

***Schisandra chinensis* and Its Main Constituent Schizandrin Attenuate Allergic Reactions by Down-Regulating Caspase-1 in Ovalbumin-Sensitized Mice**

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Abstract: *Schisandra chinensis* (SC) and its main constituent, schizandrin (SCH) exhibit anti-inflammatory and anti-allergic activities. Allergic and inflammatory reactions are aggravated via caspase-1 signaling pathway. However, the regulatory effects of SC and SCH on caspase-1 activation have not been clarified yet. In this study, we aimed to clarify the anti-allergic effects of SC and SCH using an ovalbumin (OVA)-sensitized mice and anti-CD3 and anti-CD28 antibodies-stimulated splenocytes. SC or SCH significantly inhibited the levels of immunoglobulin (Ig)E, IgG1, or interleukin (IL)-4 in serum of OVA-sensitized mice. SC or SCH significantly inhibited the levels of IL-6, tumor necrosis factor (TNF)- α , and IL-1 β in spleen of the OVA-sensitized mice. SC or SCH significantly suppressed the expression of caspase-1 and receptor-interacting protein (RIP)-2 in spleen of the OVA-sensitized mice. In activated splenocytes, SC or SCH significantly decreased the expression of caspase-1 and RIP-2 as well as the production of IL-6 and TNF- α . We suggest that SC and SCH exert an anti-allergic effect by down-regulating caspase-1 signaling.

Keywords: *Schisandra chinensis*; Schizandrin; Allergic Reaction; Caspase-1; Splenocytes.

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Introduction

The prevalence of allergic diseases including asthma, atopic dermatitis, and allergic rhinitis has been markedly increased in recent decades (Renz and Herz, 2002). Unfortunately, steroid medications cause side effects including osteoporosis, skin thinning, candidiasis, or dysphonia (Dahl, 2006). Topical steroids also induce side effects including skin atrophy, adrenal suppression, glaucoma, or growth retardation in children (Correale *et al.*, 1999). Thus, alternative medicines, such as herbal medicines, have been developed for prevention or treatment of allergic diseases (Yuan and Lin, 2000).

Medicinal plants contain substances that can be used for therapeutic purposes (Fagbohun *et al.*, 2012). Biotechnology is developing biological therapeutics and providing a novel approach to rational drug design (Yuan and Lin, 2000). The composition and efficacy of extract of medicinal plants are diversified according to a solvent-extraction procedure (Spigno *et al.*, 2007). Solid phase extract (dry extract) is obtained by dissolution of a solid in a liquid mixture, separation from a mixture into desired and undesired components, and drying. Soft phase extract (semi-solid phase extract) is obtained by partial evaporation of the solvent (Gaedcke and Steinhoff, 2003). *Schisandra chinensis* is a traditional herbal medicine has been used for the treatment of human diseases, such as oxidative stress (Panossian and Wikman, 2008), inflammation (Chun *et al.*, 2014), and allergy (Kang and Shin, 2012). The advance in extraction strategies has led to identifying lignan compounds from *Schisandra chinensis* as bioactive constituents. Schizandrin (SCH) is a major lignin isolated from *Schisandra chinensis* (Opletal *et al.*, 2004). SCH has been reported to have anti-oxidant (Ko *et al.*, 2005), anti-inflammatory (Chen *et al.*, 2013), and anti-allergic effects (Lee *et al.*, 2007, 2010).

Caspase-1 is an important target for controlling inflammatory and allergic diseases (Cunha *et al.*, 2010; Murakami *et al.*, 2006). A specific adaptor, receptor-interacting protein (RIP)-2, interacts to pro-caspase-1 and regulates the caspase-1 activity, which induces maturation or production of pro-inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α (Furlan *et al.*, 1999; Sarkar *et al.*, 2006). Caspase-1 was required for the regulation of the productions of IL-4 and immunoglobulin (Ig)E, providing a potential therapeutic target for allergic diseases (Yoshimoto *et al.*, 2000). Thus, we hypothesized that *Schisandra chinensis* or SCH could affect the pathway of caspase-1 and regulate allergic reactions.

