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Protective Effects of Acorn (*Quercus acutissima* CARR.) against IgE-mediated Allergic and Ovalbumin (OVA)-Induced Asthmatic Responses via Inhibition of Oxidative Stress

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ABSTRACT – This work was performed to investigate the protective effect of ethanol extract (AEx) from acorn (*Quercus acutissima* CARR.) against allergic mediated responses in asthma model cells and mice. The AEx inhibited antigen-stimulated cytokine production such as interleukin (IL)-4, IL-13 and tumor necrosis factor-α (TNF-α) and AEx also inhibited intracellular reactive oxygen species (ROS) generation against IgE-mediated allergic response in rat basophilic leukaemia RBL-2H3 cells. The ovalbumin (OVA)-sensitized mice were orally administered with AEx (100 or 300 mg/kg) and authentic tannic acid (75 mg/kg) every day for 15 days. Increased TNF-α production by OVA-sensitization/challenge was significantly reduced by administration of AEx. The serum triglyceride levels of asthma mice were significantly reduced after feeding for 15 days with tannic acid or AEx. The mice fed with tannic acid or AEx also exhibited a significant reduction in body weights compared to those of asthma control group. The AEx increased the heme oxygenase (HO)-1 mRNA expression in the asthma model mice and showed DPPH radical scavenging activity. These results indicate that AEx protects against IgE-mediated allergic and OVA-induced asthmatic responses via direct and indirect antioxidant activities. Reduced triglyceride and body weights may provide additional protective benefits of AEx on allergic asthma.

Key words – Acorn, Allergic response, Antioxidant activity, Asthma, Cytokines

Mast cells and basophils in allergic reaction, including asthma, are activated in response to antigen cross-linking of IgE bound to the high affinity IgE receptor (FcεRI) on the cell surface and activated mast cells and basophils induce a variety of cellular responses, including production of reactive oxygen species (ROS) and related cytokines (Itoh et al., 2008; Han et al., 2011; Itoh et al., 2011).

Recent reports demonstrated that T-helper type 2 (Th2) cytokines such as interleukin (IL)-4, IL-5 and IL-13 and proinflammatory, especially tumor necrosis factor (TNF)-α play important roles in asthma (Foster et al., 1996; Itoh et al., 2011) and the generation of Th2 cytokines and TNF-α levels are increased in asthmatic lungs (Williams and Galli, 2000; Park et al., 2007; Lee et al., 2010). Heme oxygenase (HO) catalyzes the first and rate-limiting step in the oxidative degradation of heme to bilirubin and HO-1 is highly induced by a variety of agents causing oxidative stress (Pae et al., 2003) and allergic response induced intracellular oxidative stress (Itoh et al., 2008; Han et al., 2011; Itoh et al., 2011). Recent research indicates that HO-1 is a protective gene, and its upregulation has anti-asthmatic effects (Lee et al., 2010).

Acorn (*Quercus acutissima* CARR.) has been used in food and folk medicine in Korea and dietary intake of acorn is associated with a decreased risk of obesity, hyperlipidemia and dementia (Kang et al., 2004; Lee et al., 2005). Tannic acid, along with other condensed tannins, have been reported as biologically active compounds in acorn including strong antioxidant activities (Shim et al., 2004; Gulcin et al., 2010; Tejerina et al., 2011). However, anti-allergic and anti-asthma effects of acorn remain to be unclear. In the present study, we investigated whether ethanol extract (AEx) from acorn (*Quercus acutissima* CARR.) exerts anti-allergic and anti-asthmatic effects in conjunction with possible inhibitory activity against oxidative stress and expression of Th2 cytokines in IgE-antigen complex-stimulated RBL-2H3 cells and OVA-sensitized/challenged asthma mice.

Materials and Methods

Preparation of ethanol extract from acorn

Acorn (*Quercus acutissima* CARR.) used in this study were collected in Dangjin-gun province of Chungcheongnam-do, Korea, in 2009. Four hundred kilograms of the acorn raw material was air-dried, de-shelled, lyophilized, and then

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crushed into powder, yielding 167 kg in dry mass. The freeze-dried acorn was extracted with 80% ethanol at room temperature overnight. The extraction procedure was repeated with 80% ethanol. The extracts were centrifuged at 1,500 rpm for 30 min, and the supernatants were pooled, evaporated, filtered through 10-μm pore-sized filter and then lyophilized, with a yield of 14.4% in mass. The powdered extract (named AEx) was stored at -70°C until use.

DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the AEx was measured according to the procedure described by Choi et al. (2011) with slight modifications. Different concentrations of the AEx (0.05-50 mg/mL) were placed in 96 wells. The AEx (100 μL) were mixed with 66.7 μL of ethanolic solution containing the DPPH radical (1.5×10^{-4} M). The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The reduction of the DPPH radicals was measured by an ELISA reader at 517 nm. Radical scavenging activity was measured as the decrease in DPPH absorbance and the inhibition percentage was calculated by using the following equation:

$$\text{Scavenging activity (\%)} = (1\text{-}A_{\text{sample(517nm)}}/A_{\text{control (517nm)}}) \times 100$$

Cell culture

RBL-2H3 cells, the rat basophilic leukemia cell line, were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37ºC using minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycine (PEST).

Cytotoxicity assay

Cell cytotoxicity was measured using MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to method of Chung et al. (2005). Briefly, IgE-sensitized RBL-2H3 cells were seeded to 96-well plates. After growth in MEM including 10% FBS and 1% PEST at 37°C for 24 h, various concentrations of AEx (25-100 μg/mL) were applied to the MEM. The medium was removed and 90 μL MEM supplemented with 10 μL MTT solution (5.0 mg/mL) was added. After incubation for another 4 h, the formazan formed was dissolved in DMSO. Cell viability was determined by a microplate reader at 570 nm. The density of formazan formed in control cells was taken as 100% of viability.

Measurement of ROS generation

Intracellular ROS levels were measured using H2DCF-DA, which is deacetylated by intracellular esterases, yielding non-fluorescent compound 2',7'-dichlorodihydrofluorescein (DCFH). DCFH is in turn oxidized to the fluorescent compound 2',7'-dichlorodihydrofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells were pre-incubated with H2DCF-DA for 30 min at 37ºC and then washed to remove excess H2DCF-DA. IgE-sensitized RBL-2H3 cells were then treated with AEx (0-100 μg/mL) and/or DNP-BSA for 30 min. Finally, fluorescence intensity was measured using a spectrofluorometer (excitation: 495 nm; emission: 527 nm). DCF fluorescence intensities were calculated relative to control levels.

Measurement of cytokines in cell culture supernatants and cells

RBL-2H3 cells (1×10^6 cells/well) in 6-well plates were stimulated with 0.5 μg/mL anti-DNP IgE for 24 h and then washed with Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$ and 25 mM PIPES) and incubated in Siraganian buffer containing 5.6 mM $CaCl_2$ and 0.1% BSA for an additional 10 min. Thereafter, the cells were incubated with MEM containing 0, 25, 50 and 100 μg/mL AEx for 4 h and stimulated for 2 h with DNP-HSA. The supernatant (for TNF-α and IL-4) and the cells (for IL-13) were used for Enzyme-linked immunosorbent assay (ELISA). ELISA experiments were performed according to the manufacturer's instructions. TNF-α, IL-4 and IL-5 levels in cell culture supernatants and cell lysate were measured using ELISA kits (IL-4 and TNF-α, Abcam, Cambridge, UK; IL-13, RayBiotech, Norcross, GA).

Animals, diets and experimental protocol for animal treatment

Six week-old female BALB/c mice were purchased from KOATECH (Gyeonggi-do, Korea). The mice were maintained under standard laboratory conditions and animals were provided with water and commercial diet for one week prior to their distribution into five groups (n = 10). The mice were cared for and treated following the guidelines for laboratory animals established by the Catholic University of Korea. A schematic diagram of the treatment schedule is shown in Figure 1.

A total of 500 μg/mL of ovalbumin (OVA) was complexed with 500 μg/mL of alum to the final volume ratio of alum (500 μg/mL) to OVA (500 μg/mL), 1:3. Each mouse was immunized by intraperitoneal (IP) injection of 14.7 mL/kg body weight of OVA complexed with alum on days 0, 6, and 12 (Figure 1). The control group received 14.7 mL/kg body

