Ginsenoside Rb2 promotes glucose metabolism and attenuates fat accumulation via AKT-dependent mechanisms

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ABSTRACT

Ginsenosides, the major active constituents of ginseng, have been demonstrated possess anti-diabetic, anti-inflammatory effects. Ginsenoside Rb2 (Rb2) is the most abundant saponin in Panax ginseng, this study investigates the role of Rb2 in the anti-hyperglycemic mechanism of insulin-sensitive cell lines 3T3-L1 adipocytes as well as high fat diet-induced obese mice. Glucose uptake of 3T3-L1 adipocytes was measured. The insulin signaling cascade, including insulin AKT, insulin receptor (IR) beta-subunit, IR substrate (IRS) -1, phosphatidylinositol 3-kinase (PI3K) were also examined. TNF-α-treated 3T3-L1 adipocytes were used as an insulin resistant model in which p-AKT, c-Jun NH2-terminal kinase (JNK), MAPK, and nuclear factor (NF) -κB signaling cascades were examined. As an in vivo study, C57BL/6J mice were fed with a high-fat diet for 9 weeks, with or without Rb2 supplementation. Then we investigated the effects of Rb2 on glycometabolism in these high fat diet-induced obese mice. Our results demonstrate Rb2 increases glucose uptake in 3T3-L1 adipocytes, independent of insulin receptor β-subunit (IRβ) and principally through the insulin receptor substrate (IRS)-1-phosphatidylinositol 3-kinase (PI3K)-AKT/PI3K pathway. Rb2 inhibited TNF-α-induced activation of MAPK and nuclear factor (NF)-κB signaling pathway as well as the expression of inflammatory factors. In high fat diet-induced obesity mice, Rb2 attenuated fat mass and regulated insulin resistance. In mouse adipose tissue, Rb2 phosphorylation of AKT was correlated with glycometabolism. Furthermore, Rb2 attenuates insulin resistance in 3T3-L1 adipocytes, reduces fat mass, and improves insulin sensitivity in high fat diet-obese mice.

1. Introduction

Obesity is a major cause of metabolic diseases such as type 2 diabetes (T2DM), dyslipidemia and nonalcoholic fatty liver disease, diseases which affect quality of life and life span. Insulin resistance is a characteristic of T2DM and obesity. Regulation of insulin sensitivity is an essential step to alleviate T2DM and obesity. Its therapy remains a challenge and the remission of insulin resistance is a critical target. Traditional herbal medicines have been used to treat T2DM and obesity for more than four thousand years. For examples berberine reduces insulin resistance [1], curcumin attenuates lipopenicity and the inflammation in HFD mice [2], celastrol suppresses food intake and reduces body weight in obese mice [3].

Ginsenosides, the major component of ginseng, possess multiple pharmacological activities including anti-obesity, anti-inflammatory and anti-diabetic effects [4]. Experiments in vitro and in vivo have shown that ginsenosides can lower blood glucose [5,6], increase insulin sensitivity [5,7], regulate lipid metabolism [8,9] and reduce body weight [10] and fat content [11]. Ginsenoside Rb2 (Rb2), one of the most importance saponin contained in Panax ginseng [12], was also reported to decrease glycaemia in streptozotocin-induced diabetic rats [13] and lower triacylglycerol levels in 3T3-L1 adipocytes [14]. The mechanisms by which Rb2 reduces fat accumulation have not been previously studied.

In the present study, we explored whether Rb2 could increase glucose uptake and attenuate TNF-α-induced insulin resistance in 3T3-L1 adipocytes. In addition, we treated high fat diet-induced obese (DIO) mice with Rb2 for 10 days to explore the effects of Rb2 on fat accumulation and insulin sensitivity. The results of this study provide new insights into the mechanisms of antidiabetic and anti-obesity activities of Rb2.

