Biomedical Science Letters 2013, 19(4): 285~294
eISSN : 2288-7415

Original Article

Chungyangeum Attenuated the Allergic Inflammation *in vivo* and *in vitro*

Su-Jin Kim[1,§], Jae-Ho Lee[2,§], Chung Hwan Oh[2], Sa-Rang Oh[3] and Ji-Wook Jung[2,†]

[1]*Department of Cosmeceutical Science, Daegu Hanny University, Yugok-dong, Kyungsan 712-715, Korea*
[2]*Department of Herbal Medicinal Pharmacology, Daegu Haany University, Yugok-dong, Kyungsan 712-715, Korea*
[3]*College of Pharmacy, Keimyung University, Sindang-dong, Dalseo-gu, Dae-gu 704-701, Korea*

Chungyangeum (CYE) is a newly designed herbal drug formula for the purpose of treating atopic dermatitis. The aim of the present study is to elucidate whether and how CYE modulates the allergy inflammation *in vitro* and *in vivo*. We investigate to ascertain the pharmacological effects of CYE on both compound 48/80 or histamine-induced scratching behaviors and 2, 4-dinitrochlrobenzene (DNCB)-induced atopic dermatitis in mice. Additionally, we attempted to determine the effects of CYE on lipopolysaccharide (LPS)-induced inflammatory responses in macrophages. The findings of this study demonstrated that CYE reduced compound 48/80 or histamine-induced scratching behaviors and DNCB-induced atopic dermatitis in mice. The CYE inhibited the production of inflammatory cytokines as well as the activation of NF-κB and caspase-1 in stimulated macrophages. Collectively, the findings of this study provide us with novel insights into the pharmacological actions of CYE as a potential molecule for use in the treatment of allergic inflammation diseases.

Key Words: *Chungyangeum*, Inflammatory mediators, Allergic inflammation, Nuclear factor-κB, Caspase-1

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczematous inflammation of the skin (Buske-Kirschbaum et al., 2001). The incidence of this disease has increased steadily over recent years. AD is known to be the result of an immune system dysregulation, ultimately resulting in allergic inflammation (Gold and Kemp, 2005).

Activation of macrophage is a hallmark of inflammation, especially in the AD skin where they can mediate chronic inflammation by producing cytokines. It has been previously reported that macrophage activation can be found in larger numbers in AD lesional skin (McCormick et al., 2000). In response to various stimuli, macrophage generates a variety of cytokines, including interleukin (IL)-6, and tumor necrosis factor (TNF)-α (Boero et al., 2010). The release of these cytokines may be of major importance in the develop-ment of a variety of inflammatory skin disorders (Trefzer et al., 2003). Therefore, the inhibition of cytokine secretion can aid in the development of a useful therapeutic strategy for allergic inflammatory diseases such as AD.

Nuclear factor-kappa B (NF-κB) performs a crucial function in the expression of many of the genes involved in immune and inflammatory responses (Tegeder et al., 2001). In the nucleus, NF-κB activates gene transcription; thus, NF-κB performs a pivotal function in the regulation of immune and inflammatory responses, occurring via the control of the transcription of inflammatory cytokine genes (Gadaleta et al., 2011). An increase in NF-κB activity associated with the secretion of high levels of IL-6 and TNF-α has also been noted in the context of allergic inflammatory responses (Mukaida, 2000). The results of those studies demonstrated that NF-κB activation and the

*Received: August 28, 2013 / Revised: December 24, 2013
Accepted: December 25, 2013
§These two authors equally contributed to this work as first authors.
†Corresponding author: Ji-Wook Jung. Department of Herbal Medicinal Pharmacology, Daegu Haany University, Yugok-dong, Kyungsan 712-715, Korea.
Tel: +82-53-819-1337, e-mail: jwjung@dhu.ac.kr

subsequent activation of pro-inflammatory cytokine gene expression are critically important in the initiation and perpetuation of allergic inflammation.

Caspase-1 is a member of a family of caspases with large prodomains, and its activation is involved in both apoptosis and inflammation (Lee et al., 2001; Wang et al., 2005). Activation of caspase-1 induces inflammatory response via the production of pro-inflammatory cytokines and the recruitment of neutrophils (Faubel et al., 2007). These results have implicated caspase-1 activation as an attractive target for the treatment of inflammatory diseases.

Traditional medicine has been the subject of increased interest for its potential in the treatment of inflammation. Although traditional herbal medicines have long been used effectively in treating diseases, the pharmacologic mechanisms of most herbal medicines have not been elucidated. *Chungyangeum* (CYE) is a newly designed herbal drug formula for the purpose of treating atopic dermatitis. CYE is composed of 4 oriental herbs. In the present study, we elucidate whether and how CYE modulates the allergy inflammation *in vitro* and *in vivo*. We attempted to ascertain the pharmacological effects of CYE on both compound 48/80 or histamine-induced scratching behaviors and 2, 4-dinitrochlrobenzene (DNCB)-induced atopic dermatitis in mice. In an effort to elucidate the mechanism responsible for CYE's anti-inflammatory effect, we evaluated the effects of CYE on the production of inflammatory cytokine and the activation of NF-κB and caspase-1 in lipopolysaccharide (LPS)-stimulated macrophages.

