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ABSTRACT

Backgroud: Non-alcoholic fatty liver disease (NAFLD) is currently evolving as the most common liver disease worldwide. Dyslipidemia, pathoglycemia and insulin resistance are the major risk factors for the development of NAFLD. To date, no effective drug therapies for this condition have been approved.

*Purpose:*The present study was to investigate the protective effects of yangonin, a kavalactone isolated from Kava, against NAFLD and further elucidate the mechanisms *in vivo* and *in vitro*.

Study design: A high-fat diet (HFD) induced mouse NAFLD model was used with or without yangonin treatment.

Methods: The body weight, relative liver weight and serum biochemical indicators were measured. H&E and Oil Red O staining were used to identify the amelioration of the liver histopathological changes. Serum and hepatic triglyceride, free fatty acids and total cholesterol were analyzed. siRNA, quantitative real-time PCR and Western blot assay were used to clarify the mechanisms underlying yangonin protection.

*Results:*Yangonin had obvious protective effects against NAFLD via farnesoid X receptor (FXR) activation. Through FXR activation, yangonin attenuated lipid accumulation in the liver via inhibition of hepatic lipogenesis-related protein including sterol regulatory element-binding protein 1c (SREBP-1c), fatty acid synthetase (FAS), acetyl-CoA carboxylase 1 (ACC1) and stearoyl-CoA desaturase 1 (SCD1). Besides, yangonin promoted lipid metabolism through an induction in genes required for lipoprotein lipolysis and fatty acid β-oxidation. Furthermore, yangonin modulated blood glucose homeostasis through regulation of gluconeogenesis-related gene phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and glycogen synthesis-related gene glycogen synthase kinase 3β (GSK3β)

and pyruvate dehydrogenase (PDase). Also, yangonin increased insulin sensitivity through upregulating phosphorylation of insulin responsive substrate 1, 2 (IRS-1 and IRS-2). Then, *in vivo* and *in vitro* evidence further demonstrated the involvement of FXR activation in yangonin hepatoprotection.

*Conclusions:*Yangonin protects against NAFLD due to its activation of FXR signalling to inhibit hepatic lipogenesis and gluconeogenesis, and to promote lipid metabolism and glycogen synthesis, as well as insulin sensitivity.

Keywords: NAFLD; FXR; Lipid homeostasis; Glucose homeostasis; Yangonin

Abbreviations

ACADS, acyl-coenzyme A dehydrogenase;

ACC1, acetyl-CoA carboxylase 1;

ANGPTL3, angiopoietin-like 3;

Apo C-II, apolipoprotein C-II;

Apo C-III, apolipoprotein C-III;

CDCA, chenodeoxycholic acid;

CPT1α, carnitine palmitoyl transferase 1α;

FAS, fatty acid synthesis;

FXR, farnesoid X receptor;

G6Pase, glucose-6-phosphatase;

GS, guggulsterone;

GSK3β, glycogen synthase kinase 3β;

H&E, haematoxylin & eosin;

HFD, high-fat diet;

IRS-1, insulin responsive substrate 1;

IRS-2, insulin responsive substrate 2;

ITT, insulin tolerance test;

LPL, lipoprotein lipase;

NAFLD, non-alcoholic fatty liver disease;

OGTT, oral glucose tolerance test;

PDase, pyruvate dehydrogenase;

PEPCK, phosphoenoyl pyruvate carboxykinase;

PPARα, peroxisome proliferator-activated receptor α;

SCD1, stearoyl-CoA desaturase 1;

SHP, small heterodimer partner;

SREBP-1c, sterol regulatory element-binding protein 1c;

Yan, yangonin;

Introduction

With the increase in the worldwide obesity population, non-alcoholic fatty liver disease (NAFLD) has already become one of the most common chronic liver diseases (Farinelli et al. 2015; Stal 2015; Zhu et al. 2015). NAFLD is considered as the hepatic representation of metabolic syndrome, which encompasses a spectrum of liver pathology ranging from simple steatosis to steatohepatitis, fibrosis and cirrhosis. To date, the drugs for NAFLD treatment includes insulin sensitizers, statins and fibrates (Liu et al. 2015). However, the side effects and skewed risk-benefit ratio limit their clinical application (Scorletti et al. 2015). Therefore, efforts to develop more effective drugs for the treatment of NAFLD are urgently needed.

The pathogenesis of NAFLD involves a multiple-hit process that is attributed to the disruption of lipid and glucose metabolism (Hou et al. 2016). The underlying molecular mechanisms of lipid and glucose homeostasis have recently been demonstrated to be mediated by nuclear receptors including farnesoid X receptor (FXR), pregnane X receptor and constitutive androstane receptor (CAR), *etc.* (Cave et al. 2016). Among these nuclear receptors, FXR has emerged as a new promising therapeutic target for treating NAFLD since the alterations in FXR signalling may contribute to the pathogenesis and progression of NAFLD, as well as lipid and glucose homeostasis (Cave et al. 2016; Yuan and Bambha 2015). The protective effect of FXR in NAFLD is mediated by its ability to inhibit triglyceride synthesis, to induce lipoprotein metabolism and to promote fatty acid β-oxidation (Chavez-Talavera et al. 2017; Pineda Torra et al. 2003; Watanabe et al. 2004). FXR also plays a critical role in glucose homeostasis by repressing hepatic gluconeogenesis and promoting glycogen synthesis (Kim et al. 2017; Ma et al. 2013). In fact, hepatic lipid accumulation of NAFLD represents the hepatic manifestation of impaired insulin network. FXR can

modulate insulin sensitivity through induction of phosphorylation of insulin responsive substrate 1 (IRS-1) (Chavez-Talavera et al. 2017; Zhang et al. 2006). Therefore, specific targeting FXR can represent a novel therapeutic approach to treat NAFLD.

