Research article

Processed *Panax ginseng*, sun ginseng, inhibits the differentiation and proliferation of 3T3-L1 preadipocytes and fat accumulation in *Caenorhabditis elegans*

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ABSTRACT

Background: Heat-processed ginseng, sun ginseng (SG), has been reported to have improved therapeutic properties compared with raw forms, such as increased antidiabetic, anti-inflammatory, and anti-hyperglycemic effects. The aim of this study was to investigate the antiobesity effects of SG through the suppression of cell differentiation and proliferation of mouse 3T3-L1 preadipocyte cells and the lipid accumulation in *Caenorhabditis elegans*.
Methods: To investigate the effect of SG on adipocyte differentiation, levels of stained intracellular lipid droplets were quantified by measuring the oil red O signal in the lipid extracts of cells on differentiation Day 7. To study the effect of SG on fat accumulation in *C. elegans*, L4 stage worms were cultured on an *Escherichia coli* OP50 diet supplemented with 10 μg/mL of SG, followed by Nile red staining. To determine the effect of SG on gene expression of lipid and glucose metabolism-regulation molecules, messenger RNA (mRNA) levels of genes were analyzed by real-time reverse transcription-polymerase chain reaction analysis. In addition, the phosphorylation of Akt was examined by Western blotting.
Results: SG suppressed the differentiation of 3T3-L1 cells stimulated by a mixture of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI), and inhibited the proliferation of adipocytes during differentiation. Treatment of *C. elegans* with SG showed reductions in lipid accumulation by Nile red staining, thus directly demonstrating an antiobesity effect for SG. Furthermore, SG treatment down-regulated mRNA and protein expression levels of peroxisome proliferator-activated receptor subtype γ (PPARγ) and CCAAT/enhancer-binding protein-alpha (C/EBPα) and decreased the mRNA level of sterol regulatory element-binding protein 1c in MDI-treated adipocytes in a dose-dependent manner. In differentiated 3T3-L1 cells, mRNA expression levels of lipid metabolism-regulating factors, such as amplifying mouse fatty acid-binding protein 2, leptin, lipoprotein lipase, fatty acid transporter protein 1, fatty acid synthase, and 3-hydroxy-3-methylglutaryl coenzyme A reductase, were increased, whereas that of the lipolytic enzyme carnitine palmitoyltransferase-1 was decreased. Our data demonstrate that SG inversely regulated the expression of these genes in differentiated adipocytes. SG induced increases in the mRNA expression of glycolytic enzymes such as glucokinase and pyruvate kinase, and a decrease in the mRNA level of the glycogenic enzyme phosphoenol pyruvate carboxylase. In addition, mRNA levels of the glucose transporters GLUT1, GLUT4, and insulin receptor substrate-1 were elevated by MDI stimulation, whereas SG dose-dependently inhibited the expression of these genes in differentiated adipocytes. SG also inhibited the phosphorylation of Akt (Ser473) at an early phase of MDI stimulation. Intracellular nitric oxide (NO) production and endothelial nitric oxide synthase mRNA levels were markedly decreased by MDI stimulation and recovered by SG treatment of adipocytes.

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Conclusion: Our results suggest that SG effectively inhibits adipocyte proliferation and differentiation through the downregulation of PPARγ and C/EBPα, by suppressing Akt (Ser473) phosphorylation and enhancing NO production. These results provide strong evidence to support the development of SG for antiobesity treatment.

1. Introduction

Obesity is a state of increase in adipose tissue and body weight caused by excessive fat accumulation, and has been associated with numerous metabolic disorders, including diabetes, hypertension, atherosclerosis, liver failure, and certain cancers. Adipose tissue functions in the storage of triacylglycerol and as a source of adipokines, which in turn regulate body weight [1]. An increase in the size and number of white adipocytes is a particular characteristic in patients with obesity. In general, hyperplasia of adipocytes causes obesity in early childhood, while hypertrophy is responsible for adult-onset obesity; however, hyperplasia of adipocytes can also occur in adults. Adipocyte hyperplasia is caused by preadipocyte proliferation, which results in subsequent differentiation that is referred to as *adipogenesis*. Adipogenesis in adipocytes is the process of cell differentiation by which preadipocytes become mature (i.e., fully differentiated) adipocytes that are characterized by intracellular lipid accumulation. To date, the signaling pathways and downstream mediators involved in adipogenesis have been intensively studied. Many studies have focused on the identification of potent inhibitors of adipogenesis in adipocytes for the prevention of obesity.

The mouse 3T3-L1 preadipocyte cell line is an important cell model for studying adipogenesis. Hormonal treatments with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI) stimulate the differentiation of preadipocytes to mature adipocyte by initiating adipogenesis [2]. The accumulation of lipid droplets in cells is a marker of differentiation that can be visualized using oil red O (ORO) staining. In addition, *Caenorhabditis elegans* is a valuable eukaryotic model for adipogenesis, because the core pathway of lipid metabolism is conserved with that of humans. Cholesterol that is taken up by *C. elegans* in dietary supplements is stored in lipid droplets in intestinal and hypodermal cells. Lipid droplet biogenesis in *C. elegans* can be readily observed using the vital stain Nile red, a lipophilic dye that fluoresces in hydrophobic environments. Fluorescence imaging of live worms through vital dye feeding can be used to examine the qualitative fat content in *C. elegans*. Monitoring changes in the amounts of intracellular lipid in *C. elegans* or preadipocytes may therefore be used for the discovery of novel agents to treat obesity.

Sun ginseng (SG) is a type of steamed ginseng that shows higher therapeutic efficacy than white ginseng (WG) and red ginseng (RG) [3,4]. In East Asian countries, ginseng is the most widely used traditional medicine and is considered to be a herb with multiple beneficial properties, such as antioxidant, antiaging, and inhibition of inflammation and tumorigenesis. The improved biological activities of SG compared with WG and RG are due to its increased concentration of relatively less polar ginsenosides such as Rg3, Rg5, and Rk1, which accumulate as a result of steaming WG at high temperatures.

Korean ginseng has been reported to show antiobesity effects in animal models. WG prevents the development of obesity by regulating the expression of genes involved in lipogenesis in white adipose tissue, and by slowing down the absorption of dietary fat in mice fed a high-fat diet (HFD) [5]. In addition, the administration of crude saponin prepared from Korean RG has been shown to decrease body weight, adipose tissue weight, plasma triacylglycerol, and levels of leptin and adiponectin in HFD-induced obese rats [6]. The ginsenosides (also commonly referred to as *saponins*) Re and Rg3 are regarded to be antihyperglycemic and antidiabetic agents that stimulate glucose uptake in mature adipocytes [2]. Rg3, which is an abundant ginsenoside in SG, inhibits adipocyte differentiation by the upregulation of adenosine monophosphate-activated protein kinase and the suppression of peroxisome proliferator-activated receptor gamma (PPARγ) in 3T3-L1 adipocytes, supporting the antiobesity effects of SG [7,8]. In a previous report, it was demonstrated that the protection of HepG2 cells from *t*-butyl hydroperoxide-induced cell damage by SG was attributed to its antioxidative and antiapoptotic effects [3]. Oxidative stress is known to be related to several pathological processes including obesity, diabetes, and atherosclerosis. Taken together, these data indicate that SG might have potential as an antiobesity drug.

