Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

The role of Hepassocin in the development of non-alcoholic fatty liver disease

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Background & Aims: While non-alcoholic fatty liver disease (NAFLD) is the most common risk factor of chronic liver disease, the mechanisms that initiate its development are obscure. Hepassocin (HPS) is a hepatokine that has been reported to be involved in liver regeneration. In addition to the mitogenic activity of HPS, HPS expression is decreased in patients with hepatoma. However, the role of HPS in NAFLD is still unknown.

Methods: A total of 393 subjects with (n = 194) or without (n = 199) NAFLD were enrolled to evaluate the serum HPS concentration. In order to clarify the causal inference between HPS and NAFLD, we used experimental animal and cell models. Hepatic overexpression or silencing of HPS was achieved by lentiviral vector delivery in mice and lipofectamine transfection in HepG2 cells. Lipogenesis related proteins were detected by Western blots. The expression of inflammatory factors was determined by real-time polymerase chain reaction.

Results: Subjects with NAFLD had a higher serum HPS concentration than those without it. Overexpression of HPS increased hepatic lipid accumulation and NAFLD activity scores (NAS), whereas deletion of HPS improved high fat diet-induced hepatic steatosis and decreased NAS in mice. Additionally, oleic acid, a steatogenic reagent, increased HPS expression in hepatocytes. Furthermore, overexpression of HPS in HepG2 cells induced lipid accumulation through an extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent pathway, whereas deletion of HPS decreased oleic acid-induced lipid accumulation.

Conclusions: The present study provides evidence that HPS plays an important role in NAFLD and induces hepatic lipid accumulation through an ERK1/2-dependent pathway.

Keywords: Fatty acids; Hepatic steatosis; Liver; Oleic acid; STAT3.

Received 22 February 2013; received in revised form 23 May 2013; accepted 10 June 2013; available online 18 June 2013

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Abbreviations: ACC-1, acetyl-CoA carboxylase-1; ERK1/2, extracellular signal-regulated kinase 1/2; FAS, fatty acid synthase; FFAs, free fatty acids; HPS, hepassocin; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; HNF-1, hepatocyte nuclear factor-1; HFD, high fat diet; OA, oleic acid; STAT3, signal transducer and activator of transcription 3; SREBP-1, sterol regulatory element-binding protein-1.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common risk factor of chronic liver disease, and has emerged as a growing public health problem worldwide [1–3]. It encompasses a spectrum of disorders ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) [4]. NAFLD is considered the hepatic manifestation of insulin resistance (IR) [5], and is also associated with obesity, dyslipidemia, and diabetes [6].

Hepatic steatosis is the hallmark of NAFLD, and the histological criterion for the diagnosis of NAFLD is characterized by the presence of fat in more than 5% of the hepatocytes. *De novo* lipogenesis is responsible for more than 30% of hepatic fat [7,8], and dietary lipid contributes about 10% of the hepatic lipid content [7]. The development of hepatic steatosis has the potential to progress through the more aggressive form of hepatic injury [9]. Although effective treatments are important in controlling hepatic steatosis, there are as yet no reliable forms of early diagnosis and prevention. Moreover, the basic mechanisms that trigger lipid accumulation in the liver to initiate the development of NAFLD are still obscure.

Hepassocin (HPS) is a hepatokine [10], and its expression is increased during liver regeneration [11]. HPS improves survival in rats with hepatic failure [12] and also plays an important role in the proliferation of hepatocytes through an autocrine mechanism. Silencing of HPS by RNA interference results in cell growth inhibition, while the extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor blocks HPS-induced proliferation in hepatocytes [13]. On the other hand, HPS expression is decreased in patients with HCC [14]. Although the functions of HPS in liver

failure and HCC are established, the role it plays in NAFLD is still unknown.

The current study thus aimed at clarifying the role of HPS in NAFLD. We first investigated the relationship between HPS and NAFLD in patients. Second, the role of HPS in NAFLD was determined in animals with overexpression or deletion of HPS, and the role of HPS in the regulation of hepatic lipid accumulation was also evaluated.

Materials and methods

Study subjects

The study protocol was approved by the Human Experiment and Ethics Committee of National Cheng Kung University (NCKU) Medical Center, and all eligible subjects gave written informed consent before participation. From June 2007 to July 2008, subjects who were admitted for a physical check-up to the Preventive Health Center of NCKU Hospital were screened. To avoid the confounding effects of age and sex, we selected subjects by the following algorithm: the study subjects were classified into two groups, NAFLD (−) and NAFLD (+), in the order of their admission to the study. A total of 393 subjects were enrolled (199 subjects for the NAFLD (−) group and 194 for the NAFLD (+) group) in the study cohort. Each NAFLD subject was matched to a subject of the same gender in the other group who had the closest age (within ±1 year). Subjects enrolled in this study were not taking any medication, and none of the women were pregnant when tested. Subjects with the following conditions were excluded: (1) alcohol consumption $\geqslant$20 g/d in the last year; (2) serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels more than two-fold of the normal limit; (3) a positive test for hepatitis B surface antigen, hepatitis C antibody; (4) serum creatinine >1.5 mg/L; (5) any inflammatory diseases as determined by a leukocyte count >10,000/mm^3; (6) any other major diseases, including advanced malignant diseases contraindicating this study. Diabetes was defined according to ADA criteria and hypertension was defined according to the JNC VII criteria. The smoking habit, alcohol drinking, and habitual exercise were defined as in our previous study [15].

