Reduction in activating transcription factor 4 promotes carbon tetrachloride and lipopolysaccharide/D-galactosamine-mediated liver injury in mice

XIAOFANG ZHAO1*, HONG ZHOU1*, YING CHENG1, WENJING YU1, GUOSONG LUO2, CHUNYAN DUAN1, FULI YAO1, BIN XIAO1, CHUNHONG FENG2, XIAMING XIA2, MEI WEI3, YONG WANG3, JING LI2 and RONGYANG DAI4

*Department of Biochemistry and Molecular Biology, Southwest Medical University; 2Department of Hepatobiliary Surgery, The Affiliated Hospital of Southwest Medical University; 3Department of Liver Diseases, The Affiliated Hospital of Chinese Traditional Medicine; 4Liver Diseases Laboratory, Southwest Medical University, Luzhou, Sichuan 646000, P.R. China

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Abstract. Although activating transcription factor 4 (ATF4) is involved in the regulation of numerous biological functions, whether ATF4 has a direct role in liver injury is unknown. The aim of the present study was to investigate the role of ATF4 in liver injury using mouse models. The results revealed that ATF4 protein is expressed markedly higher in the mouse liver when in comparison with other tissues. Notably, tunicamycin treatment, an endoplasmic reticulum (ER) stress inducer, induced the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), but decreased ATF4 protein levels in the mouse liver. This suggested an unconventional regulation pattern of ATF4 protein not associated with ER stress or eIF2α. In addition, it was also observed that the liver levels of ATF4 protein were significantly reduced upon chronic liver injury induced by carbon tetrachloride (CCl4). ATF4 protein was also decreased in acute liver injury induced by lipopolysaccharide/D-galactosamine (LPS/D-GalN). Furthermore, the results revealed that knockdown of ATF4 by injecting ATF4-targeting Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein 9 plasmids exacerbated CCl4 and LPS/D-GalN-induced liver injury as demonstrated by elevated serum aspartate transaminase and alanine aminotransferase levels. ATF4 suppression also enhanced CCl4 and LPS/D-GalN mediated c-Jun N-terminal kinase activation. By contrast, ATF4 overexpression alleviated CCl4 and LPS/D-GalN-induced liver injury. Taken together, these observations suggested that ATF4 may serve a protective role in the mouse liver.

Introduction

Activating transcription factor 4 (ATF4) is a transcription factor that belongs to the C/EBP transcription factor family that binds the cAMP response element (CRE) (1,2). ATF4 is a master transcription factor for which temporal expression and activity are under tight cellular control. The translation of ATF4 is regulated by eukaryotic translation initiation factor 2α (eIF2α) (3). Under normal conditions, ATF4 protein is quickly degraded by the proteasome contributing to its short half-life. Under stress conditions, the phosphorylation of eIF2α leads to general inhibition of translation, but it results in translational upregulation of specific mRNAs including ATF4 (4,5).

ATF4 is involved in the regulation of many biological processes including cellular amino acid metabolism, osteoblast differentiation, and the oxidative stress response (2,6-8). In vivo evidence has shown that ATF4 plays an important role in glucose metabolism, insulin sensitivity, and lipid metabolism (9-12). Liver injury is a common initiating process of many liver diseases, including hepatitis, cirrhosis, and hepatoma (13). There are many common risk factors which can induce liver injury, such as hepatitis virus, alcohol, and drugs. Although there are numbers of pathways reported to mediate liver injury (14,15), the precise mechanisms behind liver injury remain largely unknown.

In our current study, we observed that ATF4 protein is highly expressed in mouse livers. The liver ATF4 protein levels decreased upon carbon tetrachloride (CCl4) and lipopolysaccharide/D-galactosamine (LPS/D-GalN) induced...
liver injury. Furthermore, we show that suppressing ATF4 using CRISPR-Cas9 plasmids enhanced CCl4 and LPS/D-GalN induced liver injury in mice, while ATF4 overexpression attenuated CCl4 and LPS/D-GalN induced liver injury.

Materials and methods

Chemicals and antibodies. Tunicamycin was purchased from Tocris (Minneapolis, MN, USA). CCl4 was purchased from Guoyao (Beijing, China). LPS and D-GalN were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies against ATF4, p-eIF2α and Bip were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against eIF2α and GAPDH were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

Animals and treatments. Male C57BL/6 mice (10 weeks, 20-22 g) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The use of animals was approved by the Ethics Committee of Southwest Medical University on Animal Care (Sichuan, China).

