activity, and the cell pellet was resuspended in saline after brief hypotonic exposure to lyse red blood cells. Total cell count was determined by using a hemocytometer, and the slides were prepared using a cytospin apparatus (Cellspin, Hanil Science, Korea) and stained with Diff-Quick (Merck, Germany). Cell types were identified and scored as percentages based on the light microscopic evaluation by morphological characteristics. For measurement of EPO activity in bronchoalveolar lavage fluid, we used a colorimetric assay based on the oxidation of OPD by EPO in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Strath et al., 1985). A volume of 100 µl bronchoalveolar lavage supernatant was transferred into 96 well microplate in duplicate, and substrate solution containing 0.1 mM OPD, 0.05 M Tris-HCl (pH 8.0), Triton X-100 and 1 mM H<sub>2</sub>O<sub>2</sub> was added to each well. After incubation at room temperature for 20 min, the reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> and optical density was measured at 492 nm.

### 2.6. Analyses of lung histology

Unlabeled lungs were fixed with 3.7% formalin, and then paraffin-embedded and sectioned (4-µm). Lung inflammation was assessed by microscopic examination of sections stained with hematoxylin and eosin (H & E). All slides were read in a blinded manner, and severity of the inflammation was quantified on a 0–5 scale by both the degree of inflammation and its distribution. The appearances in negative control mice were defined as 0, and maximum changes were scored as 5. For inflammatory infiltrates, a score of 0=no inflammatory infiltrates, 1=occasional inflammatory infiltrates in a single bronchus, 2=inflammatory infiltrates in two bronchi, 3=inflammatory infiltrates around bronchi and blood vessels, 4=inflammatory infiltrates in most of the bronchi and blood vessels, and 5=wide spread inflammatory infiltrates around majority of the vessels with alteration on epithelial thickening and presence of mucus. Three lung sections were analyzed per mice, and the final values were taken as a mean of all mice studied per group.

### 2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the lung tissue using Trizol reagent as suggested by the manufacturer, and 1 µg of RNA was used for RT-PCR according to the manufacturer's instructions. The following sequence was performed for each PCR reaction. Amplification of HO-1 was initiated with 2 min of denaturation at 95 °C followed by 35 cycles at 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min (Mastcycler, Eppendorf). Primer sequences for the analysis of HO-1 and β-actin mRNA were as follows. HO-1, 5'-ACTTTCAGAAGGGTCAGGTGCC-3'

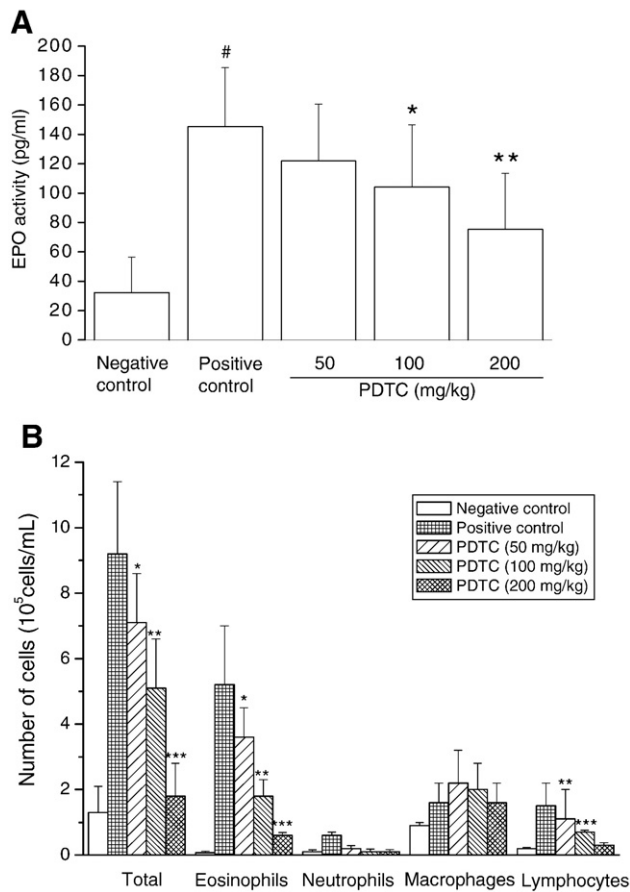


**Fig. 1.** Effect of PDTC on EPO activity and cellular composition of bronchoalveolar fluid. Ovalbumin-sensitized mice were given three daily treatments of saline or various doses of PDTC (50, 100 and 200 mg/kg, i.p.) at 12 h before aerosolized ovalbumin challenge. Bronchoalveolar lavage fluid was obtained at 62 h after last aerosolized ovalbumin challenge. (A) EPO activity was measured. (B) The number of cellular infiltrate was determined ( $n=10$  animals per group). Negative control groups were sensitized and repeatedly challenged with saline instead of ovalbumin. Results are representative of at least three similar experiments performed on different experimental days. Values are means  $\pm$  S.D. <sup>#</sup> $P<0.05$  vs negative control, <sup>\*</sup> $P<0.05$  and <sup>\*\*</sup> $P<0.01$  vs positive control group.

