Ginsenoside Rg3 ameliorated HFD-induced hepatic steatosis through downregulation of STAT5-PPAR\(\gamma\)

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Abstract

Healthy expansion of adipose tissue maintains metabolic homeostasis by storing excess chemical energy in increased fat mass. The STAT5-PPAR gamma pathway reportedly regulates adipocyte differentiation, lipid metabolism and adipogenesis. Ginsenoside Rg3 is one of the diverse groups of steroidal saponins, the major active components of ginseng, which have demonstrated pharmacological properties. In this study, we evaluated the therapeutic effects of ginsenoside Rg3 under pathological conditions in vitro and in vivo. We examined the effects of ginsenoside Rg3 on glucose level, insulin sensitivity and lipogenesis in high-fat diet-fed C57BL/6 mice. Ginsenoside Rg3 was also applied to the pre-adipocyte cell line 3T3-L1 to assess the impact on lipogenesis. Ginsenoside Rg3 reduced epididymal white adipose tissue (eWAT) size and hepatic steatosis, and the amount of triglycerides (TGs) in both eWAT and liver. Similar to the murine model, Rg3-treated 3T3-L1 cells showed a reduction in lipid accumulation and amount of total TGs. Ginsenoside Rg3 regulates the expression of PPAR gamma through STAT5 in vitro and in vivo. According to our results, lipid metabolism-related genes were downregulated in the high-fat mice and 3T3-L1 cell line. Rg3 shows potential for the amelioration of obesity-induced pathology, acting though STAT5-PPAR gamma to facilitate the healthy functioning of adipose tissue. This is the first report of evidence that obesity-induced insulin resistance and lipotoxicity can be treated with ginsenoside Rg3, which acts though the STAT5-PPAR gamma pathway in vivo and in vitro.

Key Words:
- ginsenoside Rg3
- hepatic steatosis
- diabetes
- STAT5


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Introduction

Adipose tissue is a major regulator of metabolic function, storing calories after feeding and then releasing free fatty acids (FFAs) during fasting (Rosen & Spiegelman 2014). In obesity, which can be induced by high-fat overfeeding, the size of individual adipose cells expands (hypertrophy), and new adipocytes are produced (hyperplasia) in epididymal white adipose tissue (eWAT) (Wang et al. 2013, Rosen & Spiegelman 2014). Unhealthy features of adipose tissue that occur with obesity are reduced angiogenic remodeling, increased extra-cellular matrix, persistent infiltration of immune cells and a chronic inflammatory state (Sun et al. 2011). However, the expansion of adipose tissue is not always detrimental. Healthy adipose tissue expansion is considered beneficial and is marked by improved insulin sensitivity, reduced angiogenesis, reduced dysfunctional adipocyte hypertrophy and increases in fibrosis, hypoxia and the infiltration of M1 adipose tissue macrophages and NK cells (Sun et al. 2011, Kusminski et al. 2016).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a multi-functional nuclear receptor superfamily involved in many biological processes (Lehrke & Lazar 2005, Ahmadian et al. 2013, Letterova et al. 2014). In adipocytes, PPARγ is required for differentiation, lipogenesis (Brun et al. 1996, Tontonoz & Spiegelman 2008) and survival (Imai et al. 2004). In addition to its role in adipocyte differentiation and lipid metabolism, PPARγ is also important for controlling genetic networks involved in glucose homeostasis (Ahmadian et al. 2013). Moreover, PPARγ regulates the expression of pro-inflammatory factors secreted from adipose tissue, such as tumor necrosis factor-α (TNF-α), which also influence insulin sensitivity (Hofmann et al. 1994). Recently, many reports have examined how the phosphorylation of PPARγ is a crucial factor in modulating adipose tissue function, specifically as it relates to diabetes and obesity (Hosooka et al. 2008, Choi et al. 2010, Li et al. 2011, Banks et al. 2015).

