Anti-inflammatory effect of salidroside on phorbol-12-myristate-13-acetate plus A23187-mediated inflammation in HMC-1 cells

DA-WUN YANG[1*], OK-HWA KANG[2*], YOUNG-SEOB LEE[3], SIN-HEE HAN[3], SANG-WON LEE[3], SEON-WOO CHA[3], YUN-SOO SEO[2], SU-HYUN MUN[1], RYONG GONG[1], DONG-WON SHIN[4] and DONG-YEUL KWON[2]

[1]BK21 Plus Team, Professional Graduate School of Oriental Medicine,Wonkwang University, Iksan, Jeonbuk 570-749; [2]Department of Oriental Pharmacy, College of Pharmacy and Wonkwang-Oriental Medicines Research Institute, Institute of Biotechnology, Wonkwang University, Iksan, Jeonbuk 570-749; [3]Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Chungbuk 369-873; [4]Department of Oriental Medicine Resources, Sunchon National University, Jeonnam 540-742, Republic of Korea

Received September 14, 2015; Accepted September 27, 2016

DOI: 10.3892/ijmm.2016.2781

Abstract. Salidroside [2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside (SAS)] has been identified as the most potent ingredient of the plant *Rhodiola rosea* L. Previous studies have demonstrated that it possesses a number of pharmacological properties, including anti-aging, anti-fatigue, antioxidant, anticancer and anti-inflammatory properties. In this study, to ascertain the molecular mechanisms responsible for the anti-inflammatory activity of SAS, we used phorbol-12-myristate-13-acetate (PMA) plus A23187 to induce inflammation in human mast cell line-1 (HMC-1). The HMC-1 cells were treated with SAS prior to being stimulated with PMA plus A23187. Pro-inflammatory cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). Western blot analysis was used to examine the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). SAS inhibited the mRNA expression and production of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF). In cells stimulated with PMA plus A23187, SAS suppressed the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and c-jun N-terminal kinase 1/2 (JNK1/2), but not that of p38 MAPK. SAS suppressed the expression of NF-κB in the nucleus. On the whole, our results suggest that SAS exerts an anti-inflammatory effect by inhibiting the production of pro-inflammatory cytokines through the blocking of the NF-κB and MAPK signaling pathways.

Correspondence to: Dr Dong-Yeul Kwon or Dr Ok-Hwa Kang, Department of Oriental Pharmacy, College of Pharmacy and Wonkwang-Oriental Medicines Research Institute, Institute of Biotechnology, Wonkwang University, Iksan-daero, Iksan, Jeonbuk 570-749, Republic of Korea
E-mail: sssimi@wku.ac.kr
E-mail: kangokhwa@hanmail.net

*Contributed equally

Key words: salidroside, mast cells, pro-inflammatory cytokines, nuclear factor-κB

Introduction

According to the current understanding of the pathophysiology of allergic diseases, mast cells play a key role in inflammation, and are well known for their influential effector functions in allergic disorders and reactions (1,2). The human mast cell line-1 (HMC-1) originates from a patient with mastocytosis (3) and these cells do not express the high-affinity IgE-receptor FcεR1 (4,5). The lack of FcεR1 in HMC-1 cells has led to the use of physiological stimuli, such as calcium ionophores and phorbol esters to activate these cells, by many researchers. Previous studies have demonstrated that HMC-1 cells express a wide range of cytokines, which can be synthesized, stored and released after stimulation (6,7). For example, mast cells release pro-inflammatory cytokines and chemokines, such as interleukin (IL)-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and inflammatory mediators, including histamine, leukotrienes and serotonin (8,9). The cytokines released from mast cells transform the terminal microenvironment and attract neutrophils and basophils (10). TNF-α is a pleiotropic pro-inflammatory cytokine that plays an important role in several pathological conditions related to inflammation and infection; the role of TNF in malignancies and inflammatory disorders, such as arthritis has been reviewed extensively (11). The pro-inflammatory activities of IL-6 include the recruitment of inflammatory cells, the inhibition of the apoptosis of inflammatory cells, and the inhibition of regulatory T-cell differentiation (12). IL-8 is the most potent chemokine that has been studied thus far and

is responsible for inducing chemotaxis, which is the directed migration of cells to a site of inflammation (13,14).

