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Protective effects of rutin on liver injury in type 2 diabetic db/db mice

Weishi Liang[a,b], Dandan Zhang[a,b], Jiali Kang[a,b], Xubing Meng[a,b], Jingbo Yang[a,b], Lei Yang[a,b], Ning Xue[a,b], Qingyao Gao[a,b], Shuying Han[b,c,*], Xiangbo Gou[b,c,*]

[a] Clinical Medical College, North China University of Science and Technology, Tangshan 063210, PR China
[b] Basic Medical College, North China University of Science and Technology, Tangshan 063210, PR China
[c] Department of Pharmacology, North China University of Science and Technology, Tangshan 063210, PR China

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ABSTRACT

The aim of this study was to evaluate the protective effect of rutin on the liver of type 2 diabetic mice and explore the correlation mechanism. The db/db mice, selected as the type 2 diabetes mellitus (T2DM) models, have random blood glucose (RBG) and glucose level after 2 h of oral glucose loading of more than 16.7 mmol/L. After administration of 120 mg/kg or 60 mg/kg rutin, to T2DM mice, RBG, oral glucose tolerance, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum, and advanced glycation end products (AGEs) in vivo and vitro of different groups were detected. The liver pathological changes were observed under light and electron microscopy. Western blotting was used to detect the protein expression of insulin receptor substrate 2 (IRS-2) and phosphorylation of phosphatidylinositol 3 kinase (PI3K) on p85, Akt on Ser473, glycogen synthase kinase 3β (GSK-3β) on Ser9, real-time quantitative PCR was used to detect IRS-2 mRNA expression. Moreover, dynamically observing the effect of rutin on the generation of AGEs in non-enzymatic protein glycosylated system, Cell Counting Kit-8 (CCK-8) method was used to detect the effect of rutin on proliferation activity of HepG2 liver cells. The results showed that RBG and glucose levels of oral glucose tolerance test (OGTT) of mice in model group were significantly higher than that of normal group, which were significantly reduced after the rutin treatment. Rutin could reduce the ALT, AST activities and AGEs level in serum and potentiate the expression of IRS-2, P-PI3K (p85), P-Akt (Ser473), P-GSK-3β (Ser9) protein and IRS-2 mRNA in the liver tissue of db/db mice. Moreover, rutin could significantly alleviate the structure disorder of liver, reduce the degeneration and necrosis of liver cells and formation of collagen fibers of db/db mice. The results *in vitro* also showed that rutin could obviously inhibit the generation of AGEs, and promoted the proliferation activity of high glucose-stimulating HepG2 cells. In general, the protective effect of rutin on the liver of T2DM may be mediated by facilitating signal transduction and activated state of insulin IRS-2/PI3K/Akt/GSK-3β signal pathway, promoting hepatocyte proliferation, reducing blood glucose level and generation of AGEs.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common chronic diseases in clinic, which is characterized by persistent hyperglycemia. As everyone knows, the persistence of hyperglycemia can lead to metabolic disorders, causing the disease of multiple organs such as liver, kidney, heart, especially the diabetic liver disease [1–3]. Previous studies showed that the incidence of diabetic liver injury increased year by year, which includes fatty liver, non-alcoholic fatty hepatitis, fatty liver fibrosis, fatty liver cirrhosis and late-stage liver cancer mainly [4,5]. Davis et al. reported that hepatobiliary disease and associated mortality are increased in T2DM mainly related to multiple factors including fatty infiltration, microangiopathy, and direct glucotoxicity [6]. As is known, insulin resistance is one of the main pathogenesis of T2DM, which is both the cause of its occurrence and plays an important role in the development of the disease and various complications [7]. When insulin resistance occurs, the signal transduction of insulin IRS-2/PI3K/Akt/GSK-3β signal pathway is often blocked, resulting in decreased glycogen synthesis and evaluated blood glucose [8]. The liver is the main site of insulin resistance and is also the main cause of liver injury, therefore, improving insulin resistance is the key to improve the liver injury of T2DM. In high-glucose environment, protein has a non-enzymic glycosylation reaction and forms advanced glycation end products (AGEs), which deposits in the tissues and vessels, at last causing

* Corresponding authors at: Department of Pharmacology, Basic Medical College, North China University of Science and Technology, 21 Bohai Avenue, Tangshan Bay Eco-city, Caofeidian District, Tangshan 063210, PR China.
E-mail addresses: shuyinghan59@126.com (S. Han), gouxiangbo@163.com (X. Gou).

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pathological changes of body tissues [9]. Therefore, preventing the formation of AGEs has great significance for preventing the chronic complications of T2DM.

Rutin is a flavonoid compound, which exists in various kinds of plants and has many pharmacological activities, such as reducing blood glucose, modulating insulin secretion, adjusting dyslipidemia, anti-inflammatory, anti-tumor, and attenuating reactive oxygen species (ROS) [10–13]. There are many studies shown that rutin has good therapeutic effect on liver injury caused by various reasons such as biliary obstruction, thioacetamide and high fat diet, but the mechanism of rutin on liver injury of T2DM has not been very clear [14–16]. Some studies displayed that the protection of rutin on diabetic liver may be associated with anti-inflammation, inhibiting lipogenesis and anti-oxidative effects [17–19]. However, these are obviously not sufficient to explain the mechanism of protection on liver by rutin. Therefore, this study was conducted to assess the effect of rutin on blood glucose, pathological changes of liver, signal transduction of insulin IRS-2/PI3K/Akt/GSK-3β signal pathway, generation of AGEs in vivo and vitro, proliferation activity of high glucose-stimulating HepG2 cells, and explored its possible mechanism involved.

