Journal of Ginseng Research 46 (2022) 561–571

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: https://www.sciencedirect.com/journal/journal-of-ginseng-research

Research Article

Upregulation of adiponectin by Ginsenoside Rb1 contributes to amelioration of hepatic steatosis induced by high fat diet

Yaru Li [a, b, 1], Shuchen Zhang [a, b, 1], Ziwei Zhu [a, b], Ruonan Zhou [a, b], Pingyuan Xu [a, b], Lingyan Zhou [a, b], Yue Kan [a, b], Jiao Li [a, b], Juan Zhao [b], Penghua Fang [b], Xizhong Yu [b, *], Wenbin Shang [a, b, **]

[a] Department of Endocrinology, Jiangsu Province Hospital of Chinese Medicine, the Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China
[b] Key Laboratory for Metabolic Diseases in Chinese Medicine, First College of Clinical Medicine, Nanjing University of Chinese Medicine, Nanjing, China

ARTICLE INFO

Article history:
Received 3 August 2021
Received in revised form
22 October 2021
Accepted 25 October 2021
Available online 3 November 2021

Keywords:
Adipocyte
Adiponectin
AMPK
Ginsenoside Rb1
Hepatic steatosis

ABSTRACT

Background: Ginsenoside Rb1 (GRb1) is capable of regulating lipid and glucose metabolism through its action on adipocytes. However, the beneficial role of GRb1-induced up-regulation of adiponectin in liver steatosis remains unelucidated. Thus, we tested whether GRb1 ameliorates liver steatosis and insulin resistance by promoting the expression of adiponectin.

Methods: 3T3-L1 adipocytes and hepatocytes were used to investigate GRb1's action on adiponectin expression and triglyceride (TG) accumulation. Wild type (WT) mice and adiponectin knockout (KO) mice fed high fat diet were treated with GRb1 for 2 weeks. Hepatic fat accumulation and function as well as insulin sensitivity was measured. The activation of AMPK was also detected in the liver and hepatocytes.

Results: GRb1 reversed the reduction of adiponectin secretion in adipocytes. The conditioned medium (CM) from adipocytes treated with GRb1 reduced TG accumulation in hepatocytes, which was partly attenuated by the adiponectin antibody. In the KO mice, the GRb1-induced significant decrease of TG content, ALT and AST was blocked by the deletion of adiponectin. The elevations of GRb1-induced insulin sensitivity indicated by OGTT, ITT and HOMA-IR were also weakened in the KO mice. The CM treatment significantly enhanced the phosphorylation of AMPK in hepatocytes, but not GRb1 treatment. Likewise, the phosphorylation of AMPK in liver of the WT mice was increased by GRb1, but not in the KO mice.

Conclusions: The up-regulation of adiponectin by GRb1 contributes to the amelioration of liver steatosis and insulin resistance, which further elucidates a new mechanism underlying the beneficial effects of GRb1 on obesity.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat accumulation in hepatocytes resulting from various genetic and dietary factors excluding alcohol, and is tightly associated with obesity, insulin resistance, type 2 diabetes mellitus (T2DM) and cardiometabolic diseases [1,2]. NAFLD has a spectrum of severity ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which is linked to an increased risk of hepatocellular carcinoma and cirrhosis [3]. With the globalization of obesity and its related metabolic syndrome, NAFLD has now become an important cause of chronic liver disease in developed countries, such as Europe and the United States, and in the affluent regions of Asia [4,5]. However, there are still no approved pharmacological therapies to prevent or treat this disease in clinic [6]. Thus, it is of great significance to search for the treatment strategy for NAFLD.

In the obese and/or insulin resistant state, the dysfunction of adipose tissue is a major risk factor for developing NAFLD [1,7]. Fat can be synthesized in the liver in the form of triglycerides (TG) by

* Corresponding author.
** Corresponding author. Key Laboratory for Metabolic Diseases in Chinese Medicine, First College of Clinical Medicine, Nanjing University of Chinese Medicine, Nanjing, China.
E-mail addresses: yxz@njucm.edu.cn (X. Yu), wbshang@njucm.edu.cn (W. Shang).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.jgr.2021.10.005

utilizing free fatty acids (FFAs) derived from white adipose tissue (WAT) lipolysis [8]. The elevated WAT lipolysis in obesity leads to excessive FFA efflux and the higher circulation of FFA significantly, which results in the increased uptake of fatty acids into liver. Then, liver steatosis is triggered by excessive accumulation of TG within hepatocytes [9]. Moreover, adipose tissue, as an endocrine organ, releases a large array of adipokines to regulate glucolipid metabolism and insulin sensitivity [10]. Among them, adiponectin, one of the most abundant adipokines secreted from adipose tissue, is an insulin-sensitizing cytokine possessing multiple beneficial effects on obesity-related medical complication, including NAFLD [11,12]. Adiponectin is capable of inhibiting de novo lipogenesis (DNL), stimulating fatty acids oxidation through activating AMP-activated protein kinase (AMPK) pathway in liver to prevent diet-induced liver steatosis [13]. In obesity and T2DM, the low plasma adiponectin level plays a crucial role in the pathogenesis of liver steatosis and promoting of adiponectin secretion is now considered as an attractive therapeutic strategy in the management of NAFLD [6,12].

Ginseng, as a traditional herbal medicine, has been used for the treatment of various diseases in Asian countries for thousands of years [14,15]. The bioactive ingredients of ginseng are mainly ginsenosides, which are responsible for the most pharmacological actions of ginseng [14]. Among nearly 200 ginsenosides, ginsenoside Rb1 (GRb1) is the most abundant in the roots, rhizomes, and root hairs of ginseng [16,17]. GRb1 was shown to possess antioxidant, anti-inflammatory, anti-cardiovascular disease, anti-central nervous system disorder and anticancer activity [15,17] as well as anti-obesity and anti-diabetic property [18,19]. In our previous studies, GRb1 showed its ability to increase glucose uptake in adipocytes by stimulating insulin signaling [20] and promote adipocyte differentiation by enhancing peroxisome proliferator-activated receptorγ (PPARγ) gene expression [21]. We also demonstrated that GRb1 reduced ectopic lipid deposition in the liver partly via up-regulating perilipin expression to suppress lipolysis of adipose tissue in db/db obese mice [22]. Moreover, GRb1 reversed the decreased level of adiponectin in adipoctyes and obese mice and improves symmetric insulin resistance [22]. Other study also showed that GRb1 improved fatty liver by activating hepatic 5′ adenosine monophosphate-activated protein kinase (AMPK) in obese rats [23]. Since activation of PPAR-γ in adipocyte promoted the transcription and plasma concentrations of adiponectin [24], which further activates AMPK to increase FA oxidation and decrease intracellular lipid stores and insulin resistance [13], we hypothesized that upregulation of adiponectin may involve in the therapeutic effect of GRb1 on hepatic steatosis. Therefore, in this study, we explored that a specific role of adiponectin in the amelioration of hepatic steatosis induced by GRb1 using adipocytes and hepatocytes co-culture model as well as adiponectin knockout obese mice fed with high fat diet (HFD).

2. Materials and methods

2.1. Materials

Ginsenoside Rb1 (purity more than 98%), palmitic acid (PA) and bovine serum album (BSA, FFA free) were obtained from Sigma-Aldrich (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Amresco (Radnor, PA, USA). Recombinant insulin was obtained from Lily (Fegersheim, France). Collagenase Type IV and recombinant mouse tumor necrosis factor α (TNF-α) was obtained from R&D (Minnesota, USA). AMPKα and phospho-AMPKα (Thr172) antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Adiponectin antibody was obtained from Abcam (Cambridge, UK) and IgG was obtained from RD system (Minnesota, USA). The BCA protein assay kit was purchased from Pierce (Rockford, IL, USA).