Blood samples

After an overnight 12-h fast, all subjects received a biochemistry blood tests. Blood glucose was measured by a hexokinase method (Roche Diagnostic GmbH, Mannheim, Germany). Serum insulin (intra-assay CV of 4%, inter-assay CV of 2.6%; Mercodia AB, Uppsala, Sweden), HPS (intra-assay CV <10%, inter-assay CV <12%; Uscn Life Science Inc, Wuhan, China) and hs-CRP (intra-assay CV of 2.9%, inter-assay CV of 4.7%; Immunology Consultants Laboratory, Newberg, OR, USA) were measured by ELISA. IR was defined by the homeostasis model assessment-IR index [16]. Serum lipid profiles were determined using an autoanalyzer (Hitachi 747E; Hitachi, Tokyo, Japan). LDL cholesterol was calculated using the Friedewald formula.

Analytical determinations

Each subject was examined by abdominal ultrasound by an experienced radiologist with high-resolution ultrasonography (Xario SSA-660A; Toshiba, Nasu, Japan) using a 3.5-MHz linear transducer. The NAFLD diagnostic criteria included the characteristic echo patterns of hepatorenal echo contrast, bright liver, deep (posterior beam) attenuation and vascular blurring.

Animals

Eight-week-old C57BL/6 male mice from the animal center of NCKU Medical College were housed in a temperature (25 ± 1 °C) and humidity (60 ± 5%) controlled room, and kept on a 12:12 light-dark cycle (light on at 06:00). The animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of Animal Welfare Act. NAFLD was induced in mice by feeding with HFD containing 34.9% fat (wt/wt) for twelve weeks (TestDiet, Richmond, IN, USA). Hepatic triglyceride content was measured using a kit (BioVision, Milpitas, CA, USA). Serum ALT and AST levels were determined using a kit purchased from Teco Diagnostics (Anaheim, CA, USA).

Cell culture and small interfering ribonucleic acid transfection

Primary hepatocytes were prepared according to a previous study [17]. The HepG2 cell line was purchased from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan) and maintained in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone). Cells were transfected with duplexed RNA oligonucleotides of human HPS (Invitrogen, Carlsbad, CA, USA) or scramble siRNA (as negative control) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The experiments were performed at 48 h post-transfection. Lipid accumulation was induced in HepG2 cells by oleic acid (OA), following the procedure described in a previous study [18].

Western blotting analysis

Western blotting analysis was performed following the procedure reported in a previous study [18]. Primary antibodies, such as fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC-1) (Burlingame, CA, USA), sterol regulatory element-binding protein-1 (SREBP-1) (Littleton, CO, USA), phospho-Akt and Akt, phospho-ERK1/2 and ERK1/2 (Cell Signaling Technology, Beverly, MA, USA) were used in this study.

Flow cytometry

HepG2 cells were fixed in 4% paraformaldehyde and intracellular neutral lipids were labeled with BODIPY505/515 (Invitrogen) [18]. Flow cytometry was performed using FACScan (BD Biosciences, San Diego, CA, USA) and analyzed using WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA, USA).

Lentiviral-based HPS transfection and delivery

The mouse HPS clone was purchased from Origene technologies (Rockville, MD, USA). Both HPS and GFP lenti-viral constructs (for a negative control) were used to generate recombinant lentiviral particles. The lentiviral particles were transduced in HEK-293T cells for 24 h, and then given fresh medium. The cell supernatants were then harvested and filtered with a 0.45 μm low-protein binding filter. The virus pellets were further concentrated by centrifugation at 20,000*g* at 4 °C for 2.5 h, and then resuspended with fresh medium. The short hairpin RNAs that targeted HPS (sh*HPS*) to knock down HPS was purchased from Santa Cruz Biotechnology. To deliver the viral particles, each group of mice was well-anesthetized and then injected with the constructs (8.0×10^7 TU/100 μl/mouse) via the portal vein. Daily food intake and the body weight of mice were recorded, and mice were sacrificed at day 30 after the injection, with tissue samples removed for further experiments.

Histological analysis

The tissue samples were fixed in 10% formaldehyde at 4 °C, and the fixed specimens were then dehydrated and embedded in paraffin. The specimens were cut into 5-μm thick sections at 50-μm intervals, and stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan). The sections were then observed with a microscope (100×) and analyzed on four random fields/slide. The NAFLD activity score (NAS) was determined as previously described [19].

Real-time quantitative PCR

Total RNA was extracted from the liver with TRIzol reagent (Roche Molecular Systems, Alameda, CA, USA) and cDNA synthesis was performed using Transcriptor First Stand cDNA Synthesis Kit (Roche) using 2 μg total RNA. The real-time PCR was monitored online using a LightCycler 2.0 thermocycler (Roche) according to the manufacturer's instructions. The primer sequences are available upon request.