



Ginsenoside Rg5: Rk1 Exerts an Anti-obesity Effect on 3T3-L1 Cell Line by the Downregulation of PPAR γ and CEBP α

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Received: 11 March 2016 Revised: 6 September 2017 Accepted: 18 September 2017; Published online: 29 December 2017

Background: Obesity, a global health problem and a chronic disease, is associated with increased risk of developing type 2 diabetes and coronary heart diseases. A wide variety of natural remedies have been explored for their obesity treatment potential.

Objective: The anti-adipogenic effect of ginsenoside Rg5:Rk1 (Rg5:Rk1) on 3T3-L1 mature adipocytes was investigated.

Materials and Methods: To elucidate the anti-obesity effect of Rg5:Rk1, a mixture of protopanaxadiol type ginsenosides isolated from *Panax ginseng* Meyer in a 3T3-L1 adipocytes. In order to determine the anti-obesity effect of Rg5:Rk1, based on oil Red O Staining, triglyceride (TG) content in adipose cells was assessed. Furthermore, to elucidate the possible mechanism of Rg5:Rk1 effect on lipid accumulation, mRNA and protein expression analyses of adipocyte markers such as *STAT3*, *PPAR γ* , *CEBP α* and *ap2* were carried out.

Results: Rg5:Rk1 treatment showed an inhibition of lipid droplet accumulation and decrease of TG content. In addition, expression of *STAT3*, *PPAR γ* , *CEBP α* and *ap2* were decreased in a dose dependent manner. Similarly, the Rg5:Rk1 treatment reduced *PPAR γ* and *CEBP α* protein expression.

Conclusion: Rg5:Rk1 treatment exhibits anti-adipogenic activity by down-regulation of the *STAT3/PPAR γ /CEBP α* signaling pathway in 3T3-L1 adipocyte cell line.

Keywords: Adipogenesis, Ginsenosides, Obesity, PPAR- γ 3T3-L1 cell.

1. Background

Obesity is considered a foremost health problem and major risk factor for serious metabolic diseases such as diabetes, hypertension, heart disease, dyslipidemia, atherosclerosis, stroke, nonalcoholic fatty liver disease, neuronal injury and cancer (1-4). Moreover, obesity is defined as the imbalance between energy intake and expenditure that eventually leads to the accumulation of the excess amount of lipids (triglycerides) in a major cellular component adipocytes cell. Adipocytes play an important role in regulating lipid metabolism (5, 6). However, adipogenic differentiation is a complex process where pre-adipocytes are converted into adipocytes by regulating multiple signaling pathways. In this process, genes such as peroxisome proliferator-

activated receptor gamma (PPAR γ) and CCAAT enhancer binding proteins (C/EBP) are required to be transcribed for adipogenic differentiation. These so-called master regulators of adipogenesis control the expression of a variety of transcriptional factor to the formation of mature adipocytes (7). In addition to these transcription factors, recent studies have shown that the signal transducer and activator of transcription 3 (STAT3) and Jak2 (Janus kinase 2) play important roles in adipogenic differentiation by regulating PPAR γ (8). Therefore, suppression of this process is essential to control adipogenesis. Many studies have aimed to reduce obesity by focusing on decreasing pre-adipocyte differentiation, inhibiting adipogenesis and increasing lipolysis (9). Although, a lot of drugs are available in

market to treat obesity, but their prolonged exposure may cause severe side effects. Thus, scientists are looking forward for new natural sources which have a strong potential of anti-adipogenic activity.

Natural compounds from different sources have gained attention owing to safety and extended efficacies in the development of pharmaceutical agents against obesity. Several natural plants, have been shown to exert anti-adipogenic through the modulation of different signaling pathway (10). *Panax ginseng* is one of the ancient beneficial medicinal plant has reported for the various pharmacological effect such as anti-cancer, anti-inflammation, anti-obesity, bone osteoporosis, sexual enhancement and diabetics (11). Ginsenoside Rg5:Rk1 is a mixture of protopanaxadiol-type ginsenoside, isolated from *P. ginseng* root, via a nine-time repetitive steaming and drying process (black ginseng), and was shown to possess many biological effects such as osteoporosis and inflammation (12, 13). However, the effect of Rg5:Rk1 in adipogenesis and the related underlying mechanism have not yet been investigated.

