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TRC8 downregulation contributes to the development of non-alcoholic steatohepatitis by exacerbating hepatic endoplasmic reticulum stress

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ABSTRACT

Endoplasmic reticulum (ER) stress is implicated in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). TRC8 is an ER-resident E3 ligase with roles in modulating lipid and protein biosynthesis. In this study we showed that TRC8 expression was downregulated in steatotic livers of patients and mice fed with a high fat diet (HFD) or a methionine and choline deficient (MCD) diet. To investigate the impact of TRC8 downregulation on steatosis and the progression to non-alcoholic steatohepatitis (NASH), we placed TRC8 knockout (KO) mice and wild type (WT) controls on a HFD or MCD diet and the severities of steatosis and NASH developed were compared. We found that TRC8 deficiency did not significantly affect diet-induced steatosis. Nevertheless, MCD diet-induced NASH as characterized by hepatocyte death, inflammation and fibrosis were exacerbated in TRC8-KO mice. The hepatic ER stress response, as evidenced by increased eIF2α phosphorylation and expression of ATF4 and CHOP, and the level of activated caspase 3, an apoptosis indicator, were augmented by TRC8 deficiency. The hepatic ER stress and NASH induced in mice could be ameliorated by adenovirus-mediated hepatic TRC8 overexpression. Mechanistically, we found that TRC8 deficiency augmented lipotoxic-stress-induced unfolded protein response in hepatocytes by attenuating the arrest of protein translation and the misfolded protein degradation. These findings disclose a crucial role of TRC8 in the maintenance of ER protein homeostasis and its downregulation in steatotic liver contributes to the progression of NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease associated with metabolic syndrome in civilized countries [1–3]. Although steatosis caused by the accumulation of triglyceride (TG) in liver is a benign clinical course, it can progress to non-alcoholic steatohepatitis (NASH) characterized by hepatic injury, inflammation and fibrosis, and eventually advance to cirrhosis and hepatocellular carcinoma [1–3]. Nevertheless, the pathological mechanisms underlying NASH are not completely understood. Over the past decade, accumulative evidence has supported the involvement of endoplasmic reticulum (ER) stress in the progression of NAFLD [4–6]. In addition to regulating the quantity of secretary and membrane protein synthesis, ER is well known for its crucial role in protein quality control by which the unfolded or misfolded proteins are removed and degraded via a process called ER-associated degradation [7]. Disruption of the ER homeostasis characterized by the accumulation of unfolded or misfolded proteins within ER is commonly seen in the livers of experimental animals and human patients with NAFLD [4–6]. When ER stress is induced, it activates the unfolded protein response (UPR) to reduce global protein synthesis and facilitate the capacity of protein folding and degradation to restore ER homeostasis. However, prolonged UPR caused by the aggravated ER stress fails to promote cell survival but activates the apoptotic pathway ultimately leading to cell death and inflammatory response seen in various pathophysiological conditions, including NASH [8,9]. Apparently, increasing our understanding of the mechanism underlying the persistent ER stress in NASH could pave the way to a new therapeutic strategy for NAFLD.

Abbreviations: Adv, adenovirus; CHX, cycloheximide; Dgat1, DAG acyltransferase; ER, endoplasmic reticulum; FAS, fatty acid synthase; FBS, fetal bovine serum; GST, glutathione-s-transferase; HA, hemagglutinin; HFD, high-fat diet; KO, knockout; MCD, methionine and choline deficient; MCP-1, monocyte chemoattractant protein-1; MMP-2, matrix metalloproteinase-2; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBA, sodium 4-phenylbutyrate; pfu, plaque-forming unit; Ppap2a, phosphatidate phosphatase 2a; RT-PCR, reverse transcription-polymerase chain reaction; SREBPs, sterol regulatory element-binding proteins; SUnSET, surface sensing of translation; TG, triglyceride; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UPR, unfolded protein response; WT, wild type.

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TRC8 is an ER-resident E3 ligase and its gene disruption has been associated with hereditary renal cancer [10]. In addition to its tumor suppressive function [11,12], TRC8 has been shown to regulate cholesterol and fatty acid biosynthesis by stimulating the destabilization of the ER-bound sterol regulatory element-binding proteins (SREBPs) [13], hindering ER to Golgi transport of SREBP-2 [14], and mediating the degradation of HMG-CoA reductase [15]. Moreover, studies in *Drosophila* and mammalian cells revealed that TRC8 suppresses protein translation through targeting eIF3f and eIF3h for degradation [11]. In view of its multifaceted roles, dysregulation of TRC8 expression may have an impact on cellular homeostasis. In the present study, we show that TRC8 expression was downregulated in steatotic livers of human patients and mice placed on a high-fat diet (HFD) or methionine and choline deficient (MCD) diet. Moreover, MCD diet-induced NASH was more severe in TRC8 knockout (KO) mice comparing to their wild type (WT) controls, supporting the implication of TRC8 in NAFLD progression. Additional experiments were performed to disclose the mechanisms involved.

