



Inhibitory effects of Pycnogenol® (French maritime pine bark extract) on airway inflammation in ovalbumin-induced allergic asthma



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ABSTRACT

Pycnogenol® (PYC) is a standardized extracts from the bark of the French maritime pine (*Pinus maritime*) and used as a herbal remedy for various diseases. In this study, we evaluated the effects of PYC on airway inflammation using a model of ovalbumin (OVA)-induced allergic asthma and RAW264.7 cells. PYC decreased nitric oxide production and reduced the interleukine (IL)-1 β and IL-6 levels in LPS-stimulated RAW264.7 cells. PYC also reduced the expression of inducible nitric oxide synthase (iNOS) and matrix metalloproteinase (MMP)-9 and enhanced the expression of hemeoxygenase (HO)-1. In the in vivo experiment, PYC decreased the inflammatory cell count and the levels of IL-4, IL-5, IL-13, and immunoglobulin (Ig) E in BALF or serum. These results are consistent with the histological analysis findings, which showed that PYC attenuated the airway inflammation and mucus hypersecretion induced by OVA challenge. In addition, PYC enhanced the expression of HO-1. In contrast, PYC inhibited the elevated expression of iNOS and MMP-9 proteins induced by OVA challenge. In conclusion, PYC exhibits protective effects against OVA-induced asthma and LPS-stimulated RAW264.7 cells. These results suggest that PYC has potential as a therapeutic agent for the treatment of allergic asthma.

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1. Introduction

Airway inflammation is an important feature in allergic asthma. In the pathogenesis of asthma, airway inflammation is a very complex response that is associated with various factors, such as reactive oxygen species (ROS), proinflammatory mediators, and growth factors (Lee et al., 2011). According to several previous studies, the overproduction of ROS has been implicated in the pathogenesis of asthma (Rahman, 2003; Park et al., 2009 and Rosanna and Salvatore, 2012). Increased ROS production induces proinflammatory mediators, including inducible nitric oxide synthase (iNOS), nuclear factor (NF)- κ B, and matrix metalloproteinase (MMP)-9 (Lee and Yang, 2012). These mediators are closely associated with inflammatory cell recruitment and proinflammatory cytokines, such as interleukin (IL)-4, IL-5, and IL-13 (Lee et al., 2011). Therefore, the phenotypes of allergic asthma, such

as airway inflammation, mucus overproduction, and airway hyperresponsiveness, are aggravated through a sequence of responses.

Pycnogenol® (PYC), a standardized bioflavonoid extracts from bark of the French maritime pine (*Pinus maritime*) has been traditionally used for the treatment of inflammatory disease in Europe and North America. This herbal medicine is utilized throughout the world as an herbal remedy for the treatment of various diseases ranging from chronic inflammation to circulatory diseases (D'Andrea, 2010 and Lee et al., 2011). PYC consists of approximately 70% procyanidins, which exhibit antioxidant, anti-inflammatory, anticarcinogenic and immuno-modulatory effects (Maimoona et al., 2011). The anti-inflammatory mechanism of PYC has been elucidated in many in vitro and in vivo experiments (Kim et al., 2011 and Peng et al., 2012). It is well-known that the anti-inflammatory effect of PYC is closely related with the enhancement of antioxidant mediator.

Although many studies have investigated the properties of PYC, its protective effects and its mechanism against the allergic asthma have not been elucidated. Therefore, in this study, we investigated

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the protective effects of PYC on allergic asthma using a murine model of ovalbumin (OVA) induced allergic asthma and RAW264.7 cells.

2. Materials and methods

2.1. Cell culture and MTT methods

The RAW264.7 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC; MD, USA). The cells were incubated in 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Gibco Inc., NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 5.5% fetal bovine serum (FBS; Gibco Inc., NY, USA). The cells were also treated with 0.05% DMSO as a vehicle control. Cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated with serum-free medium in the presence of different concentrations of PYC. The PYC was obtained from Prof. Jong Choon Kim (Chonnam National University, Gwangju, Republic of Korea). After incubation for 24 h, the cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) methods. All of the experiments were performed in triplicate.

