Research Article

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Targeting hepatic TRAF1-ASK1 signaling to improve inflammation, insulin resistance, and hepatic steatosis

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Background & Aims: Tumor necrosis factor receptor-associated factor 1 (TRAF1) is an important adapter protein that is largely implicated in molecular events regulating immunity/inflammation and cell death. Although inflammation is closely related to and forms a vicious circle with insulin dysfunction and hepatic lipid accumulation, the role of TRAF1 in hepatic steatosis and the related metabolic disorders remains unclear.

Methods: The participation of TRAF1 in the initiation and progression of hepatic steatosis was evaluated in high fat diet (HFD)-induced and genetic obesity. Mice with global *TRAF1* knockout or liver-specific *TRAF1* overexpression were employed to investigate the role of TRAF1 in insulin resistance, inflammation, and hepatic steatosis based on various phenotypic examinations. Molecular mechanisms underlying TRAF1-regulated hepatic steatosis were further explored *in vivo* and *in vitro*.

Results: TRAF1 expression was significantly upregulated in the livers of NAFLD patients and obese mice and in palmitate-treated hepatocytes. In response to HFD administration or in *ob/ob* mice, TRAF1 deficiency was hepatoprotective, whereas the overexpression of TRAF1 in hepatocytes contributed to the pathological development of insulin resistance, inflammatory response and hepatic steatosis. Mechanistically, hepatocyte TRAF1 promotes hepatic steatosis through enhancing the activation of ASK1-mediated P38/JNK cascades, as evidenced by the fact that ASK1 inhibition abolished the exacerbated effect of TRAF1 on insulin dysfunction, inflammation, and hepatic lipid accumulation.

Conclusions: TRAF1 functions as a positive regulator of insulin resistance, inflammation, and hepatic steatosis dependent on the activation of ASK1-P38/JNK axis.

Introduction

The liver is a key insulin-responsive tissue and is actively involved in maintaining whole-body glucose and lipid metabolism. Non-alcoholic fatty liver disease (NAFLD), a clinical pathological alteration characterized by lipid accumulation in hepatocytes, has become a major health issue due to its high prevalence worldwide (approximately 46% in the middle-aged population) and its serious complications and/or sequelae, which include obesity, type 2 diabetes mellitus, hyperlipidemia, and insulin resistance [1–3]. The prevalence of NAFLD increases to 58% in overweight individuals and reaches 98% in non-diabetic obese individuals [4]. During the initiation and progression of hepatic steatosis, the dysregulation of lipid metabolism is frequently associated with an inflammatory condition [5], and the elevated inflammatory status plays an important role in the development of insulin resistance, which in turn further promotes ectopic fat accumulation in the liver, forming a vicious cycle [6]. Accumulating studies have provided evidence that hepatocyte activation of the IKKβ/NF-κB and the JNK/P38 MAPK signaling pathways in mice causes hepatic and systemic insulin resistance and increased hepatic production of inflammatory cytokines [7,8]. However, the molecular mechanism underlying NAFLD progression is not completely understood, hindering the development of effective treatments for this disease [9].

Tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) is a member of the TRAF family, which includes 7 known members (TRAF1-7) in mammals. TRAF proteins can function as signaling adapters by binding directly to TNFR superfamily receptors [10]. In contrast to other TRAFs, which feature a RING domain in the N-terminal region, there is no RING domain in

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Abbreviations: TRAF1, Tumor necrosis factor receptor-associated factor 1; NAFLD, non-alcoholic fatty liver disease; HFD, high fat diet; KO, knockout; LTG, liver-specific TRAF1 transgenic; NTG, non-transgenic; TNFR, Tumor necrosis factor receptor; GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, areas under the curve; TG, triglyceride; TC, total cholesterol; NEFA, non-esterified fatty acid; HOMA-IR, homeostasis model assessment of the IR index; ALT, alanine amino transferase; AST, aspartate amino transferase; ANOVA, analysis of variance; LW/BW, liver weight/body weight.

TRAF1 [10,11]. Extensive studies have indicated that TRAF1 is involved in multiple signaling pathways, including the NF-κB and MAPK pathways [12,13], and thus influences inflammatory and apoptotic responses to tightly regulate the development of rheumatoid arthritis and chronic infection [14,15]. Notably, most previous studies on TRAF1 have focused on its role in immune cells, particularly T cells and B cells. We recently demonstrated that neuronal TRAF1 overexpression can significantly exacerbate neuron death during stroke via interaction with ASK1 and subsequent enhancement of JNK and inhibition of Akt signaling [16]. In hepatocytes, TRAF1 deficiency inhibits both the inflammatory responses mediated by NF-κB and cell death controlled by the ASK/JNK axis [17]. Xu *et al.* [5] reported that TRAF1 expression can be induced by TNF-α treatment to mediate the crosstalk between inflammation and lipid metabolism, thereby suggesting a role of TRAF1 in metabolic disorders. However, the precisely functional and biological relevance of hepatocyte TRAF1 in hepatic steatosis and related pathologies remains to be clarified.

Based on the well-established role of TRAF1 in inflammatory response [18] and the close interaction between immunity and the metabolic system [19], we hypothesized a role of TRAF1 in regulating obesity-related metabolic diseases including NAFLD. Accordingly, the current study investigated the following: 1) whether TRAF1 expression is altered during the development of NAFLD; 2) the consequences of defective or augmented expression of hepatocyte TRAF1 in high fat diet (HFD)-induced and genetic obesity; 3) the underlying mechanisms by which TRAF1 exerts its roles in hepatic steatosis and related metabolic disorders.