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway responds to the actions of hormones, interferons, colony-stimulating factors and interleukins in immune responses and immune-mediated diseases (O’Shea & Plenge 2012). In the fibroblast precursor 3T3-L1 cell line, the expression of STAT proteins is markedly elevated in the cell and influences differentiation into adipocytes (Stephens et al. 1996). The phosphorylation of STAT5 promotes adipogenesis, is associated with the glucocorticoid receptor (GR) (Floyd & Stephens 2003) and increases the level of PPARγ stimulated by growth hormone regulation (Kawai et al. 2007). Increased expression of STAT5 proteins correlates with the induction of both PPARγ and C/EBPα. Thus, PPARγ and C/EBPα are important transcription factors in adipocyte development (Richard & Stephens 2011, Letterova et al. 2014). In pre-adipocytes, PPARγ enhancer regions lead to the recruitment of a transcriptional activation complex, which includes the transcription factors GR, signal transducer and activator of transcription 5A (STAT5A) and retinoid X receptor, as well as a co-activator complex (Cristancho & Lazar 2011). Overall, STAT5 is one of the factors regulating expression of PPAR in adipocytes and adipose tissue.

Ginsenoside Rg3 is one of the diverse groups of biologically active steroidal saponins found in ginseng, which has demonstrated the ability to elicit pharmacological responses (Attele et al. 1999, Jia et al. 2009). Rg3, a natural compound, has been reported to have diverse functions, including as an anti-cancer agent (Kim et al. 2014, Shan et al. 2015, Tian et al. 2016), a regulator of the NLRP3 inflammasome (Yoon et al. 2015), an antioxidant (Wei et al. 2012) and as a provider of protection against adriamycin-induced cardiotoxicity and LPS-induced acute lung injury (Wang et al. 2015a, Cheng & Li 2016). In type 2 diabetes, Rg3 inhibits the apoptosis of β-cells that is induced by palmitate through modulating p44/p42 MAPK activation and increased glucose in rat myoblast L6 cells (Kim et al. 2009, 2010). In addition, it has been reported that Rg3 downregulates the level of PPARγ though modulation of AMPK activity and the level of CCAAT/enhancer binding protein alpha (C/EBP-α); it was also suggested that Rg3 acts as an anti-obesity effector (Hwang et al. 2009, Zhang et al. 2017). In our study, we found that Rg3 improved insulin sensitivity and ameliorated hepatic steatosis in the mouse model and reduced lipid accumulation in the cell culture model, acting though as the STAT5-PPAR axis. Based on the results, we suggest that this natural compound may be useful in supporting healthy adipose tissue expansion and protecting against pathological expansion, which is a part of obesity-associated diabetes.

Materials and methods

Animal study and tissue staining

All animal-related procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology.
(KRIBB-IACUC), and all procedures were performed in accordance with institutional (National Institutes of Health) guidelines for animal care. All C57BL/6 male mice were housed in a specified-pathogen-free animal facility under a standard light–dark cycle with standard rodent chow or high-fat chow (ENVIGO, Madison, WI, USA) containing 60% calories from fat; water was provided ad libitum. Ginsenoside Rg3, with approximately 98% purity, was obtained from Hanwool Life Sciences (Daejeon, Korea). Male mice, 6 weeks old, were placed on a high-fat diet (HFD) for 4 weeks, and then daily injected intraperitoneally (i.p.) with 1 mg/kg of ginsenoside Rg3 for 8 weeks, while maintaining their HFD. After 12 weeks, all assays were determined from HFD mice (Control; n = 5) and Rg3-treated HFD mice (Rg3; n = 5). Tissues were fixed in formalin and embedded in paraffin prior to sectioning (5 µm thick). Paraffin sections were counterstained with hematoxylin–eosin. For Oil Red O staining, cryo-sections were prepared 15-µm thick and then incubated 12 h in the stain.

Cell culture and cytotoxicity

Mouse 3T3-L1 (CL-173, ATCC) pre-adipocyte cells were cultured in DMEM supplemented with 10% newborn calf serum (Gibco), penicillin G (100 IU/mL) and streptomycin (100 µg/mL). Differentiation of the cell was induced by treating confluent cells with an adipogenic mixture consisting of 10 µg/mL insulin (Sigma), 500 µM isobutylmethylxanthine (Sigma) and 1 µM dexamethasone (Sigma) in the presence of 10% fetal bovine serum (FBS), at 37°C in a 5% CO₂ atmosphere (Duteil et al. 2014). The medium containing the adipogenic mixture was replaced 3 days later with medium supplemented with 10 µg/mL insulin and incubated for 2 days. The medium was exchanged every 2 days until 9 days after the start of differentiation. 3T3-L1 cells were treated with ginsenoside Rg3 (0, 5, 25 and 50 µM) combined with the adipogenic mixture given at the start of differentiation. For determining the cytotoxicity of ginsenoside Rg3, cells were cultured in 96-well plates for 24 h. The MultiTox-Glo Multiplex Cytotoxicity Assay (G9270, Promega) was used to assess the viability of the cells after treatment; the medium was exchanged every 2 days until 9 days after differentiation. For determining the cytotoxicity of ginsenoside Rg3, cells were cultured in 96-well plates for 24 h. The MultiTox-Glo Multiplex Cytotoxicity Assay (G9270, Promega) was used to assess the viability of the cells after treatment; the medium was exchanged every 2 days until 9 days after differentiation.