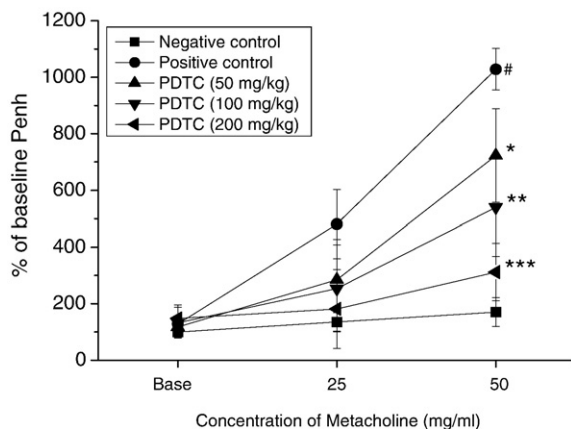


**Fig. 2.** Effect of PDTC on airway hyperresponsiveness. Ovalbumin-sensitized mice were given three daily treatments of saline or various doses of PDTC (50, 100 and 200 mg/kg, i.p.) at 12 h before aerosolized ovalbumin challenge. Using whole body plethysmography, airway hyperresponsiveness was measured at 48 h after last aerosolized ovalbumin challenge by increasing doses of inhaled methacholine. Values are means  $\pm$  S.D. from three independent experiments of the response from 8–10 animals. <sup>#</sup> $P<0.05$  vs negative control, <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$  and <sup>\*\*\*</sup> $P<0.001$  vs positive control group.

and 5'-TTGAGCAGGAAGGCGGTCTTAG-3' (524 bp);  $\beta$ -actin, 5'-CCTTCTACAATGAGC-3' and 5'-ACGTCACACTTCATG-3' (594 bp). After amplification, the samples were separated on a 1.5% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide (EtBr) and photographed. Digital photographs were assessed with image analysis software, and mRNA expression of HO-1 was evaluated as the ratio to  $\beta$ -actin.

## 2.8. Assay of IL-4 and IL-5

The levels of IL-4 and IL-5 were measured in bronchoalveolar lavage fluid using BD bioscience ELISA kit (San Jose, CA).