Materials and methods

Mice and diets

TRAF1 knockout mice (KO) were obtained from the Jackson Laboratory (in C57BL/6 genetic background; Stock No. 008076). Liver-specific *TRAF1* transgenic (LTG) mice (C57BL/6J background) were generated, and genotyping was performed as previously reported [17]. For specific knockdown of *TRAF1* in hepatocytes *in vivo*, recombinant adenovirus containing a short hairpin RNA targeting *TRAF1* (Adsh*TRAF1*; 5×10^9 pfu) was constructed as previously described [20] and injected into mice via the jugular vein. Adsh*TRAF1* was injected into 8 weeks old female *ob/ob* mice fed with normal chow (NC) diet or male C57 mice after 20 weeks of HFD administration for another 4 weeks. Liver-specific overexpression of constitutively kinase-active *ASK1* (ca*ASK1* LTG) mice were generated by crossing albumin-cre mice (Jackson laboratory) with *ASK1* flox mice. ca*ASK1* LTG mice were further crossed with *TRAF1* knockout (KO) mice to create ca*ASK1* LTG/*TRAF1* KO (CTTK) mice. To generate dominant-negative *ASK1* transgenic mice (dn*ASK1* TG), a kinase-negative (K709M) mutant of *ASK1* was injected into fertilized mouse embryos, and the resulting dn*ASK1* TG mice were crossed with *TRAF1* LTG mice to obtain dn*ASK1* TG/*TRAF1* LTG (DTG) mice. All mice obtained by genetic engineering were confirmed by Western blotting.

Mice were maintained in a temperature-controlled (23 ± 2 °C) environment with a 12 h light-dark cycle. Five-week-old mice were fed *ad libitum* either a NC or a HFD (Research Diets Inc.) for the indicated durations. On the caloric basis, the HFD consisted of 60% fat (90.8% lard and 9.1% soybean oil), 20% carbohydrate, and 20% protein, for a total of 5.24 kcal per 1 g of diet. Cholesterol quantitates 279.6 mg per 1 kg of diet. The NC consisted of 10% fat, 70% carbohydrate, and 20% protein, for a total of 3.85 kcal per 1 g of diet. Food intake was recorded weekly, and body weight and fasting blood glucose were measured every 4 weeks [21]. All experiments were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University. All experiments and subsequent analyses were performed in a blinded manner for genotyping.

Human liver tissue samples

Human liver samples were obtained from adult NAFLD patients who underwent liver biopsy or transplantation. Control liver samples were collected from donors whose livers were unsuitable for transplantation for non-hepatic reasons [22]. Histological slides were scored by two pathologists using standard criteria used before [23]. The clinical information for the NAFLD patients and donors were provided in Supplementary Table 4. This study complied with the Declaration of Helsinki, and informed consent was obtained from each participant or their families. The research protocol was approved by the Ethics Committee of Renmin Hospital at Wuhan University.

Statistical analysis

All data are presented as the mean ± SD. Differences between two groups were compared using a two-tailed Student's *t* test, and differences among more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

Additional experimental procedures are provided in the Supplementary methods.

Results

Hepatic TRAF1 expression is increased in NAFLD

To determine the relevance of TRAF1 in hepatic metabolism, we first measured its expression levels in liver biopsy samples from normal donors and NAFLD patients. As shown in Fig. 1A, robustly enhanced expression of TRAF1 was observed in NAFLD patients compared with normal donors. Immunofluorescence experiments also revealed the increased expression of TRAF1 in the livers of NAFLD patients (Fig. 1B). To specify whether this increase occurs in hepatocytes, primary cells were isolated from wild type (WT) mice and stimulated with vehicle or palmitate, an inducer of lipid accumulation and insulin resistance in hepatocytes [24]. Compared with vehicle-treated cells, palmitate-triggered a marked increase in TRAF1 expression in hepatocytes (Fig. 1C). Moreover, both 24 weeks HFD treatment-induced and genetic obese mice (*ob/ob*) also exhibited an increase in TRAF1 expression in the liver compared with lean controls (Fig. 1D and E). Taken together, these results suggest a plausible role for TRAF1 in mediating obesity-associated pathologies.

TRAF1 deficiency attenuates HFD-induced obesity and insulin resistance

The effects of TRAF1 deficiency in obesity was subsequently examined using *TRAF1* KO mice (Supplementary Fig. 1A). After continuous administration of HFD for 24 weeks, a marked increase in body weight was observed in WT mice, whereas mice with *TRAF1* KO displayed a slight increase in body weight (Fig. 2A). HFD-fed WT mice exhibited consistently higher fasting glucose levels, plasma insulin levels, and HOMA-IR indexes than *TRAF1* KO mice (Fig. 2B). In addition, *TRAF1* KO mice exhibited improved glucose tolerance and enhanced insulin sensitivity compared with WT mice as indicated by intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) (Fig. 2C and D).

To evaluate the role of hepatocyte TRAF1 in diet-induced pathologies, we introduced adenoviruses expressing shRNA or sh*TRAF1* into the livers of 20-week-old HFD-fed WT mice. Significant adenovirus-mediated downregulation of *TRAF1* was confirmed in the livers of Adsh*TRAF1*-injected mice compared with AdshRNA controls (Supplementary Fig. 1B). Following continuous HFD treatment for 4 weeks, although body weight failed to be significantly influenced by Adsh*TRAF1* injection (Fig. 2E), fasting glucose levels, plasma insulin levels, and HOMA-IR index were dramatically reduced in Adsh*TRAF1*-treated mice compared