Western blot analysis

Cell lysate was prepared in RIPA buffer containing a protease inhibitor cocktail (Millipore), and tissues were homogenized using a Precellys24 homogenizer (Bertin Technologies, Paris, France). The protein contents of the cells and tissues were determined using a BCA protein assay kit (Pierce) and 20 µg was used for the Western blot analysis. Primary antibodies specific for PPARγ (2435), FABP4 (3544), SCID1 (2438), CPT1α (12,252), Phospho-STAT5 (9359) and STAT5 (9363) were purchased from Cell Signaling Technology. Antibodies against β-actin (sc-47778) were purchased from Santa Cruz.

mRNA analysis

Total RNA was prepared from each cell and tissue sample using RNA prepkit (Qiagen), as recommended by the manufacturer’s instructions. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using ReverTra Ace qPCR Master Mix (Toyobo, Osaka, Japan), according to the manufacturer’s instructions. For the PCR amplification of target mRNAs, 2 µL of cDNA was used for each 20µL. The following primers were used:

- Ppar, 5′-GGAGGCTAAGTTGAGTTGTGCTGTG-3′ (forward), 5′-TGAGCAGGGTCTGTGGAGATG-3′ (reverse);
- FABP4 5′-TGAGAGCTGAAGTGTGTCTC-3′ (forward), 5′-GAATTCACCGCCACCCGGTGA-3′ (reverse);
- SCID1 5′-TCACACACTCAGGGCCAAC-3′ (forward), 5′-CTGGATTGGGCATAGGGCAAGA-3′ (reverse);
- CPT1A 5′-TCATTGGGCCACAGTTCCAITA-3′ (forward), 5′-CCAATGCCTGCCACACTCTC-3′ (reverse).

Knock-down of Stat5 in 3T3-L1 cell

We cultured the 3T3-L1 cell for 2 days, and transfected siRNA using Dynamic TransIT-X2 Dynamic Delivery System (Mirus, Madison, WI, USA). After 1 day, we treated MDI and incubated for 1 h or 3 days and then prepared the sample of protein and total RNA. In the case of siPparγ, we cultured 3T3L-1 cell for 3 days, and incubated with MDI. siRNA was transfected into the cell using Dynamic TransIT-X2 Dynamic Delivery System (Mirus). After 1 day, we prepared the sample of total RNA. siRNA specific for control, Stat5 and Pparγ, were purchased from Cell Signaling Technology.

Blood analysis

Mice were fasted for 12 h and then injected i.p. with 2 g/kg of 20% diluted d-glucose (JW-pharma, Seoul, Korea). Blood glucose was measured from the tail vein at various intervals (0, 30, 60 and 120 min). Blood glucose levels of each
individual mouse and the mean ± standard deviation (s.d.) for each group are presented within the glucose tolerance test (GTT) graph. For the insulin tolerance test (ITT), each group of mice was fasted for 5 h and then injected i.p. with 0.75 units/kg of insulin. Blood glucose was measured from the tail vein at various intervals (0, 30, 60 and 120 min). Blood glucose levels in each individual mouse and the mean ± s.d. for each group are presented within the ITT graph. Blood aspartate transaminase (AST) and alanine transaminase (ALT) were analyzed using an automated blood chemistry analyzer (Hitachi 7150; Hitachi).

Quantification of FFAs and triglycerides (TGs) in cells and tissues

The levels of FFAs were determined using the Free Fatty Acid Quantification Assay Kit (ab65341, Abcam). Cells were incubated in chloroform/1% Triton X-100 for 30 min at room temperature and centrifuged at 16,000 g. The supernatant was discarded and the pellet was dried to remove residual chloroform before being resuspended with the buffer provided by the assay kit. Tissues were homogenized using chloroform/1% Triton X-100 and then processed according to the same procedure used with the cells. The assay kit was utilized according to the manufacturer’s protocol. The levels of TGs were determined using the Triglyceride Quantification Assay Kit (ab65336, Abcam). Cells were mixed with 5% NP-40/distilled H2O and incubated in boiling water for 5 min, and the mixing and boiling were repeated once. The samples were then centrifuged at 400 g, and the supernatants were retained. Tissues were homogenized using 5% NP-40/distilled H2O in boiling water for 5 min and homogenizing and boiling were repeated once, after which the procedures were the same as with cell sample preparation. The assay kit was utilized according to the manufacturer’s protocol.