The mitogen-activated protein kinases (MAPKs) include extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK). Each MAPK signaling pathway consists of at least three components, a MAP kinase kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. The MAPK pathways are activated by numerous extracellular and intracellular stimuli, including peptide growth factors, cytokines, hormones and various cellular stressors. Studies on the effects of dominant-interfering or constitutively activated forms of various components of the JNK, p38 and ERK signaling pathways have reported that the activation of JNK and p38 and the coincident inhibition of ERK are significant for the induction of apoptosis. The JNK and p38 signaling pathways are activated by pro-inflammatory cytokines, such as TNF-α in response to cellular stresses (15,16).

Over the past decade, the pharmacological activities have been reported for plants of the *Rhodiola* genus, such as anti-hypoxic, antifatigue, antioxidant and anticancer effects, and their effects on anti-apoptotic processes in cells have also been reported (17,18). The *Rhodiola rosea* L. roots contain a variety of compounds that may contribute to its pharmacological effects, including phenols, polyphenols, rosavin, rosin, rosarin, organic acids, terpenoids, phenolcarbonic acids and their derivatives, flavonoids, anthraquinones and alkaloids (19-23). Salidroside [2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside (SAS)] is a glucoside of tyrosol found in the plant, *Rhodiola rosea* L. and it possesses a number of pharmacological properties, including anti-aging, anti-fatigue, antioxidant, anticancer and anti-inflammatory properties (24-26). SAS is a powerful anti-inflammatory agent in asthma (27,28). However, the potential effects of SAS against phorbol-12-myristate-13-acetate (PMA) plus A23187-induced inflammation in HMC-1 cells have not yet been fully investigated. Thus, in this study, to elucidate the molecular mechanisms responsible for the pharmacological and biochemical activities of SAS, we examine the effects of SAS on pro-inflammatory mediators in HMC-1 cells stimulated with PMA plus A23187.

Materials and methods

Reagents and antibodies. Salidroside (chemical structure shown in Fig. 1) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO was adjusted to <0.01% (v/v) in the culture medium. PMA and the calcium ionophore, A23187 (calcymycin; $C_{29}H_{37}N_3O_6$), were purchased from Sigma-Aldrich. The CellTiter 96® AQueous One Solution Cell Proliferation assay (MTS) system was purchased from Promega (Madison, WI, USA). Iscove's modified Dulbecco's medium (IMDM) was obtained from Welgene (Daegu, Korea). Anti-human TNF-α (555212), anti-IL-6 (555220) and anti-IL-8 (555244) antibodies, biotinylated anti-human TNF-α (51-26372E), anti-IL-6 (51-26452E) and IL-8 (51-26542E) antibodies, and recombinant human TNF-α (51-26376E), IL-6 (51-26456E) and anti-IL-8 (51-26546E) antibodies were obtained from BD Pharmingen (San Diego, CA, USA). Anti-phosphorylated (p-)ERK1/2 (sc-7383), anti-p-JNK1/2 (sc-6254), anti-p-p38 (sc-7973), anti-ERK1/2 (sc-93), anti-JNK1/2 (sc-571), anti-p38 (sc-535), anti-β-actin (sc-47778), anti-NF-κB (sc-8008) and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The reverse transcription kit was purchased from Qiagen (Valencia, CA, USA), and nuclear and cytoplasmic extraction reagents were purchased from Thermo Scientific (Waltham, MA, USA).

Cell culture. The human leukemic mast cell line, HMC-1, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) and grown in IMDM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin and 1.2 mM α-thioglycerol at 37˚C under 5% CO_2 in air.

MTS assay. For the analysis of cell viability by MTS assay, we used the manufacturer's procedure for the AG Protocol-CellTiter 96® AQueous One Solution Cell Proliferation assay. Cell aliquots ($5x10^4$) were seeded in microplate wells and treated with SAS (10, 25, 50 and 100 μM) for 30 min. The following day, the cells were incubated with 20 μl of MTS solution for 2 h at 37˚C under 5% CO_2 and 95% air. An automatic microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to read the absorbance of each well at 490 nm.