Plasmid hydrodynamic injection. Hydrodynamic injection was performed as described in the report of Chen and Calvisi (16). In brief, 10 µg ATF4-targeting CRISPR-Cas9 plasmid, ATF4 overexpression plasmid or empty vector were diluted in 2 ml saline (0.9% NaCl), filtered through a 0.22 µm filter and injected into the lateral tail vein of 10-week-old male C57BL/6 mice in 5 to 7 sec.

CCl4-induced liver injury model. Male C57BL/6 mice (6 mice per group) were injected intraperitoneally with CCl4 (4 ml/kg, 5% w/v dissolved in olive oil) three times a week for 2 weeks as previously described (17). The mice were killed 24 h after the final injection of CCl4, and liver tissues were harvested for analysis.

LPS/D-GalN-induced liver injury model. Male C57BL/6 mice (6 mice per group) were injected intraperitoneally with LPS (50 µg/kg) and D-GalN (800 mg/kg, phosphate buffer saline as control) and killed 6 h after LPS/D-GalN injection (18).

Histological analysis. Liver tissues of mice were fixed in 4% formalin at room temperature for at least 24 h, embedded in paraffin and cut into 5 µm sections. Liver sections were deparaffinized and stained with hematoxylin and eosin (H&E) for morphologic analysis. Sirius red staining was performed according to the usual method and the positive area was quantified with Image J software.

Semi-quantitative (sq-) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fischer Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The reverse transcription reactions were carried out using the M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. sqPCR was performed by running the products on a 1% (for ATF4 and 18S) or 4% (for XBP1) agarose gel. RT-qPCR analyses were performed using SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) as previously described (17). Results were normalized with 18S and quantified using the 2^ΔΔCq method (19). The primers used are as follows: Mouse ATF4-forward: 5'-TCCTGAACAGCGAAGTGTTG, and mouse ATF4-reverse: 5'-AGAGGCTCATCTGGCATGGTT-3'; mouse XBPI-forward: 5'-TGCTGAGTCCCAGACAGAGTGTG-3', and mouse XBPI-reverse: 5'-ACTAGCAGCTCTGGGGAGAAG-3'; mouse 18S-forward: 5'-CGGCTACCACTCCTAGGAA-3', and mouse 18S-reverse: 5'-GCTGGAATTACCCGGCCT-3'.

Western blot analysis. Mouse tissues were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycercol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/ml aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 4°C, 12,000 x g for 15 min. Protein concentrations of the supernatant were measured using the BCA assay. Protein samples were denatured with 4x SDS-loading buffer (200 mM Tris, pH 6.8, 8% SDS, 400 mM DTT, 0.4% bromophenol blue, 40% glycercol) at 100°C for 5 min and subjected to standard SDS-PAGE and western blot analysis as previously described (17).

Statistical analysis. Results are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's t-test and Excel software (version 2010; Microsoft Corporation, Redmond, WA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

ATF4 protein is highly expressed in the mouse liver. To investigate the expression of ATF4 in vivo, we evaluated both protein and mRNA levels of ATF4 in mouse tissues, including liver, heart, kidney, lung, stomach, spleen, and small and large intestine. Interestingly, the western blot results showed ATF4 protein is highly expressed in mouse liver, while being almost nondetectable in other tissues (Fig. 1A). However, the RNA levels of ATF4 in these tissues are comparably high (Fig. 1B).

High levels of ATF4 protein in the liver are independent of ER stress or eIF2α. Considering that ATF4 is conventionally regulated by eIF2α, we analyzed the phosphorylation level of eIF2α in mouse tissues by western blotting. Our results showed that phospho-eIF2α levels are very low in the tissues tested (Fig. 2A), which seemed contradictory with the high protein level of ATF4 in the liver. To clarify whether ATF4 can be upregulated by ER stress in the mouse liver, we treated mice with tunicamycin. As shown in Fig. 2B, tunicamycin treatment caused XBPI mRNA splicing, suggesting the induction of the unfolded protein response. Next, we determined the eIF2α/ATF4 signal in mouse liver and lung upon tunicamycin treatment. The results showed that tunicamycin significantly promoted eIF2α phosphorylation, and increased ATF4 and Bip protein levels in mouse lungs (Fig. 2C). In the liver tissue, phosphorylation of eIF2α and expression of Bip were increased upon tunicamycin administration as expected. However, the ATF4 protein level decreased in a time-dependent manner after tunicamycin treatment
These results indicated that the high levels of liver ATF4 protein present in the liver are independent of eIF2α or ER stress.