Immunostaining

Cells were seeded on cover slides in 24-well plates. Cells were treated with IBMX (0.5 mM), dexamethasone (1 µM) and insulin (10 µg/mL) for 30 min in DMEM supplemented with 10% FBS, penicillin G (100 IU/mL) and streptomycin (100 µg/mL); treated cells received ginsenoside Rg3. Then, cells were washed with cold phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. Cells were treated with 0.2% Triton X-100 for 15 min. Then, the cells were incubated with STAT5 antibody (9363, CST) at 4°C overnight. The next day, the cells were washed with PBS and incubated with Alex Fluor 546-conjugated goat-anti-rabbit IgG (Invitrogen) for 2 h at room temperature with DAPI (Life Technologies) and images were taken using florescence microscopy (Olympus) and analyzed using Metamorph software.

Statistical analysis

Quantitative data are presented as the mean ± s.d. from representative experiments (n=3 or 5). Statistical analyses were carried out using one-tailed Student’s t-tests. Values of P<0.05 and P<0.01 were considered statistically significant.

Results

Ginsenoside Rg3 reduced lipogenesis in 3T3-L1 cells

The chemical structure of the natural compound ginsenoside Rg3 is shown in Fig. 1A. It has been reported to regulate adipocyte differentiation (Hwang et al. 2009), but it is not clear how Rg3 regulates lipogenesis. Healthy adipose tissue expansion, which involves the accumulation of lipid inside adipose cells, functions in stark contrast with obesity-induced pathologies such as inflammation and insulin resistance (Sun et al. 2011, Kusminski et al. 2016). We first assessed whether Rg3 influences lipogenesis in 3T3-L1 cells. Rg3 has been reported to induce apoptosis and cause cytotoxicity (Kim et al. 2014, Shan et al. 2015, Tian et al. 2016); therefore, we analyzed cell viability to rule out such side effects. The high concentration of 100-µM Rg3 induced cytotoxicity and reduced the viability of 3T3-L1 cells. The LC50 value of Rg3 in 3T3-L1 cell lipogenesis is 200 µM, and our working concentrations (5, 25 and 50 µM) of Rg3 reduced lipogenesis without cell toxicity (Fig. 1B and C). To investigate the inhibiting effect on lipid accumulation by Rg3, we treated 3T3-L1 cells for 3 or 9 days. Oil Red O staining confirmed lipid accumulation in the cell. At 3 days, the lipogenic cocktail MDI (insulin, dexamethasone and isobutylmethylxanthine) induced in pre-adipocytes a very low increase in their accumulated fat deposits. When Rg3 was combined with MDI-induced cells, their lipid accumulation was suppressed (Fig. 1D), in an Rg3 dose-dependent manner, as assessed at 9 days after treatment using Oil Red O staining microscopy data and Oil Red O absorbance at 520 nm (Fig. 1E). In adipocytes, lipid accumulation is related to the amount of TGs in the cell. Similar to the Oil Red O staining results, TG storage was reduced by Rg3 treatment in 3T3-L1 cells in a
dose-dependent manner (Fig. 1F). These data suggest that Rg3 can prevent lipogenesis and lipid accumulation in the 3T3-L1 cell line.

Ginsenoside Rg3 modulates PPAR gamma and lipogenesis genes in 3T3-L1 cells

PPARγ is a key transcription factor of lipogenesis and lipid accumulation in the adipocyte (Brun et al. 1996, Imai et al. 2004, Tontonoz & Spiegelman 2008). Under HFD conditions, PPAR gamma directly binds to PPAR response elements and regulates genes related to adipogenesis, lipid metabolism and glucose homeostasis. Some studies have reported that ginsenosides can reduce fat storage and adipogenesis. In addition, PPARγ is known to be one of the targets for ginsenoside-derived compounds (Alkhouri et al. 2010, Trauner et al. 2010, Wang et al. 2015b). However, the mechanism by which ginsenosides regulate PPARγ-dependent lipogenesis is unclear. To elucidate the mechanism, we treated Rg3 and MDI with 3T3L-1 cells and confirmed the level of protein and mRNA. Because PPARγ was expressed by adipocyte differentiation stimuli at 3 days, we prepared the sample at the 24-, 48- and 72-h time point to assess the pattern for PPARγ level. We assessed the level of PPARγ protein in 3T3-L1 cells and found that the level in cells treated with MDI alone was similar to that in cells treated with MDI and 5-µM Rg3; the same was true for the Pparγ mRNA level. However, Rg3 effectively reduced the level of PPARγ protein and mRNA in 3T3-L1 cells treated with 25-µM and 50-µM Rg3 at the 48-h and 72-h time points (Fig. 2A and B). In addition, we assessed PPARγ target lipogenesis proteins related to lipid accumulation in adipocytes, such as Fabp4, Scd1 and Cpt1. Western blot data showed that Rg3 reduced the levels of Fabp4, Scd1 and Cpt1 (Fig. 2C). Moreover, real-time PCR data indicated lowered levels of PPARγ target genes in Rg3-treated 3T3-L1 cells (Fig. 2D). These data suggest that Rg3 can modulate PPARγ and inhibit the expression of target genes such as Fabp4, Scd1 and Cpt1 in 3T3-L1 cells.
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Research

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Ginsenoside Rg3 reduced the activity of STAT5 at an early stage of the signal cascade for lipogenesis in 3T3-L1 cells

PPARγ is a master modulator of adipogenesis and a therapeutic target in obesity-induced type 2 diabetes (Tontonoz & Spiegelman 2008, Ahmadian et al. 2013, Kusminski et al. 2016). Until now, our data showed that Rg3 reduced the level of PPAR gamma in 3T3-L1 cells and we sought to elucidate how Rg3 regulated PPARγ. Among the PPARγ regulatory transcription factors, we focused on STAT5 (Stephens et al. 1996, Floyd & Stephens 2003, Kawai et al. 2007, Richard & Stephens 2011). Based on the function of STAT5 as a regulator of PPARγ, we hypothesized that Rg3 regulates STAT5 activity at an early stage of the signal cascade for lipogenesis and that this regulation would influence lipid accumulation. The 3T3-L1 cells were treated with MDI and Rg3 for 3 h to assess the impact on STAT5 activity. Western blot data from the analysis of cells collected at different time points while incubating with 25-µM and 50-µM Rg3 showed that Rg3 does downregulate STAT5 phosphorylation at early time points (Fig. 3A and B). Because Rg3 modulates the phosphorylation of STAT5 in MDI-induced 3T3-L1 cells, we next evaluated the translocation of STAT5 from the cytosol to the nucleus at 30 min, using confocal microscopy. We identified a significant decrease in the nuclear translocation of STAT5 in Rg3/MDI-treated 3T3-L1 cells compared to MDI-treated 3T3-L1 cells (Fig. 3C). Next, we conducted siRNA knockdown regulation for STAT5 to confirm the Rg3-mediated
Ginsenoside Rg3 and STAT5-PPARγ signaling pathway in lipogenesis.

**Figure 3**

Ginsenoside Rg3 reduced the activity of STAT5 in 3T3-L1 cells. (A) Ginsenoside Rg3 was incubated with the adipogenic mixture and samples were collected at six time points (0, 10, 20, 30, 60, and 180 min) for the detection of phosphorylated STAT5, using the Western blot analysis. Actin was used as a loading control. The bottom panel shows a graph of relative phospho-STAT5 to STAT5 ratios. (B) The various doses (0–50 µM) of ginsenoside Rg3 were incubated with the adipogenic mixture MDI and then the samples were collected at 30 min for the detection of phosphorylated STAT5 to confirm the dose-dependent effect of Rg3, using the Western blot analysis. Actin was used as a loading control. (C) Confocal microscopy images of immunostained STAT5 with nuclear stain (blue = nucleus, red = STAT5). 3T3-L1 cells were incubated in medium with or without the lipogenic mixture MDI and with or without Rg3 for 30 min. After incubation, the cells were fixed and permeabilized before staining with STAT5 antibody and DAPI. Scale bar = 10 µm. (D) The scrambled control siRNA and STAT5-specific siRNA were transfected into 3T3-L1 cell for 24 h and the cells were incubated with the adipogenic mixture and ginsenoside Rg3 (50 µM) for 30 min, and cell lysates from 3T3-L1 were analyzed to determine the protein level of pSTAT5 and STAT5. (E) After siRNA transfection for STAT5 and PPARγ using siRNA, the mRNA levels of PPARγ and target genes, such as Fabp4, Scd1, and Cpt1α, were measured using real-time qRT-PCR analysis for 9 days. Hprt was used as the housekeeping gene for analysis (*P<0.05, **P<0.01, compared with the Rg3(-)). MDI consists of insulin (10 µg/mL), dexamethasone (1 mM) and isobutylmethylxanthine (500 mM).