2.2. Cell culture

Mouse 3T3-L1 fibroblast cells and HepG2 cells were obtained from ATCC (Manassas, VA, USA). 3T3-L1 fibroblast cells were cultured and induced to differentiated adipocytes as previously described [20]. Between 8 and 10 days after the initiation of differentiation, more than 90% of the cells presenting the adipocyte phenotype were used for the next experiments. GRb1, dissolved in DMSO as a 1000-fold stock, and added into 0.2% BSA-DMEM (20 μM) for treating differentiated adipocyte in the presence of TNF-α (10 ng/ml). DMSO was present in the control culture at less than concentration 0.1% (v/v). After 24 h incubation, the conditioned medium (CM) was harvested for ELISA and next experiment in the liver cells.

HepG2 cells were grown in DMEM, supplemented with 10% FBS. The hepatocytes were isolated from mice as previously described [25]. Mice were anesthetized using 2% pentobarbital sodium, and liver were perfused with D-HBSS (5.33 mM KCL, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.34 Mm Na2HPO4, 5.56 mM D-glucose, 15 mM HEPES) containing 0.5 mM EGTA (pH 7.4) and then with 80 ml DMEM containing 40 mg collagenase type IV at a rate of 5–6 ml/min. The hepatocytes were dissociated and washed with DMEM. Hepatocytes were plated at 1×10^5 cells per cm^2 on dishes in DMEM, 10% FBS, 64 μg/ml penicillin, and 100 μg/ml streptomycin and kept in a humidified cell-culture incubator at 37 °C and 5% CO_2 until use the following day. Hepatocytes and HepG2 cells were pretreated with 0.67% BSA-DMEM containing 500 μM palmitic acid for 24 h, then treated with either GRb1 (20 μM) or CM collected from adipocytes culture.

2.3. Animals experiments

Male C57BL/6J mice (WT mice) were obtained from SLAC Laboratory Animal Co. Ltd (Shanghai, China). Adiponectin homozygous knockout mice (KO mice) in a C57BL/6 background were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China). Mice were free access to standard rodent chow (SLAC, Shanghai, China) and tap water, maintained on a 12/12 h light/dark cycle at 25 °C. The WT mice and the KO mice were randomized, then fed ad libitum with either a standard rodent chow containing approximately 10% fat (SLAC, Shanghai) or high fat diet (HFD) containing 60% fat (Research Diets, USA), respectively. After 12 weeks, the mice were still fed with chow or HFD and randomly assigned to receive either vehicle (0.9% saline) or Rb1 at dosage of 10 mg/kg/body weight per day via intraperitoneal injection for 2 weeks. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed. After 3 days, the blood samples were collected from the orbital vein of mice after anesthetized by diethyl ether. Epididymal fat and liver were also surgically collected and stored at −80 °C. The animal experiment was approved by the Animal Care and Use Committee at the University Of Nanjing University Of Chinese Medicine (No.ACU170702).

2.4. Glucose and insulin tolerance tests

OGTT was performed in the mice fasted overnight for 16 h. Mice were given of glucose (2 g/kg body weight) by gavage. Blood glucose levels at 0, 15, 30, 60 and 120 min were measured using a blood glucose meter (Johnson & Johnson Medical, Shanghai, China) by collecting blood samples from the tail vein.

3 days after the OGTT, insulin tolerance test (ITT) in mice was performed after fasting overnight for 16 h. The mice were injected

intraperitoneally with insulin at 0.75IU/kg bodyweight and blood glucose measurement was performed as described in the OGTT.

2.5. Biochemical measurement and ELISA

The fasting insulin levels were measured using an ELISA kit (Mercodia, Uppala, Sweden). The homeostasis model assessment of basal insulin resistance (HOMA-IR) was calculated (the product of the fasting concentrations of blood glucose (mmol/L) and fasting serum insulin (mIU/L) divided by 22.5). Serum EFA was analyzed using NEFA kit (wako, Osaka, Japan). Serum fasting blood glucose levels, TG, ALT and AST were measured using biochemical methods. The levels of adiponectin from serum and cell supernatant were measured using an ELISA kit (Millipore, St Charles, MO, USA).

2.6. Measurement for TG in cells and liver

Approximately 100 mg of liver tissue was added into ethanolic KOH at 55 °C overnight, and the digest was extracted twice with 50% ethanol. Then, the TG content in the supernatant neutralized

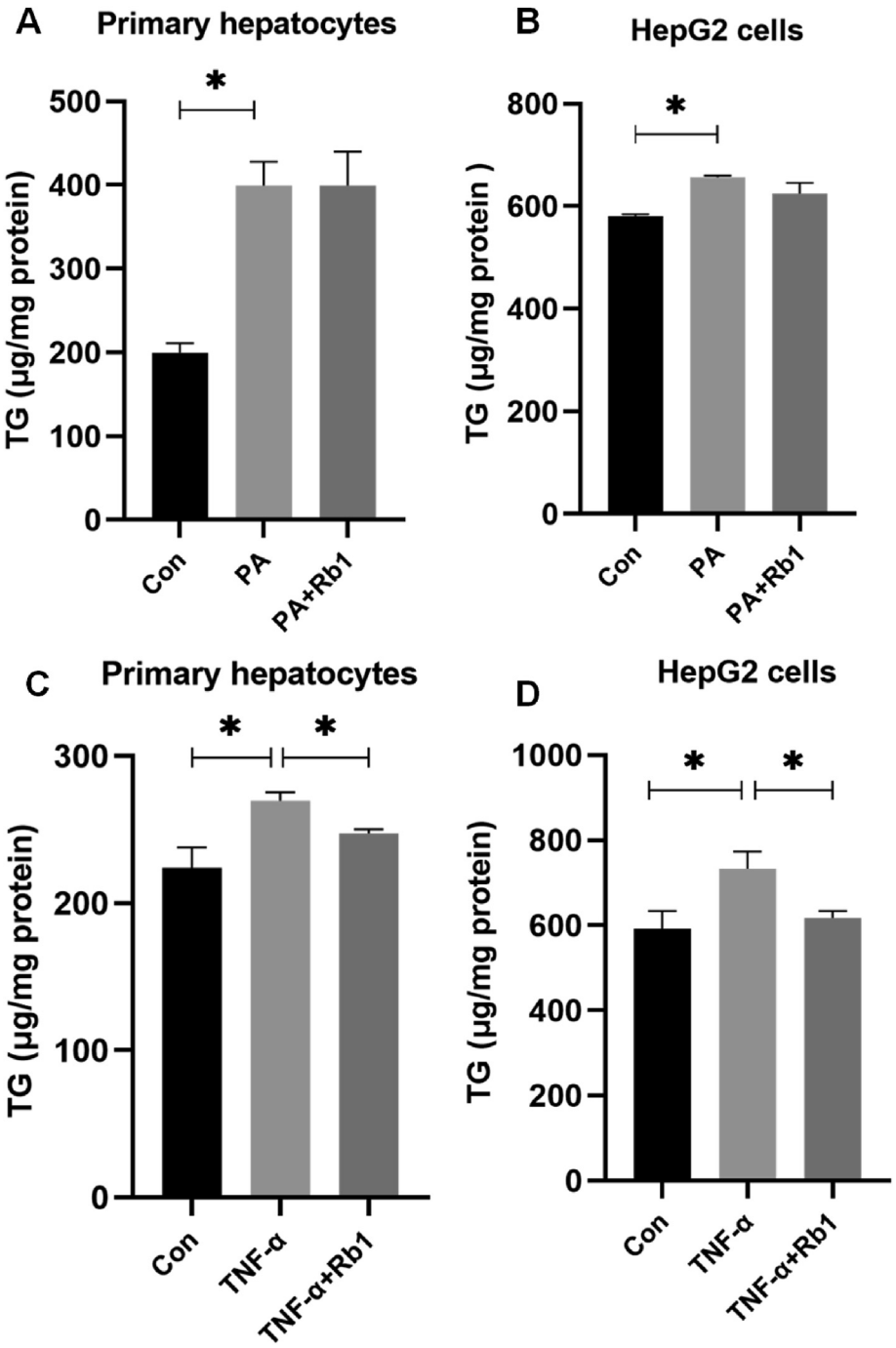


Fig. 1. GRb1 reduced TG accumulation in hepatocytes depending on its action on adipocytes. After the treatment of 500 μM palmitic acid (PA) for 24 h, primary hepatocytes (A) and HepG2 (B) cells were treated with or without Rb1 (20 μM) for 24 h. Primary hepatocytes (C) and HepG2 cells (D) were pretreated with 500 μM PA for 24 h, then incubated with the conditioned medium (CM) collected from 3T3-L1adipocytes treated with TNF-α (10 ng/ml) in the presence or absence of Rb1 for 24 h. The TG in cells was measured. Values are expressed as mean ± SD. *$P < 0.05$. Experiments were performed in three replicates.

with $MgCl_2$ was measured using a TG measurement kit (Sigma, Saint Louis, USA).