2. Materials and methods

2.1. Human liver samples

Fresh frozen non-tumorous liver samples, including nine samples with non-alcoholic fatty liver disease: 4 with mild (10–20%) fatty change and 5 with moderate to severe (30–75%) fatty change, and another five samples with minimal (0–1%) fatty change were retrieved from the Human Biobank of National Cheng Kung University Hospital. The severity of fatty change was evaluated and graded corresponding to the percentage of macrovesicular fat accumulation in liver parenchyma. These samples were collected from patients who were serologically negative for HBsAg and HCV antibody and had primary liver cancer undergoing hepatectomy (12 cases had HCC and 2 cases had cholangiocarcinoma). Eleven patients were male and three were female and the mean age was 65.9 years old (range 53 ~ 83 years old). Studies were approved by the Institutional Review Boards of both National Cheng Kung University (A-ER-103-285) and Academia Sinica (AS-IRB-BM 14064 v.1) Taiwan.

2.2. Animal experiments

The mouse ES cells (C57BL/6N-A origin) carrying *TRC8* deletion alleles (Rnf139 DPGS00168_A_D07) were purchased from KOMP (knockout mouse project repository) (http://www.komp.org) in UC Davis, USA and used for the establishment of a mouse line with TRC8 deficiency in a C57BL/6J genetic background. Briefly, the ES cells were injected into mouse blastocysts to generate chimeric mice. The germline transmission and genotype of the mouse line containing the targeted deletion were confirmed by a genomic DNA-polymerase chain reaction (PCR) assay using two pairs of primers to amplify the inserted neomycin resistance gene (5′-TGTTCGGCTATGACTGGGCACA-3′ and 5′-GAGCAAGGTG AGATGACAGGAGAT-3′) and a deleted TRC8 fragment from the WT gene (5′-GTTCTGATCTTGTCACAGCGATC-3′ and 5′-AGGTTAGCTGCAGA ACGATGAGT-3′), respectively. PCR products were then subjected to 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining (Supplementary Fig. 1). To induce steatosis, C57BL/6J mice, TRC8-KO mice and their WT control mice (male, 8 weeks old) were fed with chow or HFD (60 kcal% fat, D12492, Research Diets) for 12 weeks. To induce NASH, mice were fed with chow or a MCD diet for 6 weeks. For the sodium 4-phenylbutyrate (PBA) treatment experiment, MCD diet-fed mice in each genotype were divided into two groups with one group of mice receiving intraperitoneal injection of saline and another group receiving PBA dissolved in saline (Sigma-Aldrich, St. Louis, MO; 100 mg/kg body weight) every three days starting from the second week of MCD diet feeding. For the adenovirus (Adv)-mediated gene transfer experiment, C57BL/6J mice were fed with the MCD diet for 3 weeks, followed by intravenous injection of saline or 1×10^9 plaque-forming units (pfu)/mouse of empty control Adv or Adv bearing human TRC8 cDNA (Adv-TRC8) via tail vein. Mice were continuously fed with the MCD diet for 3 weeks prior to sacrifice. All mice were kept on a 12 h light–dark cycle and allowed free access to food and water. All experimental procedures with animals were approved by the Institutional Animal Care and Utilization Committee of the Academia Sinica, Taiwan (IACUC protocol ID: 12-02-320).

2.3. DNA constructs

pIRES-NHK-HA vector bearing misfolded α1-antitrypsin variant-null Hong Kong was kindly provided by Dr. Xin Chen of the National Health Research Institutes, Taiwan. Control Adv and the Adv-TRC8 bearing human TRC8 gene were prepared using AdEasy™ Adenoviral Vector System (Agilent Technologies, La Jolla, CA, USA) according to the manufacturer's instruction.

2.4. Cell culture

Human HepG2 and mouse Hepa 1-6 hepatoma cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS). Primary hepatocytes were isolated from WT and TRC8-KO mice using collagenase perfusion as described previously [16] seeded on a collagen-coated plate (0.5 μg type I collagen/cm^2) and cultured in Dulbecco's modified Eagle's medium/F12 medium containing 10% FBS.

2.5. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated and quantitative RT-PCR was performed as described previously [17]. The primers used were listed in Supplementary Table 1.

2.6. Hepatic TG and cholesterol measurements

Hepatic lipid was extracted as described previously [17]. TG and cholesterol concentrations were determined using TG and cholesterol assay kits (Cayman, Ann Arbor, MI).

2.7. Anti-mouse TRC8 antibody production

A cDNA fragment encoding amino acids 591-668 of mouse TRC8 was subcloned into pGEX-4T-1 vector. The recombinant TRC8 peptide fused with glutathione-s-transferase (GST) at N-terminus was induced in *E. coli*, purified by glutathione affinity resin, and used as an antigen for antibody production in rabbit. TRC8 specific antibody was purified from the serum of an immunized rabbit with an affinity column conjugated with recombinant GST-TRC8 and stored in aliquots in 50% glycerol at −20 °C. The specificity of the antibody was confirmed by Western blot analysis using recombinant GST and GST-TRC8 proteins and liver tissue lysates from WT and TRC8-KO mice (Supplementary Fig. 2).

2.8. Western blot analysis

Tissue and cell lysates were prepared as described previously [17]. The protein samples were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed as described previously [17]. Antibodies used included anti-GRP78, anti-ubiquitin, anti-ATF4 (from Santa Cruz, Santa Cruz, CA), anti-p-eIF2α, anti-eIF2, anti-CHOP, anti-cleaved caspase 3 (from Cell Signaling, Danvers, MA), anti-α-smooth muscle actin (α-SMA) (DAKO, Carpinteria, CA), and anti-hemagglutinin (HA) (Abcam, Cambridge, MA).