



# Anthocyanins inhibit airway inflammation and hyperresponsiveness in a murine asthma model

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## Abstract

Asthma is a common chronic inflammatory disease regulated by coordination of T-helper cell type 2 (Th2) cytokines and inflammatory signal molecules. Additionally, oxidative stress may play an important role in airway inflammation such as eosinophilia, mucus hypersecretion, and airway hyperresponsiveness (AHR). In the present report, we investigated whether anthocyanins would reduce airway inflammation in a mouse asthma model immunized and challenged with ovalbumin (OVA). OVA inhalation elicited inflammatory responses characterized by eosinophilia and increased lipid hydroperoxide (LPO) in bronchoalveolar lavage (BAL) fluid, enhanced pause (Penh), increased glycoprotein and proliferating cell nuclear antigen (PCNA) expressions in mucus hypersecretion, and an increased expression of various cytokines and cyclooxygenase (COX) 2 in lung tissues. All parameters were attenuated in a dose-dependant manner by the administration of anthocyanins. These results suggest that anthocyanins may attenuate the development of asthma by downregulating Th2 cytokines, proinflammatory cytokines, and COX-2. Our findings suggest that anthocyanins have positive contributions as a dietary supplement for the prevention of asthma.

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**Keywords:** Anthocyanins; Asthma; Airway inflammation; Interleukin-13

## 1. Introduction

Bronchial asthma is an inflammatory disease of the airways, characterized by eosinophilia, mucus hypersecretion and airway hyperresponsiveness (AHR). Oxidative stress may play an important role in the pathogenesis of asthma such as enhancing AHR, mucus secretion, and broncho-

constriction (Talati et al., 2006). Superoxide dismutase (SOD) activity was lower in asthmatic individuals than that seen in healthy controls (Comhair et al., 2000). In population-based study, a higher intake of selenium and apples may protect against asthma, and a higher consumption of red wine might reduce asthma severity in some individuals (Shaheen et al., 2001). In addition, vitamin C and E have been shown to decrease the severity of pollutant-induced bronchial responsiveness (Trenga et al., 2001).

Anthocyanins are natural pigments that belong to the flavonoid family and widely distributed in the human diet such as beans, fruits, vegetables, and red wines. It has been reported that anthocyanins have positive effects as antioxidants. Cyanidin-3-*O*-glucoside (C3G), a large part of anthocyanin fractions, has protective effects as a scavenger of active oxygen species in ischemia/reperfusion damage (Tsuda et al., 1999; Amorini et al., 2003). Especially, anthocyanins have been shown to reduce the levels of inflammatory mediators in a lung inflammatory disease model

**Abbreviations:** AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; C3G, cyanidin-3-*O*-glucoside; CFA, complete Freund's adjuvant; CINC-1, cytokine-induced neutrophil chemoattractant-1; COX-2, cyclooxygenase-2; H&E, haematoxylin and eosin; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; MDA, malondialdehyde; OVA, ovalbumin; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SOD, superoxide dismutase; STAT, signal transducers and activators of transcription; Th2, T-helper cell type 2; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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(Rossi et al., 2003). These study results suggested that anthocyanins have protective effects in inflammation and oxidative stress-mediated asthma models.

Asthma is an allergic disease that is strongly associated with an infiltration of inflammatory cells such as mast cells, eosinophils, neutrophils and lymphocytes in lung (Elias et al., 2003). Oxidative stress can be associated with asthma by enhancing the production of cytokines (Crapo and Day, 1999; Crapo, 2003). T-helper cell type 2 (Th2) response is known to be essential for the development and perpetuation of asthma by releasing Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13. Especially, these cytokines are indicated to play the dominant role in the induction and effector phases of allergic inflammation. IL-13 produced by T cells, eosinophils, mast cells, and basophils is known to have various biological properties associated with asthma and be present at an increased level in asthmatic airways and lungs. IL-13 can induce mucus hypersecretion, airway inflammation, airway hyperresponsiveness, and tissue fibrosis through its receptors (Kips, 2001; Wills-Karp, 2004). Many studies have suggested that the blockade of IL-4, IL-13 or IL-13 signaling pathway may have a therapeutic potential for asthma. Recently, the adaptive response of Th2 cytokines (IL-4 and IL-13) has been reported to significantly decrease synovocyte and monocyte COX-2 mRNA and protein levels in vitro (Sugiyama et al., 1996; Endo et al., 1996). COX-2 regulation by Th2 cytokines may have considerable functional implications in diseases such as asthma.

In the present study, we examined whether anthocyanins would have protective effects on allergic airway inflammation and AHR through regulation by various cytokines and COX-2 in a mouse model of asthma.