In this study, we investigated the inhibitory effect of SG on adipocyte differentiation and lipid accumulation in *C. elegans*, and assessed the expression level of genes contributing to adipogenesis. We showed that the Akt pathway is activated in adipogenesis mediated by MDI stimulation, and that Akt phosphorylation is suppressed by SG in 3T3-L1 preadipocytes.

2. Materials and methods

2.1. Reagents

Isobutyl methylxanthine, dexamethasone, insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ORO, 1,3,3-tetraethoxypropane, Nile red [9-diethylamino-5-benzo(α)phenoxazinone], and Griess reagent were purchased from Sigma (St. Louis, MO, USA). RNAiso Plus, PrimeScript first-strand complementary DNA (cDNA) synthesis kit, and SYBR Premix Ex Taq real-time PCR kit were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Antibodies against PPARγ, CCAAT/enhancer-binding protein-alpha (C/EBPα), phospho-Akt (Ser473), and Akt were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibody for β-actin was obtained from Sigma. Horseradish peroxidase (HRP)-conjugated antimouse and anti-rabbit immunoglobulin G (IgG) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Extracts of SG, RG, and WG were provided by Ginseng Science Inc. (Seoul, Korea).

2.2. Cell culture and preadipocyte differentiation

Mouse 3T3-L1 preadipocytes (obtained from American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; WELGENE, Daegu, Korea) supplemented with 10% newborn calf serum (Gibco BRL Life Technology, Grand Island, NY, USA) without antibiotics in a 5% CO_2 atmosphere. Two days after cells reached confluence [differentiation Day 0 (D0)], the medium was replaced with DMEM containing MDI medium (1 μg/mL 3-isobutyl-1-methylxanthine, 1μM dexamethasone, and 1 μg/mL insulin). After 2 d (differentiation D2), the MDI medium was replaced with insulin-containing DMEM. During differentiation,

cells were maintained by replenishing with new DMEM every 2nd d. SG was dissolved in dimethyl sulfoxide (DMSO) and added to the cells at 0.4 μg/mL, 2 μg/mL, or 10 μg/mL during each differentiation period. Comparative effects of SG, RG, and WG on the differentiation of 3T3-L1 cells were assessed by treatment with each extract at 10 μg/mL plus MDI stimulation for 7 d.

2.3. Oil red O staining and micrographs of lipid drop formation in 3T3-L1 cells

The lipid content of differentiated 3T3-L1 cells was evaluated by ORO staining on D2, D4, and D7 of differentiation. The 3T3-L1 preadipocytes were plated at a density of 2.5×10^5 cells in a six-well plate and images were taken on D7. Cells were washed with phosphate-buffered saline (PBS) and fixed in 10% formalin in PBS for 1 h at 4°C, and then washed two times with PBS and stained with 0.5% ORO in 60% isopropanol for 1 h at 4°C. To quantify the intracellular lipid content, excess stain was removed by washing with 70% ethanol, and cells were extracted with 4% Nonidet P-40 (NP-40) in isopropanol. The absorbance of the extract solution was measured at 520 nm using a GENios microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Lipid drop accumulation in 3T3-L1 cells was photographed using an Olympus IX51 inverted microscope (Olympus Corp., Tokyo, Japan) at 100× magnification.

2.4. MTT assay and nitric oxide determination

To determine the effect of SG on proliferation of preadipocytes, 3T3-L1 cells were plated at a density of 1.5×10^4 cells/well in 96-well plates and incubated with increasing concentrations of SG for 24 h. The cells were washed two times with PBS and treated with 100 μL of MTT solution (5 mg/mL) at 37°C. After 3 h, the MTT solution was removed and 100 μL of DMSO was added to extract MTT formazan crystals. The absorbance at 570 nm was measured on a microplate reader.

Estimation of nitric oxide (NO) production in 3T3-L1 cells was performed using Griess reagent on D5. After incubation, the culture media were transferred to a new plate and mixed with the same volume of Griess reagent. To measure NO production, the mixture was incubated for 20 min at room temperature and absorbance was measured at 548 nm using a microplate reader. The extracellular release of NO was calculated from a sodium nitrite standard curve.

2.5. RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction

Cultures of 3T3-L1 preadipocytes were plated at a density of 2.5×10^5 cells in six-well plates and lysed with 400 μL of RNAiso reagent after 5 d. Total RNA was isolated using RNAiso according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using the PrimeScript first-strand cDNA synthesis kit. The cDNA was amplified to estimate gene expression levels during adipocyte differentiation by reverse transcription-polymerase chain reaction (RT-PCR). PCR was carried out using the SYBR premix Ex Taq kit and a Thermal Cycler Dice Real Time System (Takara). In brief, cDNA was denatured at 95°C for 10 s, followed by 40 amplification cycles (5 s at 95°C, 30 s at 60°C). RNA concentrations and purity were determined using NanoDrop ND-1000 (NanoDrop Technologies Inc., Rockland, DE, USA). All messenger RNA (mRNA) levels were normalized using β-actin mRNA as an internal control. Primers used for amplifications are shown in Table 1.

Table 1
Oligonucleotide primer sequences used in quantitative real-time polymerase chain reaction (5′ to 3′)

Gene name	Forward primer	Reverse primer
PPARγ	AGGCCGAGAAGGAGAAGCTGTTG	TGGCCACCTCTTTGCTCTGCTC
C/EBPα	TTACAACAGGCCAGGTTTCC	GGCTGGCGACATACAGTACA
SREBP1c	TGTTGGCATCCTGCTATCTG	AGGGAAAGCTTTGGGGTCTA
Leptin	GACACCAAAACCCTCAT	CAGTGTCTGGTCCATCT
LPL	TCCAAGGAAGCCTTTGAGAA	CCATCCTCAGTCCCAGAAAA
aP2	AACACCGAGATTTCCAA	ACACATTCCACCACCAG
FATP1	TGCCTCTGCCTTGATCTTTT	GGAACCGTGGATGAACCTAA
FAS	TTGCTGGCACTACAGAATGC	AACAGCCTCAGAGCGACAAT
CPT1	CAACCCAGAACCATGGAAGT	GGCCTCTGTGGTACACGACAA
HMG-CoA	GATTGTGGCAGTCAGTGGGAA	GTTGTAGCCGCCTATGCTCC
Glk	TACGACCGGATGGTGGATGA	ACCAGCTCGCCCATGTACTTTC
Pyk	TGCAATTGGAGCCGTGGA	CCTGGGCAGAACGAGTCACA
PEPC	GTGTTTGTAGGAGCAGCCATGAGA	GCCAGGTATTTGCCGAAGTTGTAG
GLUT1	CCATCCACCACACTCACCAC	GCCCAGGATCAGCATCTCAA
GLUT4	CTTCTTTGAGATTGGCCCTGG	AGGTGAAGATGAAGAAGCCAAGC
IRS1	CAAGACGCTCCAGTGAGGATTTAAG	GACGTGAGGTCCTGGTTGTGAA
eNOS	CCTTCCGCTACCAGCCAGA	CAGAGATCTTCACTGCATTGGCTA
β-actin	ACATCTGCTGGAAGGTGGAC	GGTACCACCATGTACCCAGG

aP2, amplifying mouse fatty acid-binding protein 2; C/EBPα, CCAAT/enhancer-binding protein-alpha; CPT1, carnitine palmitoyltransferase 1; eNOS, endothelial nitric oxide synthase; FAS, fatty acid synthase; FATP1, fatty acid transporter protein 1; Glk, glucokinase; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IRS1, insulin receptor substrate-1; LPL, lipoprotein lipase; PPARγ, peroxisome proliferator-activated receptor subtype γ; Pyk, pyruvate kinase; SREBP1, sterol regulatory element-binding protein 1; PEPC, phosphoenolpyruvate carboxylase.

