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Rutin exhibits hepatoprotective effects in a mouse model of non-alcoholic fatty liver disease by reducing hepatic lipid levels and mitigating lipid-induced oxidative injuries

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive accumulation of hepatic lipids and oxidative injury of hepatocytes. Rutin is a natural flavonoid with significant roles in combating cellular oxidative stress and regulating lipid metabolism. The current study aims to investigate the molecular mechanisms underlying rutin's hypolipidemic and hepatoprotective effects in nonalcoholic fatty liver disease. Rutin treatment was applied to male C57BL/6 mice maintained on a high-fat diet and HepG2 cells challenged with oleic acid. Hepatic lipid accumulation was evaluated by triglyceride assay and Oil Red O staining. Oxidative hepatic injury was assessed by malondialdehyde assay, superoxide dismutase assay and reactive oxygen species assay. The expression levels of various lipogenic and lipolytic genes were determined by quantitative real-time polymerase chain reactions. In addition, liver autophagy was investigated by enzyme-linked immunosorbent assay. In both fat-challenged murine liver tissues and HepG2 cells, rutin treatment was shown to significantly lower triglyceride content and the abundance of lipid droplets. Rutin was also found to reduce cellular malondialdehyde level and restore superoxide dismutase activity in hepatocytes. Among the various lipid-related genes, rutin treatment was able to restore the expression of peroxisome proliferator-activated receptor alpha (PPAR-α) and its downstream targets, carnitine palmitoyltransferase 1 and 2 (CPT-1 and CPT-2), while suppressing those of sterol regulatory element-binding protein 1c (SREBP-1c), diglyceride acyltransfase 1 and 2 (DGAT-1 and 2), as well as acyl-CoA carboxylase (ACC). In addition, rutin was shown to repress the autophagic function of liver tissues by down-regulating key autophagy biomarkers, including tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β). The experimental data demonstrated that rutin could reduce triglyceride content and mitigate oxidative injuries in fat-enriched hepatocytes. The hypolipidemic properties of rutin could be attributed to its ability to simultaneously facilitate fatty acid metabolism and inhibit lipogenesis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), which afflicts approximately 20% to 30% of the western population [1], is characterized by excessive fat accumulation in liver that is unrelated to alcohol consumption. NAFLD is widely considered as the most universal chronic liver disease and acts as an independent risk factor for cardiovascular diseases [2]. The initial stage of NAFLD generally involves the development of simple fatty liver, which then gradually progresses to increasingly severe forms of fat-related liver diseases, including nonalcoholic steatohepatitis (NASH), hepatic fibrosis and cirrhosis [3]. Recently, there has also been mounting evidence linking NAFLD to hepatocellular carcinoma [4,5].

Despite the broad pathological implications of NAFLD, the mechanism underlying its pathogenesis remains inadequately elucidated. In 1998, Day et al. [6] proposed the so-called “two-hit” hypothesis, which became the theoretical basis for a series of subsequent studies. Based on the hypothesis, the buildup of triglycerides (TG) in hepatocytes

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constitutes the first “hit” in the development of NAFLD. Some of the most significant contributing factors for this step include excessive calorie intake and obesity. In addition, insulin resistance that arises from elevated levels of free fatty acids in liver has also been suggested to play an essential role [7,8]. The second “hit” is associated with oxidative stress resulting from the mitochondrial oxidation of free fatty acids, as well as increased inflammation caused by dysregulation of pro-inflammatory cytokines and adipokines [6]. These pathological changes can promote the apoptosis of hepatocytes, inflict widespread damage on liver tissues, and eventually cause NAFLD to progress toward more advanced stages.

It is generally agreed that early forms of NAFLD, particularly simple steatosis, are reversible. Since hyperlipidemia is one of the leading causes of steatosis, there is considerable interest in ascertaining whether dietary modification could be employed as a preventative measure and a treatment strategy against NAFLD. For example, Xu and colleagues studied the beneficial effects of dioscin on a murine model of NAFLD using a combination of iTRAQ labeling and nano-liquid chromatography-time of flight-mass spectroscopy/mass spectroscopy [9]. Similar results were also obtained in a study by Liu et al., in which the authors demonstrated that dioscin could alleviate diet-induced obesity by altering the balance between lipid synthesis and metabolism [10]. Rutin is a plant-derived flavonoid commonly found in fruits, tea and wine [11]. It has been shown to possess a multitude of desirable therapeutic, particularly antioxidant and anticarcinogenic, properties [12–14]. In addition, rutin can also reduce capillary permeability, prevent atherogenesis and alleviate the cytotoxicity of oxidized LDL-cholesterol [15–19]. Recently, Xin et al. demonstrated that rutin can significantly decrease hepatic TG and cholesterol levels in rats maintained on a high-fat diet [20]. Despite these findings, rutin's roles in regulating hepatic lipid homeostasis and protecting liver against NAFLD remain poorly understood. Herein, we seek to investigate the hypolipidemic and hepatoprotective effects of rutin, and shed light on the underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Aqueous solution of rutin (30 mg/ml, Biochemical reagent, endotoxin free) was purchased from Yabao Pharmaceutical Group Co. Ltd., Ruicheng, China.