## 2. Materials and methods

### 2.1. Animals and treatment

Male BALB/c mice (25–31 g, Oriental Co., Ltd. Kyounggi, Korea) were used in this study. The animals were kept in a storage room under the conditions of constant temperature ( $23 \pm 3^\circ\text{C}$ ), relative humidity ( $50 \pm 10\%$ ), and illumination (12 h light/dark cycles) until the initiation of the experiment. All animals were fed with standard animal chow daily and had access to drinking water ad libitum. For sensitization, the asthma control group and anthocyanin treated groups received 20  $\mu\text{g}$  of ovalbumin (OVA, Sigma, St. Louis, MO) with 1 mg of aluminum hydroxide (alum, Pierce biotechnology, Inc., Rockford, IL) in 500  $\mu\text{L}$  of saline, whereas the normal group received 500  $\mu\text{L}$  of saline intraperitoneally on day 1. Each group consisted of seven animals. Anthocyanins (150 or 300 mg/kg, Medox, Medpalett Pharmaceuticals AS, Sandnes, Norway) was orally administered to animals of the anthocyanin treated groups, whereas distilled water was orally administered to the normal and asthma control groups on days 14 to days 20. Animals were challenged with OVA on days 14 to days 20 by daily exposure to an aerosol of 5% OVA in saline, using an ultrasonic nebulizer (3 ml/min, Omron, Tokyo, Japan). Mice of the normal group were challenged with nebulized saline.

### 2.2. Assessment of airway hyperresponsiveness (AHR)

Airway hyperresponsiveness (AHR) was assessed by methacholine-induced airflow obstruction from conscious unrestrained mice placed in a

whole body plethysmograph (model PLY 3211, Buxco Electronics Inc., Wilmington, NC). Mice were challenged for 3 min with a series of aerosolized methacholine inhalation. Enhanced pause (Penh) was monitored for 3 minutes after each aerosol challenge by transducer (model TRD5100, Buxco Electronics Inc., Wilmington, NC) connected to preamplifier modules (model MAX2270, Buxco Electronics Inc., Wilmington, NC) and Biosystem XA software (Buxco Electronics Inc., Wilmington, NC). Penh is a function of total pulmonary airflow during the respiratory cycle and is described by the following equation:

$$\text{Penh} = \text{pause} \times (\text{PEP}/\text{PIP}),$$

Pause, PEP and PIP are expiration time, the peak expiratory pressure, and peak inspiratory pressure, respectively.

### 2.3. Collection of BAL (bronchoalveolar lavage) fluid and differential cell count

Immediately following assessment of airway responsiveness, mice were injected intraperitoneally with 60 mg/kg of pentobarbital. The trachea was cannulated. The right lung was then lavaged with 0.4 ml of PBS three times and the fluid was pooled. The BAL fluid was centrifuged at 3000 rpm for 5 min and the pellet was re-suspended with 200  $\mu\text{L}$  of PBS. Total and differential cell counts were performed using a coulter counter (T-540 Coulter counter, Coulter Electronics, Hialeah, FL). LPO was extracted from 1 ml of BALF using the extract solution and the concentration of LPO was measured as an indicator of oxidative stress by Lipid Hydroperoxide Assay kit (Cayman Chemical, Ann Arbor, MI).

### 2.4. Histological examination

Lung tissues were fixed for 2 days in a 10% formaldehyde solution at room temperature and then were paraffin-embedded. The tissues were sectioned at a thickness of 4  $\mu\text{m}$  for histology. The sections were deparaffinized with xylene, and stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stain. All stained tissue sections were analyzed under a bright-field microscopy.

### 2.5. Immunohistochemistry

Sections were deparaffinized with xylene and washed in serial ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Slides were then blocked with 1% bovine serum albumin in PBS for 1 h. A primary antibodies for PCNA (1:100 dilution, Santa Cruz Biotechnology) and COX-2 (1:100 dilution, Cayman Chemical, Ann Arbor, MI) were then applied and incubated at  $4^\circ\text{C}$  for overnight. After washing in PBS, slides were treated with biotinylated secondary antibody for 20 min, streptavidin-horse radish peroxidase (HRP) for 20 min. Signals for immunoreactivity was visualized with DAB substrate (Zymed Laboratories, South San Francisco, CA). Slides were counterstained with hematoxyline (Sigma–Aldrich, St. Louis, MO) and finally mounted using mounting medium (Sigma–Aldrich, St. Louis, MO).

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

Lungs were rapidly dissected out and frozen on liquid nitrogen. For extraction of total RNA, tissues were homogenized in 1 ml of ice cold RNA-Bee (TEL-TEST, Inc., Friendswood, TX) on ice. Reverse transcription was carried out using M-MLV reverse transcriptase (Promega corporation, Madison, WI) according to the manufacturer's instructions. Total RNA (2  $\mu\text{g}$ ) was used as a template for the reverse transcription (RT) reaction. For the semi-quantitative polymerase chain reaction (PCR) of Th2 and proinflammatory cytokines, the primers were synthesized as follows: TNF- $\alpha$  forward, 5'-GGCAGGTCTACTTTGGAGT-CATTGC-3' and reverse, 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'; IL-1 $\beta$  forward, 5'-TCATGGGATGATGATGATAACCTGCT-3' and reverse, 5'-CCCATACTTTAGGAAGACACGGATT-3'; IL-4 forward, 5'-TCATCGGCATTTTGAACGAG-3' and reverse, 5'-GAATCCAGG-