MATERIALS AND METHODS

Reagents

Compound 48/80, histamine, terfenadine, lipopolysaccharide (LPS), avidin peroxidase (AP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-chloro-2,4-dinitrochlorobenzene (DNCB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco Modified Eagles Medium (DMEM) and Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Somerset, NJ, USA). Anti-human TNF-α/IL-6, recombinant TNF-α/IL-6, biotinylated TNF-α/IL-6, anti-mouse IgE, recombinant IgE and biotinylated IgE were purchased from Pharmingen (San Diego, CA, USA). NF-κB, histone and iNOS antibodies (Abs) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

Male ICR mice (4 weeks, 18~20 g) and BALB/c mice (5 weeks, 19~20 g) were purchased from the Daehan biolink Co., Ltd. (Chungbuk, Korea). Animals were housed 6 heads per cage, allowed spontaneous take in food and water. Animals were kept under a 12-h light/dark cycle (light on 08:00~20:00) at room temperature (23 ± 2℃) and humidity (55 ± 10%). Animal treatment and maintenance were carried into effect in accordance with the animal care and use guidelines of Daegu haany University, Korea.

Preparation of CYE

CYE extract which is a mixture of four traditional drugs obtained from the Omniherb (Daegu, Korea). The ingredients of 100 g CYE include 20 g of *Panax ginseng*, 20 g of *Schizandra chinensis*, 40 g of *Liriope platyphylla*, and 20 g of *Glycyrrhiza uralensis*. The ingredients were chopped using a blender with 2 L of 70% ethanol solution under room temperature for 24 h and then concentrated under a vacuum. Then the extract solution obtained was filtered, concentrated on a water bath under vacuo, frozen and lyophilized to yield ethanol extracts (yield: 26.34%). Dilutions were made in saline and filtered through 0.22-μm syringe filter.

Scratching behavioral experiment

Before the experiment, the ICR mice (n=6) were put into acrylic cages (22 × 22 × 24 cm) for about 30 min for acclimation. The behavioral experiments were performed according to the method of Sugimoto et al.. The rostral part of the skin on the back of mice was clipped, and compound 48/80 (50 μg/kg) or histamine (100 μg/kg) for each mouse was intradermally injected. The scratching agents were dissolved in tween 80 and then used. Control mice received a tween 80 injection in place of the scratching agent. Immediately after the intradermal injection, the mice (one

animal/cage) were put back into the same cage for the observation of scratching. Scratching of the injected site by the hind paws was counted and compared with that of the other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches for 1 s, and a series of these behaviors was counted as one incident of scratching for 30 min. CYE (200 mg/kg) and terfenadine (10 mg/kg) was orally administered 1 h before the scratching agents.

DNCB-induced atopic dermatitis

Experiments were conducted in accordance with a previously described protocol. The dorsal skin of the BALB/c mice (n=6) was shaved and treated with a depilatory prior to the experiment. The mice were sensitized with 100 μl of 0.15% DNCB in acetone-olive oil (3:1) applied to the dorsal skin twice per week for 5 weeks. Control mice received vehicle (acetone/olive oil=3:1). After 3 weeks, CYE (200 mg/kg) and terfenadine (10 mg/kg) was orally administered 2 weeks until the end of the experiment.

Cell culture

The murine macrophage cell line RAW 264.7, cells were grown in DMEM medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37℃ in 5% CO_2.

MTT assay

To test the cell viability by each concentration of CYE, the MTT colorimetric assay was performed. Briefly, RAW 264.7 cells (3×10^5 cells/well) were pre-treated with CYE (0.01~1 mg/ml) for 1 h before stimulation with LPS (1 μg/ml) for 24 h. 50 μl of MTT solution (5 mg/ml) was added and then cells were incubated at 37℃ for 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Cytokine assay

TNF-α, IL-6, and IgE secretion were measured by modification of an enzyme-linked immunosorbent assay (ELISA). 96 well plates were coated with 100 μl aliquots of anti-mouse TNF-α, IL-6, and IgE monoclonal Abs at 1.0 μg/ml in PBS at pH 7.4 and were incubated overnight at 4℃. After additional washes, 100 μl of cell medium or TNF-α, IL-6, and IgE standards were added and incubated at 37℃ for 2 h. After 2 h incubation at 37℃, the wells were washed and then 0.2 μg/ml of biotinylated anti-mouse TNF-α, IL-6 and IgE was added and again incubated at 37℃ for 2 h. After washing the wells, AP was added and plates were incubated for 30 min at 37℃. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6, and IgE in serial dilutions.

Measurement of nitrite concentration

RAW 264.7 cells (3×10^5 cells/well) were pre-treated with CYE for 1h, and then treated with LPS (1 μg/ml) for 24 h. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamidey/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloridey/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a plate reader. NO_2^- was determined using sodium nitrite as a standard. This value was determined in each experiment and subtracted from the value obtained from the medium with RAW 264.7 cells.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasmic extracts were prepared as described previously. Briefly, after the cells were activated with LPS and then washed with ice-cold phosphate-buffered saline (PBS). These cells were resuspended in 60 μl of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonide P (NP)-40, and centrifuged at 2,000 × g for 10 min at 4℃. The supernatant