Pharmacological studies have significantly expanded to include a massive screening of natural products in search for novel drug candidates. Yangonin (Yan) is one of the bioactive kavalactones isolated from Kava, a well-known perennial tropical shrub widely cultivated in the South Pacific Island Countries (Ligresti et al. 2012; Wang et al. 2015). Yangonin has been demonstrated to have several pharmacological activities such as antiproliferative activity of cancer cell lines, antioxidant and hepatoprotective properties (Kong et al. 2018; Liu et al. 2017; Wruck et al. 2008). However, whether yangonin exerts protection against NAFLD remains unknown. Therefore, the present study aimed to investigate whether yangonin can protect against NAFLD and whether FXR signalling plays a role in its hepatoprotection against NAFLD.

Materials and methods

Chemicals

Yangonin, chenodeoxycholic acid (CDCA) and guggulsterone (GS) (purity > 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, free fatty acids and total cholesterol kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other biochemical indicators kits and other chemicals were commercially available.

Animals and treatments

Male 8-week-old C57BL/6 mice were housed in cages and maintained under 12-h light-dark cycles with free access to food and water. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. Mice were randomly divided into seven groups (n=8 per group). Mice were fed either a normal diet consisting of 12% kcal fat content (Research Diets D12450H, New Brunswick, USA) or a high-fat diet (HFD) consisting of 45% kcal fat content (Research Diets D12451, New Brunswick, USA) for 16 weeks. Yangonin (10, 20 or 40 mg/kg) or vehicle alone was treated to mice by oral gavage once a day. Four hours before vehicle or yangonin administration, mice were injected with 10 mg/kg GS intraperitoneally. The food intake was monitored daily, and body weight was measured weekly. After exposure to HFD for 16 weeks, blood was collected from suborbital veins and liver tissues were excised, weighed, processed for subsequent analyses.

Biochemical analysis

Liver injury was evaluated by measuring serum ALT and AST. Lipid profiles were evaluated by determining serum triglyceride, free fatty acids, total cholesterol, and hepatic triglyceride, free fatty acids and total cholesterol according to the manufacturer's instructions.

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

Three days before the termination of the experiment, glucose (2 g/kg) was given to mice by oral gavage. Blood samples were collected over the time course of 0 to 120 min after given with glucose. In insulin tolerance test, mice were injected

intraperitoneally with 1U/kg of insulin (Novolin 30R, Novo Nordisk, Bagsvaerd, Denmark). Blood samples were obtained over the time course of 0 to 120 min after insulin injection. The levels of blood glucose were measured by an ACCU-CHEK Performa blood glucose meter (Roche Diagnostic, Mannheim, Germany).

Histopathology

The isolated left lateral segment of the liver lobes was fixed in 10% neutral buffered formalin. Paraffin-embedded liver sections were stained with H&E for pathological evaluation. The frozen sections of formalin-fixed livers were stained with Oil Red O. Then, the samples were analyzed by light microscopy (Nikon Eclipse TE2000-U, NIKON, Japan).

Mice primary hepatocytes culture

Hepatocytes from C57BL/6 mice were isolated using two-step collagenase digestion method (Klaunig et al. 1981). The isolated hepatocytes were cultured in the William's E medium which contains 10% heat-inactivated fetal bovine serum, 1× insulin-transferrin-selenium-sodium pyruvate solution, 0.1 μM dexamethasone and 1× glutamine for 4 h. Then hepatocytes were incubated with the fresh medium.

RNA silencing experiment

Mice primary cultured hepatocytes (2×10^5) were seeded in six-well plates and divided into four groups: si-control, si-control+ yangonin (10 μM), si-FXR and si-FXR+ yangonin (10 μM). Cells were transiently transfected with specific siRNA (200 nM) using lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocols. After transfection, the cells were incubated for 6 h and then

treated with 10 μM yangonin for 24 h. Finally, genes were extracted from the cells for quantitative real-time PCR.

Quantitative real-time PCR

Total RNA was extracted from tissues or cells and reverse-transcribed to cDNA using PrimeScript RT reagent kit. The mRNA expression of mouse lipid metabolism-related genes including CPT1α, ACADS, PPARα, LPL, Apo C-II, Apo C-III and ANGPTL3, and gluconeogenesis-related gene PEPCK, as well as glycogen synthesis-related gene PDase, GSK3β were detected by qRT-PCR, which was performed by SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems, USA). Relative mRNA expression of target genes was obtained by normalizing to control group and the level of β-actin.

Protein isolation and Western blot

Liver tissues were homogenized and lysed in an appropriate cold lysis buffer containing 1 mM PMSF. The homogenates were centrifuged at 13,000× g for 10 min. Protein concentrations were determined by BCA protein assay. 20 μg of protein extracts were separated in 10% SDS-PAGE, transferred onto PVDF membranes and blocked with 5% dried milk in Tris-buffered saline. Membranes were incubated overnight with primary antibodies, including SREBP-1c, FAS, ACC1 and SCD1 (Santa Cruz Biotechnology, Santa Cruz, CA). The specific bands were visualized using an ECL detection kit.

Statistical analysis

Values were reported as means ± S.D. Statistical comparison between different

groups was made using one-way analysis of variance and Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Results

Effects of yangonin on body weight and relative liver weight in mice fed a HFD

To investigate whether yangonin has protective effects on NAFLD, male C57BL/6 mice were fed with a HFD for 16 w. CDCA is a known FXR agonist used as a positive control drug. After 16 w of HFD feeding, the mice had significantly increased body weight compared to Control mice. Yangonin treatment markedly reduced the increases in body weight induced by HFD (Fig. 1A). There was no significant difference in food intake among groups. In addition, yangonin treatment significantly decreased liver weight to body weight ratio in a dose-dependent manner (Fig. 1B). These results suggested that yangonin could inhibit the occurrence of obesity in mice.

