Saikosaponin a, an active compound of Radix Bupleuri, attenuates inflammation in hypertrophied 3T3-L1 adipocytes via ERK/NF-κB signaling pathways

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Abstract. *Bupleurum falcatum* L. is employed in oriental medicine in Korea. This root has been used for anti-inflammatory, anti-pyretic, and anti-hepatotoxic effects in the treatments of common cold, fever, and hepatitis. One of major bioactive compounds of Radix Bupleuri is the saikosaponin a (SSNa). However, little is known concerning the effects of SSNa on obesity associated with a state of low-grade inflammation. Consequently, this study was conducted to determine the inhibition of the inflammation pathway of SSNa in obesity. MTT assay was conducted for cytotoxicity and viability; nuclear and cytoplasmic fractions were extracted from adipocytes for translocation of nuclear factor-κB cells (NF-κB); nitric oxide (NO) production and secretion using Griess reagent; reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting for mRNA and protein levels associated with inflammation in the hypertrophied adipocytes. The results revealed that SSNa significantly decreased the expression of tumor necrosis factor-α (TNFα), interleukin (IL)-1β and IL-6 as proinflammatory cytokines, compared to that of non-treated control cells. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) as inflammatory factors were reduced by treatment of these cells with SSNa and also suppressed NO production. Phosphorylation of IκBα was inhibited and translocation of NF-κB was suppressed via the ERK pathway in response to SSNa treatment. In conclusion, the results demonstrated that SSNa can inhibit the expression of inflammatory-associatied genes in hypertrophied 3T3-L1 adipocytes and is a potent inhibitor of NF-κB activation. Thus these results suggest that SSNa is a novel therapeutic agent against that can be used against obesity-associated inflammation.

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Key words: saikosaponin a, inflammation, hypertrophy, adipocytes, MAPK pathway, NF-κB

Introduction

Koreans are 1.5-fold more likely to be obese as compared to 10 years ago, according to the Ministry of Health and Welfare (1). A >4-fold increase in health-care expenditure for the treatment of obesity-associated diseases such as diabetes, heart disease, bone disorders and cancer, has been observed. Therefore, obesity is now considered a social concern (http://www.oecd.org/health/prevention. 2012).

Previous findings have conclusively demonstrated that inflammation plays a major role in obesity. Obesity is closely associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and activation of the inflammatory signaling pathway in adipose tissues (2,3). Obese inviduals commonly have many elevated markers of inflammation, including major proinflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6; nitric oxide (NO) production, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) as an immune-associated cytotoxic factor; and transcription factor of nuclear factor-κB cells (NF-κB) through activation of the mitogen-activated protein kinase (MAPK) pathway including ERK, JNK and p38 signaling pathways in obese adipocytes (4-7). Inflammation is a response of a tissue to injury associated with biological, chemical or physical stimuli. It is a defense mechanism aimed to remove the injurious stimuli and initiates the tissue healing process (8). However, an excessive inflammatory response itself constitutes a dilemma. It is also an important pathogenic mediator for the development of obesity. Thus, using bioactive compounds to regulate the expression of inflammatory-associated cytokines is significant in the prevention and treatment of obesity.

Bioactive compound extracts from natural products are known to possess some therapeutic potential for inflammation (9-12). Saikosaponin a (SSNa) is one of the major triterpenoid saponins derived from *Bupleurum falcatum* L. (13), and has various pharmacological activities such as anti-inflammatory, immunoregulating, antibacterial, antiviral, and anticancer activities (9,14-18). However, the anti-inflammatory effects of

SSNa, especially the underlying mechanisms in hypertrophied 3T3-L1 adipocytes have not been studied. In the present study, we assessed SSNa control proinflammatory-associated cytokines (TNFα, IL-1β, and IL-6) and immune-related cytotoxic factors (NO, iNOS, and COX-2) through the NF-κB signaling pathway in 3T3-L1 hypertrophied adipocytes. In addition, the possible mechanisms involved in the inhibitory responses were examined.

Materials and methods

Cell and reagents. Mouse embryo fibroblast 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin/streptomycin (P/S), 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO), rosiglitazone, Oil Red O, and sulforaphane, used as the positive control compound, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). SSNa was purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan).

