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Protective effects of glycyrrhizic acid against non-alcoholic fatty liver disease in mice

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) has become a predictive factor of death from many diseases. The purpose of the present study is to investigate the protective effect of glycyrrhizic acid (GA), a natural triterpene glycoside, on NAFLD induced by a high-fat diet (HFD) in mice, and further to elucidate the mechanisms underlying GA protection. GA treatment significantly reduced the relative liver weight, serum ALT, AST activities, levels of serum lipid, blood glucose and insulin. GA suppressed lipid accumulation in liver. Further mechanism investigation indicated that GA reduced hepatic lipogenesis via downregulating SREBP-1c, FAS and SCD1 expression, increased fatty acids β-oxidation via an increase in PPARα, CPT1α and ACADS, and promoted triglyceride metabolism through inducing LPL activity. Furthermore, GA reduced gluconeogenesis through repressing PEPCK and G6Pase, and increased glycogen synthesis through an induction in gene expression of PDase and GSK3β. In addition, GA increased insulin sensitivity through upregulating phosphorylation of IRS-1 and IRS-2. In conclusion, GA produces protective effect against NAFLD, due to regulation of genes involved in lipid, glucose homeostasis and insulin sensitivity.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease with the prevalence of up to 30% in developed countries and nearly 10% in developing nations (Smith and Adams, 2011). NAFLD is a serious medical problem and a rising threat to public health, which encompasses a spectrum of hepatic disease ranging from fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (Araújo et al., 2016; Francque et al., 2016). Though life-style changes and exercise to reduce body weight are appropriate for some NAFLD patients, effective drug therapies are limited to convincingly reduce the risk of disease progression. Therefore, there is an urgent medical need to develop drugs for treatment of this common disease.

The pathogenesis of NAFLD is multifactorial and only partially understood; however, lipid homeostasis, insulin resistance and glucose homeostasis play critical roles in the disease process (El-Karaksy et al., 2015; Elshazly, 2015; Kim et al., 2016; Pawlak et al., 2015). In NAFLD patients, liver fat (mainly triglyceride) derives from free fatty acids that are mainly from diet, de novo synthesis or adipose tissue. In the liver, free fatty acids can be esterified and stored as triglyceride in lipid droplets or used for energy and ketone body production via β-oxidation (Chen et al., 2011). Once the capacity of the liver to store triglyceride is overwhelmed, NAFLD starts to develop (Yoon and Cha, 2014). Many genes have been reported to play crucial roles in lipid homeostasis, including sterol regulatory element-binding protein 1c (SREBP-1c) which is an important regulatory factor in hepatic lipogenesis, carnitine palmitoyl transferase 1α (CPT1α) and lipoprotein lipase (LPL) that are two key enzymes in lipid metabolism (Li et al., 2013; Yin et al., 2009). The hepatic gluconeogenesis correlates with the expression of phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Ma et al., 2013). Besides, insulin sensitivity corresponds to phosphorylation of insulin responsive substrate 1 and 2 (IRS-1 and IRS-2) (Zhang et al., 2006).

Glycyrrhizic acid (GA) is a triterpene glycoside isolated from *Glycyrrhiza glabra* which is an edible and medicinal plant widely used as a traditional Chinese medicine for a long history. Its chemical structure is shown in Fig. 1. Many pharmacological studies have revealed that GA has several pharmacological activities, such as anti-inflammatory, antiviral and antiallergic effects (Wang et al., 2013; Zhao et al., 2016). Furthermore, it has been recently demonstrated that GA has markedly hepatoprotective effect (Orazizadeh et al., 2014).

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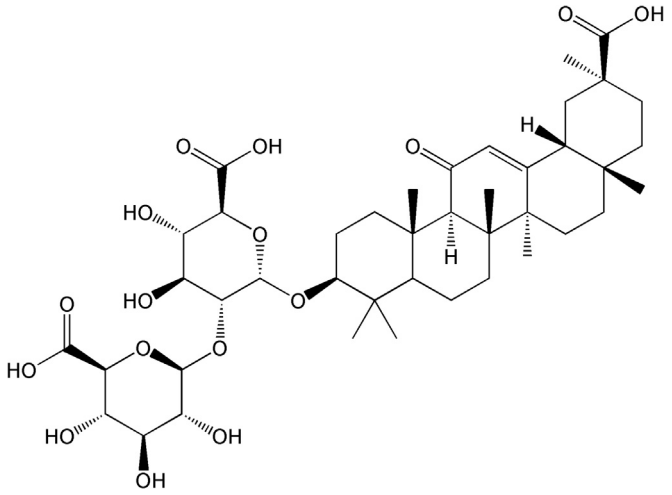

Fig. 1. The chemical structure of GA.

