Research Article

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Hepatic steatosis exacerbated by endoplasmic reticulum stress-mediated downregulation of FXR in aging mice

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Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is characterized by an increase in hepatic triglyceride (TG) contents. The prevalence of NAFLD is increased with aging. However, the molecular mechanism for aging-induced fatty liver remains poorly understood.

Methods: Hepatic TG contents and gene expression profiles were analyzed in body weight-matched young (2 months), middle (8 months) and old (18 months) C57BL/6 mice. Endoplasmic reticulum (ER) stress and farnesoid X receptor (FXR) expression were examined. The mechanism of ER stress activation in the regulation of FXR expression was further investigated.

Results: In the present study, we found that TG was markedly accumulated and lipogenic genes were up-regulated in the liver of C57BL/6 mice aged 18 months. FXR, a key regulator of hepatic lipid metabolism was down-regulated in these old mice. At molecular levels, ER stress was activated in old mice and repressed FXR expression through inhibition of hepatocyte nuclear factor 1 alpha (HNF1α) transcriptional activity.

Conclusions: Our findings demonstrate that FXR down-regulation plays a critical role in aging-induced fatty liver.

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by aberrant triglyceride accumulation in the liver has become one of the most common liver diseases all over the world and affects up to one-third of adults in developed countries [1]. Growing evidence suggests that NAFLD prevalence is markedly increased in aging humans [2,3]. The prevalence of NAFLD in population aged above 60 years was twice more than those between 20 and 40 years [4]. One study compared the age in subjects with or without NAFLD and showed that the average age was higher in individuals with hepatic steatosis than in those without [5]. However, the molecular mechanism underlying the initiation and progression of NAFLD in elderly people still remains unknown.

The imbalance between triglyceride synthesis and clearance results in triglyceride accumulation in the liver. Hepatic lipogenesis is mainly regulated by SREBP-1c, which increases the expression of genes involved in *de novo* lipogenesis such as *Fasn*, *SCD-1*, and *Acly* [6]. Increased expression of *SREBP-1c* has been implicated in many cases of hepatic steatosis [7,8]. Moreover, *SREBP-1c* expression was tightly regulated by many nuclear receptors, including Liver X receptor (LXR) and farnesoid X receptor (FXR), which regulate SREBP-1c in an opposite direction, respectively [9,10].

Initially, FXR was considered a central regulator of bile acid homeostasis [11–13]. Subsequent studies demonstrated that FXR also played a crucial role in the regulation of hepatic

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Abbreviations: NAFLD, Non-alcoholic fatty liver disease; TG, triglyceride; ER, endoplasmic reticulum; THA, thapsigargin; Tun, tunicamycin; TUDCA, tauroursodeoxycholate; MPH, mouse primary hepatocyte; FXR, farnesoid X receptor; HNF1α, hepatocyte nuclear factor 1 alpha; SREBP-1c, sterol regulatory element binding transcription factor 1c; FASN, fatty acid synthase; SCD-1, stearoyl-Coenzyme A desaturase 1; Acly, Acetyl-CoA ylase; LXR, liver X receptor; PPARα, peroxisome proliferative activated receptor; CPT-Iα, carnitine palmitoyltransferase 1α; MCAD, medium-chain specific acyl-CoA dehydrogenase; ACOX1, acyl-coenzyme A oxidase 1; LRH-1, liver receptor homolog-1; SHP, small heterodimer partner; BSEP, bile salt export pump; PERK, pancreatic eIF-2alpha kinase; Grp78, 78 kDa glucose-regulated protein; ATF6, activating transcription factor 6; JNK, c-Jun amino-terminal kinase; IRE1, inositol-requiring enzyme-1; XBP1, X-box-binding protein 1.

triglyceride metabolism [9]. Mice with *FXR* deficiency exhibited hepatic steatosis and hyperlipidemia, whereas overexpression or activation of FXR could efficiently improve hepatic triglyceride accumulation and hyperlipidemia in obese rodents [14–16]. Consistently, our previous study revealed that *FXR* heterozygous mice also displayed fatty liver under a short-term high-fat-diet feeding [17]. In addition, hepatic FXR expression was markedly reduced in obese rodents and NAFLD patients, suggesting that FXR dysfunction might play a causal role in the development of hepatosteatosis in obesity [17,18].

In the present study, we speculate that FXR dysfunction might be involved in the development of hepatosteatosis in aging mice. Our study provides a novel insight for the mechanism of aging-induced fatty liver and proposed a promising therapeutic target for the related diseases.

Materials and methods

Animal treatment

C57BL/6 mice were purchased from the Shanghai Laboratory Animal Company (SLAC, Shanghai, China). *JNK1* knockout mice were obtained from Jackson Laboratories Bar (Harbor, Maine, USA). All mice were maintained in a temperature and light-controlled environment. Mice were housed in ventilated microisolator cages under a 12-h light and dark cycle with free access to food and water, and fasted overnight before they were sacrificed. GW4064, thapsigargin, tunicamycin, tauroursodeoxycholate (TUDCA) and 4-phenyl butyric acid (PBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mice were given tunicamycin (1 mg/kg) via intraperitoneal injection and sacrificed at the indicated time. Old mice were treated with TUDCA (250 mg/kg) or PBA (1 g/kg) via intraperitoneal injection or gavage for 7 or 10 days, respectively. All the animals received human care and experimental procedures were approved by the Animal Care Committee of School of Medicine, Shanghai Jiao Tong University.