Enzyme-linked immunosorbent assay (ELISA). The HMC-1 cells were treated with various concentrations of SAS (10, 25 and 50 μM) for 1 h prior to stimulation with PMA (50 nM) plus A23187 (1 μM). An ELISA was used to assay the protein levels of IL-6, IL-8 and TNF-8 in the culture supernatants. To measure the cytokine levels, we used a modified ELISA. First, we conducted a sandwich ELISA for IL-6, IL-8 and TNF-8 in triplicate in 96-well ELISA plates (Nunc, Roskilde, Denmark). The supernatant was then transferred to a new microcentrifuge tube, and the cytokines were quantified using ELISA. ELISA plates (Falcon; Becton-Dickinson Labware, Franklin Lakes, NJ, USA) were coated overnight at 4˚C with anti-human IL-6, anti-IL-8 and anti-TNF-α monoclonal antibodies in coating buffer (0.1 M carbonate, pH 9.5) and then washed 3 imes with phosphate-buffered saline (PBS) containing 0.05% Tween-20. The non-specific protein binding sites were blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. After washing the plates again, the test sample or recombinant IL-6, IL-8 and TNF-α standards were added. Following incubation for 2 h, a working detector (biotinylated anti-human IL-6, anti-IL-8 and anti-TNF-α monoclonal antibodies and streptavidin-horseradish peroxidase reagent) were added followed by incubation for 1 h. The non-specific protein binding sites were blocked. Subsequently, substrate solution [tetramethylbenzidine (TMB)] was added to the wells followed by incubation for 30 min in the dark before the reaction was terminated by the addition of 1 M H_3PO_4. The absorbance was read at 450 nm. All subsequent steps were carried out at room temperature, and all standards and samples were assayed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR). Using an Easy Blue total RNA extraction kit (Intron Biotechnology, Gyeonggi-do, Korea) we isolated total RNA from the HMC-1 cells in accordance with the specifications of the manufacturer. The total RNA was dissolved in DEPC-treated distilled water. A spectrophotometer (NanoDrop Technologies,

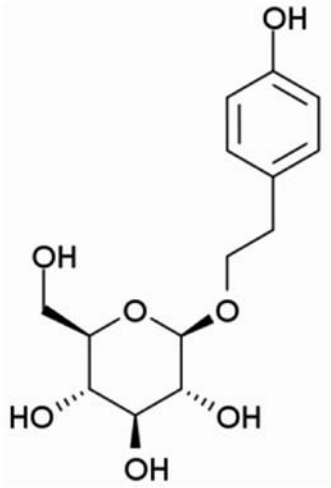

Figure 1. Chemical structure of salidroside (SAS).

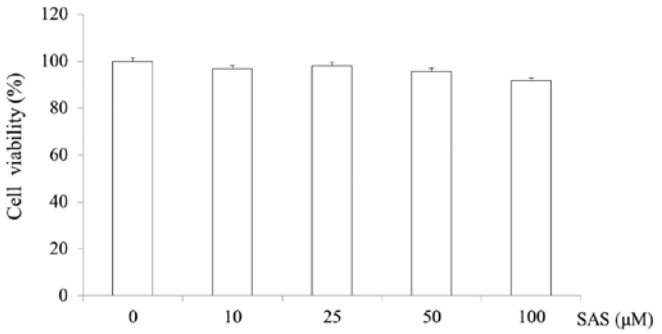

Figure 2. Effect of salidroside (SAS) on the viability of the human mast cell line-1 (HMC-1). Cell viability was evaluated by MTS assay. HMC-1 cells were treated with the indicated concentrations of SAS for 24 h. Data are the means concentration of SAS for 2 measurements from 3 separate experiments.