CATCGA AAA GC-3'; IL-6 forward, 5'-CTGGTGACAACCACGGC-CTTCCCTA-3' and reverse, 5'-ATGCTTAGGCATAACGCACTAG-GTT-3'; IL-13 forward, 5'-TCAGCCATGAAATAACTTATTGTTT TGT-3' and reverse, 5'-CCTTGAGTGTAACAGGCCATTCT-3'; IL-13R2 $\alpha$  forward, 5'-GGTTATGCCAAATGCACCTTGAG-3' and reverse, 5'-ATGGCTTTTGTGCATATCAGAT-3'. The PCR cycles consisted of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 60 s for 32 cycles.  $\beta$ -Actin was also amplified as an internal PCR control using the following primers, forward, 5'-GGGCATTGTGATG-GACTCCG-3' and reverse, 5'-TGAGGCCAAGATGGAGCCAC-3'. The temperature cycling conditions were as follows: 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 5 min. PCR products were separated by electrophoresis through a 2% agarose gel, stained with ethidium bromide, and then detected using UV light. For semiquantitative analysis of PCR bands, the density of each band was measured with a computer imaging device and

accompanying software (Bio-Rad, Hercules, CA). The background value was subtracted from all other readings.

## 2.7. Western blot analysis

Lung tissues were homogenized with 1 ml of ice-cold RIPA lysis buffer (Upstate, VA, USA) containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). Aliquots of total tissue homogenate were frozen and kept at –80 °C. The protein concentration was determined using Bradford method. Equal amounts of protein were loaded in each lane with 5 $\times$  sample loading buffer. Samples were boiled at 100 °C for 5 min before gel loading. Samples were electrophoresed on 10% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Invitrolon, Invitrogen, CA, USA) using an electrophoretic transfer system (Bio-Rad, CA, USA). The membranes were