2. Methods

2.1. Reagents and antibodies

Ginsenoside Rb2 (purity > 98.0%) was purchased from Shanghai Yuanye Biotech Co, Ltd (Shanghai, MO, China). Dexamethasone,
isobutylmethylxanthine (IBMX) were obtained from Sigma-Aldrich Co. (St. Louis, MO), while fetal bovine serum (FBS) and DMEM were from Gibco Laboratory (Gaithersburg, MD). Anti-IRβ antibody, Anti-PY20 antibody and Anti-β-actin antibody bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The following items were purchased from Cell Signaling Technology Inc. (Beverly, MA): Polyclonal anti-IκBα antibody, anti-p38 antibody, anti-phospho-p38 (Thr180/Tyr182) antibody, anti-ERK1/2 antibody, anti-phospho-ERK1/2 (Thr202/Tyr204) antibody, anti-AKT antibody, anti-phospho-AKT (Ser473) antibody, anti-IKKβ antibody, anti-phospho-IKKβ (Ser181) antibody, anti-SAPK/JNK antibody, anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody, anti-IRS-1 antibody, anti-phospho-IRS-1 (Ser307) antibody.

2.2. Animals

All C57BL/6J mice were housed under a constant 12 h light/dark cycle with free access to water and high-fat diet (60% Kcal fat, MD12033, China) for 9 weeks. The mice were obtained from Shanghai Slake Experimental Animal CO. LTD and were maintained at 22 ± 2 °C with 60 ± 5% relative humidity. The DIO mice (n = 5/group) were administrated with Rb2 (40 mg/kg/d) or PBS (vehicle) daily (intraperitoneal injection) between 16:00 and 17:00. Bodyweight was monitored once a day over the 10 days course of treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee at Wenzhou Medical University (No: SYXK-2015-0009).

2.3. Cell culture

3T3-L1 adipocytes from the American Type Culture Collection (Rockville, MD) were grown in DMEM medium containing 10% FBS with streptomycin and penicillin at 37 °C. Two day-old post-confluent cells were incubated with 10% FBS and 1 uM dexamethasone, 0.5 mM IBMX, and 10 g/ml insulin, then medium was replaced with DMEM containing 10% FBS and 10ug/mL insulin for another 6 days. In the chronic TNF-α treatment assay, 3T3-L1 adipocytes were treated with TNF-α for 72 h and Rb2 for the last 24 h. TNF-α was added every 24 h.

2.4. Glucose uptake measurement

Glucose uptake was evaluated by measuring cellular incorporation of [3H]-2-deoxy-D-glucose ([3H]-2-DOG). Adipocytes were cultured in 48-
well plates to 80% confluence and differentiated. After treatment Rb2 or otherwise, adipocytes were serum-starved for 4 h and then washed with Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 4.7 mM KCl, 136 mM NaCl, 1.25 mM MgSO4, 1.25 mM CaCl2 and 10 mM Na3PO4. The cells were placed in KRP buffer that contained insulin (100 nM) for 20 min, then incubated with 2-DOG (0.1 mM) and 3H-2-DOG (1 mCi/mL) at 37°C for an additional 10 min. To terminate reaction, the cells were washed 3 times with PBS to remove the unconjugated dye and lysed in 0.5 M NaOH and 0.1% SDS at 37°C.

Nonspecific uptake was subtracted from all values, which was determined in the presence of 20 µM cytochalasin B.

2.5. Glucose tolerance test and insulin tolerance test

Methods previously described were used to carryout glucose tolerance test (GTT) and insulin tolerance test (ITT). GTT was performed in male C57BL/6J mice after fasting overnight. The blood glucose concentrations from the tail vein were measured immediately at 0, 15, 30, 45, 60, 90 and 120 min, after intraperitoneal injection of glucose (0.75 g/kg) [15]. Food was removed from the cage 2 h before ITT was performed. After intraperitoneal injection of human insulin (Eli Lilly) at 0.75 U/kg, the glucose concentrations were measured at 0, 15, 30, 45, 60, 90 and 120 min [16].

2.6. Western blot

3T3-L1 adipocytes were collected in modified RIPA buffer. Samples were centrifuged at 12,000 x g for 20 min at 4°C, insoluble pellets were discarded, and the supernatants were boiled in SDS loading buffer. After SDS-polycrylamide gel electrophoresis and transfer to a nitrocellulose transfer membrane (Whatman, GE Healthcare), the membranes were incubated with different primary antibodies overnight at 4 °C, followed by a secondary antibody conjugated with horseradish peroxidase for 2 h at room temperature. The antigen–antibody complexes were detected by enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia).