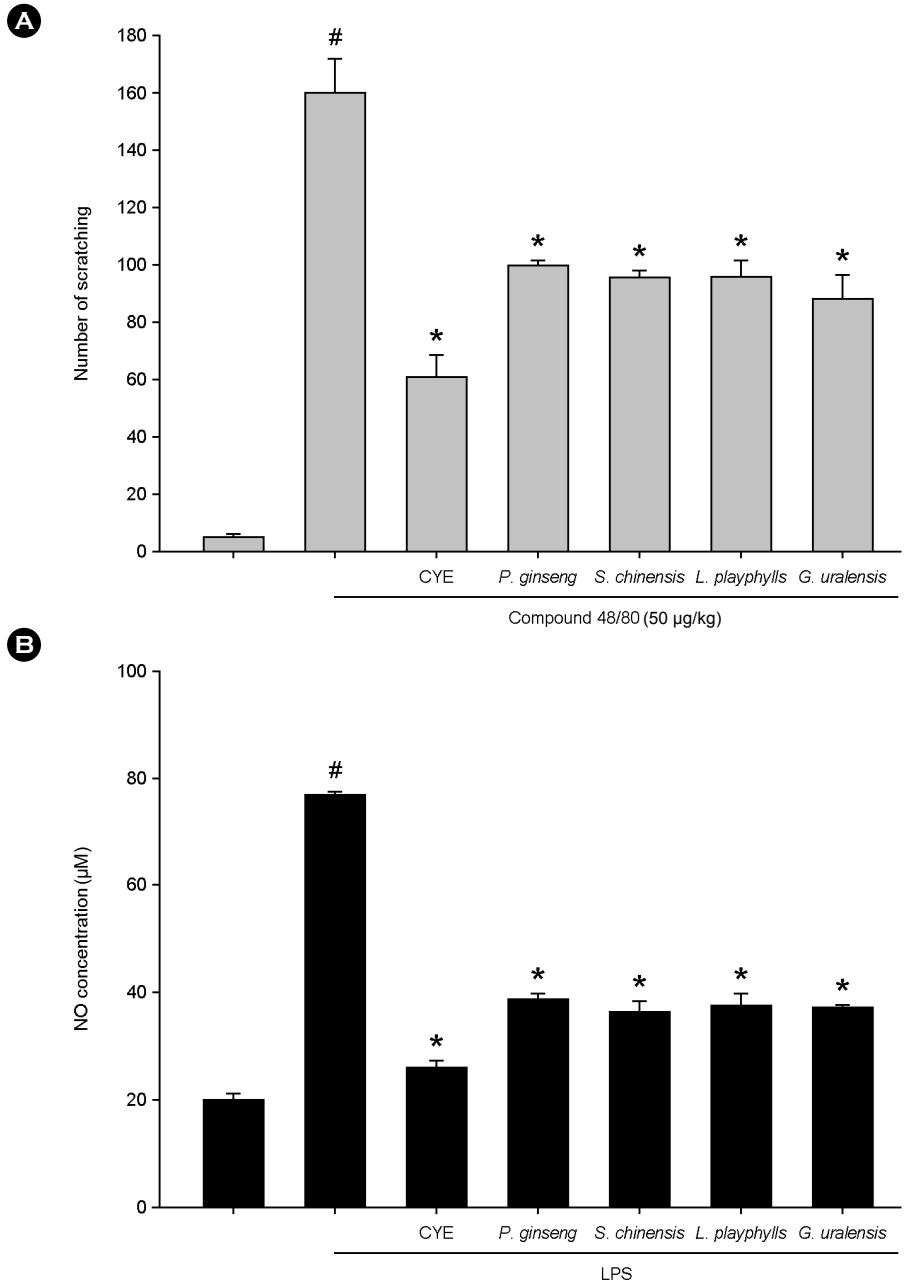

Fig. 1. The effect of CYE and its herbs on scratching behavior and NO production. (A) CYE or its herbs, respectively was orally administered 1 h before compound 48/80 (50 μg/kg) intradermal injection. Scratching behavior was counted as one incident of scratching for 30 min. (B) RAW264.7 cells were pre-treated with CYE or its herbs, respectively for 1 h and then stimulated with LPS for 24 h. NO production in the medium was measured via the Griess reaction. All data were represented in the mean ± S.E.M. of triplicate determinations from triplicate separate experiments (#$P < 0.05$ vs. control, *$P < 0.05$ vs. LPS alone).

was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, inverted and the nuclear debris was spun down at 15,000 × g for 15 min to remove nuclear debris. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at -70℃ until ready for analysis.

Western blot analysis

Nuclear extract combined with an equal volume of sodium dodecyl sulfate sample loading buffer, boiled for 5 min for denaturation. Samples of protein were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. The membrane was blocked in 5% skim milk for 1 h, washed and incubated overnight at 4℃ with primary antibodies in 3% bovine serum albumin in PBS. After washing the membranes, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit immunoglobulin (secondary antibodies). After three washes in PBST/0.1% Tween 20 for 30 min, the protein bands were visualized by an enhanced chemiluminesence detection system according to the recommended producere (Amersham Corp. Newark). The quantity of protein was evaluated by using a bicinchoninic acid (BCA) protein assay (Sigma. st. Louis, MO, USA).

Caspase-1 activity assay

The enzymatic activity of caspase-1 was assayed using a caspase colorimetric assay kit (R&D System Inc., MN, USA) according to the manufacturer's protocol. The lysed cells were centrifuged at 14,000 rpm for 5 min. The protein supernatant was incubated with 50 μl reaction buffer and 5 μl caspase substrates at 37℃ for 2 h. The absorbance was measured was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a BCA protein assay.

Statistical analysis

The experiments were shown a summary of the data from at least-three experiments and presented as the mean ± S.E.M. Statistical evaluation of the results was performed by independent *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Comparative activity of CYE and its constituent herbs

In the first step, we compared the effect of CYE and individual herbs (*Panax ginseng*, *Liriope platyphylla*, *Schisandra chinensis*, *Glycyrrhiza uralensis*), respectively on scratching behavior and NO production. As shown in Fig. 1, we observed that CYE attenuated the scratching behavior and NO production more effectively than ingredient herbs. On the basis of this result, we tried to investigate the effect and mechanism of CYE on atopic and allergic reaction.

