Original Article

Effects of Ginsenoside Rb1 on Skeletal Muscle Insulin Resistance and Adenosine Monophosphate-activated Protein Kinase Signaling Pathway in Obese Mice

Dan-Dan Zhao[a], Ying Bai[a], Rui Wu[b], Fang-Fang Mo[a], Chen-Yue Liu[a], Ru-Yuan Zhu[a], Guang-Jian Jiang[a], Jia-Xian Liu[c], Dong-Wei Zhang[a], Si-Hua Gao[a]

[a]School of Chinese Medicine, Beijing University of Chinese Medicine, [b]Department of Endocrinology, South Area of Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing, China, [c]Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA, USA

Abstract

Objectives: The objective of the study is to observe the effects of ginsenoside Rb1 on indexes of body weight, body composition, blood lipid, skeletal muscle endurance, and insulin sensitivity in obese mice, probe into its pharmacological action, and further explore its effects on adenosine monophosphate-activated protein kinase (AMPK) signaling pathway in skeletal muscle. **Materials and Methods:** Eight-week-old C57BL/6J mice were fed with high-fat diet for 12 weeks to establish obese mouse model. The model-establishment obese mice were randomly divided into three groups including model control group, metformin group, and ginsenoside Rb1 group. In the normal control group, normal diet was administered. The intervention period was 8 weeks. Body weight and food intake of the mice were measured regularly every week. The treadmill test was performed at weeks 3 and 7, and the oral glucose tolerance test was carried out at weeks 4 and 8. Body composition of the mice was detected by applying NMR Animal Body Composition Analyzer at week 8. Four parameters of blood lipids and free fatty acid (FFA) levels were detected. The mRNA expression of AMPKα and proliferator-activated receptor gamma coactivator-1α (PGC-1α) in skeletal muscle was examined by real-time fluorescence quantitative polymerase chain reaction, and the influence of ginsenoside Rb1 on protein expression of AMPKα, p-AMPKα, and PGC-1α was observed by western blotting. **Results:** The body weight (since the 5th week of drug administration) and food intake of the mice in the ginsenoside Rb1 group were significantly lower than those in the model control group ($P < 0.05$) in a time-dependent manner. Ginsenoside Rb1 could significantly reduce the levels of triglyceride and low-density lipoprotein cholesterol, while increase the high-density lipoprotein cholesterol level ($P < 0.05$). In addition, ginsenoside Rb1 could reduce the serum FFA level ($P < 0.05$). After the administration of ginsenoside Rb1 for 8 weeks, the body fat mass of obese mice decreased and the lean mass increased ($P < 0.05$). The skeletal muscle endurance and the oral glucose tolerance of the obese mice improved using ginsenoside Rb1. At the molecular level, ginsenoside Rb1 could up-regulate the mRNA and protein expression of AMPKα in skeletal muscle, and increase the content of p-AMPK protein significantly ($P < 0.01$). At the same time, the mRNA and protein level of PGC-1α was also un-regulated, correspondingly ($P < 0.01$). **Conclusion:** Ginsenoside Rb1 exerts effects on reducing body weight, decreasing blood lipid levels, enhancing the skeletal muscle endurance, and increasing the insulin sensitivity in obese mice by activating the related proteins in AMPK signaling pathway in skeletal muscle.

Keywords: Adenosine monophosphate-activated protein kinase signaling pathway, ginsenoside Rb1, insulin resistance, obesity, skeletal muscle

INTRODUCTION

Insulin resistance (IR) refers to a decrease in the ability of insulin to maintain normal blood sugar with its biological effect inferior to normal, or a decrease in the reactivity of surrounding target tissues such as liver, muscle, and adipose tissue to insulin leading to a decrease of glucose intake and utilization efficiency giving rise to a series of clinical manifestations. IR is one of the important pathogenesis of

Address for correspondence: Prof. Si-Hua Gao,
School of Chinese Medicine, Beijing University of Chinese Medicine,
Beijing 100029, China.
E-mail: gaosihua1216@163.com