Wilmington, DE, USA) was used to assess RNA purity by measuring the ratio of the absorbance at 260 and 280 nm; only RNA samples with a value in the 1.6-2.0 range were used. A cDNA synthesis kit (Qiagen, Valencia, CA, USA) was used for 2 min at 42˚C, 30 min at 42˚C, and 30 min at 95˚C to reverse transcribe each sample into cDNA. The primer sequences were as follows: IL-6 forward, 5'-GATGGATGCTTCCA ATCTGGAT-3' and reverse, 5'-AGTTCTCCATAGAGAA CAACATA-3'; IL-8 forward, 5'-TGTGCTCTCCAAAT TTTTTTTACTG-3' and reverse, 5'-CTCTCTTTCCTCTTT AATGTCCAGC-3; TNF-α forward, 5'-CACCAGCTGGTTA TCTCTCAGCTC-3' and reverse, 5'-CGGGACGTGGA GCTGGCCGAGGAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'CCATGTTCGTCAT GGGTGTGAACCA-3' and reverse, 5'-GCCAGTAGAGG CAGGGATGATGTTC-3'. Finally, following electrophoresis on a 2% agarose gel, the expression levels were confirmed using a UV detector (ImageQuant LAS 500; GE Healthcare Life Science, Chicago, IL, USA).

Preparation of cytoplasmic and nuclear extracts. Nuclear extraction reagent (NER) and cytoplasmic extraction reagent (CER) were used to extract the nucleus and cytoplasm. A cell volume of 20 μl corresponded to a volume ratio of CER I:CER II:NER (200:11:100 μl, respectively). A tube containing CER I was first vortexed vigorously on the highest setting for 1 sec to fully suspend the cell pellet. The tube was then incubated on ice for 10 min. Ice-cold CER II was then added to the tube, and the tube was vortexed for 5 sec on the highest setting. The tube was then incubated on ice for 1 min before being vortexed for 5 sec on the highest setting. The tube was then centrifuged at 13,000 rpm for 5 min on a microcentrifuge (Centrifuge 5415F; Eppendorf, Hamburg, Germany). The supernatant (cytoplasmic extract) was then immediately transferred to a clean, pre-chilled tube and stored until needed. Ice-cold NER was added to the pellet, and the tube was vortexed on the highest setting for 15 sec. The sample was placed on ice and vortexed for 15 sec every 10 min, for a total of 40 min. The tube was then centrifuged at 13,000 rpm for 5 min on a microcentrifuge. The supernatant (nuclear extract) was then immediately transferred to a clean, pre-chilled tube.

Western blot analysis. The HMC-1 cells ($5x10^6$ cells/well) were stimulated with PMA (50 nM) plus A23187 (1 μM). The cell lysates were prepared in a sample buffer containing sodium dodecyl sulfate (SDS). The samples were heated at 95˚C for 5 min and briefly cooled on ice. Following centrifugation at 13,000 rpm for 5 min, the proteins in the cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and then incubated with anti-MAPKs, anti-β-actin and anti-NF-κB antibodies overnight. After washing the blot in Tris-buffered saline and Tween-20 (TBST) 3 times, it was incubated with a secondary antibody for 1 h, and then the antibody-specific proteins were visualized using an ECL™ prime western blotting detection reagent in accordance with the recommended procedure (Amersham Corp., Newark, NJ, USA).

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons and the Student's t-test for single comparisons. The data from the experiments are presented as the means ± SEM. The numbers of independent experiments assessed are provided in the figure legends.

Results

Cytotoxicity of SAS in HMC-1 cells. The cytotoxicity of SAS was evaluated by MTS assay. SAS was found to not affect the viability of the HMC-1 cells at concentrations of 10, 25, 50 and 100 μM. The cell viability of the cells treated with 100 μM SAS was 91.6% (Fig. 2).