Cell culture. The mouse embryo fibroblast 3T3-L1 cells were obtained from ATCC, and 3T3-L1 preadipocytes were differentiated into mature adipocytes. Briefly, 3T3-L1 preadipocytes were cultured at 37˚C in a humidified 5% CO_2 atmosphere and grown in DMEM supplemented with 10% (v/v) BCS, 1% (v/v) of P/S until confluency was reached. For the induction of differentiation into mature adipocytes, the cells were grown to 4 days post-confluence (0 day). Subsequently, the completely confluent preadipocytes were induced with adipogenic hormone mixture, DMEM medium containing 10% (v/v) FBS, 0.5 mM of IBMX, 2 μM of DEX, and 2 μg/ml of insulin for 2 days. The cells were then maintained in DMEM containing 10% FBS and 2 μg/ml of insulin for 2 days, followed by culturing for an additional 2 weeks, at which time cells reached full differentiation as hypertrophied adipocytes.

Cell viability. Cells ($5x10^4$ cells/ml) were cultured in a 96-well plate. After the supernatants were removed, the cells were treated with 200 μl of various concentrations of SSNa for 24 h, and then MTT (5 mg/ml in serum-free medium, 10 μl/well) was added. The medium was discarded after 2 h and the formazan crystals were solubilized in 50 μl of DMSO. The optical density was measured at 570 nm with microplate reader (Tecan Austria GmbH, Grödig, Austria).

NO assay. Hypertrophied 3T3-L1 cells were treated with 100 nM SSNa and 50 nM sulforaphane for 1 h, respectively. The cell supernatants were collected and assayed for NO production using Griess reagent (Molecular Probes, Eugene, OR, USA). Briefly, the samples were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was measured at 570 nm on a microplate reader. Sodium nitrite (0-100 μM) was used as a standard to assess nitrite concentrations.

Reverse transcription-polymerase chain reaction (RT-PCR). Total-RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and was then used for RT-PCR with one step RT-PCR premix (Intron Biotechnology Co., Seongnam, Korea) according to the manufacturer's instructions. PCR was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) with the primers indicated in Table I. Conditions for PCR were 1x (94˚C for 3 min); 35x (94˚C for 45 sec; 58˚C for 45 sec; and 72˚C for 1 min), and 1x (72˚C for 10 min). As a control of sample loading and normalization between samples, PCR amplification of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was included for each sample at each run. *GAPDH* was used as an internal control. Amplification products obtained by PCR were electrophoretically separated on 1.5% agarose gel and visualized by ethidium bromide (Sigma-Aldrich Co.) staining.

Western blot analysis. After the differentiated 3T3-L1 cells were harvested, total protein lysates were prepared and ice-cold lysis buffer was added. The supernatant was obtained following centrifugation at 9,000 x g for 15 min at 4˚C. Cytoplasmic proteins were separated from the nuclear fraction using a NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL, USA). β-actin was used as an internal control for the cytosolic fraction and lamin A (Table II) was applied as an internal control for the nuclear fraction. The protein concentrations were determined by the Bradford assay (Bio-Rad protein assay; Hercules, CA, USA). The proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF; Millipore, Billerica, MA, USA) membranes. Non-specific binding was blocked by 1-h incubation of the membranes in 5% (w/v) non-fat dry milk (or 5% BSA) in Tris-buffered saline (pH 7.5). The blots were then incubated overnight with primary antibodies (Table II) in the antibody buffer containing 1% (w/v) non-fat dry milk in 0.05% (v/v) Tween-20 in Tris-buffered saline (TBST), washed three times with TBST, and incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. The blots were developed for visualization using an enhanced chemiluminescence detection kit (Pierce).

Statistical analysis. Data were presented as ± standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was set at $p \leq 0.05$. Band intensities in the immunoblots were quantified by densitometry using L Process (Version 2.01; Fujifilm, Stamford, CT, USA) and Multi Gauge software (Version 2.02; Fujifilm). Band intensities were normalized relative to the internal control and background. Multiple experiments were combined and all the experiments were repeated a minimum of three times.

Results

SSNa does not affect cytotoxicity of 3T3-L1 cells. The metabolic-dye-based MTT assay was performed to determine the

Table I. Oligonucleotide sequence used in PCR.

Name	Sequence of primers (5→3)	Annealing temp (˚C)
TNFα	F: ACGGCATGGATCTCAAAGAC R: GTGGGTGAGGAGCACGTAGT	58
IL-6	F: AGTTGCCTTCTTGGGACTGA R: CAGAATTGCCATTGCACAAC	58
iNOS	F: CTC CTT CAA AGA GGC AAA AAT A R: CAC TTC CTC CAG GAT GTT GT	54
NF-κB	F: GCACGAGGCTCCTTTTCTCAA R: CGTTTTTCTTCAATCCGGTGG	53
IL-1β	F: GCT GAC AGA CCC CAA AAG ATT R: TGTGCAGACTCA AACTCC ACTT	55
COX-2	F: CATTCTTTGCCCAGCACTTC R: CCTGAGTGTCTTTGACTGTG	54
GAPDH	F: TGCCTBCTTCACCACCTTC R: TGCCTCCTGCACCACCAACT	53

Table II. List of antibodies used in western blot analysis.