2.2. Measurement of nitric oxide (NO) production

Cells (2.5×10^5 cells/mL) were seeded in 96-well plates in phenol red-free DMEM, treated with different concentrations (25, 50, 75, and 100 ng/mL) of PYC for 1 h, and then incubated in the presence of LPS (1 µg/mL) for 24 h. The nitrite accumulation in the culture medium was measured using the Griess reagent (Promega Corporation, WI, USA). The absorbance at 535 nm was measured using a microplate reader (Bio-Rad Laboratories, CA, USA).

2.3. Measurement of proinflammatory cytokine production

Cells were treated as described above, and the concentrations of IL-1β and IL-6 in the culture medium were quantified using a competitive ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories). The absolute concentrations were obtained by running standard curves on the same ELISA plates.

2.4. Western blotting

The cells were treated as described above and then incubated in the presence of LPS (1 µg/mL) for 1 h or 24 h. The cells were collected by centrifugation, washed twice with phosphate-buffer saline (PBS), and suspended in an extraction lysis buffer (Sigma-Aldrich) containing protease inhibitors. The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Equal amounts of total cellular protein (30 µg) were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution (5% skim milk) and then incubated overnight at 4 °C with the appropriate primary antibody. The following primary antibodies and dilutions were used: anti-β-actin (1:2000 dilution; Cell Signaling, MA, USA), anti-iNOS (1:200 dilution; Abcam, MA, USA), anti-HO-1 (1:250 dilution; Abcam), and anti-MMP-9 (1:1000 dilution; Cell Signaling). The blots were washed three times with Tris-buffered saline containing Tween 20 (TBST) and then incubated with a 1:10000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research, PA, USA) for 30 min at room temperature. The blots were washed three times with TBST, and then developed using an enhanced chemiluminescence (ECL) kit (Thermo, CA, USA).

2.5. Gelatin zymography

The cell supernatant was loaded for gelatin zymography. To determine the gelatinase activity, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) zymography was performed according to the method described by Heussen and Dowdel (1980). Briefly, the zymogram gels consisted of 10% SDS–PAGE containing 1% gelatin as the MMP substrate. The gels were washed in 2.5% Triton X-100 for 1 h to remove SDS and then incubated at 37 °C for 16 h in developing buffer (1 M Tris-HCl, pH 7.5 with CaCl₂). Thereafter, the gels were stained with 25% methanol/8% acetic acid containing Coomassie brilliant blue. The gelatinase activity was visualized as white bands on a blue background, which represent areas of proteolysis.

2.6. OVA-induced allergic asthma

Specific pathogen-free 6-week-old female BALB/c mice were purchased from Koatech Co. (Pyeongtaek, Korea) and used after two weeks of quarantine and acclimatization. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology.

The mice were sensitized on days 0 and 14 through an intraperitoneal injection of 20 µg OVA (Sigma-Aldrich) emulsified with 2 mg of aluminum hydroxide in 200 µL of phosphate-buffered saline (PBS) buffer (pH 7.4). On days 21, 22, and 23, the mice received an airway challenge with OVA (1% (w/v)) for 1 h using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). PYC and montelukast (positive control, Sigma-Aldrich, CA, USA) was orally administered to the mice at a dose level of 30 mg/kg body weight one hour before the OVA challenge. Montelukast was developed as a cysteinyl leukotriene (cys-LT)-1 receptor antagonist (Lynch et al., 1999) and was introduced into the market after successful clinical evaluation in patients with aspirin-sensitive asthma, nocturnal exacerbation of asthma, and allergic asthma (Korenblat, 2001). The airway responsiveness (AHR) was assessed 24 h after the last challenge indirectly through single-chamber, whole body plethysmography (Allmedicus, Seoul, Korea). Bronchoalveolar lavage fluid (BALF) samples were obtained for analysis. The mice were sacrificed 48 h after the last challenge through the intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharm. Co., Seoul, Korea), and a tracheostomy was performed. To obtain the BALF, ice-cold PBS (0.4 mL) was infused into the lung three times and each time withdrawn via tracheal cannulation (total volume of 1.2 mL). After the exclusion of dead cells through Trypan blue staining, the total inflammatory cell numbers were determined by counting the cells in at least five squares of a hemocytometer. The differential cell count of the BALF was performed using the Diff-Quik® staining reagent (B4132-1A; IMEB Inc., CA, USA), according to the manufacturer's instructions.