Therefore an important and intriguing question arises whether GA has protective effect against a high-fat diet (HFD)-induced NAFLD. Another further question is that what the mechanisms are if GA possesses protective effect against NAFLD.

In the present study, we aimed to investigate the hepatoprotective and antihyperlipidemic effects of GA against NAFLD, and further to explore the potential mechanisms.

2. Materials and methods

2.1. Reagents

GA (purity > 98%) was purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Silymarin (SM) and guggulsterone (GS) were purchased from Sigma-Aldrich (St. Louis, MO). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, free fatty acids, total cholesterol detection kits were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Enhanced BCA Protein Assay Kit and Tissue Mitochondria Isolation Kit were provided by Beyotime Institute of Biotechnology (Jiangsu, China). Tissue Protein Extraction Kit was purchased from KeyGEN Biotech. CO., Ltd. (Nanjing, China). Tris and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). RNAiso Plus, PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) and SYBR® *Premix Ex Taq*™ II were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

2.2. Animals and treatments

All animal maintenance and treatment protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. Male C57BL/6 mice (8–9 weeks) were housed in laboratory animal facilities under a 12-hr light/dark cycle with access to standard chow and water ad libitum. After 1 week of acclimatization, the mice were divided into 7 experimental groups (n=8 per group), fed and treated as follows for 12 weeks: (1) Control group with a control chow diet (Control, 12% kcal fat content, Research Diets D12450H, New Brunswick, USA), (2) model group fed a high-fat diet (HFD, 45% kcal fat content, Research Diets D12451, New Brunswick, USA) with a daily oral gavage of vehicle (distilled water), (3) the mice fed a high-fat diet with a daily oral gavage of low-dose GA (15 mg/kg, HFD+GA15), (4) the mice fed a high-fat diet with a daily oral gavage of medium-dose GA (30 mg/kg, HFD+GA30), (5) the mice fed a high-fat diet with a daily oral gavage of high-dose GA (60 mg/kg, HFD+GA60), (6) the mice fed a high-fat diet with a daily oral gavage of SM (60 mg/kg, HFD+SM60) and (7) the mice fed a normal diet with a daily oral gavage of GA (60 mg/kg, GA60). GS was dissolved in 100 mM DMSO and diluted in methylcellulose 1%. The mice were injected intraperitoneally with 10 mg/kg of GS 4 h before vehicle or GA administration. We monitored food intake and found no difference between experimental groups. On the last day, mice were orally administered with vehicle or compounds in the morning, fasted for 3.5 h, then blood was collected by cardiac puncture, and livers were dissected and fixed in 10% formalin for histological analysis or snap frozen in lipid nitrogen for future analyses.

2.3. Biochemical analysis

Serum ALT, AST, triglyceride, free fatty acids, total cholesterol and hepatic triglyceride, free fatty acids, total cholesterol were analyzed using commercial kits according to the manufacturer's protocols.

2.4. Histopathology

Liver fragments were fixed in 10% neutral buffered formalin, embedded in paraffin, sliced for 5 μm, stained with H & E using standard protocols and examined microscopically for structural changes. The sum of the scores (degree of steatosis, hepatocyte ballooning, lobular inflammation, and portal inflammation) was considered as the total pathology grade. For oil red O staining, fresh liver tissues were embedded in optimum cutting temperature (OCT) compound and cryosectioned. The sections were fixed in 4% paraformaldehyde in PBS, and were stained with 0.3% oil red O according to standard procedures.

2.5. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

During the last week of treatment, oral glucose tolerance test and insulin tolerance test were performed. For glucose tolerance test, the mice were administered oral glucose (2 g/kg) and blood samples were obtained at 0, 30, 60, 90 and 120 min after glucose administration. For insulin tolerance test, 1 U/kg of recombinant human insulin (Novolin 30R, Novo Nordisk, Bagsvaerd, Denmark) was injected intraperitoneally and blood glucose were measured at 0, 30, 60, 90 and 120 min after insulin injection. Blood glucose levels were tested using ACCU-CHEK touch test paper on an ACCU-CHEK Performa blood glucose meter (Roche Diagnostic, Mannheim, Germany).

2.6. Quantitative real-time PCR

Total RNAs from mouse hepatic, intestinal tissue or mice primary hepatocytes were extracted by RNAiso Plus reagent according to the manufacturer's instructions. Total RNAs (1 μg) was reverse-transcribed to cDNA using PrimeScript® RT reagent kit. The levels of mRNA expression were quantified using SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems, USA). The quantity of mRNA was normalized with an internal standard mouse β-actin.

2.7. Protein isolation and western blot

Liver tissues were homogenized in protein lysis buffer containing 1 mM PMSF. Fifty μg of total protein were resolved with 8–12% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated