Ginsenoside Rg3 ameliorated HFD-induced hepatic steatosis through downregulation of STAT5-PPAR γ

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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 through 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 through 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 (PPAR γ) is a multi-functional nuclear receptor superfamily involved in many biological processes (Lehrke & Lazar 2005, Ahmadian *et al.* 2013, Lefterova *et al.* 2014). In adipocytes, PPAR γ 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, PPAR γ is also important for controlling genetic networks involved in glucose homeostasis (Ahmadian *et al.* 2013). Moreover, PPAR γ 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 PPAR γ 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 PPAR γ stimulated by growth hormone regulation

(Kawai *et al.* 2007). Increased expression of STAT5 proteins correlates with the induction of both PPAR γ and C/EBP α . Thus, PPAR γ and C/EBP α are important transcription factors in adipocyte development (Richard & Stephens 2011, Lefterova *et al.* 2014). In pre-adipocytes, PPAR γ 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 PPAR γ through 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 through 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 μ m thick). Paraffin sections were counterstained with hematoxylin–eosin. For Oil Red O staining, cryo-sections were prepared 15- μ m 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 μ g/mL). Differentiation of the cell was induced by treating confluent cells with an adipogenic mixture consisting of 10 μ g/mL insulin (Sigma), 500 μ M isobutylmethylxanthine (Sigma) and 1 μ M 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 μ g/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 μ M) 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; all steps were performed according to the manufacturer's instructions.

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 μ g was used for the Western blot analysis. Primary antibodies specific for PPAR γ (2435), FABP4 (3544), SCD1 (2438), CPT1 α (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 μ g of total RNA was reverse-transcribed using ReverTra Ace qPCR RY Master Mix (Toyobo, Osaka, Japan), according to the manufacturer's instructions. For the PCR amplification of target mRNAs, 2 μ L of cDNA was used for each 20 μ L. The following primers were used:

Ppar γ 5'-GGAGCCTAAGTTTGAGTTTGCTGTG-3' (forward), 5'-TGCAGCAGGTTGTCTTGGATG-3' (reverse); Fabp4 5'-TGGGAACCTGGAAGCTTGTCTC-3' (forward), 5'-GAATCCACGCCCAGTTTGA-3' (reverse); Scd1 5'-TCACACAGTCCAGGGCCAAC-3' (forward), 5'-CTGGATTGGGCTATAGGGACAAGA-3' (reverse); Cpt1a 5'-TCATTGGCCACCAGTTCCATTA-3' (forward), 5'-CCAATGGCTGCCACACTCTC-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 *siPpar γ* , 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 *Ppar γ* , 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 $\pm$ 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 $\pm$ 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 16,000g. 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 400g, 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 μ M) and insulin (10 μ g/mL) for 30 min in DMEM supplemented with 10% FBS, penicillin G (100 IU/mL) and streptomycin (100 μ g/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 fluorescence microscopy (Olympus) and analyzed using Metamorph software.

Statistical analysis

Quantitative data are presented as the mean $\pm$ 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- μ M Rg3 induced cytotoxicity and reduced the viability of 3T3-L1 cells. The LC₅₀ value of Rg3 in 3T3-L1 cell lipogenesis is 200 μ M, and our working concentrations (5, 25 and 50 μ M) 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

Figure 1

Ginsenoside Rg3 attenuates lipid synthesis and has no cytotoxicity in 3T3-L1 cells. (A) The structure of ginsenoside Rg3. (B) Cytotoxicity due to ginsenoside Rg3. (C) Viability of 3T3-L1 cells was determined after 24h. The LC₅₀ value was calculated to be 20.01 mM. (D) 3T3-L1 cells were differentiated using an adipogenic mixture consisting of 10 μg/mL insulin, 1 mM dexamethasone and 500 mM isobutylmethylxanthine in the presence of 10% FBS. After incubation with the adipogenic mixture, Oil Red O staining was applied on Days 3 and 9. (E) Oil Red O was detected in the cell lysate using absorbance at 520 nm (**P*<0.05, ***P*<0.01, compared with the control). (F) After incubation with the adipogenic mixture, cell lysates from 3T3-L1 cells were analyzed for their triglyceride levels, on Day 9 (**P*<0.05, ***P*<0.01, compared with the control).

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

PPAR γ 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, PPAR γ is known to be one of the targets for ginsenoside-derived compounds (Alkhoury *et al.* 2010, Trauner *et al.* 2010, Wang *et al.* 2015b). However, the mechanism by which ginsenosides regulate PPAR γ -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 PPAR γ 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 PPAR γ level. We assessed the level of PPAR γ 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 PPAR γ 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 PPAR γ 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 PPAR γ target genes in Rg3-treated 3T3-L1 cells (Fig. 2D). These data suggest that Rg3 can modulate PPAR γ and inhibit the expression of target genes such as *Fabp4*, *Scd1* and *Cpt1* in 3T3-L1 cells.