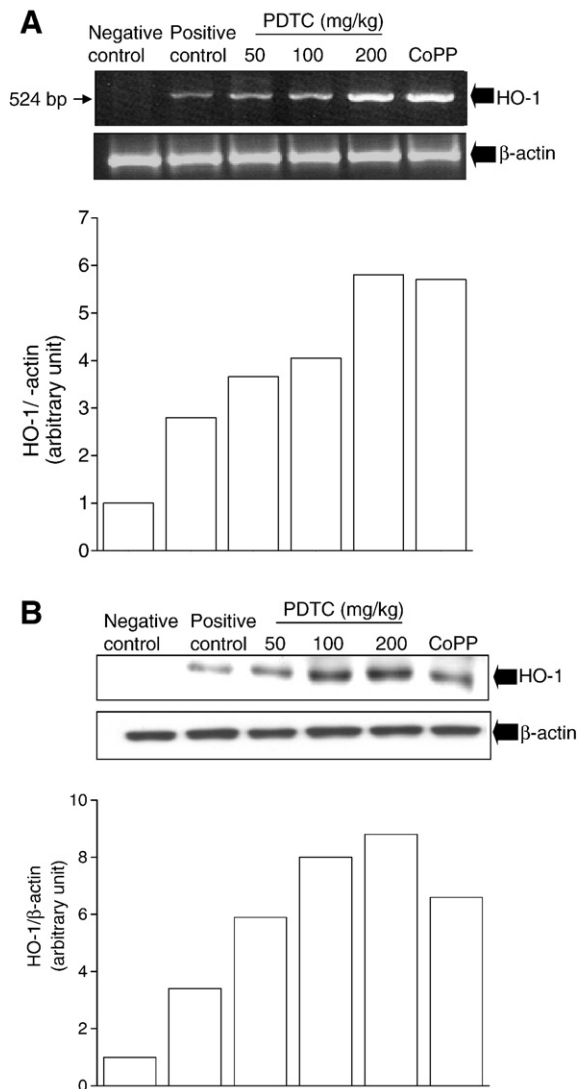


**Fig. 3.** Effect of PDTC on HO-1 mRNA and protein levels in ovalbumin-challenged mice. Ovalbumin-sensitized mice were given three daily treatments of saline or various doses of PDTC (50, 100 and 200 mg/kg, i.p.) at 12 h before aerosolized ovalbumin challenge. On 3rd day after last aerosolized ovalbumin challenge, mice were sacrificed and the lung was excised. (A) Total cellular RNA was isolated from the lung treated with PDTC as described above, and RT-PCR was performed using specific primers for HO-1 and  $\beta$ -actin. (top). HO-1 mRNA expression was normalized to  $\beta$ -actin mRNA (bottom). (B) Protein expression of HO-1 in the lung was determined by western blot analysis after ovalbumin challenge with or without PDTC (top). HO-1 protein was normalized to  $\beta$ -actin (bottom). CoPP (30 mg/kg, i.p.) was used as a HO-1 inducer. Negative control groups were sensitized, and repeatedly challenged with saline instead of ovalbumin. Results are representative of at least three similar experiments performed on different experimental days ( $n=10$ ).