Effect of SAS on the production of IL-6, IL-8 and TNF-α. To evaluate the effects of SAS on the production of IL-6, IL-8 and TNF-α, we treated the cells with SAS (10, 25 and 50 μM) prior to stimulation with PMA (50 nM) plus A23187 (1 μM) for 8 h and analyzed the levels using ELISA. The levels of IL-6, IL-8, and TNF-α in the HMC-1 cells significantly increased following stimulation with PMA plus A23187 (Figs. 3-5). Pre-treatment of the cells with SAS (10, 25 and 50 μM) significantly inhibited the increase in the levels of these cytokines in a concentration-dependent manner. The maximal inhibition of

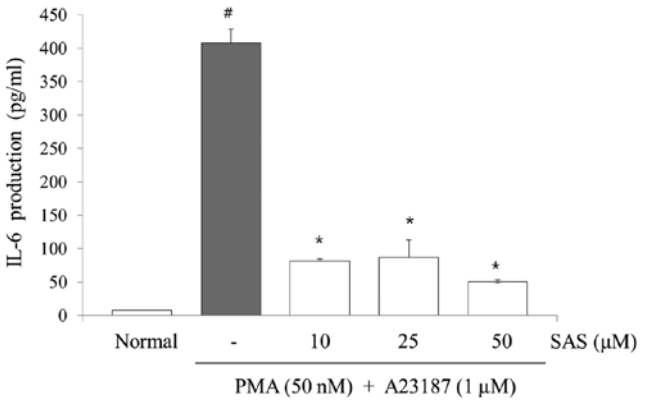

Figure 3. Effect of salidroside (SAS) on interleukin-6 (IL-6) production. HMC-1 cells were treated with SAS (10, 25 and 50 μM) before being stimulated with phorbol-12-myristate-13-acetate (PMA, 50 nM) plus A23187 (1 μM) for 8 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

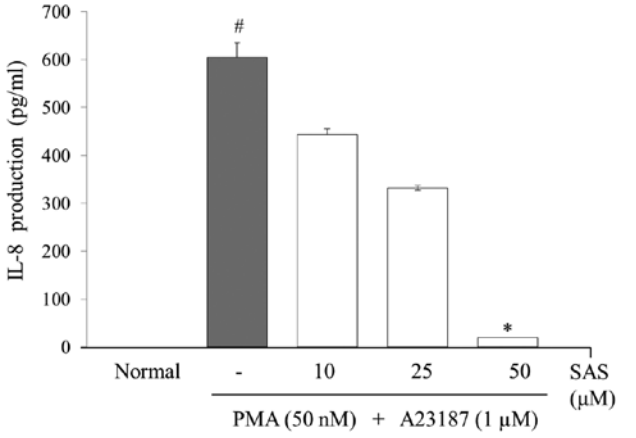

Figure 4. Effect of salidroside (SAS) on interleukin-8 (IL-8) production. HMC-1 cells were treated with SAS (10, 25 and 50 μM) 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA) (50 nM) plus A23187 (1 μM) for 8 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

IL-6, IL-8 and TNF-α production by SAS (50 μM) was 88, 94 and 63%, respectively (Figs. 3-5).

Effect of SAS on IL-6, IL-8 and TNF-α gene expression. The IL-6, IL-8 and TNF-α gene expression levels were analyzed by RT-PCR. The increase in the expression of IL-6, IL-8 and TNF-α induced by PMA plus A23187 was inhibited by pretreatment of the cells with SAS. In particular, SAS (50 μM) significantly inhibited the PMA plus A23187-induced increase in the expression of IL-6 and IL-8 (Fig. 6).

Effect of SAS on the activation of MAPKs. In order to elucidate the mechanisms underlying the effect of SAS, we examined the effect of SAS on MAPK activation. The stimulation of the HMC-1 cells with PMA plus A23187 resulted in an increased phosphorylation of all three types of MAPKs (ERK, JNK and p38) after 1 h. SAS reduced the PMA plus A23187-induced expression of p-ERK1/2 and p-JNK1/2 (Fig. 7). However, SAS had no effect on the levels of p-p38 (Fig. 7).

