Inactivation of Cystein-Aspartic Acid Protease (Caspase)-1 by Saikosaponin A

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This work investigates the anti-inflammatory mechanism of saikosaponin A (SA), a major component of Bupleurum falcatum LINNE. SA significantly inhibited phorbol myristate acetate (PMA) plus A23187-induced production and expression of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in human mast cell (HMC)-1 cells. SA suppressed PMA plus A23187-induced phosphorylation of extracellular signal-regulated kinase and p38. When HMC-1 cells were treated with SA, translocation of nuclear factor (NF)-κB/Rel A into nucleus and degradation of inhibitor of NF-κB (IκB) in cytoplasm were inhibited. SA decreased PMA plus A23187-induced cystein-aspartic acid protease (caspase)-1 activity. IL-1β production was also inhibited by SA. Finally, SA significantly decreased the number of nasal rubs and serum TNF-α level in the ovalbumin-sensitized allergic rhinitis mouse model. The underlying mechanism involves, at least in part, inactivation of caspase-1, which provides new evidence for therapeutic application of SA to target inflammatory processes.

Key words saikosaponin A; mast cell; interleukin-6; tumor necrosis factor-α; cystein-aspartic acid protease-1; allergic rhinitis

Allergic rhinitis (AR) is a common manifestation of allergic inflammatory diseases and a heterogeneous disorder characterized by one or more of the following nasal symptoms: sneezing, itching, rhinorrhea, and/or nasal congestion. The inflammatory process in AR involves many different inflammatory cells (mast cells and eosinophils), cytokines, chemokines, and other regulatory molecules.1 Inflammatory reactions are currently regulated with steroidal and non-steroidal anti-inflammatory agents.2 Both of these widely prescribed drug classes have negative side effects, limiting their use in certain segments of the population.3–5 There is a need to develop new agents with novel modes of action. Natural product-based anti-inflammatory agents with a transcriptional mode of action, good efficacy, and lower risk of side effects offer promising control of inflammatory reactions. Saikosaponin A (SA) is a triterpene saponin isolated from Bupleurum falcatum LINNE (Umbelliferae), which has been traditionally used to regulate inflammatory diseases, such as hepatitis and nephritis and so on.6–8 However, the effect mechanism of SA on the inflammatory reaction is not well understood.

Mast cells are critical effector cells of the allergic inflammatory reaction. Activated mast cells release inflammatory mediators, such as interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor.6 The expression of these inflammatory cytokines depends on the activation of the transcription factor, nuclear factor (NF)-κB or mitogen-activated protein kinases (MAPKs), c-Jun NH2-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK).9 In mammalian cells, MAPKs signaling cascade plays an essential role in the initiation of inflammatory responses.9–11 Three major MAPKs have been defined: the ERK pathway, the stress-activated pathways of the JNK, and the p38 MAPKs.9 These pathways are central components of the intracellular signaling networks that control many aspects of mammalian cellular physiology, including cell proliferation, differentiation, and apoptosis.10 In most cell types, NF-κB is present in the cytosol in an inactive form and is associated with its inhibitor proteins, called inhibitor of NF-κB (IκB). IκBs family members include IκBα, IκBβ, IκBε, and IκBζ, with IκBα being the prototypical member of this family.11 Activation of NF-κB by extracellular stimuli leads to rapid phosphorylation, ubiquitination, and proteolytic degradation of IκBα, thereby exposing the nuclear localization signals on NF-κB and resulting in the nuclear translocation of NF-κB complex and phosphorylation of NF-κB. The binding of NF-κB to a specific sequence in the promoter region of a gene triggers the transcriptional activation of NF-κB regulated genes.12–14

Cystein-aspartic acid protease (caspase)-1 activation is mediated by cytosolic protein complexes termed inflammasomes, which function in various immune cells. Caspase-1 is characterized by its ability to activate the inactive precursors of IL-1β and IL-18.15 IL-1β is a proinflammatory cytokine which is proteolytically processed to its active form by caspase-1. Nuclear translocation of NF-κB is seen after activation of mast cells by IL-1β.16 Therefore, caspase-1 could theoretically contribute to NF-κB activation through the autocrine action of IL-1β on cell surface receptors.17

We examined the regulatory effects of SA on the production and mRNA expression of IL-6 and TNF-α in phorbol myristate acetate (PMA) plus A23187-stimulated human mast cell line (HMC-1) cells. We next investigated the effect of SA on the MAPKs and NF-κB pathways in PMA plus A23187-stimulated HMC-1 cells. We also investigated the effect of SA on IL-1β production as well as caspase-1 expression and activity. Finally, we investigated the effect of SA on an AR mouse model.