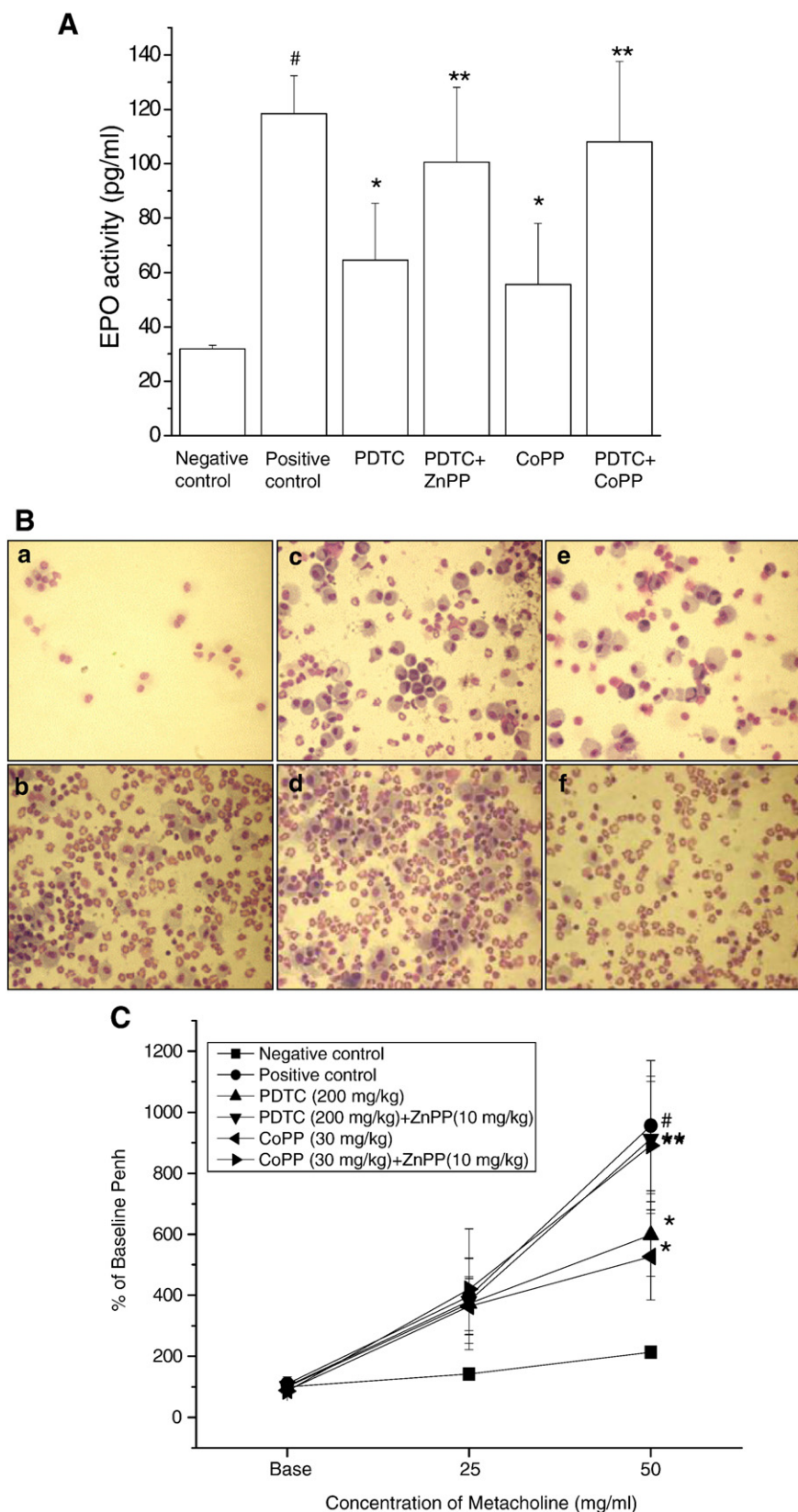


**Fig. 4.** Effect of ZnPP on EPO activity and eosinophil infiltrations in ovalbumin-challenged mice. Mice were pre-treated with ZnPP (10 mg/kg) plus PDTC (200 mg/kg, i.p.) or CoPP (30 mg/kg, i.p.) at 12 h before challenges with aerosolized ovalbumin. Bronchoalveolar lavage fluid was obtained at 62 h after last aerosolized ovalbumin challenge. (A) EPO activity was measured. (B) Detection of eosinophils in negative control (a), saline (b), PDTC (c), PDTC plus ZnPP (d), CoPP (e) and CoPP plus ZnPP (f) is shown (Diff-Quick staining, original magnification  $\times 100$ ). (C) Airway hyperresponsiveness was determined by whole body plethysmography as depicted in Fig. 2. Results are representative of at least three similar experiments performed on different experimental days ( $n=8-10$  per group). Values are means  $\pm$  S.D. #  $P<0.05$  vs negative control, \* $P<0.05$  vs positive control group, and \*\* $P<0.01$  vs PDTC or CoPP-treated group.

### 2.9. Statistical analysis

Data are represented as means  $\pm$  S.D. of more than three separate experiments with  $n=8-10$  in each group. The significance of difference from the respective control for each experimental test condition was examined by using unpaired Students' *t*-test and one way ANOVA. A *P* value  $<0.05$  was regarded as a statistical significance.

## 3. Results

### 3.1. PDTC treatment reduces EPO activity and the number of inflammatory cells in bronchoalveolar lavage fluid

To evaluate whether PDTC treatment resulted in anti-inflammatory effects on ovalbumin-induced airway inflammation model, we assessed the EPO activity in bronchoalveolar lavage fluid after PDTC

treatment. EPO activity was markedly increased in ovalbumin model (positive control group) when compared with negative control group. PDTC treatment decreased EPO activity in a dose dependent manner as shown in Fig. 1A.

The analysis of the cellular contents of bronchoalveolar lavage fluids is depicted in Fig. 1B. After ovalbumin challenge, total cell numbers and eosinophils in the bronchoalveolar lavage fluid were greatly increased (87% and 52%, respectively), as compared with negative control group. Similarly, the numbers of lymphocytes and macrophages were increased in the bronchoalveolar lavage fluid whereas neutrophil numbers were not significantly increased after challenge. PDTC treatment (50, 100 and 200 mg/kg) reduced total cell numbers (22.8%, 44.5% and 80.2%, respectively) and eosinophils (30.7%, 65.3% and 88.4%, respectively) when compared with positive control group. PDTC also reduced the number of lymphocytes at 200 mg/kg, but its effects on macrophages were minimal (Fig. 1B).