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type 2 diabetes mellitus (T2DM). Studies have shown that 80% of T2DM patients accompanying with obesity. The pathological changes are characterized by disorder of lipid metabolism due to obesity leading to IR.[1] Skeletal muscle is the main target of insulin action, and responsible for more than 80% of glucose metabolism after meal.[2] Adenosine monophosphate-activated protein kinase (AMPK) is the main kinase regulating metabolism of skeletal muscle cells and gets involved in multiple metabolic pathways such as regulation of glucose consumption.[3] AMPK, a metabolic sensor, gatekeepers of the activity of the master regulator of mitochondria, proliferator-activated receptor gamma coactivator-1α (PGC1α), are vital links in a regulatory network for metabolic homeostasis. It was accepted that the development of metabolic disorders such as obesity and type 2 diabetes-related closely to the improper function of the energy regulating network, including PGC-1α and AMPK.[4] Moreover, researchers find that the caloric restriction leads to phospho-activation of AMPK in muscle and increase expression of PGC-1 in skeletal muscle, and vice versa.[5]

Ginsenoside Rb1 is one of the representative components of ginsengdiol saponins. It is mainly distributed in traditional Chinese herbal medicines such as *Radix et Rhizoma Ginseng* (rén shēn), *Radix et Rhizoma Notoginseng* (sān qī), and *Radix Panacis Quinquefolii* (xī yáng shēn), which is widely used to treat DM, coronary heart disease, and tumor. Studies have shown that ginsenoside Rb1 exhibits a variety of pharmacological actions such as central nervous system, cardiovascular system, immune system, and anti-tumor,[6] but few reports on lowering blood glucose and regulating IR, especially in skeletal muscle energy regulation using ginsenoside Rb1. Therefore, we would like to observe the effects of ginsenoside Rb1 on body composition, blood lipids, glucose tolerance, and AMPK signaling pathway in obese mice, and to provide a scientific basis for further elucidation of ginseng for DM and its complications.

MATERIALS AND METHODS
Materials
Animal and fodder
Eight-week-old male C57BL/6J mice were purchased from Beijing Sibefu Biotechnology Co., Ltd. (license No. SCXK [Beijing] 2014-0004) and were raised in the Animal Laboratory with Barriers of Beijing University of Chinese Medicine. The mice were housed in single cages, in a 12-h light/dark cycle, at a constant 23°C with 45% relative humidity. The mice were free to water during feeding. As ordinary feed, AIN-96G full-price nutrient feed was used, and high-fat purified feed was provided by Jiangsu Medison Biomedical Co., Ltd. (Yangzhou, China).

Drugs
Ginsenoside Rb1 was purchased from Chengdu Puruifa Technology Development Co., Ltd (Chengdu, China).

Metformin hydrochloride tablet was from Sino-America Shanghai Squibb Pharmaceutical Co., Ltd. (Shanghai, China). They all stored at 4°C. Prepare the suspension of the desired concentration with ultrapure water before gavage.

Reagents and equipment
The blood lipid kits were all products of Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Free fatty acid (FFA) kits were provided by Beijing Bainuo Co., Ltd (Beijing, China). The automatic biochemical analyzer was Hitachi 7080 (Tokyo, Japan). The NMR Animal Body Composition Analyzer was from Shanghai Newmai Electronic Technology Co., Ltd (Shanghai, China). AMPK, p-AMPK, PGC-1α, and GAPDH primary antibody were all purchased from CST (Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-labeled secondary antibody was from Zsbio, Co., Ltd (Beijing, China).

Methods
Model establishment, grouping, and drug administration
Fifty male C57/B6J mice (8-week-old) were randomly selected 10 ones as the normal control group after 1-week adaptive feeding. The remaining 40 mice were fed with a high-fat diet for 12 weeks, and the body weight was 20% greater than the average weight of the normal control group as a model for obese mice. Successful establishment of model obese mice was randomly divided into model control group, metformin group and ginsenoside Rb1 group, with eight mice in each group.

Drug administration
Continuous intragastric administration for 8 weeks, 20 mg/kg/day ginsenoside Rb1 was used in the ginsenoside Rb1 group, and the dosage was determined according to the previous document;[7] in the metformin group, 75 mg/kg/day with the volume of 0.1 mL–10 g body weight; in the normal control and model control groups, the sterile water with the same volume was used for gastric lavage.

Detected indicators and methods
Body composition detection
At the 8th week of drug intervention, the body composition of mice in each group was tested by NMR Animal Body Composition Analyzer, and the body fat content and body muscle content were recorded.

Oral glucose tolerance test
Oral glucose tolerance test (OGTT) was performed at weeks 4 and 8 after drug intervention. Before the experiment, the mice were fasted overnight, and 50% glucose was intraperitoneally injected at 2 g/kg body weight. The blood glucose of the mice before the injection (0 min) and 30 min, 60 min, and 120 min after the injection was measured. The OGTT time curve was plotted, and the curve was calculated by the approximate trapezoidal method. The area under the Curve (AUC) is calculated as follows: AUC = 0.5 × (BG 0 min + BG 30 min)/2 + 0.5 × (BG 30 min + BG 60 min)/2 + 1 × (BG 60 min + BG 120 min)/2.