Primary antibodies	Antibody information	Dilution factor
TNFα	Santa Cruz Biotechnology, Inc.	1:500
IL-6	Santa Cruz Biotechnology, Inc.	1:500
IL-1β	Millipore	1:500
iNOS	Santa Cruz Biotechnology, Inc.	1:1,000
COX-2	Santa Cruz Biotechnology, Inc.	1:1,000
IκBα	Santa Cruz Biotechnology, Inc.	1:1,000
pIκBα	Santa Cruz Biotechnology, Inc.	1:1,000
NF-κB p65	Santa Cruz Biotechnology, Inc.	1:1,000
NF-κB p50	Santa Cruz Biotechnology, Inc.	1:1,000
β-actin	Santa Cruz Biotechnology, Inc.	1:1,000
Lamin A	Santa Cruz Biotechnology, Inc.	1:1,000
ERK	Cell Signaling Technology, Inc.	1:1,000
pERK	Cell Signaling Technology, Inc.	1:1,000

cytotoxicity of SSNa on 3T3-L1 cells with 0-1 μM SSNa. No cytotoxicity on 3T3-L1 cells was observed at the concentration of 0-1 μM SSNa (Fig. 1). Therefore, 100 nM SSNa was used for treatments in this experiment. This concentration did not inhibit cell growth and cytotoxicity compared to that of the control.

SSNa suppresses NO production. To determine the effect of SSNa on NO production and iNOS expression, 3T3-L1 hypertrophied adipocytes were treated with SSNa (100 nM) for 1 h. The culture medium from the cells was collected and assayed for NO generation using the Griess reagent assay. The production of NO in hypertrophied cells increased in the medium by ~5-fold (26.3±2.8 μM) compared to that of preadipocytes (5.3±2.1 μM) (Fig. 2A). However, treatment with SSNa markedly attenuated NO generation, such that the concentration was only 15.2±1.3 μM. Thus, we assessed whether SSNa of NO production was associated with iNOS mRNA and protein levels. As expected, treatment of SSNa significantly decreased excessive iNOS mRNA (Fig. 2B) and protein expressions (Fig. 2C) compared with those of the control cells. These results showed that excessive lipogenesis significantly induces NO generation and iNOS expression. However, SSNa suppressed NO production by inhibiting iNOS expression in hypertrophied adipocytes.

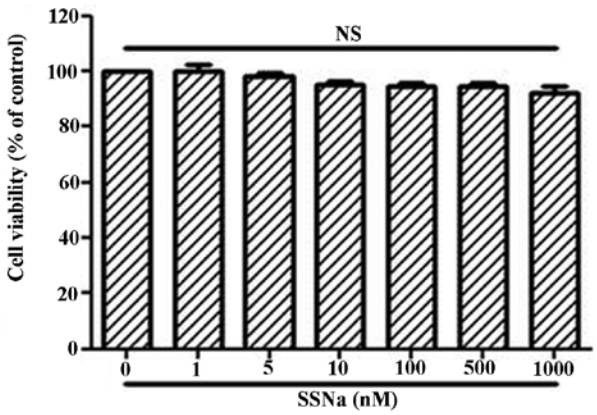

Figure 1. Effect of saikosaponin a (SSNa) on cell viability in 3T3-L1 cells. Cells were seeded in a 96-well plate and grown for one day. The cell viability was measured by the metabolic-dye-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are expressed as a percentage of the vehicle-treated control ± standard error of the mean (SEM) of three separate experiments. Statistical significance (Student's t-test) analyses were performed using GraphPad Prism 5. NS, no significance.

Downregulation of levels of proinflammatory mediator genes. To determine whether SSNa inhibits the production of TNFα, IL-6, IL-1β, COX-2 and NF-κB, RT-PCR and immunoblot analysis were performed on 3T3-L1 hypertrophied adipocytes. As shown in Fig. 3, mRNA and protein levels of TNFα, IL-6 and IL-1β as proinflammatory cytokines were significantly reduced by SSNa in the cells ($p<0.05$) compared to those of the control. Tho mRNA and protein levels of inflammatory factor COX-2 were also reduced by the treatment of SSNa compared to those of the control. SSNa also strongly prevented the expression of NF-κB, a central role in general inflammatory 3T3-L1 hypertriphied adipocytes compared to that of control ($p<0.05$).