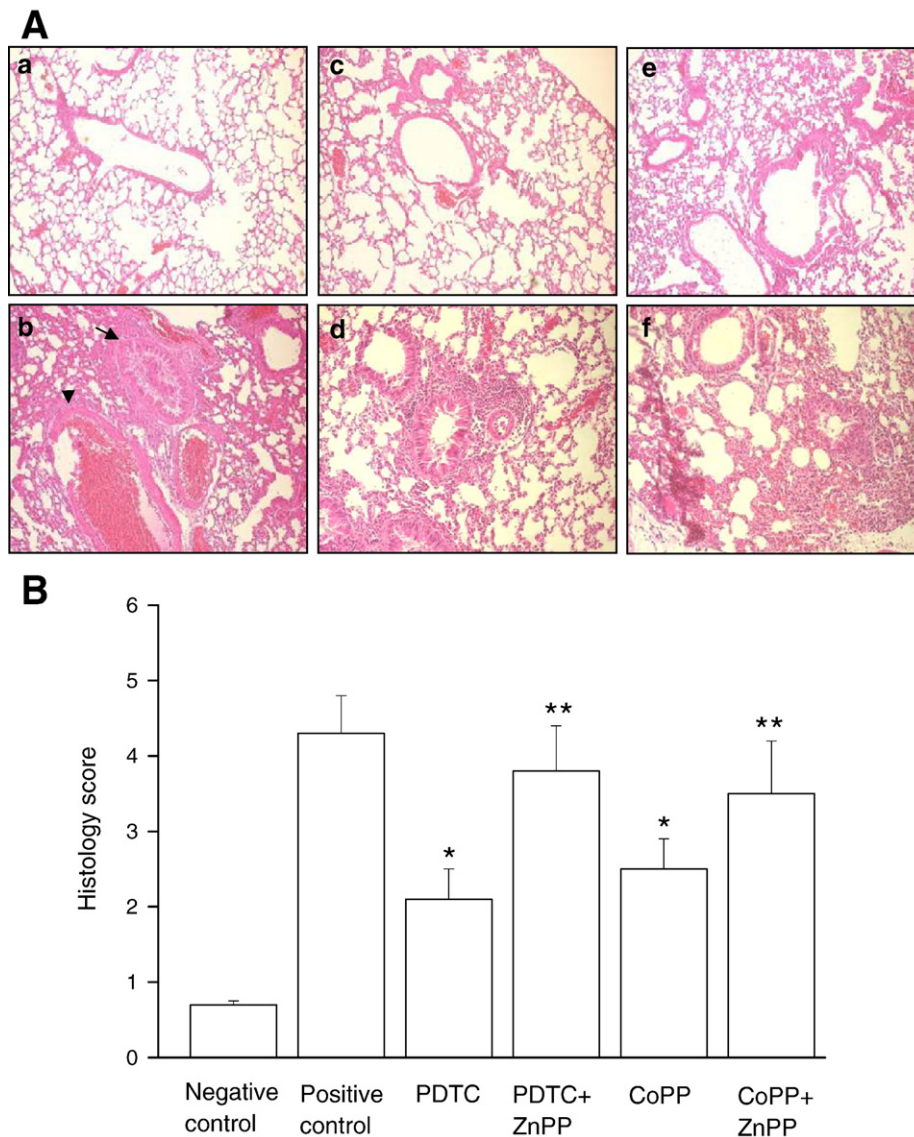


**Fig. 5.** Effect of ZnPP on histologic examination of airway inflammation. Mice were pre-treated with ZnPP (10 mg/kg) plus PDTC (200 mg/kg, i.p.) or CoPP (30 mg/kg, i.p.) at 12 h before challenges with aerosolized ovalbumin. On 3rd day after last aerosolized ovalbumin challenge, lung was fixed and histologic analysis was performed. (A) Representative photographs of mice in saline (b), PDTC (200 mg/kg, i.p.) (c), PDTC plus ZnPP (d), CoPP (e) and CoPP plus ZnPP (f). In positive control mice, peribronchial (arrowhead) and perivascular (arrow) inflammatory cell infiltrates were prominent (b), which were absent in negative control mice (a). Administration of PDTC ameliorates ovalbumin-induced peribronchial and perivascular inflammation (c), as reflected by thinner inflammatory cell infiltrates in PDTC-treated group than in positive control group. Results are representative of at least three similar experiments performed on different experimental days (H & E stain, original magnification  $\times 100$ ). (B) Quantification of histological scores. Values are mean  $\pm$  S.D. # $P < 0.05$  vs negative control, \* $P < 0.05$  vs positive control group, and \*\* $P < 0.01$  vs PDTC or CoPP-treated group. Results are representative of at least three experiments performed on different experimental days ( $n=8-10$ ).

### 3.2. PDTC treatment reduces the development of airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of the airway allergic response, and has been associated with the recruitment of inflammatory cells to the lung. To investigate the effects of PDTC on airway function, airway hyperresponsiveness was measured by whole body plethysmography with increasing doses of methacholine. Fig. 2 shows that the positive control group demonstrated increased airway hyperresponsiveness compared with the negative control group, and that PDTC dose dependently decreased airway hyperresponsiveness at a dose of 50 mg/ml methacholine, compared with the positive control group (Fig. 2).