2.7. Real-time PCR

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) and was reverse transcribed to first-strand cDNA using the Reverse Transcription System (A3500, Promega, Madison, WI). To analyze expression of genes, Real-time quantitative PCR were carried out using an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA).
The primers of genes were as follows:

IL-6 sense, 5′-TTCCATCCAGTTGCCTTCTT-3′, antisense 5′-CAGAATTGCCATTGCACAAC-3′;
SOCS3 sense 5′-AGAGCGGATTCTACTGGAGC-3′, antisense 5′-TGGATGCGTAGGTTCTTGGTC-3′;
Adiponectin sense: 5′-GCAGGCATCCCAGGACATC-3′, antisense 5′-GCGATACATATAAGCGGCTTCT-3′;
Actin Sense 5′-GGCTGTATTCCCCTCCATCG-3′, antisense 5′-CCAGTTGGTAACAATGCCATGT-3′.

After normalized of values with β-actin, the expression level of each gene was quantified.

2.8. Histopathology

Adipose tissues were fixed in 10% formalin for 48 h. General morphological observations were made with the staining of hematoxylin and eosin. A total of three mice were used for the analysis. The images were acquired using an Olympus BX51 system.

2.9. Statistical analysis

Results are expressed as mean ± SME or mean ± SD from at least three independent experiments. Data were analyzed by two-tailed Student’s t-test or one-way ANOVA by GraphPad7 (San Diego, CA). P < .05 was considered to be significant.

3. Results

3.1. Ginsenoside Rb2 increased glucose uptake via AKT activation in 3T3-L1 adipocytes

2-DG uptake was used to measure the influence of Rb2 on glucose uptake in 3T3-L1 adipocytes. Basal glucose uptake in 3T3-L1 adipocytes was gradually elevated after treatment with different concentrations of Rb2 for 2 h (Fig. 1A). During the period of study, the maximal glucose uptake occurred at 2 h of incubation (Fig. 1B). Rb2 could further enhance glucose uptake at concentration of 25 μM (Fig. 1C). In addition, the insulin signaling pathway was examined to explore how Rb2 might increase glucose transport. The data demonstrated that Rb2, at concentration of 25 μM, increased phosphorylation of AKT on Ser473, thus activating the PI3K/AKT signal pathway. Furthermore, pretreatment with LY294002, a PI3K inhibitor, attenuated the activation of AKT induced by Rb2 (Fig. 1D). The engagement of insulin to its receptor activates its intrinsic tyrosine-kinase activity, which consequently phosphorylates insulin-receptor substrate (IRS) proteins on tyrosine residues. These phosphorylated proteins then serve as anchoring sites for the p85 regulatory subunits of p85/PI3K kinase at the cell membrane [17]. This development facilitates recruitment and interaction between the AKT protein kinases. Using this mechanism, we investigated the effects of Rb2 on IRS protein, which showed Rb2 increased the phosphorysine content of IRS-1 and the recruitment of p85 to IRS-1 (Fig. 1F), but did not affect phosphorylation of IRβ (Fig. 1E). These findings demonstrate that Rb2 may promote PI3K/AKT...
signaling pathway and phosphotyrosine of IRS-1 then enhance the glucose transport.