Yangonin attenuates HFD-induced NAFLD

The function of the liver is indicated by specific enzymes which act as hallmarks of liver injury. The liver function parameters ALT and AST were found to be elevated by chronic HFD feeding. These elevations in serum ALT and AST were dose-dependently reversed by yangonin treatment (Fig. 1C-D). Besides, histological analysis of H&E staining indicated that the liver of the HFD-fed mice had markedly lipid droplet accumulation, acinar and portal inflammation, infiltration of macrophages and lymphocytes. These hepatic pathological changes were significantly attenuated by yangonin treatment (Fig. 1E). To further evaluate the effects of yangonin on hepatic lipid accumulation, the liver sections were stained with Oil red O. Yangonin dose-dependently decreased hepatic neutral lipids in HFD mice. These

results indicated that yangonin could attenuate HFD-induced NAFLD.

Effects of yangonin on serum and hepatic lipid profiles

To further investigate the effects of yangonin on lipid changes, serum and hepatic lipid were determined. As shown in Fig. 2A-C, HFD mice had higher plasma levels of triglyceride, free fatty acids and total cholesterol compared to Control mice, while yangonin dose-dependently decreased their plasma levels. Also, hepatic concentrations of triglyceride, free fatty acids and total cholesterol were increased by HFD and decreased by yangonin treatment (Fig. 2D-F). Yangonin treatment also reversed HFD-induced increases in blood glucose and insulin (Fig. 2G-H). These data indicated that yangonin can markedly lower the increases in lipid induced by HFD in mice. Since 40 mg/kg of yangonin had the best hepatoprotective effects, this dose was chosen for the subsequent study.

Yangonin modulates lipogenesis through regulating the related protein expression

To elucidate the mechanism why yangonin inhibits excessive lipid deposition in the liver, the levels of the lipogenesis-related protein were determined. The protein levels of sterol regulatory element-binding protein 1c (SREBP-1c), and its downstream genes fatty acid synthetase (FAS), acetyl-CoA carboxylase 1 (ACC1) and stearoyl-CoA desaturase 1 (SCD1) were increased in HFD mice. However, yangonin reduced the protein levels of SREBP-1c, FAS, ACC1 and SCD1 (Fig. 3A). The results suggested that yangonin repressed hepatic lipogenesis through down-regulating the expression of the related protein.

Yangonin modulates lipid metabolism via altering the expression of the related genes

To further clarify the mechanism of decreased lipid by yangonin, the expression levels of genes in fatty acid β-oxidation was examined. Carnitine palmitoyl transferase 1α (CPT1α) and acyl-coenzyme A dehydrogenase (ACADS) were decreased in HFD mice and were increased in yangonin-treated mice. We further determined their upstream gene peroxisome proliferator-activated receptor α (PPARα) which is a nuclear hormone receptor and regulates CPT1α and ACADS expression. As expected, yangonin treatment increased PPARα expression (Fig. 3B).

In addition, the lipoprotein lipolysis is also important for lipid homeostasis. Lipoprotein lipase (LPL) which is regulated by apolipoprotein C-II (Apo C-II), apolipoprotein C-III (Apo C-III) and angiopoietin-like 3 (ANGPTL3), is a critical enzyme in the process of lipoprotein lipolysis. As shown in Fig. 3C, yangonin treatment through up-regulating Apo C-II and down-regulating Apo C-III, ANGTPL3 expression, induced LPL level. Taken together, these results suggested that yangonin promoted lipid metabolism through the regulation of lipid metabolism-related gene expression.

Effects of yangonin on glucose tolerance and insulin sensitivity

To further investigate the effect of yangonin on blood glucose, we performed oral glucose tolerance test and insulin tolerance test. As illustrated in Fig. 4A, HFD-fed mice had higher glucose levels, and this elevation was reduced by yangonin treatment. In addition, yangonin restored insulin sensitivity (Fig. 4B). These results indicated that yangonin could restore the changes in glucose tolerance and insulin sensitivity induced by HFD.

To clarify the mechanism of yangonin-decreased blood glucose, the expression levels of gluconeogenesis- and glycogen synthesis-related genes were determined.

The critical enzymes in gluconeogenesis, phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), were observed to be increased in HFD mice and decreased in yangonin-treated mice (Fig. 4C). Besides, two important enzymes involved in glycogen synthesis, pyruvate dehydrogenase (PDase) and glycogen synthase kinase 3β (GSK3β), were induced by yangonin (Fig. 4D). These findings suggested that yangonin through regulating the expression of key enzymes suppressed gluconeogenesis and promoted glycogen synthesis.

To clarify the mechanism underlying changed insulin sensitivity, we examined the expression of two important genes in insulin signalling. Yangonin treatment reversed the decreases in phosphorylation of IRS-1 and IRS-2 induced by HFD (Fig. 4E). These data indicated that yangonin induced IRS-1 and IRS-2 phosphorylation, resulting in the increase in insulin sensitivity.