### 3.3. HO-1 is induced by PDTC treatment in ovalbumin-challenged mice

To determine whether the inhibitory effects of PDTC on airway inflammation are related to the HO-1 inducing activities, HO-1 expression was measured by RT-PCR and Western blot. RT-PCR was performed after i.p. administration of PDTC at 12 h prior to aerosolized ovalbumin exposure. As shown in Fig. 3A, treatment of PDTC resulted in the increase of HO-1 mRNA levels in a dose dependent manner. PDTC exhibited a marked increase at a dose of 200 mg/kg by 6 fold as compared with positive control mice (Fig. 3A). In accordance with this mRNA increase, HO-1 protein levels were significantly increased in PDTC-treated lung while barely detectable in positive control group (Fig. 3B). As a positive control, CoPP (30 mg/kg, i.p.), a potent HO-1 inducer was included, and as expected, it also induced HO-1 mRNA and protein expression in ovalbumin-sensitized mice lung.

### 3.4. Blockade of HO-1 by ZnPP treatment abrogates the beneficial effects of PDTC on the EPO activity and airway hyperresponsiveness

Next, we examined the effect of ZnPP, a potent HO-1 inhibitor on PDTC actions including EPO activity and airway hyperresponsiveness. Mice were pre-treated with ZnPP (10 mg/kg, i.p.) plus PDTC (200 mg/kg, i.p.) at 12 h before challenge with aerosolized ovalbumin. As shown in Fig. 4, ZnPP significantly reversed the inhibitory effects of PDTC on EPO activity and eosinophil number (Fig. 4A and B). Furthermore, ZnPP administration abolished the inhibitory effects of PDTC on airway hyperresponsiveness (Fig. 4C), suggesting that HO-1 is an important mediator of PDTC in ameliorating airway inflammation.

To further check whether HO-1 was involved in the protective effects against airway inflammation, we tested the effects of CoPP in ovalbumin-challenged mice. In our system, CoPP (30 mg/kg, i.p., given at 12 h before ovalbumin challenge) mimicked the protective effects of PDTC (Fig. 4), supporting that HO-1 induction may be important in protecting from ovalbumin-induced airway inflammation. ZnPP alone tends to modestly increase both EPO activity and airway hyperresponsiveness as compared with the positive control group, but this change was statistically insignificant (data not shown).

### 3.5. Effects of ZnPP on PDTC action in histological analysis

Airway inflammation is characterized by peribronchiolar and perivascular inflammatory cell infiltrates. To investigate the extent and anatomical localization of inflammation, we performed histological analyses in the lung. The positive control group showed characteristics of wide spread peribronchiolar and perivascular inflammatory infiltrates when compared to the negative control group (Fig. 5A-a and b). These infiltrates are consisted predominantly of eosinophils with a significant number of lymphocytes and macrophages. On the other hand, PDTC treatment (200 mg/kg) clearly decreased inflammatory infiltrates including eosinophils in peribronchiolar, perivascular and alveolar airspaces (Fig. 5A-c). ZnPP coadministration increased airway inflammation in lung as compared with mice treated with PDTC alone (Fig. 5A-d). Also, CoPP