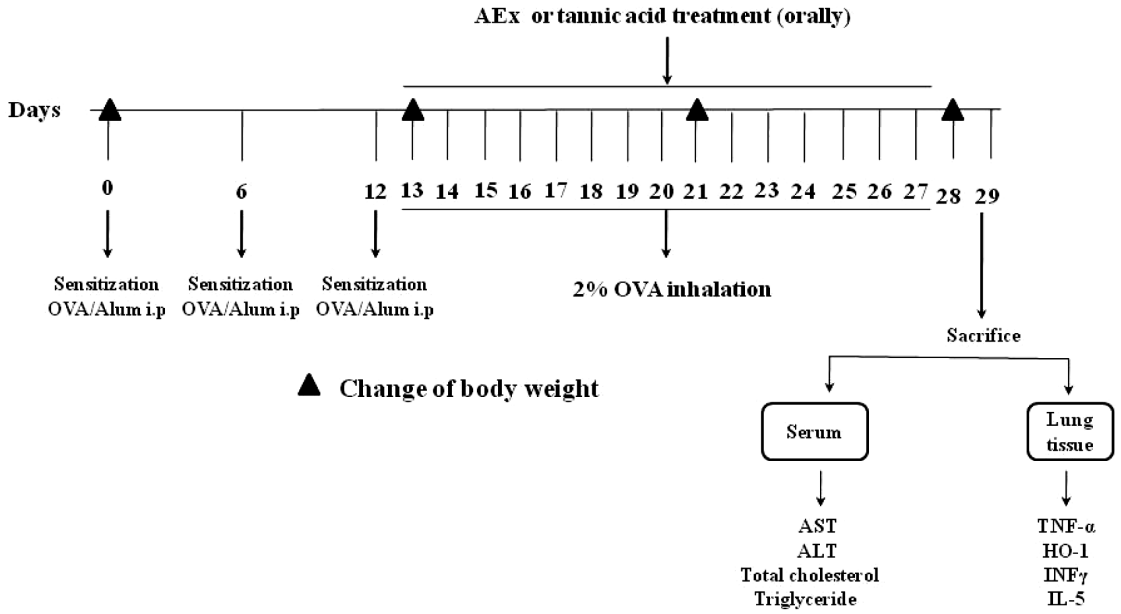

Figure 1. Mouse model of airway inflammation induced by ovalbumin (OVA) and treatment with acorn ethanol extract (AEx) or tannic acid. i.p., intraperitoneal.

weight of phosphate-buffered saline (PBS) with alum by IP injection.

The OVA-challenged mice were exposed to 2% OVA (w/v, in PBS) for 10 min (keeping for 5 min) by inhalation using a Compressor Nebulizer (0.4 mL/min, NE-C28, Omrom, Tokyo, Japan) and were orally administered with water (asthma group, n = 10), AEx (100 or 300 mg/kg body weight/day, n = 10) and authentic tannic acid (75 mg/kg body weight/day, n = 10) were orally administered everyday from day 13 to day 27 consecutively. After feeding, the mice were fasted overnight (16-19 h) and sacrificed on day 29. The mice were euthanized with an IP injection of a Zoletil 50 (Virbac S.A, France) and Rompun (Bayer, Germany) mixture (3:2 mixture). The blood samples were collected into tubes. The lungs were rapidly removed, frozen on liquid nitrogen and stored at -80°C for total RNA extraction.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitron, Carlsbad CA, USA) as described by the manufacturer's instruction. For cDNA preparation, total RNA was reversed-transcribed using a Power cDNA synthesis kit (Intron, Gyeonggi-do, Korea) with oligo $(dT)_{15}$ primer according to the manufacturer's recommendations. The PCR was performed with a Maxime PCR PreMix kit (Intron, Gyeonggi-do, Korea) in a 20-μL total reaction mixture containing 1 μL of the RT-reaction mixture and 2 μL of each primer (forward and reverse, 10 pmol/μL). The following primer sequences were used: mouse TNF-α, 5'-GGCAGGTCTACTTTGGAGTCATTGC-3' and 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'; mouse HO-1, 5'-GAGAATGCTGAGTTCATG-3' and 5'-ATGTTGAGCAGGAAGGC-3'; mouse INFγ, F 5'-TGTTTCTGGCTGTTACTG-3' and 5'-TTGCTGTTGCTGAAGAAG-3'; mouse IL-5, F 5'-AAGCAATGAGACGATGAG-3' and 5'-CATCACACCAAGGAACTC-3'; mouse β-actin, F 5'-TGCTGTCCCTGTATGCCTCT-3' and 5'-AGGTCTTTACGGATGTCAACG-3'. Amplification was performed as follows: initial denaturation at 94°C for 2 min, followed by 22 cycles (β-actin) or 38 cycles (IL-5, INF-γ, TNF-α, HO-1) of denaturation at 94 °C for 20 sec, annealing at 50°C (IL-5 and IFN-γ), 58°C (TNF-α and HO-1) or 60°C (β-actin) for 10 sec, extension at 72°C for 30 sec, and final extension at 72°C for 5 min. The β-actin was used as the internal control for each reaction. The final PCR products were separated on 1% agarose gels, and visualized by ethidium bromide staining. Densitometric analysis was performed using SigmaGel software (Jandel Scientific, San Rafael, CA).