Exhaustion time determination using treadmill test for mice

At weeks 3 and 7 of the gavage, mice in each group underwent 6-day treadmill adaptation training, and then, a formal test was performed to record the exhaustion time of each animal per day. The exhaustion speed was 20 m/min, and the running platform slope is 5°. The exhaustion standard included continuous drop into the power grid with shock, being always in the last third of the runway, resting in abdomen position, shortness of breath, and inability to finish the treadmill test. The exhaustion time was recorded.

Detection of blood lipids and free fatty acid levels

After 8 weeks of administration, fasting overnight, the animals were anesthetized the next morning. The blood was collected from the abdominal aorta, and the serum was separated after centrifugation. The levels of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined using the biochemical analyzer. The level of serum FFA was determined according to the kit instructions.

Collection of skeletal muscle tissue

After animal anesthesia, the hind limb skeletal muscles were exposed, and the gastrocnemius muscles were cut out as skeletal muscle collection. The skeletal muscles were washed with 4°C saline for subsequent electron microscopy (fresh tissue fixation) and molecular biological indicators detection (80°C freezing save).

Real-time fluorescence quantitative polymerase chain reaction

The total RNA was extracted from skeletal muscle tissue using the Trizol method. Following the reverse transcription kit instruction, total RNA was transcribed into single-stranded cDNA. Further, reaction system of SYBR MIX (10 μL) + cDNA template (2 μL) + upstream/downstream primers (1 μL each) + nuclease-free ultrapure water (6 μL) was subjected to amplification reaction on a real-time polymerase chain reaction instrument, pre-denaturation at 95°C for 2 min, followed by denaturation at 95°C for 20 s, annealing for 15 s, extension at 72°C for 30 s, and thus repeated 40 cycles. After that, it was stored at 4°C. Three replicate wells were set up for each sample. AMPK upstream primers were AAACCCACAGAAATCCAAACAC, downstream primers were CCTTCCATTCATAGTCCAACTG; PGC-1α upstream primers were CCCTGCCATTGTTAAGACC, and downstream primers were TGTTGCTGTTCCTGCTCCT.

Western blotting

After cutting the skeletal muscle tissue, pyrolysis in a lower temperature was carried on in the pre-cooled RIPA lysate containing 1% PMSF, centrifugation, and the total protein was extracted and determined according to the BCA kit. SDS-PAGE separation gel and concentration gel were prepared, after that, electrophoresis, transmembrane, blocking were performed. The PVDF membrane was incubated with diluted AMPK, p-AMPK, PGC-1α (1:1000, 5% skim milk powder diluted), shaking at 4°C overnight, and then incubated for 1.5 h using fluorescent secondary antibody (dilution in 1: 5000) at room temperature. The color development was by ECL luminescence, and imaging was exposed by gel imager. ImageJ software analyzed the gray scale of the strip and performed the semi-quantitative analysis.

Statistical analyses

Statistical analysis was performed using SPSS17.0 software. The measurement data were expressed as mean ± standard error of the mean. The data of multiple groups were compared using analysis of variance. The comparison between the two groups was performed by Dunnett or independent sample *t*-test. $P < 0.05$ was considered as statistical significance. GraphPad Prism6 software was used for data management and charting (GraphPad Software Inc., San Diego, Calif, USA).

RESULTS

Effects on body weight and food intake in obese mice

As shown in Figure 1a, after ginsenoside Rb1 intervention, the difference in body weight between the ginsenoside Rb1 group and the model control group on week 5 after drug administration was statistically significant ($P < 0.05$), which was similar to the positive drug metformin. As shown in Figure 1b, the body weight of the model control group increased by 9.50 g at average, significantly higher than that of the normal control group, 1.40 g ($P < 0.05$). The mice in the metformin group lost weight by 0.63 g, while in the ginsenoside Rb1 group, the body weight of mice increased by 1.04 g, which were displayed statistical differences between the metformin group and the model control group, and between the ginsenoside Rb1 and the model control group ($P < 0.05$). As shown in Figure 1c, the food intake of mice in the model control group was significantly higher than that of the normal control group ($P < 0.05$). Compared with the model control group, the food intake of the metformin group (from the 6th week) and the ginsenoside Rb1 group (from the 1st week) differed significantly ($P < 0.05$).