2.6. Western blot analysis

Western blot analysis was performed to determine the effect of SG on PPARγ and C/EBPα protein levels and phosphorylation of Akt in 3T3-L1 cells. To estimate the protein levels of PPARγ and C/EBPα, 3T3-L1 preadipocytes were seeded onto six-well plates and differentiated in MDI medium in the presence or absence of SG as described earlier. On D5 of differentiation, cells were collected and resuspended in lysis buffer [25mM Tris—HCl (pH 7.5), 100mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktails (Calbiochem, Darmstadt, Germany)] and centrifuged at 5,000 rpm for 10 min. A total of 30 μg of protein was electrophoresed in SDS—polyacrylamide gels and transferred onto polyvinyl fluoride membranes. The membrane was first probed with primary antibodies against PPARγ and C/EBPα and then with secondary antibody (HRP-conjugated antimouse or antirabbit Ig). Proteins were visualized with the enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA). Protein loading was normalized by probing blots with monoclonal anti-β-actin antibody. For detection of Akt activation, 3T3-L1 preadipocytes were preincubated for 1 h with SG and stimulated with MDI treatment, followed by incubation for 30 min. Membranes were incubated with antibody solution containing phospho-Akt (Ser473) antibody. Activation of phospho-Akt was normalized by the total Akt content.

2.7. Analysis of body fat accumulation in C. elegans

Wild-type *C. elegans* (strain N2) was obtained from the Laboratory of Animal Genetics, Konkuk University (Gwangjin-gu, Seoul, Korea) and maintained on nematode growth medium plates supplied with *Escherichia coli* OP50 as a food source at 18°C as previously described [9]. To assay the fat content of *C. elegans*, L4 stage worms grown on nematode growth medium plates were inoculated into S liquid media containing SG at 10 μg/mL and incubated on a shaking incubator (105 rpm, 18°C) in the dark for 24 h [10]. Nile

red in acetone (5 μg/mL) was added to the culture media and incubated for an additional 2 d. The stained worms were transferred to a new tube and centrifuged at 400*g* at 4°C. Worms were fixed in 3% NaN$_3$ on glass slides and observed by fluorescence microscopy (650nM emission) at 400× magnification.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation and the significance of the results was assessed using the SigmaPlot program (version 6.0). All experiments were conducted at least three times.

3. Results

3.1. SG treatment inhibited differentiation of preadipocytes

Several concentrations of SG were tested to investigate its inhibitory effect on adipocyte differentiation. SG was added to 3T3-L1 cells together with MDI stimulation, and lipid droplets that accumulated in differentiated cells were observed by microscopy on D7 of differentiation (Fig. 1A). To assess the degree of differentiation, levels of stained intracellular lipid droplets were quantified by measuring the ORO signal in lipid extracts of cells on D2, D4, and D7. Stained cells were also photographed at these time points. As previously described, MDI exposure augmented the differentiation of adipocytes. On D4 and D7, differentiated cells showed significant lipid accumulation. However, SG at 2 μg/mL and 10 μg/mL showed an inhibitory effect on adipocyte differentiation by 36.4% and 24.8%, respectively, compared with cells stimulated with MDI alone. Interestingly, SG increased lipid content by 125% compared with MDI-stimulated cells on D2.

To determine whether SG could alter cell proliferation, preadipocytes were treated with SG during differentiation. On D2, D4, and D7, the degree of cell proliferation was assessed by the MTT assay (Fig. 1B). Consistent with previous studies, MDI stimulated log-phase growth of adipocytes during MDI-induced differentiation [11]. As shown in Fig. 1C, cell proliferation was not affected by 0.4 μg/mL or 2 μg/mL of SG as compared with cells stimulated with MDI alone. By comparison, 10 μg/mL of SG markedly decreased proliferation by 29.5%, 26.8%, and 42.6%, respectively, on D2, D4, and D7. Taken together, these data revealed that SG efficiently inhibited adipocyte differentiation as well as proliferation without toxicity, suggesting that SG may be a safe antiobesity agent.

The effects of SG on adipocyte differentiation were compared with those of other processed ginsengs. Preadipocytes were treated with SG, RG, or WG at 10 μg/mL plus MDI stimulation for 7 d. Cells were stained with ORO to detect intracellular lipid accumulation and photographed. As shown in Fig. 2, among the ginseng extracts, SG showed the most significant inhibition of differentiation in 3T3-L1 adipocytes. ORO staining indicated that RG and WG also blocked adipocyte differentiation, but much less effectively than SG.

3.2. SG diminished body fat content in C. elegans

Nile red staining of the nematode *C. elegans* provides a qualitative measurement of fat content, and can be used as a model for studying fat metabolism. To investigate whether SG treatment diminished body fat content in *C. elegans*, L4 stage worms were cultured on an *E. coli* OP50 diet supplemented with 10 μg/mL of SG, followed by Nile red staining.

Wild-type worms grown in the absence of SG (control group) showed an increase in fat content based on light and fluorescence microscopy observations, whereas worms fed with SG showed an obvious decrease in fat droplets compared with control worms

(Fig. 3). These findings were consistent with the antiadipogenic effect of SG demonstrated in adipocytes.

3.3. SG regulated the mRNA level of genes involved in lipid and glucose homeostasis

MDI stimulation has previously been shown to significantly increase the expression of transcriptional factors involved in lipid and glucose metabolism, such as PPARγ, C/EBPα, and sterol regulatory element-binding protein 1c (SREBP1c) [12–14]. To determine whether SG affects the expression of these genes, mRNA levels were evaluated by RT-PCR analysis in 3T3-L1 adipocytes. As shown in Fig. 4A, treatment with 0.4 μg/mL, 2 μg/mL, and 10 μg/mL of SG resulted in decreased PPARγ mRNA levels by 76.5%, 90.2%, and 90.2%, respectively, compared with differentiated cells. In addition, the gene expressions of C/EBPα and SREBP1c were inhibited by SG treatment up to 85% and 83%, respectively. Because PPARγ, C/EBPα, and SREBP1c are known to induce expression of genes that govern energy metabolism in adipocytes, we examined the effect of MDI and SG on mRNA levels of several downstream target genes of these transcription factors. MDI stimulation resulted in increased expression of mRNAs for leptin, lipoprotein lipase (LPL), amplifying mouse fatty acid-binding protein 2 (aP2), fatty acid transporter protein 1 (FATP1), fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), as expected. By contrast, SG treatment showed an inhibitory effect on mRNA expression in a dose-dependent manner; SG at 0.4 μg/mL, 2 μg/mL, and 10 μg/mL decreased the expression of leptin (as a representative adipokine) by 11.3%, 20.7%, and 52.6%, respectively. Levels of mRNA for the lipogenic enzymes LPL, FATP1, FAS, and HMG-CoA, and lipogenic factor aP2 were downregulated by 83.5%, 73.9%, 94.5%, 33.1%, and 99.4%, respectively, in SG-treated adipocytes compared with cells stimulated with MDI alone. Conversely, mRNA level for the lipolytic enzyme carnitine palmitoyl transferase 1 (CPT1) was decreased by MDI stimulation and increased to nearly the same level observed in nondifferentiated (ND) cells treated with SG (Fig. 4B).

