

Original Article

IL-17 enhances oxidative stress in hepatocytes through Nrf2/keap1 signal pathway activation

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Abstract: Hepatic disease is common worldwide. As apoptosis of hepatocytes is a hallmark of hepatic disease, strategies targeting apoptosis are an effective way to prevent and treat the disease. Oxidative stress is a classic pathway of apoptosis. Increased expression of IL-17 has been found in diverse human liver diseases. The aim of this research was to investigate whether the function of IL-17 influenced hepatocyte-induced H_2O_2 . We found that the expression of IL-17 was enhanced in hepatocyte-induced H_2O_2 and that IL-17 was down-regulated by siRNA. A TUNEL assay showed that apoptosis was decreased in Δ IL-17-mutant hepatocyte-induced H_2O_2 . Additionally, the ratio of Bax/Bcl-2 was decreased in Δ IL-17-mutant hepatocyte-induced H_2O_2 . Finally, Nrf2/keap1 signal pathway was analyzed, the expression of Nrf2 and keap1 was decreased in Δ IL-17-mutant hepatocyte-induced H_2O_2 . These results suggest that oxidative stress-induced apoptosis was induced and that IL-17 could enhance oxidative stress-induced apoptosis by the Nrf2/keap1 signal pathway in hepatocytes.

Keywords: Hepatic disease, IL-17, Nrf2/keap1 pathway, hepatocytes

Introduction

Hepatic diseases, which have a high prevalence worldwide, include a broad spectrum of diseases ranging from early fatty liver to severe hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma [1]. Moreover, patients with hepatic diseases show a higher risk for cardiovascular disease [2]. Unfortunately, hepatic diseases lack effective and specific treatments. This underscores the importance of understanding the mechanisms of hepatic diseases.

In recent years, accumulating evidence indicates that oxidative stress plays important roles in hepatic diseases [3]. The liver is a major organ attacked by oxidative stress, and it has been reported that the augmented generation of reactive oxygen species (ROS) induce lipid peroxidation leading to fibro-genesis through the activation of stellate cells [4]. Oxidative stress triggers hepatic damage by inducing the irretrievable alteration of lipids, protein and DNA contents, and it also modulates the pathways with control normal biological functions [5]. These pathways regulate gene transcription, protein expression, cell apoptosis, and

hepatic stellate cell activation. Oxidative stress is regarded as one of the pathological mechanisms that results in the initiation and progression of various hepatic diseases, for example, chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis [6]. Antioxidant enzymes such as SOD, GSH-Px are essential for the cellular response needed to deal with oxidative stress. The Nrf2/keap1 pathway is a major regulator of cellular redox balance. Under physiological conditions, the nuclear factor erythroid-2 related factor 2, (Nrf2) binds to the epoxy chloropropane Kelch sample related protein-1 (Keap1) in the cytoplasm [7]. However, under oxidative stress, phosphorylation of Nrf2 is translocated into the cell nucleus and interacts with an antioxidant response element (ARE), promoting the expression of cytoprotective target genes [8]. The enhanced activation of Nrf2 has been shown to protect the liver in different oxidative stress models [9]. Swardfager et al. found that IL-17 can increase oxidative stress [10].

IL-17, belonging to the IL-17 signaling axis, is a pro-inflammatory cytokine protein [11]. An increased expression of IL-17 has been found in

diverse human liver diseases, including alcoholic liver disease, acute liver injury and chronic hepatitis B and C, primary biliary cirrhosis, hepatocellular carcinoma and acute liver transplant rejection [12]. But the precise mechanism of how IL-17 regulates hepatic diseases is unclear. In the present study, we first investigated the expression of IL-17 in hepatocyte-induced H₂O₂ and then studied the potential mechanisms and signaling pathways involved in this expression. Our results showed that the expression of IL-17 was up-regulated in hepatocyte-induced H₂O₂ and activated oxidative stress-initiated cellular apoptosis. Our study provides a theoretical foundation for targeting apoptosis in the pathogenesis of hepatic diseases.