2.7. Measurement of the levels of cytokines in the BALF and IgE in serum

The levels of IL-4, IL-5, and IL-13 in the BALF were measured using ELISA kits (R&D System) according to the manufacturer's protocols. The levels of total IgE and OVA-specific IgE in serum were also measured by ELISA. Microtiter plates were coated with anti-IgE antibodies (anti-mouse IgE; 10 g/mL; Serotec, Oxford, UK) in PBS-Tween 20, and incubated with the serum. The plates were then washed four times, and 200 µL of o-phenylenediamine dihydrochloride (Sigma-Aldrich) was added to each well. The plates were incubated for 10 min in the dark and the absorbance was measured at 450 nm.

2.8. Immunoblotting

The lung tissue was homogenized (1/10 w/v) using a homogenizer in a Tissue lysis/extraction reagent (Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich). Each protein concentration was determined using the Bradford reagent (Bio-Rad). Western blotting was performed as described above, and the levels of MMP-9, iNOS and HO-1 were determined.

2.9. Histology

After the BALF samples were obtained, the lung tissue was fixed in 4% (v/v) paraformaldehyde. Tissues were embedded in paraffin, sectioned to 4-µm thickness, and stained with a H&E solution (Sigma-Aldrich) and periodic acid–Schiff (PAS) (IMEB Inc., CA, USA) to estimate the inflammation and mucus production, respectively.

2.10. Statistical analysis

The data are expressed as the means ± standard error of the mean (SEM). The statistical significance was determined using analysis of variance (ANOVA) followed by a multiple comparison test with Dunnett's adjustment. Differences with a *P* value of less than 0.05 were considered to be significant.

3. Results

3.1. Effects of PYC on NO, IL-1β and IL-6 in LPS-stimulated RAW264.7 cells

LPS-stimulated RAW264.7 cell exhibited increased NO production, whereas the treatment of RAW264.7 cells with PYC significantly decreased the elevated NO production stimulated by LPS in concentration dependent manner (Fig. 1A). The effects of PYC on the production of IL-1β and IL-6 were similar to that obtained for NO production. The PYC-treated RAW264.7 cells exhibits a marked reduction in the production of IL-1β and IL-6 in concentration dependent manner compared with that found for LPS-stimulated RAW264.7 cells (Fig. 1B and C).

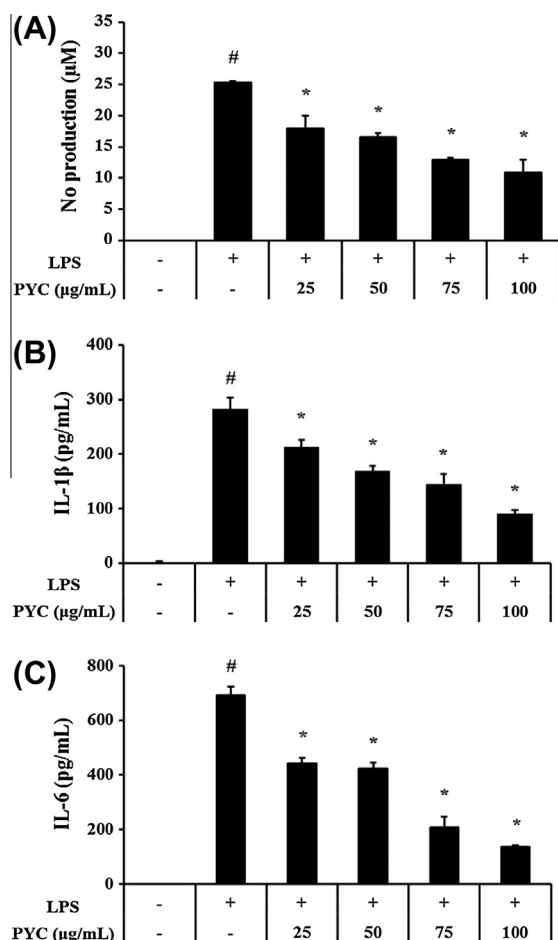


Fig. 1. PYC reduces the production of proinflammatory mediators in RAW264.7 cells stimulated with LPS. The cells were treated with different concentrations (25, 50, 75, and 100 μg/mL) of PYC for 1 h, and then incubated in the presence of LPS (1 μg/mL) for 24 h. (A) NO production, (B) IL-1β, and (C) IL-6. *Significantly different from the control, $P < 0.05$; *Significantly different from cells treated with LPS alone, $P < 0.05$.