Effects on the fat content and the content of muscle tissues

As shown in Figure 2a and b, the content of muscle tissue in mice fed with high-fat diet was significantly lower than that in the normal control group, and the body fat content was significantly higher compared with that in the normal control group ($P < 0.05$). After 8-week drug intervention, the body fat content in the metformin and ginsenoside Rb1 groups was 19.50% and 20.91%, respectively, which decreased significantly compared with that of the model control group, 30.46% ($P < 0.05$). The body muscle content was 72.64% and 72.61%, respectively in the metformin and ginsenoside Rb1 groups, significantly higher than that of the model control group, 60.02%, suggesting that ginsenoside Rb1 can reduce the total amount of fat in obese mice, increase the muscle tissue content, and have a certain effect on improving body composition.
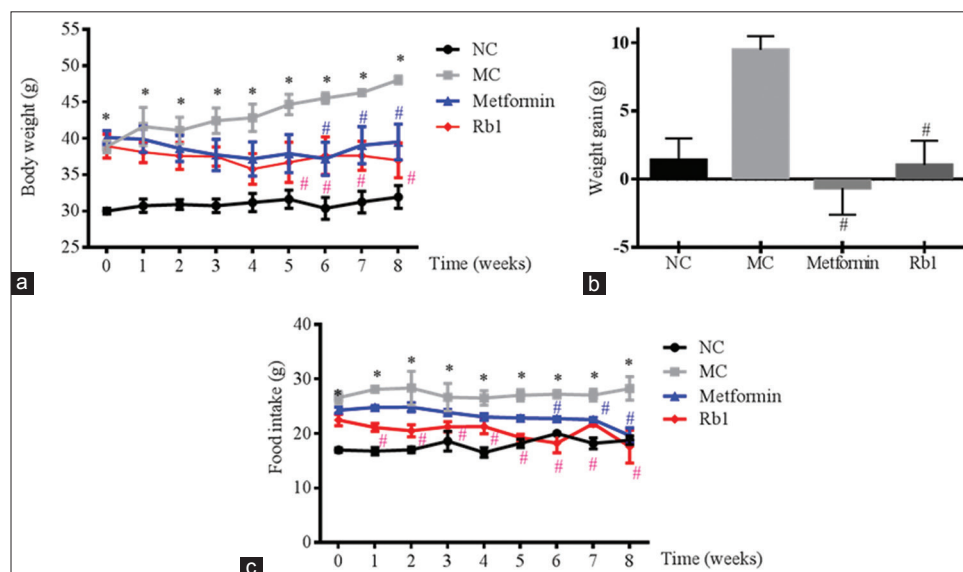
Figure 1: Comparison of the body weight (a), body weight gain (b) and food intake (c). *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control
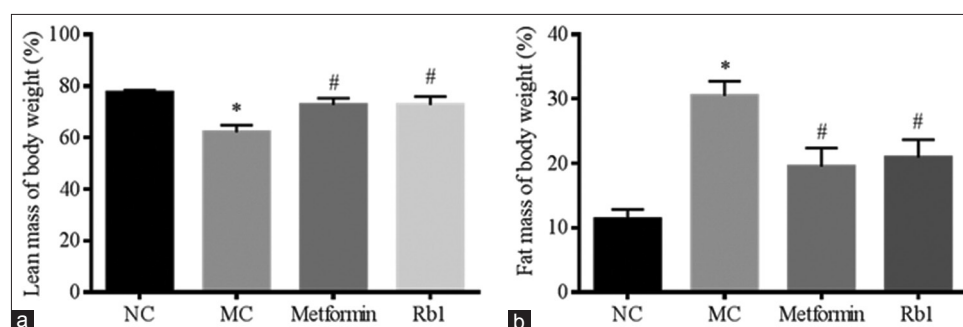

Figure 2: Comparison of the lean mass (a) and the fat mass (b). *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control

Regulate blood lipid levels and free fatty acid levels in obese mice

As shown in Figure 3a-d, the blood lipid levels of the model control group were abnormal. Specifically, the levels of TC, TG, LDL-C elevated, while the level of HDL-C was in decrease. Compared with the normal control group, the differences were significant ($P < 0.05$). Eight weeks after drug intervention, ginsenoside Rb1 could significantly reduce TG, LDL-C levels, and increase serum HDL-C level, compared with the model control group, the differences were significant ($P < 0.05$). As shown in Figure 3e, compared with the normal control group, the FFA level of the model control group increased significantly ($P < 0.05$). After 8 weeks of drug intervention, both metformin and ginsenoside Rb1 could significantly reduce the FFA level of obese mice. Compared with the model control group, the difference in FFA level was displayed statistical significance ($P < 0.05$).