Suppression of NF-κB activation. We investigated whether SSNa prevents the expression of NF-κB (p65 and p50) in hypertrophied adipocytes. As shown in Fig. 4A, NF-κB (p65 and p50) was highly expressed in non-treated 3T3-L1 cells whereas SSNa-treated cells were significantly decreased ($p<0.05$). Following treatment with SSNa, phosphorylation of IκB and ERK was significantly decreased compared to that of the controls. To confirm the effects of SSNa on the prevention of NF-κB translocation from the cytosol to the cell nucleus in fully mature 3T3-L1 adipocytes, the expression of NF-κB in the cytosol and nucleus were analyzed using immunoblots. As shown in Fig. 4B, translocation of NF-κB (p65 and p50) was decreased in 3T3-L1 cell nucleus treated with 100 nM SSNa compared to that of the controls. Consequently, SSNa inhibited the translocation of NF-κB from the cytosol to the nucleus by inhibiting the degradation of IκBα in cytoplasm.

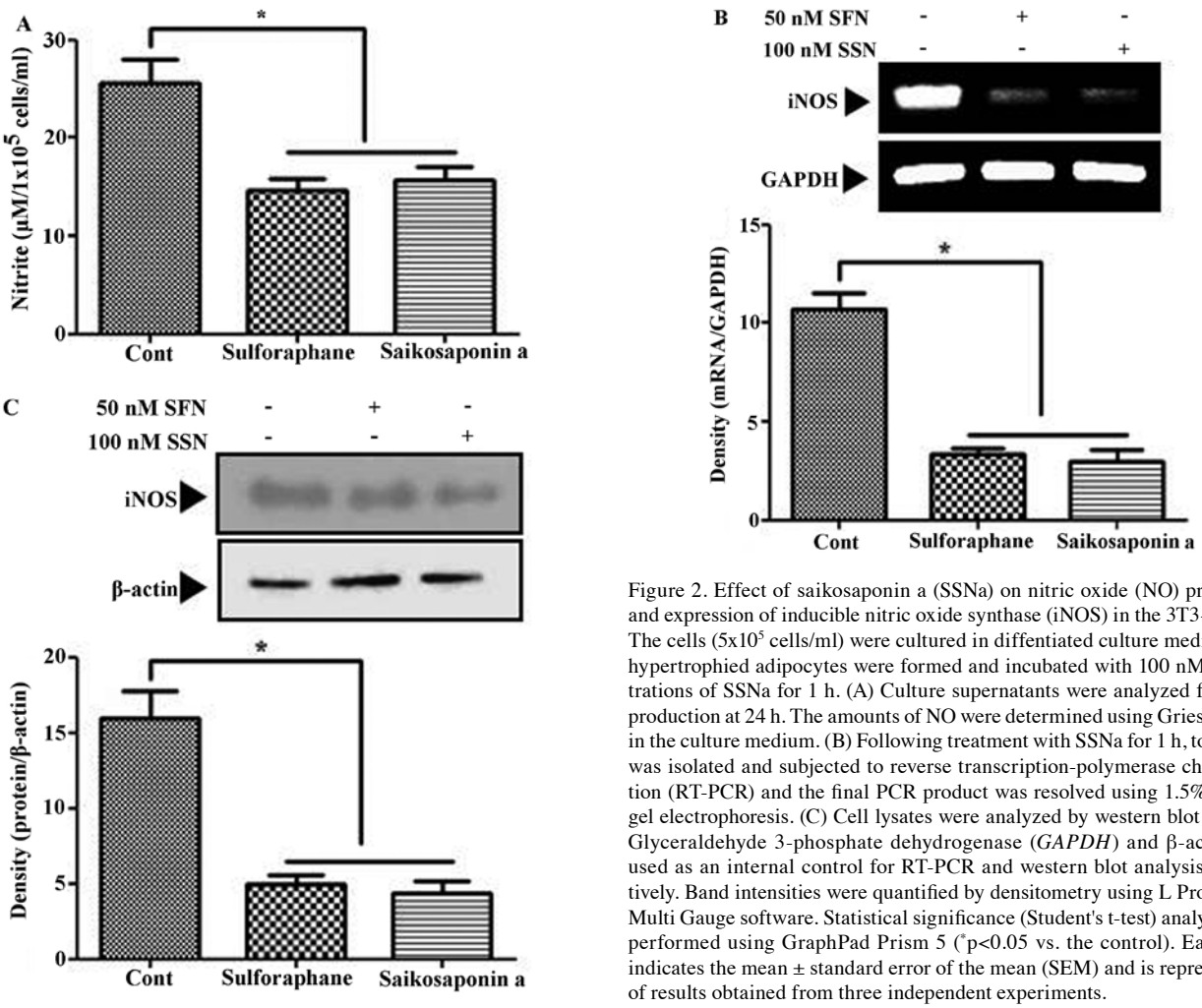

Figure 2. Effect of saikosaponin a (SSNa) on nitric oxide (NO) production and expression of inducible nitric oxide synthase (iNOS) in the 3T3-L1 cells. The cells ($5x10^5$ cells/ml) were cultured in diffentiated culture medium until hypertrophied adipocytes were formed and incubated with 100 nM concentrations of SSNa for 1 h. (A) Culture supernatants were analyzed for nitrite production at 24 h. The amounts of NO were determined using Griess reagent in the culture medium. (B) Following treatment with SSNa for 1 h, total RNA was isolated and subjected to reverse transcription-polymerase chain reaction (RT-PCR) and the final PCR product was resolved using 1.5% agarose gel electrophoresis. (C) Cell lysates were analyzed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin were used as an internal control for RT-PCR and western blot analysis, respectively. Band intensities were quantified by densitometry using L Process and Multi Gauge software. Statistical significance (Student's t-test) analyses were performed using GraphPad Prism 5 ($^*p<0.05$ vs. the control). Each value indicates the mean ± standard error of the mean (SEM) and is representative of results obtained from three independent experiments.