A variety of different allergen challenge can induce several different types of allergic reactions through complicated signaling. Ovalbumin (OVA) derived from chicken egg is a T cell-dependent antigen and a common allergen that induces robust allergic inflammation (Nials and Uddin, 2008; Jacobsen *et al.*, 2008). OVA is frequently used to elucidate the pathogenetic mechanisms underlying allergic disease as well as identify novel therapeutic targets (Kumar *et al.*, 2008). T cells play a pivotal role in the regulation of OVA-induced allergic reactions (Nieuwenhuizen *et al.*, 2007). T cells are activated by antigens through the CD3/T cell receptor (TCR) and co-stimulatory signals from antigen presenting cells (Call and Wucherpfennig, 2005). CD28 is a co-stimulatory molecule expressed on T cells

and required for the T cell activation. Combination with anti-CD3 and anti-CD28 antibodies in naïve T cells results in developing into T helper (Th)2 cells (Ci *et al.*, 2010).

The effects of the soft phase extract of *Schisandra chinensis* (SE1), solid phase extract of *Schisandra chinensis* (SE2), and SCH on OVA-induced allergic reaction through regulating caspase-1 signaling have not been clarified yet. In this study, we investigated the anti-allergic effects of SE1, SE2, or SCH in OVA-sensitized mice and anti-CD3 and anti-CD28 antibodies-challenged splenocytes.

Materials and Methods

Reagents

OVA and aluminum hydroxide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); Anti-IL-4, IFN- γ , IgE, IgG1, IL-6, and TNF- α antibodies from BD PharMingen (San Diego, CA, USA); Anti-IL-1 β antibody from R & D Systems, Inc. (Minneapolis, MN, USA); Anti-caspase-1, RIP-2, α -tubulin, and actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of SE1, SE2, or SCH

Schisandra chinensis was obtained from NOA Oriental Pharmacy (Seoul, Republic of Korea). *Schisandra chinensis* was extracted with 70% ethanol at 80°C for 3 h and filtered. The residue was extracted with 70% ethanol at 80°C for 3 h again and filtered. The filtered crude extracts were concentrated in compression. We obtained 60 brix of SE1. The yield of SE1 from the starting materials was about 58.82% (w/w). To obtain SE2, SE1 was lyophilized and reduced to powder. The yield of SE2 from starting materials was about 37.04% (w/w). SCH was isolated from ethanolic solution of *Schisandra chinensis* according to a previous report (Panossian and Wikman, 2008).

HPLC Analysis

SE1, SE2, or SCH were passed through a 0.45 μ m membrane filter and 20 μ l aliquots of the filtrate were injected into the HPLC. The standard solution of SCH (Sigma-Aldrich Co.) was prepared to be dissolved in ethanol. ProntoSIL C18 ace-EPS 5 μ m (250 $\times$ 4.6 mm) was used as an analytical column. The mobile phases consisted of 40% water and 60% acetonitrile. The analysis was carried out at a flow rate of 1.0 ml/min. The injection volume was 20 μ l. Constituents were detected on UV 254 nm. SCH from SE1 was included about 1.3%. SCH from SE2 was included about 2.0%.

Mice and Experimental Protocol

Mice (5-week Balb/c) were purchased from Dae-Han Experimental Animal Center (Eumsung, Republic of Korea). Mice were sensitized by intra-peritoneal injection of 100 μ g OVA emulsified in 20 mg aluminum hydroxide on days 1, 5, and 14. Mice were

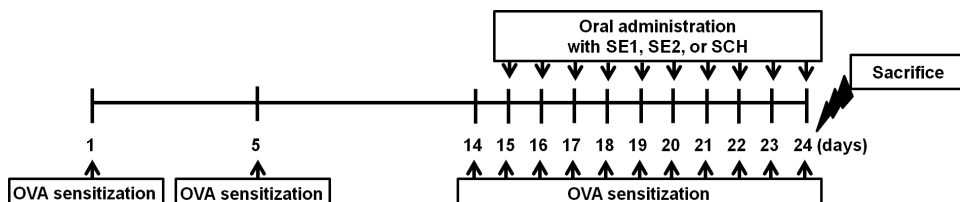


Figure 1. A mouse model of OVA-induced allergic reactions and treatments with SE1, SE2, or SCH.

divided into five groups ($n = 5$ per group): distilled water (DW)-administered blank mice; OVA-sensitized and DW-administered mice; OVA-sensitized and SE1 (100 mg/kg)-administered mice; OVA-sensitized and SE2 (100 mg/kg)-administered mice; OVA-sensitized and SCH (10 mg/kg)-administered mice. The doses were decided on the basis of previous reports (Egashira *et al.*, 2008; Jeong *et al.*, 2013). SE1, SE2, or SCH was orally administered to OVA-sensitized mice once daily for 10 days (Fig. 1). Blood was obtained from the heart and stored at -70°C until analyzed for levels of IL-4, IFN- γ , IgE, and IgG1. Spleen from each mouse was excised and stored at -70°C until analyzed for levels of IL-6, TNF- α , and IL-1 β . All animal experiments were conducted according to the guidelines established by the institutional animal care committee of Kyung Hee University (approval no. KHUASP (SE)-12-019).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-4, IFN- γ , IgE, IgG1, IL-6, TNF- α , and IL-1 β in serum or homogenized spleen were determined using a sandwich ELISA method according to the manufacturer's specifications (BD PharMingen; R & D Systems, Inc.). The protein levels of IL-6, TNF- α , and IL-1 β measured in spleen homogenate were presented as a ratio to the total protein level.