2. Objective

In this study, the anti-adipogenic effect of ginsenoside Rg5:Rk1 was investigated. Cytotoxicity and lipid accumulation or TG content in 3T3-L1 mature adipocytes were evaluated. Furthermore, the ability of ginsenoside Rg5:Rk1 to reduce the expression of different adipogenic markers in 3T3-L1 mature adipocytes was assessed.

3. Materials and Methods

3.1. Chemicals

3T3-L1 fibroblast cells were purchased from the ATCC, American Type culture collection. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin streptomycin (P/S). Cell culture media Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum (BCS) was obtained from Welgene (Gyeongsangbuk-do, South Korea), the antibiotic solution was purchased from GeneDEPOT (Barker, TX, USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone were purchased from Wako (Tokyo, Japan), PPAR γ (cat no: sc 7273, mouse monoclonal), CEBP α (cat no: sc-61) and Beta actin (cat no: sc-47778, mouse monoclonal) primary antibody were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The ginsenoside Rg5:Rk1 mixture (95% purity) was

obtained from Ginseng Genetic Resource Bank (Kyung Hee University, Korea) and dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C.

3.2. Cell Culture and Differentiation of Adipocytes

3T3-L1 fibroblast was grown and differentiated into the adipocyte. The cells were maintained in DMEM supplemented with 10% BCS and 1 % penicillin-streptomycin (P/S) (complete medium) at 37 °C in a CO₂ incubator. Cells were plated at density 1×10^4 in a 24 well plate complete medium. Two or three days after confluency (day 0) of the cells were stimulated with differentiation medium (MDI) containing 1 μ M dexamethasone, 0.5 mM IBMX, and 10 μ g.mL⁻¹ insulin added into Complete media. After two days later, 3T3-L1 cells were supplemented with insulin in complete media defined as differentiation medium (DM) in the presence or absence of ginsenoside Rg5:Rk1 and further incubated for additional 5-8 days. Approximately the day 8 adipocyte cell displayed the characteristic of the differentiated cell (with lipid droplet) under the microscope.

3.3. Cytotoxicity Assay

Cytotoxicity of ginsenoside Rg5:Rk1 was determined by the MTT assay. 3T3-L1 pre-adipocytes were seeded in a 96 well plate at a density of 1×10^4 cells/well. After confluency (24 h incubation), the media was fully removed and the cells were treated with various concentrations (1-100 μ g.mL⁻¹) of ginsenoside Rg5:Rk1 for 72 h. MTT assay solutions (20 μ L) were added to the treated cells to stain the pre-adipocyte cell. Cell viability was determined using an ELISA reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA) at 570 nm as previously described (14).

3.4. Oil Red O Staining and TG Content

To investigate the excess lipid accumulation in adipocyte cell Oil red O (ORO) staining assay was performed. After differentiation of 3T3-L1 pre-adipocyte to mature adipocyte on day 8, cells were washed with PBS and fixed with 3.7% (v/v) formaldehyde for 1 h. Cells were stained with 60% isopropanol in filtered ORO solution (6:4 of oil red stock solution: distilled water) for 30 min. The excess stain was removed by washing with distilled water 3 to 4 times before observation under an inverted light microscope (Nikon Instruments, Melville, NJ, USA). For the quantitative determination of excess lipid (TG content) in adipocytes, ORO staining eluted with 100% isopropanol and the absorbance was measured at 520 nm using Synergy™2 microplate reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA).