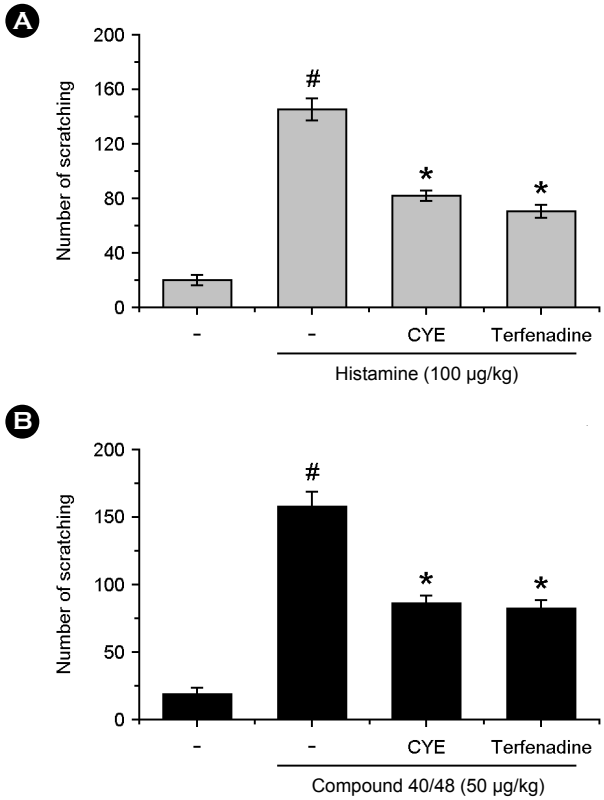

Fig. 2. The effect of CYE on scratching behavior in ICR mice. CYE (200 mg/kg) was orally administered 1 h before compound 48/80 (50 μg/kg) or histamine (100 μg/kg) intradermal injection. Scratching behavior was counted as one incident of scratching for 30 min. Each datum represents the means ± S.E.M. of three independent experiments (#$P < 0.05$ vs. control group, *$P < 0.05$ vs. compound 48/80 or histamine-treated group).

Effect of CYE on scratching behaviors in mice

The anti-scratching behavior effects of CYE were investigated on the compound 48/80-induced scratching behavior animal model. When the CYE was orally administered 1 h before compound 48/80 injections, the scratching behaviors were reduced. The inhibition rate of CYE (200 mg/kg) was

approximately 44.4% (Fig. 2A). In addition, we investigated the contribution of CYE in histamine-induced scratching behavior. As shown in Fig. 2B, orally administered CYE inhibited the scratching behaviors by 42.7%. Terfenadine was used as a positive control in this study.

Effect of CYE on DNCB-induced atopic dermatitis and IgE levels in serum

In order to evaluate the regulatory effects of CYE in an atopic dermatitis *in vivo* model, DNCB was administered to BALB/c mice. As shown in Fig. 3A, when mice were treated for 2 weeks with CYE, the atopic dermatitis was recovered to a significant extent. To evaluate the effects of CYE on IgE levels in serum, blood samples were collected. The levels of IgE were measured via ELISA. The results showed that IgE levels were increased as the result of DNCB exposure, but this phenomenon was significantly reduced in the CYE group (Fig. 3B).

Effect of CYE the inflammatory cytokines production in LPS-stimulated Raw 264.7 cells

In an effort to determine the molecular mechanism of CYE, the murine macrophage cell line, Raw 264.7, was employed in this study. We determined whether CYE modulates the LPS-induced production of TNF-α and IL-6. The levels of TNF-α and IL-6 in culture supernatants were measured via ELISA. As is shown in Fig. 4, the production

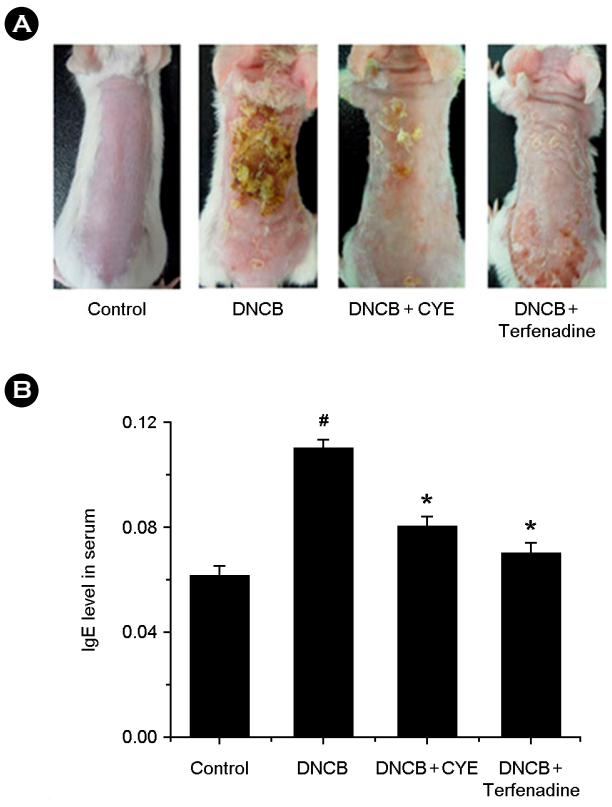

Fig. 3. The effect of CYE on the DNCB-induced dermatitis and serum IgE level. (A) The BALB/c mice (n=6) were sensitized with 100 μl of 0.1% DNCB in acetone-olive oil (3:1) or vehicle (acetone/olive oil=3:1) applied to the dorsal skin twice each week for a total period of 5 weeks. After 3 weeks, CYE (200 mg/kg) was orally administered 2 weeks prior to the end of the experiment. (B) Blood samples were collected and then levels of serum IgE in the indicated groups were measured using the ELISA method (#P < 0.05 vs. control group, *P < 0.05 vs. DNCB-treated group).