We then examined the effect of SG on the mRNA expression of genes mediating glucose metabolism, such as glucokinase (Glk), pyruvate kinase (Pyk), and phosphoenol pyruvate carboxylase (PEPC; Fig. 4C). The expression of the glycolytic enzymes Glk and Pyk was significantly decreased by MDI stimulation, whereas mRNA levels of these genes were increased by SG treatment up to 1.5- and 3.5-fold, respectively. By contrast, treatment with 0.4 μg/mL, 2 μg/mL, or 10 μg/mL of SG dramatically decreased the expression of the glycogenic enzyme PEPC by 41.4%, 34.7%, and 58.6%, respectively, compared with MDI-treated adipocytes.

Insulin present in MDI results in phosphorylation of insulin receptor (IR), insulin receptor substrate-1 (IRS1), and other downstream effectors. Activation of the insulin signaling pathway leads to glucose transporter (GLUT) expression, resulting in glucose uptake. We showed that MDI-stimulated 3T3-L1 preadipocytes induced the expressions of GLUT1, GLUT4, and IRS1 (Fig. 4C) [15,16]. By contrast, increasing concentrations of SG suppressed the expression of these genes by up to 75.9%, 75.7%, and 81.3%, respectively, compared with cells cultured in MDI medium only. Interestingly, mRNA expression levels of aP2, GLUT1, and IRS1 in SG-treated cells were lower than those in ND adipocytes (negative control). SG-treated adipocytes also showed higher Pyk mRNA levels than the ND group.

Collectively, these observations suggest that the antiobesity effects of SG are mediated via regulation of genes that control lipid/glucose homeostasis in 3T3-L1 adipocytes.
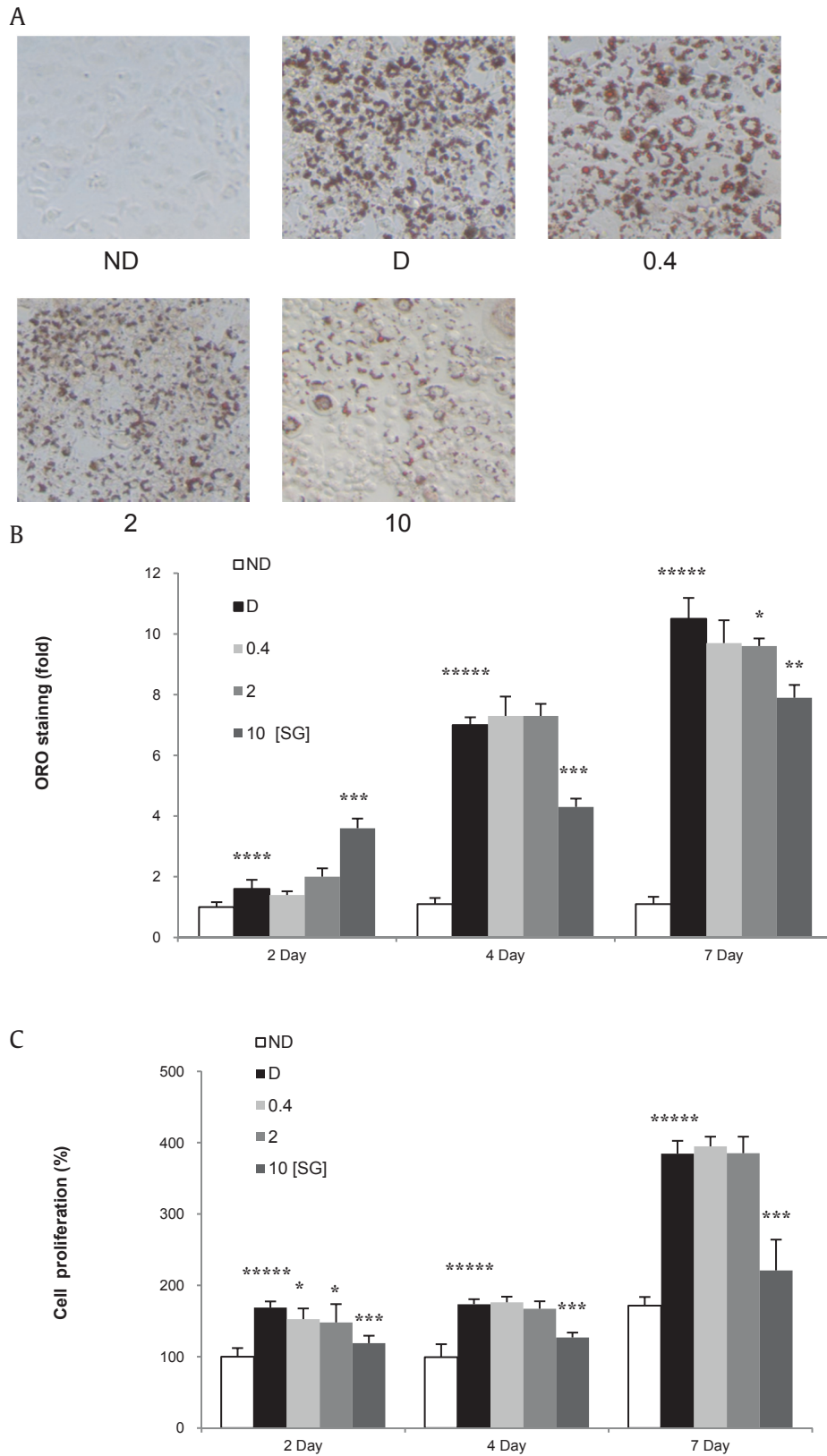
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Fig. 1. Effect of SG on differentiation and proliferation of 3T3-L1 adipocytes. (A) 3T3-L1 preadipocytes were stimulated with MDI in the presence or absence of SG (0.4 μg/mL, 2 μg/mL, or 10 μg/mL). On Day 2, Day 4, and Day 7 of differentiation, cells were stained with ORO and lipid accumulation was quantified by measuring the absorbance at 520 nm. (B) Cells were incubated with or without SG for 24 h, and cell viability was determined by the MTT assay. Data represent the mean ± standard deviation of triplicate experiments. Statistically significant differences from the negative control of differentiation (ND, marked as ****/*****) or the MDI-induced differentiated control (D) (*) are indicated. (C) Decreased the mRNA level of sterol regulatory element binding proteins. * $p < 0.05$, ****/** $p < 0.01$, *****/*** $p < 0.001$. MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ND, nondifferentiated; ORO, oil red O; SG, sun ginseng.
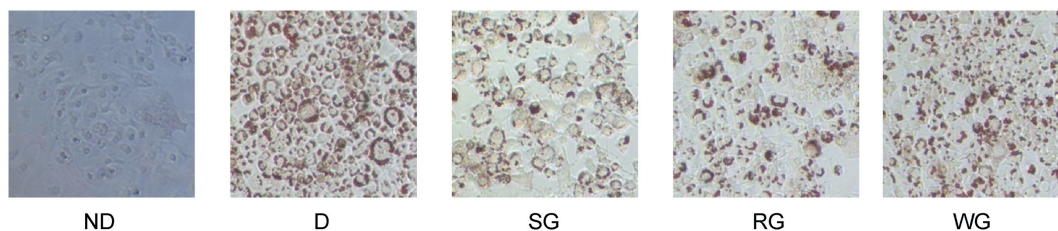
Fig. 2. Comparative effect of SG, RG, and WG on differentiation of 3T3-L1 adipocytes. Preadipocytes were stimulated with MDI plus SG, RG, or WG (10 μg/mL) for 7 d. Cells were stained and photographed to assess intracellular lipid accumulation. Representative images of stained cells are shown. D, MDI-induced differentiated control; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; ND, nondifferentiated; RG, red ginseng; SG, sun ginseng; WG, white ginseng.