Measurement of body weight, serum lipids, ALT and AST

During the experimental period, the food intake of mice was checked twice a week, and their body weights were measured on days 0, 13, 21, and 28 after initial sensitization. The total cholesterol, triglyceride, alanine transaminase (ALT) and aspartate transaminase (AST) levels in serum of OVA-induced asthma mice were measured using commercial kits (Asan

Chemical, Seoul, Korea), according to the manufacturer's instructions, after feeding the mice for 15 days with tannic acid (75 mg/kg/day) or AEx (100 or 300 mg/kg/day).

Statistical analysis

Data from three independent experiments were expressed as mean ± S.D. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the results from different treatments. Data were considered to have statistical significances at $P < 0.05$.

Results and Discussion

Effects of AEx on cytotoxicity and ROS production in IgE-sensitized RBL-2H3 cells

As shown in Figure 2A, the concentrations (25-100 μg/mL) of AEx had no detectable level of effect on the viability of RBL-2H3 cells compared with the control.

Antigen treatment stimulated ROS production in IgE-sensitized RBL-2H3 cells and AEx suppressed the ROS production in IgE-sensitized RBL-2H3 cells (Figure 2B). We also measured the radical-scavenging activity of AEx by the DPPH radical-scavenging method. The result of DPPH radical-scavenging test showed that AEx has a strong radical-scavenging activity in a dose-dependent manner. These results suggest that suppression of intracellular ROS production by AEx is mediated by the strong direct antioxidant activity.

Several reports indicated that endogenous ROS is a critical regulator of mast cells and basophils response and antioxidants blocked intracellular ROS generation induced by FcεRI activation (Itoh et al., 2008; Han et al., 2011; Itoh et al., 2011). The AEx blocks ROS production caused by IgE-antigen complex and the strong antioxidant activity suggests that AEx may be effective in the treatment of allergies like asthma because ROS are involved in allergic inflammation (Springer et al., 2007).

AEx inhibits antigen-induced TNF-α, IL-4 and IL-13 protein production in IgE-sensitized RBL-2H3 cells

The inhibitory effects of AEx against protein expression of TNF-α, IL-4 and IL-13 were also measured using ELISA analysis in RBL-2H3 cells stimulated by the IgE-antigen complex. As shown in Figure 3, the treatment of the IgE-antigen complex to RBL-2H3 cells increased protein expression of TNF-α, IL-4 and IL-13 but the treatment with 50 μg/mL AEx resulted in significant suppression of the production of TNF-α, IL-4 and IL-13 proteins. However, no significant changes were observed in the production of TNF-α and IL-3 proteins at the concentration of 100 μg/mL AEx.

Mast cells and basophils produce proinflamatory cytokines, especially TNF-α which plays a critical role in late-phase reactions of hypersensitivity, and Th2 cytokines (such as IL-4, IL-5 and IL-13) due to allergic asthma. Antigen-induced actions of RBL-2H3 cells induce production of Th2 cytokines, which are involved in immediate allergic responses (Lee et al., 2007; Huang et al., 2008). The ELISA assay showed that AEx inhibited the protein production of TNF-α, IL-4 and IL-13 in RBL-2H3 cells stimulated by IgE-antigen complex and also the production of cytokines associated with allergic asthma reactions.

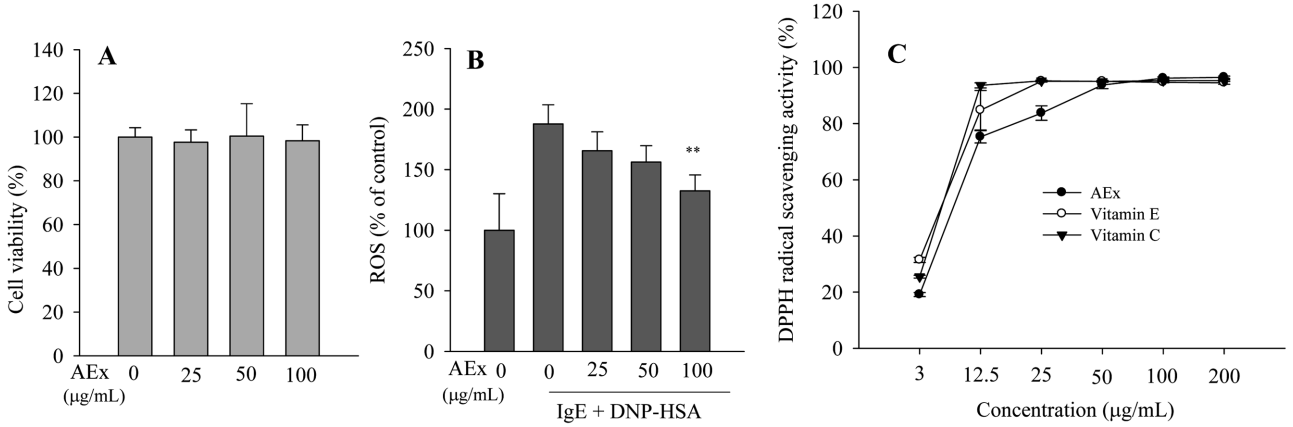

Figure 2. Effects of AEx on cytotoxicity, DPPH radical-scavenging activity and antigen-induced intracellular reactive oxygen species (ROS) production in IgE-antigen complex stimulated rat basophilic leukemia RBL-2H3 cells. (A) Cytotoxicity levels were assessed by MTT cell viability assays at different concentrations. Cells were incubated with the test samples for 24 h. (B) Intracellular ROS levels were measured using non-fluorescent H2DCF-DA. *P<0.05 vs. positive control (DNP-BSA); **P<0.01 vs. positive control (DNP-BSA). (C) The DPPH radical-scavenging activities of AEx were measured at indicated concentrations. The data are the means ± SD (n = 4).

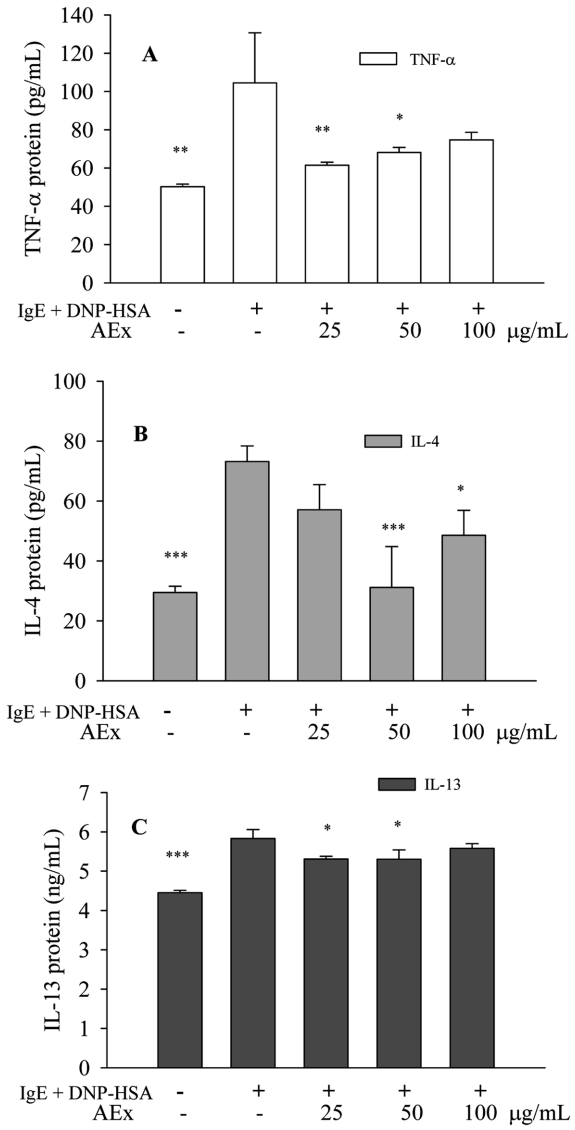

Figure 3. Effects of AEx on inflammatory cytokine production in IgE-antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells. The production of TNF-α (A) and IL-4 (B) were determined in cultured media and the production of IL-13 (C) was determined in cell lysate using a commercial ELISA kit. The data are the means ± SD (n = 4). **P*<0.05, ***P*<0.01, and ****P*<0.001 vs. positive control (DNP-BSA).