2.7. Histological examination of liver

Liver sections were cut using a microtome (Leica, Wetzlar, Germany) and mounted on gelatin-coated slides. The sections were fixed with 4% pareformaldehyde at room temperature for 24 h, and then at 4 °C for 24 h. Samples were dehydrated with ethanol and cleared with xylene, then embedded in paraffin. The sections were stained with Hematoxylin and Eosin (HE). The sections were photographed at 100 × magnification.

2.8. Western blotting

Total proteins of liver, 3T3-L1 adipocytes and primary hepatocytes were extracted by lysis buffer (containing 100 μM Na_3VO, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, pH 8.8). Protein concentration was measured. The samples was resolved with SDS-PAGE, and then transferred to PVDF membranes. The membranes were incubated in blocking buffer (1 × Tris-

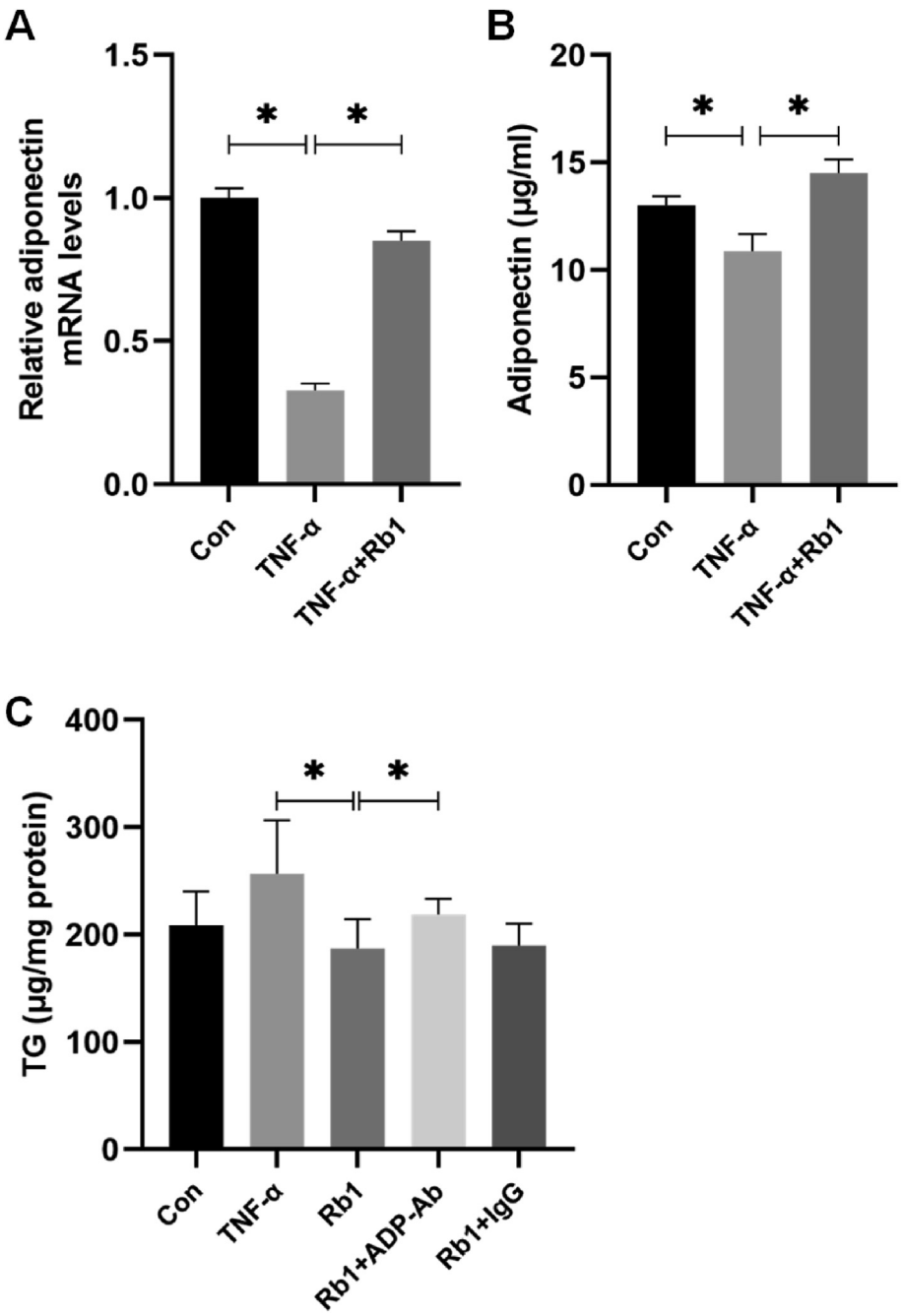
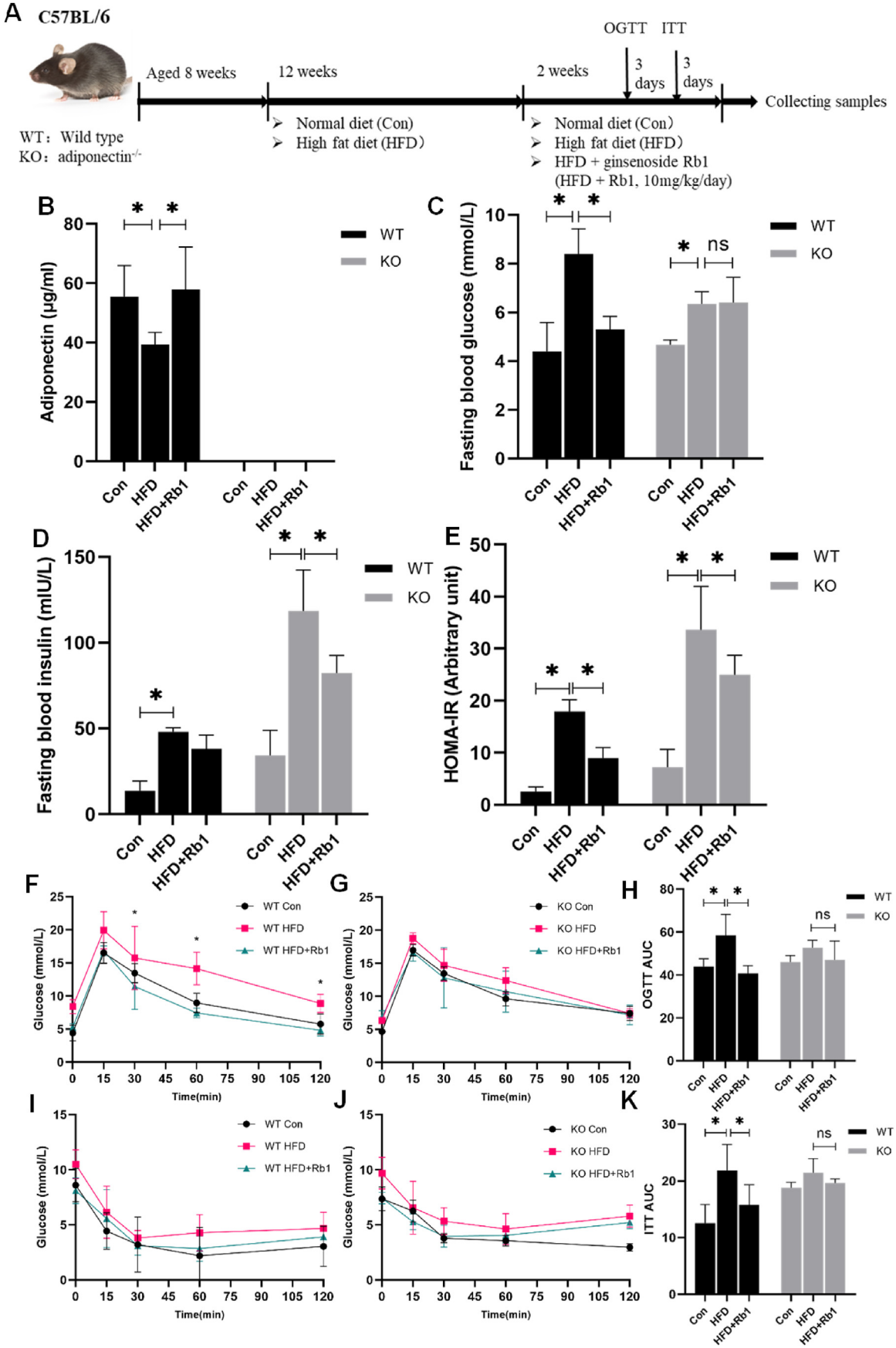