3.2. Effects of PYC on the expression of proinflammatory and antioxidant protein in LPS-stimulated RAW264.7 cells

LPS-stimulated RAW264.7 cells exhibited increased expression of iNOS and MMP-9. However, the treatment of these cells with PYC significantly reduced the iNOS and MMP-9 expression in concentration dependent manner compared with LPS-stimulated RAW264.7 cells (Fig. 2A and C). In addition, the PYC-treated RAW264.7 cells over-expressed HO-1 protein in concentration dependent manner. As shown in Fig. 2B, the MMP-9 activity increased in the LPS-stimulated RAW264.7 cells and was reduced in the PYC-treated RAW264.7 cells.

3.3. Effects of PYC on the inflammatory cell count in the BALF of OVA-sensitized/challenged mice

The OVA-sensitized/challenged mice showed significant increases in the numbers of eosinophils and other inflammatory cells compared with the negative controls. In contrast, the PYC-treated mice exhibited a significant reduction in the numbers of inflammatory cells in the BALF, particularly eosinophils, compared with the OVA-sensitized/challenged mice (Fig. 3). These reductions were similar to the results obtained with montelukast-treated mice.

3.4. Effects of PYC on the airway hyperresponsiveness (AHR) of OVA-sensitized/challenged mice

The AHR significantly increased in the OVA-sensitized/challenged mice with increasing methacholine concentration. The treatment of these mice with montelukast decreased the AHR induced by OVA challenge. The PYC-treated mice also exhibited markedly reduced the AHR compared with the OVA-sensitized/challenged mice (Fig. 4).

3.5. Effects of PYC on IL-4, IL-5, and IL-13 in the BALF and IgE in the serum of OVA-sensitized/challenged mice

The OVA-sensitized/challenged mice showed a marked elevation of IL-4, IL-5, and IL-13 in the BALF compared with the negative controls. In contrast, the treatment of these mice with PYC

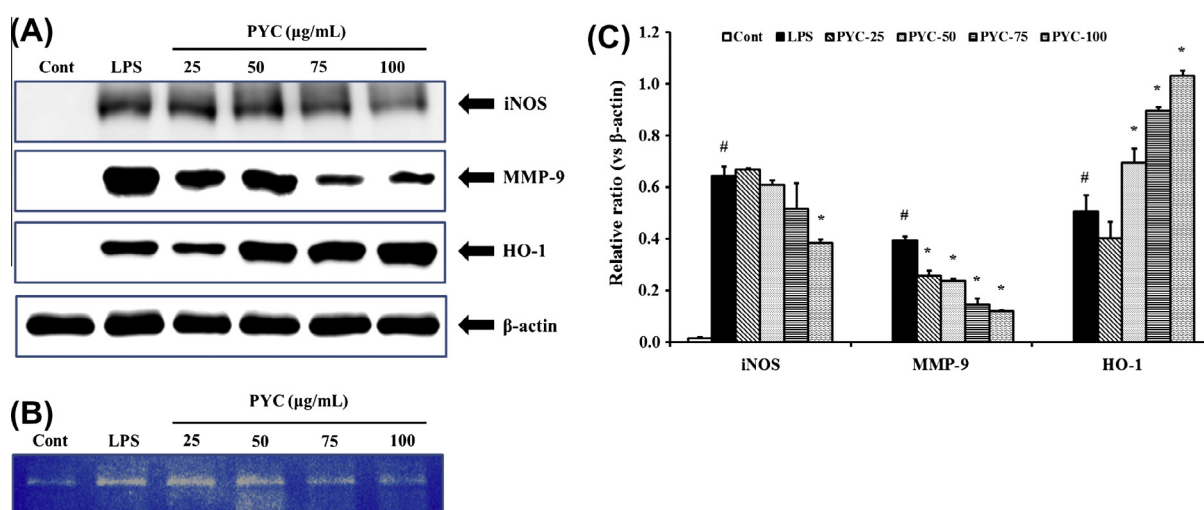


Fig. 2. PYC decreases iNOS and MMP-9 expression and increases HO-1 expression in RAW264.7 cell stimulated with LPS. (A) Western blotting, (B) gelatin zymography, and (C) relative ratio. The cells were pretreated with PYC for 1 h and then stimulated with LPS for 24 h. Equal amounts of protein in the cell lysates were electrophoresed to determine the levels of iNOS, MMP-9 and HO-1 protein expression. In addition, the MMP-9 activity was assessed through gelatin zymography. *Significantly different from the control, $P < 0.05$; *Significantly different from cells treated with LPS alone, $P < 0.05$.

