Ginsenoside Rh1 Ameliorates High Fat Diet-Induced Obesity in Mice by Inhibiting Adipocyte Differentiation

Wan Gu, Kyung-Ah Kim, and Dong-Hyun Kim*

Department of Life and Nanopharmaceutical Sciences, Kyung Hee University; Seoul 130–701, Republic of Korea.
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Ginseng (the root of Panax ginseng C. A. Meyer), which contains protopanaxadiols and protopanaxatriols as its main constituents, has been used for many disorders, such as cancer, diabetes, inflammation, and hyperlipidemia. Of these ginsenosides, protopanaxadiol ginsenoside Rh2 alone is reported to inhibit adipogenesis in 3T3-L1 in vitro. Therefore, we investigated the effect of protopanaxatriol ginsenoside Rh1 on adipogenesis in 3T3-L1 cells and high fat diet-induced obesity (DIO) mice. Treatment with ginsenoside Rh1 inhibited adipogenesis, as evidenced by Oil red O staining and lipid droplet extraction assay. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that ginsenoside Rh1 decreased the expressions of peroxisome proliferator-activated receptor (PPAR)-γ, CCAAT/enhancer-binding protein (C/EBP)-α, fatty acid synthase, and adipocyte fatty acid-binding protein. Oral administration of ginsenoside Rh1 (20 mg/kg) suppressed body and epididymal fat weight gains and plasma triglyceride level in DIO mice. Ginsenoside Rh1 also inhibited the expressions of PPAR-γ, C/EBP-α, fatty acid synthase, adipocyte fatty acid-binding protein, as well as F4/80, CD68, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in DIO mice by real time PCR analysis. Based on these findings, ginsenoside Rh1 may ameliorate obesity, by inhibiting adipocyte differentiation and inflammation.

Key words Panax ginseng; ginsenoside Rh1; adipogenesis; obesity

Obesity is a chronic, stigmatized, and highly-priced disease that is intractable and its prevalence is increasing throughout most of the world.\(^1,2\) It is an abnormal condition accumulating lipid in adipose tissues, which secrete adipokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, leptin, resistin and adiponectin) closely associated with inflammation. Obesity is caused by various environmental and genetic factors. Of them, a main environmental factor is the consumption of a high-fat diet. The obesity is an important risk factor for various diseases such as cardiovascular diseases, type 2 diabetes and some cancers caused by hyperglycemia, insulin resistance, dyslipidemia and hypertension. Therefore, it is very important to prevent obesity for a healthy life.

Ginseng (the root of Panax ginseng C. A. Meyer), which contains ginsenosides classified into protopanaxtriols and protopanaxadiols as main constituents, has been used in Chinese traditional medicines for type 2 diabetes, inflammation, cancer and hyperlipidemia.\(^3,4\) Of ginsenosides, protopanaxtriols such as ginsenoside Rb1, ginsenoside Rh2 and compound K, have anti-cancer effect, enhancement of proliferation and differentiation of bone marrow cells.\(^5,6\) Ginsenoside Rh2 also exhibits the inhibitory effect against 3T3-L1 cells differentiation in in vitro study.\(^7,8\) However, in vivo study of ginsenoside Rh2 has not been performed. Among protopanaxadiols, ginsenosides Re and Rg1 are main constituents in ginseng, followed by ginsenoside Rh1. However, ginsenosides Re and Rg1 are metabolized to ginsenoside Rh1 or protopanaxatriol by human intestinal microflora.\(^9\) The ginsenoside Rh1 exhibits anti-allergic and anti-inflammatory and estrogenic effects.\(^10,11\) However, its anti-obesity effect has not been studied.

As a part of our studies on anti-obesity constituents from natural medicines, we isolated the main constituents, protopanaxatriol ginsenosides Re, Rg1 and Rh1, from ginseng in our preliminary experiment. Therefore, we examined the effect of ginsenoside Rh1 on the adipogenesis in 3T3-L1 cells and diet-induced obesity (DIO) mice.