2. Materials and methods

2.1. Main reagents

Rutin (purity: > 98%) was supplied by Sigma Company (USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination kit were purchased from Beijing Ruizhengshanda Bioengineering Technology Co. Ltd. (Lot number: 20150706, China). AGEs determination kit was purchased from Beijing Kainuo Spring Biotechnology Co. Ltd. (Lot number: 20151201A, China). Reverse transcription kit was produced by Invitrogen Company (Lot number: C11744-100, USA). Anti-IRS-2 antibody (Cat number: ab134101), Anti-P-PI3K (p85) antibody (Cat number: ab182651), Anti-P-Akt (Ser473) antibody (Cat number: ab81283) and Anti-P-GSK-3β (Ser9) antibody (Cat number: ab131097) was produced by Abcam Company. β-actin antibody was produced by Bioworld Technology Co. Ltd. (Cat number: AP0060, USA). PVDF membrane was produced by Milipore Company (Lot number: K5EA5857D, USA). BCA protein concentration determination kit was produced by Biyuntian Biotechnology (Lot number: 122515160426, China). RIPA lysis buffer was purchased from Beijing Solaibao Technology Co. Ltd. (Lot number: 20160104, China). HepG2 cells was supplied by Shanghai Fudan University Cell Library (China). RPMI-1640 cell culture medium was produced by Gibco Company (Lot number: 8116118, USA). Fetal calf serum was produced by Gibco Company (Lot number: A79E00G, USA). Cell counting Kit-8 (CCK-8) was produced by Dojindo Laboratories (Japan).

2.2. Experimental animal care

Male db/db mice (33–38 g, 7–8 weeks) and male db/m mice (18–22 g, 7–8 weeks) were purchased from Changzhou Cavens Laboratory Animal Co. Ltd. (License key: SCXK (Su) 2016-0010, China). ^{60}Co radiation mice granule feedstuff was purchased from Nanjing Beisifu Feed Co. Ltd. (Lot number: 20151030001MF01, China). All mice were raised and tested in the SPF barrier laboratory of the animal experiment center of North China University of Science and Technology. All animal experimental procedures were approved by the Animal Ethics Committee of North China University of Science and Technology according to the guidelines of the European Union (Directive 2010/63/EU for animal experiments) and the National Institute of Health of the USA (NIH Publications No. 8023, revised 1978).

2.3. Grouping and administration

After a week's adaptive feeding of db/db mice, the blood was taken from the tail and the blood glucose meter (Abbott, USA) was used to determine the random blood glucose (RBG). Thirty experimental db/db mice were selected based on the RBG and glucose concentration after 2 h of oral glucose administration. Ten male db/m mice were treated as normal group, and 30 db/db mice were randomly divided into three groups (n = 10). The Specific treatment methods were as follows: rutin high-dose group and rutin low-dose group was orally administered with rutin at the dose of 120 mg kg^{-1} d^{-1} and 60 mg kg^{-1} d^{-1}, respectively, rutin dissolved in 1% carboxymethylcellulose sodium solution, normal group and model group was orally administered with 1% carboxymethylcellulose sodium solution at the same dose, all groups were administered at 9 a.m. every day, and the whole experimental time was 8 weeks.

2.4. Measurement of weight, blood glucose and oral glucose tolerance

Weight and RBG were measured (60 min after dosing) in the 0 w and 8 w, and the oral glucose tolerance of the mouse was measured after fasting 16 h in the 8 w.

2.5. Detection of serum biochemical indexes

Sixteen hours after the last administration, the blood samples were collected from the eye socket and the serum was separated. ALT and AST activities were determined by a 7150-automatic biochemical analyzer (Hitachi, Japan). Serum AGEs level was determined by enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer instructions.

2.6. Liver sampling and microscopical examination

The liver tissue was removed immediately after the mice was executed, and rinsed with cool saline water. The left lobe of the liver was immediately taken into the liquid nitrogen tank, and then placed in the −80 °C refrigerator for western blotting and PCR. The right lobe of the liver was cut into the size of 1–2 mm^3, fixed in 2.5% glutaraldehyde for 4 h, fixed in 1% osmic acid for one hour, dehydrated by ethanol, step by step, at last covered with epoxy resin. Then cutting it into ultrathin sections (70 nm thick), stained with lead citrate, and the liver's ultrastructural changes were observed by a transmission electron microscopy (Hitachi, Japan). The remaining liver tissue was fixed in 4% polyformaldehyde solution, then carried out the conventional paraffin embedding, at last cut into the sections of 4 μm thickness. After hematoxylin-eosin (H&E) and Masson trichrome staining, the morphological structure of the liver tissue was observed under a BX50 microscope (Olympus, Japan).

2.7. Western blot analysis

Proteins were separated on gradient RunBlue SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred electrophoretically to Immobilon-P polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat dry milk for 2–4 h and incubated with primary antibodies overnight at 4 °C. After being washed, membranes were incubated with goat anti-rabbit fluorescently labeled secondary antibodies (1:5000). Quantification of the signals was performed by the Odyssey Infrared Imaging System (LICOR 9120, Li-COR, USA). The protein bands were normalized to the β-actin band in each sample. The following primary antibodies were used: anti-IRS-2 (1:1000), anti-P-PI3K (1:1000), anti-P-Akt (1:1000), anti-P-GSK-3β (1:1000) and anti-β-actin (1:1000).

2.8. Real-time quantitative PCR to detect IRS-2 mRNA expression

Quantification of IRS-2 mRNA expression by real-time quantitative PCR. Total RNA was extracted from the liver of mice using Trizol