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Deficiency of CKIP-1 aggravates high-fat diet-induced fatty liver in mice

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ABSTRACT

Casein kinase 2 interacting protein-1(CKIP-1) is widely expressed in a variety of tissues and cells, and plays an important role in various critical cellular and physiological processes including cell growth, apoptosis, differentiation, cytoskeleton and bone formation. Here, we found: (1) CKIP-1 deficient mice exhibited increased body weight, liver weight, number and size of lipid droplets, and TG content comparing with WT mice after being exposed to high fat diet (HFD); (2) the levels of serum insulin, liver glycogen, phosphorylated C-Jun-N-terminal kinase-1 (pJNK1) and phosphorylated insulin receptor substrate −1(pIRS1) in CKIP-1$^{-/-}$ mice were higher than those of WT mice; (3) CKIP-1 interacted with JNK1 *in vitro*. Our results indicate that CKIP-1 deficiency in mice aggravates HFD-induced fatty liver by upregulating JNK1 phosphorylation and further upregulating IRS-1 phosphorylation and RI.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), with a 25% global prevalence, is the most common chronic liver disease in the world [1–3]. With an increase of the population with obesity and diabetes, the incidence of NAFLD will increase further in the future, with an expected 50% prevalence in the US by 2030 [4,5]. A growing body of evidence suggests that NAFLD is a potential independent risk factor for cardiovascular disease, which is the leading cause of mortality among NAFLD patients [6,7]. In addition, some forms of NAFLD (especially non-alcoholic steatohepatitis) can progress to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [8], and NALFD is predicted to be the leading cause of end-stage liver disease requiring liver transplantation by 2020 [9]. Despite the emergence of NAFLD and the health burden caused by this epidemic, no pharmacological therapy has been developed to treat NAFLD specifically partially because of our incomplete understanding of its pathogenesis. We therefore aimed to study NAFLD at the mechanistic level to provide a future framework for developing a potential effective treatment.

Insulin resistance (IR) plays a key role in the pathogenesis of NAFLD by enhancing the uptake and synthesis of fatty acids and inhibiting the β-oxidation of fatty acids in hepatocytes [10,11]. IR is mediated by tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), the insulin receptor adapter protein, and C-Jun-N-terminal kinase-1 (JNK1), a member of the mitogen-activated protein kinase family. It is known that JNK1 deficiency protects mice against HFD-induced IR. JNK1 induces the phosphorylation of IRS-1 on Ser-307, which disrupts the interaction of the phosphotyrosine binding domain of IRS-1 with the tyrosine phosphorylated insulin receptor [12]. The PH-domain-containing casein kinase 2 interacting protein-1 (CKIP-1) was originally identified as an interacting partner of the α-subunit of casein kinase 2 [13]. Recently, we showed that the targeted deletion of CKIP-1 causes the upregulation of phosphorylated JNK1 without changing the total JNK1 in mouse embryonic fibroblasts [14]. We therefore speculated that CKIP-1 might play a role in the development of NAFLD *via* regulating JNK1 phosphorylation, IRS-1 phosphorylation, and IR. In this study, we demonstrated that CKIP-1 deficiency exacerbates fatty liver induction in mice fed with high-fat diet (HFD). In addition, the levels of serum insulin, liver glycogen, phosphorylated JNK1 (pJNK1), and phosphorylated IRS1 (pIRS1) also increased in CKIP-1 deficient mice. Finally, we found there was an interaction between CKIP-1 and JNK1 in hepatocytes.

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2. Material and methods

2.1. Animals

CKIP-1 knockout (CKIP-$1^{-/-}$) mice in C57BL/6 background were prepared as previously described [14], and CKIP-$1^{+/+}$(wild-type, WT) C57BL/6 mice were used as control. The mice were housed under pathogen-free conditions in a temperature-controlled animal facility with a 12 h light/dark illumination cycle. All procedures for handling animals were in accordance with protocols approved by Chinese Academy of Military Medicine Science. CKIP-$1^{-/-}$ mice and the WT control mice were fed with an HFD (D12492, 60% of kilocalories from fat; Research Diet) for 8 weeks. Their body weight was measured at the beginning and the end of the experiment. Mice were then sacrificed, their liver tissues were weighed, and blood serum samples were collected. The liver tissues were frozen in liquid nitrogen and maintained at −80 °C until further analyses. A portion of each liver was fixed in 10% buffered formalin for histopathology. The collected blood samples were kept on ice for 30 min and then separated into serum by centrifugation at 4◦C. The serum was then kept at −20 °C until the examination.

2.2. Analysis of blood insulin

Serum insulin was measured using Ultra Sensitive Mouse Insulin ELISA Kit (Shanghai Elisa Biotech Co., Ltd., China) according to manufacturer's instructions.

2.3. Liver histopathology test

After embedded in paraffin, the live tissues were cut into 5-micrometer thin sections and processed for hematoxylin and eosin staining to evaluate the extent of lipid accumulation in hepatocytes.