MATERIALS AND METHODS

Materials Insulin, isobutylmethylxanthine, dexamethasone, Oil red O reagent, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and antibiotics (AB), were all purchased from Sigma Chemical Co. (MO, U.S.A.). Anti-PPARγ antibody were purchased from Santa Cruz Biotechnology (CA, U.S.A.). Ginsenoside Rh1 was isolated from the root of Panax ginseng according to a previously published method.\(^9,10\)

Cell Culture Mouse embryo fibroblasts 3T3-L1 cells were obtained from American Type Culture Collection (VA, U.S.A.) and incubated in DMEM, containing 10% FBS and 1% AB, at 37°C and 5.6% CO₂ atmosphere. To induce differentiation, two days after confluence, preadipocytes (designated day 0) were cultured in the differentiation medium (DM), which is consisted of DMEM, 10% FBS, 1% AB, and DMI (0.28 unit/mL insulin, 0.5 mM isobutylmethylxanthine and 1 μM dexamethasone) for 2 d in the presence or absence of 50 μM and 100 μM of ginsenoside Rh1, and switched to DM containing 10% FBS and 10 μg/mL insulin and then changed to DMEM medium with 10% FBS for every 2 d.

Oil-Red O Staining Oil red O staining was performed on day 8. Briefly, cells were washed twice, with phosphate-buffered saline (PBS), and were fixed with 4% paraformaldehyde for 1h. They were washed three times with water and stained with Oil red O (6 parts of 0.6% Oil red O dye in isopropanol and 4 parts of water) for 1h. Excess stain was removed by washing with water and the stained cells were dried. Spectrophotometric quantification of the stain was performed by dissolving the stained oil droplets in 100% isopropanol for 10 min. Optical density was then measured at 500 nm.

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: dhkim@khu.ac.kr© 2013 The Pharmaceutical Society of Japan
**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**  
Total RNA was extracted from the 3T3-L1 cells and epididymal fat, using a total RNA extraction kit (Qiagen, Germany). cDNA synthesis for PPAR-γ, CCAAT/enhancer-binding protein (C/EBP)-α, fatty acid synthase (FAS), CD68, F4/80, TNF-α, IL-1β, and IL-6 was performed with 2 µg of total RNA, oligo-dT primers, and a reverse transcriptase in total volume of 40 µL. PCR reactions were performed in a total volume of 50 µL, comprising 4 µL of cDNA product, and 25 µL of Premix EX Taq (Takara Bio Inc.). PPAR-γ for, 5′-ACT GCC TAT GAG CTC TTC AC-3′, and rev, 5′-CAA TGG GAT GGT TCT TCG GA-3′; C/EBP-α for, 5′-TGG ACA AGA ACA GCA AGC-3′, rev 5′-AAT CTC CTA GTC CTG GCT TG-3′; FAS for, 5′-TGC TCC CAG CTG CAG GC-3′, rev, 5′-GCC CGG TAG CTC TGG GTG TA-3′; adipose fatty acid binding protein (aFABP) for, 5′-ATG AGT ACT ACA TGG CTA-3′, rev, 5′-CAA TGG GGG CCA GAT CAT-3′; GAPDH for, 5′-CAT CAC CTT CCA GGA GGC-3′, rev, 5′-TGA CCT TGC CCA CAG CCT GT-3′; CD68 for, 5′-TTG TCC GGT GTG AGG AGG AGT TG-3′; TNF-α for, 5′-AAT CTT CCC AAA AGG GCT TAT-3′, F4/80 for, 5′-TCT CCA AAG AGT GGA TGA TGC AC-3′, rev, 5′-AAT CAC GGT GAG TGC GCA AGC-3′; C/EBP-α for, 5′-CCA GAG CAT GGT GCC TTC GCT-3′, and rev, 5′-GCC TGC ACC TTC AGA CTG GTG AC-3′, 5′-GAA TGT CCA CAA ACT GAT ATG CCT-3′. The reaction conditions were as follows: denaturation at 94°C for 30 s; annealing at 50°C (aFABP), 56°C (PPAR-γ, FAS, GAPDH, IL-6, CD68), 58°C (TNF-α, F4/80), 60°C (EBP-α) for 30 s; and extension at 72°C for 1 min. Varying cycles of PCR were carried out to determine the linear ranges of PCR products. PCR products were electrophoresed on a 2% agarose gel and visualized with UV.