3.2. Ginsenoside Rb2 ameliorated insulin resistance induced by TNF-α

Insulin-stimulated glucose uptake was significantly decreased in TNF-α-treated 3T3-L1 adipocytes. Rb2 co-incubated with insulin could further increase glucose uptake. This decreased glucose uptake could be ameliorated by Rb2 treatment at 25 μM for 24 h, especially in the insulin treated group (Fig. 2A). Consistently, either AKT phosphorylation is induced by TNF-α or insulin, but not both, and Rb2 could attenuate the insulin-stimulated phosphorylation levels of AKT induced by TNF-α (Fig. 2B), suggesting that Rb2 could improve the insulin sensitivity in 3T3-L1 adipocytes, which was impaired by TNF-α. The MAPK signaling pathway plays a crucial role in TNF-α-induced insulin resistance. Phosphorylation levels of the key components in the MAPK pathways, including c-Jun N-terminal kinases (JNK), ERK and P38, were dramatically increased by TNF-α. However, Rb2 impressively reduced TNF-α-mediated activation of JNK, ERK and P38 (Fig. 2C). Previously, studies identified that JNK could induce insulin resistance by phosphorylation of IRS-1 at Ser 307. Our results indicate Rb2 reduces the phosphorylation of IRS-1 at Ser307 which was increased by TNF-α (Fig. 2D). Rb2 ameliorates insulin resistance through phosphorylation of AKT and phosphorylation of MAPK pathways in 3T3-L1 adipocytes insulin resistant models.

3.3. Ginsenoside Rb2 inhibited the expression of inflammation induced by TNF-α

To investigate whether Rb2 could inhibit inflammation, we analyzed the IKK/α/β signal pathway and several inflammation factors. As shown in Fig. 3A, TNF-α induced nuclear factor-κB kinases (IKK) α/β phosphorylation, and the activation of IKKα/β was significantly inhibited by Rb2. In addition, Rb2 suppresses the degradation of NF-κB inhibitors usually induced by TNF-α. TNF-α treated 3T3-L1 adipocytes produced several cytokines and chemokines, such as interleukin (IL)-6, adiponectin and the suppressor of cytokine signaling (SOCS)-3, which might relate to TNF-α mediated insulin resistance. The expression of IL-6 and SOCS-3 were found to be significantly reduced by Rb2 (Fig. 3B, C). Furthermore, Rb2 treatment weakly enhanced TNF-α-induced the suppression of adiponectin mRNA expression (Fig. 3D), indicating Rb2 may inhibit cytokines to attenuate TNF-α mediated insulin resistance.

3.4. Ginsenoside Rb2 reduced body weight and improved glucose metabolism in DIO mice

HFD feeding increased adipose mass and insulin resistances. After 10 days of treatment with 40 mg/kg Rb2, mouse body weight was significantly suppressed (Fig. 4A). Consistent with this reduced body weight, the Rb2-treated group had a reduced body fat weight compared with the control group (Fig. 4B). Insulin resistance is primarily
characterized by high blood glucose level, so to investigate whether Rb2 lowered blood glucose levels and improved its metabolism, GTT and ITT were performed with mice after treatment with Rb2. Glucose levels during the intraperitoneal GTT were reduced by Rb2 at all time points from 0 to 120 min (Fig. 4C). The area under curve (AUC) of glucose was significantly reduced (Fig. 4D). The results of ITT were similar to GTT (Fig. 4E, F). The results show that Rb2 potently decreased the fat accumulation and improved insulin sensitivity in HFD mice.

3.5. Ginsenoside Rb2 reduced fat accumulate and activated phosphorylation of AKT in DIO mice

New findings have connected insulin resistance to adipose tissue which play a crucial role in T2DM. So we examined the effects of Rb2 on the adipose tissue in HFD mice. Ratio of epididymal white adipose tissue (eWAT) (Fig. 5A), subcutaneous white adipose tissue(iWAT) (Fig. 5C), interscapular brown adipose tissue (BAT) (Fig. 5E) to body weight in HFD mice after injection with Rb2 were significantly decreased. The eWAT ratio had the most remarkable change. In parallel with these findings, Rb2 reduced eWAT (Fig. 5B), iWAT (Fig. 5D) and BAT (Fig. 5F) mass compared with PBS-treated HFD mice. Furthermore, western blotting analysis showed that the Rb2 group expressed higher levels of p-AKT (Ser473) (Fig. 5G). These data indicate that Rb2 reduced fat accumulation in BAT and WAT, and may be activated via AKT signal flow.