ATF4 protein was decreased in CCI4 and LPS/D-GalN induced mouse liver injury. It was interesting to find that ATF4 protein displayed high levels of expression in the mouse liver and was nonconventionally regulated. We therefore investigated the role of ATF4 in liver injury. Animal models of liver injury are commonly used in research, for example CCI4 is a classical hepatotoxicant, which is used to induce chronic liver injury and liver fibrosis (20). Similarly, LPS plus D-GalN is a well-known acute liver injury model (21). Thus, CCI4 was used to establish chronic liver injury while LPS/D-GalN was used to induce acute liver injury. The western blot assay demonstrated that ATF4 protein was decreased significantly following repeated CCI4 exposure, while the mRNA level of ATF4 was not significantly changed (Fig. 3A and B). In
addition, the ATF4 protein decreased markedly after 6 h of LPS/D-GalN treatment (Fig. 3C). In contrast, the mRNA of ATF4 did not change significantly (Fig. 3D). These results suggested that ATF4 protein is downregulated in response to both chronic and acute liver injury.

**ATF4 suppression aggravated CCl4 and LPS/D-GalN induced liver injury.** Next, to investigate effects of ATF4 on liver injury, ATF4 targeting a CRISPR-Cas9 plasmid (ATF4-cri) was constructed and injected through the tail vein to knockdown the expression of ATF4 in the liver. As shown in Fig. 4A and B, the ATF4-cri plasmid efficiently lowered the liver ATF4 expression at both mRNA and protein levels. After injection with ATF4-cri plasmid or control plasmid, mice were challenged with CCl4 or LPS/D‑GalN. Serum transaminase analysis revealed that knockdown of ATF4 by ATF4-cri significantly increased CCl4-induced levels of AST and ALT compared with controls (Fig. 4B). Similar results were obtained in the LPS/D-GalN model (Fig. 4C). These data demonstrated that ATF4 suppression augmented hepatocyte damage and the inflammatory response in both the CCl4 and LPS/D-GalN models. The c-Jun-N-terminal kinase (JNK) is a mitogen-activated protein kinase family member that plays important roles in the regulation of cell death, survival, and inflammation (22). We therefore explored a possible role for JNK in our model. The results showed that both CCl4 and LPS/D-GalN treatment lead to the activation of JNK (Fig. 5D). More importantly, ATF4 suppression increased the activation of JNK induced by CCl4 and LPS/D-GalN (Fig. 5D). These results suggested that ATF4 plays a protective role of in the liver, in part, through regulating JNK signaling.

**ATF4 overexpression alleviated CCl4 and LPS/D-GalN induced liver injury.** To verify the protective role of ATF4 in the liver, we investigated the effects of ATF4 overexpression on liver injury induced by CCl4 and LPS/D-GalN. After injection with ATF4 overexpression plasmid (ATF4-ov) or control plasmid, mice were challenged with CCl4 or LPS/D-GalN. Serum transaminase analysis revealed that overexpression of ATF4 significantly decreased CCl4 induced AST and ALT elevation compared with controls (Fig. 6A). Similar results were obtained in the LPS/D-GalN model (Fig. 6B). These data thus further confirm the protective role of ATF4 in the liver.

**Discussion**

In this study, we characterized the expression pattern of ATF4 *in vivo* at both the protein and mRNA level. We firstly discovered that ATF4 maintained high protein levels in the...
mouse liver under normal conditions. Considering there is no difference in mRNA levels of ATF4 between the tissues we tested, the difference in ATF4 protein levels could be due to variation in translation or stability between tissues. It is well known that ATF4 protein is usually upregulated by stress conditions and plays a crucial role in the stress response. Multiple intracellular stress pathways including endoplasmic reticulum stress, amino acid deprivation, and oxidative stress can induce the phosphorylation of eIF2α, which both leads to a general inhibition of protein synthesis but also the translational upregulation of ATF4 mRNA (4). Here, we observed high protein levels of ATF4 in mouse livers but not in other tissues. However, the phosphorylation levels of eIF2α are uniformly low in the mouse tissues we tested, inconsistent with the high protein levels of ATF4 in the liver. We hypothesized that liver ATF4 protein levels are not associated with eIF2α activation. To confirm this speculation, tunicamycin, an ER stress inducer, was used to trigger ER stress and eIF2α phosphorylation. Notably, tunicamycin induced ER stress in mouse liver and lung, as demonstrated by spliced XBP1 mRNA, increased Bip