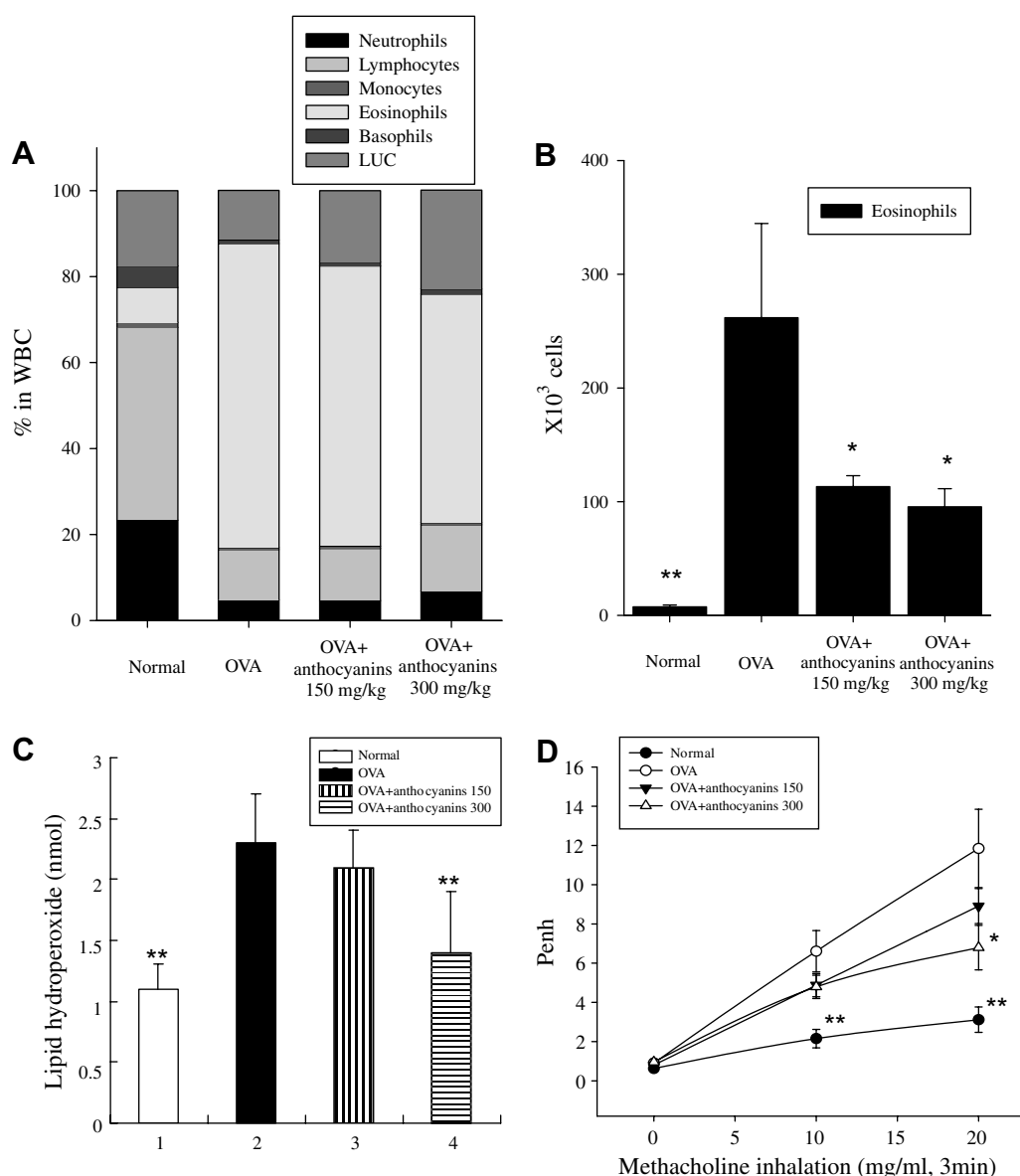


Fig. 1. Effects of anthocyanins on OVA-induced eosinophils recruitment, Lipid peroxidation and airway hyperresponsiveness (AHR) to inhaled methacholine in the lungs. The bronchoalveolar lavage (BAL) fluid was collected and analysis. (A) The percentage of each cell type in white blood cells (WBC) is shown. LUC; large unstained cells. (B) Total numbers of eosinophils are presented. (C) The levels of lipid hydroperoxide was determined in the normal, OVA, and OVA + anthocyanins-treated groups. (D) The degree of broncho-constriction, expressed as Penh was determined in the normal, OVA, and OVA + anthocyanins-treated groups. Values are means  $\pm$  SEM,  $n = 7$ . Significantly different at \* $P < 0.05$ , \*\* $P < 0.01$  compared to the OVA group.

then blocked in TBS buffer (in mM: 20 Tris–HCl, 500 NaCl, pH 7.4) containing 5% skim milk for 1 h with gentle shaking followed by incubation overnight at 4 °C with the primary antibodies in TBS containing 5% skim milk and 0.05% Tween 20. COX-2 levels were monitored using antibody to COX-2 (1:1000 dilution, Cayman Chemical, Ann Arbor, MI). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Calbiochem, CA, USA) diluted 1:1000 for 1 h at room temperature and washed again. The blots were developed with an enhanced chemiluminescence kit.

### 2.8. Statistical analysis

Numerical data obtained during the conduct of the study was subjected to calculation of group means and standard error of the mean. The significance of inter-group differences was analyzed using Student–Newman–Keuls test. Data was considered to be significant when  $P < 0.05$  or  $P < 0.01$ . These statistical data were analyzed by using the GraphPad InStat program (Version 3.05, GraphPad Software Inc., San Diego, CA).

## 3. Results

### 3.1. Collection of BAL (bronchoalveolar lavage) fluid and differential cell count

To determine whether anthocyanins could change pulmonary immune responses, inflammatory cell recruitment to the lung in response to anthocyanins (150 or 300 mg/kg) in an OVA sensitized asthma model was compared with non-treated normal group. Differential cell counts indicated that the changes in total cell number were accounted for by an increase in the representation of eosinophils as a proportion of total white blood cells in asthma relative to normal animals. The percentages of eosinophils were  $8.14 \pm 1.63\%$  in normal mice,  $70.73 \pm 4.25\%$  in asthmatic mice,  $65.17 \pm 3.88\%$  in OVA-allergic mice administered 150 mg/kg of anthocyanin,  $53.14 \pm 5.46\%$  in OVA-allergic

mice administered 300 mg/kg of anthocyanin, respectively (Fig. 1A). Correspondingly, the numbers of eosinophils in BAL fluid in mice treated with 150 ( $113.14 \pm 9.71$ ) and 300 mg/kg ( $95.43 \pm 15.94$ ) anthocyanins were significantly reduced in comparison with the asthmatic animals ( $261.71 \pm 82.88$ ) (Fig. 1B). However, there were no differences in the ratio of neutrophils, lymphocytes, monocytes, and basophils among the asthma control group and 150 mg/kg or 300 mg/kg-anthocyanins treated groups. We measured the levels of lipid hydroperoxide. There was a significant decrease in the concentration of lipid hydroperoxide (1.4 nmol) in mice treated with 300 mg/kg anthocyanins as compared to that (2.3 nmol) of OVA-allergic mice (Fig. 1C).

### 3.2. Airway hyperresponsiveness (AHR)

Airway responsiveness, as determined in the basis of Penh, was increased in the asthmatic mice that had been sensitized by and challenged with OVA and subsequently challenged with methacholine. Increased Penh was observed in the asthma group following inhalation of 10 mg/ml and 20 mg/ml of methacholine as compared to the normal control group. In comparison with untreated asthmatic mice, significantly decreased AHR was observed in the mice administered 300 mg/kg anthocyanins after inhalation of 20 mg/ml of methacholine (Fig. 1D).

### 3.3. Histological studies on effects of anthocyanins in OVA-induced asthma model

The morphological features of haematoxylin and eosin (H&E) stained lung sections are shown in Fig. 2. Since