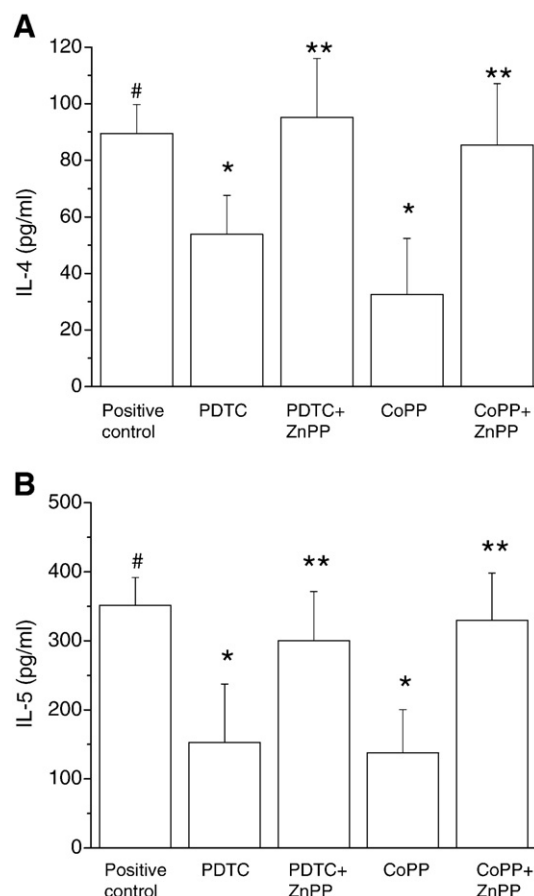


**Fig. 6.** Effect of PDTC on IL-4 and IL-5 secretion in bronchoalveolar lavage fluid. Mice were pre-treated with ZnPP (10 mg/kg) plus PDTC (200 mg/kg, i.p.) or CoPP (30 mg/kg, i.p.) at 12 h before challenges with aerosolized ovalbumin. Bronchoalveolar lavage fluid was collected at 12 h after the last ovalbumin challenge and analyzed for IL-4 and IL-5 contents by ELISA. Results are representative of at least three similar experiments performed on different experimental days. Values are means  $\pm$  S.D. #  $P < 0.05$  vs negative control, \*  $P < 0.05$  vs positive control group, and \*\*  $P < 0.05$  vs PDTC or CoPP-treated group.

(30 mg/kg, i.p.) was shown to decrease inflammatory infiltrates, which was reversed by ZnPP (Fig. 5A-e and F). For quantification of the histological changes, all sections were examined and scored in a blinded manner on a scale of 0–5. PDTC or CoPP-treated groups significantly reduced the scores of the inflammatory infiltrates around perivascular and peribronchiolar areas when compared with the positive control groups, and these were reversed by ZnPP (Fig. 5B).

### 3.6. PDTC inhibits IL-4 and IL-5 secretion in a HO-1 dependent manner

To determine whether HO-1 induction by PDTC treatment in lung affects Th<sub>2</sub> cytokine (IL-4 and IL-5) secretion, cytokine levels in bronchoalveolar lavage fluid were measured at 12 h after the final airway challenge. IL-4 and IL-5 secretion were increased in the positive control group, but PDTC (200 mg/kg, i.p.) administration significantly reduced their secretion. Of note, the inhibitory effects of PDTC on IL-4 and IL-5 secretion in bronchoalveolar lavage fluid were abrogated when ZnPP was administered concomitantly with PDTC, suggesting that HO-1 induction by PDTC may result in the inhibition of Th<sub>2</sub> cytokine secretion, leading to the attenuation of airway inflammation (Fig. 6).

## 4. Discussion

PDTC is a widely used pharmacological agent in molecular and cell biology, and it has been reported to have both pro-oxidant and anti-

oxidant properties (Li et al., 2001; Muller et al., 2000; Liu et al., 1999). Recently, it has been demonstrated that PDTC is a potent inducer of HO-1 and attenuates hepatic ischemia/reperfusion injury by inhibiting proinflammatory cytokines (Hata et al., 2003; Muhl et al., 2004). However, the effects of PDTC on airway inflammation and possible involvement of HO-1 in PDTC action have not yet been reported. In the present study, we demonstrate that PDTC exerts protective effects against airway inflammation, and that HO-1 induction may be at least partly responsible for its action.

HO-1 is induced in a mouse model of asthma, and its deficiency leads to an increase in chronic inflammation and leukocyte recruitment (Vogt et al., 1996; Wilis et al., 1996). Several studies now show that the induction of HO-1 helps to ameliorates tissue injury and inflammation in a variety of experimental animal models and in humans (Shibahara et al., 1987; Otterbein and Choi, 2000; Kitada et al., 2001; Amersi et al., 1999), suggesting that HO-1 induction occurs to protect cells from injury or inflammation. This interpretation is also consistent with the finding that the overexpression of HO-1 reduces ocular inflammation by downregulating proinflammatory cytokines (Ohta et al., 2003). Thus, we examined whether PDTC-induced HO-1 actually could attenuate airway inflammation. In this report, we show that exogenously administered PDTC protects against allergic airway inflammation, characterized by decreased eosinophilia, airway hyperresponsiveness and Th<sub>2</sub> cytokine secretion, and these effects are, at least in part, mediated via HO-1 induction.

