Research Article

JOURNAL OF HEPATOLOGY

Hepatic estrogen receptor α improves hepatosteatosis through upregulation of small heterodimer partner

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Background & Aims: Estrogen participates in the control of energy homeostasis and lipid metabolism. However the role of hepatic estrogen receptor α (ERα) in triglyceride (TG) homeostasis remains poorly understood. This study aims to investigate the roles of estrogen and ERα in the regulation of hepatic TG metabolism.

Methods: Liver TG metabolism was analyzed in female mice with ovariectomy or tamoxifen treatment, and in hepatic ERα knockdown or overexpression. Phenotypes and expression of genes were compared in male and female mice with farnesoid X receptor deficiency. The mechanism of ERα in the regulation of small heterodimer partner (SHP) expression was further investigated.

Results: Female mice receiving ovariectomy or tamoxifen treatment exhibited hepatic TG accumulation. Ablation of ERα using adenoviral shRNA markedly increased hepatic TG accumulation, while overexpression of ERα ameliorated hepatosteatosis in obese mice. At the molecular level, estrogen upregulated hepatic SHP expression through binding to its proximal promoter. In addition, the roles of estrogen were largely blunted in mice with SHP deficiency.

Conclusion: These findings reveal a novel role of estrogen in improving hepatosteatosis through upregulation of SHP expression.

Keywords: Hepatosteatosis; Estrogen; ERα; FXR; SHP.

Received 27 June 2014; received in revised form 13 February 2015; accepted 15 February 2015; available online 24 February 2015

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Abbreviations: NAFLD, Non-alcoholic fatty liver disease; ERα, estrogen receptor α; FXR, farnesoid X receptor; SHP, small heterodimer partner; TG, Triglyceride; LXR, liver X receptor; ERβ, estrogen receptor β; HFD, High-Fat Diet; SREBP-1c, sterol regulatory element binding transcription factor 1c; ChREBP, carbohydrate response element binding protein; SCD-1, stearoyl-Coenzyme A desaturase 1; FASN, fatty acid synthase; AceCS, Acetyl-coenzyme A synthetase; MCAD, medium-chain specific acyl-CoA dehydrogenase; ACOX1, acyl-coenzyme A oxidase 1; Cyp7A1, cholesterol 7alpha-hydroxylase; Cyp8B1, Sterol 12alpha-hydroxylase; MPH, mouse primary hepatocytes; ChIP, chromatin immunoprecipitation; SRC-1, Steroid Receptor Co-activator 1; ERE, Estrogen-Responsive Element; LBD, ligand binding domain; DBD, DNA binding domain; HNF4, hepatocyte nuclear factor-4α; LRH-1, liver receptor homologue 1; PPARγ, peroxisome proliferative activated receptor γ; VLDL, very-low-density lipoprotein.

Introduction

Non-alcoholic fatty liver disease (NAFLD), a burgeoning health problem worldwide, is characterized by triglyceride (TG) accumulation in hepatocytes and can progress to steatohepatitis, cryptogenic liver cirrhosis and even hepatocellular carcinoma [1]. Several population-based studies have demonstrated the gender difference in the prevalence of NAFLD which is generally higher in men than in women [2], although, hepatosteatosis is more prevalent in women after menopause [3,4]. Thus, these findings suggest that estrogen deficiency may be associated with the development of NAFLD.

Estrogen plays an important role in the systemic glucose, lipid, and energy homeostasis by reducing food intake and lipogenesis, while increasing insulin secretion, and insulin sensitivity [5–7]. Indeed, estrogen deficiency is associated with the increase of central obesity and type 2 diabetes in postmenopausal women, which may be reversed by estrogen replacement therapy [8,9]. However, the roles of estrogen in the regulation of hepatic TG homeostasis and the development of NAFLD remain poorly understood.

Farnesoid X receptor (FXR), a member of the nuclear receptor superfamily, also plays a critical role in the regulation of hepatic TG homeostasis [10]. At the molecular level, FXR inhibits hepatic lipogenesis through upregulation of small heterodimer partner (SHP), which suppresses SREBP-1c expression by interacting with liver X receptor (LXR) [11]. In agreement, we and others have shown that ablation of FXR in male mice resulted in hepatosteatosis and insulin resistance [12,13]. In contrast, expression

of FXR was reduced in rodents and humans with liver steatosis [13,14]. Therefore, pharmaceutical compounds that target FXR/SHP signaling have been proposed as promising therapeutic approaches against NAFLD and related metabolic disorders [15].

In the present study, we investigated the association of estrogen deficiency with hepatosteatosis and the molecular link between estrogen receptor and FXR/SHP.

Materials and methods

Human subjects

A total of 1043 adult participants were enrolled from the Lian Qian Community, Xiamen China from April to August 2011, as described previously [16]. Subjects received type B ultrasonography to determine fatty liver. Subjects with an alcohol consumption $\geqslant$140 g/week for men or 70 g/week for women in the past 6 months were excluded from the study. Menopause is defined as the absence of menses for one or more years. The study was approved by the Human Research Ethical Committee of the Xiamen First Hospital, Xiamen University. Written informed consent was obtained from each subject.