Splenocytes Culture

Single cell suspension was obtained from the spleen of normal Balb/c mice. Splenocytes (2.5×10^6) were cultured with either RPMI 1640 media alone or plate-bound anti-CD3 antibody (2 $\mu\text{g}/\text{ml}$) and soluble anti-CD28 antibody (4 $\mu\text{g}/\text{ml}$). And SE1, SE2, or SCH was treated for 48 h in the splenocytes. Supernatant was harvested for cytokines (IL-6 and TNF- α) measurements. For Western blot analysis, splenocytes (1×10^7) were cultured with either RPMI 1640 media alone or plate-bound anti-CD3 antibody (2 $\mu\text{g}/\text{ml}$) and soluble anti-CD28 antibody (4 $\mu\text{g}/\text{ml}$). And SE1, SE2, or SCH was treated for 1 h and then harvested.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Using an easy-BLUETM RNA extraction kit (iNtRON Biotech, Republic of Korea), total RNA was isolated from the spleen in accordance with the manufacturer's instructions. PCR

was performed with the following primers: IL-6 (5' CGG GAT CCA TGT TCC CTA CTT CAC AA 3'; 5' CCC AAG CTT CTA CGT TTG CC 3'); TNF- α (5' TAC AGG CTT GTC ACT CGA AT 3'; 5' ATG AGC ACA GAA AGC ATG AT 3'); IL-1 β (5'AGG CCA CAG GTA TTT TGT CG 3'; 5' GCC CAT CCT CTG TGA CTC AT 3'). Reference gene actin (5' CGC TCA GGA GGA GCA ATG 3'; 5' TGA CAG GAT GCA GAA GGA GA 3') was used to verify that equal amounts of RNA. The annealing temperature for IL-6, IL-1 β , and actin was 50°C. The annealing temperature for TNF- α was 60°C.

Western Blot Analysis

Spleen homogenate or splenocyte lysate was prepared for Western blot analysis. Western blot analysis was performed as previously described (Go *et al.*, 2013).

Statistical Analysis

The *ex vivo* data is a summary from at least three experiments. All data were expressed as the means $\pm$ S.E.M. Statistical evaluation was performed by independent *t*-test. The statistical analysis was performed using SPSS v12.00 statistical analysis software (SPSS Inc.). The significance was considered at a value of $p < 0.05$.

Results

SCH Regulated Levels of IgE, IgG1, and IL-4 in Serum of OVA-Sensitized Mice

First, to determine whether SE1, SE2, or SCH could regulate allergic reactions, we measured the levels of IgE, IgG1, IL-4, and IFN- γ in serum of OVA-sensitized mice. SE2 or SCH significantly inhibited the levels of IgE and IgG1 in serum of OVA-sensitized mice ($p < 0.05$, Figs. 2A and 2B). SE1 significantly inhibited the level of IgG1 in serum of OVA-sensitized mice ($p < 0.05$, Fig. 2B). As shown in Fig. 2C, the level of serum IL-4 was up-regulated by OVA sensitization, but the level of serum IL-4 was significantly inhibited by SCH administration in OVA-sensitized mice ($p < 0.05$). However, the level of serum IFN- γ was not increased by SE1, SE2, or SCH in OVA-sensitized mice (Fig. 2D).