Administration of PDTC at 12 h before ovalbumin challenge significantly enhanced HO-1 mRNA and protein levels in lung. In addition, there was a significant decrease in EPO activity and eosinophilia along with HO-1 induction. Previous report demonstrated that airway eosinophilia is related to airway hyperresponsiveness in allergic airway inflammation (Hogan et al., 1997). Similarly, we found that PDTC administration decreased airway hyperresponsiveness to inhaled methacholine in ovalbumin-challenged mice. Recently, tiotropium, an anticholinergic agent was shown to inhibit allergen-induced eosinophilia in guinea pigs (Bos et al., 2007). Thus, we examined the effects of PDTC on EPO activity and lung histology in the absence of methacholine treatment, and found that PDTC inhibited both EPO activity and inflammatory changes on histology, similar to results obtained with methacholine treatment (results not shown), supporting that PDTC has direct suppressive action on eosinophilia. Since ZnPP is a known HO-1 inhibitor (Maines, 1981; Choi et al., 2003; Chakder et al., 1996), we employed ZnPP in our study to confirm the role of HO-1 in PDTC-induced protection against airway inflammation. Treatment with ZnPP abolished the beneficial effects of PDTC, indicating that HO-1 is involved in the protection by PDTC from eosinophilia and airway hyperresponsiveness in this airway inflammation model.

To further elucidate the cell types involved in HO-1 induction by PDTC, we tried to fractionate total cells obtained from bronchoalveolar lavage fluid into different cell types, and to determine HO-1 mRNA and protein levels. Unfortunately, we were unable to collect enough cells of each type for HO-1 measurement. However, we were able to determine the effects of PDTC on different cell types by using cell lines *in vitro*. As expected, HO-1 mRNA and protein levels were induced by PDTC in eosinophils (differentiated EoL-1 cells) and Jurkat cells in a concentration dependent manner (results not shown), suggesting that HO-1 induction by PDTC *in vivo* may occur in various inflammatory cells infiltrated by ovalbumin stimulation.

To date, several discrete mechanisms have been suggested regarding the molecular mechanisms by which HO-1 attenuates airway inflammation. One possible mechanism of HO-1 upregulation by PDTC may occur via inhibition of Th<sub>2</sub> responses. It is widely accepted that mast cells, eosinophils and T-lymphocytes are important inflammatory cells in the onset and progression of asthma. (Lee et al., 2004; Wills-Karp and Karp, 2004). Recently, effector CD<sub>4</sub><sup>+</sup> T cells have been recognized to play dominant roles in initiating allergic pulmonary inflammation. Following allergen challenge, the T cell

population in the lungs markedly increases, with the bulk of the expansion due to CD<sub>4</sub><sup>+</sup> cells producing Th<sub>2</sub> cytokines, such as IL-4, IL-5 and IL-13, which are involved in pulmonary eosinophilia, mucus hypersecretion and airway hyperresponsiveness (Ritz et al., 2000). On the other hand, inhibition of HO-1 by ZnPP administration increases Th<sub>2</sub> cytokine production such as IL-4 and IL-10 during pregnancy (Sollwedel et al., 2005). Based on such findings, we tested whether HO-1 induction by PDTC would have similar effects in our model. According to our data, PDTC administration downregulated Th<sub>2</sub> cytokine IL-4 and IL-5 in bronchoalveolar lavage fluids, and these were reversed by ZnPP, consistent with the previous findings (Sollwedel et al., 2005). However, the signaling pathway linking HO-1 and Th<sub>2</sub> cytokine secretion remains to be elucidated.

Many studies have reported that PDTC inhibits gene expression of various proinflammatory cytokines via NF- $\kappa$ B inactivation (Muller et al., 2000; Liu et al., 1999). The findings of our study do not eliminate the possibility that NF- $\kappa$ B inactivation might contribute to the attenuation of airway inflammation by PDTC. However, the protective effects of PDTC were almost completely abolished with a known HO-1 inhibitor ZnPP treatment, which was paralleled by the reduction of IL-4 and IL-5 secretion. Although ZnPP may possibly inhibit other HO isoforms besides HO-1, our results support the hypothesis that HO-1 induction is one mechanism mediating the protective effect of PDTC via inhibition of Th<sub>2</sub> cytokine secretion.

In conclusion, the principal findings of this work are as follows: (a) PDTC prevents allergic airway inflammation as measured by eosinophil infiltration and airway hyperresponsiveness. (b) Cotreatment with ZnPP, the HO-1 inhibitor, abolishes these beneficial effects, supporting the involvement of HO-1 in the protection against airway inflammation. (c) Induction of HO-1 by PDTC is associated with the inhibition of Th<sub>2</sub> cytokine IL-4 and IL-5 secretion, which might be responsible for the protective effects against airway inflammation. To our knowledge, this is the first experimental evidence that documents the potential utility of PDTC as a novel therapeutic approach for the treatment of airway inflammation via HO-1 induction.

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