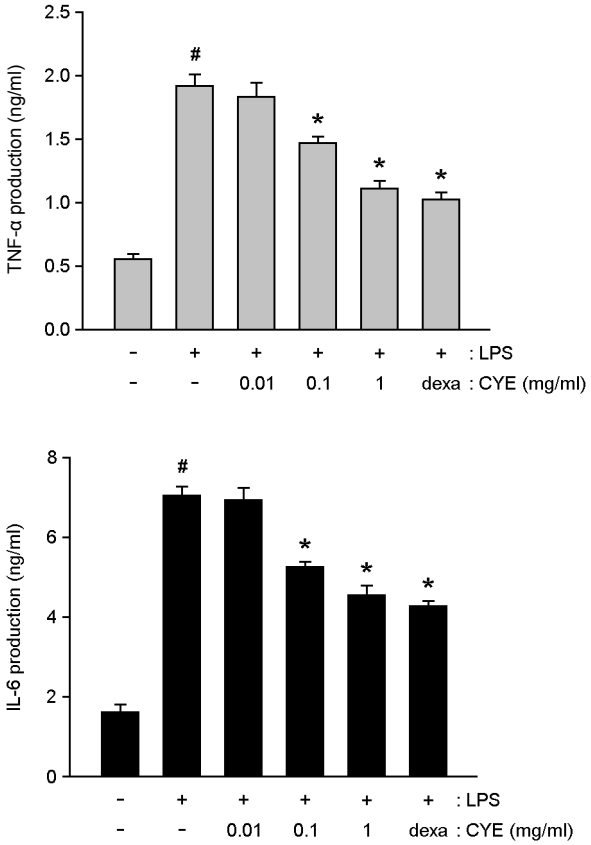

Fig. 4. The effects of CYE on the production of inflammatory cytokines in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with CYE (0.01~1 mg/ml) for 1 h and then stimulated with LPS for 24 h. The levels of inflammatory cytokines (TNF-α and IL-6) were measured from cell supernatant using ELISA. Dexamethason was used as a positive control. All data were represented in the mean ± S.E.M. of triplicate determinations from triplicate separate experiments (#P < 0.05 vs. control, *P < 0.05 vs. LPS alone).

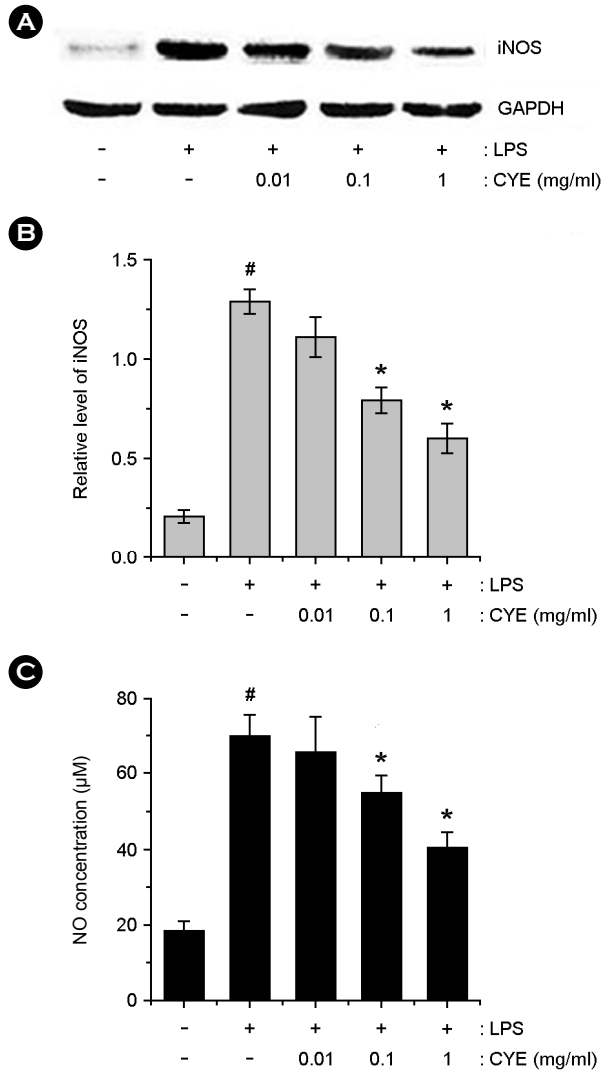

Fig. 5. The effect of CYE on the LPS-stimulated iNOS expression and NO production in RAW264.7 cells. RAW264.7 cells were pre-treated with CYE (0.01~1 mg/ml) for 1 h and then stimulated with LPS for 24 h. (A) Total cellular proteins were resolved by SDS-PAGE, and iNOS was detected using specific antibodies. (B) The relative levels of iNOS were represented. (C) Nitrite level in culture media were determined using Griess assays. All data were represented in the mean ± S.E.M. of triplicate determinations from triplicate separate experiments (#$P < 0.05$ vs. control, *$P < 0.05$ vs. LPS alone).

of TNF-α and IL-6 in response to LPS was inhibited as the result of pre-treatment with CYE in a dose-dependent manner. The maximal rates of TNF-α and IL-6 inhibition by CYE were approximately 45.41% and 34.84%, respectively. Additionally, we observed that CYE did not affect cell viability (data not shown).