Animal studies

Male or female C57BL/6 and *ob/ob* mice at age of 8–12 weeks were purchased from Shanghai Laboratory Animal Company (SLAC, Shanghai, China). FXR null mice were obtained from the Jackson Laboratories Bar (Harbor, Maine, USA). SHP null mice were kindly provided by Dr. David D. Moore (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA). Obese male mice were maintained with free access to a high-fat diet (D12492, Research Diets, New Brunswick, New Jersey, USA), containing 60 kCal % fat, 20 kCal % carbohydrate and 20 kCal % protein. The normal diet (ND) contains 10 kCal % fat, 70 kCal % carbohydrate and 20 kCal % protein. In separate experiments, mice received daily intraperitoneal injections of estrogen (E2; 50 μg/kg, Sigma-aldrich, St. Louis, MO, USA), tamoxifen (TMX; 1 mg/kg, Sigma-Aldrich) or vehicle controls. For adenoviral injections, 4×10^9 plaque-forming units (pfu) for each recombinant adenovirus were administrated via tail vein injection. All animal experiments were conducted in accordance with the guideline of the Animal Care Committee of Shanghai Jiao Tong University School of Medicine.

Biochemical measurements

For insulin tolerance tests, mice were injected with regular human insulin (Eli Lily, Indianapolis, Indiana, USA) at a dose of 0.75 U/kg body weight after a 6 h fast. Blood glucose levels were measured using a portable blood glucose meter (Lifescan, Johnson & Johnson, USA). Serum or hepatic TGs were extracted and quantified using commercial kits (Biovision, Milpitas, California, USA), according to the manufacturer's instructions.

Cell culture

HepG2 and L02 cells were cultured in DMEM (Gibco, Shanghai, China) containing 10% fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). Mouse primary hepatocytes (MPH) were isolated from adult mice and maintained in hepatocyte medium (Sciencell, USA). To test the effect of estrogen in inducing SHP expression, MPH were pre-incubated with DMEM medium in the absence of FBS and phenol red for 24 h, followed by incubation with 100 nM E2 for another 24 h. To induce the hepatic TG accumulation *in vitro*, MPH were exposed to 0.5 mM palmitic acid (PA), oleic acid (OA) or bovine serum albumin (BSA) as a control. Cellular TG contents were extracted and determined using commercial kits (Biovision).

Transfection and luciferase assays

All the transient transfections were conducted using Lipofectamine 2000 (Invitrogen, Shanghai, China). The *SHP* promoter (mouse: from −1000 bp to +10 bp, human: from −1200 bp to +12 bp) was amplified from the mouse or human genomic DNA templates and inserted into pGL4.15 empty vector (Promega). For the luciferase assays, cells were transfected with the *SHP* promoter reporter together with ERα expression plasmids or empty vectors. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Real-time PCR and Western blotting

Total RNA was isolated from hepatic tissues or cell lysates using the TRIzol method according to the manufacturer's instructions (Invitrogen, Shanghai). In order to quantify the transcripts of the genes of interest, quantitative real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland). For Western blotting experiments, liver homogenates were prepared with radioimmunoprecipitation (RIPA) lysis buffer containing protease and phosphatase inhibitors (Millipore, USA), and then immunoblotted with antibodies as indicated. Antibodies targeting AKT (4060) and GAPDH (5174) were purchased from Cell Signaling Company (Danvers, Massachusetts, USA). Anti-ERα antibodies recognizing human and mouse ERα were obtained from Santa Cruz Company (sc-8005, sc-787) (California, USA).

Chromatin immunoprecipitation assays

A chromatin immunoprecipitation (ChIP) assay kit was employed (Upstate, Billerica, Massachusetts, USA). In short, MPH or nuclear lysates from homogenized mouse liver tissues were fixed with formaldehyde. Chromatin was incubated and precipitated with antibodies against ERα, SRC1 (Santa Cruz), Ac-H3 (Millipore), or control IgG (Santa Cruz). DNA fragments were subjected to real-time PCR using primers flanking ERα binding site in the *SHP* promoter. The primer sequences are listed below: Forward (5′-CATGGAAATGGGCATCAATA-3′), Reverse (5′-CGTGGCCTTG CTATCACTTT-3′).

Statistical analysis

Values were shown as mean ± SEM. Significant differences were determined by 2-tailed Student's *t* test or by 2-way ANOVA with Bonferroni-adjusted post test. Statistical significances were displayed as $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

Results

NAFLD in women before or after menopause

Firstly, we investigated the prevalence of NAFLD in obese women before and after menopause (Table 1). As expected, the prevalence of NAFLD was significantly higher in postmenopausal women (60.2%) than that in premenopausal women (42.9%) (p <0.001), suggesting that estrogen deficiency is associated with NAFLD in women.

Estrogen deficiency leads to hepatic TG accumulation in female mice

To determine the role of estrogen deficiency in hepatic TG homeostasis, we performed ovariectomy (OVX) or sham operation in female C57BL/6 mice to remove endogenous estrogen. Hepatic TG contents were markedly increased in ovariectomized mice, and serum TG levels were increased as well, compared with the mice with sham operation (Supplementary Fig. 1A and B).

Next, female mice were treated with TMX, an anti-estrogen drug widely used in the treatment of hormone-sensitive breast cancer [17]. As a result, TMX treatment significantly increased hepatic and serum TG levels, compared with vehicle controls (Supplementary Fig. 1C and D).

To further explore whether estrogen can improve hepatosteatosis, male high-fat diet-induced obese mice were treated with estrogen (E2) or vehicle control for 4 weeks. Body weight and food intake remained unaffected in these mice (Supplementary Fig. 1E and F). However, estrogen treatment