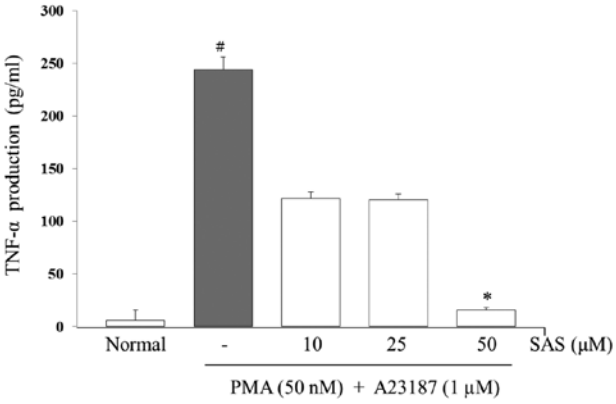

Figure 5. Effect of salidroside (SAS) on tumor necrosis factor (TNF)-α. HMC-1 cells were treated with SAS (10, 25 and 50 μM) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA) (50 nM) plus A23187 (1 μM) for 8 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

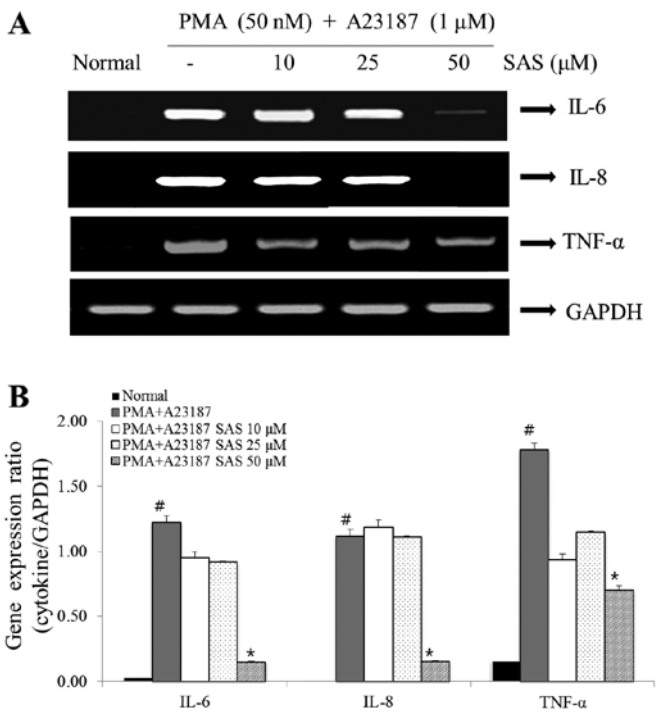

Figure 6. (A and B) Effect of salidroside (SAS) on the mRNA expression of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α as analyzed by RT-PCR. HMC-1 cells were treated with SAS (10, 25 and 50 μM) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA) (50 nM) plus A23187 (1 μM) for 8 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

Effect of SAS on the expression of NF-κB. To evaluate the mechanisms through which SAS affected the gene expression of pro-inflammatory cytokines, we examined the effects of SAS on NF-κB expression. The expression of pro-inflammatory cytokines is regulated by the transcription factor, NF-κB (8). Stimulation of the HMC-1 cells with PMA plus A23187 induced the nuclear translocation of NF-κB p65 after 2 h of incubation (8). SAS inhibited the PMA plus A23187-initiated nuclear translocation of NF-κB p65 (Fig. 8). In order