Inhibition of ERK axis. To confirm SSNa-suppressed translocation of NF-κB through phosphorylation of ERK as a TNFα downstream, U0126 as ERK inhibitor was pretreated in 3T3-L1 adipocytes for 1 h. Phosphorylation of ERK was inhibited compared to that of the controla and that of NF-κB was also significantly inhibited in 3T3-L1 cell nucleus pretreated with U0126 compound (Fig. 5). Therefore, SSNa controlled the suppression of phosphorylation of ERK, suggesting that SSNa-suppressed inflammation is associated with NF-κB via the ERK signaling pathway in 3T3-L1 adipocytes.

Discussion

Obesity is a chronic disease and its prevalence is on the increase worldwide (http://www.oecd.org/health/prevention. 2012). Obesity is an important risk factor for various diseases such as cardiovascular diseases, type 2 diabetes by hyperglycemia and insulin resistance, dyslipidemia and hypertension (19-21). It is an abnormal condition accumulating lipid in adipose tissues, which produce and secrete adipokines (TNFα, IL-1β, IL-6, leptin, resistin and adiponectin) closely involved with inflammation (22,23). Thus, prevention of obesity and adopting a healthy lifestyle is crucial. Consequently, studies on pharmaceutical natural compounds associated with the prevention of obesity are on the increase. In this study, we demonstrated that SSNa has inhibitory effects on the induction and secretion of adipokines to modulate proinflammatory cytokines and inflammatory factors in 3T3-L1 hypertrophied cells that accumulated complete large lipid droplets.

Inflammatory mediator NO generated by iNOS in adipocyte-cultured media treated with 100 nM SSNa was significantly decreased compared to that of the controla and the mRNA and protein level of iNOS was decreased. This result shows that SSNa inhibits NO production through suppression of iNOS expression in 3T3-L1 hypertrophied cells. COX-2, an inflammatory biomarker, is activated to produce prostaglandins as potent lipid mediators, such as PGE2 and is involved in the regulation of neurodegeneration, inflammation and cancer (24,25). Accordingly, decreases in the level of COX-2 have been considered as a therapeutic effect on inflammation (26,27). Results of the COX-2 mRNA and protein revealed that treatment with SSNa resulted in a significantly inhibited expression of COX-2 protein. Consequently, SSNa is useful in exerting an inflammatory therapeutic effect in obesity-induced inflammatory diseases. Lu *et al* reported that SSa and SSd significantly inhibited the expression of iNOS and COX-2 in LPS-induced RAW264.7 cells (9). Results of that study support our findings showing that, SSNa plays inhibitory roles in the inflammation effect of 3T3-L1 hypertrophied adipocyte cells.