**STAT5 mechanism.** Western blot data from the analysis of cells collected for siRNA sample while incubating with 50-µM Rg3 showed that siSTAT5-transfected 3T3-L1 cell downregulates total form and phosphorylated STAT5 (Fig. 3D). To elucidate the effect of STAT5, we analyzed the level of Pparg mRNA in STAT5 knock-down 3T3-L1 cells and compared with 50-µM Rg3 at the 72-h time points. In the case of Stat5 knock-down cell, the level of Pparg mRNA is reduced in Stat5 knock-down cell and Rg3-treated cell. Also, the data of Stat5 knock-down cell show that the mRNA level of Pparg target genes, such as Fabp4, Scd1 and Cpt1α is reduced by Rg3 treatment (Fig. 3E). According to our data, Rg3 regulates PPARγ expression through the downregulation of STAT5 phosphorylation.

**Effect of ginsenoside Rg3 on the type 2 diabetes model of HFD-induced obesity**

Lipogenesis is related to the pathological features of type 2 diabetes, insulin resistance and increased serum glucose...
levels (Sun et al. 2011, Kusminski et al. 2016). We used an HFD mouse model to assess the effect of Rg3 on lipogenesis and related pathology. The time line of Rg3 treatment of the mice is shown in Fig. 4A. C57BL/6 mice that were 6 weeks old were fed an HFD for 4 weeks to induce obesity. Control mice were fed a normal diet. Each group of treated mice received 1 mg/kg Rg3 i.p. for 8 weeks. The Rg3-treated mice had lower body weights compared to the HFD mice that received no Rg3 (Fig. 4B). Next, we used the GTT to examine the efficiency of glucose uptake in the mice. Our data showed that HFD mice treated with Rg3 had lower serum glucose levels compared to other HFD mice (Fig. 4C). Next, we used the ITT to evaluate whether the improved glucose uptake ability of the Rg3-treated mice was related to insulin usage (Fig. 4D). The data demonstrated that Rg3-treated mice had better insulin sensitivity compared with untreated HFD mice. We assessed the protein levels of phosphorylated AKT in liver and epididymal white adipose tissue from both HFD mice and Rg3-treated HFD mice. Actin was used as a loading control. The arrow represents the real size of phosphorylated AKT (60 kDa).

**Effect of ginsenoside Rg3 on the pro-inflammatory response and hepatic steatosis in HFD mice**

Obesity-induced insulin resistance is another target in the therapy of type 2 diabetes. The concentration of the

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**Figure 4**

Ginsenoside Rg3 ameliorated insulin sensitivity in high-fat diet (HFD)-fed mice. (A) Timeline and scheme of the in vivo experiment. After 6-week-old male C57BL/6 mice were fed HFD for 4 weeks, the mice were injected with 1 mg/mL ginsenoside Rg3 while maintaining their HFD for 8 additional weeks. After 12 weeks, one group of HFD mice was subjected to the glucose tolerance test or the test for insulin tolerance (n=5) and from another group, epididymal white adipose tissue, liver tissue and serum were harvested for biochemical analysis. (B) The weight of each mouse was determined at 12 weeks (n=7 for HFD mice and n=7 for Rg3-treated HFD mice). (C) Glucose tolerance tests were performed after each mouse fasted overnight and was injected with 20% glucose i.p. After the injection, the level of blood glucose was analyzed at each time point (0, 30, 60, 90 and 120 min) (n=5 for HFD mice, n=5 for Rg3-treated mice) (*P<0.05, **P<0.01, compared with HFD mice). (D) Insulin tolerance tests were performed after each conditioned mouse fasted for 6 h and were injected with 0.75 U/kg insulin i.p. After the injection, the level of blood glucose was analyzed at each time point (0, 30, 60, 90 and 120 min) (n=5 for HFD mice, n=5 for Rg3-treated mice) (*P<0.05, **P<0.01, compared with HFD mice). (E) The protein levels of AKT and phosphorylated AKT were determined by the Western blot analysis of mouse liver and epididymal white adipose tissue from both HFD mice and Rg3-treated HFD mice. Actin was used as a loading control. The arrow represents the real size of phosphorylated AKT (60 kDa).
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pro-inflammatory cytokine TNF-α and fatty liver status are valuable indicators in the evaluation of this disease, and all are associated with insulin resistance. Therefore, we analyzed the effect of Rg3 on obesity-related insulin resistance. We analyzed FFA levels in eWAT and serum because FFA is an important indicator of obesity-induced type 2 diabetes. In our mouse model, Rg3 did not affect the level of FFA in eWAT; however, the levels in blood showed that Rg3 did cause a reduction in FFA (Fig. 5A). Next, we examined the mRNA levels of cytokines, including TNF-α, IL-1β, IL-6 and IL-10, in the white fat tissue of HFD mice. TNF-α, especially, was found to be lower in Rg3-treated HFD mice. However, other cytokines such as IL-1β, IL-6 and IL-10 displayed slight differences between treated and untreated groups (Fig. 5B). These results suggest that Rg3 has a role in reducing the amount of FFA in blood and the mRNA levels of the pro-inflammatory cytokine TNF-α in white fat tissue.