Fig. 2. GRb1 attenuated TG accumulation in hepatocytes through increasing adiponectin expression and secretion in adipocytes. Adipocytes were treated with TNF-α and TNF-α+Rb1 for 24 h. (A) The mRNA levels of adiponectin in adipocytes were measured by qPCR. (B) The levels of adiponectin in the supernatants were analyzed by ELISA. (C) After the pretreated with 500 μM PA for 24 h, primary hepatocytes were incubated with CM in the presence or absence of adiponectin antibody (10 μg/ml). The level of TG in primary hepatocytes was measured. Values are expressed as mean ± SD. *$P < 0.05$. Experiments were performed in three replicates.


A
C57BL/6
OGTT ITT
Aged 8 weeks
12 weeks
2 weeks
3 days
3 days
Collecting samples
WT: Wild type
KO: adiponectin-/-
Normal diet (Con)
High fat diet (HFD)
Normal diet (Con)
High fat diet (HFD)
HFD + ginsenoside Rb1
(HFD + Rb1, 10mg/kg/day)
B
Adiponectin (μg/ml)
C
Fasting blood glucose (mmol/L)
D
Fasting blood insulin (mIU/L)
E
HOMA-IR (Arbitrary unit)
WT
KO
Con
HFD
HFD+Rb1
ns
F
G
H
I
J
K
Glucose (mmol/L)
Time(min)
WT Con
WT HFD
WT HFD+Rb1
KO Con
KO HFD
KO HFD+Rb1
OGTT AUC
ITT AUC

buffered saline containing 0.1% Tween-20 and 5% BSA) for 2 h. Then, the membranes incubated with a primary antibody overnight at 4 °C. The membranes were then washed and incubated at room temperature with HRP-conjugated secondary antibodies for 2 h. The protein signal was detected with the ECL Plus system (GE Healthcare).

2.9. Real-time PCR

Total RNA was extracted from 3T3-L1 cells using RNAiso Plus (TaKaRa, Dalian, China) and reverse-transcribed using SuperScript III Reverse Transcriptase and Oligo (dT) primer (Invitrogen, Shanghai, China). Quantitative real-time PCR was performed by using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primer sequences were: adiponectin, 5′-ACA AGG CCG TTC TCT TCA CC-3' (forward) and 5′-CCA GAT GGA GGA GCA CAG AG-3' (reverse); β-actin, 5′CAT CCG TAA AGA CCT CTA TGC CAA C-3' (forward) and 5′-ATG GAG CCA CCG ATC CAC A-3' (reverse); Quantitation of adiponectin expression was adjusted by normalization to β-actin expression, and the results were calculated using the $2^{-\Delta\Delta}$Ct method.

2.10. Statistical analysis

All values were expressed as mean ± standard deviation. The data were analyzed using Graphpad prism 8.0. Student's *t*-test was used to determine the differences between two groups. The differences among multiple groups were determined by one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. GRb1 reduced TG accumulation in hepatocytes depending on its action on adipocytes

To explore whether direct action of GRb1 on TG content in hepatocytes, the primary hepatocytes and HepG2 cells were pretreated with 500 μM palmitic acid (PA) for 24 h, then treated with GRb1 (Rb1) at a concentration of 20 μM (the optimal dosage in cells as previous reports [20,23]. After 24 h treatment, GRb1 did not reduce the elevated TG accumulation induced by PA in both types of hepatocytes (Fig. 1A and B). However, when primary hepatocytes and HepG2 cells were incubated with the conditioned medium collected from adipocytes treated with 20 μM GRb1 in the presence or absence of TNF-α, GRb1 significantly inhibited the TG accumulation in primary hepatocytes (Fig. 1C, $P < 0.05$) and HepG2 cells (Fig. 1C, $P < 0.05$). These results suggested the inhibitory effect of GRb1 on TG deposit in hepatocytes via its action on adipocytes.

3.2. GRb1 attenuated TG accumulation in hepatocytes through increasing adiponectin in adipocytes

The mRNA level of adiponectin in adipocytes and the concentration of adiponectin in the conditioned medium were assessed. While the treatment of TNF-α decreased the expression of adiponectin and GRb1 recovered the suppressed mRNA expression of adiponectin in adipocytes (Fig. 2A, $P < 0.05$). GRb1 also elevated the decreased concentration of adiponectin induced by TNF-α in the conditioned medium (Fig. 2B, $P < 0.05$). To elucidate whether the decrease of the TG content in hepatocytes treated with the CM was associated with the elevated adiponectin, 10 μg/ml adiponectin antibody (ADP-Ab) was added into the CM, then primary hepatocytes were incubated with the above CM containing ADP-Ab after pretreated with PA. The result showed that the inhibitory action of the Rb1-treated CM on TG accumulation was partly attenuated by ADP-Ab (Fig. 2C, $P < 0.05$), suggesting that adiponectin mediates between reduced TG deposit in hepatocytes and Rb1's action on adipocytes.

3.3. The deletion of adiponectin weakened GRb1's insulin-sensitizing activity in obese mice

In order to verify the role of adiponectin in the regulation of lipid and glucose metabolism by GRb1, adiponectin knock out mice (KO mice) fed with HFD were used in the next experiments (Fig. 3A). In the wild type mice (WT), the level of serum adiponectin was reduced in HFD mice compared with that in the normal chow mice, and GRb1 treatment restored the decreased level of serum adiponectin induced by HFD (Fig. 3B, $P < 0.05$). There was no adiponectin detected in serum of the KO mice (Fig. 3B). The fasting blood glucose (FBG) of HFD group was higher than that of the control group in WT and KO mice, respectively. After the GRb1 treatment, the FBG was significantly decreased in the WT mice (Fig. 3C, $P < 0.05$), but there was no significant change was observed in the KO mice (Fig. 3C). The treatment of GRb1 group significantly reduced the level of serum insulin in both WT and KO mice, which was increased by HFD (Fig. 3D, $P < 0.05$). HOMA-IR index was calculated to assess insulin resistance (Fig. 3E). GRb1 also decreased the HOMA-IR in both WT and KO mice, respectively (Fig. 3E, $P < 0.05$). However, GRb1 made the HOMA-IR reduced by 50% in the WT mice, in contrast to 26% in the KO mice (Fig. 3E). In OGTT (Fig. 3F—H) and ITT (Fig. 3I—K), GRb1 decreased the area under curve (AUC) ambulated by HFD in the WT mice, but not in the KO mice. (Fig. 3H and K, $P < 0.05$). These results suggest that GRb1 improves insulin resistance and glucose tolerance partially depended on adiponectin.