Effects of CYE on NO production and iNOS expression

To determine the effect of CYE on LPS-induced iNOS expression in Raw 264.7 cells, western blotting was performed. Data in Fig. 5, LPS caused a significant increased of iNOS expression. Pretreatment of CYE (0.01~1 mg/ml) resulted inhibition of iNOS expression in a dose-dependent manner (Fig. 5A). The relative levels of iNOS were represented in Fig. 5B. To investigate effect of CYE on LPS-induced NO production, cells were pretreated with CYE (0.01~1 mg/ml) for 1 h and then treated with LPS for 24 h. We showed that CYE decreased NO production in dose-dependent manner (Fig. 5C). The inhibition rate reached up to 48.57%.

Effect of CYE on NF-κB activation in the nuclei of LPS-stimulated Raw 264.7 cells

As the suppression of NF-κB activation has been linked with anti-inflammation, we theorized that the effects of CYE might be mediated, at least in part, via the suppression of NF-κB activation. Additionally, because NF-κB activation requires the nuclear translocation of the RelA/p65 subunit of NF-κB, we evaluated the effects of CYE on the nuclear pool of RelA/p65 protein via western blot analysis. In LPS-stimulated cells, the expression level of Rel/p65 was increased. However, pre-treatment of CYE decreased the expression level of Rel/p65 (Fig. 6A). The relative levels of NF-κB (in nucleus) were represented in Fig. 6B.

Effect of CYE on caspase-1 activation in LPS-stimulated Raw 264.7 cells

Activation of caspase-1 induces inflammatory response via stimulation of inflammatory cytokines. In order to determine the regulatory mechanism of CYE on allergic inflammation, we evaluated the effects of CYE on LPS-induced caspase-1 activation. We showed that the enhanced caspase-1 activity induced by LPS was significantly reduced by CYE in a dose-dependent manner (Fig. 7).

DISCUSSION

In this study, we demonstrated that molecular mechanisms

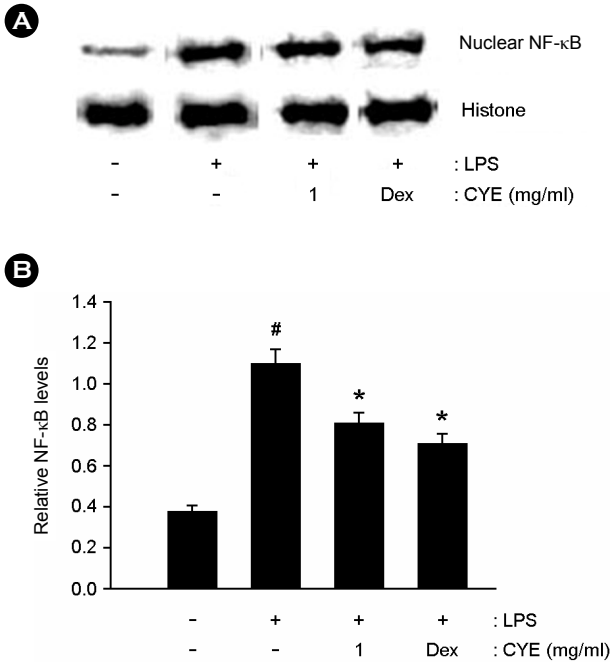

Fig. 6. The effect of CYE on the NF-κB activation in the nuclei of LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with CYE (1 mg/ml) for 1 h and then stimulated with LPS for 2 h. (A) Nuclear extracts were prepared as described in the materials and methods section and evaluated for RelA/p65 via Western blot analysis. (B) The relative levels of NF-κB were represented. All data were represented in the mean ± S.E.M. of triplicate determinations from triplicate separate experiments (#P < 0.05 vs. control, *P < 0.05 vs. LPS alone).

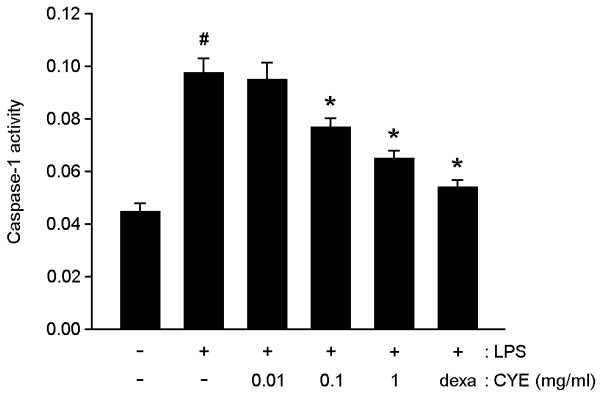

Fig. 7. The effect of CYE on caspase-1 activation in LPS-stimulated Raw 264.7 cells. The cells were pretreated with CYE (0.01~1 mg/ml) for 1 h prior to LPS stimulation for 4 h. The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. All data were represented in the mean ± S.E.M. of triplicate determinations from triplicate separate experiments (#P < 0.05 vs. control, *P < 0.05 vs. LPS alone).