Fat tissue is not a simple energy storage organ but rather exerts important endocrine and immune functions (2). These functions are achieved primarily through the release of adipo-

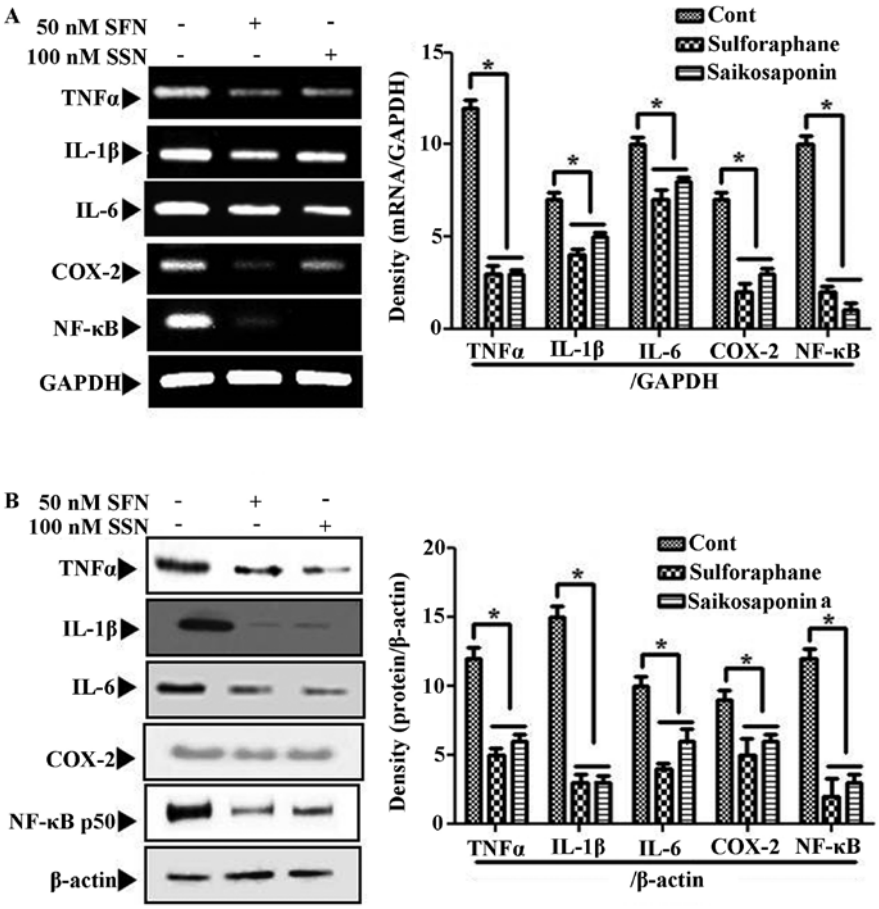

Figure 3. Effect of saikosaponin a (SSNa) on expression of tumor necrosis factor-α (TNFα), IL-6, IL-1β, and cyclooxygenase-2 (COX-2) in the 3T3-L1 hypertrophied adipocytes. (A) Following treatment with SSNa for 1 h, total RNA was isolated and subjected to reverse transcription-polymerase chain reaction (RT-PCR) and the final PCR product was resolved using 1.5% agarose gel electrophoresis. (B) Cell lysates were analyzed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin were used as an internal control for RT-PCR and western blot analysis, respectively. Band intensities were quantified by densitometry using L Process and Multi Gauge software. Statistical significance (Student's t-test) analyses were performed using GraphPad Prism 5 ($^{*}p<0.05$ vs. the untreated control). Each value indicates the mean ± standard error of the mean (SEM) and is representative of results obtained from three independent experiments.

cytokines by white adipose tissues (WAT), which include leptin, resistin, adiponectin and inflammatory cytokines as TNFα, IL-6, monocyte chemotactic protein (MCP)-1, and IL-1. These cytokines and chemokines are critically involved in insulin resistance and chronic inflammation (19,20). Evidence suggesta that TNFα is a major mediator of inflammation in general and of obesity and insulin resistance in particular. TNFα and IL-6 also increase lipolysis and are involved in hypertriglyceridemia and increased serum FFA levels associated with obesity (28,29). Abe *et al* reported that saikosaponin (10 mg/kg x 4 days) decreased the increase in triglyceride and body weights in the rat (30). Those reports support that obesity-accumulated excessive body fat can be prevented by inhibition of inflammation treated with SSNa. Wang *et al* showed that curcumin suppressed the transcription and secretion of TNFα and IL-6 induced by palmitate through inhibition of the activation of NF-κB in 3T3-L1 adipocytes (31). Gonzales and Orlando examined the effect of curcumin on NF-κB and on the expression of NF-κB-regulated gene products for TNFα, IL-6, and COX-2 in adipocytes (32). Treatment of TNFα activated NF-κB signaling, IL-1β, IL-6 and COX-2 in differentiated adipocytes. We demonstrated that SSNa significantly decreased pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 in 3T3-L1 hypertrophied cells. These data suggest that SSNa has potent anti-inflammatory activity in 3T3-L1 hypertrophied adipocytes.