3.4. The inhibition of liver fat accumulation by GRb1 was attenuated by knockout of adiponectin in obese mice

GRb1 reduced the increase of liver weight and liver TG content induced by HFD in the WT mice (Fig. 4A and C, $P < 0.05$). However, in the KO mice, but this reducing effect of GRb1 in KO mice was weaker than that in the WT mice, approximately 24% less (Fig. 4A and C, $P < 0.05$). HE staining of liver sections showed that HFD induced liver steatosis in both WT and KO mice. GRb1 improved liver steatosis in the WT mice, while the reduced degree of liver steatosis in the GRb1-treated KO mice was not noticeable compared with that in the WT mice (Fig .4D).

Besides, the changes of body weight (BW) and the weight of epididymal fat (eWAT) were also observed after GRb1 treatment. In the WT mice, GRb1 significantly reduced the BW and eWAT weight of obese mice (Fig. 4E-H, $P < 0.05$). In the KO mice, the BW was increased by HFD, but not eWAT weight. GRb1 did not affect the BW and eWAT weight in the KO mice (Fig. 4E—H). This result indicates that the absence of adiponectin attenuates GRb1- induced amelioration of hepatic steatosis in obese mice.

Fig. 3. The deletion of adiponectin weakened GRb1's insulin-sensitizing activity in obese mice. (A) Schematic figure illustrating the animal experiment. (B) The serum levels of adiponectin in mice were measured by ELISA. (C) The fasting blood glucose level was measured. (D) The level of fasting insulin was analyzed. (E) The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated. (F-G) Oral glucose tolerance test (OGTT) was performed. (H) Area under curve (AUC) of OGTT was calculated. (I-J) insulin tolerance test (ITT) was performed. (K) AUC of ITT was calculated. Values are expressed as mean ± SD, n = 7, *$P < 0.05$; ns means no significance.

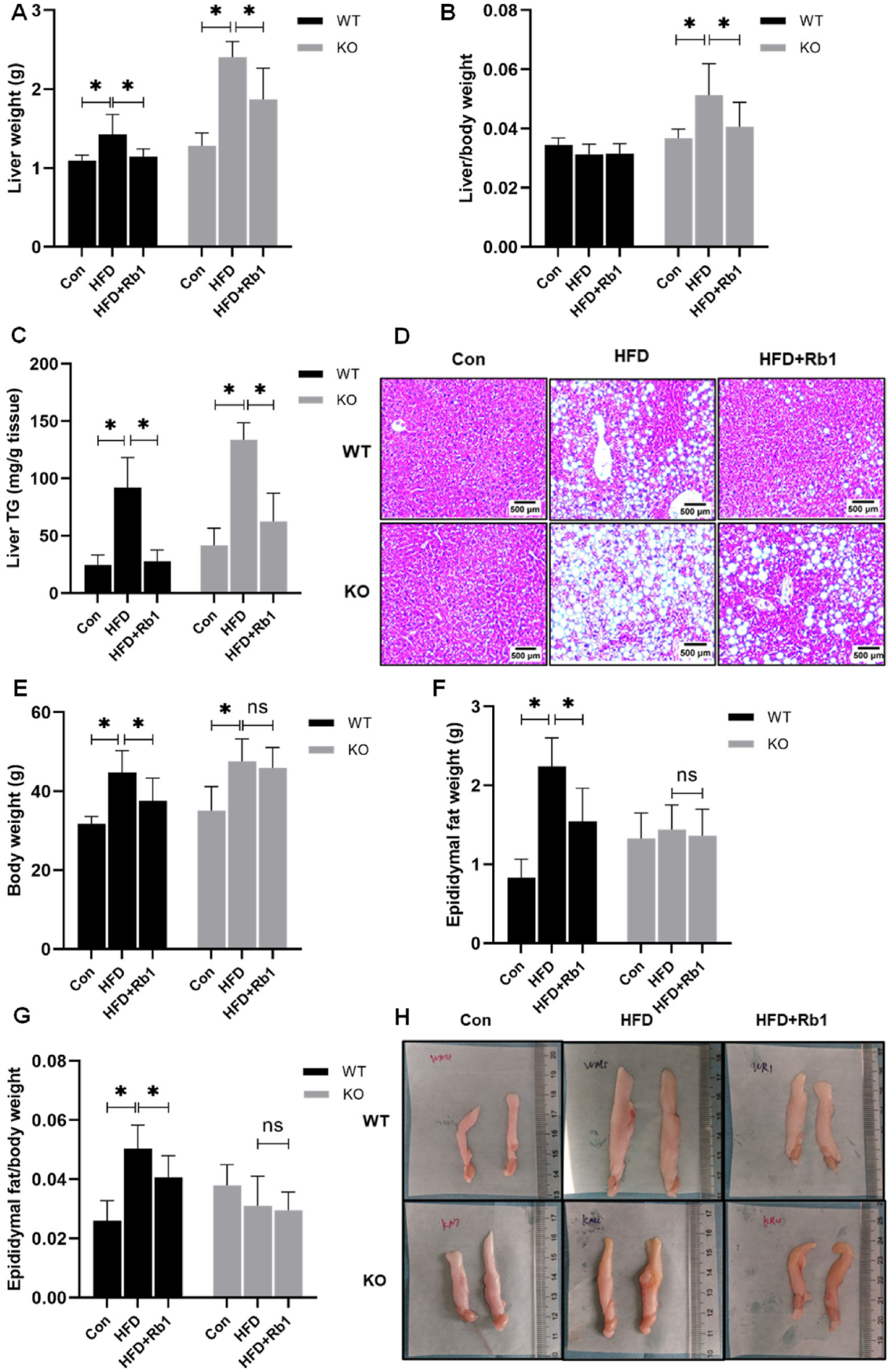


Fig. 4. The inhibition of liver fat accumulation by GRb1 was attenuated by knockout of adiponectin in mice. The obese mice were treated with GRb1 for 2 weeks then sacrificed. (A) and (B) The liver was weighed. (C) The TG content in liver was measured. (D) The liver sections were stained with Hematoxylin and Eosin (HE). (E) The body weight was recorded. (F) and (G) The epididymal fat was weighed. (H) The representative pictures of epididymal fat of mice. Values are expressed as mean ± SD, n = 7. *$P < 0.05$; ns means no significance.

3.5. GRb1 enhanced the improvement of liver dysfunction in the presence of adiponectin

The serum FFA was elevated by HFD, and further augmented by the knockout of adiponectin in mice. GRb1 treatment significantly decreased the level of serum FFA in both WT and KO mice (Fig. 5A, $P < 0.05$). The serum TG was affected by GRb1 treatment in both WT and KO mice (Fig. 5B). The serum ALT and AST, which are considered to be indicators of liver function, were increased by HFD in the WT mice and the KO mice. GRb1 treatment suppressed the elevated ALT and AST in the WT mice (Fig. 5C and D, $P < 0.05$). The decreased degree of both indexes by GRb1 treatment did not reach significant in the KO mice (Fig. 5C and D), suggesting that GRb1-induced improvement of liver dysfunction was enhanced by the presence of adiponectin.

3.6. Up-regulation of adiponectin by GRb1 activates AMPK in liver

To testify whether the increased adiponectin by GRb1 treatment in adipocytes activates AMPK in liver, the proteins of liver tissue from mice and primary hepatocytes were extracted to detect the phosphorylated AMPK by Western blot. In the WT mice, GRb1 treatment increased the phosphorylation of AMPK in the liver (Fig. 6A and B, $P < 0.05$). However, in the liver from KO mice, GRb1 lost this AMPK activating effect (Fig. 6A and B). In primary hepatocytes treated with the CM from adipocytes incubated with GRb1 and TNF-α, the phosphorylation of AMPK increased significantly in comparison with that in cells treated with the CM from adipocytes incubated with TNF-α alone (Fig. 6C and D, $P < 0.05$). The activation of AMPK was also detected in the primary hepatocytes directly treated with GRb1 and PA. GRb1 did not show a significant effect on the phosphorylation of AMPK in the presence of PA (Fig. 6E and F). These results suggest that the up-regulation of adiponectin links GRb1 with the amelioration of hepatic steatosis through the activation of AMPK pathway in hepatocytes.