Materials and methods

Cell culture

All experiments in this study were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China. Mice hepatocytes were isolated from C57BL/6 mice. After washing with PBS, the livers were cut into 1-3 mm pieces. Then the pieces were digested by a collagenase type II and trypsin mixture. The isolated hepatocytes were plated in culture dishes and cultured in DMEM (high glucose) with 10% fetal bovine serum (FBS), and 1% antibiotics. The medium was replaced every 2 days and cells were subcultured or subjected to experimental procedures at an amount so as to achieve a 70-80% confluency. H₂O₂ induced toxicity with a concentration of 100 µmol/L, 200 µmol/L, 400 µmol/L.

siRNA design and transfection

For siRNA transfection, mice hepatocytes were cultured in culture plates overnight and transfected with control and calumenin siRNA oligonucleotides (5'-TGG ACT TCC TCC AGA ATG TGT TCA AGA GAC ACA TTC TGG AGG AAG TCC TTT TTT C-3' and 5'-TCG AGA AAA AAG GAC TTC CTC CAG AAT GTG TCT CTT GAA CAC ATT CTG GAG GAG TCC A-3'). The medium was changed with a fresh culture medium after 24 h, and the cells were used for subsequent experiments after 72 h of siRNA transfection.

Cell viability assay

The toxicity of the mice hepatocytes was tested by a colorimetric assay using 3-(4,5)-dimethyl-

thiazazo(-z-yl)-3, 5-di-phenyltetrazoliumromide (MTT) (Sigma-Aldrich). Mice hepatocytes were seeded into 96-well plate and exposed to DMEM or H₂O₂ within/without IL-17. Experiments were done during the exponential phase of cell growth.

Measurement of GSH-Px and SOD activity in mice hepatocytes

The cellular activities of GSH-Px and SOD were measured using commercial assay kits according to the manufacturers' instructions. Then they were determined by commercially available, enzyme-linked immunosorbent assays (ELISAs). All samples were assayed six times.

TUNEL staining

A TUNEL kit was used to detect the apoptotic cells following the manufacturer's instructions. After the mice hepatocytes were cultured for 24 h, the cells were fixed in a 4% paraformaldehyde solution for 30 min. 0.3% H₂O₂ was used to block the endogenous peroxidase activity in the cells. The TUNEL reaction was used to incubate the cells. The results were detected by microscopy.

Western blot analysis

The Western blot technique was used to analyze the expression of Bax, Bcl-2, Nrf2, Keap1, β-actin and IL-17. Mice hepatocytes were washed in cold PBS and lysed in a RIPA buffer with a protease inhibitor mixture (Sigma-Aldrich, USA). Cell lysates were solubilized in an SDS loading buffer then separated by electrophoresis on 10% gel and transferred to nitrocellulose (NC) membranes. After closure, the membranes were incubated with primary antibodies (Bax 1:1000; Bcl-2 1:1000; Nrf2 1:1000; Keap1 1:1000; β-actin 1:1000 and IL-17 1:1000) overnight at 4°C.

Statistical analysis

All values were expressed as mean ± SEM. Two-group comparisons of the means were carried out by a matched t-test using SPSS 17.0. The number of stars (*/#) indicate the p value range: *p value <0.05, **p value <0.01, ***p value <0.001; #P<0.05, ##P<0.01, ###P<0.001.

IL-17 affected Nrf2/keap1 pathway

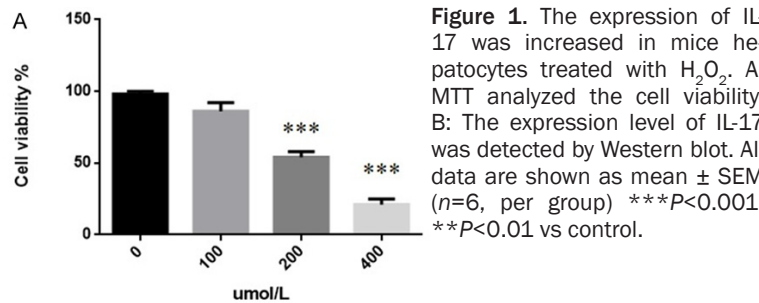


Figure 1. The expression of IL-17 was increased in mice hepatocytes treated with H₂O₂. A: MTT analyzed the cell viability. B: The expression level of IL-17 was detected by Western blot. All data are shown as mean ± SEM (n=6, per group) ***P<0.001, **P<0.01 vs control.