Improve insulin sensitivity in obese mice

As shown in Figure 4, the blood glucose at various points-in-time and the AUC in the model control group were significantly higher than those of the normal control group ($P < 0.05$), suggesting that the obese mice suffered from impaired glucose tolerance in this experiment. At 4 weeks after administration, the blood glucose level in mice at 30 min and 60 min markedly decreased in the metformin group, and the AUC was also greatly less than that of the model control group. However, for the ginsenoside Rb1, the OGTT at different points-in-time and the AUC in obese mice were not significant from those in the model control group ($P > 0.05$). At the 8th week of administration, ginsenoside Rb1 significantly decreased the blood glucose level after glucose load in mice and reduced the area under the glucose tolerance test (GTT) curve ($P < 0.05$). The effect of improving GTT in obese mice was equivalent to that of metformin, indicating that ginsenoside Rb1 can improve the glucose tolerance of obese mice, and this effect shows a certain time-effect manner.

Enhance mice exercise tolerance

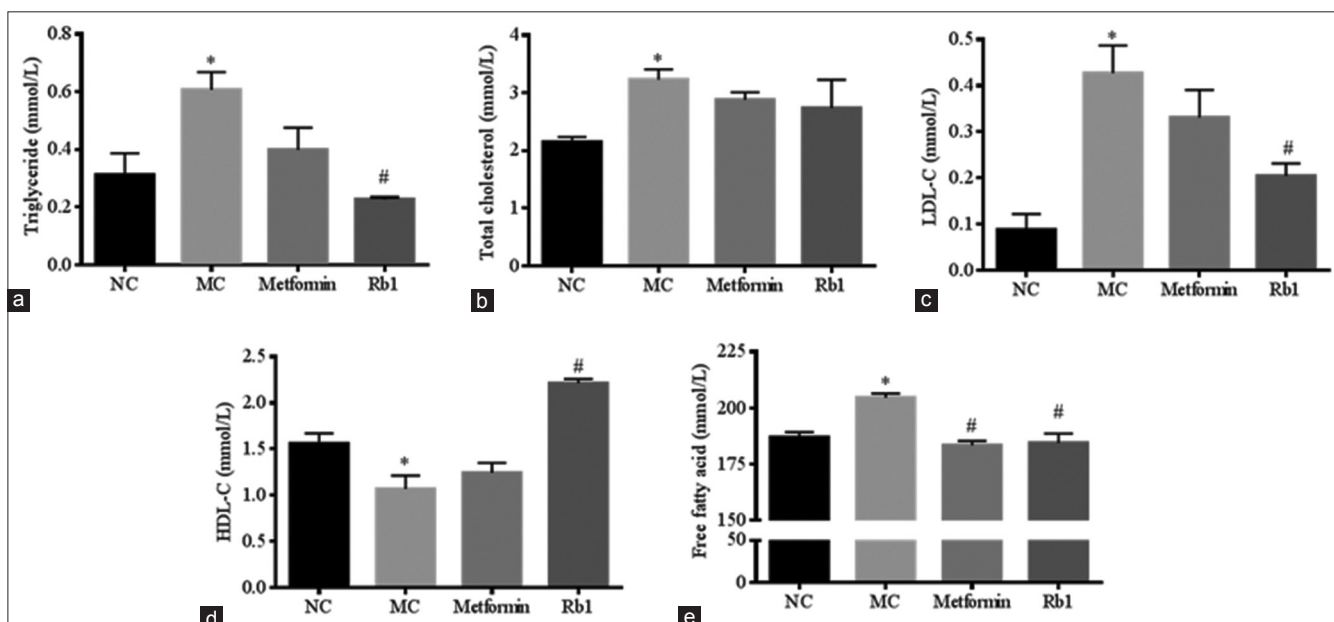As shown in Figure 5, in the model control group, the exhaustion time of the mice significantly decreased on weeks