3.5. RNA Isolation and RT-PCR

Total RNA was extracted from differentiated 3T3-L1 on day 8 using TriZol LS reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. First strand cDNA was synthesized with 1 µg of total RNA by using Thermo Scientific cDNA synthesis kit (Onebio, Lithuania, EU) according to the protocol. The Reverse-transcription polymerase chain reaction (RT-PCR) was performed on the cDNA using gene-specific primers (Table 1). For amplification of *PPAR-γ* and *STAT3* the PCR cycles (31) were 94 °C for 40 s; 58 °C; 72 °C for 5 min. PCR amplicons were loaded in 1% agarose gel for electrophoresis.

3.6. Real-Time PCR

The gene expression levels were analyzed by quantitative real-time RT-PCR using a real-time rotary analyzer (Rotor-Gene 6000; Corbet Life Science, Sydney, Australia). The reaction was performed by using SYBER® Green SensiMix plus Master Mix (Quantace, England), with gene specific primers (Table 1). The thermo reaction (10-14 µL) was contained 1-2 µg of cDNA. Real-Time PCR was carried out for 40 cycles of 95 °C: 10 sec; 60 °C: 10 sec; 72 °C: 20 sec. β-actin was used as housekeeping gene to normalize the data (15).

3.7. Western Blotting

Cells were seeded in 6 well plate at a density of 1×10^4 and adipocyte differentiation was induced as described above with the presence or absence of ginsenoside Rg5:Rk1 at concentration of 100 µg.mL⁻¹. After differentiation on day 8, cells were rinsed twice with PBS and incubated with 2X sodium dodecyl sulfate (SDS)

loading buffer containing 100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM β-mercaptoethanol for 5 min at 22 °C (17). Cell lysates were collected and denatured at 95 °C for 10 min. Protein samples were loaded to 10% SDS-polyacrylamide gel and electrophoresed. The separated proteins were transferred to PVDF membrane (ATTO Corporation, Tokyo, Japan) at 100 V for 2 h. Membranes were blocked with 5% skimmed milk for 2 h and incubated with specific primary antibodies such as PPARγ, CEBPα (Santa Cruz Biotechnology, Inc) for 16 h at 4 °C. The membranes were washed 7 times with Tris-buffered saline and Tween 20 (TBST). The blot was probed by anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc) for 2 h at 22 °C and immuno-reactivity was detected through Enhanced Chemiluminescence system (Amersham Bioscience Inc., Piscataway, NJ, USA) and exposed to X-ray film (Fuji photo Film Co. Ltd Minato, Tokyo, Japan). Band densities were measured using ImageJ software (16).

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad 6.04 software (La Jolla, CA). Data are expressed as means ± standard error of mean (SEM) of three independent experiment. The statistical significance of the differences between values was evaluated by one-way ANOVA. Statistical significance was accepted at a level of $p \leq 0.05$.

4. Results

Effects of Ginsenoside Rg5:Rk1 on Cell Viability

Ginsenoside Rg5:Rk1 exerted no cytotoxic effect at

Table 1. Primers used for RT-PCR and quantitative real-time RT-PCR.

Primer Name	Sequence	Tm (°C)
<i>PPAR-γ</i>	Forward: 5'-ATGGGTGAAACTCTGGGAGATT-3'	58.4
	Reverse: 5'-AGCTTCAATCGGATGGTTCTT-3'	55.9
<i>CEBPα</i>	Forward: 5'-TTCATGGAGAATGGGGGCAC-3'	59.3
	Reverse: 5'-TAGACGTGCACACTGCCATT-3'	57.3
<i>STAT3</i>	Forward: 5'-GCCACGT TGGTGTTCATAATC-3'	58.4
	Reverse: 5'-TTCGAAGTTGTGCTGATAGAG-3'	58.4
<i>ap2</i>	Forward : 5'-AAAGACAGCTCCTCCTCGAAGGTT-3'	66.7
	Reverse: 5'-TGACCAAATCCCCATTTACGC-3'	57.9
β-actin	Forward: 5'-ATGAAGTGTGACGTTACGC-3'	52.94
	Reverse: 5'-CCTAGAAGCATTTCGGGTGCAC-3'	64.75

concentration up to 100 $\mu\text{g}\cdot\text{mL}^{-1}$ whereas more than 98% cells were viable (Data not shown).