MATERIALS AND METHODS

Reagents We purchased PMA, A23187 (Calcinycin; C24H15N3O8), amin-peroxidase, dexamethasone (DEX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), SB203580, PD98059, pyrroolidine-dithiocarbamate (PDTC), ovalbumin (OVA), bicinchoninic acid (BCA), alu-
minum hydroxide and 2′-AZINO-bis(3-ethylbenzthiazoline-6-sulfonic acid) tablets substrates from Sigma Chemical Co. (St. Louis, MO, U.S.A.); caspase-1 inhibitor (CI) and caspase-1 assay kit from R&D Systems (Minneapolis, MN, U.S.A.); SA from Wako Pure Chemical Industries (Osaka, Japan); Isocove’s modified Dulbecco’s medium (IMDM) from Gibco BRL (Grand Island, NY, U.S.A.); IL-1ß, IL-6, TNF-α antibodies, and recombinant human TNF-α from Pharmingen (San Diego, CA, U.S.A.); NF-κB, p38, ERK, JNK, phosphorylated (p) p38, pERK, pJNK, and caspase-1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); tubulin antibody from Assay Designs Inc. (Ann Arbor, MI, U.S.A.).

Cells HMC-1 cells were grown in IMDM and supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% fetal bovine serum at 37°C in 5% CO2 with 95% humidity.

Preparation of SA SA was prepared by dissolving with dimethyl sulfoxide (DMSO). Dilutions were made in phosphate buffered saline (PBS) and filtered through 0.22-µm syringe filter.

MTT Assay Cell viability was determined by MTT assay. Briefly, 500 µl of HMC-1 cells suspension (4×105 cells) was cultured in 4-well plates for 8 h after treatment by each concentration of SA. Twenty microliters of MTT solution (5 mg/ml) was added and the cells were incubated at 37°C for an additional 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 an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm.

OVA-Induced AR Mouse Model We maintained 4-week-old female BALB/c (Charles River Technology) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University. We sensitized mice on days 1, 5, and 14 by intraperitoneal (i.p.) injection of 100 µg OVA emulsified in 20 mg aluminum hydroxide and we challenged mice with 1.5 mg OVA. SA was administrated orally before intranasal (i.n.) OVA challenge for 10 d. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation for 3 d before sacrifice. Serum was obtained after counting the number of nasal rubs.

Cytokines Assay HMC-1 cells were pretreated with various concentrations of SA (0.1 to 10 µM) for 2 h before PMA plus A23187 stimulation. The production of IL-1ß, IL-6, and TNF-α and serum TNF-α level were measured from cells culture supernatant and serum according to the manufacturer’s specifications (R & D System Inc., Minneapolis, MN, U.S.A.).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Republic of Korea), we isolated the total RNA from HMC-1 cells in accordance with the manufacturer’s specification. We performed a PCR with the following primers: IL-6 (5′-GAT GGATGC TTC CAATCT GGAT-3′; 5′-AGT TCT CCATAG AGA ACA ACA TA-3′); TNF-α (5′-CAC CAG CTG GTT ATC TCT CAG CTC-3′; 5′-CGG CAG GTG GAG CTG GCC GAG GAG-3′). To verify if equal amounts of RNA were used for the reverse transcription and PCR amplification from different experimental conditions, we also used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-CAA AAG GGT CAT CAT CTC TG-3′; 5′-CCT GCT TCA CCA CCT CCT TG-3′). The annealing temperature was 56°C for IL-6 and 60°C for TNF-α and GAPDH. The PCR products increased as the concentration of RNA increased. The amplified fragment sizes were 443 bp for IL-6, 355 bp for TNF-α, and 446 bp for GAPDH.