Figure 3: Comparison of the serum triglyceride (a), total cholesterol (b), low density lipoprotein cholesterol (c), high density lipoprotein cholesterol (d) and free fatty acid (e). *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control
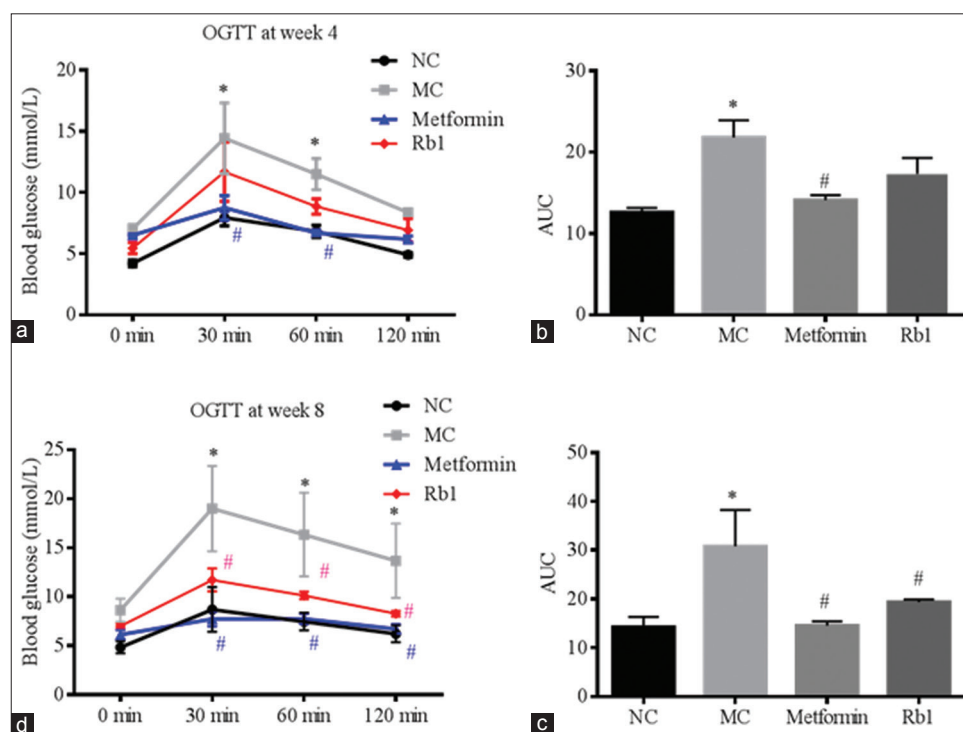

Figure 4: Comparison of glucose (a) and the area under the curve (b) during oral glucose tolerance test at the fourth week; comparison of blood glucose (c) and the area under the curve (d) during oral glucose tolerance test at the eighth week. *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control

3 and 7 compared with that in the normal control group ($P < 0.05$). Compared with the model control group, the exhaustive time of mice in the metformin and ginsenoside Rb1 groups was significantly prolonged ($P < 0.05$), indicating that ginsenoside Rb1 can markedly improve the skeletal muscle exercise tolerance in obese mice.

Influence on gene expression of skeletal muscle adenosine monophosphate-activated protein kinase α and proliferator-activated receptor gamma coactivator-1α in mice

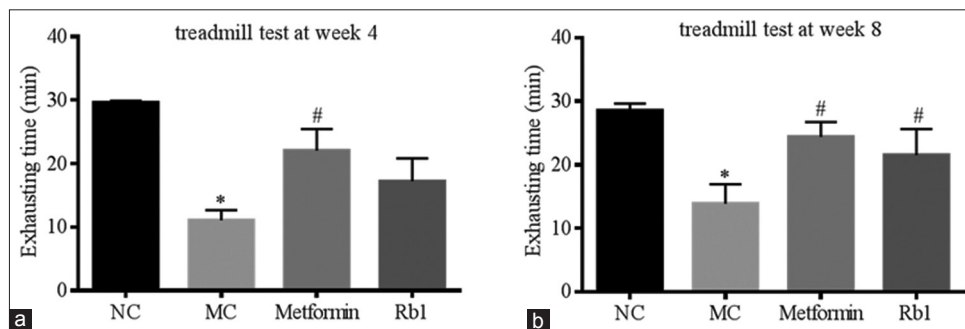As shown in Figure 6, taking the untreated normal control sample as a reference, the expression of AMPKα and PGC-1α