4.1. Effect of Ginsenoside Rg5:Rk1 on Intracellular Lipid Accumulation During Differentiation in 3T3-L1 Pre-adipocyte

The effect of Rg5:Rk1 was tested to determine if it can suppress adipogenesis differentiation in 3T3-L1. Cells were incubated with adipocyte hormonal cocktail with the presence or absence of Rg5:Rk1 at different concentration. Inside fat droplet, formation was analyzed by ORO assay. As shown in Figure 1a, intracellular lipid accumulation reduced to 100 $\mu\text{g}\cdot\text{mL}^{-1}$ compared with MDI-treated cells used as the positive control. In addition, TG content suppressed at same concentration (Fig. 1b).

4.2. Effect of Ginsenoside Rg5:Rk1 on Adipogenic Markers *STAT3*, *PPAR γ* , *CEBP α* , *ap2*

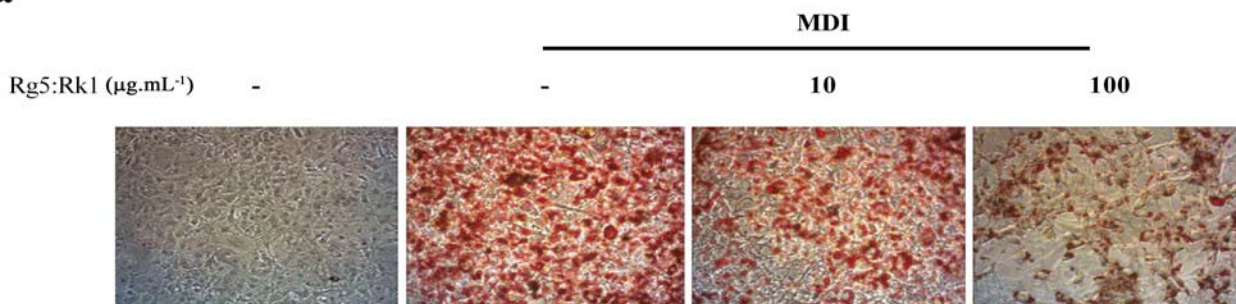
Adipocyte differentiation is inflicted by the master regulator *PPAR γ* involved in triglyceride synthesis and pre-adipocyte to adipocytes. Therefore, 3T3-

L1 cells were differentiated into mature adipocyte in the presence/absence of ginsenoside Rg5:Rk1. The expression of adipocyte markers, *STAT3*, *PPAR γ* , *CEBP α* , and *ap2*, were analyzed by both RT-PCR and qRT-PCR. *STAT3*, *PPAR γ* , *CEBP α* , and *ap2* were significantly suppressed in a dose-dependent manner (Figs. 2a-2d). RT-PCR data showed that the expression of *STAT3* and *PPAR γ* were significantly decreased by the treatment with ginsenoside Rg5:Rk1 at the 100 $\mu\text{g}\cdot\text{mL}^{-1}$. Therefore, ginsenoside Rg5:Rk1 may have a natural anti-adipogenic compound.

4.3. Inhibitory Effect of Ginsenoside Rg5:Rk1 on the Expression of Adipogenic Protein Expression

The effect of Rg5:Rk1 (100 $\mu\text{g}\cdot\text{mL}^{-1}$) on the expression of *PPAR γ* and *CEBP α* involved in adipogenesis were examined. Western blot analysis revealed that a time-dependent increase in the protein expression levels of *PPAR γ* and *CEBP α* in 3T3-L1 pre-adipocytes induced by the treatment of MDI in positive control compared with control (cell culturing complete media containing 10% BCS, 1% penicillin streptomycin in DMEM).