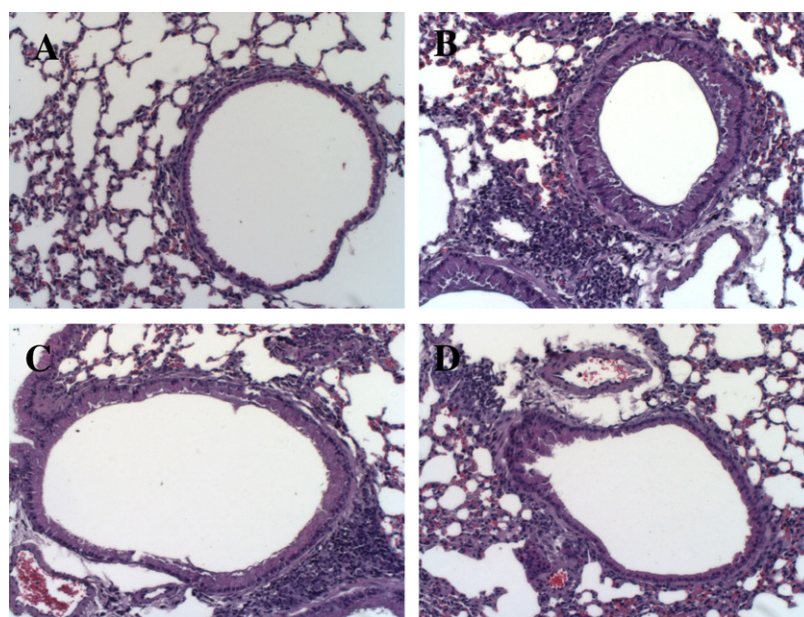


Fig. 2. Effects of anthocyanins on lung histopathology in a mouse model of OVA-induced asthma. Tissue sections stained with H&E and examined under bright-field microscopy (magnification 200 $\times$ ). Representative photographs of mice in the normal (A), OVA (B), OVA + anthocyanins 150 mg/kg (C), and OVA + anthocyanins 300 mg/kg (D) groups are shown.

anthocyanins markedly reduced inflammatory cell recruitment into bronchoalveolar lavage fluid, we further examined changes in inflammatory cell recruitment and mucus secretion in the lung tissue sections from OVA-allergic mice. Inflammatory cell infiltration in peribronchial and perivascular areas was observed in OVA-allergic mice (Fig. 2B) as compared to the normal controls (Fig. 2A). Furthermore, many goblet cells containing mucus and cell proliferation were increased in the bron-

chial epithelium of OVA-allergic mice (Fig. 3B and F) as compared to the normal controls (Fig. 3A). Anthocyanins treatment (300 mg/ml) markedly reduced inflammatory cell infiltration in the peribronchial and perivascular areas (Fig. 2D). Mucus hypersecretion of goblet cells (Fig. 3C and D) and hyperplasia of bronchial epithelial cells (Fig. 3G and H) were also reduced in mice treated with anthocyanins in a dose-dependant manner.