Yangonin protects against HFD-induced NAFLD via FXR in vivo

Since the above genes involved in lipid and glucose homeostasis are FXR downstream target genes, we hypothesize that yangonin may activate FXR to exert the hepatoprotective effects against HFD-induced NAFLD *in vivo*. To verify this hypothesis, FXR was blocked by antagonist GS in mice. GS decreased the gene expression of small heterodimer partner (SHP), a classical target gene of FXR and PEPCK (Fig. 5A). Also, the decreases in protein levels of SREBP-1c, FAS, ACC1 and SCD1 were abrogated by GS (Fig. 5B). And H&E staining result further demonstrated that GS abrogated the hepatoprotective effects of yangonin (Fig. 5C). These findings suggested that yangonin exerted protection against NAFLD through FXR activation *in vivo*.

FXR gene silencing abrogated yangonin-regulated FXR target gene expression in vitro

The effect of yangonin on FXR activation was subsequently examined using FXR gene silencing experiment in mice primary cultured hepatocytes *in vitro*. As illustrated in Fig. 6A, yangonin dose-dependently induced the gene expression of GSK3β and PEPCK, with a maximal change at the dose of 10 μM. Thus, the dose of 10 μM was chosen for the subsequent study. The protein expression of FXR was decreased by approximately 80% after specific siRNA targeting FXR mRNA transfection, (Fig. 6B). And the changes in GSK3β, PEPCK, PPARα, LPL and G6Pase induced by yangonin were abrogated by siFXR (Fig. 6C-D). These data further demonstrated that the involvement of FXR activation in the hepatoprotective effect of yangonin.

Discussion

NAFLD, an emerging metabolic-related disease characterized by hepatic fatty infiltration, has become to be the most common chronic liver disorder which is strongly associated with obesity. Currently, there is no approved pharmacological treatment that can convincingly reverse NAFLD. Thus, studies on potential therapeutic interventions of NAFLD are urgent.

FXR, a member of the nuclear receptor super family of ligand-activated transcription factors (Forman et al. 1995), has been recognized as a novel therapeutic target for NAFLD. Most importantly, it has been reported that FXR expression is decreased in NAFLD patients (Yang et al. 2010), indicating that as the key regulatory transcription factor for lipid, FXR may become the focus of targeted therapies in NAFLD. FXR has at least two roles in lipid homeostasis. The first role is to modulate hepatic lipogenesis via inhibition of the genes involved in lipid synthesis. The second

role is to promote lipid metabolism via an induction in genes required for lipolysis of lipoproteins and fatty acid β-oxidation. In the present study, yangonin through FXR activation reduced the hepatic protein levels of SREBP-1c, a leading regulator of lipogenesis, and its downstream genes including FAS, ACC1 and SCD1 (Watanabe et al. 2004). Besides, through FXR activation, yangonin increased the expression of key enzymes involved in fatty acid β-oxidation such as PPARα, CPT1α and ACADS. Also, yangonin treatment through up-regulating Apo C-II and down-regulating Apo C-III, ANGTPL3 expression, induced LPL level, resulting in the increase in the lipolysis of lipoproteins, which is also attributed to FXR activation.

FXR also has an important role in glucose homeostasis via regulating gluconeogenesis, glycogen synthesis and insulin sensitivity (Samuel and Shulman 2018). It has been demonstrated that FXR deletion results in glucose intolerance and insulin resistance (Samuel and Shulman 2018). However, liver adenovirus overexpression of FXR or treatment with FXR synthetic agonists lowers blood glucose by repressing hepatic gluconeogenesis and inducing glycogen synthesis. In the present study, yangonin decreased the mRNA levels of G6Pase and PEPCK that are the key enzymes in gluconeogenesis, resulting in the decrease in gluconeogenesis. Besides, yangonin induced the gene expression of glycogen synthesis-related enzymes including PDase and GSK3β. And yangonin elevated insulin sensitivity through up-regulating IRS-1 and IRS-2 phosphorylation.

To further demonstrate the involvement of FXR activation in the yangonin hepatoprotection, FXR antagonist GS *in vivo* and FXR gene silencing experiment *in vitro* was performed. *In vivo* evidence demonstrated that GS abrogated the decrease in SREBP-1c, FAS, ACC1, SCD1 and PEPCK, and the hepatoprotective effects of yangonin. *In vitro* evidence demonstrated that the yangonin-mediated regulation of

GSK3β, PEPCK, PPARα, LPL and G6Pase was abrogated by FXR silencing.

In general, NAFLD animal models can be divided into two types: genetic mutations and those induced by dietary or pharmacological modifications. **NAFLD animal models induced by diet are usually made using three diets: a methionine and choline-deficient (MCD) diet, a choline-deficient diet (CD) and a HFD. The differences between the three diets are as follows: rodents fed MCD diets lose weight and do not become insulin resistant; CD diets induce steatosis, inflammation and fibrosis without any difference in body weight; HFD are well-known to increase body weight, body fat and induce insulin resistance in rodent models. In the present study, we want to investigate the effect of yangonin on hepatic lipogenesis and gluconeogenesis as well as insulin sensitivity, therefore, HFD was used.** And since caloric overconsumption is a major factor in NAFLD development in the clinical condition, recent studies have successfully established mouse NAFLD model using HFD (Feng et al. 2017). Mice fed with a long-term HFD can serve as an experimental model of NAFLD, with typical hepatic lesions including hepatomegaly, hepatocyte ballooning and steatosis (Ma et al. 2017). This liver injury is accompanied by increased serum liver function markers and elevated levels of serum triglyceride, free fatty acids and total cholesterol compared with the mice fed a standard chow diet. These results suggested that HFD caused dyslipidemia, hepatic steatosis, insulin resistance and impaired liver function. Treatment with yangonin significantly reduced the liver index and the accumulation of lipid in liver. Furthermore, the pathological injury was attenuated following yangonin treatment.