SCH Regulated Expression of IL-6, TNF- α , and IL-1 β in Spleen of OVA-Sensitized Mice

The spleen serves an important role in immune function because it helps the immune system to recognize allergens and mediate most of the systemic inflammatory response (Martelli *et al.*, 2014). Thus, we determined whether SE1, SE2, or SCH could regulate the expression of IL-6, TNF- α , and IL-1 β in spleen of OVA-sensitized mice. The protein levels of IL-6 and TNF- α were up-regulated by OVA sensitization, but SE1, SE2, or SCH significantly inhibited the protein levels of IL-6 and TNF- α in spleen of OVA-sensitized

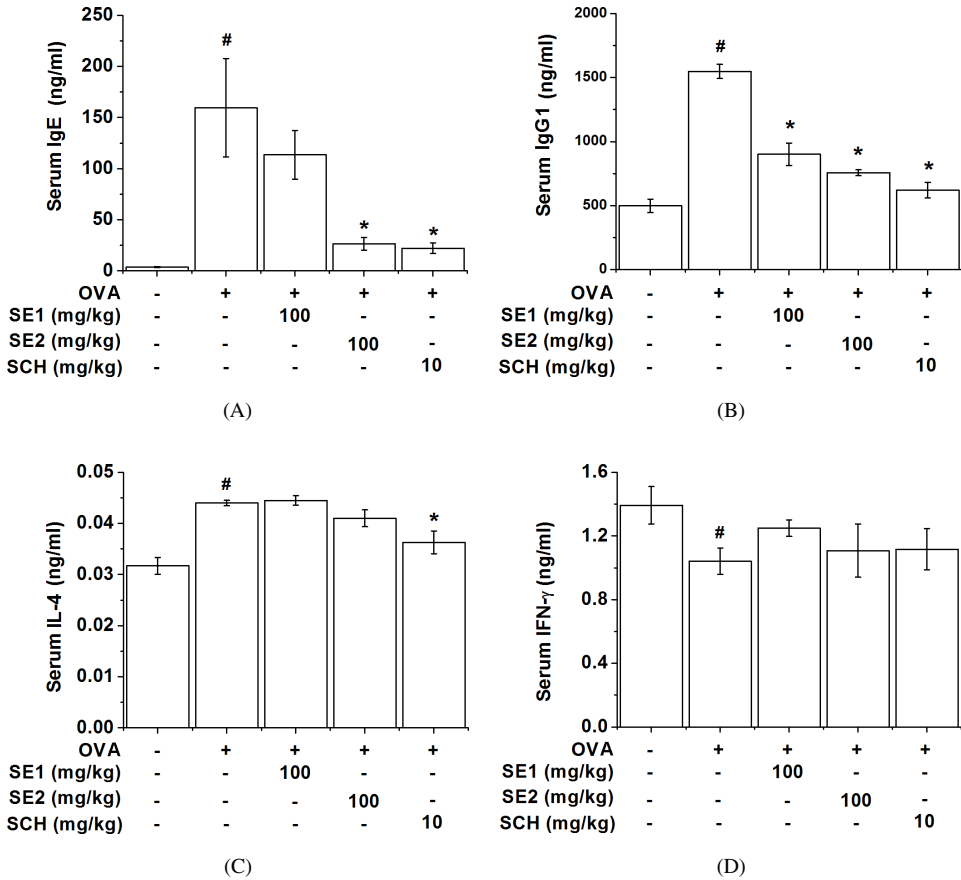


Figure 2. Effect of SE1, SE2, or SCH on the levels of IgE, IgG1, and IL-4 in serum of OVA-sensitized mice. The levels of (A) IgE, (B) IgG1, (C) IL-4, and (D) IFN- γ in serum were measured by ELISA. Each datum represents the means $\pm$ S.E.M. SE1, soft phase extract of *Schisandra chinensis*; SE2, solid phase extract of *Schisandra chinensis*; SCH, schizandrin. # $p < 0.05$; significantly different from blank mice value. * $p < 0.05$; significantly different from the control mice (OVA) value.

mice ($p < 0.05$, Figs. 3A and 3B). Also, the protein levels of IL-1 β were significantly inhibited by SE2 or SCH in spleen of OVA-sensitized mice ($p < 0.05$, Fig. 3C). However, SE1 did not improve the protein levels of IL-1 β in the spleen. Next, we analyzed the mRNA expression of IL-6, TNF- α , and IL-1 β in the spleen of OVA-sensitized mice. As shown in Figs. 3D and 3E, SE1, SE2, or SCH significantly suppressed the mRNA expression of IL-6, TNF- α , and IL-1 β in the spleen of OVA-sensitized mice ($p < 0.05$).