NF-κB, a family of transcription factors, regulates the expression of a number of immune-related cytotoxic factors, including iNOS and COX-2, and pro-inflammatory cytokines, including TNFα, IL-1β and IL-6. The MAPK family also induces the production of immune-related cytotoxic factors and pro-inflammatory cytokines (31-33). NF-κB and MAPKs are well-recognized as targets of anti-inflammatory agents (33,34). In this study, to elucidate the molecular mechanism of the inhibitory effect of SSNa on inflammatory mediators, we investigated the effects of SSNa on the activation of two signaling pathways, NF-κB and MAPK, in 3T3-L1 hypertrophied adipocytes. These results show that SSNa inhibits the NF-κB signaling pathway as a core regulator of inflammation through suppression of the phosphorylation of ERK (the downstream of TNFα) and IκBα, preventing NF-κB translocation to the nucleus in 3T3-L1 hypertrophied adipocytes. However, activation of the JNK and p38 pathways did not have a greater effect than that of ERK (data not shown). These pathways were verified with U0126 (a specific inhibitor of ERK), i.e., a decrease of NF-κB translocation by treatment of SSNa. Thus, we consider

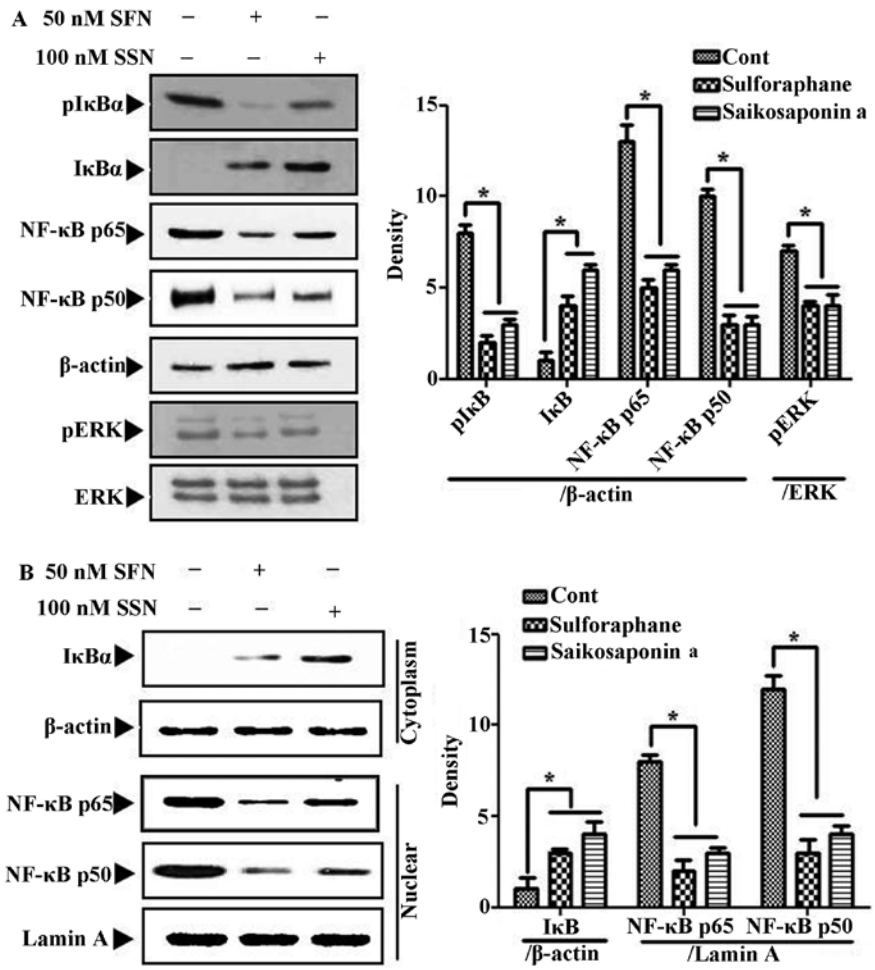

Figure 4. Effect of saikosaponin a (SSNa) on nuclear factor-κB cells (NF-κB) activation in 3T3-L1 hypretriphied adipocytes. (A) Following treatment with SSNa for 1 h, total RNA was isolated and subjected to reverse transcription-polymerase chain reaction (RT-PCR) and the final PCR product was resolved using 1.5% agarose gel electrophoresis. Cell lysates were analyzed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin were used as an internal control for RT-PCR and western blot analysis, respectively. (B) Nuclear and cytosolic fractions were extracted by commercial extraction kit. Each extract was prepared to determine the levels of p65, p50 and IκBα by western blot analysis. β-actin was used as an internal control for the cytosolic fraction and lamin A was applied as an internal control for the nuclear fraction. Band intensities in the immunoblots were quantified by densitometry using L Process and Multi Gauge software. Band intensities were normalized relative to the internal control (β-actin and lamin A) and background, respectively. Statistical significance (Student's t-test) analyses were performed using GraphPad Prism 5 (*$p<0.05$ vs. the untreated control). Each value indicates the mean ± standard error of the mean (SEM) and is representative of results obtained from three independent experiments.