Conclusions

Yangonin dose-dependently protects against NAFLD. The hepatoprotection of

yangonin is due to its activation of FXR signalling to inhibit hepatic lipogenesis and gluconeogenesis and to promote lipid metabolism and glycogen synthesis, as well as insulin sensitivity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Legend to Figures

Fig. 1. Hepatoprotection of yangonin (Yan) against HFD-induced NAFLD. Effects of yangonin on body weight gain (A) and liver weight to body weight ratio (B) in mice. Serum ALT (C) and AST (D) levels elevated by HFD were significantly reduced by treatment with different doses of yangonin. Data are the mean ± S.D. (n=8). *$p<0.05$ versus Control; #$p<0.05$ versus HFD alone. (E) The images of representative H&E or Oil Red O stained liver sections (400 ×magnification).

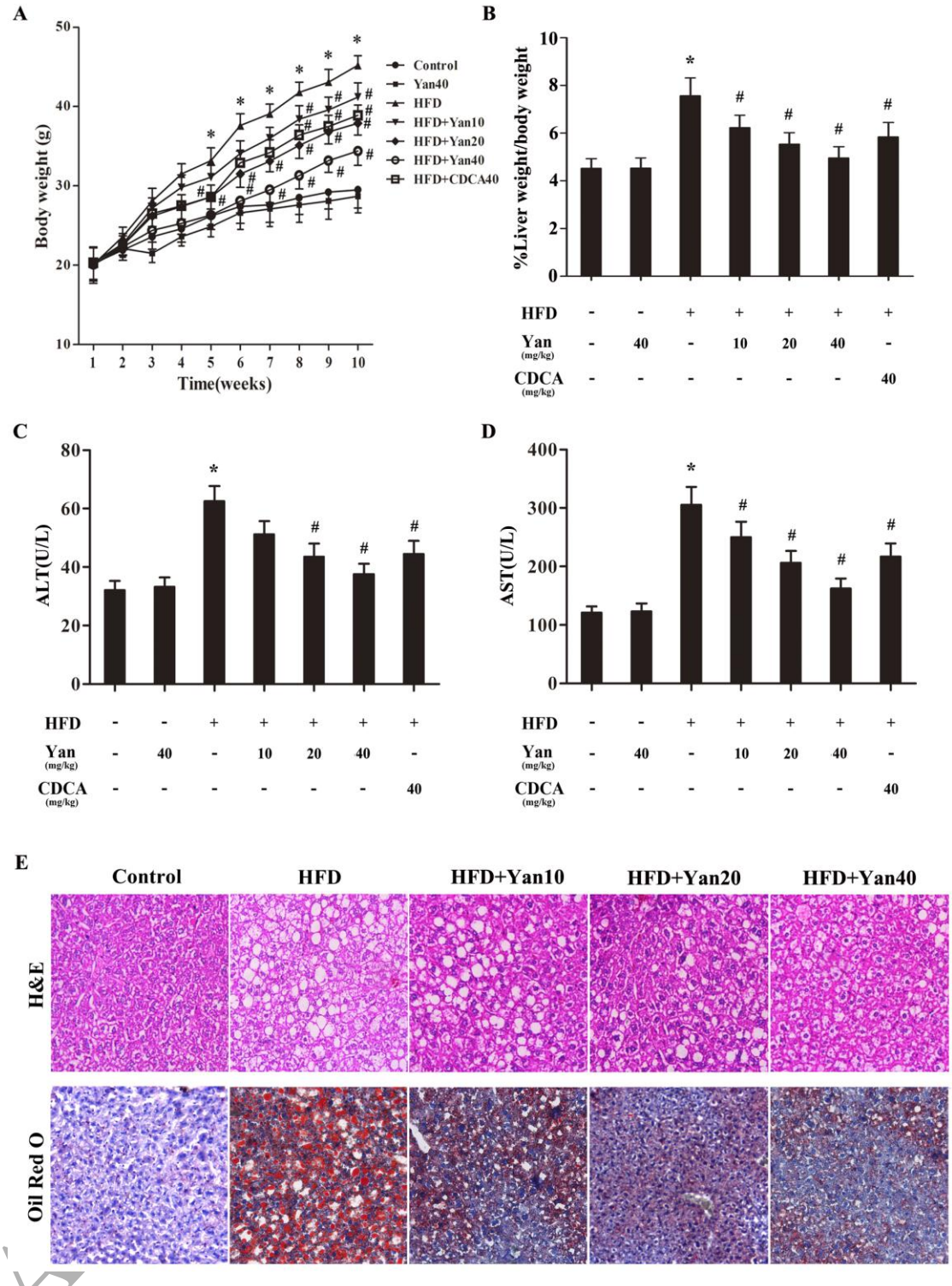


Fig. 2. Effects of yangonin (Yan) on lipid profiles. Serum levels of triglyceride (A), free fatty acids (B) and total cholesterol (C) were increased by HFD and were reduced by yangonin treatment. Yangonin reversed the increases in hepatic levels of triglyceride (D), free fatty acids (E) and total cholesterol (F) induced by HFD. Yangonin also reduced the basal blood glucose (G) and insulin (H). Data are the mean

± S.D. (n=8). *p<0.05 versus Control; #p<0.05 versus HFD alone.