Figure 2

Ginsenoside Rg3 modulates PPAR γ and PPAR γ -dependent lipogenesis gene expression in 3T3-L1 cells. (A) After incubation with the adipogenic mixture, cell lysates from 3T3-L1 were analyzed to determine the protein level of PPAR γ that resulted from treatment with ginsenoside Rg3 in each indicated time (24, 48 and 72 h) and dose. Actin was used as the loading control. (B) The levels of *Ppary* mRNA in the 3T3-L1 cells were analyzed using real-time PCR (* P <0.05, ** P <0.01, compared with the control). *Hprt* was used as the housekeeping gene for analysis. (C) After incubation with the adipogenic mixture and different ginsenoside Rg3 concentrations (5, 25 and 50 μ M) for 9 days, the expression levels of PPAR γ -dependent protein were determined by the Western blot analysis. Actin was used as the loading control. (D) After incubation with the adipogenic mixture and different ginsenoside Rg3 concentrations (5, 25 and 50 μ M) for 9 days, the mRNA levels of PPAR γ -dependent *Fabp4*, *Scd1* and *Cpt1 α* were determined using real-time PCR analysis. *Hprt* was used as the housekeeping gene for analysis (* P <0.05, ** P <0.01, compared with the control).

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

PPAR γ 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 PPAR γ . Among the PPAR γ 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 PPAR γ , 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- μ M and 50- μ M 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 knock-down regulation for STAT5 to confirm the Rg3-mediated

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 μ M) 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 μ m. (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 μ M) 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 PPAR γ using siRNA, the mRNA levels of PPAR γ and target genes, such as *Fabp4*, *Scd1* and *Cpt1 α* , 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 μ g/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- μ M 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 *Ppar γ* mRNA in STAT5 knock-down 3T3-L1 cells and compared with 50- μ M Rg3 at the 72-h time points. In the case of Stat5 knock-down cell, the level of *Ppar γ* 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 *Ppar γ* target genes, such as *Fabp4*, *Scd1* and *Cpt1* is reduced by Rg3 treatment (Fig. 3E). According to our data, Rg3 regulates PPAR γ 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

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).

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 eWAT. The phosphorylation of AKT, which is activated by insulin signaling and is an indicator of insulin resistance, was elevated in each tissue of the Rg3-treated HFD mice compared to the tissues of HFD mice in the absence of Rg3 (Fig. 4E). These results show that ginsenoside Rg3 is an insulin sensitizer in the type 2 diabetes model of obesity induced by HFD.

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

Figure 5

Ginsenoside Rg3 ameliorates obesity-induced pro-inflammatory responses and hepatic steatosis in mice fed a high-fat diet (HFD). (A) The levels of free fatty acid were determined in epididymal white adipose tissue and blood from HFD mice (Control; $n=5$) and Rg3-treated HFD mice (Rg3; $n=5$) (* $P<0.05$, ** $P<0.01$, compared with Control HFD mice). (B) The mRNA levels of pro- and anti-inflammatory cytokines in epididymal white adipose tissue were analyzed using real-time PCR. *Hprt* was used as a housekeeping gene to normalize the expression for all samples (* $P<0.05$, ** $P<0.01$, compared with HFD mice). (C) Liver tissues were extracted from HFD mice and HFD mice that had been treated with Rg3. (D) The liver cryo-section samples were stained with Oil Red O. Scale bar = 100 μ m. (E) Liver paraffin section samples were analyzed for histopathological changes after staining with hematoxylin-eosin. Scale bar = 100 μ m. (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).

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-1 β , 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-1 β , 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 or not with Rg3. In addition, histological Red O and hematoxylin-eosin staining data showed that

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 μ m, and in the group of HFD mice not treated with Rg3, adipocyte size increased dramatically to 150 μ m (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 PPAR γ from primary eWAT and liver tissues of Rg3-treated or non-treated HFD mice. STAT5 phosphorylation and PPAR γ in Rg3-treated HFD mice were markedly decreased compared to non-treated HFD mice (Fig. 6D). Additionally, we assessed PPAR γ , *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 PPAR γ 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.

Figure 6

Ginsenoside Rg3 reduced lipid accumulation and the amount of phosphorylated STAT5 *in vivo*. (A) hematoxylin–eosin-stained epididymal white adipose tissue (eWAT) in mice fed high-fat food with or without Rg3 treatment. Scale bar=100 μm. (B) Adipocyte diameter was quantified in eWAT. (C) The amount of lipid accumulation was determined by measuring the triglyceride level in eWAT from normal fat diet (NFD) mice (NFD control; $n=5$), Rg3-treated NFD mice (NFD Rg3; $n=5$), high-fat diet (HFD) mice (HFD Control; $n=5$) and Rg3-treated HFD mice (HFD Rg3; $n=5$) (** $P<0.01$, compared with HFD mice). (D) The levels of phospho-STAT5 and PPAR γ protein in eWAT and liver were determined by the Western blot analysis of both high-fat food (control; $n=5$) and HFD plus Rg3 (Rg3; $n=5$) mice. Actin was used as a loading control. (E) The mRNA expression levels of *Pparγ* and the target genes were analyzed using real-time PCR on samples of eWAT from both HFD (Control; $n=5$) and HFD plus Rg3 (Rg3; $n=5$) mice. *Hprt* was used as a housekeeping gene to normalize the expression for all samples (* $P<0.05$, ** $P<0.01$, compared with HFD mice). (F) A schematic model explaining the inhibitory effect of ginsenoside Rg3 on the STAT5-PPAR γ mediated lipid accumulation pathway. In our study, ginsenoside Rg3 inhibited the phosphorylation of STAT5 and reduced the expression of PPAR γ . Ginsenoside Rg3 downregulated PPAR γ -dependent lipogenesis related genes, including *Fabp4*, *Scd1* and *Cpt1α*.

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 *Cpt1* 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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