4. Discussion

GRb1, as the most abundant ingredient in *Panax ginseng*, has been proven to possess the properties of anti-diabetes and anti-obesity [18,19,26]. NAFLD is a prominent complication of obesity and T2DM with the features of insulin resistance and excess visceral adiposity [1]. In the present study, the results show that GRb1 improves liver steatosis as well as insulin resistance and glucose tolerance induced by HFD through up-regulating adiponectin expression and secretion in adipocytes. These findings elucidate a new mechanism underlying GRb1's beneficial effect on obesity and the obesity-related metabolic disorders.

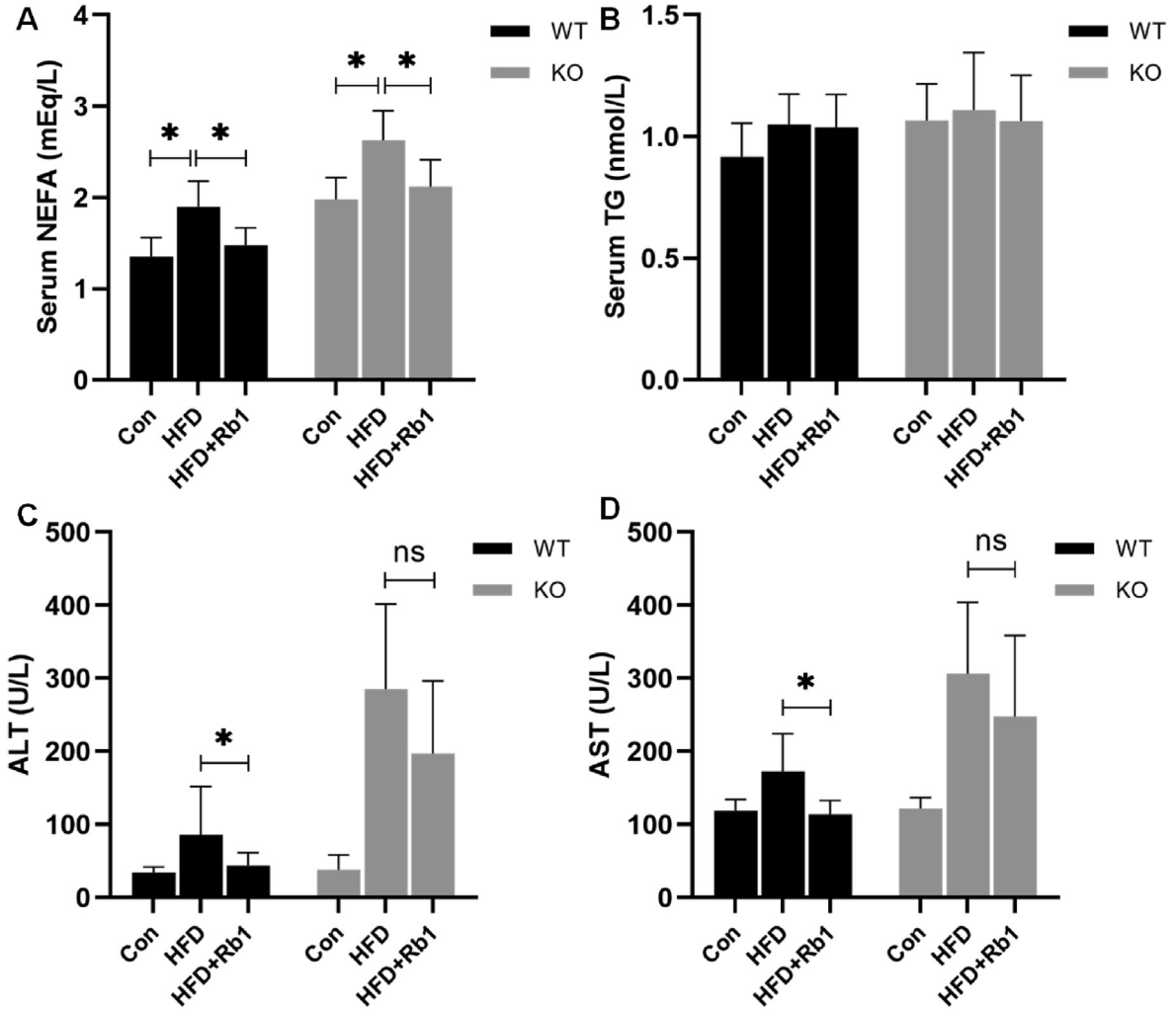


Fig. 5. GRb1 improved liver dysfunction in the presence of adiponectin. The obese mice were treated with GRb1 for 2 weeks then sacrificed. (A) Nonestesterified fatty acid (NEFA) was measured. (B) Serum TG, (C) ALT and (D) AST of were measured. Values are expressed as mean ± SD, n = 7. *$P < 0.05$; ns means no significance.