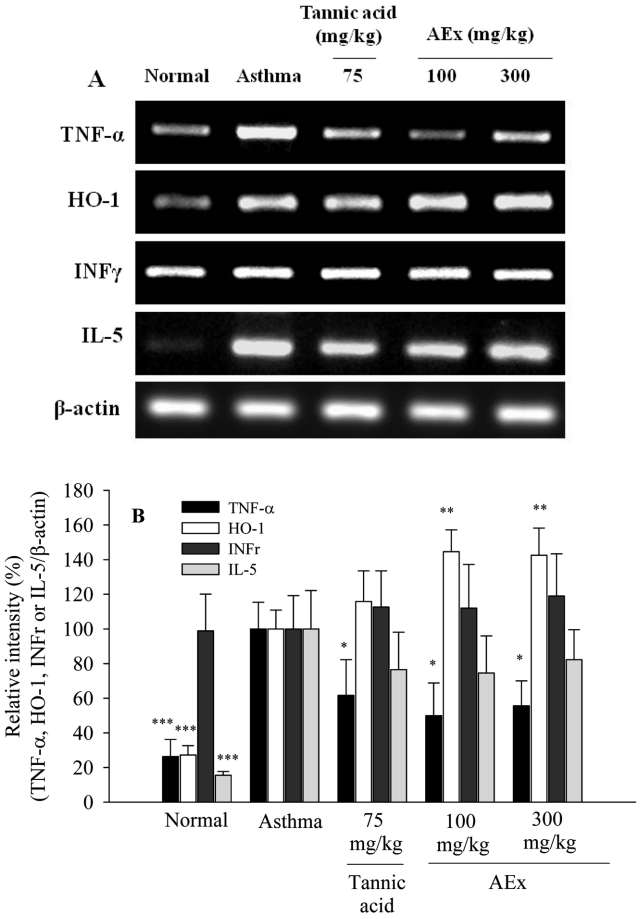

Figure 4. Effects of AEx and tannic acid on the expression of TNF-α, HO-1, INFγ and IL-5 in lung tissues. The total RNA was extracted from each lung tissue, and the mRNA expression of TNF-α, HO-1, INFγ and IL-5 were analyzed by RT-PCR. β-Actin was used as an internal control for each PCR reaction. The density of each mRNA was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA). Values are mean ± SD, n = 6. Significantly different at **P*<0.05, ***P*<0.01 or ****P*<0.001 compared to the OVA (asthma) group.

Effects of AEx on cytokine expression and production in lung tissue and serum from OVA-induced asthma mice

Gallic acid and tannic acid have been reported to be effective antioxidant components of acorn (Lee at al. 1992). We analyzed the major compounds in our AEx mainly by HPLC and the major phenolic compound was shown to be tannic acid (Data not shown). Thus, we used authentic tannic acid (Sigma-Aldrich, St Louis, MO, USA) as a reference compound for subsequent *in vivo* studies using asthma model mice.

Because AEx reduced cytokines production in allergic asthma model cells, we examined the mRNA expression and protein production of cytokines associated with asthma in lung tissue and serum from OVA-induced asthma mice. In addition, we investigated whether AEx upregulates HO-1 expression against OVA-sensitized/challenged lung tissue injury.

The mRNA expressions of TNF-α, HO-1 and IL-5 were increased in lung tissues of the OVA-sensitized/challenged asthma control (without AEx or tannic acid) group (Figure 4). Significantly decreased mRNA expression of TNF-α was observed after treatment with tannic acid (75 mg/kg) or AEx (100 mg/kg and 300 mg/kg) as compared to OVA-sensitized/challenged asthma control group (Figure 4). AEx treatment led to increase in HO-1 mRNA expression. INF-γ and IL-5