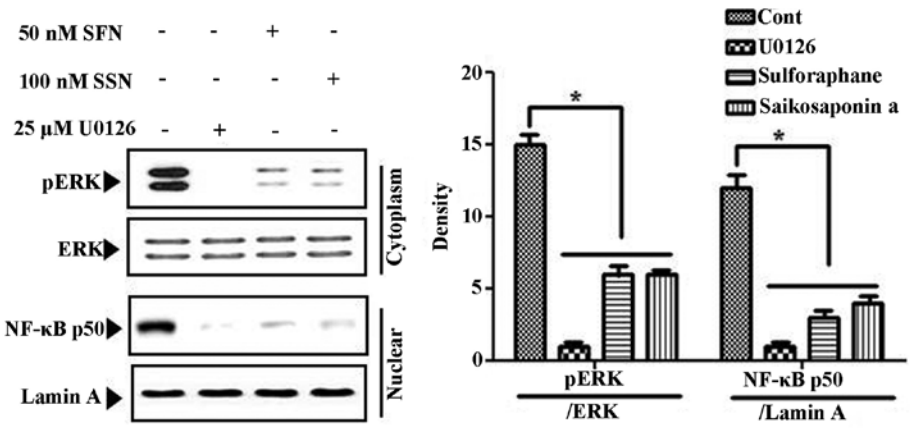

Figure 5. Inhibition of ERK axis with U0126 as ERK inhibitor-suppressed translocation of NF-κB into nuclear in 3T3-L1 hypertrophied adipocytes. Cells were pretreated for 30 min with U0126 and then treated with saikosaponin a (SSNa). Nuclear and cytosolic fractions were extracted by commercial extraction kit. Each extract was prepared to determine the levels of phospholyated ERK and NF-κB p50 by western blot analysis, respectively. Total ERK was used as an internal control for the cytosolic fraction and lamin A was applied as an internal control for the nuclear fraction. Band intensities in the immunoblots were quantified by densitometry using L Process and Multi Gauge software. Band intensities were normalized relative to the internal controls (total ERK and lamin A) and background, respectively. Statistical significance (Student's t-test) analyses were performed using GraphPad Prism 5 (*$p<0.05$ vs. the untreated control). Each value indicates the mean ± standard error of the mean (SEM) and is representative of results obtained from three independent experiments.

that the anti-inflammatory effect of SSNa is associated with the inhibition of NF-κB translocation to the nucleus via an inhibitory effect on ERK signaling pathway.

In conclusion, these results show that SSNa can regulate the expression of proinflammatory and inflamatory-associated genes in 3T3-L1 hypertrophied adipocytes and is a potent inhibitor of NF-κB activation. Inhibition of the ERK pathway by SSNa prevents the induction of inflammation in hypertrophied adipocytes. Thus these results suggeste that SSNa serves as a novel therapeutic agent against obesity-associated inflammation. The anti-inflammatory effects of SSNa appear to be controlled through inhibition of the NF-κB/IκB signaling cascade. However, future *in vivo* studies are required to precisely clarify the mechanisms of action of SSNa and its role in the relationship between activation of NF-κB and production of inflammatory cytokines in obese individuals.

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