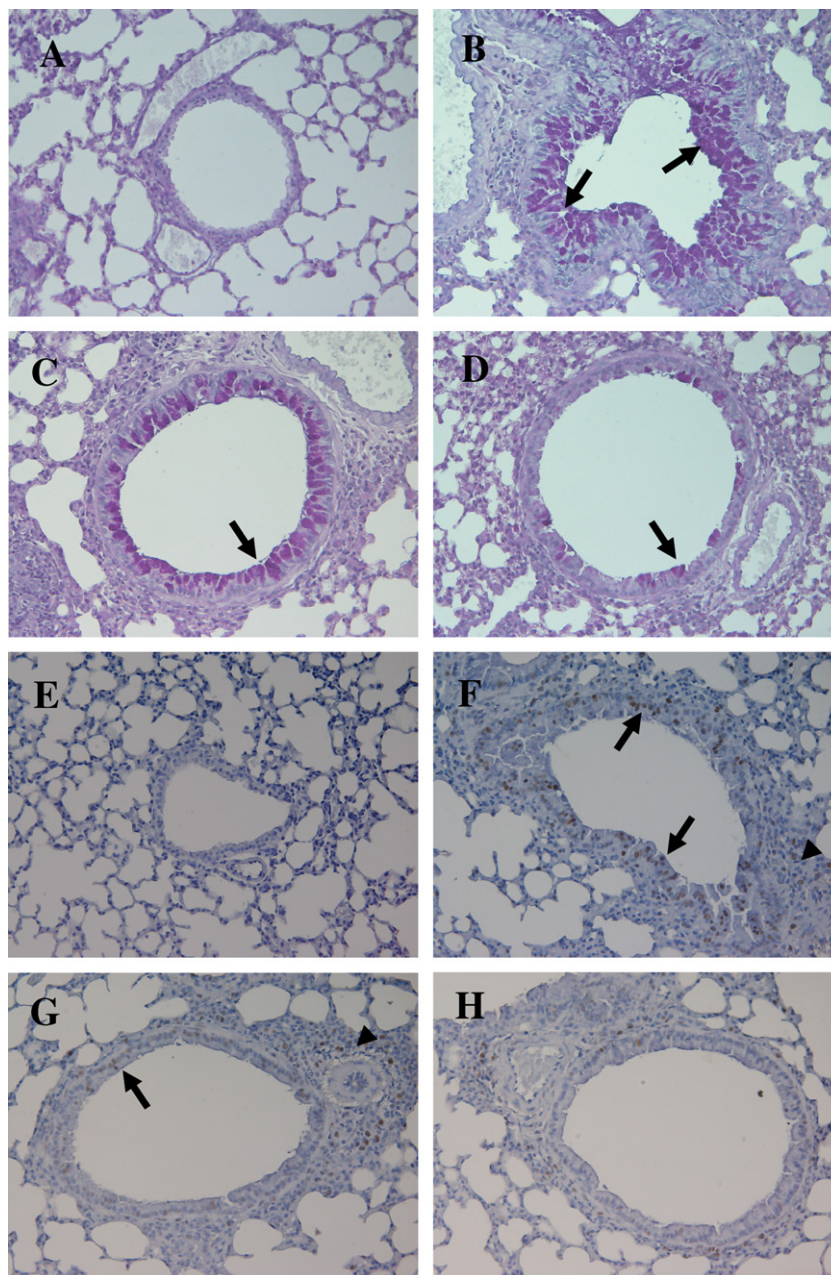


Fig. 3. Effects of anthocyanins on mucus secretion and cell proliferation in the lung tissues of OVA-induced asthma. Tissue sections stained with periodic acid-Schiff (PAS) stain and PCNA antibody (magnification 200 $\times$ ). Representative photographs of mice in the normal (A), OVA (B), OVA + anthocyanins 150 mg/kg (C), OVA + anthocyanins 300 mg/kg (D) groups for PAS stain and normal (D), OVA (E), OVA + anthocyanins 150 mg/kg (F), OVA + anthocyanins 300 mg/kg (G) groups for PCNA immunostaining are shown. Arrows indicate the mucus hypersecretion by PAS stain (B, C and D) and proliferated cells in the bronchiolar epitheliums (F, G and H). Arrow heads indicate the infiltrated inflammatory cells around the bronchiols and arterioles (F and G).

### 3.4. Anthocyanins inhibit Th2 and proinflammatory cytokines mRNA expression

Allergic asthmatic inflammation and airway remodeling are caused by the secretion of a series of Th2 and pro-inflammatory cytokines. To investigate this effect, we examined whether anthocyanins would be capable of altering mRNA expressions of Th2 cytokines (IL-4, IL-13, IL-13R1 $\alpha$ , IL-13R2 $\alpha$ ) and proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) in a mouse model of OVA-induced asthma. RT-PCR analysis showed the increase of TNF- $\alpha$ , IL-6, IL-13, and IL-13R2 $\alpha$  mRNA expression after OVA inhalation. Significantly decreased mRNA expressions of TNF- $\alpha$ , IL-6, IL-13, and IL-13R2 $\alpha$  were observed after treatment with anthocyanins (150 and 300 mg/kg) as compared to OVA-allergic controls in mouse asthma models (Fig. 4).

### 3.5. Anthocyanins reduce COX-2 expression

Cyclooxygenase is one of the key enzymes that catalyze the formation of prostaglandins and thromboxanes. Prostaglandins and thromboxanes are important modulators of airway physiology. In particular, COX-2 expression has been reported in various inflammatory diseases (Hou et al., 2005). Therefore, we examined where COX-2 was localized in the lung tissue of allergic asthma and whether COX-2 expression would be affected by the treatment of

anthocyanins in mouse model of OVA-induced asthma. COX-2 protein levels, measured by Western blot analysis, were low in the control animals but were increased in OVA-induced asthma group. Increased COX-2 expression in OVA-induced asthma was reduced gradually by the treatment of anthocyanin (Fig. 5A). Therefore, we used the immunohistochemistry to know the localization of COX-2 protein. COX-2 was mainly localized in the bronchial epithelial cells, bronchial mucus cells, and bronchial smooth muscle cells in the asthmatic lung as well as normal lung (Fig. 5B). The immunoreactivity for COX-2 was increased in the bronchial mucosa and infiltrated inflammatory cell around the peribronchiole in asthmatic lungs as compared to the normal control (Fig. 5B).

## 4. Discussion

In this study, we have demonstrated that the intake of anthocyanins effectively suppressed allergic inflammation and AHR in a model of asthma. Furthermore, we found that these effects of anthocyanins were related to decrease of Th2 and pro-inflammatory cytokines. Asthma is a chronic disease that causes bronchoconstriction, inflammation and hyperresponsiveness to cholinergic agonists, such as methacholine (Chung, 1986; Barnes, 1989). Airway inflammation and hyperreactivity in asthma are likely to involve oxidative stress to the lung. Excess production of reactive oxygen species (ROS) by immune cells may play an important role in the airway injury in asthma (Talati et al., 2006; Dworski, 2000). Previous studies showed that the generation of ROS is increased in the airways (Nishida et al., 2002; Zhang et al., 2002) and BAL fluid (Lee et al., 2004) in OVA-induced asthma models.

Anthocyanins are considered as antioxidants because they were shown to possess many beneficial characteristics in various animal models. In the isolated ischemic and reperfused rat heart, C3G attenuated lipid peroxidation and the generation of malondialdehyde (MDA) induced by oxidative damage (Amorini et al., 2003). The intake of anthocyanins enhanced plasma antioxidant capacity and decreased hydroperoxides and 8-Oxo-deoxyguanosine concentrations in the liver of vitamin E-depleted rats (Ramirez-Tortosa et al., 2001). In agreement with these antioxidant effects, recent studies have suggested that anthocyanins have anti-inflammatory properties as well. Anthocyanins are effective as indomethacin in suppressing carrageenan and complete Freund's adjuvant (CFA)-induced inflammation (Tall et al., 2004). In zymosan-treated rats, the elevation of inducible nitric oxide synthase (iNOS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and the cytokine-induced neutrophil chemoattractant-1 (CINC-1) were reduced by C3G during the zymosan-induced inflammatory response (Tsuda et al., 2002). Anthocyanins suppressed nitrate/nitrite and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in a rat model of the carrageenan-induced pleurisy (Rossi et al.,