2.4. Analysis of Liver glycogen content

400 μl of 5% trichloroacetic acid was added into 150 mg of liver tissue. After a centrifugation at 4000 rpm for 3 min, the supernatant was mixed with 400 μl of 95% ethanol. The precipitate was then removed by centrifugation at 4000 rpm for 5 min. The amount of glycogen in the digested samples was measured using a Glycogen Assay Kit (Sigma), following the manufacturer's instructions.

2.5. Analysis of liver triglyceride (TG) content

50 mg frozen liver tissue was homogenized in 1 ml of tissue lysis solution (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton). After centrifugation at 2000 rpm for 5 min at 37◦C, the supernatants were used to determinate the liver TG levels using an EnzyChrom™ TG assay kit (Kampenhout, Belgium) and normalized by the protein concentration, according to the protocol provided by the manufacturer. Data were expressed as milligrams of TG per gram of liver.

2.6. Western blot analyses

Liver lysates were prepared by homogenization of liver samples with RIPA buffer containing 1% phosphatase inhibitor. Protein concentrations were measured using the BCA assay. Protein from each sample was separated by 10% SDS-PAGE and electro-transferred to nitrocellulose filter membranes. The membranes were blocked with 5% skim milk, incubated at 4 °C overnight using the indicated primary antibodies, followed by detection with the related secondary antibody and the Super Signal chemiluminescence kit (Thermo Fisher). Primary antibodies used in this study are anti-CKIP-1 (Santa Cruz, anti-JNK1, anti-pJNK1 (Abcam), anti-pIRS1 (Ser^{307}) (Abcam) and anti-GAPDH (MBL). GAPDH was used as an internal control. The relative band intensity in the scanned images was analyzed with Scion Image software.

2.7. Analysis of pJNK1 in isolated hepatocyte

Hepatocytes were isolated by collagenase digestion method. Briefly, 1 weeks old CKIP-$1^{-/-}$ and WT mice were killed to obtain the liver tissues. The liver sample was washed 3 times with PBS, then minced and digested by 0.02% IV collagenase for 20 min at 37 °C. The suspension was centrifuged at 1000 rpm for 10 min. The obtained hepatocytes were collected and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were treated with anisomycin (10 ng/ml) for 48 h to induce JNK1 phosphorylation. Then, the cells were collected for testing pJNK1 protein by western blot analyses as above.

2.8. Co-immunoprecipitation (Co-IP)

The human embryonic kidney (HEK293T) cells were transfected with CKIP-1 plasmid. The CKIP-1 plasmids were constructed as described previously [14]. After 48 h, cells were harvested and lysed in HEPES (N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid) lysis buffer (20 mM HEPES, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF and 1 mM dithiothreitol). Lysates were incubated with either anti-CKIP-1 or anti-JNK1 antibody overnight at 4 °C in the presence of 50 μl Protein-G/A beads. Beads were collected, washed, and resuspended in equal volumes of 5×SDS loading buffer. The immunoprecipitated proteins were separated by SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% skim milk, incubated at 4 °C overnight with indicated antibodies, and followed by detection with the related secondary antibody and the Super Signal chemiluminescence kit (ThermoFisher).

2.9. Confocal microscopy, immuno-fluorescence analysis

For immuno-staining of endogenous JNK-1, HEK293T cells transfected with GFP-CKIP-1 were fixed in 4% PFA (paraformaldehyde) for 10 min. Images of endogenous JNK-1 were then fixed in 0.1% PBST (containing 0.5% Triton X-100) for 15 min. Further processing included incubating cells in 5% BSA for 30 min before incubations with primary for 3 h at 37 °C and with secondary antibody for 1 h at room temperature. Cells were analyzed in PBS when the nucleus was stained with 0.1 g/ml DAPI. Images of fixed cells were acquired on a confocal microscope using LaserSharp software.

2.10. Statistical Analysis

The results were expressed as the mean ± SD. The significance of differences was determined by *t*-test using the SPSS 17.0 software (SPSS, Chicago, IL, USA). Difference with a p value of less than 0.05 was considered statistically significant.

3. Results

3.1. CKIP-1 deficiency increases body weight and liver weight in mice fed with HFD

At the end of experiment, the body weight of CKIP-$1^{-/-}$ mice significantly increased compared to those of WT mice ($p < 0.05$) (Table 1), showing that the deficiency of the CKIP-1 gene enhances the degree of obesity in mice fed with HFD. The liver weight of CKIP-$1^{-/-}$ mice also significantly increased compared to those of WT mice ($p < 0.05$) (Table 1), suggesting that the CKIP-1 deficiency probably increases the degrees of fatty liver.

3.2. CKIP-1 deficiency aggravates the degree of fatty liver in mice fed with HFD

After 8 weeks of HFD consumption, lipid droplets were observed in liver tissues for both CKIP-$1^{-/-}$ and CKIP-$1^{+/+}$ mice by hematoxilin & eosin