4. Discussion

Ginsenosides are the major active constituents of ginseng, with Rb2 is the main compound of Panax ginseng root [4]. Rb2 has displayed anti-obesity and anti-hyperglycemic effects by reducing food intake and body weight in rats [18], highlighting its anti-diabetic effect [19]. In this study, our results demonstrate that Rb2 influences glucose...
metabolism in 3T3-L1 adipocytes and HFD mice as well as elucidate the possible mechanism for this phenomenon.

Previous studies have shown that ginseng has beneficial effects on key components in the insulin signaling pathway. Insulin activity is initiated by binding and activating an insulin receptor (IR). This phosphorylation activates downstream of PI3K, AKT and protein kinase C (PKC)ζ/λ, induces the translocation of glucose transporter type 4 (GLUT4) and then the transportation of glucose into 3T3-L1 adipocytes [20]. AKT protein kinase is required for insulin regulation of the pathways controlling systemic glucose homeostasis, including glucose transport in adipocytes and muscle [21,22], inhibition of hepatic gluconeogenesis [23,24] and cell-autonomous activation of hepatic lipogenesis [24]. Previous studies have shown that human monogenic mutations in insulin receptor, PI3-kinase and AKT cause severe insulin resistance [25]. Our results show that Rb2 improves the IRS-1/PI3K/AKT insulin signaling pathway and subsequently increases glucose uptake.

Insulin resistance is the critical mechanisms in type 2 diabetes to which chronic low-grade inflammation is relevant [26,27]. TNF-α, a major pro-inflammatory factor, induces chronic low-grade inflammation which results in insulin resistance [26]. Therefore, 3T3-L1 adipocytes treated with were used as an insulin resistance model to explore the anti-diabetic mechanisms of Rb2. The present study showed Rb2 enhanced glucose uptake and alleviated the insulin signal pathway in 3T3-L1 adipocytes impaired by TNF-α. The MAPK signaling pathways, including JNK, ERK and P38, are the critical participants in the pathogenesis of insulin resistance induced by TNF-α [28]. JNK promoted insulin resistance via phosphorylation of IRS-1 at Ser 307 [29]. It has been reported that suppression of JNK improves insulin resistance and could be a therapeutic target for T2DM. This study demonstrates that Rb2 inhibits MAPK activities, especially in JNK, and decreased phosphorylation of IRS-1 at Ser 307, which was consistent with other reports, suggesting that Rb2 might attenuate TNF-α induced insulin resistance by inhibiting MAPK signaling pathway.

Due to the important role inflammation plays in the development of insulin resistance [30,31], we further explored whether Rb2 could inhibit inflammation. By blocking inflammasome activation, Rb2 inhibited TNF-α-induced phosphorylation of IKKβ and the consequent degraded IκBα, as well as decreased IL-6 and SOCS-3. IKKβ is an important kinase of inflammation. Phosphorylated IKKβ activates IκBα protein, inhibitors of NF-κB, leading to its proteasomal degradation. NF-κB then moves into the nucleus where it induces the expression of numerous target genes, producing insulin resistance [32]. NF-κB will up-regulate inflammatory mediators to participate in creating insulin resistance. Activated NF-κB increases the production of IL-6 and SOCS-3, two of important cytokines and chemokines correlating with TNF-α-induced insulin resistance [33]. Hence, these results indicate that Rb2 may ameliorate TNF-α-induced insulin resistance by down-regulating the NF-κB signaling cascade and expression of related downstream inflammation factors.

Obesity is closely associated with T2DM. In recent years, adipose tissue has been identified as a key factor to promote T2DM [34]. This study shows that Rb2 has positive effects on glycometabolism, by reducing the amount and size of adipocytes in vivo and then improving insulin sensitivity. We demonstrated that Rb2 affected these changes via the AKT signal pathway.

5. Conclusion

Rb2 activated the AKT pathway, inhibited TNF-α-induced the activation of MAPK, NF-κB pathway, in addition to upregulating of inflammatory factors, reducing fat accumulation, and improving glycometabolism. These findings suggest Rb2 attenuates insulin resistance in 3T3-L1 adipocytes and DIO mice. Rb2 thus can be a potential therapeutic target in T2DM.

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Disclosure

The authors declared no conflicts of interest.

Author contributions

All the authors have significantly contributed to this work.

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