**Immunoblotting**  
Epididymal fat pad of mice was homogenized in radio immunoneutralization assay (RIPA) lysis buffer (Pierce, IL, U.S.A.). Total proteins (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and then hybridized overnight, at 4°C with 1:1000 diluted PPAR-γ, C/EBP-α, FAS, aFABP antibodies. After incubation with 1:2000 diluted anti-rabbit or anti-mouse immunoglobulin G secondary antibody for 1 h at room temperature, it was then washed 3 times with PBST (PBS+Tween 20 0.1%) for 10 min. Bands were visualized with enhanced chemiluminescence reagent. **Enzyme-Linked Immunosorbent Assay (ELISA)**  
For the ELISA of IL-1β, IL-6 and TNF-α, the epididymal fats were homogenized in a 600 µL of ice-cold RIPA lysis buffer, which contained 0.1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (10000×g, 4°C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. IL-1β, TNF-α and IL-6 concentrations were determined, using commercial ELISA kits (Pierce, IL, U.S.A.).

**Animals**  
Male C57BL/6j mice were purchased from Central Lab. Animal Inc. (Seoul, Korea) at the age of 4 weeks. After 3 d of acclimation, mice were either placed in a low-fat diet (LFD, 10% of calories from fat; Research, NJ, U.S.A.) or a high-fat diet (HFD, 60% of calories from; Research, NJ, U.S.A.). All mice were maintained under standardized conditions with the cycle of 12 h light, and 12 h dark.

Mice were separated into 3 groups, LFD, HFD and HFD-Rhl. Each group is consisted of ten mice. LFD group fed LFD for 8 weeks, HFD group fed HFD for 8 weeks. HFD-Rhl group fed HFD diet for 4 weeks and then simultaneously treated with HFD and 20 mg/kg/d ginsenoside Rh1, which was orally administrated. Weight and food intake of mice were measured daily. After finishing treatment for 4 weeks, blood and epididymal fats were collected for further analysis.

For histological analysis, the epididymal fat pad was removed and fixed in 4% paraformaldehyde. The fat pads were subsequently dehydrated by the exposure to increasing ethanol concentrations, and embedded in paraffin, sectioned with 7 µm thickness, and stained with hematoxylin-eosin for microscopic assessment.

All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use. The study was approved by the committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

**Real Time-Polymerase Chain Reaction**  
Reverse transcription was performed with 2 µg of total RNA. Real time PCR for PPAR-γ, C/EBP-α, FAS, CD68, F4/80, TNF-α, IL-1β, IL-6, and β-actin was performed as described previously, utilizing Takara thermal cyclers, which used SYBER premix agents, as per the instructions from Takara Bio Inc. PPAR-γ for, 5′-CCA GAG CAT GGT GCC TTC GCT-3′, rev, 5′-CAC CAA TCT GGT GAG GCT-3′, C/EBP-α for, 5′-GAA CAG CAA GTG GCC AGG GGT-3′, rev, 5′-GCC ATG GGC TGT GCT TAC TAG TGA TAA-3′; aFABP for, 5′-TAC CGG TCC CTA AAC GAA TTG TGG-3′; IL-6, 5′-CAC AGC CTG GTC ATG GAC TTC CTA AAC GAA CC-3′, rev, 5′-GCC GAG TGT GCT GAC ATG CCT CGT ACC TCT GTC-3′; CD68 for, 5′-TCC AGG GTG GAA TGG TAA GAA AA-3′, rev, 5′-CAA TGG GAG CCA GCA AGA GG-3′; F4/80 for, 5′-TCA GAG CAT GGT GCC TTC GCT-3′, rev, 5′-GTG GCA TGA GCT GCA AGC-3′; GAPDH for, 5′-GAA TGC TGA AAA GAT GAC CCA GAT C-3′, rev, 5′-GCT GCA TGA GGT GCC TTC GCT-3′, IL-1β for, 5′-CCA GAG CAT GGT GCC TTC GCT-3′; cytokines (IFN-γ, TNF-α, IL-1β, IL-6) were determined, via the use of ultrasensitive ELISA kit (ALPCO, Salem, NH, U.S.A.).