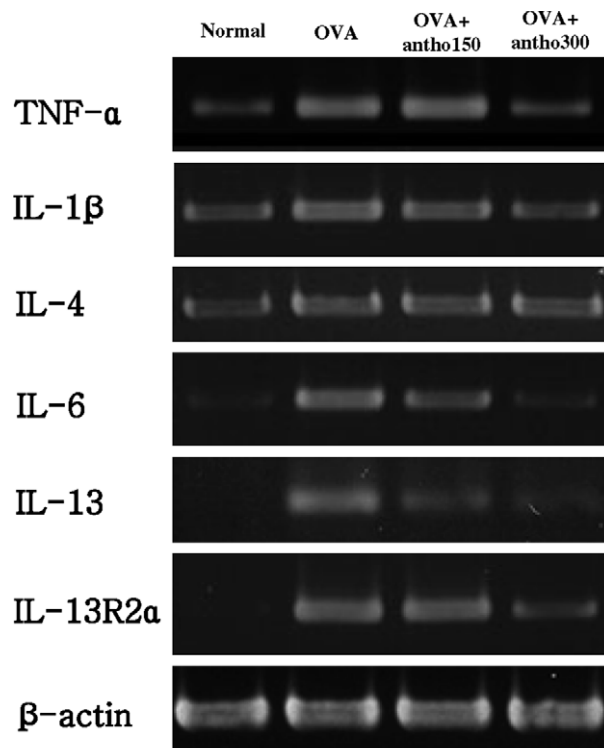


Fig. 4. Effects of anthocyanins on various cytokines mRNA expression in lung tissues. RT-PCR of TNF- $\alpha$ , IL-1 $\beta$ , IL-4R, IL-6, IL-13, IL-13R1 $\alpha$ , IL-13R2 $\alpha$  and  $\beta$ -actin in the lung of normal, OVA, OVA + anthocyanins 150 mg/kg, and OVA + anthocyanins 300 mg/kg treated mouse.