The reduction in both FFA and TNF-α mRNA levels in Rg3-treated mice suggested that Rg3 might be involved in the regulation of hepatic steatosis. Hepatic steatosis is one of the important indicators of obesity-induced pathology (Lee et al. 2006, Chen et al. 2012, Gu et al. 2013, Zhang et al. 2014). Therefore, we examined whether Rg3 could reduce hepatic steatosis in the HFD mouse model. We analyzed liver samples from mice with HFD-induced obesity that had been treated with Rg3. Liver cryo-section samples were stained with Oil Red O. Scale bar = 100 μm. (F) The amounts of accumulated lipid were determined by measuring triglyceride levels in liver samples from HFD mice and Rg3-treated HFD mice (**P<0.01, compared with HFD mice). (G) AST and ALT levels were measured in the serum (*P<0.05, **P<0.01, compared with HFD mice).
Rg3-treated mice had lower lipid compositions compared to HFD mice (Fig. 5C, D and E). Next, we investigated hepatic steatosis by the determination of TG amount in the liver and found that, consistent with the histological data, the level of TG was lower in Rg3-treated mice (Fig. 5F). To access hepatic damage degree, measurement of AST or ALT levels in blood samples from mice with HFD-induced obesity that had been treated or not with Rg3 revealed that the levels of both were much higher in HFD mice (Fig. 5G). Finally, our mouse model showed that Rg3 lowered the signs of hepatic steatosis, which is an obesity-induced pathological feature (Figs 2 and 3). Our in vivo results suggest that Rg3 reduced the amount of FFA in blood and TNF-α mRNA levels and prevented the hepatic steatosis induced by HFD.

Ginsenoside Rg3 reduced the size of adipose tissue and the activity of STAT5 and PPAR gamma target genes in vivo

Obesity is characterized by the expansion of adipocytes. In the Rg3-treated HFD mice group, the average adipocyte size was 90 μm, and in the group of HFD mice not treated with Rg3, adipocyte size increased dramatically to 150 μm (Fig. 6A and B). This indicates that Rg3 had an inhibitory effect on adipose tissue expansion in HFD mice. We measured TG content of adipose tissue and found that it was lower in the Rg3-treated HFD mice (Fig. 6C).

To assess the suppression of STAT5 by Rg3 in vivo (Fig. 3A), we measured the STAT5 phosphorylation and PPARγ from primary eWAT and liver tissues of Rg3-treated or non-treated HFD mice. STAT5 phosphorylation and PPARγ in Rg3-treated HFD mice were markedly decreased compared to non-treated HFD mice (Fig. 6D). Additionally, we assessed PPARγ, Fabp4, Scd1 and Cpt1 levels in the adipocytes of Rg3-treated or non-treated HFD mice. The real-time PCR data revealed that these genes were downregulated in the Rg3-treated HFD mice compared to Rg3 non-treated HFD mice (Fig. 6E). Overall, these data suggest that Rg3 can modulate obesity-induced type 2 diabetes in vivo through PPARγ regulation by suppressing STAT5 phosphorylation (Fig. 6F).