Figure 4. Suppression of ATF4 promotes CCl₄ and LPS/D-GalN-induced liver injury. (A) Mice were injected with the ATF4-cri or control plasmid through the tail vein and were sacrificed 72 h post-injection. The liver ATF4 mRNA levels were analyzed by reverse transcription-quantitative polymerase chain reaction and protein levels were analyzed by western blotting. (B) Mice were injected with ATF4-cri plasmid or empty vector once a week via the tail vein. Two days following the first plasmid injection, the mice were injected intraperitoneally with CCl₄ (4 ml/kg) or vehicle control for 2 weeks. Serum AST and ALT levels were determined at 24 h following the last CCl₄ injection. (C) Mice were injected with ATF4-cri plasmid or empty vector through the tail vein. Two days following plasmid injection, the mice were injected intraperitoneally with LPS (50 µg/kg) and D-GalN (800 mg/kg). Serum AST and ALT levels were determined following 6 h. Data are presented as the means ± standard deviation. *P<0.05, as indicated. ATF4, activating transcription factor 4; LPS, lipopolysaccharide; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; ATF4-cri, ATF4-targeting CRISPR-CRISPR associated protein 9 plasmid; CCl₄, carbon tetrachloride; D-GalN, D-galactosamine; AST, aspartate transaminase; ALT, alanine aminotransferase; ctl, control.
protein and eIF2α phosphorylation. The ATF4 protein in the lung was consistently induced by tunicamycin, indicating a conventional regulation of ATF4 by eIF2α. Nevertheless, the liver expression of ATF4 protein decreased upon tunicamycin treatment. This demonstrated a unique regulation pattern of ATF4 protein in the liver tissue that is not associated with eIF2α. Another possible mechanism is that the stability of ATF4 is different between the liver and other tissues. It has been reported that ATF4 degradation is mediated by the E3 ubiquitin ligase SCFβTrCP (23). Additional reports have shown that p300 modulates ATF4 stability and its transcriptional activity (24). Whether the stability of ATF4 contributes to the difference in tissue ATF4 protein levels requires further investigation.

We wondered whether the high protein level of ATF4 expression in mouse livers hinted at an important role in the liver. It has been reported that ATF4 mutations resulted in severe fetal anemia and fetal liver hypoplasia (25). Additional reports have suggested that ATF4 plays an important role in hepatic lipid metabolism (10-12). Liver injury is the most common liver disorder resulting in aggressive liver diseases. In the present study, we investigated the role of ATF4 in liver injury using two models, CCl4-mediated chronic liver injury and LPS/D-GalN-induced acute liver injury. Intriguingly, we found decreased ATF4 protein levels in mouse livers following both CCl4 and LPS/D-GalN administration without recognizable mRNA changes. This indicated posttranscriptional
regulation of ATF4 in CCl4 and LPS/D-GalN models, possibly via regulation of translation or stability. Our future research will focus on the regulatory mechanisms of ATF4 in these liver models. However, the question remained whether the reduction in ATF4 influences liver injury. Our data showed that inactivation of ATF4 by CRISPR significantly aggravated CCl4 and LPS/D-GalN induced liver injury, as demonstrated by elevated serum AST and ALT. In addition, the overexpression of ATF4 attenuated CCl4 and LPS/D-GalN mediated liver injury. These results implied a protective role for ATF4 during liver injury. The JNK pathway has been reported to regulate cellular stress responses, apoptosis, malignant transformation, and hepatocarcinogenesis (22,26). We demonstrated that ATF4 suppression promoted CCI4 and LPS/D-GalN induced JNK activation. This may suggest that the inhibition of ATF4 aggravated liver injury, at least partly, through the upregulation of the JNK pathway. In a previous study by Masuoka and Townes (25), ATF4 was identified as critical for normal cellular proliferation, especially for the high-level proliferation required during fetal-liver hematopoiesis. The liver is a highly regenerative tissue, as hepatocytes are able to proliferate in response to injury to restore liver function (27). Here in our models, a high level of cell proliferation was required after CCI4 and LPS/D-GalN treatment. Thus, a reasonable explanation for our results is that downregulation of ATF4 inhibited compensatory cell proliferation during liver repair response, resulting in more serious liver injury. Further studies are needed to investigate the detailed mechanisms linking ATF4 and liver injury.

In summary, we revealed a nonconventional expression pattern of ATF4 protein in mouse livers. Chemical-induced liver injury caused a decrease in liver ATF4 protein. Moreover, we demonstrated that ATF4 suppression aggravated CCI4 and LPS/D-GalN induced liver injury, while ATF4 overexpression attenuated CCI4 and LPS/D-GalN induced liver injury, indicating a hepatoprotective role for ATF4.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and HZ designed the experiments, and performed the animal experiments and data analyses. YC performed the experiments, and performed the analysis of the main relevant signaling molecules. JD and YL performed the cell and murine studies. YW, ZT, and YL participated in data analysis and interpreting the results. RD and JL designed the experiments, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Southwest Medical University on Animal Care (Sichuan, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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