SCH Regulated Expression of Caspase-1 and RIP-2 in Spleen of OVA-Sensitized Mice

Caspase-1 and RIP-2 signaling mainly affects the secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6, or TNF- α (Furlan *et al.*, 1999; Sarkar *et al.*, 2006). Thus, we supposed

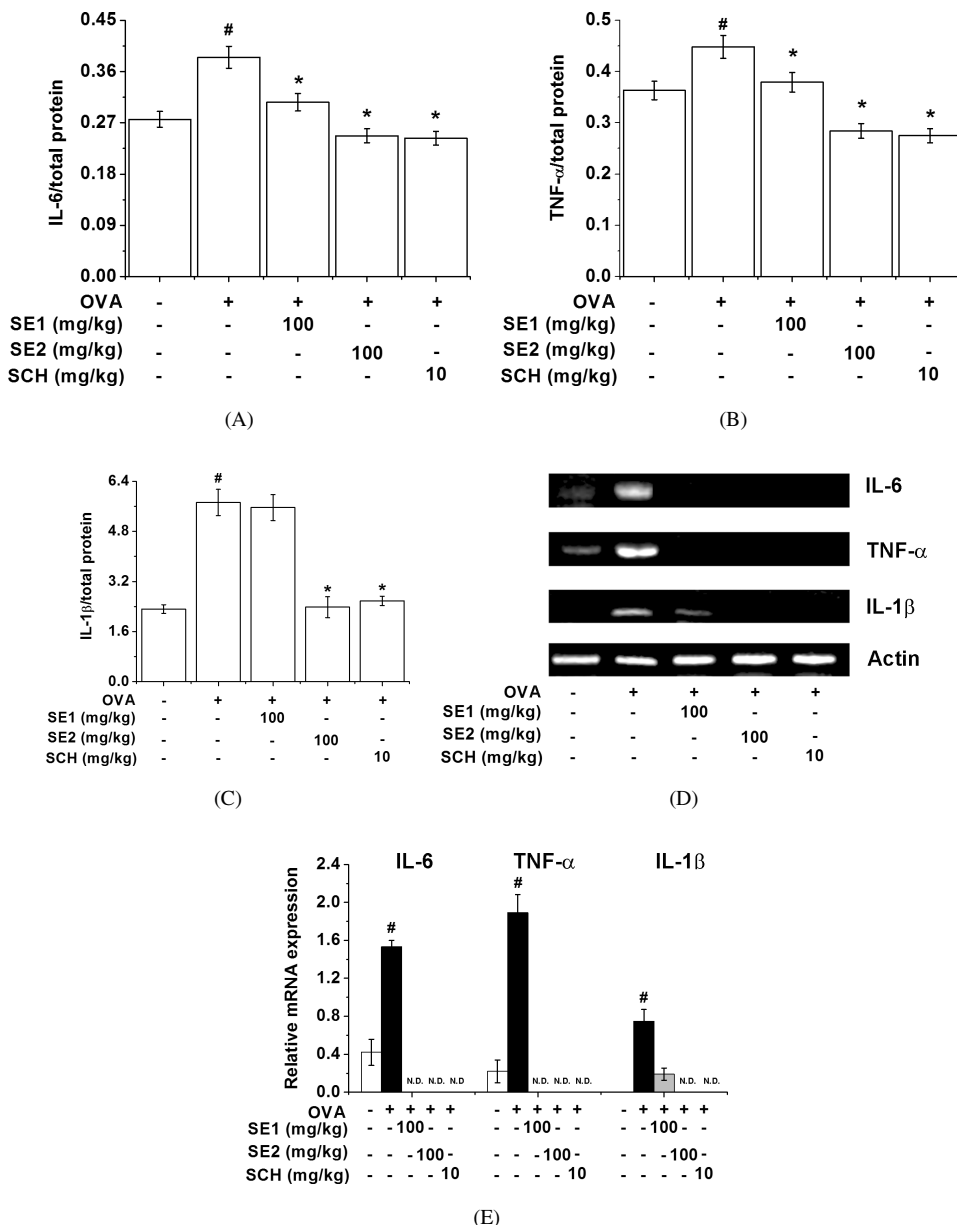


Figure 3. Effect of SE1, SE2, or SCH on the levels of IL-6, TNF- α , and IL-1 β in the spleen of OVA-sensitized mice. The protein levels of (A) IL-6, (B) TNF- α , and (C) IL-1 β in homogenized spleen were measured by ELISA. (D) The mRNA expression of IL-6, TNF- α , and IL-1 β was measured by RT-PCR. (E) All levels were quantified by densitometry. Each datum represents the means $\pm$ S.E.M. SE1, soft phase extract of *Schizandra chinensis*; SE2, solid phase extract of *Schizandra chinensis*; SCH, schizandrin; N.D., not detectable. [#] $p < 0.05$; significantly different from blank mice value. ^{*} $p < 0.05$; significantly different from the control mice (OVA) value.