3.4. SG recovered NO production and endothelial nitric oxide synthase expression during adipocyte differentiation

MDI stimulation is known to suppress NO production by downregulating endothelial nitric oxide synthase (eNOS) in adipocytes [17]. Consistently, stimulation with MDI led to a decrease in NO production by nearly 87% compared with undifferentiated cells (Fig. 5A). However, SG increased NO production. On differentiation D5, NO production was increased by more than threefold by SG (10 μg/mL) as compared with MDI stimulation alone. Moreover, eNOS expression positively correlated with NO production (Fig. 5B). eNOS was expressed at low levels in MDI-stimulated adipocytes, and SG increased eNOS mRNA expression by up to 50% compared with MDI alone. These results suggest that SG may inhibit adipogenic differentiation through the recovery of intracellular NO levels.

3.5. SG downregulated protein levels of PPARγ and C/EBPα during adipocyte differentiation

Western blot analysis was conducted to determine the effect of SG on protein levels of PPARγ and C/EBPα during adipocyte differentiation. As shown in Fig. 6, PPARγ and C/EBPα were abundantly expressed in MDI-stimulated 3T3-L1 adipocytes on D5. Treatment with increasing concentrations of SG decreased protein levels of PPARγ and C/EBPα, which was consistent with the RT-PCR data for these genes. Our data suggested that SG regulated not only mRNA expression but also protein levels of PPARγ and C/EBPα to inhibit adipocyte differentiation.

3.6. SG suppressed Akt phosphorylation during adipocyte differentiation

The Akt signaling pathway has been reported to play a role in MDI-driven differentiation in adipocytes [18,19]. To examine the involvement of Akt in the inhibitory mechanism of adipocyte differentiation by SG, 3T3-L1 preadipocytes were pretreated with different concentrations of SG for 1 h followed by MDI stimulation for 30 min, and cells were analyzed for Akt phosphorylation by Western blot analysis. As shown in Fig. 7, SG inhibited the phosphorylation of Ser473 in Akt, indicating Akt activation [20]. These findings demonstrate that the prevention of adipocyte differentiation by SG was associated with inhibition of the Akt signaling pathway.
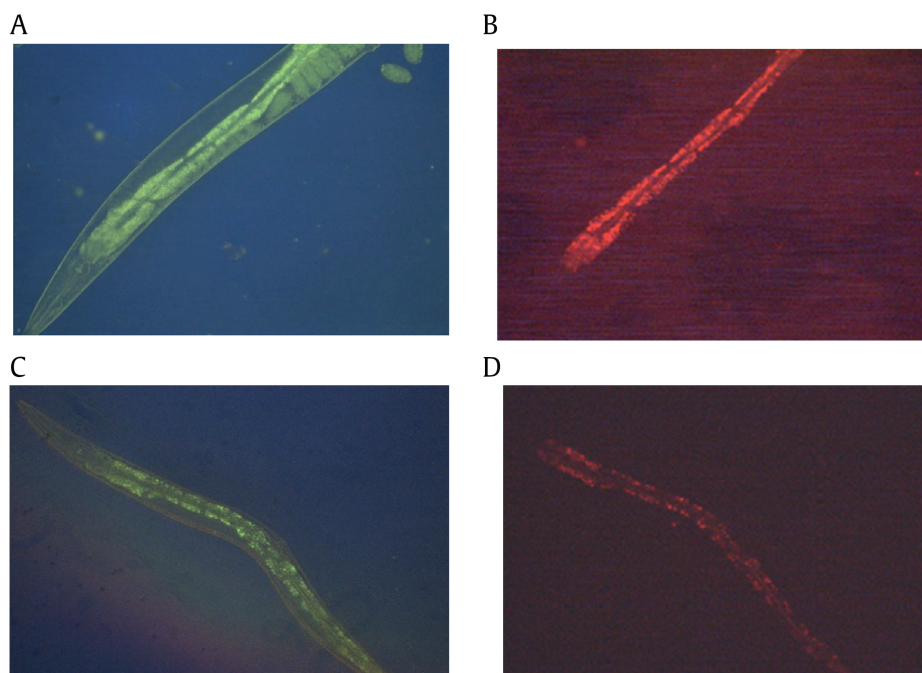