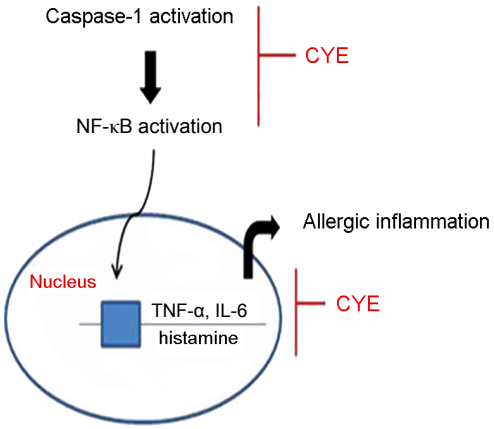

Fig. 8. Proposed anti-allergic mechanism of CYE in LPS-stimulated macrophage.

of CYE on allergic inflammation *in vivo* and *in vitro*. The findings of this study showed that CYE attenuated the compound 48/80 or histamine-induced scratching behaviors and inhibited DNCB-induced atopic dermatitis under *in vivo* conditions. Additionally, CYE inhibited the production of TNF-α and IL-6, and also inhibited the activation of NF-κB and caspase-1 in LPS-simulated macrophage.

AD is a chronic inflammatory skin disease and is characterized by erythema, edema, and scaling (Leung and Bieber, 2003). Generally, steroid therapy is a crucial factor in the treatment of AD, but it cannot be administered over the long-term, owing to its deleterious side-effects. Therefore, several researchers have attempted to find a new drug, which is effective in the treatment of AD (Shiohara et al., 2004). The AD was characterized by a potent skin inflammation associated with an elevated level of IgE against many types of allergens (Allam and Novak, 2006; Brenninkmeijer et al., 2008). According to traditional theory, increasing fever in the body induces the loss of body fluid and it cause the skin dryness and itching. As already described, CYE consists of four different herbs. *Panax ginseng* and *Schisandra chinensis* of CYE were used to supply the body fluid. *Liriope platyphylla* and *Glycyrrhiza uralensis* have been used to alleviate a fever. Additionally, each of them have anti-inflammatory activity. Therefore, we can speculate that CYE exert anti-atopic effects. In this study, we observed that inhibited the scratching behavior and NO production more effectively than ingredient herbs. On the base of these

studies, we have focused to evaluate the effects of CYE on DNCB-induced allergic reactions *in vivo*. The findings of this study revealed that CYE significantly reduced DNCB-induced atopic dermatitis. Additionally, CYE caused a reduction in IgE levels in serum induced by DNCB. These results demonstrate CYE's potential effect on anti-allergic responses via the regulation of IgE levels.

In pathological skin conditions, histamine is involved in the induction of itching and edema (Minami and Kamei, 2004). This study focused on the manner in which CYE regulate the scratching behaviors in mice. In this study, we showed that CYE inhibited the compound 48/80 or histamine-induced scratching behaviors in mice.

Macrophage is an important effecter cell in allergic inflammatory diseases, such as AD. Inflammatory cytokines derived macrophage play an important role in the development of inflammatory reaction. To gain further insights into the mechanisms of CYE-mediated inhibition of LPS-induced inflammatory mediators (TNF-α and IL-6), we examined the regulatory effect of CYE on intracellular signaling molecules involved in the LPS signaling pathways in macrophage. In this research, we demonstrated that CYE inhibited the secretion of TNF-α and IL-6 in LPS-simulated macrophage. The maximal rates of TNF-α and IL-6 inhibition by CYE were approximately 45.41% and 34.84%, respectively. These results demonstrate that CYE exerts an anti-inflammatory effect via the regulation of inflammatory cytokine production.

These cytokines production is associated with increased activation of the gene transcription regulators NF-κB (Gilmore and Garbati, 2011). After a variety of stimuli, the IκB proteins are phosphorylated, and degraded, allowing for NF-κB to translocate into the nucleus where it can bind specific DNA sequences located in the promoter regions of target genes and activate gene transcription, thereby indicating its pivotal function in the regulation of inflammatory responses, via the control of the transcription of inflammatory cytokine genes. From this, inhibition of NF-κB activation has been suggested as an anti-inflammatory strategy in AD. Therefore, we attempted to determine whether the anti-inflammatory effect of CYE is through the regulation of NF-κB activation. The results demonstrated that CYE inhibited the NF-κB translocation into nucleus in stimulated macrophage. Therefore, we hypothesized that macrophage might exert anti-inflammatory effects via NF-κB activation. Although macrophage attenuated the activation of NF-κB, the effect of macrophage on the pathways involving NF-κB (phosphorylation of IκB-α and IKK activation) was not determined. Therefore, further studies will be necessary in order to clarify more precisely the role of CYE on the NF-κB pathway.

The increase of inflammatory mediators is associated with increased activation of caspase-1 (Faubel et al., 2007). It was reported that caspase-1-/- mice evidenced reduced IL-6 production (Druilhe et al., 2001; Humke et al., 2000). In another study, it was revealed that activation of caspase-1 induced NF-κB and MAPK-signaling pathways leading to activation of p38 and ERK (Bauernfeind et al., 2009; Taxman et al., 2011). Previously, we showed that caspase-1 inhibitor reduced the production of TNF-α and IL-6 in LPS-stimulated mouse peritoneal macrophages (Kim et al., 2011). Correctly, these studies suggested that the activation of caspase-1 is an attractive target for therapies for the treatment of inflammatory diseases. In this study, we noted that CYE suppressed the LPS-induced activation of caspase-1. This finding demonstrated that the inhibitory effects of CYE on inflammation might derive from the regulation of caspase-1 activation. Although CYE attenuated caspase-1 activation, the effect of CYE on other pathways that involved caspase-1 upstream/downstream is not elucidated in the present study. Thus, further investigation is necessary to clarify the role of CYE on caspase-1 associated pathways in LPS-stimulated macrophages. A model of the anti-inflammatory mechanism of CYE is provided in Fig. 8. These results suggested that down-regulation caspase-1 by CYE might reduced NF-κB activation, and then ultimately suppressed the levels of inflammatory cytokines.