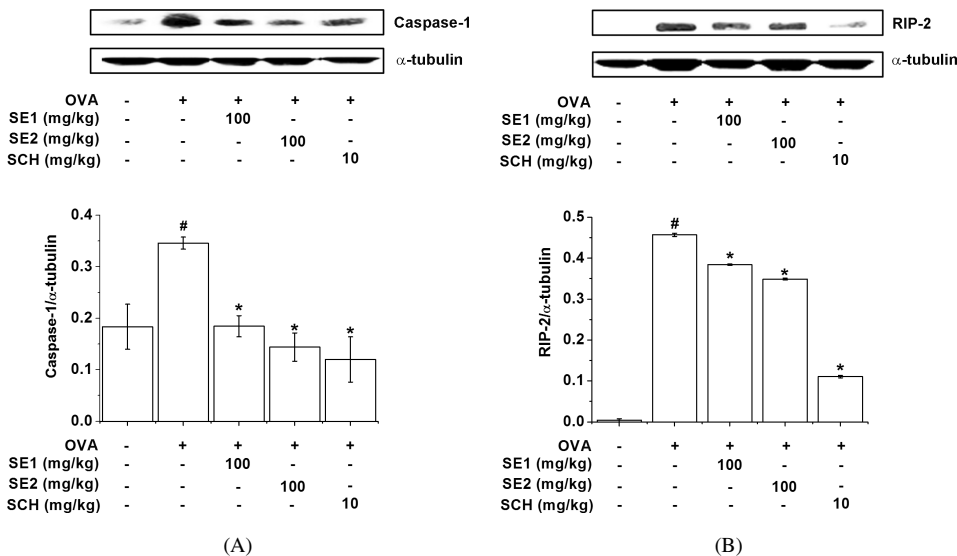


Figure 4. Effect of SE1, SE2, or SCH on the expression of caspase-1 and RIP-2 in the spleen of OVA-sensitized mice. The spleen from each group was prepared and analyzed for (A) caspase-1 and (B) RIP-2 by Western blot analysis as described in the experimental procedures. All protein levels were quantified by densitometry. Each datum represents the means $\pm$ S.E.M. SE1, soft phase extract of *Schisandra chinensis*; SE2, solid phase extract of *Schisandra chinensis*; SCH, schizandrin. # $p < 0.05$; significantly different from blank mice value. * $p < 0.05$; significantly different from the control mice (OVA) value.

that SE1, SE2, or SCH would regulate the levels of the IL-1 β , IL-6, and TNF- α through the blockade of caspase-1/RIP-2 signaling. We analyzed the caspase-1 and RIP-2 expression in the spleen of OVA-sensitized mice. As expected, SE1, SE2, or SCH significantly inhibited the expression of caspase-1 and RIP-2 in the spleen of OVA-sensitized mice ($p < 0.05$, Fig. 4).

SCH Regulated the Levels of IL-6 and TNF- α by Inhibiting Caspase-1/RIP-2 in Activated Splenocytes

Based on the regulatory effect of SE1, SE2, or SCH on the levels of inflammatory cytokines and caspase-1/RIP-2 in the spleen of OVA-sensitized mice, we investigated whether SE1, SE2, or SCH would regulate the levels of inflammatory cytokines and caspase-1/RIP-2 in activated splenocytes. As shown in Figs. 5A and 5B, anti-CD3 and anti-CD28 antibodies increased the production of IL-6 and TNF- α . However, SE1, SE2, or SCH significantly decreased the production of IL-6 and TNF- α in anti-CD3 and anti-CD28-stimulated splenocytes (Figs. 5A and 5B, $p < 0.05$). And SE1, SE2, or SCH significantly reduced the expression of caspase-1 and RIP-2 in activated splenocytes ($p < 0.05$, Figs. 5C and 5D).