Fig. 3. Effect of SG on fat accumulation in *Caenorhabditis elegans*. Photographs of lipid droplets in *C. elegans* treated with SG (10 μg/mL) for 24 h followed by Nile red staining for an additional 2 d. (A) Control *C. elegans* as observed by light microscopy, (B) fluorescence microscopy images of lipid droplets in *C. elegans* controls stained with Nile red, (C) SG (10 mg/mL)-fed *C. elegans* as viewed by light microscopy, (D) fluorescence microscopy images of lipid droplets in *C. elegans* fed with SG. SG, sun ginseng.
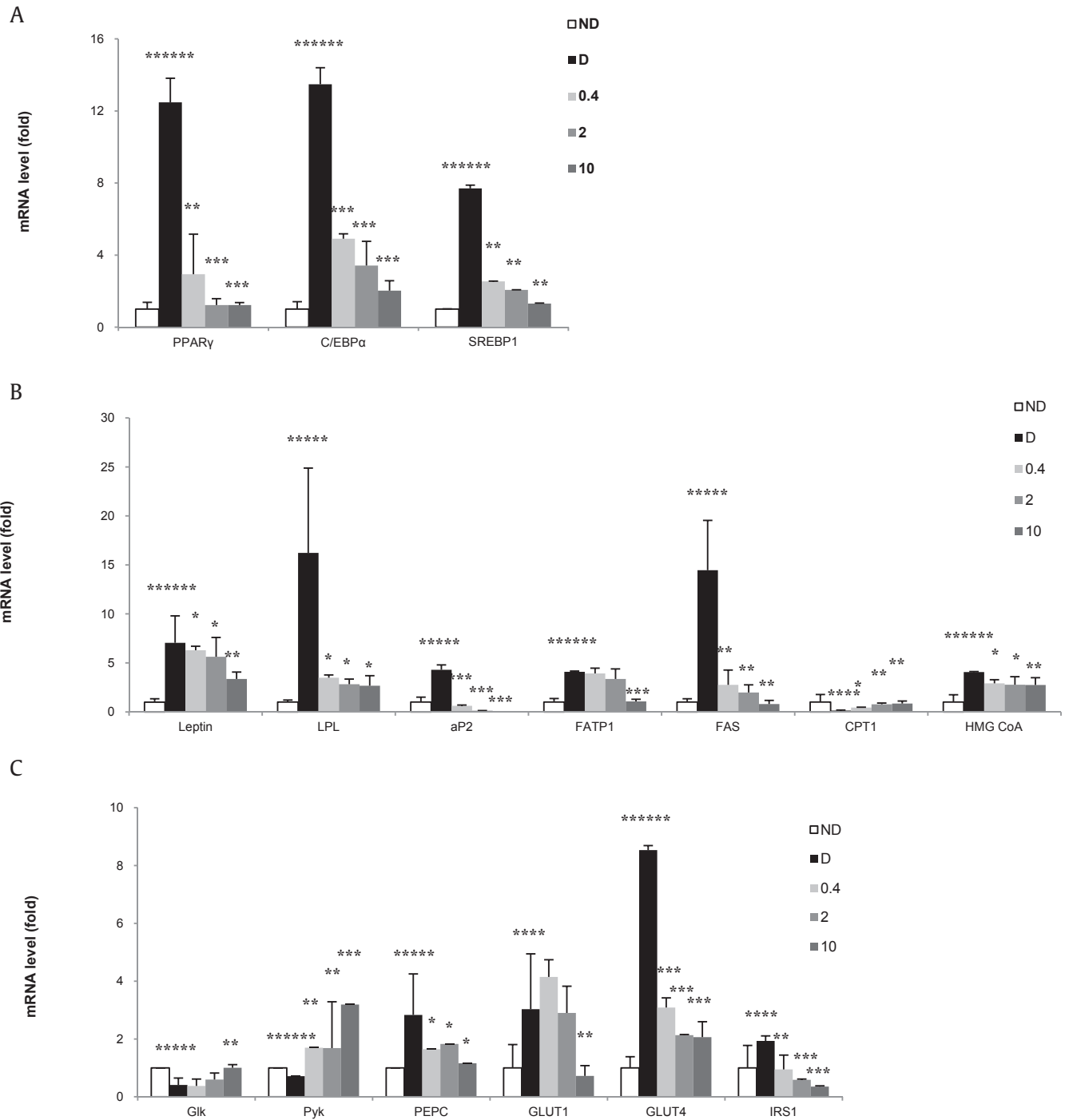
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Fig. 4. Effects of SG on mRNA level of genes related to lipid/glucose homeostasis in differentiated adipocytes. 3T3-L1 preadipocytes were incubated with MDI in the presence or absence of SG (0.4 μg/mL, 2 μg/mL, or 10 μg/mL). On Day 5, cells were harvested and total RNA was extracted for RT-PCR analysis. Gene expression levels of (A) PPARγ, C/EBPα, SREBP1c; (B) leptin, LPL, aP2, FATP1, FAS, CPT1, HMG-CoA; and (C) Glk, Pyk, PEPC, GLUT1, GLUT4, and IRS1 were determined as described in the "Materials and methods" section. Data represent the mean ± standard deviation of triplicate experiments. Statistically significant differences from the ND (****) or D (*) are indicated. ****/* $p < 0.05$, *****/** $p < 0.01$, *****/*** $p < 0.001$. aP2, amplifying mouse fatty acid-binding protein 2; C/EBPα, CCAAT/enhancer-binding protein-alpha; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; FATP1, fatty acid transporter protein 1; Glk, glucokinase; GLUT, glucose transporter; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; IRS1, insulin receptor substrate-1; LPL, lipoprotein lipase; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; mRNA, messenger RNA; ND, nondifferentiated; PEPC, phosphoenol pyruvate carboxylase; PPARγ, proliferator-activated receptor gamma; Pyk, pyruvate kinase; RT-PCR, reverse transcription-polymerase chain reaction; SG, sun ginseng; SREBP1c, sterol regulatory element-binding proteins 1c.

4. Discussion

SG acquires high levels of unique ginsenosides by steaming at high temperature, resulting in more potent pharmacological properties, such as anticarcinogenic, free radical scavenging, nephroprotective, and antiplatelet effects, compared with RG and WG [21−25]. Although SG is expected and has been shown to be more effective than RG and WG for treatment of various diseases, the antiobesity effect of SG has remained unclear compared with that of RG and WG [3,4]. In this study, we demonstrated that SG
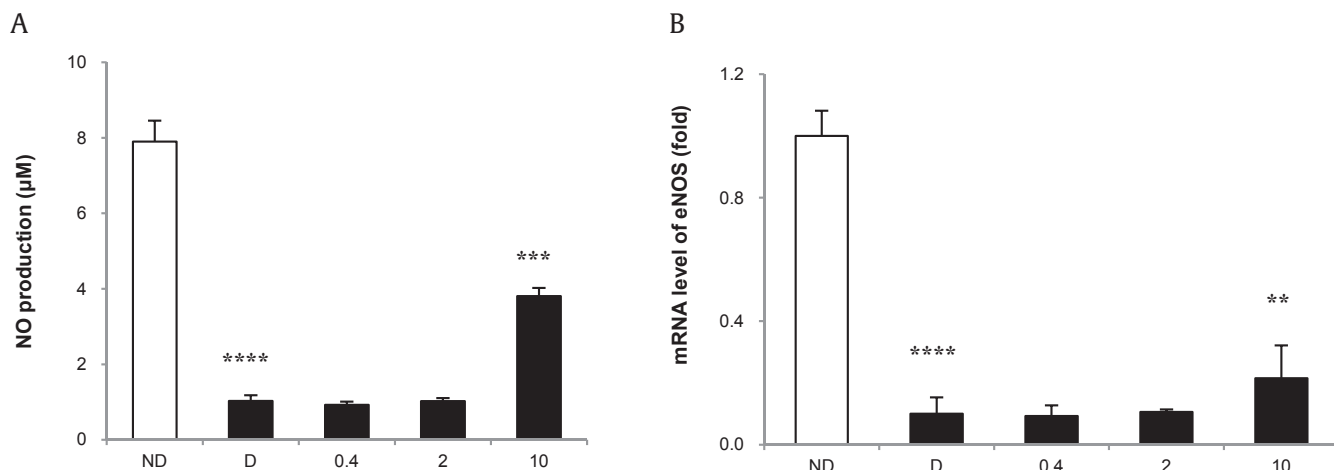
A

B

Fig. 5. Effects of SG on NO production and eNOS expression in differentiated adipocytes. (A) 3T3-L1 preadipocytes were incubated with MDI in the presence or absence of SG (0.4 μg/mL, 2 μg/mL, or 10 μg/mL). On Day 5, the culture media were mixed with Griess reagent. (B) Cells were incubated and treated as described in the text. On Day 7, cells were harvested and total RNA was extracted for RT-PCR analysis. Data represent the mean ± standard deviation of triplicate experiments. Statistically significant differences from the ND (marked as ****) or D (*) are shown. ** $p < 0.01$; ****/*** $p < 0.001$. eNOS, endothelial nitric oxide synthase; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; ND, nondifferentiated; NO, nitric oxide; RT-PCR, reverse transcription-polymerase chain reaction; SG, sun ginseng.

inhibits adipogenesis and revealed the potential mechanisms by which SG modulates adipocyte differentiation.

Mouse 3T3-L1 preadipocytes have been commonly used as a model to understand the regulation of adipogenesis, lipid/glucose metabolism, and apoptosis in adipocytes of adipose tissue [26]. Although adipose tissue contains various cell types, including mesenchymal stem cells, endothelial precursor cells, preadipocytes, and macrophages, changes in the rate of proliferation

and/or differentiation in adipocytes have been the focus of studies aimed at the identification of antiobesity agents.

