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Invited Perspective

Cycloastragenol improves hepatic steatosis by activating farnesoid X receptor signalling

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) has become a global health problem. However, there is no approved therapy for NAFLD. Farnesoid X receptor (FXR) is a potential drug target for treatment of NAFLD. In an attempt to screen FXR agonists, we found that cycloastragenol (CAG), a natural occurring compound in Astragali Radix, stimulated FXR transcription activity. In animal studies, we demonstrated that CAG treatment resulted in obvious reduction of high-fat diet induced lipid accumulation in liver accompanied by lowered blood glucose, serum triglyceride levels and hepatic bile acid pool size. The stimulation of FXR signalling by CAG treatment in DIO mice was confirmed *via* gene expression and western blot analysis. Molecular docking data further supported the interaction of CAG and FXR. In addition, CAG alleviated hepatic steatosis in methionine and choline deficient L-amino acid diet (MCD) induced non-alcoholic steatohepatitis (NASH) mice. Our data suggest that CAG ameliorates NAFLD *via* the enhancement of FXR signalling.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver metabolic disease encompassing a spectrum of medical condition, from hepatic steatosis to non-alcoholic steatohepatitis (NASH) followed by progression to fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD is often accompanied by multifaceted metabolic disorder factors including obesity, insulin resistance, dyslipidemia and *etc* [1,2]. Due to the prevalence of over-nutritious lifestyle, concealed disease progression, and eruptible obesity morbidity, NAFLD has been a leading killer worldwide [3,4]. Although many attempts were made to find an appropriate molecule target and related chemical drug to treat NAFLD, there is no therapy approved for NAFLD currently [5].

Farnesoid X receptor (FXR) is a ligand-activated nuclear receptor transcription factor which serves as an intracellular bile acid sensor in enterohepatic system [6]. FXR plays roles in correcting multiple metabolic disorders including cholestasis, dyslipidaemia, IR, inflammation, NAFLD *via* regulating a series of genes expression [7–11]. On the other hand, loss of FXR in mice lead to development of impaired glucose tolerance and insulin resistance, elevated circulating lipids levels and severe liver steatosis [12,13]. Obeticholic acid (OCA), a lipophilic bile acid derivative and a potent FXR activator, has been the focal point in drug research field due to its significantly beneficial effects on NAFLD in both animal experiments and human trials [14–16]. Thus, FXR activation is a potential therapeutic approach for NAFLD.

Astragali Radix, the roots of *Astragalus membranaceus (Fisch) Bunge*, is widely distributed in Europe and Asia [17,18]. In China, Astragali Radix has been used as an herbal medicine to treat vari-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; BA, bile acid; DIO, diet-induced obesity; CA, cholic acid; DMSO, dimethylsulfoxide; FXR, farnesyl X receptor; HDL-c, high density lipoprotein cholesterol; HF, high-fat; IPITT, intraperitoneal insulin tolerance test; LDL-c, low density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa B; NR, nuclear receptor; MS, metabolic disease; Rosi, rosiglitazone; TBA, total bile acid; TC, total cholesterol; TCA, taurocholic acid; TCDCA, tauro-chenodeoxycholic acid; TG, triglyceride; TUDCA, tauro-ursodeoxycholic acid.

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ous diseases such as diabetes, hyperlipidemia, atherosclerosis and cancers [19–21]. Astragaloside IV, the major constituent of Astragali Radix, has been identified to be mostly responsible for the effect observed with Astragali Radix [17,18,22]. However, astragaloside IV is metabolized as cycloastragenol (CAG) mostly [23], which is also natural occurred in Astragali Radix [22]. Recently CAG has been demonstrated to activate telomerase and shown potential pharmacological effects including anti-aging, anti-oxidation, relieving endoplasmic reticulum (ER) stress and *etc* [23–26]. However, whether CAG could act on controlling MS and NAFLD remains elusive.

In screening for FXR agonists from natural compounds, we found that CAG stimulated the FXR transcription activities. Here we aim to reveal whether CAG treatment improves liver steatosis and other metabolic disorders.

2. Methods

2.1. Chemicals and diets

Cycloastragenol (Pusi Biotech, Chengdu, China) and Ursodeoxycholic Acid (UDCA, Zhongxin Biotech, Tianjin, China) were dissolved in dimethylsulfoxide (DMSO) to the final concentration of 50 mM for cell culture. GW4064, T090173, Rosiglitazone (Rosi), int-777, WY14643, rifampicin, and bile acid standard references were purchased from Sigma–Aldrich (St. Louis, MO, USA). High-fat diets (HF, 60% of calories derived from fat), Chow diets (Chow, 10% of calories derived from fat) were purchased from Research Diet (D12492 and D12450B, New Brunswick, NJ, USA). Methionine and choline deficient L-amino acid diets (MCD, 20% of calories derived from fat) and methionine and choline supplement diet (MCS) were purchased from Qifa Biol Comp (A02082002B and A02082003B, Shanghai, China).