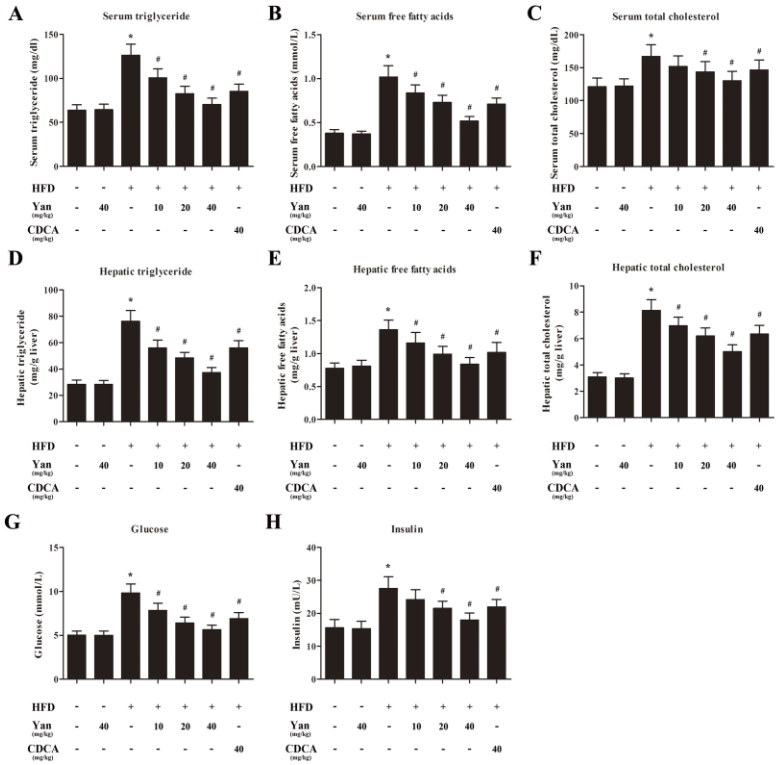


Fig. 3. Yangonin (Yan) alters hepatic expression of genes and proteins involved in lipid homeostasis. (A) Effects of yangonin on the expression of protein involved in lipid synthesis, including SREBP-1c, FAS, ACC1 and SCD1. (B) Effects of yangonin on the expression of genes involved in fatty acid β-oxidation, including CPT1α, ACADS and PPARα. (C) Effects of yangonin on the expression of genes involved in lipid metabolism, including LPL, Apo C-II, Apo C-III and ANGTPL3. Data are the mean ± S.D. (n=5). *p<0.05 versus Control; #p<0.05 versus Vehicle + HFD.

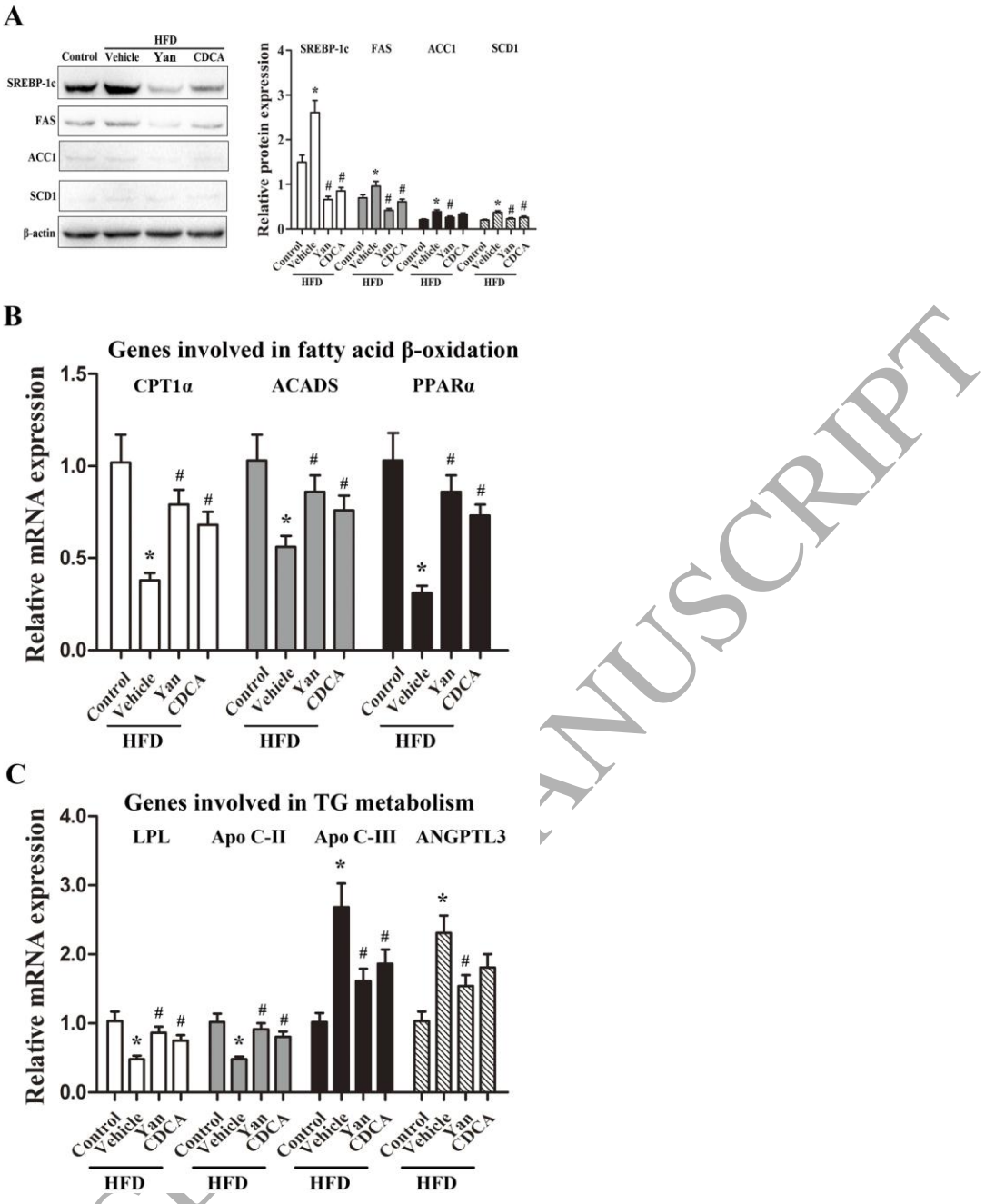


Fig. 4. Yangonin (Yan) improves glucose tolerance and insulin sensitivity in mice. Effects of yangonin on blood glucose in OGTT (A) and ITT (B). Yangonin altered hepatic expression of genes involved in gluconeogenesis (C) and glycogen synthesis (D). In addition, yangonin increased phosphorylation of IRS-1 and IRS-2 which are involved in insulin sensitivity (E). Data are the mean ± S.D. (n=5). *p<0.05 versus Control; #p<0.05 versus Vehicle + HFD.