Western Blot Analysis The stimulated cells were lysed and separated through 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred to nitrocellulose membranes and then the membranes were blocked and incubated with primary and secondary antibodies. Finally, the protein bands were visualized by an enhanced chemiluminescence assay purchased from Amersham Co. (Newark, NJ, U.S.A.) following the manufacturer’s instructions.

Preparation of Nuclear and Cytoplasmic Extracts Nuclear and cytoplasmic extracts were prepared as described previously. Briefly, after cell activation for the times indicated, cells were washed with ice-cold PBS and suspended in 60 µl of buffer A (10 mM N-(2-hydroxyethyl)piperezine-N′-2-ethanesulfonic acid (HEPES)/KOH, 2 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 µl of 10% Nonidet P-40, and centrifuged at 2000×g for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellets were 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 dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9), left on ice for 20 min and inverted. The nuclear debris was then spun down at 15000×g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen, and stored at −70°C until conducting the analysis.

Transient Transfection and Luciferase Assay For the transfection, we seeded HMC-1 cells (1×105) in a 100 mm culture dish. We then used Lipofectamine™2000 purchased from Invitrogen (Carlsbad, CA, U.S.A.) to transiently transfect pNF-κB luciferase (LUC) and pSV40-LUC reporter gene constructs into HMC-1 cells. To measure the luciferase activity, we used a luminometer 1420 luminescence counter purchased from Perkin Elmer (Waltham, MA, U.S.A.) in accordance with the manufacturer’s protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity.

Caspase-1 Activity Assay Caspase-1 activity was measured according to the manufacturer’s specification by using caspase assay kit. Catalytic activity of caspase-1 from Whole-cell lysate and nose skin was measured by proteolytic cleavage of WEHD-p-nitroaniline (pNA, caspase-1 colorimetric substrate) for 24 h at 37°C. An equal amount of total protein was quantified by BCA protein quantification kit purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The plates were read at 405 nm.

Statistics All results are expressed as the mean±standard deviation (S.D.). The statistical evaluation of the results
was performed by an independent t-test and an ANOVA with a Tukey post hoc test. The results are significant with a value of \( p < 0.05 \).

RESULTS

Effect of SA on the Production and mRNA Expression of IL-6 and TNF-\( \alpha \) in HMC-1 Cells

To assess the regulatory effect of SA on the production of IL-6 and TNF-\( \alpha \), we stimulated HMC-1 cells with PMA plus A23187 for 8 h and used the ELISA method to analyze the supernatants for IL-6 and TNF-\( \alpha \). We compared inhibitory effects on the production of IL-6 and TNF-\( \alpha \) of SA with those of PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), PDTC (NF-\( \kappa \)B inhibitor), and CI (caspase-1 inhibitor). Furthermore, DEX was used as a reference drug. Stimulation with PMA (0.05 \( \mu \)M) plus A23187 (1 \( \mu \)M) increased the production of IL-6 and TNF-\( \alpha \) in HMC-1 cells. However, SA significantly inhibited the production of IL-6 and TNF-\( \alpha \) (\( p < 0.05 \), Fig. 1A). The maximal inhibition rates of production of IL-6 and TNF-\( \alpha \) were 35.98 \pm 2.38\% and 51.98 \pm 1.30\%, respectively. To determine whether SA could modulate the PMA plus A23187-induced mRNA expression of IL-6 and TNF-\( \alpha \), we performed RT-PCR analysis. The mRNA expression of IL-6 and TNF-\( \alpha \) was up-regulated by PMA plus A23187, but the up-regulated mRNA expression was significantly inhibited by pretreatment with SA (0.1 to 10 \( \mu \)M, \( p < 0.05 \)), Figs. 1B, C). Inhibitors (PD98059, SB203580, and PDTC) significantly inhibited the production of IL-6 and TNF-\( \alpha \) (\( p < 0.05 \), Fig. 1D). However, CI (500 nm) significantly inhibited IL-6 production and did not inhibit TNF-\( \alpha \) production. We assessed the effect of SA on the viability in HMC-1 cells. When SA was given as a pretreatment at concentrations ranging from 0.1 to 20 \( \mu \)M, cytotoxicity of SA was not shown at any doses except 20 \( \mu \)M (Fig. 2).