a



b

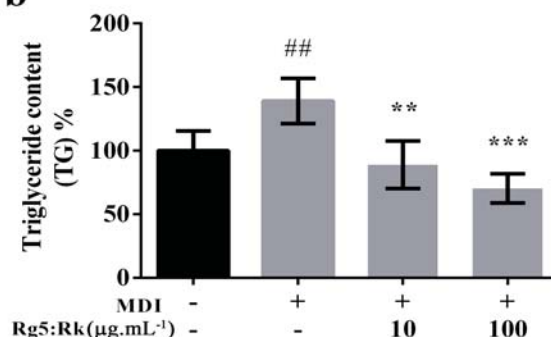


Figure 1. Effect of different concentration of ginsenoside Rg5:Rk1 on MDI-induced intracellular lipid accumulation in 3T3-L1 cells. The data shown are representatives of triplicate experiments. a: Demonstration of reduction of lipid accumulation in the cultured cells treated with ginsenoside Rg5:Rk1, in a dose dependent manner. b: Comparative study of the Triglyceride content of the cells, treated with various amount of ginsenoside Rg5:Rk1. Data are expressed as a percentage of control. ## (MDI treated cell) $p < 0.01$ compare with control (absence of MDI and only given complete media) and **: $p < 0.01$; ***: $p < 0.001$ versus MDI treated cell (differentiated cell).