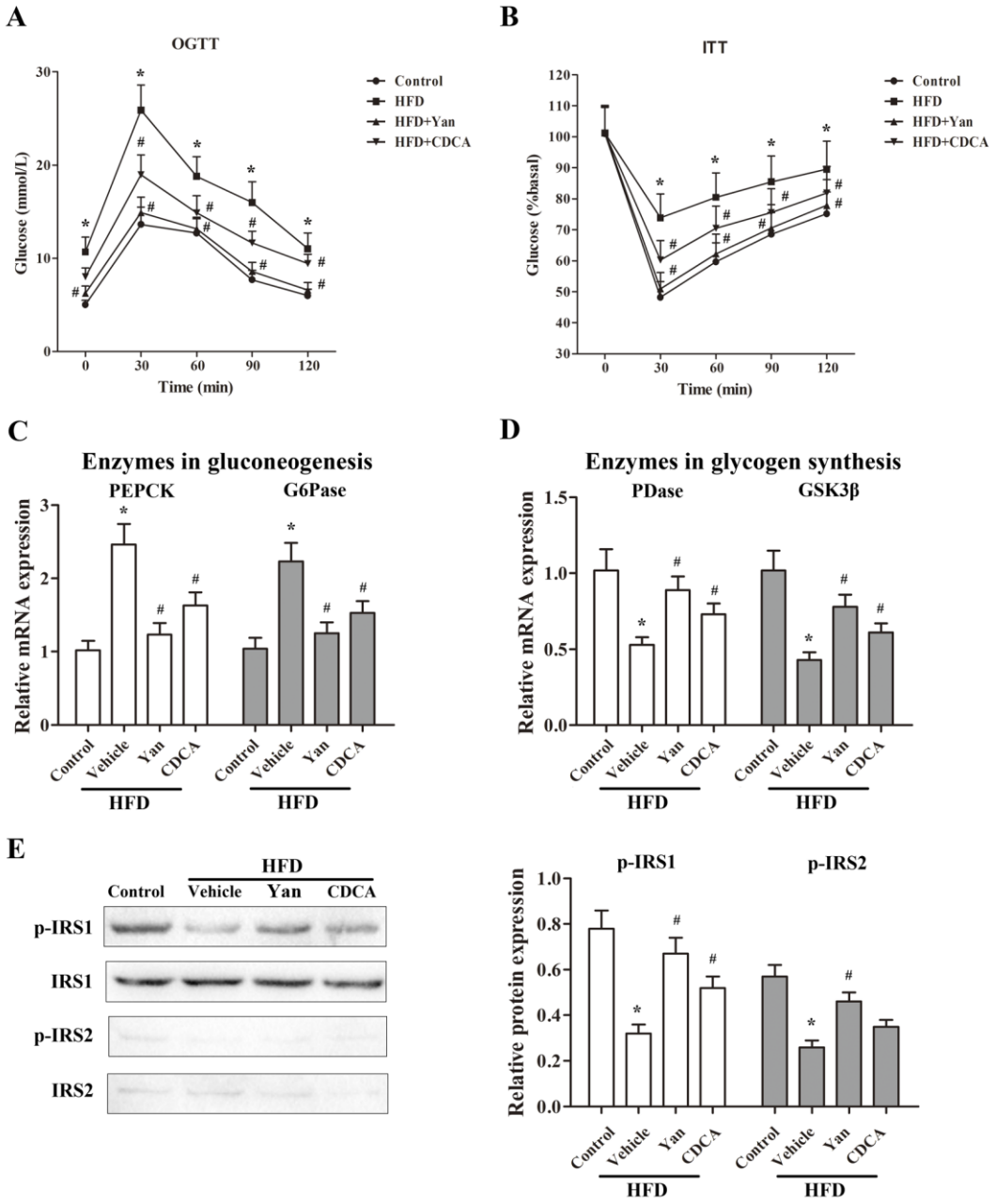


Fig. 5. Yangonin (Yan) protects against HFD-induced NAFLD via FXR in mice. (A) The hepatic expression of SHP and PEPCK in mice with vehicle, Yan, FXR antagonist GS or GS + Yan. (B) The changes in protein expression of SREBP-1c, FAS, ACC1 and SCD1 in yangonin-treated mice were abrogated by GS. Data are the mean ± S.D. (n=5). *p<0.05 versus Control; #p<0.05 versus HFD alone. (C) The images of representative H&E stained liver sections (400 ×magnification) after GS administration were shown.