Effect of SA on MAPKs Phosphorylation in HMC-1 Cells

To investigate whether SA would influence phosphorylation of p38, ERK, and JNK induced by PMA plus A23187, we pretreated SA for 2 h before stimulation with PMA plus A23187 for 30 min. Then, we performed Western blot analysis for the phosphorylation of p38, ERK, and JNK using phospho-specific antibodies. The results showed that

![Fig. 1. Effect of SA on the Production and mRNA Expression of IL-6 and TNF-\( \alpha \) in HMC-1 Cells](image_url)
SA inhibited the phosphorylation of ERK and p38 in PMA plus A23187-stimulated HMC-1 cells (p<0.05, Fig. 3). However, SA did not affect the phosphorylation of JNK (data not shown).

Effect of SA on NF-κB Activation and IκBα Degradation in HMC-1 Cells
SA showed an inhibitory effect on PMA plus A23187-induced cytokine expression (Fig. 1). Thus, we evaluated the mechanism of SA on the mast cell-mediated inflammatory reaction. First, to determine the regulatory effect of SA on the translocation of NF-κB, we performed Western blot analysis. In the group that was stimulated by PMA plus A23187, the expression level of NF-κB/Rel A was increased in the nucleus. However, the expression level of NF-κB/Rel A in the nucleus was decreased by treatment with SA (p<0.05, Fig. 4B). Then, to determine whether SA affects IκBα degradation, we used Western blot analysis to examine the cytoplasmic level of IκBα that existed after PMA plus A23187 stimulation. Figure 4A shows that PMA plus A23187 stimulation effectively induced IκBα degradation, however, pretreatment with SA caused an inhi-
bition of IκBα degradation in HMC-1 cells (*p*<0.05, Fig. 4B). Next, we examined whether SA could modulate the luciferase expression specifically via NF-κB activation. We transiently transfected pNF-κB-LUC and pSV40-LUC into HMC-1 cells and pretreated with SA for 2 h before stimulation with PMA plus A23187 or recombinant TNF-α. As shown in Fig. 4C, PMA plus A23187 or recombinant TNF-α stimulation increased the reporter gene activity. However, this increased activity was significantly decreased by treatment with SA (*p*<0.05).

**Effect of SA on Caspase-1 Activation in HMC-1 Cells**

We investigated whether SA could modulate caspase-1 expression and activity. Caspase-1 expression and activity were significantly decreased by pretreatment with SA in PMA plus A23187-stimulated HMC-1 cells (*p*<0.05, Figs. 5A, B). Caspase-1 is known to generate IL-1β and IL-18.15 We investigated the effect of SA on IL-1β production. As shown in Fig. 5C, SA significantly inhibited IL-1β production (*p*<0.05).

**Effect of SA on Clinical Symptom and TNF-α Level in the AR Mouse Model**

Finally, we investigated the inhibitory effect of SA in the AR mouse model. The number of nasal rubs after the OVA challenge in OVA-sensitized mice was significantly higher than those in the OVA-unsensitized mice. The increased rub score was inhibited by treatment with SA (8 mg/kg, *p*<0.05, Fig. 6A). Increased level of serum TNF-α in the OVA-sensitized mice was significantly decreased by treatment with SA (*p*<0.05, Fig. 6B).

**DISCUSSION**

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. The accumulation of mast cells takes place in relation to inflammatory conditions.19,20 Oh et al. reported that the number of mast cells in the nasal mucosa of AR mice was significantly higher than those in the OVA-unsensitized mice.21 In the inflammatory process, proinflammatory cytokines recruit activated immune and inflammatory cells to the sites of lesions, thereby amplifying and perpetuating the inflammatory condition.22,23 A proinflammatory cytokine, IL-6, plays a prominent role in inflammatory disease processes.24 TNF-α promotes inflammation, leukocyte infiltration, and tissue fibrosis and is thought to be an initiator of cytokine-related inflammatory states via stimulation of cytokine production.25 In a previous study, SA inhibited chemical-induced liver inflammation and fibrosis in rats.26 SA also
inhibited the proliferation and activation of T cells through cell cycle arrest and induction of apoptosis. 27 In this study, we showed that SA inhibits PMA plus A23187-induced proinflammatory cytokines (IL-6 and TNF-α) production and mRNA expression in HMC-1 cells (Fig. 1). SA also inhibited TNF-α level in an AR in vivo model (Fig. 6). These results suggest that SA exerts an anti-inflammatory effect through the regulation of IL-6 and TNF-α levels.