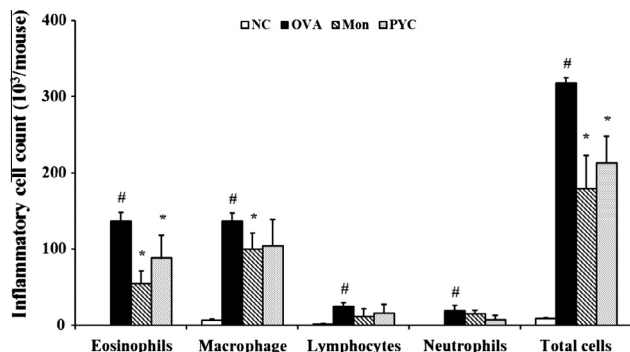


Fig. 3. PYC inhibits the recruitment of inflammatory cells in the BALF of mice. The cells were isolated by centrifugation and stained with the Diff-Quik[®] stain reagent. After excluding the dead cells using Trypan blue, the cell numbers were determined using a light microscope to count the cells in at least five squares of a hemocytometer. NC; normal control mice treated with PBS only; OVA; OVA-sensitized/challenged mice; Mon; OVA sensitized/challenged mice treated with montelukast (30 mg/kg); PYC; OVA-sensitized/challenged mice treated with PYC (30 mg/kg). [#]Significantly different from NC, $P < 0.05$; ^{*}Significantly different from OVA, $P < 0.05$.

Table 1

The levels of total IgE and OVA-specific IgE in serum.

Groups	Total IgE ($\mu\text{g/mL}$)	OVA-specific IgE (ng/mL)
NC	0.3 ± 0.13	–
OVA	$3.0 \pm 0.34^{\#}$	$438.8 \pm 90.75^{\#}$
Mon	$2.0 \pm 0.49^*$	$198.2 \pm 42.65^*$
PYC	$2.4 \pm 0.30^*$	$251.4 \pm 63.81^*$

NC, normal control mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice; PYC, PYC (30 mg/kg) + OVA-sensitized/challenged mice.

[#] Significantly different from NC, $P < 0.05$.

^{*} Significantly different from OVA, $P < 0.05$.

3.6. Effects of PYC on the inflammatory response and mucus production in the lung tissue of OVA-sensitized/challenged mice

The OVA-sensitized/challenged mice exhibited extensive infiltration of inflammatory cells into the peribronchial and perivascular lesions in the lung tissue and the PYC-treated mice showed reduced airway inflammation compared with the OVA-sensitized/challenged mice (Fig. 6A and B). The mucus production increased in the bronchial airway of OVA-sensitized/challenged mice. However, the PYC-treated mice exhibited a reduction in the mucus production (Fig. 6A and C).

3.7. Effects of PYC on the expression of iNOS, MMP-9 and HO-1 in the lung tissue of OVA-sensitized/challenged mice

The OVA-sensitized/challenged mice exhibited increased expression of iNOS and MMP-9 in the lung tissue compared with the negative controls. However, treatment of these mice with PYC decreased the expression of proinflammatory proteins in the lung tissue compared with the OVA-sensitized/challenged mice (Fig. 7A and B). In contrast to the expression of proinflammatory proteins, HO-1 expression in lung tissue was increased in the PYC-treated mice compared with the OVA-sensitized/challenged mice.