2.2. Animal model

The murine NAFLD model reported by Wang et al. was used in this study [21]. All animal experiments were approved by institutional guidelines of Zhejiang Chinese Medical University. Male C57BL/6 mice (8 week) weighing 25 ± 0.5 g were housed in the Experimental Animal Center of Zhejiang Traditional Chinese Medical University. All mice were initially housed under conventional conditions on a standard diet and water *ad libitum* for 1 week prior to the diet program. The mice were subsequently divided into four groups (n = 6 per group) and fed with water and different diets *ad libitum*. Control group and high-fat (HF) group were maintained on a control diet (D12450B, Research Diets, New Brunswick, NJ) and a high-fat diet (60% fat, D12492, Research Diets, New Brunswick, NJ), respectively, for eight weeks. In comparison, mice in rutin (RT) group and high-fat/rutin (HR) group were fed the same control diet and high-fat diet as above, respectively, and were each given an intraperitoneal injection of rutin at a dose of 200 mg/kg body weight every day from week 4 until the end of the diet program. The body weight of each mouse was recorded on a weekly basis and the amount of food consumed was logged daily. At the end of the diet program, the mice were subjected to fasting for 4 h and anesthetized with Avertin at a dose of 300 mg/kg body weight before being sacrificed. Plasma, liver and epididymal fat pad were harvested.

2.3. Cell culture

The human hepatocarcinoma cell line HepG2 and murine macrophage like cell line RAW 246.7 were obtained from Shanghai Institute of Cell Bank. The cells were cultivated at 37 °C under a humidified atmosphere of O_2/CO_2 (19:1) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 2 mmol/l glutamine, 5 U/ml penicillin and 50 μg/ml streptomycin, until the culture reached 80% confluency. One milliliter of cells was subsequently seeded to each well of a 24-well petri plate at a density of 2×10^5 cells/ml. Four incubation groups were prepared in parallel, namely, the untreated control group (UT), rutin group (Rutin), oleic acid group (OA) and rutin/oleic acid group (Rutin/OA). The Rutin and Rutin/OA groups were first treated with DMEM containing 10–40 μM rutin for 2 h, whereas the OA group was incubated in plain DMEM over the same period of time. Subsequently, the OA and Rutin/OA groups were treated with 0.5 mM of OA. The cultures were then incubated overnight under the same conditions specified above.

2.4. Oil Red O (ORO) staining

Lipid droplets in mature adipocytes were stained with Oil Red O. Briefly, the cells were fixed with 10% formalin and incubated with a filtered solution of 60% (wt/wt) Oil Red O (Sigma-Aldrich, St louis, MO, USA) in pure isopropanol for 1 h at 60 °C. The stained cells were subsequently washed twice with distilled water to remove excess dye and visualized under an optical microscope.

2.5. Malondialdehyde (MDA) assay

MDA assay was performed on the treated HepG2 cells and harvested liver tissues using a commercial MDA Assay Kit (A003–4, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For sample preparation, DMEM was removed following the overnight incubation described above (see Cell culture) and 0.5 ml of lysis buffer was added to each well. The plate was allowed to stand for 2 min and the resultant lysate was directly used. For the processing of liver tissues, 100 mg of tissue samples were mixed with 0.5 ml of lysis buffer and disrupted using a glass tissue grinder (K749510–1501, Kimble Chase, Vineland, NJ, USA) at room temperature for 30 min. After complete tissue homogenization, the resultant suspension was centrifuged at 12000 rpm and 4 °C for 15 min. The supernatant was collected and directly used for the assay following the manufacturer's instructions.

2.6. Statistical analysis

All experiments were performed in triplicate and data were expressed as mean ± standard error. Student's t-test was used for comparing two unpaired groups and one-way ANOVA was employed for three or more groups. All statistical analyses were executed using SPSS 17.0. Differences were considered statistically significant at $P < 0.05$.

Evaluation of liver injury and hepatic fat content, lactate dehydrogenase release (LDH) assay, superoxide dismutase (SOD) assay, reactive oxygen species (ROS) assay, quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting and enzyme-linked immunosorbent assay (ELISA) were conducted as described in Supporting Information.