Typically, patients with obesity store excessive fat in adipocytes of adipose tissue. In this study, 3T3-L1 preadipocytes were stimulated with an MDI hormonal mixture to differentiate cells, which resulted in intracellular lipid accumulation. Through the use of ORO staining, we confirmed that lipid had accumulated after the differentiation period, and that intracellular lipid content was reduced
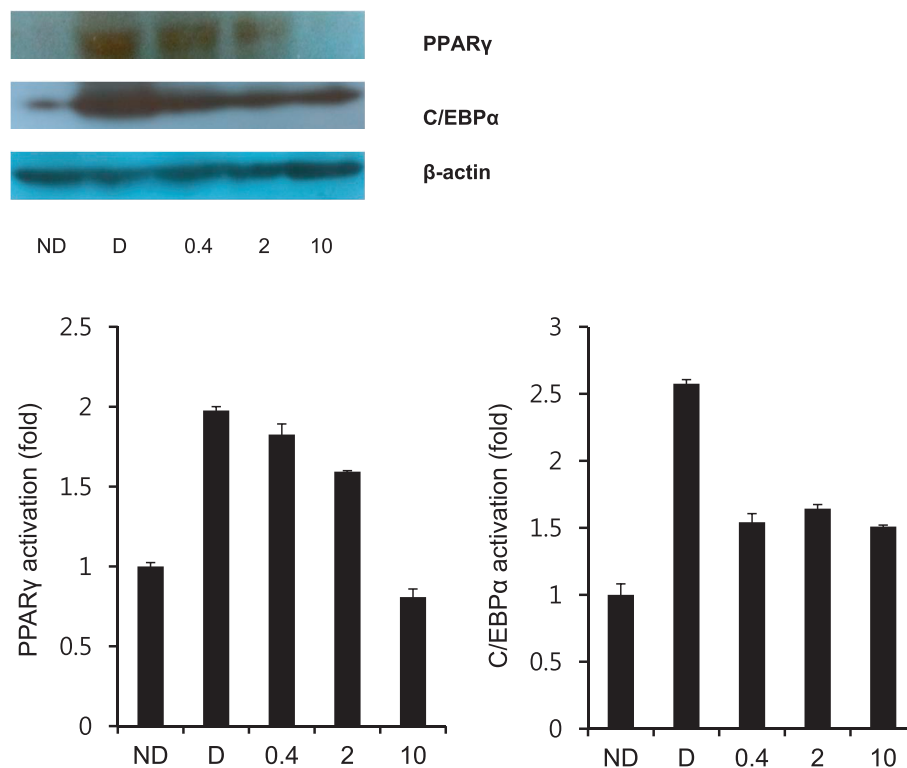

Fig. 6. Effects of SG treatment on protein levels of PPARγ and C/EBPα during adipocyte differentiation. Western blots showing the effects of SG on PPARγ and C/EBPα protein levels in 3T3-L1 cells. Preadipocytes were differentiated with or without SG at indicated concentrations as described in the text. Cells were harvested on Day 5 of differentiation and protein extracts (30 μg) were analyzed for PPARγ and C/EBPα expression. Actin was used as a loading control. C/EBPα, CCAAT/enhancer-binding protein-alpha; SG, sun ginseng; PPARγ, proliferator-activated receptor gamma.
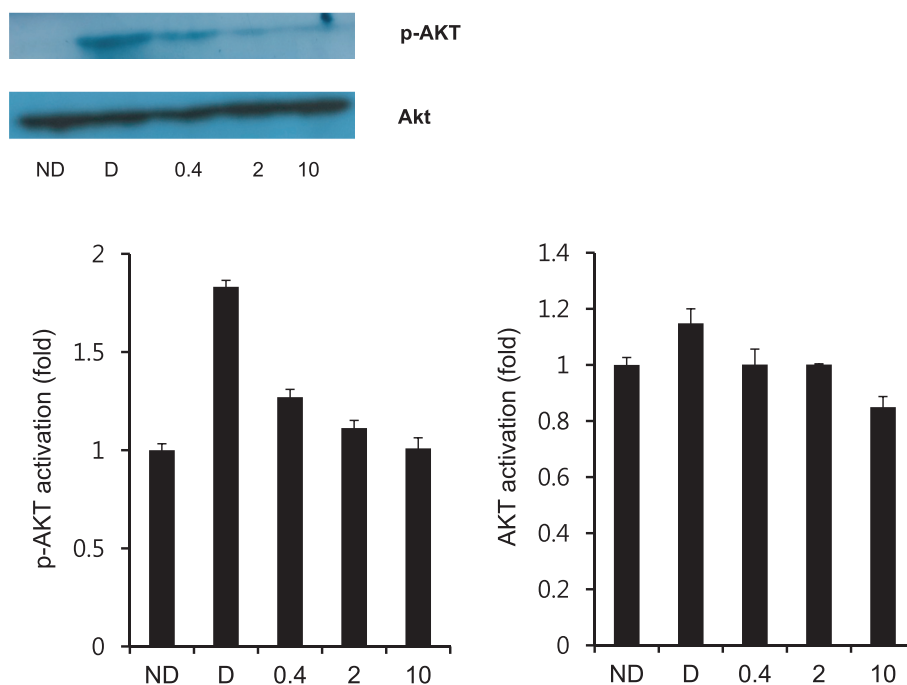
Fig. 7. Effects of SG treatment on Akt phosphorylation in MDI-stimulated adipocytes. Western blots showing the effect of SG and MDI on Akt phosphorylation in 3T3-L1 cells. Preadipocytes were seeded and cultured for 24 h. Cells were exposed to SG at indicated concentrations for 30 min, followed by MDI stimulation for 30 min. Protein blots were incubated with phospho-Akt (Ser473) antibody. Activation of phospho-Akt was normalized by the total Akt content. SG, sun ginseng; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; p-AKT, phospho-Akt.

in adipocytes by treatment with increasing concentrations of SG (Fig. 1A). Reduction in adipocyte lipid content precisely correlated with inhibition of adipocyte differentiation, suggesting that SG may be a more effective antiobesity agent than either RG or WG (Fig. 2) [27].

Interestingly, treatment with SG significantly increased lipid accumulation on D2 of differentiation despite the suppression of cell proliferation. This finding suggests that SG treatment may enhance the insulin-induced glucose uptake at an early stage of differentiation. SG has been reported to relieve the symptoms of diabetes in diabetic animal models [22,28]. Obesity has been known as the most important factor in development of Type 2 diabetes. Potentially, initial exposure to SG could protect against various symptoms of obesity and diabetes, although this requires further investigation.

The antimitogenic effects of natural compounds on 3T3-L1 preadipocyte have already been reported. Green tea catechins, such as (−)-epigallocatechin gallate, exhibited an inhibitory effect on preadipocyte proliferation via activation of the ERK and Cdk2 signaling pathways, suggesting that these compounds may be used to prevent obesity [29]. In addition, Yun et al. [30] reported that widdrol, a natural sesquiterpene known as an anticancer and antifungal agent, suppressed preadipocyte proliferation by p21- and Rb-dependent G_1 arrest in 3T3-L1 preadipocytes. In this study, SG inhibited adipocyte proliferation during differentiation despite an early increase in the number of adipocytes (Fig. 1B). Because obesity is associated with adipocyte size and number in adipose tissue, the control of adipocyte proliferation by SG suggests that it may have potential as a treatment for obesity.

