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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_2 cytokines such as IL-4 and IL-5, whereas ZnPP blocked the inhibitory effects of PDTC on Th_2 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_2 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_2 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_2 cells induces the growth and development of Th_2 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 neurodegenerative 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 endogeneous 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 [$Al(OH)_3$] 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 [$Al(OH)_3$]. A second injection was given 10 days later with 10 μg ovalbumin with the same concentration of [$Al(OH)_3$]. 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. $Penh=(Te/Tr-1)\times(pef/pif)$ (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 hyperresponsiveness), 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^{2+}- and Mg^{2+}-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_2O_2) (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_2O_2 was added to each well. After incubation at room temperature for 20 min, the reaction was stopped by adding H_2SO_4 and optical density was measured at 492 nm.

2.6. Analyses of lung histology

Unlavaged 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′-ACTTTCAGAAGGGTCAGGTGTCC-3′

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