**Statistical Analysis**  
The data were expressed as the
RESULTS AND DISCUSSION

First, we examined the effect of ginsenoside Rh1 on adipogenesis in 3T3-L1 cells (Fig. 1A). Ginsenoside Rh1 potently inhibited adipogenesis, as assessed by Oil-red O staining and lipid contents in 3T3-L1 adipocytes. Ginsenoside Rh1, at concentrations of 50 µM and 100 µM, inhibited the adipogenesis by 50% and 63%, respectively (Fig. 1B).

Next, we examined the expression levels of adipocyte-specific genes such as PPAR-γ, C/EBP-α, FAS, aFABP and some genes during early phase of differentiation such as Pref-1, C/EBP-δ and GR. After the treatment with ginsenoside Rh1 in 3T3-L1 cells, mRNA was extracted on 18 h and 24 h for Pref-1, C/EBP-δ and GR and day 8 for PPAR-γ, C/EBP-α, FAS, aFABP. Then, the expression profiles of adipocyte-specific genes were investigated by RT-PCR (Fig. 1C). PPAR-γ, C/EBP-α, FAS, and aFABP expressions were significantly increased in DMI-stimulated differentiated adipocyte compared to those of non-stimulated adipocyte cells. However, treatment with DMI in the presence of ginsenoside Rh1 significantly suppressed the expression levels of PPAR-γ, C/EBP-α, FAS, and aFABP in a dose-dependent manner, whereas the expression levels of Pref-1, C/EBP-δ and GR were not affected (data not shown). Ginsenoside Rh1 at tested concentrations (within 150 µM) did not affect viability of 3T3-L1 cells by crystal violet staining.

When HFD fed mice for 8 weeks, body and epididymal fat weight gains were significantly increased compared to those of LFD-fed mice (Table 1). However, when ginsenoside Rh1 was treated in HFD-fed mice, body and epididymal fat weight gains were significantly decreased compared with those of the HFD-fed mice (Table 1). TG, glucose, insulin, total cholesterol, and HDL levels in the blood were significantly increased in HFD-fed mice group compared to LFD-fed mice group (Table 1). However, treatment with ginsenoside Rh1 in HFD-fed mice significantly lowered TG level alone (Table 1).

We also investigated epididymal fat pad by histological
The number of bigger epididymal cells was significantly increased in the HFD group as compared to the LFD group. However, treatment with HFD in the presence of ginsenoside Rh1 reduced number of bigger epididymal cells, as well as their sizes (Fig. 2B). Next, we extracted mRNA in epididymal fat tissue, and measured adipogenesis markers by real time-PCR. HFD increased PPAR-γ and C/EBP-α, the main transcriptional factors that are involved in the differentiation of preadipocyte. However, treatment with ginsenoside Rh1 reduced the expression levels of these markers (Figs. 2C).