In conclusion, CYE can regulate the allergy response *in vivo*, including in compound 48/80 or histamine-induced scratching behaviors and DNCB-induced atopic dermatitis. Additionally, we demonstrated in this study that the anti-inflammatory activities of CYE could be attributed, at least in part, to the inhibition of inflammatory cytokine production (TNF-α and IL-6). These effects of CYE are caused by the

inhibition of LPS-induced the activation of NF-κB and caspase-1 activation in macrophage. These results provide experimental evidence demonstrating that CYE may prove useful in the treatment of allergic inflammatory diseases.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0022703).

REFERENCES

Allam JP, Novak N. The pathophysiology of atopic eczema. Clin Exp Dermatol. 2006. 31: 89-93.

Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol. 2009. 183: 787-791.

Boero S, Silvestri M, Ullmann N, Rossi GA. Modulation by flunisolide of tumor necrosis factor-alpha-induced stimulation of airway epithelial cell activities related to eosinophil inflammation. J Asthma. 2010. 47: 381-387.

Brenninkmeijer EE, Spuls PI, Legierse CM, Lindeboom R, Smitt JH, Bos JD. Clinical differences between atopic and atopiform dermatitis. J Am Acad Dermatol. 2008. 58: 407-414.

Buske-Kirschbaum A, Geiben A, Hellhammer D. Psychobiological aspects of atopic dermatitis: an overview. Psychother Psychosom. 2001. 70: 6-16.

Druilhe A, Srinivasula SM, Razmara M, Ahmad M, Alnemri ES. Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. Cell Death Differ. 2001. 8: 649-657.

Faubel S, Lewis EC, Reznikov L, Ljubanovic D, Hoke TS, Somerset H, Oh DJ, Lu L, Klein CL, Dinarello CA, Edelstein CL. Cisplatin-induced acute renal failure is associated with an increase in the cytokines interleukin (IL)-1beta, IL-18, IL-6, and neutrophil infiltration in the kidney. J Pharmacol Exp Ther. 2007. 322: 8-15.

Gadaleta RM, Oldenburg B, Willemsen EC, Spit M, Murzilli S, Salvatore L, Klomp LW, Siersema PD, van Erpecum KJ, van Mil SW. Activation of bile salt nuclear receptor FXR is repressed by pro-inflammatory cytokines activating NF-κB signaling in the intestine. Biochim Biophys Acta. 2011. 1812: 851-858.

Gilmore TD, Garbati MR. Inhibition of NF-κB signaling as a strategy in disease therapy. Curr Top Microbiol Immunol. 2011. 349: 245-263.

Gold MS, Kemp AS. Atopic disease in childhood. Med J Aust. 2005. 182: 298-304.

Humke EW, Shriver SK, Starovasnik MA, Fairbrother WJ, Dixit VM. ICEBERG: a novel inhibitor of interleukin-1beta generation. Cell. 2000. 103: 99-111.

Kim SJ, Chung WS, Kim SS, Ko SG, Um JY. Antiinflammatory effect of *Oldenlandia diffusa* and its constituent, hentriacontane, through suppression of caspase-1 activation in mouse peritoneal macrophages. Phytother Res. 2011. 25: 1537-1546.

Lee SH, Stehlik C, Reed JC. Cop, a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. J Biol Chem. 2001. 276: 34495-34500.

Leung DY, Bieber T. Atopic dermatitis. Lancet. 2003. 361: 151-160.

McCormick TS, Stevens SR, Kang K. Macrophages and cutaneous inflammation. Nat Biotechnol. 2000. 18: 25-26.

Minami K, Kamei C. A chronic model for evaluating the itching associated with allergic conjunctivitis in rats. Int Immunopharmacol. 2004. 4: 101-108.

Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. Int J Hematol. 2000. 72: 391-398.

Shiohara T, Hayakawa J, Mizukawa Y. Animal models for atopic dermatitis: are they relevant to human disease? J Dermatol Sci. 2004. 36: 1-9.

Taxman DJ, Holley-Guthrie EA, Huang MT, Moore CB, Bergstralh DT, Allen IC, Lei Y, Gris D, Ting JP. The NLR adaptor ASC/PYCARD regulates DUSP10, mitogen-activated protein kinase (MAPK), and chemokine induction independent of the inflammasome. J Biol Chem. 2011. 286: 19605-19616.

Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J. 2001. 15: 2057-2072.

Trefzer U, Hofmann M, Sterry W, Asadullah K. Cytokine and anticytokine therapy in dermatology. Expert Opin Biol Ther. 2003. 3: 733-743.

Wang X, Wang H, Figueroa BE, Zhang WH, Huo C, Guan Y, Zhang Y, Bruey JM, Reed JC, Friedlander RM. Dysregulation of receptor interacting protein-2 and caspase recruitment domain only protein mediates aberrant caspase-1 activation in Huntington's disease. J Neurosci. 2005. 25: 11645-11654.