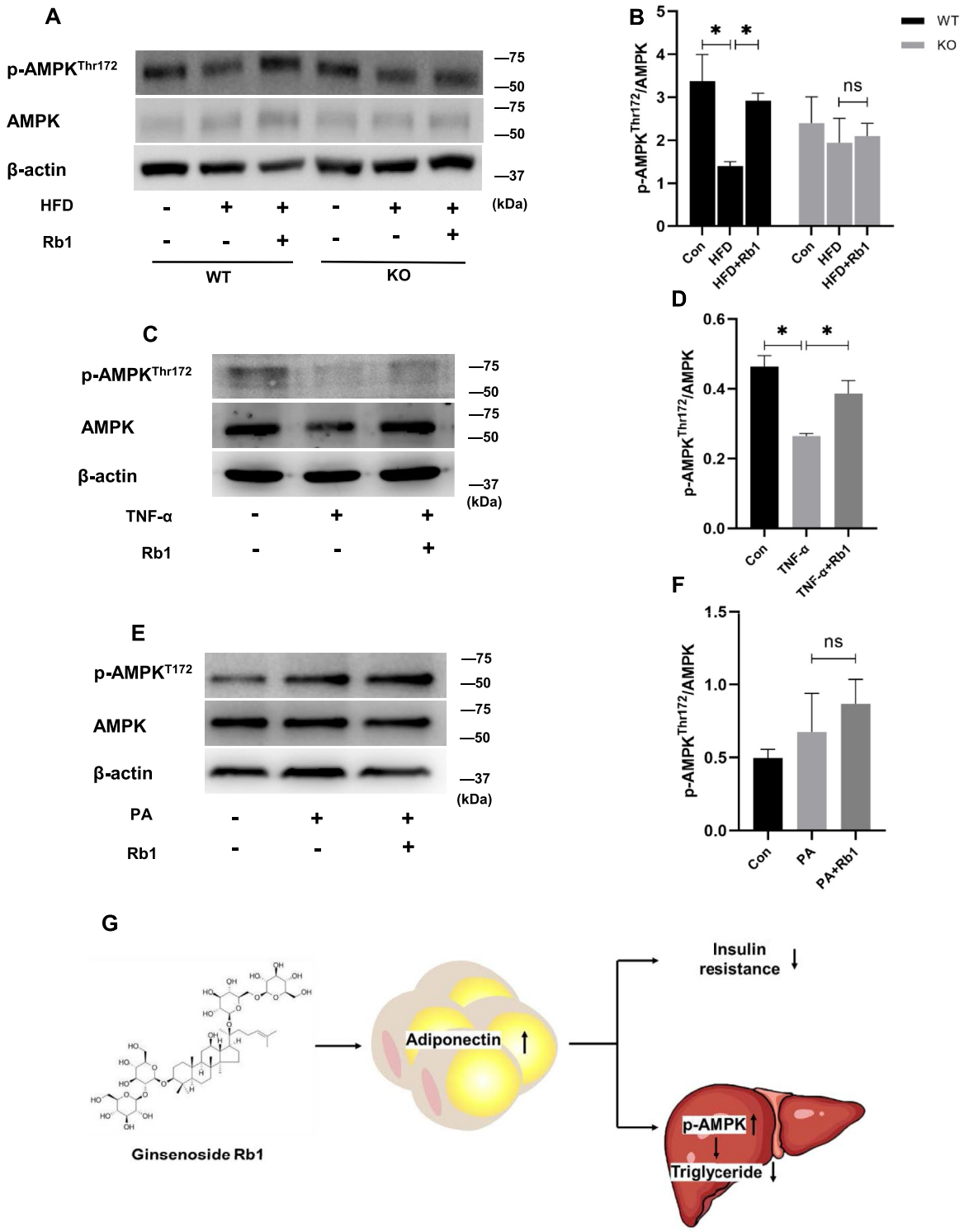


Fig. 6. Up-regulation of adiponectin by GRb1 activates AMPK in liver. (A) and (B) The obese mice were treated with GRb1 for 2 weeks, AMPK and *p*-AMPK^{Thr172} in the liver tissues were analyzed by western blotting and their density were quantified by ImageJ program. (C) and (D) After the pretreated with PA for 24 h, primary hepatocytes were incubated with CM collected from adipocytes for 24 h, AMPK and *p*-AMPK^{Thr172} of primary hepatocytes were analyzed by western blotting and their density were quantified. (E) and (F) After the treated with PA for 24 h, primary hepatocytes were incubated with or without Rb1 for 24 h, AMPK and *p*-AMPK^{Thr172} of primary hepatocytes were analyzed by western blotting their density were quantified. Values are expressed as mean ± SD. n = 3, $^{*}P < 0.05$; ns means no significance. (G) Schematic depiction of a role in GRb1-induced amelioration of hepatic steatosis and insulin resistance.

Adiponectin, one of insulin-sensitizing adipocyte-specific cytokines, decreases in the circulation of obese mice and humans, correlating well with diminished insulin sensitivity [11,27]. The regulation of adiponectin gene expression is tightly controlled by some transcription factors in adipocytes. Peroxisome proliferator-activated receptor γ (PPAR-γ) which is expressed mainly in adipose tissue, is the major positive regulator of adiponectin gene expression. CCAAT/enhancer-binding protein alpha (C/EBPα) has also been identified as an important adiponectin transcription factor in various adipocyte [27]. The previous studies have demonstrated that GRb1 promoted the transcriptional activity of PPAR-γ and the expression of C/EBPα in adipocytes to increase insulin sensitivity [21,28,29], suggesting that the increase of adiponectin in adipocytes and obese mice may result from the action of GRb1 on PPAR-γ and C/EBPα activity in this study. On the other hand, obesity is manifested by low-grade inflammation with a high level of pro-inflammatory factors in adipose tissue and plasma, such as TNF-α, which contributes to the development of insulin resistance [30]. TNF-α significantly reduces adiponectin gene expression by inhibiting the inflammatory pathway in adipocytes [31]. Contrariwise, adiponectin treatment suppresses hepatic production of TNF-α and inflammation to alleviate hepatomegaly and steatosis in obese mice [32]. GRb1 is able to decrease the expression of inflammatory cytokines, such as monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6) and TNF-α by inhibiting the NF-κB and MAPK pathway in adipocytes and macrophages [33,34]. In vivo, GRb1 reduced serum TNF-α in obese mice. In the present study, GRb1 reversed the impaired expression and secretion of adiponectin in adipocytes treated with TNF-α. These results imply that the anti-inflammatory activity of GRb1 is also responsible for the up-regulation of adiponectin and the amelioration of hepatic steatosis.

Adiponectin stimulates AMPK in skeletal muscle and liver to achieve the insulin-sensitizing activity by binding to adiponectin receptor 1 (AdipoR1) or 2 (AdipoR2) [27,35]. In liver, enhanced AMPK phosphorylation and peroxisome proliferator-activated receptor α (PPARα) ligand activity induced by adiponectin result in decreased gluconeogenesis and de novo lipogenesis as well as increased fatty acid oxidation, which reduces the TG accumulation and insulin resistance [27]. In the present study, the conditioned medium from adipocytes treated with GRb1, which containing adiponectin, increased AMPK phosphorylation in hepatocytes, but GRb1 did not show the ability to directly increase AMPK phosphorylation on hepatocytes. In obese mice, the action of GRb1 on AMPK phosphorylation was also attenuated in absence of adiponectin. These results suggest that adiponectin may mediate between GRb1 and the activation of AMPK. The in vivo data in the present study is consistent to the two previous reports, which showed that GRb1 increased the phosphorylation of AMPK in the skeletal muscle and liver of obese rats fed with HFD [23,36]. However, GRb1 did not significantly increase the phosphorylation of AMPK in the cultured hepatocytes, which is different from that in the previous study [23]. This inconsistency may be due to the different experimental condition, the normal primary cultured hepatocytes were used in the previous report [23], but in the present experiment, the hepatocytes were pretreated with PA, then GRb1 were added.

In obesity, elevated levels of circulating FFAs from increased adipocyte adipose lipolysis is a major contributor to NAFLD [1]. Our previous study showed that GRb1 reduced hepatic fat accumulation in the obese mice through inhibiting the lipolysis in adipocytes [22]. The increased FFA release induced by TNF-α from 3T3-L1 adipocyte was partly attenuated by GRb1, however, which was not in proportion to the reduced degree of TG in liver of the obese mice, implying that besides inhibition of FFA release from adipocytes, other factors may be involved in the GRb1 induced decrease of TG accumulation in liver [22]. In this study, the knockout of adiponectin failed to completely abolish the GRb1-induced amelioration of liver steatosis, and the inhibitory action of GRb1 on serum NEFA was not affected by the deletion of adiponectin, suggesting that the inhibition of lipolysis by GRb1 is independent of adiponectin. Therefore, in addition to suppressing FFA release, increasing the secretion of adiponectin from adipose tissue is another important mechanism underlying the GRb1-induced amelioration of hepatic steatosis.

GRb1, as the main protopanaxadiol type (PPD) of ginsenosides, is poorly absorbed from gut after oral administration [37]. Thus, we treated the mice with GRb1 via intraperitoneal injection to observe its action on adiponectin expression in *in vivo* condition. However, oral administration of GRb1 is mainly metabolized into compound K (CK), a major metabolite via transformation to ginsenoside Rd and F2 by the intestinal microbes [38,39]. CK is rapidly absorbed from gastrointestinal tract and exhibits multiple pharmacological properties, such as anti-allergic, anti-diabetic, anti-carcinogenic, anti-inflammatory, anti-aging effects, etc. [40,41]. CK suppresses the hepatic gluconeogenesis and attenuates hepatic steatosis by activating AMPK [42,43]. The anti-inflammatory effect of CK leads to the improvement of insulin resistance [44], which may result from the inhibition of the activity of NF-κB in macrophages and NLPR3 inflammasomes in adipose tissue [44,45]. These pharmacological functions of CK are similar to that of GRb1 [18]. Therefore, adiponectin may be also the major factor to mediate the alleviating effect of CK and other PPDs on metabolic dysregulation, which needs further investigation.

In summary, this study revealed that the up-regulation of adiponectin by GRb1 contributes to the amelioration of liver steatosis and insulin resistance induced by high fat diet. The findings elucidate a novel molecular mechanism of the beneficial effects of GRb1 on obesity and its related metabolic diseases.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (grant numbers 81503312, 81873060) and the Open Projects of the Discipline of Chinese Medicine of Nanjing University of Chinese Medicine Supported by the Subject of Academic priority discipline of Jiangsu Higher Education Institutions (ZYX03KF058). This study was also partly supported by Jiangsu Province Graduate Students Research and Innovation Plan (KYCX21_1665).

References

[1] Bence KK, Birnbaum MJ. Metabolic drivers of non-alcoholic fatty liver disease. Mol Metab 2020:101143.
[2] Birkenfeld AL, Shulman GI. Nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes. Hepatology 2014;59:713–23.
[3] Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism 2016;65:1038–48.
[4] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73–84.
[5] Fan JG, Kim SU, Wong VW. New trends on obesity and NAFLD in Asia. J Hepatol 2017;67:862–73.
[6] Ferguson D, Finck BN. Emerging therapeutic approaches for the treatment of NAFLD and type 2 diabetes mellitus. Nat Rev Endocrinol 2021;17(8):484–95.
[7] Azzu V, Vacca M, Virtue S, Allison M, Vidal-Puig A. Adipose tissue-liver cross talk in the control of whole-body metabolism: implications in nonalcoholic fatty liver disease. Gastroenterology 2020;158:1899–912.