Figure 5: Comparison of the exhausting time during the treadmill test at the fourth week (a) and the eighth week (d). *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control
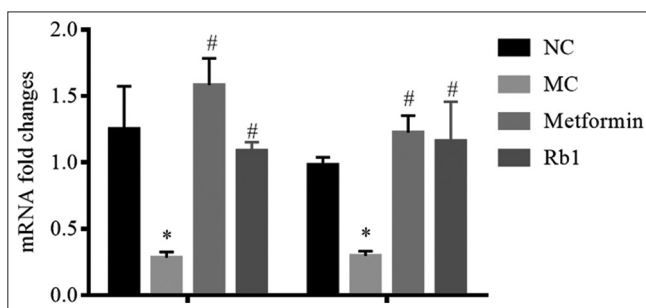

Figure 6: Ginsenoside Rb1 on gene expression of adenosine monophosphate-activated protein kinase α and proliferator-activated receptor gamma coactivator-1α in skeletal muscle in obese mice. *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control

in skeletal muscle of obese mice was down-regulated. Compared with the model control group, the expression of AMPKα and PGC-1α mRNA in mice skeletal muscle in both the metformin and the ginsenoside Rb1 groups was significantly upregulated ($P < 0.05$).

Influence on protein expression of skeletal muscle adenosine monophosphate-activated protein kinase α, p-adenosine monophosphate-activated protein kinase α and proliferator-activated receptor gamma coactivator-1α in mice

As shown in Figure 7, compared with the normal control group, the expression of AMPKα, p-AMPKα, and PGC-1α proteins in the mice skeletal muscle of the model control group significantly decreased ($P < 0.05$). Compared with the model control group, in both the metformin and ginsenoside Rb1 groups, the expression of AMPKα, p-AMPKα, and PGC-1α proteins in skeletal muscle of mice significantly increased ($P < 0.05$).

Discussion

IR is a condition in which the dose of insulin produced by our body is insufficient for the normal biological effect. From a quantitative point of view, skeletal muscle is the most important tissue for blood glucose utilization in the body and is the main target of peripheral IR. Solving the skeletal

IR is of great significance on improving the metabolic state of individuals such as obesity and DM.[8] This study showed that after 12 weeks of a high-fat diet in mice, compared with normal-fed mice, obesity is obvious in morphology. In the physical constitutional analysis, the content of fat increased presented with marked IR, hyperlipidemia, and elevated level of serum FFA.

Ginsenoside Rb1 serves as one of the main active ingredients in ginseng, which gets involved in multiple links in the regulation of glucolipid metabolism on exploring the anti-diabetic mechanism of traditional Chinese herbal medicine ginseng.[9] In the current study, we found that ginsenoside Rb1 could markedly reduce the body weight, decrease the body fat content, regulate the disorder of dyslipidemia, and improve insulin sensitivity in obese mice, and its effect is equivalent to the positive drug metformin, which is in accord with the previous study.[10] On this basis, we also found that ginsenoside Rb1 could improve the body composition of obese mice, have a tendency of increasing muscle tissue content, and strengthen the skeletal muscle tolerance, indicating that ginsenoside Rb1 improves the IR status of obese mice. This effect is not only associated with up-regulation of adipocyte peroxisome proliferator-activated receptor gamma promoting adipocyte differentiation,[11] up-regulation of the expression of glucose transporters in adipocytes promoting glucose consumption,[10] inhibition of inflammatory responses and oxidative stress,[12] but also with the regulation of skeletal muscle energy substance metabolism.

The elevation of plasma FFA is a clinical feature of T2DM and obesity, and plays a decisive role in leading to IR.[13] Increased FFA levels in skeletal muscle cells due to elevated FFA levels in blood circulating can obviously reduce insulin-stimulated skeletal muscle glucose uptake and utilization, affect skeletal muscle energy metabolism, and trigger the skeletal muscle IR.[14,15] In the current study, compared with the model control group, ginsenoside Rb1 can remarkably reduce the serum FFA levels, thereby further improving the metabolism of skeletal muscle energy substances.