Discussion

Obesity is a leading risk factor for the development of type 2 diabetes. Several studies support recommendations to reduce the intake of excess calories and to exercise more for better health (Kastorini & Panagiotakos 2009, Asif 2014, Zhao et al. 2015). However, many supplements or drugs also have the potential to help reduce obesity. Not all phenomena related to obesity are always pathological. It was recently reported that healthy adipose tissue expansion is different from pathological obesity (Kusminski et al. 2016). The maintenance of healthy adipose tissue can help to prevent obesity-induced type 2 diabetes (Rosen & Spiegelman 2014, Kusminski et al. 2016). The natural compounds called ginsenosides have been used in traditional medicine. Many studies have shown clinical effects on diverse biological processes, such as anti-inflammatory, anti-cancer, anti-angiogenic, anti-aging and anti-diabetes (Attele et al. 1999, Yang et al. 2015). Ginsenoside Rg3 has also been shown to influence the pro-inflammatory response (Shin et al. 2013), been used in cancer therapy (Kim et al. 2014, Shan et al. 2015, Tian et al. 2016), been shown to affect NLRP3-dependent inflammasome formation (Yoon et al. 2015), displayed a protective effect against tissue damage (Wang et al. 2015a, Cheng & Li 2016) and been studied as an anti-diabetes agent (Hwang et al. 2009, Kim et al. 2009, 2010). However, it is not clear how ginsenoside might regulate type 2 diabetes-induced pathology. We have investigated the possible mechanisms of action for Rg3 in obesity-related type 2 diabetes.

In our studies, ginsenoside reduced adipose tissue size and TG amount in vivo and in vitro. The smaller size of adipose tissue and the reduction of TG accumulation are indicators of a healthy process of lipogenesis (Sun et al. 2011, Kusminski et al. 2016). The primary function of adipose tissue is the storage of absorbed lipid from food. However, an unhealthy increase of fat storage can induce lipotoxicity, which is a metabolic syndrome, in adipose tissue, along with cellular dysfunction, cell death and hepatic steatosis (Kusminski et al. 2009, Alkhouri et al. 2010, Trauner et al. 2010, Unger et al. 2010). In addition, ginsenoside has been reported to improve insulin sensitivity and uptake of glucose (Hwang et al. 2009, Kim et al. 2009, 2010, Zhang et al. 2017). However, its effects on adipose tissue size and TG levels in vivo have not been reported. In our studies, Rg3 reduced the blood glucose level, TNF-α amount in eWAT and hepatic steatosis. Consistent with our GTT and ITT results, the level of phosphorylated AKT was increased in both eWAT and liver tissues from Rg3-treated HFD mice. These results show that Rg3 can ameliorate insulin sensitivity and reduce lipogenesis in metabolic tissues such as liver and eWAT.

Next, we sought to identify the upstream signal molecule affecting PPAR gamma during the early phase.
Ginsenoside Rg3 and STAT5-PPARγ

We focused on STAT5, which regulates the ability of PPAR gamma to bind to the promoter of this gene (Floyd & Stephens 2003, Kawai et al. 2007, Cristancho & Lazar 2011, Richard & Stephens 2011). Our data show that Rg3 regulates the level of PPAR gamma, mediated by STAT5 activity, in vitro and in vivo. Rg3 reduced the phosphorylation of STAT5 at 25-µM and 50-µM concentrations in 20 min. In addition, translocation of STAT5 to the nucleus was reduced by Rg3, combined with the adipogenic cocktail, in 3T3-L1 cells. As shown in Fig. 6, the phosphorylation of STAT5 was reduced in the eWAT and liver tissues of Rg3-treated mice. These findings support the conclusion that Rg3 regulates the level of PPAR gamma through the suppression of STAT5 phosphorylation in vitro and in vivo.

In these studies, we have shown that Rg3 specifically prevents STAT5 phosphorylation and suppresses PPAR gamma expression in these HFD models. This leads to downregulation of lipogenesis proteins, such as Fabp4, Scd1 and Cpt1α in cells. We suggest that Rg3 is a candidate for the treatment of obesity-induced disease (Li et al. 2016, Zhang et al. 2016). We have demonstrated that Rg3 facilitates insulin sensitivity and ameliorates hepatic steatosis, and this natural compound may be useful in supporting healthy adipose tissue expansion and
protecting against pathological expansion, which is a part of obesity-associated diabetes.

Declaration of interest
The authors declare that the research was conducted with no commercial or financial relationship that could be interpreted as a potential conflict of interest.

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