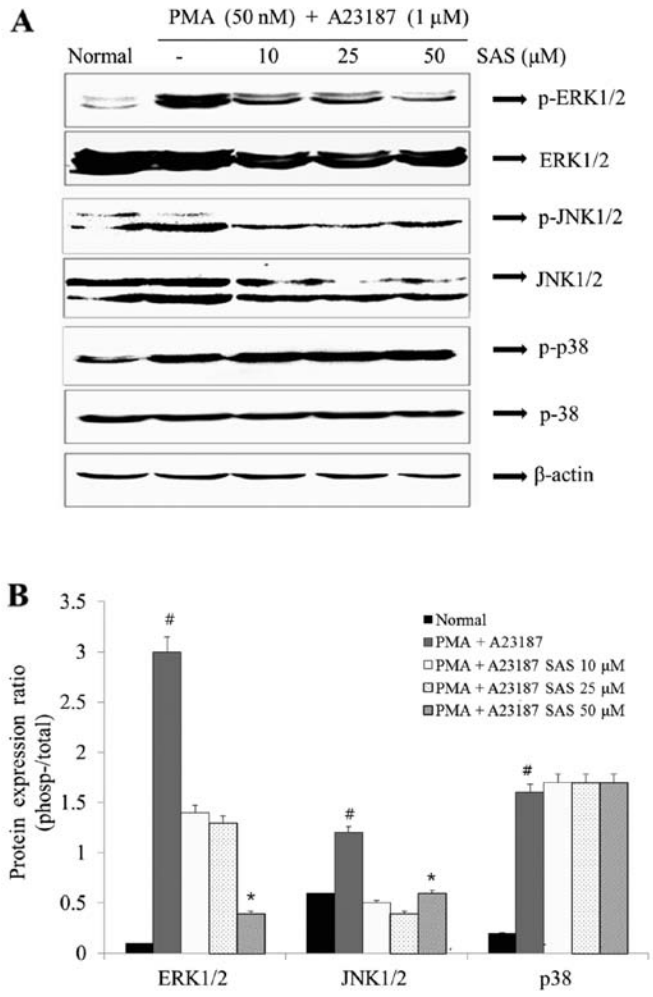

Figure 7. (A and B) Effect of salidroside (SAS) on the activation of mitogen-activated protein kinases (MAPKs) as analyzed by western blot analysis. HMC-1 cells were treated with SAS (10, 25 and 50 μM) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA) (50 nM) plus A23187 (1 μM) for 8 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

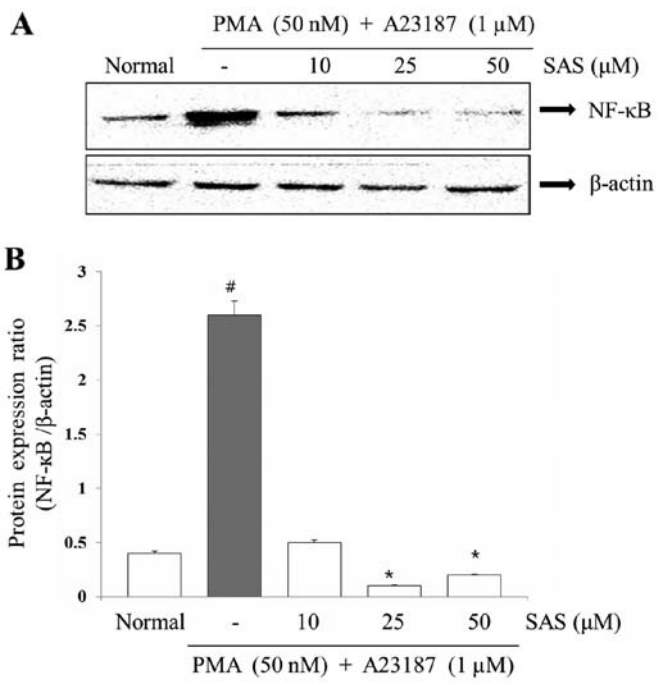

Figure 8. (A and B) Effect of salidroside (SAS) on the expression of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) was analyzed by western blot analysis. HMC-1 cells were treated with SAS (10, 25 and 50 μM) for 1 h prior to being stimulated with phorbol-12-myristate-13-acetate (PMA) (50 nM) plus A23187 (1 μM) for 2 h. Values are expressed as the means ± SE (n=3 experiments). #P<0.05. vs. normal (untreated cells); *P<0.05. vs. PMA (50 nM) plus A23187 (1 μM)-stimulated cells.

to confirm the inhibitory effect of SAS on NF-κB expression, we examined the effect of SAS on NF-κB-dependent protein expression (Fig. 8).

Discussion

Previous studies on plant-derived anti-inflammatory compounds have investigated the potential inhibitory effects of natural products using *in vivo* and *in vitro* methods. These studies suggest an important role for SAS as a potential chemo-prevention agent due to its anti-inflammatory effects, and anticancer effects (24-26). The aim of the present study was to examine the effects of SAS on the production of TNF-α, IL-6 and IL-8 in PMA plus A23187-stimulated HMC-1 cells, since these cytokines have potent inflammatory effects.