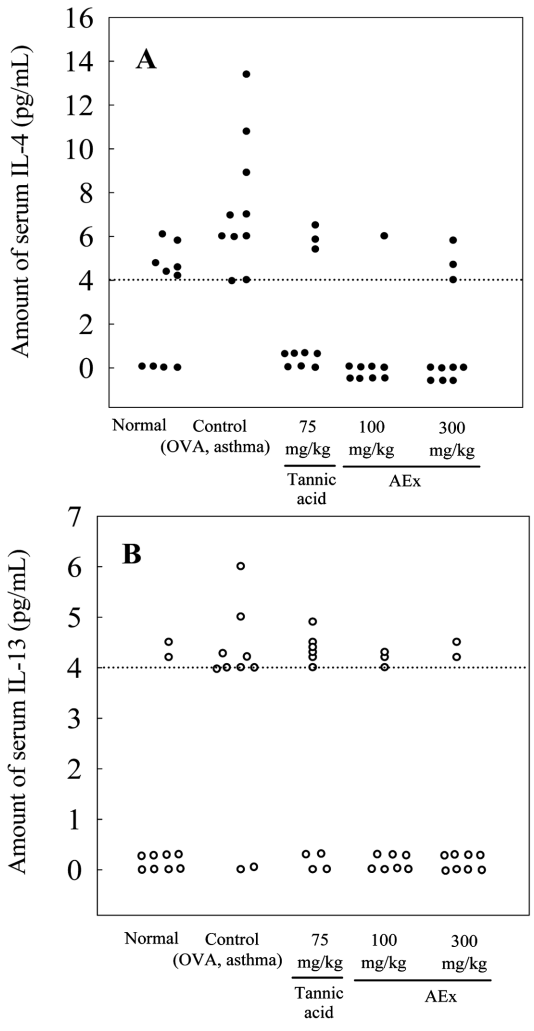

Figure 5. Effects of AEx on the cytokines levels in serum of asthma model mice. Serum was obtained on day 29 from PBS-treated mice (normal group), OVA-treated mice [control (OVA, asthma) group], and OVA-treated mice administered with AEx (100 mg/kg or 300 mg/kg) or tannic acid (75 mg/kg). The cytokines in serum were analyzed using ELISA. The detection limit of the ELISA is approximately 4 pg/mL (eBioscience, San Diego, CA). The detection limit for the serum cytokines was thus set at 4 pg/mL.

mRNA levels by AEx were not changed as compared to OVA-sensitized/challenged asthma control group.

The Th2-type cytokine (IL-4 and IL-13) protein levels in serum were measured using ELISA. Mice in OVA-sensitized/challenged asthma control group exhibited an increased production of serum IL-4 and IL-13 but administration of AEx led to a reduction in these protein levels, compared with those of OVA-sensitized/challenged asthma control group (Figure 5).

Interferons including INF-γ are Th1-type cytokines that have a role in inhibiting IgE production (Busse and Rosenwasser, 2003). Th2-type cytokines (IL-4, IL-5 and IL-13) and proinflammatory cytokine (TNF-α) play a role in the pathogenesis of allergy and asthma (Mosmann and Sad, 1996; Choi et al., 2009). Biological balance between Th1-cytokines and Th2-cytokines is needed for maintenance of health and prevention of autoimmune disease (Busse and Rosenwasser, 2003). In clinical study, IFN-γ has no effect in asthma (Szabo et al., 2002). As shown in Figure 5, orally administered AEx reduced Th2 cytokines (such as IL-4 and IL-13) and proinflammatory cytokine (TNF-α) levels in lung tissue and/or serum from OVA-induced asthma mice. However, the AEx increased HO-1 mRNA level in the OVA-sensitized/challenged asthma mice. Lee et al. (2010) reported that *Ulmus davidiana* var. *Japonica* showed protective effects against OVA-induced murine asthma model via upregulation of HO-1.

Our results suggest that AEx inhibit Th2-type cytokines (IL-4 and IL-13) and proinflammatory cytokine (TNF-α)-dependent pathways and AEx may have beneficial effects on asthma-related inflammation via HO-1 upregulation. These results strongly support the utility of AEx as potential anti-oxidative and anti-inflammatory agent in asthma treatment.

Effects of AEx on serum AST, ALT, total triglyceride and cholesterol levels in OVA-induced asthma mice

To confirm the absence of liver toxicity by the AEx, its effects on AST and ALT levels in the blood of the OVA-induced asthma mice were investigated. The results showed that both tannic acid and AEx had no effect on alteration of serum AST and ALT levels, known indicators of hepatocellular damage, as compared to the control (Normal) group (Figure 6A). The data indicate that the doses of AEx used in the present study had no cytotoxic effects on mice.