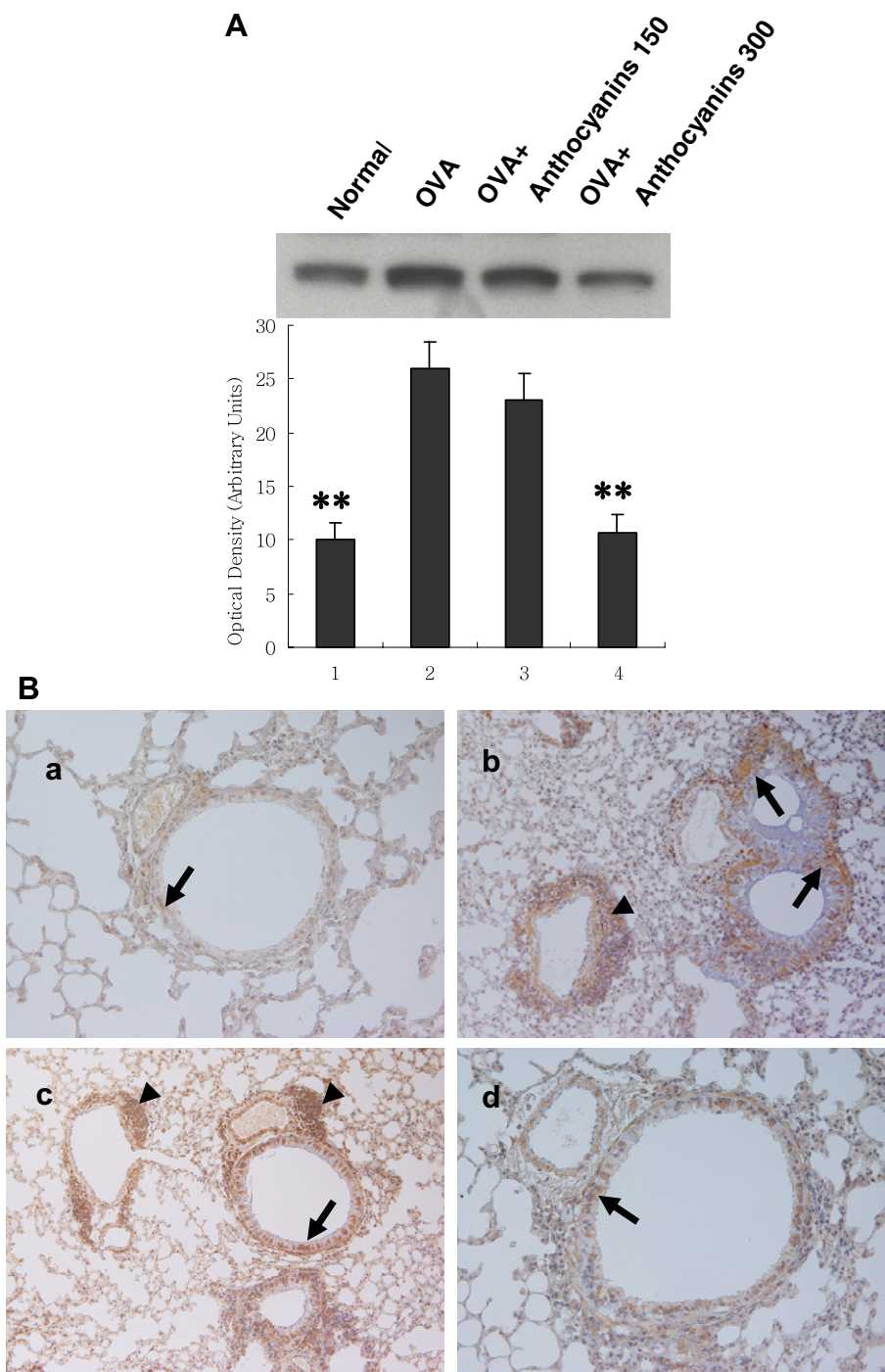


Fig. 5. Effects of anthocyanins on COX2 expression in the lung tissues of OVA-induced asthma. (A) Western blot analysis of COX-2. Graphic representation of the mean of optical density. Values are means  $\pm$  SEM,  $n = 3$ . Significantly different at  $**P < 0.01$  compared to the OVA group. (B) Tissue sections stained with COX-2 antibody (magnification 100 $\times$  and 200 $\times$ ). Representative photographs of mice in the normal (a), OVA (b), OVA + anthocyanins 150 mg/kg (c), and OVA + anthocyanins 300 mg/kg (d) groups are shown. Arrows and arrow heads indicate COX-2 positive bronchiolar epithelium and infiltrated inflammatory cells, respectively.

2003). However, the anti-asthmatic activities of anthocyanins are poorly defined in animal models.

Neurogenic abnormalities and airway inflammation, characterized by eosinophil infiltration, and the release of cytokine have been implicated in the development of AHR (Bousquet et al., 1990; Djukanovic et al., 1990). The development and perpetuation of asthmatic inflamma-

tion involve various factors and cytokines that promote immune responses. In this study, we demonstrated that administration of anthocyanins reduces mucus hypersecretion, recruitment of inflammatory cells, and mRNA levels of various cytokines in an OVA-induced murine asthma model. Numerous previous studies reported that Th2 cells promoted allergic diseases such as asthma and that Th2

and proinflammatory cytokines such as IL-4, IL-5, IL-13, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 would play the leading roles in generating the asthmatic phenotype (Kips, 2001; Schuh et al., 2003; Cohn et al., 2004). Especially, IL-4 and IL-13, in particular, exert various pathobiological activities by binding to IL-13 receptors expressed on various immune cells such as B cells, monocytes/macrophages, dendritic cells, eosinophils, fibroblasts, endothelial and epithelial cells. IL-13 signal is transduced intracellularly, mainly via Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathway (Corry and Kheradmand, 2002; Izuhara and Arima, 2004; Wills-Karp, 2004). Intratracheal IL-13 delivery can induce lung eosinophilia and airway hyperresponsiveness in mice (Blanchard et al., 2005). Overexpression of IL-13 was reported in asthma patients (Kotsimbos et al., 1996; Bodey et al., 1999). Moreover, IL-13 stimulated airway fibrosis and promoted epithelial damage, mucus production, and eosinophilia (Cohn et al., 2004). As IL-13 is the key factor in pathogenesis of asthma, it is reasoned that pursuing IL-13 or the inhibition of its biological activities may be effective in the treatment of asthma. In the present study, anthocyanins markedly reduced the expressions of elevated Th2 cytokines (IL-13 and IL-13R2 $\alpha$ ) and proinflammatory cytokines (TNF- $\alpha$  and IL-6), suggesting many anti-inflammatory effects of anthocyanins in an OVA-induced murine asthma model.

A previous report demonstrated that repeated challenges with allergen upregulated the level of COX-2 protein and caused a corresponding increase in PGE<sub>2</sub> in BALF in murine lungs (Gavett et al., 1999). The inhibition of NF- $\kappa$ B activity reduced the production of Th2 cytokines, IL-4, IL-5 and IL-13, in p50-deficient mice (Das et al., 2001). Additionally, MOL 294, a potent inhibitor of NF- $\kappa$ B, decreased the level of IL-13 and eotaxin in OVA-treated mice (Henderson et al., 2002). A number of reports demonstrated similar effects with antioxidants such as vitamin C and E (Tan et al., 2005) and green tea extract (Lee et al., 2005). Hou and colleagues demonstrated that anthocyanin extracts inhibited the expression and transcriptional levels of cyclooxygenase-2 (COX-2) involved in many inflammatory processes *in vitro* (Hou et al., 2005). In the present study, COX-2 protein production was inhibited by anthocyanins in an OVA-induced murine asthma model. COX-2 expression could be easily induced by proinflammatory IL-1 $\beta$ , and the enhanced COX-2 expression led to increased PGE<sub>2</sub> formation in human bronchial smooth muscle cells (Vingano et al., 1997). Elements of the adaptive Th2 immune response (IL-4 and IL-13) have been reported to significantly decrease synovocyte and monocyte COX-2 mRNA and protein levels *in vitro* (Sugiyama et al., 1996; Endo et al., 1996). COX-2 regulation by Th2 cytokines may have considerable functional implications in diseases such as asthma.

Our study demonstrated the protective effects of anthocyanins against asthmatic inflammation and also showed that these effects are related to downregulation of Th2

and proinflammatory cytokines mRNA. These results suggest that anthocyanins can be suitable candidates for a dietary supplement to attenuate the initial development as well as the symptoms of asthma.

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