4. Discussion

In this study, we investigated the protective potentials of PYC on airway inflammation using a murine model of OVA-induced asthma and RAW264.7 cells. PYC effectively suppressed the enhanced production of NO, IL-1 β and IL-6 in LPS-stimulated RAW264.7 cells. In addition, PYC inhibited the elevated expression or activity of iNOS and MMP-9 and enhanced the HO-1 expression in LPS-stimulated RAW264.7 cells. In an OVA-induced asthma model, PYC markedly suppressed the OVA challenge-induced asthmatic response, including airway inflammation, mucus overproduction, and AHR. PYC also inhibited the expression of proinflammatory proteins and enhanced the antioxidant protein in OVA-challenged lung tissue.

NO plays an important role in the airway inflammation of allergic asthma and is produced from L-arginine by iNOS. A previous study demonstrated that iNOS aggravates airway inflammation in allergic asthma. The blockage of iNOS-derived NO attenuates asthmatic responses such as airway constriction, inflammation, and remodeling processes, by reducing both collagen and elastic fibers (Prado et al., 2006). In contrast, the overexpression of iNOS results in increases in MMP-9 activity and expression (Wang et al., 2011). MMP-9 is involved in the breakdown of the extracellular matrix and is considered a hall marker in airway remodeling. MMP-9 is modulated by a variety of cytokines and is associated with tissue remodeling and the infiltration of inflammatory cells into the airway (Di Girolamo et al., 2006). In several studies, the MMP-9

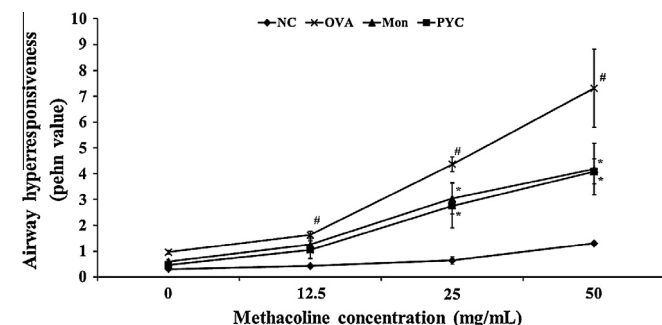


Fig. 4. PYC reduces the AHR induced by OVA challenge. The AHR was indirectly assessed 24 h after the last challenge through using single-chamber, whole body plethysmography (Allmedicus, Seoul, Korea). [#]Significantly different from NC, $P < 0.05$; ^{*}Significantly different from with OVA, $P < 0.05$.

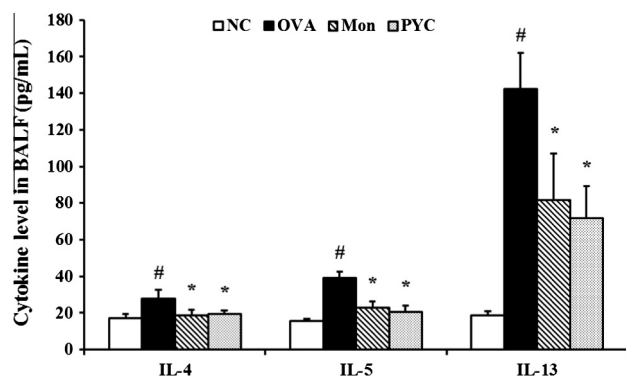


Fig. 5. PYC decreases the levels of IL-4, IL-5 and IL-13 in the BALF. The levels of these Th2 cytokines in the BALF were determined using ELISA. [#]Significantly different from NC, $P < 0.05$; ^{*}Significantly different from OVA, $P < 0.05$.

significantly decreased the elevated proinflammatory cytokines induced by OVA-challenge (Fig. 5). The effects of PYC on total and OVA-specific IgE in the serum were consistent with the results obtained for the proinflammatory cytokines. The treatment of these mice with PYC significantly decreased the elevated total and OVA-specific IgE levels in the serum induced by OVA challenge (Table 1).