On the other hand, since many studies have reported an association between asthma and obesity in human (Figueroa-Munoz et al., 2001), the effects of AEx on the serum total triglyceride and cholesterol levels in OVA-induced asthma mice were also determined. Interestingly, as shown in Figure 6B, while the serum total cholesterol levels were almost unchanged, the serum triglyceride levels of OVA-sensitized/challenged mice were significantly reduced after feeding for 15 days with tannic acid (75 mg/kg/day) or AEx (100 or 300 mg/kg/day). Furthermore, as shown in Figure 6C, the mice fed with tannic acid (75 mg/kg/day) or AEx (300 mg/kg/day) exhibited a significant reduction in body weights after 15 days of feeding, compared to those of asthma control group ($P < 0.05$). There was no significant difference in food intake (data not shown). These results suggested that both tannic acid and the AEx have positive hypolipidemic and anti-obese effects *in vivo* in the mice with asthma symptoms and these effects are mediated at least by reducing serum triglyceride levels. A

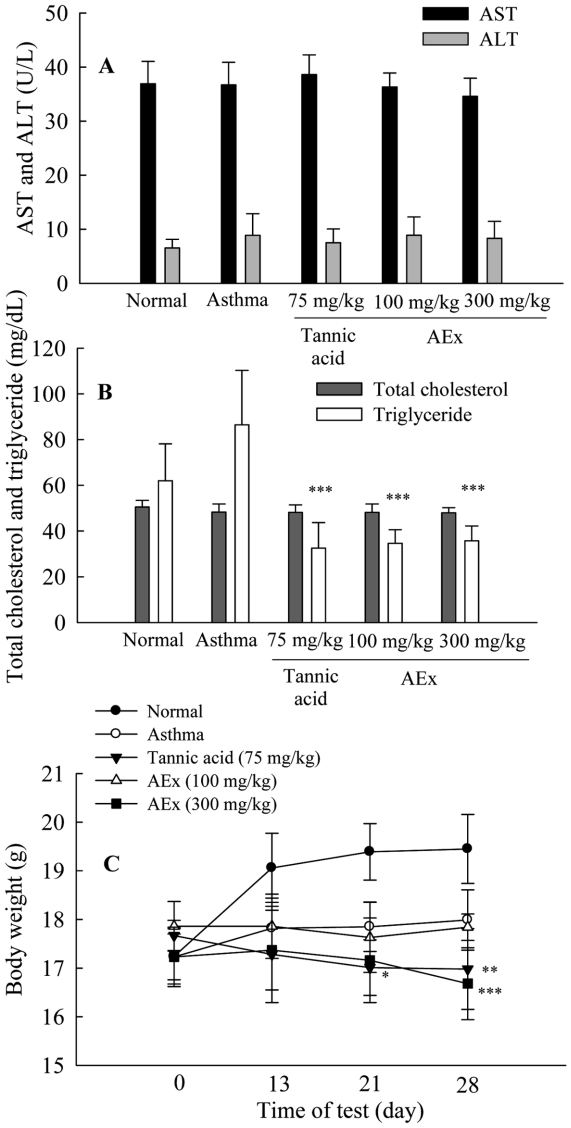

Figure 6. Effects of AEx on body weight change, serum total cholesterol, triglyceride, ALT and AST levels in OVA-induced asthma mice. Normal, mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Tannic acid (75 mg/kg body weight) + OVA-sensitized/challenged mice; AEx, acorn ethanol extracts (100 or 300 mg/kg body weight) + OVA-sensitized/challenged mice. Body weights were weighed prior to sacrifice (Fig. 1). Serum samples were prepared and assayed for total cholesterol, triglyceride, ALT and AST levels as described in the text. The data are the means ± SD (n = 10). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. OVA-sensitized control asthma group.

recently published article demonstrated the relationship between triglyceride and obesity (Choi et al., 2010). Obesity leads to a state of low-grade systemic inflammation that may act on the lung to exacerbate asthma. Data from animal models also support a relationship between obesity and asthma (Shore, 2007). Thus, the body weight reduction by AEx appears to be highly correlated to the reduced triglyceride levels and the observed anti-obesity effect of AEx in the present study may provide additional benefits of AEx.

Conclusion

This study is first to show that AEx, ethanol extract of acorn, inhibits the production of asthma-specific cytokines such as IL-4, IL-13 and TNF-αs and intracellular ROS generation in IgE-antigen complex-stimulated RBL-2H3 cells. Oral administration of AEx also inhibits the production of asthma-specific cytokines in the OVA-induced asthma model mice. Protective effects of AEx against IgE-mediated allergic and OVA-induced asthmatic responses via inhibition of oxidative stress may be beneficial for alleviating symptoms of allergic disease.

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