[8] Alves-Bezerra M, Cohen DE. Triglyceride metabolism in the liver. Comp Physiol 2017;8:1–8.
[9] Ipsen DH, Lykkesfeldt J, Tveden-Nyborg P. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. Cell Mol Life Sci 2018;75:3313–27.
[10] Cao H. Adipocytokines in obesity and metabolic disease. J Endocrinol 2014;220:T47–59.
[11] Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. Endocr Rev 2005;26:439–51.
[12] Polyzos SA, Kountouras J, Zavos C, Tsiaousi E. The role of adiponectin in the pathogenesis and treatment of non-alcoholic fatty liver disease. Diabetes Obes Metabol 2010;12:365–83.
[13] Combs TP, Marliss EB. Adiponectin signaling in the liver. Rev Endocr Metab Disord 2014;15:137–47.
[14] Ratan ZA, Haidere MF, Hong YH, Park SH, Lee JO, Lee J, Cho JY. Pharmacological potential of ginseng and its major component ginsenosides. J Ginseng Res 2021;45:199–210.
[15] Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685–93.
[16] Lim W, Mudge KW, Vermeylen F. Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (Panax quinquefolium). J Agric Food Chem 2005;53:8498–505.
[17] Mohanan P, Subramaniyam S, Mathiyalagan R, Yang DC. Molecular signaling of ginsenosides Rb1, Rg1, and Rg3 and their mode of actions. J Ginseng Res 2018;42:123–32.
[18] Zhou P, Xie W, He S, Sun Y, Meng X, Sun G, Sun X. Ginsenoside Rb1 as an anti-diabetic agent and its underlying mechanism analysis. Cells 2019;8.
[19] Lin N, Cai DL, Jin D, Chen Y, Shi JJ. Ginseng panaxoside Rb1 reduces body weight in diet-induced obese mice. Cell Biochem Biophys 2014;68:189–94.
[20] Shang W, Yang Y, Zhou L, Jiang B, Jin H, Chen M. Ginsenoside Rb1 stimulates glucose uptake through insulin-like signaling pathway in 3T3-L1 adipocytes. J Endocrinol 2008;198:561–9.
[21] Shang W, Yang Y, Jiang B, Jin H, Zhou L, Liu S, Chen M. Ginsenoside Rb1 promotes adipogenesis in 3T3-L1 cells by enhancing PPARgamma2 and C/EBPalpha gene expression. Life Sci 2007;80:618–25.
[22] Yu X, Ye L, Zhang H, Zhao J, Wang G, Guo C, Shang W. Ginsenoside Rb1 ameliorates liver fat accumulation by upregulating perilipin expression in adipose tissue of db/db obese mice. J Ginseng Res 2015;39:199–205.
[23] Shen L, Xiong Y, Wang DQ, Howles P, Basford JE, Wang J, Xiong YQ, Hui DY, Woods SC, Liu M. Ginsenoside Rb1 reduces fatty liver by activating AMP-activated protein kinase in obese rats. J Lipid Res 2013;54:1430–8.
[24] Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, et al. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 2001;50:2094–9.
[25] Li WC, Ralphs KL, Tosh D. Isolation and culture of adult mouse hepatocytes. Methods Mol Biol 2010;633:185–96.
[26] Xiong Y, Shen L, Liu KJ, Tso P, Xiong Y, Wang G, Woods SC, Liu M. Antiobesity and antihyperglycemic effects of ginsenoside Rb1 in rats. Diabetes 2010;59:2505–12.
[27] Fang H, Judd RL. Adiponectin regulation and function. Comp Physiol 2018;8:1031–63.
[28] Guo R, Wang L, Zeng X, Liu M, Zhou P, Lu H, Lin H, Dong M. Aquaporin 7 involved in GINSENOSIDE-RB1-mediated anti-obesity via peroxisome proliferator-activated receptor gamma pathway. Nutr Metab 2020;17:69.
[29] Mu Q, Fang X, Li X, Zhao D, Mo F, Jiang G, Yu N, Zhang Y, Guo Y, Fu M, et al. Ginsenoside Rb1 promotes browning through regulation of PPARγ in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2015;466:530–5.
[30] Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. J Clin Invest 2017;127:1–4.
[31] Chang E, Choi JM, Kim WJ, Rhee EJ, Oh KW, Lee WY, Park SE, Park SW, Park CY. Restoration of adiponectin expression via the ERK pathway in TNFalpha-treated 3T3-L1 adipocytes. Mol Med Rep 2014;10:905–10.
[32] Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. J Clin Invest 2003;112:91–100.
[33] Wang M, Chen Y, Xiong Z, Yu S, Zhou B, Ling Y, Zheng Z, Shi G, Wu Y, Qian X. Ginsenoside Rb1 inhibits free fatty acids-induced oxidative stress and inflammation in 3T3-L1 adipocytes. Mol Med Rep 2017;16:9165–72.
[34] Gao H, Kang N, Hu C, Zhang Z, Xu Q, Liu Y, Yang S. Ginsenoside Rb1 exerts anti-inflammatory effects in vitro and in vivo by modulating toll-like receptor 4 dimerization and NF-kB/MAPKs signaling pathways. Phytomedicine 2020;69:153197.
[35] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288–95.
[36] Shen L, Haas M, Wang DQ, May A, Lo CC, Obici S, Tso P, Woods SC, Liu M. Ginsenoside Rb1 increases insulin sensitivity by activating AMP-activated protein kinase in male rats. Phys Rep 2015;3:e12543.
[37] Odani T, Tanizawa H, Takino Y. Studies on the absorption, distribution, excretion and metabolism of ginseng saponins. III. The absorption, distribution and excretion of ginsenoside Rb1 in the rat. Chem Pharm Bull (Tokyo) 1983;31:1059–66.
[38] Akao T, Kanaoka M, Kobashi K. Appearance of compound K, a major metabolite of ginsenoside Rb1 by intestinal bacteria, in rat plasma after oral administration–measurement of compound K by enzyme immunoassay. Biol Pharm Bull 1998;21:245–9.
[39] Akao T, Kida H, Kanaoka M, Hattori M, Kobashi K. Intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb1 from Panax ginseng. J Pharm Pharmacol 1998;50:1155–60.
[40] Sharma A, Lee HJ. Ginsenoside compound K: insights into recent studies on pharmacokinetics and health-promoting activities. Biomolecules 2020;10.
[41] Yang XD, Yang YY, Ouyang DS, Yang GP. A review of biotransformation and pharmacology of ginsenoside compound K. Fitoterapia 2015;100:208–20.
[42] Wei S, Li W, Yu Y, Yao FAL, Lan X, Guan F, Zhang M, Chen L. Ginsenoside Compound K suppresses the hepatic gluconeogenesis via activating adenosine-5'monophosphate kinase: a study in vitro and in vivo. Life Sci 2015;139:8–15.
[43] Hwang YC, Oh DH, Choi MC, Lee SY, Ahn KJ, Chung HY, Lim SJ, Chung SH, Jeong IK. Compound K attenuates glucose intolerance and hepatic steatosis through AMPK-dependent pathways in type 2 diabetic OLETF rats. Korean J Intern Med 2018;33:347–55.
[44] Chen W, Wang J, Luo Y, Wang T, Li X, Li A, Li J, Liu K, Liu B. Ginsenoside Rb1 and compound K improve insulin signaling and inhibit ER stress-associated NLRP3 inflammasome activation in adipose tissue. J Ginseng Res 2016;40:351–8.
[45] Lee JO, Choi E, Shin KK, Hong YH, Kim HG, Jeong D, Hossain MA, Kim HS, Yi YS, Kim D, et al. Compound K, a ginsenoside metabolite, plays an antiinflammatory role in macrophages by targeting the AKT1-mediated signaling pathway. J Ginseng Res 2019;43:154–60.