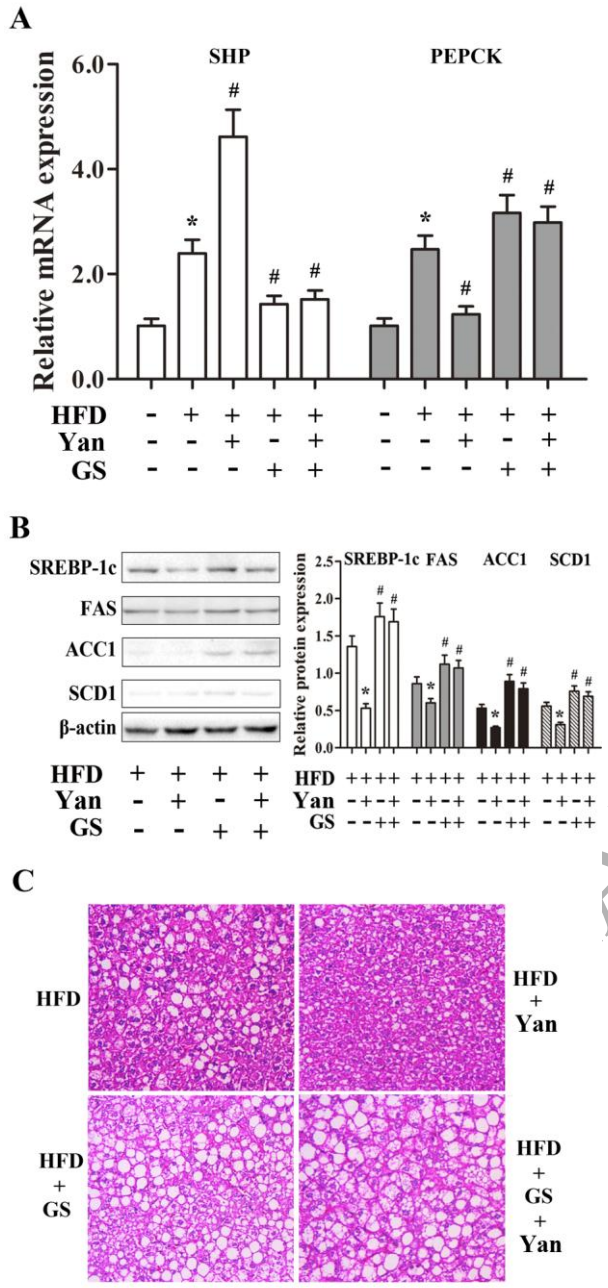
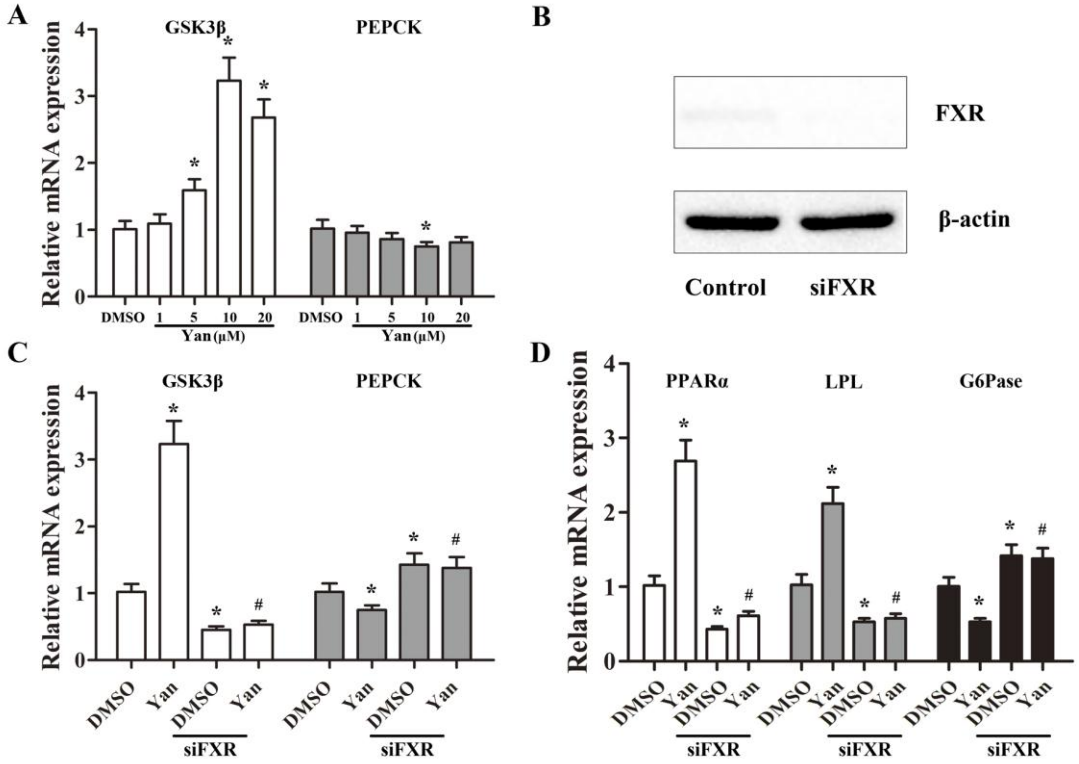


Fig. 6. *In vitro* evidence on FXR activation by yangonin (Yan). (A) Yangonin dose-dependently induced GSK3β and PEPCK in mice primary cultured hepatocytes. Quantitative real-time PCR analysis was performed to measure the gene expression and data are expressed as mean ± S.D. (n=5). * $p<0.05$ versus DMSO. (B) FXR silencing efficiency was measured by Western blot. FXR silencing abrogated the regulation of GSK3β, PEPCK (C) and PPARα, LPL, G6Pase (D) by yangonin in mice primary hepatocytes. Data are expressed as mean ± S.D. (n = 5). *$p < 0.05$ versus

DMSO alone; #p < 0.05 versus Yan alone.



Graphical abstract