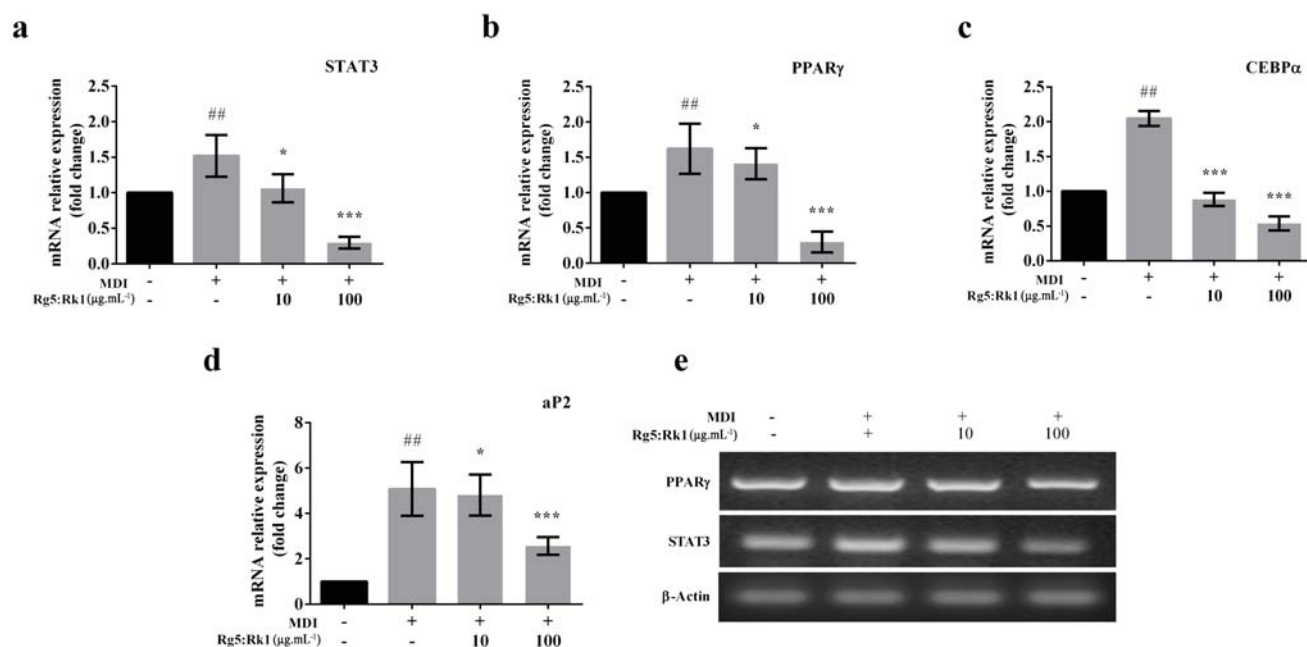


Figure 2. Effect of different concentration of ginsenoside Rg5:Rk1 on adipogenic transcriptional factors in 3T3-L1 adipocytes. Panels a-d: The quantification of mRNA levels of *STAT3*, *PPAR γ* , *CEBP α* and *aP2* genes were analyzed by qRT-PCR and normalized with β -actin as house keeping gene. Each value is expressed as the mean \pm standard error of three independent experiments. ## (MDI treated cell) $p < 0.01$ compare with control (absence of MDI and only given complete media) and *: $p < 0.01$; ***: $p < 0.001$ versus MDI treated cell (differentiated cell). Panel e: The mRNA expression levels of *PPAR γ* and *STAT3* by RT-PCR.

Whereas, treatment with Rg5:Rk1 (100 $\mu\text{g.mL}^{-1}$) significantly inhibited the adipogenic protein expression such as *PPAR γ* and *CEBP α* (Fig. 3).

5. Discussion

This study exposed for the first time the anti-adipogenic effect of Rg5:Rk1 on the suppression of adipocyte differentiation through down-regulation of transcriptional factors such as *PPAR γ* , *STAT3*, *aP2* and *CEBP α* . Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes to mature adipocytes in adipose tissue (18). Therefore, in order to interpret the potential anti-obesity effect and the mechanisms involved, the optimal working concentration of ginsenoside Rg5:Rk1 (Rg5:Rk1) was checked by evaluating its cytotoxicity on 3T3-L1 pre-adipocytes. The result showed no significant cytotoxicity effect on 3T3-L1 up to 100 $\mu\text{g.mL}^{-1}$.

The development of fat cells from pre-adipocyte to mature adipocyte includes morphological changes, increase in cell numbers, expansion of cell size, expression of lipogenic enzyme and extensive

accumulation of lipid droplet (19-21). In addition, accumulation of lipid droplet is an index of adipogenesis and it can be confirmed by ORO staining assay, which stains TGs and lipids in the mature adipocytes (22). So far, many studies have been reported that ginsenosides from *Panax ginseng* Meyer can reduce lipid accumulation in mature adipocyte by up and/or down regulation of several signaling pathways at *in vitro* level (23-25). Our data showed that, Rg5:Rk1 effectively inhibited the adipogenic differentiation (reduced lipid droplet size) in 3T3-L1 pre-adipocytes at 100 $\mu\text{g.mL}^{-1}$ (Fig. 1a). Similarly, the TG content in mature adipocytes significantly reduced at 100 $\mu\text{g.mL}^{-1}$ (Fig. 1b). Taken together, these data suggest that Rg5:Rk1 may have anti-adipogenic effect by reducing lipid droplet accumulation in 3T3-L1 mature adipocyte. Adipogenesis is the procession of the reflection of lipid accumulation of intracellular adipocytes through several signaling pathway by inducing overexpression of transcriptional factors such as *STAT3*, *PPAR γ* , *CEBP α* and *aP2* (26, 27). Among them, *PPAR γ* and *CEBP α* are the major transcriptional factors regulating adipogenesis through modulating the expression of its target genes during early to middle stage of differentiation. Specially,