2.2. Cell culture and FXR deletion in HepG2 cell

HepG2 (ATCC) cells were seeded on six-well plates (1×10^6 cells/well) and grown to 80% confluence with high-glucose DMEM containing 10% FBS at 37 °C in 5% CO2. The following day, cells were treated with vehicle control, CAG (25 μM). After 24-h treatment, the cells were collected for RNA isolation.

To generate the FXR deletion cell, HepG2 cells were transfected with pX330-U6-Chimeric_BB-CBh-hSpCas9 containing a single-guiding nucleotide sequence (Forward sequence: taggtaacaaagaagccccgcat, reverse sequence: aaacatgcggggcttctttgtta) for targeting human FXR for 48 h. Then, cells were treated with vehicle or CAG (25 μM). After 24-h treatment, the cells were collected for RNA analysis.

2.3. Gene reporter assays

The reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega, USA) as previously described [27]. For nuclear receptor transcription activity assay, the expression plasmids for phFXR, phRXR and FXR-response reporter (EcRE-LUC), pCMXGal-hPPARα, γ, LXRα, β, PXR–LBD and the Gal4 reporter vector MH100 × 4-TK-Luc were co-transfected with a reporter construct so that 1 μg of the relevant plasmid combined with 1 μg of reporter plasmids and 0.1 μg of pREP7 (*Renilla luciferase*) reporter could be used to normalize transfection efficiencies. The transfection mixture, which contained 10 μg of total plasmids and 15 μl FuGENE-HD (Roche) per ml of DMEM, was added to HEK293T cells (ATCC) for 24 h and then removed. The FXR, PPARα, PPARγ, LXRs, and PXR agonists (GW4064, WY14643, Rosiglitazone, T090173 and Rifampicin respectively), CAG were added to fresh media and the cells were incubated for another 24 h to determine luciferase activity.

2.4. Animal experiment

All animal protocols used in this study were approved by Shanghai University of Traditional Chinese Medicine (Approval Number: SZY20150522). Female C57BL/6 mice were purchased from the SLAC Laboratory (Shanghai, China). Animals were housed and bred according to standardized procedures, under controlled temperature (22–23 °C) and on a 12-h light, 12-h dark cycle. Six-week-old female mice were fed with high-fat diet for 12 weeks to induce obesity and these mice were then randomly divided into four groups according to body weight: Chow group (10% of calories derived from fat), High-fat group (HF, 60% of calories derived from fat), UDCA group (HF diet supplemented with UDCA powder (Ursofalk, Losan Pharma GmbH, Germany), at dose of 80 mg/100 g diet) and CAG group (HF diet supplemented with CAG powder, at dose of 100 mg/100 g diet). Mice were treated for additional 6 weeks. Food intake amount was measured by recording food weight every two days throughout the experiment. The amount of food intake over a 24-h period was calculated.

For MCD-diet fed animals experiment, female C57BL/6 mice were managed as the same methods in HF diet-fed experiment. Six-week-old female mice randomly divided into three groups according to body weight: MCS group (fed MCS diet), MCD (fed MCD diet), and CAG group (MCD diet supplemented with CAG powder at 100 mg/100 g diet). Mice were fed with corresponding diets for additional 6 weeks before the experiment ending.

2.5. Intraperitoneal glucose tolerance and insulin tolerance tests

At the end of the HF-diet treatment, mice were fasted overnight (12 h). The baseline glucose values (0 min), prior to the injection of glucose (1 g/kg body weight), were measured through tail vein. Additional blood samples were collected at regular intervals (15, 30, 60, and 90 min) during glucose tolerance tests.

2.6. Serum chemistry analysis

At the end of animal experiment study, mice were anesthetized 20% urethane (Sigma, St. Louis, MO) and cardiac blood was taken. Serum alanine aminotransferase (ALT), aspartate transaminase (AST), triglyceride (TG), total cholesterol (TC), HDL cholesterol (HDL-c), and LDL cholesterol (LDL-c) were measured using a Hitachi 7020 Automatic Analyzer (Hitachi, Ltd., Tokyo, Japan) with 100 μl of heart blood serum.

2.7. Histochemistry

Liver tissues were fixed in formalin, and paraffin-embedded sections were cut at 5 μm. Sections were stained with haematoxylin and eosin according to a standard procedure. For Oil red O staining, the frozen liver tissues were embedded in O.C.T. compound (Sakura Finetek, Torrance, CA), sliced, and then stained with Oil red O.

2.8. Hepatic lipid content analysis

Lipid content was measured as described [28]. Briefly, 100 mg of the liver tissue was homogenized with 2 ml chloroform-methanol and then agitated overnight on an orbital shaker at 4 °C. The homogenate was then centrifuged (5 min at 2300 *g*), 0.9% NaCl solution was subsequently added to the liquid phase before the samples were vortexed. Phase separation was induced by centrifugation (800 *g* for 10 min), and the bottom phase was removed to a new tube and evaporated to dryness, Samples were then