Mast cells contain potent mediators, including histamine, heparin, proteinases, leukotrienes and pro-inflammatory cytokines; all of which potentially contribute to inflammatory processes (29). Pro-inflammatory cytokines, particularly TNF-α, IL-6 and IL-8, play critical biological roles in allergic inflammation. These cytokines are released as stored cytokines and can be newly synthesized during mast cell activation (30). TNF-α promotes inflammation, granuloma formation and tissue fibrosis and is thought to be an initiator of cytokine-related inflammatory responses by promoting cytokine production (31). Previous studies have indicated that reduced amounts of TNF-α and IL-6 released from mast cells is key to reducing the symptoms of allergic inflammation (32,33). IL-8 released from mast cells acts on surrounding cells, such as neutrophils and eosinophils, and induces the migration and activation of inflammatory effector cells (34). In this study, we found that SAS reduced the production of TNF-α, IL-6 and IL-8 in PMA plus A23187-stimulated HMC-1 cells. Increases in levels of intracellular calcium induce the release of biological mediators, including TNF-α, IL-8 and IL-6. It has also been reported that the release of intracellular calcium from internal stores is required for MAPK activation (35,36). MAPKs have been reported to be involved in important pathways associated with the differentiation, activation, proliferation, degranulation and migration of various immune cells, airway smooth muscle and epithelial cells (37). We also investigated whether pre-treatment SAS would interfere with the MAPK signaling pathways. The results from western blot analysis indicated that the expression of p-ERK, p-JNK, and p-p38 considerably increased in the PMA plus A23187-stimulated mast cells, as compared to the untreated mast cells. However, the expression of p-ERK and p-JNK proteins significantly decreased following treatment with SAS, but no decreases were observed in p-p38 expression; therefore, it is thought that SAS is not

involved in the p38 pathway. The expression of inflammatory cytokines requires the phosphorylation of MAPKs. In this study, no significant changes were found in the expression of total ERK, JNK and p38 between the different groups. The ERK pathway is predominantly activated by mitogenic and proliferative stimuli, whereas the JNK and p38 MAPK pathways respond to environmental stresses (38). While the exact nature of the involvement of the ERK1/2 pathway remains elusive, nuclear retention, dimerization, phosphorylation and release from cytoplasmic anchors have been shown to play a role (39). The JNKs are greatly activated in response to cytokines, growth factor deprivation, DNA-damaging agents, some G protein-coupled receptors, serum and growth factors. In mammalian cells, the p38 pathway is strongly activated by environmental stresses and inflammatory cytokines, but not appreciably by mitogenic stimuli. Additionally, p38 participates in macrophage and neutrophil functional responses, including respiratory burst activity, chemotaxis, granular exocytosis, apoptosis and also mediates T-cell differentiation and apoptosis by regulating γ-interferon production and regulates the immune response by stabilizing specific cellular mRNAs (40).

NF-κB is a transcription factor that induces the transcription of a variety of genes. Many of these genes encode molecules important in inflammatory processes, such as cytokines and adhesion molecules. The role of NF-κB, in particular its regulation of cytokine production, in allergic inflammation has previously been characterized (41). NF-κB regulates the expression of multiple inflammatory and immune genes and plays a critical role in chronic inflammatory diseases (42). To evaluate the mechanisms of the inhibition of SAS on the expression of pro-inflammatory cytokines, we examined the effect of SAS on the NF-κB pathway. In this study, SAS decreased the nuclear translocation of NF-κB p65. These results indicate that the inhibitory effects of SAS on inflammatory cytokines were due to the regulation of the NF-κB pathway.

In conclusion, our study demonstrates pre-treatment with salidroside significantly inhibits the increase in the levels of TNF-α, IL-6 and IL-8 induced by PMA plus A23187 in mast cells, suppresses NF-κB p65, ERK1/2 and JNK1/2 expression, and inhibits the upregulation of pro-inflammatory cytokines. Therefore, salidroside may prove to be an effective therapeutic agent for the treatment of inflammation resulting from mast cell-mediated inflammatory responses.

Acknowledgements

This research was supported by Wonkwang University in 2015.

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