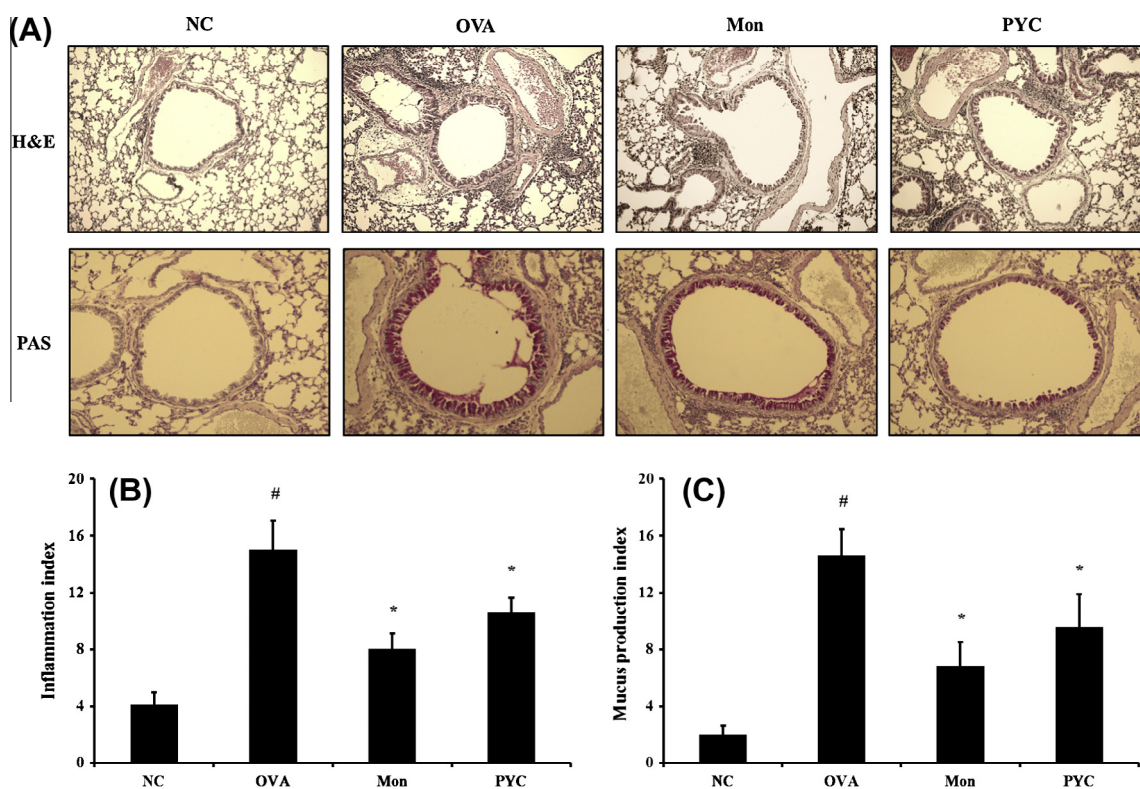


Fig. 6. PYC attenuates airway inflammation and mucus production. (A) Histological examination of the airway inflammation and mucus production in lung tissue using H&E and PAS staining, respectively. (B) Quantitative analysis for airway inflammation. (C) Quantitative analysis for mucus production. [#]Significantly different from NC, $P < 0.05$; ^{*}Significantly different from OVA, $P < 0.05$.

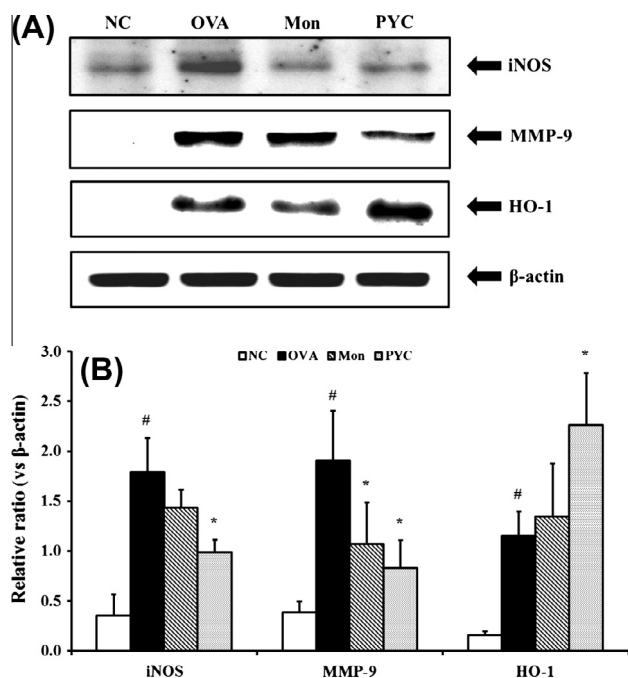


Fig. 7. PYC inhibits the expression of proinflammatory proteins and enhances the expression of antioxidant protein. (A) The expression of iNOS, MMP-9, and HO-1 in lung tissue. (B) Relative ratio. [#]Significantly different from NC, $P < 0.05$; ^{*}Significantly different from OVA, $P < 0.05$.