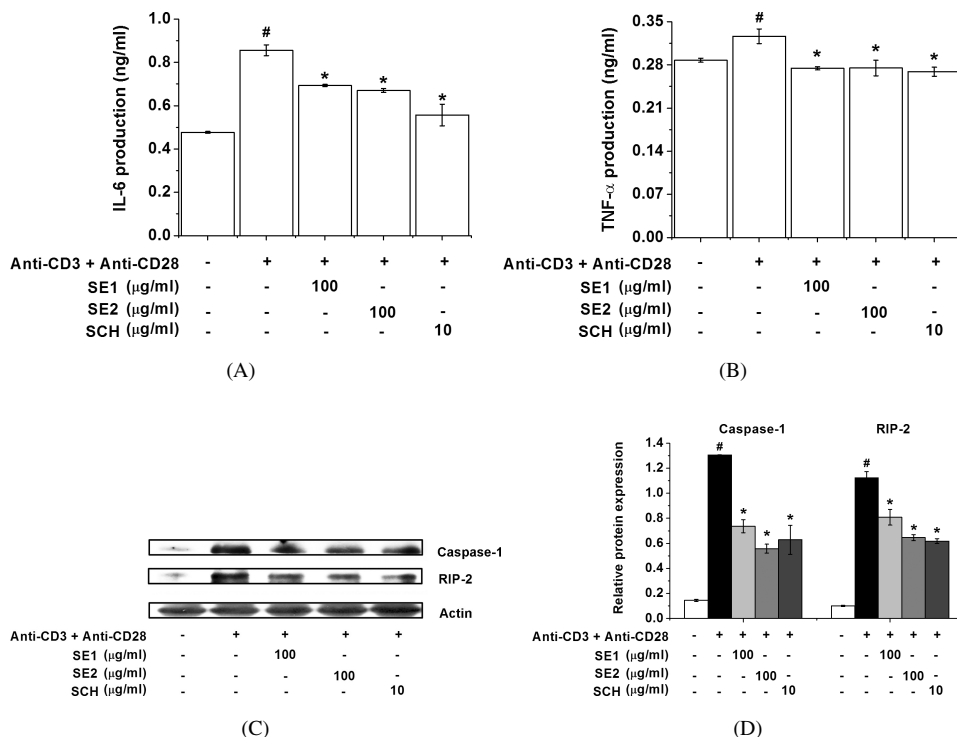


Figure 5. Effect of SE1, SE2, or SCH on the levels of IL-6, TNF-α, and caspase-1/RIP-2 in activated splenocytes. The splenocytes were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies and treated with SE1, SE2, or SCH for 48 h. The production of (A) IL-6 and (B) TNF-α were measured in culture supernatants by ELISA. (C) The splenocytes stimulated with immobilized anti-CD3 and soluble anti-CD28 were treated with SE1, SE2, or SCH for 1 h. Whole cell extract were prepared and subjected to Western blotting. The blots were analyzed with antibodies against caspase-1, RIP-2, and actin. (D) All protein levels were quantified by densitometry. Each datum represents the means ± S.E.M of three independent experiments. SE1, soft phase extract of *Schizandra chinensis*; SE2, solid phase extract of *Schizandra chinensis*; SCH, schizandrin. [#]*p* < 0.05; significantly different from unstimulated cells value. ^{*}*p* < 0.05; significantly different from the anti-CD3 and anti-CD28-stimulated cells value.

Discussion

In this study, we showed that SE1 and SE2, extracted via different extraction techniques of *Schizandra chinensis*, and SCH, significantly regulated OVA-induced allergic reactions. SE2 and SCH particularly had regulatory effects on the levels of IgE and IgG1 in serum and the levels of IL-6, TNF-α, and IL-1β in the spleen of OVA-sensitized mice. Also, the expression of caspase-1 and RIP-2 was decreased by SE2 or SCH in the spleen of OVA-sensitized mice. SE1 inhibited the level of IgG1 in serum and the levels of IL-6, TNF-α, caspase-1, and RIP-2 in the spleen of OVA-sensitized mice. In addition, SE1, SE2, and SCH suppressed the production of IL-6 and TNF-α, and the expression of caspase-1 and RIP-2 in activated splenocytes.

The spleen is a primary immune organ and mediates the systemic inflammatory responses (Bronte and Pittet, 2013). IL-6, which is secreted in response to antigens, was implicated in the development of the allergic reaction (Park *et al.*, 2014). TNF- α contributed to the development of allergic reaction via the production of antigen-specific IgE and Th2 cytokines (Iwasaki *et al.*, 2003). TNF- α produced in the spleen induced immunological responses that participate in allergic reactions (Tarkowski *et al.*, 2010). IL-1 β is a pluripotent cytokine that is implicated in pathophysiology of systemic inflammatory response syndrome (Dinarello *et al.*, 1993). IL-1 β overexpressed in the spleen participated in immune-inflammation of allergy (Li *et al.*, 2013). IL-1 β deficient mice showed significantly less allergic reactions compared to wild-type mice (Nakae *et al.*, 2003). Seo *et al.* (2013) reported that cordycepin inhibited the levels of IL-6 and TNF- α in the spleen and regulated the immediate hypersensitivity reaction. Taxifolin was demonstrated to have anti-inflammatory effects through inhibiting the level of IL-1 β in the spleen (Kim *et al.*, 2008). In this study, SE1, SE2, or SCH also inhibited the levels of IL-6, TNF- α , and IL-1 β in the spleen of OVA-sensitized mice.