To investigate the *in vivo* function of SG, we examined a model organism, the nematode *C. elegans*, for which genetic and molecular biological methods are well-established [31]. Several studies have used Nile red staining to explore regulators of fat accumulation in *C. elegans*. As shown in Fig. 3, wild-type worms grown in liquid media showed increased fat accumulation. By contrast,

worms fed with SG accumulated fewer fat droplets compared with control worms. These results are consistent with our data describing the antiadipogenic effect of SG in adipocytes.

We evaluated changes in the expression of transcriptional factors and downstream molecular mediators required for adipocyte differentiation. PPARγ, C/EBPα, and SREBP1c are considered to be important markers of adipocyte differentiation [12−14]. PPARγ is a key adipogenic transcriptional factor, and governs several molecular pathways in lipid and glucose metabolism. As such, PPARγ has been used as a target molecule in the identification of drugs for treatment of metabolic diseases. PPARγ belongs to the nuclear receptor superfamily (including PPARα and δ), and forms a heterodimer with other nuclear receptors to regulate the expression of peroxisome proliferator response elements-containing genes involved in lipid/carbohydrate metabolism in adipocytes [32]. During adipocyte differentiation, PPARγ cooperates with C/EBPα to transactivate adipocyte-specific genes. At early stages of differentiation, C/EBPδ and β upregulate the expression of C/EBPα and PPARγ for terminal adipogenesis [13,14]. The antiobesity effects of various isolated compounds and extracts from medicinal herbs that function through the suppression of C/EBPα and PPARγ have been reported [33,34]. To evaluate the effect of SG on PPARγ and C/EBPα expression, 3T3-L1 cells were incubated in the presence or absence of SG during differentiation. MDI-stimulated PPARγ and C/EBPα mRNA level gradually diminished as a result of SG treatment in a dose-dependent manner (Fig. 4A). Western blot analysis also showed decreases in protein levels of PPARγ and C/EBPα by SG treatment in adipocytes, which correlated with the reductions in mRNA levels of these genes (Fig. 6).

Other transcriptional factors implicated in insulin-induced adipogenesis regulation include SREBPs. There are three subtypes of SREBPs, namely, SREBP1a, 1c, and 2. Each subtype is encoded by a different gene and has a unique function. Among these isoforms of SREBPs, SREBP1c is thought to regulate lipid and glucose metabolism in adipose tissue, whereas SREBP2 is a ubiquitously

expressed transcription factor that functions in cholesterol homeostasis [12]. In addition, it has been reported that SREBP1c can directly control the expression of PPARγ and C/EBPα [12,35]. In this study, the elevated mRNA level of SREBP1c was reversed by SG treatment in differentiated adipocytes, in agreement with our data for PPARγ and C/EBPα (Fig. 4A). These results indicate that SG might play an inhibitory role in adipocyte differentiation through the downregulation of PPARγ, C/EBPα, and SREBP1c expressions.

We further analyzed changes in mRNA levels of other genes that regulate lipid/glucose metabolism. During adipocyte differentiation, elevated PPARγ expression can lead to changes in the expression of genes mediating fatty acid synthesis, lipid hydrolysis, fatty acid transport, β-oxidation, glycolysis, gluconeogenesis, and glucose transport. Consistent with other studies, MDI stimulation modulated mRNA levels of genes involved in these processes in differentiated 3T3-L1 cells (Figs. 4B and 4C) [12,36]. To examine the effect of SG on the progression of lipogenesis and lipolysis, we determined mRNA levels of lipogenic molecules such as LPL, aP2, FAS, FATP1, and HMG-CoA by RT-PCR analysis. On D5 of differentiation, we found that SG remarkably suppressed mRNA levels of these genes in differentiated adipocytes. By contrast, the mRNA level of CPT1, a lipolytic enzyme, was decreased by MDI exposure and was increased by SG treatment.

MDI stimulation induces gene expression of leptin as a marker of differentiation in 3T3-L1 cells [37]. Leptin is a peptide hormone and is produced by adipocytes in proportion to intracellular lipid accumulation. In this study, SG strongly suppressed the mRNA expression of leptin that had been induced by MDI stimulation (Fig. 4B).

MDI strongly activates the lipogenesis of adipocytes through the insulin signaling pathway. Insulin phosphorylates IR and IRS1, leading to GLUT4 expression and translocation to the plasma membrane, which subsequently promotes glucose uptake into adipocytes [15,16]. Insulin and dexamethasone can also increase gene expression of GLUT1 and GLUT4, both of which play a role in lipogenesis of adipocyte [16,38]. In this study, we demonstrated that SG can dose dependently reduce the expressions of IRS1, GLUT1, and GLUT4, suggesting that SG may have the potential to suppress MDI-stimulated adipogenesis in adipocytes.

To investigate the molecular mechanisms by which SG inhibited MDI-mediated signaling activities in 3T3-L1 adipocytes, Akt phosphorylation was measured by Western blot analysis. Recently, the roles of the Akt pathway and its downstream mediators in adipocyte differentiation were reported [39]. In brief, insulin triggers the autophosphorylation of tyrosine in IRS1 followed by the phosphorylation of Ser473 in Akt. This event activates PPARγ- and C/EBPα-located downstream of Akt, and subsequently regulates PPARγ target genes during adipocyte differentiation. We found that MDI treatment increased the amount of phosphorylated Akt (Ser473). Not surprisingly, SG induced the downregulation of Akt phosphorylation (Fig. 7). According to our data, SG-mediated suppression of adipocyte differentiation might be due to the inhibition of PPARγ and C/EBPα expression via decreased Akt (Ser473) phosphorylation.

NO is a multifunctional molecule related to inflammation, cell proliferation, and differentiation. NO is synthesized from L-arginine by NOS, which exists in three isoforms: neuronal NOS, eNOS, and inducible NOS. Although NO has been shown to have conflicting effects on adipogenesis, previous studies reported that MDI treatment inhibited NO production by suppression of NOS in adipocytes [17,40]. In *in vivo* studies, both endogenous and exogenous NO mediated lipolysis in adipocytes [41,42]. Furthermore, Kawachi et al. [43] reported that NO inhibits adipocyte differentiation through the *S*-nitrosylation of PPARγ, leading to defective transcriptional activity. Therefore, it was not surprising that SG

dramatically recovered decreases in NO production in differentiated adipocytes (Fig. 5A). To determine which NOS isoform might be involved in the regulation of NO production in adipocytes, we examined the expression levels of NOS mRNA by RT-PCR analysis. We evaluated eNOS expression, because differentiating 3T3-L1 cells have been shown to lack expression of inducible NOS [44]. As shown in Fig. 5B, treatment with SG increased eNOS expression, indicating that SG may play role in regulating NO production. These results agree with a previous report showing increased eNOS protein levels in differentiated adipocytes [17].

Our study has provided compelling scientific evidence that SG can be developed as a safe and potent antiadipogenic agent for prevention of obesity. SG may suppress adipocyte differentiation by enhancing NO production via eNOS expression, thereby regulating the expression of genes related to energy metabolism and inhibition of Akt activation.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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