### Table 1. Effect of Ginsenoside Rh1 on Metabolic Parameters in Mice Treated with High Fat Diet (HFD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LFD</th>
<th>HFD</th>
<th>HFD+Rh1 (20 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>1.89±0.22</td>
<td>8.94±0.54</td>
<td>3.77±0.77**</td>
</tr>
<tr>
<td>Epididymal fat weight (g)</td>
<td>0.39±0.03</td>
<td>2.14±0.14</td>
<td>1.78±0.18**</td>
</tr>
<tr>
<td>Food intake (g/d/mouse)</td>
<td>2.26±0.034</td>
<td>2.26±0.045</td>
<td>1.91±0.054</td>
</tr>
<tr>
<td>Glucose in plasma (mg/dL)</td>
<td>92.72±6.02</td>
<td>179.02±9.71</td>
<td>175.67±13.47</td>
</tr>
<tr>
<td>Insulin in plasma (ng/mL)</td>
<td>0.11±0.03</td>
<td>1.56±0.25</td>
<td>1.36±0.22</td>
</tr>
<tr>
<td>TG in plasma (mg/dL)</td>
<td>92.54±5.36</td>
<td>136.53±12.26</td>
<td>79.45±12.71**</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>85.09±4.42</td>
<td>135.25±4.34</td>
<td>129.92±4.04</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>49.60±6.32</td>
<td>86.71±9.30</td>
<td>91.85±7.05</td>
</tr>
</tbody>
</table>

* Body weight gain was calculated by differences between initial body weight of when ginsenoside Rh1 treated and final body weight of when experiment finished. LFD, low fat diet; HFD, high fat diet; HFD+Rh1, HFD with ginsenoside Rh1 (20 mg/kg). Values are mean±S.E. (n=10). ‡p<0.01 vs. LFD group. **p<0.01 vs. HFD group.

![Figure 2](image-url)
Ginsenoside Rh1 also inhibited PPAR-γ, C/EBP-α, FAS, aFABP protein expression, even when they were measured by immunoblotting (Fig. 2C). FAS and aFABP, which are involved in the synthesis of fatty acids, and CD68 and F4/80, a transmembrane protein present on the cell-surface of macrophages, were measured (Figs. 3C–F). HFD increased these expression levels. However, treatment with ginsenoside Rh1 significantly down-regulated these expression levels. Furthermore, the expression levels of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β were measured. HFD increased these gene expression levels by 50%, 62%, and 76%, respectively. However, ginsenoside Rh1 group suppressed these gene expression levels (Figs. 3G–I). Ginsenoside Rh1 also inhibited TNF-α, IL-6, and IL-1β in blood, even when they measured by ELISA (Figs. 2D–F). However, mice treated with ginsenoside Rh1 did not show the toxicity.

Many studies report that obesity may be related to inflammation.1,2,3 For example, Hwang et al. suggested that ginsenoside Rh2, a representative protopanaxadiol, may suppress adipogenesis by inhibiting PPARγ expression.7 Niu et al. reported that ginsenoside Rh2 can induce differentiation of preadipocyte.8 Nevertheless, its in vivo studies have not been studied. Therefore, to understand the possible role of protopanaxatriol ginsenosides, we measured the inhibitory effects of protopanaxdiol ginsenosides Re, Rgl and Rh1 against adipocyte differentiation (data not shown). Of them, ginsenoside Rh1 inhibited adipocyte differentiation most potently. Furthermore, ginsenosides Re and Rgl were metabolized to ginsenoside Rh1 by human intestinal microflora.9 Ginsenoside Rh1 suppressed the adipogenesis and inflammation markers and the body and epididymal fat weight gains in HFD-treated mice. Ginsenoside Rh1 inhibited the expression of TNF-α, IL-6 and IL-1β, as well as macrophage antigen, CD68 and F4/80. Thus, ginsenoside Rh1 inhibited the infiltration of macrophage into the epididymal tissue of HFD-treated mice. Although the detailed inhibitory mechanism of ginsenoside Rh1 in 3T3-L1 cell adipocyte differentiation was not fully elucidated, ginsenoside Rh1 potently inhibited the dominant adipogenic markers. Based on these findings, ginsenoside Rh1 may inhibit obesity by inhibiting adipocyte differentiation and inflammation.

REFERENCES