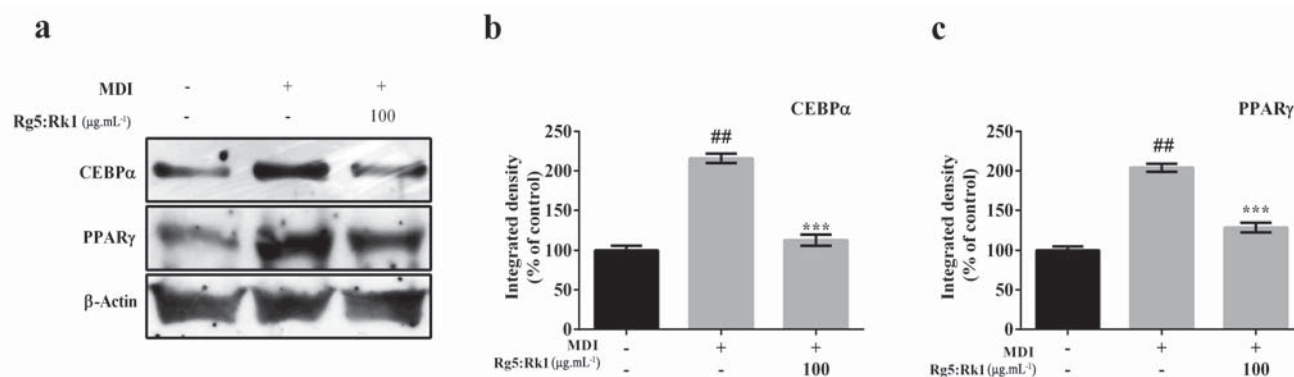


Figure 3. Protein expression levels of PPAR and CEBP were measured by western blotting (panel a), and integrated density was analyzed by ImageJ software (panels b and c). Integrated density values were expressed as % of control. All experiment were performed in triplicate.

PPAR γ (peroxisome proliferator-activated receptor-gamma) is highly expressed in adipocyte tissue compare with other tissues, and highly up-regulated when pre-adipocytes are converted into mature adipocyte (28). Besides, CEBP α (CCAAT/enhancer binding protein-alpha) primarily expressed in fat, liver and intestinal tissues, and is also reported as a key regulator of adipogenesis. Particularly, PPAR γ and CEBP α were reported to act as a cross-regulator on adipogenesis. These transcription factors control the adipogenic target gene such as *ap2* (activating protein), which is directly implicated in lipogenic pathways in 3T3-L1 adipocytes (7). In addition, recent studies showed that signal transducer and activator of transcription 3 (STAT3) plays an imperative role in adipogenesis process (29). Furthermore, several studies have shown adipocyte differentiation is largely influenced by the expression of PPAR γ and CEBP α (30-32). We found that transcript analysis of *STAT3*, *PPAR γ* , *CEBP α* and *ap2* genes, were significantly enhanced by the stimulation of hormone cocktail (MDI) once compared with undifferentiated cells. On the other hand, mRNA expression levels of these genes were decreased in differentiated cells after treatment with Rg5:Rk1 at 100 $\mu\text{g.mL}^{-1}$ (Figs. 2a-d). In addition, RT-PCR data showed that *STAT3*, *PPAR γ* genes were significantly blocked by the treatment of Rg5:Rk1 at concentration 100 $\mu\text{g.mL}^{-1}$ (Fig. 2e). Therefore, these results support that Rg5:Rk1 is capable of blocking adipogenic transcriptional factors. Subsequently, we investigated the protein expression level of major adipogenic proteins, PPAR γ and CEBP α . It was found that Rg5:Rk1 significantly down-regulated protein expression levels at 100 $\mu\text{g.mL}^{-1}$ (Figs. 3a-c). Results indicates that expression of mRNA level of

adipogenic genes and adipogenic proteins are similarly downregulated by the treatment of Rg5:Rk1 at 100 $\mu\text{g.mL}^{-1}$. Taken together, this data suggest that reduction of lipid accumulation in 3T3-L1 cells after Rg5:Rk1 treatment might be regulated by down-regulation of the STAT3/ PPAR γ /CEBP α signaling pathway. However, this study is limited by the nature of pre-adipocytes growth cycle that remains to be elucidated.

In conclusion, our results demonstrate that Rg5:Rk1 from *Panax ginseng* Meyer efficiently inhibited adipogenesis in 3T3-L1 adipocytes by a significant reduction in intracellular lipid accumulation without induction of cytotoxicity in a dose-dependent manner. Furthermore, the inhibition of adipogenesis by Rg5:Rk1 might be mediated by major transcriptional activators PPAR γ and CEBP α . Therefore, ginsenoside Rg5:Rk1 may provide a possible therapeutic approach for the treatment of obesity.

Acknowledgements

This research was supported by iPET (313038-03-1-SB010), Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea. The ginseng sample used in this study was provided by Ginseng Bank Kyung Hee University.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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