Hepatic lipid measurement

Liver tissues were homogenized in chloroform/methanol (2:1 v/v) using a Polytron tissue grinder (Kinematica AG, Luzern, Switzerland). Lipid extracts were prepared by the classical Folch method. Extracts were dried under N_2 flow and dissolved in isopropanol. Triglyceride and cholesterol contents were measured using commercial kits (Biovision, USA) according to the manufacturer's instructions.

Primary hepatocyte isolation

Mouse primary hepatocytes were isolated from C57BL/6 mice aged 8–12 weeks by collagenase perfusion and purified by centrifugation. Fresh prepared hepatocytes were seeded at a final density of 0.5×10^6 cells per well in 6-well plates in attachment media (Science Cell, USA). The media were replaced with DMEM (Gibco, USA) within 24 h.

RNA isolation and real-time PCR

Total RNA was extracted from liver tissues or cell lysates using TRIzol according to the manufacturer's instructions (Invitrogen, USA). 2 μg of total RNA was reversely transcribed into cDNA using oligo-dT primers (Promega, USA). Real-time PCR analysis was carried out using SYBR Green Premix Ex Taq (Takara, Otsu, Japan) on Light Cycler 480 (Roche, Basel, Switzerland) Switzerland). Relative mRNA levels were calculated using the comparative ΔCt values. The *36B4* gene was used as an internal reference for normalization.

Cell culture and luciferase assay

HepG2 cell lines were purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences (CAS, Shanghai) and maintained in DMEM medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). For luciferase assays, HepG2 cells were seeded into 24-well plates and transfected with the indicated plasmids in duplicate wells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. pRL-TK expressing renilla luciferase (Promega, USA) was used to normalize the luciferase activity. Cells were cultured 36 h and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega, USA).

Western blot

Hepatic tissues and cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM NaF, 1% NP40 and 0.1% SDS. 50 μg lysates were loaded onto 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The proteins were visualized with enhanced chemiluminescence (ECL) reagents (GE LifeScience, little Chalfont Buckinghamshire, UK) according to the manufacturer's protocol.

Co-immunoprecipitation

HEK293T cells were transfected with indicating constructs and were then harvested 48 h later. Cells were resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TRITON X-100) containing protease and phosphatase inhibitors. The lysates were immunoprecipitated with anti-HA (Santa Cruz biotechnology, USA) or anti-myc (Santa Cruz biotechnology, USA) antibodies overnight and then incubated with protein A/G plus beads (Santa Cruz biotechnology, USA).

Chromatin immunoprecipitation

A chromatin immunoprecipitation (ChIP) assay kit was used (Millipore, USA). In brief, lysates from mouse primary hepatocytes or homogenized mouse liver tissues (nuclear lysates) were fixed with formaldehyde (Sigma, USA). DNA was sheared to fragments of 200–1000 bp by sonication. The chromatin was incubated and precipitated with antibodies against HNF1α (Santa Cruz Biotechnology, USA), or IgG (Santa Cruz Biotechnology, USA). The mouse *FXR* gene promoter fragment containing the HNF1α motif (−0.3 ∼ −0.2 kb) was amplified using the following primers: 5′-TGGATTTATTAAATTTATTT-3′ (forward) and 5′-GGTTAATCAGTAAACCACA-3′ (reverse). The distal region of the *FXR* gene promoter (−1.3 ∼ −1.1 kb) was amplified using the following primers: 5′-GATCTGTCACATGGGTG CTG-3′ (forward) and 5′-GGAAATTGGAGCCAGAGA-GA-3′ (reverse). Primers for the *Aldolase* promoter (−0.26 ∼ −0.11 kb) are: 5′-CTATGAGTGTAAGGAGTGTG-3′ (forward) and 5′-CTCTTATCAAGGTCTGGTG-3′ (reverse). Primers for the *GAPDH* promoter (−0.33 ∼ −0.23 kb) are: 5′-CTATCCTG GGAACCAT CA-3′ (forward) and 5′-AAGCGTGTGGGCTCCG AA-3′ (reverse).

Histological analysis

Liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were subjected to standard hematoxylin and eosin staining. For oil red O staining, liver tissues were fixed in 4% paraformaldehyde in PBS, embedded in optimum cutting temperature compound (OCT), and cryosectioned. Frozen liver sections were stained with 0.15% oil red O according to standard procedures.

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Statistical differences were calculated by using Student's *t* test. Statistical significance was shown as $^{*}p$ <0.05, $^{**}p$ <0.01 or $^{***}p$ <0.001.

Results

Increased hepatic triglyceride content in aging mice

To determine the hepatic triglyceride (TG) accumulation in aging mice, we measured TG contents in C57BL/6 male mice aged 2 months (young), 8 months (middle) and 18 months (old). We and others have shown that expression of FXR was reduced in obese animals and humans [17–19]. Therefore, to exclude the