expression has been found to be increased in the BALF and lung tissue of asthmatic animals, and elevated MMP-9 activity and expression has been found to be upregulated in association with the accumulation of inflammatory cells into the airway (Locke et al.,

2007; Gueders et al., 2008 and Kong et al., 2008). In this study, PYC effectively inhibited NO production and the levels of proinflammatory cytokines and proteins in LPS-stimulated RAW264.7 cells. PYC also suppressed asthmatic phenotypes, including airway inflammation, mucus overproduction, and AHR through a reduction in the levels of Th2 cytokines and IgE, and the expression of iNOS and MMP-9 in OVA-induced asthmatic mice. These findings indicate that PYC attenuates the asthmatic responses induced by OVA challenge via the suppression of iNOS and MMP-9 expression. HO-1 catalyzes the rate-limiting step of heme oxidation to biliverdin, carbon monoxide (CO), and iron. Biliverdin is converted to bilirubin, which exhibits potent antioxidant activity by efficiently scavenging peroxyl radicals (Lee et al., 2011). CO also has various biological properties, including anti-inflammatory and anti-apoptotic effects and smooth muscle relaxation (Almolki et al., 2004). HO-1 is induced by several stimuli associated with oxidative stress and inflammation, such as cytokines, NO, hypoxia, hydrogen peroxide, and thiol-reactive substances (Carter et al., 2004). Asthma is closely related with increased ROS and NO production and several inflammatory mediators in the airway (Lee and Yang, 2012). All of these stimuli may be inducers of HO-1 expression in asthma. Additionally, the lower oxidative stress related with HO-1 upregulation in asthmatic animals is attributed to a reduction in the inflammatory cell count in the airway (Raval and Lee, 2010). It has been reported that HO-1 reduces the recruitment of inflammatory cells, apoptosis, adhesion, and migration (Ryter et al., 2007). According to previous studies, HO-1 induction leads to the attenuation of airway inflammation, mucus hypersecretion, oxidative stress, and AHR (Almolki et al., 2004 and Lee et al., 2011). In this study, PYC treatment caused the induction of HO-1 in OVA-challenged mice, and this finding is consistent with that the results of the in vitro experiment. PYC increased the HO-1 expression in RAW264.7 cells stimulated with LPS. These findings indicate that PYC enhances the expression of HO-1. Many previous studies have

demonstrated that PYC exhibits protective effects on various experimental animal models through its antioxidant property (Ishrat et al., 2009; Parveen et al., 2010; Lee et al., 2012 and Peng et al., 2012). PYC enhances the activity of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione reductase, and glutathione-S-transferase and thereby reduces lipid peroxidation. Therefore, the HO-1 induction observed in this study is likely caused by the antioxidant property of PYC.

Montelukast, a cystenyl leukotriene (CysLT)₁ antagonist blocks the action of LTD₄ on the CysLT₁ in lung tissue by binding to it (Nayak and Langdon, 2007). CysLT₁ receptor expressed in inflammatory cells and other structural cells. LTD₄ binds to CysLT₁ receptor, which induces inflammatory cell recruitment into airway, mucus hypersecretion, and smooth muscle hyperplasia Espinosa et al., 2003. Therefore, montelukast exhibits antiasthmatic effects via a cystenyl leukotriene antagonistic action. In this study, we found that the efficacy of PYC on asthma is similar to that of montelukast. However, we did not confirm that PYC plays a CysLT₁ antagonist. These will be investigated in further study.

Recently, Choi and Yan (2009) demonstrated that PYC effectively inhibits anti-dinitrophenyl IgE-mediated passive cutaneous anaphylaxis and reduces histamine release through suppression of tumor necrosis factor- α and IL-6 in mast cells. These previous findings are supported by the results of the present study. In conclusion, PYC decreases asthmatic responses through its anti-inflammatory and anti-oxidant properties. Our results suggest that PYC may be useful as a therapeutic agent for the treatment of asthmatic patients.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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