Caspase-1 converts pro-IL-1 β to its active form (Sarkar *et al.*, 2006). Caspase-1 and IL-1 β are required for allergic airway disease (Martin *et al.*, 2013). Caspase-1 activation caused inflammation via the production of pro-inflammatory cytokines, such as IL-6 or TNF- α (Furlan *et al.*, 1999). Caspase-1 transgenic mice overexpressing caspase-1 spontaneously had chronic allergic reactions and significant increases in levels of serum histamine and IgE (Murakami *et al.*, 2006). Caspase-1 deficient mice showed an alteration in the production of several pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α (Kuida *et al.*, 1995). In addition, caspase-1 deficiency in the spleen reduced the levels of pro-inflammatory cytokines (Faubel *et al.*, 2004). RIP-2 interacts with caspase-1 and plays a critical role in NF- κ B activation (Sarkar *et al.*, 2006). The knockdown of RIP-2 ameliorated experimental allergic inflammation, markedly suppressing the levels of serum IgE and IL-4 in OVA-challenged mice (Goh *et al.*, 2013). These studies demonstrate that the activation of caspase-1 or RIP-2 would be an attractive target for the therapeutic agents against allergic diseases. In this study, SE1, SE2, or SCH inhibited the levels of caspase-1 and RIP-2 as well as the levels of IL-6, TNF- α , and IL-1 β in the spleen of OVA-sensitized mice. In addition, SE1, SE2, or SCH inhibited the levels of IL-4, IgE, and IgG1 in serum of OVA-sensitized mice. The anti-allergic effect of SE1, SE2, or SCH would result from less expression of caspase-1 and RIP-2 in the spleen of OVA-sensitized mice.

The spleen consists of T cell, B cell, or natural killer cells, etc. (Gupta, 2011). T cells play an important role in allergic inflammation. Dexamethasone inhibited inflammatory responses through the down-regulation of TCR/CD3 signaling in T cells (Baus *et al.*, 1996). Zinc oxide nanoparticles in dietary supplements had an immunomodulatory effect, decreasing the level of TNF- α in activated splenocytes (Roy *et al.*, 2014). The activated caspase-1 in splenocytes mediated inflammation and hypersensitivity to infection (Ataide *et al.*, 2014). Splenocytes treated with a caspase-1 inhibitor reduced the production of pro-inflammatory cytokines (Fantuzzi *et al.*, 1999). In this study, SE1, SE2, or SCH also inhibited the levels of IL-6 and TNF- α in activated splenocytes. SE1, SE2, or SCH reduced the levels of caspase-1 and RIP-2 in activated splenocytes. Therefore, these data imply that

SE1, SE2, or SCH inhibits the levels of IL-6 and TNF- α through inhibition of caspase-1/RIP-2 signaling in activated splenocytes.

Adjuvants for acute sensitization, such as aluminum hydroxide promote the development of the Th2 phenotype when it is exposed to an antigen (Nials and Uddin, 2008). Wu *et al.* (2014) demonstrated an anti-allergic effect of mushroom by inhibiting the levels of IL-4, IgE, and IgG1 in serum of an OVA/adjuvant-sensitized allergy mouse model. Go *et al.* (2013) reported an anti-allergic effect of herbal mixture by inhibiting the levels of IgE, IL-4, and IL-1 β in an OVA/adjuvant-induced allergy mouse model. In this study, we showed that SE1, SE2, or SCH significantly regulates caspase-1 signaling and has an anti-allergic effect using an OVA/aluminum hydroxide-sensitized allergic model for the first time.

In conclusion, the present study showed that SE1, SE2, or SCH has an anti-allergic effect via blockade of caspase-1 signaling pathway. Treatment with SE1, SE2, or SCH inhibited the levels of IL-6, TNF- α , and IL-1 β through down-regulation of expression of caspase-1/RIP-2 in the spleen of OVA-sensitized mice. In addition, SCH inhibited the levels of IgE, IgG1, and IL-4 in the serum of OVA-sensitized mice. SE1, SE2, or SCH inhibited the levels of IL-6 and TNF- α through inhibiting caspase-1/RIP-2 signaling in activated splenocytes. However, the more research needs to be done to explore the role of *Schizandra chinensis* and SCH in various allergic reactions.

Acknowledgments

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