Therefore, on the basis of pharmacodynamics, we further detected changes in AMPK and PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α), important
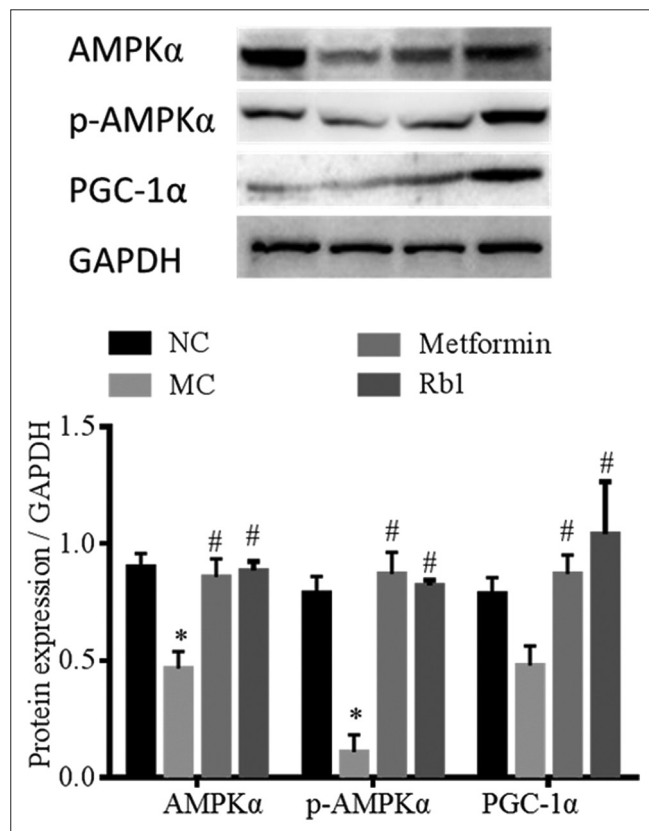
Figure 7: Ginsenoside Rb1 on expression of adenosine monophosphate-activated protein kinase α, p-adenosine monophosphate-activated protein kinase α, and proliferator-activated receptor gamma coactivator-1α proteins in skeletal muscle in obese mice. *versus normal control group, $P < 0.05$, #versus model control group, $P < 0.05$. NC: Normal control; MC: Model control

regulators of skeletal muscle energy metabolism. Skeletal muscle is the main tissue of aerobic oxidation of glucose and lipids and plays an important role in regulating systemic energy metabolism. Copious studies displayed that AMPK is an energy receptor for skeletal muscle and can regulate the body's energy metabolism,[16] so some scholars believe that AMPK is a potential target in metabolic diseases and energy metabolism disorders.[17] As a key mediator of energy metabolism regulation, AMPK activation plays a vital role in the skeletal muscle energy metabolism through enhancing glucose uptake and oxidation of skeletal muscle cells, increasing the oxidation rate of fatty acids, and inhibiting muscle glycogen synthesis and promoting glycogen decomposition,[18] which is closely associated with skeletal muscle IR. Studies proved overexpression of AMPK in skeletal muscle tissue can increase fatty acid oxidation and prevent skeletal muscle lipid deposition induced by high-fat diet.[19] PGC-1α can regulate energy metabolism and induce mitochondrial biogenesis. Research exhibits that PGC-1α in skeletal muscle maintains glucose metabolism balance and establishes a cytokine-mediated association between skeletal muscle and islets.[20] AMPK, an important regulator of PGC-1α, activates PGC-1α, enhances skeletal muscle mitochondria and promotes fatty acid

oxidation.[4] Therefore, activated AMPK can not only enhance glucose uptake by skeletal muscle cells through the increase of glucose transporter expression and transportation to its vesicle of the cell membrane,[21] but also improve the IR status by regulating PGC-1α which promotes mitochondrial oxidative respiratory chain activity.[22] In the present study, the expression of AMPK and PGC-1α genes in skeletal muscle tissue of model control mice was in a down-regulation tendency, and the tendency of PGC-1α activity changes was consistent with the activation trend of AMPK. After 8 weeks of ginsenoside Rb1 intervention, the two indicators were up-regulated remarkably. The results of protein levels were similar to the gene levels, and the expression of activated AMPK (p-AMPK) protein was significantly increased, indicating that ginsenoside Rb1 can activate the linkage reaction of AMPK-PGC-1α, affects the mitochondrial state and energy metabolism of skeletal muscle, thereby improving IR in an obese mouse model.

To sum up, ginsenoside Rb1 exerts remarkable effects on weight loss, lipid-lowering and improvement of skeletal muscle IR in obese mice. The mechanism may be related with FFA lowering, upregulation of skeletal muscle mitochondrial AMPK and PGC-1α gene expression, which activates AMPK, thereby enhancing mitochondrial function and fatty acid β oxidation, and ultimately reducing skeletal muscle lipid deposition. This study has laid a good foundation for the later research on ginsenoside Rb1 in the treatment of obesity, diabetes, and other metabolic diseases and the development of AMPK agonists.

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

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