MAPKs (p38 and ERK) and NF-κB signaling pathways are involved in the anti-inflammatory properties of a glucocorticoid receptor agonist. 28 In addition, it has been reported that SB203580 suppressed the production of IL-6 and TNF-α in mast cells. 29 PD98059 decreased the production of IL-6, TNF-α, and IL-8 in stimulated HMC-1 cells. 30 In the present study, we also confirmed that PD98059, SB203580, and PDTC significantly inhibit the production of IL-6 and TNF-α in stimulated HMC-1 cells. SA inhibits MAPKs phosphorylation in stimulated HMC-1 cells (Fig. 3). Therefore, we can conclude that SA only acts as a specific inhibitor of MAPKs.

Expression of various inflammatory cytokines was regulated by transcription factors, NF-κB, nuclear factor activated T cells, and NF-IL-6. The activation of NF-κB is dependent on the phosphorylation and degradation of IκB, an endogenous inhibitor that binds to NF-κB in the cytoplasm. 31,32 Previously, glucocorticoids were reported to suppress immune inflammation by interacting with NF-κB. 33 DEX-induced anti-inflammatory responses are associated with the inhibition of NF-κB. 34 In this study, we observed that SA inhibited PMA plus A23187 or TNF-α-induced NF-κB activation in HMC-1 cells (Fig. 4). Therefore, we believe that the anti-inflammatory effect of SA is regulated through the inhibition of NF-κB/IκBα signal cascade.

Caspase-1 activation regulates inflammation via production of proinflammatory cytokines and recruitment of neutrophils. 35 Transgenic mice overexpressing caspase-1 in the epidermis showed spontaneous recurrent dermatititis and skin ulcers. 36 Moreover, a caspase-1 inhibitor, VX-765, blocked the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. 37 Caspase-1−/− mice decreased IL-6 production after stimulation with lipopolysaccharide. 38 Furthermore, it was reported that caspase-1 activates NF-κB and p38 MAPK, but NF-κB is independent of p38 MAPK. 39 Grzegorczyk et al., have reported a significant increase in caspase-1 levels in serum from allergic asthmatic patients as compared to those of a control group. 40 Caspase-1 contains an N-terminal caspase recruitment domain (CARD). 41 Specific adaptor molecules of a receptor interacting protein-2 (RIP2, CARD containing kinase) regulate the activation of caspase-1 through CARD–CARD interaction. 15 Other studies have shown that RIP2 knockout reduces the secretion of pro-inflammatory cytokines, such as TNF-α and IL-6. 42 Therefore, we postulated that SA mediates these effects at least partly through the suppression of RIP2/caspase-1 activation. In this study, we confirmed that SA suppressed PMA plus A23187-induced caspase-1 activation in HMC-1 cells for the first time. However, CI (500 ms) significantly inhibited IL-6 production but did not significantly inhibit TNF-α production in stimulated HMC-1 cells. Therefore, further studies will be needed to precisely clarify the mechanism of SA on the relationship between caspase-1 activation and inflammatory cytokine production in the AR mice.

In conclusion, we have shown that SA inhibited the production and mRNA expression of IL-6 and TNF-α in stimulated HMC-1 cells. Moreover, SA suppressed PMA plus A23187-induced ERK and p38 phosphorylation in stimulated HMC-1 cells. SA inhibited the translocation of NF-κB/Rel A into the nucleus and degradation of IκBα in the cytoplasm. SA decreased PMA plus A23187-induced caspase-1 activity and IL-1β production. Finally, SA significantly inhibited the serum TNF-α level and the number of nose rubs in the AR mouse model. Given these findings, the inhibition of caspase-1 activation by SA in HMC-1 cells could form the basis of a new strategy for the treatment of mast cell-mediated inflammatory diseases.

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