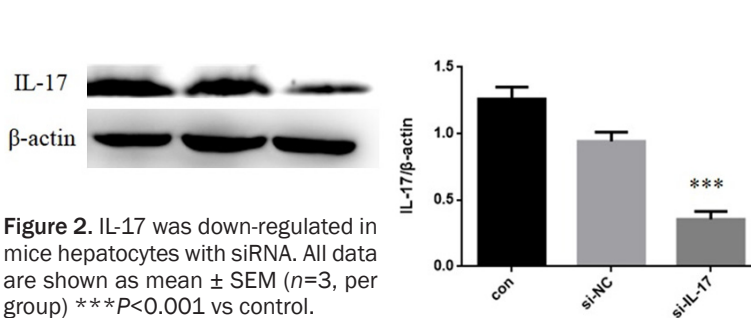
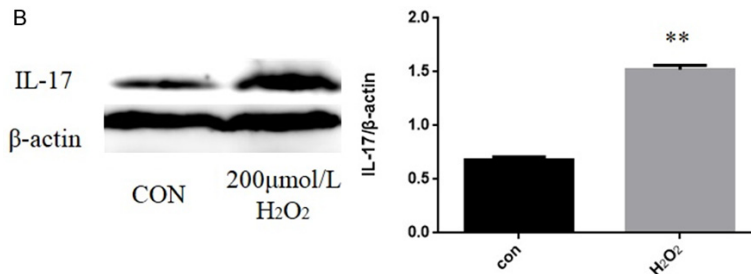


Figure 2. IL-17 was down-regulated in mice hepatocytes with siRNA. All data are shown as mean ± SEM (n=3, per group) ***P<0.001 vs control.

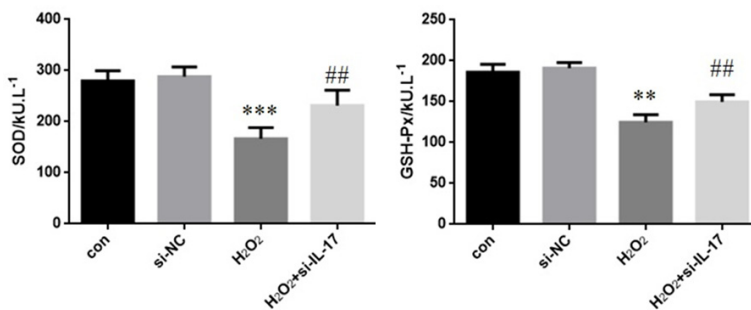


Figure 3. si-IL-17 in mice hepatocytes prevents H₂O₂-induced oxidative stress. All data are shown as mean ± SEM (n=6, per group) ***P<0.001, **P<0.01 vs control; ##P<0.01 vs H₂O₂.

Results

Increased expression of IL-17 upon H₂O₂ treatment

After applying different concentrations (100 μmol/L, 200 μmol/L, 400 μmol/L) of H₂O₂ to treated mice hepatocytes over about 4 h, cell viability was determined using MTT. The results showed that the damage of mice hepatocytes increased more with different concentrations

H₂O₂. Cells were treated with 200 μmol/L of H₂O₂ for 24 h and had a 54±4% survival rate (**Figure 1A**). Therefore, 200 μmol/L of H₂O₂ for 24 h was used in subsequent experiments. The expression of IL-17 was tested by Western blotting with different treatments. In our study, the expression level of IL-17 in mice hepatocytes treated with H₂O₂ was significantly higher than the control group (**Figure 1B**).

Down-regulation of IL-17 decreased damage of mice hepatocytes

After treatment with 200 μmol/L of H₂O₂ for 24 h, the mice hepatocytes were transfected with siRNA-IL-17. Western blotting proved the expression of IL-17 was down-regulated in si-IL-17 mice hepatocytes (**Figure 2**).

The levels SOD and GSH-Px were increased in si-IL-17 mice hepatocytes

Mice hepatocytes were evaluated by ELISA. As an indicator of lipid peroxidation, the levels of GSH-Px and SOD were analyzed (**Figure 3**). The levels of SOD and GSH-Px were decreased in H₂O₂ treatment, while SOD and GSH-Px was increased in H₂O₂+si-IL-17 group.

Apoptosis was decreased by IL-17 in mice hepatocytes treated with H₂O₂

In order to examine whether IL-17 influenced the survival of mice hepatocytes, a terminal deoxynucleotidyltransferase-mediated dUTP Nick-End Labeling (TUNEL) assay was performed (**Figure 4**). The TUNEL assay results revealed an increase in the number of TUNEL-positive mice hepatocytes by 20±4.2% in the H₂O₂ treatment compared to the control. However, in the H₂O₂+si-IL-17 group, the TUNEL-positive mice hepatocytes were decreased.

IL-17 affected Nrf2/keap1 pathway

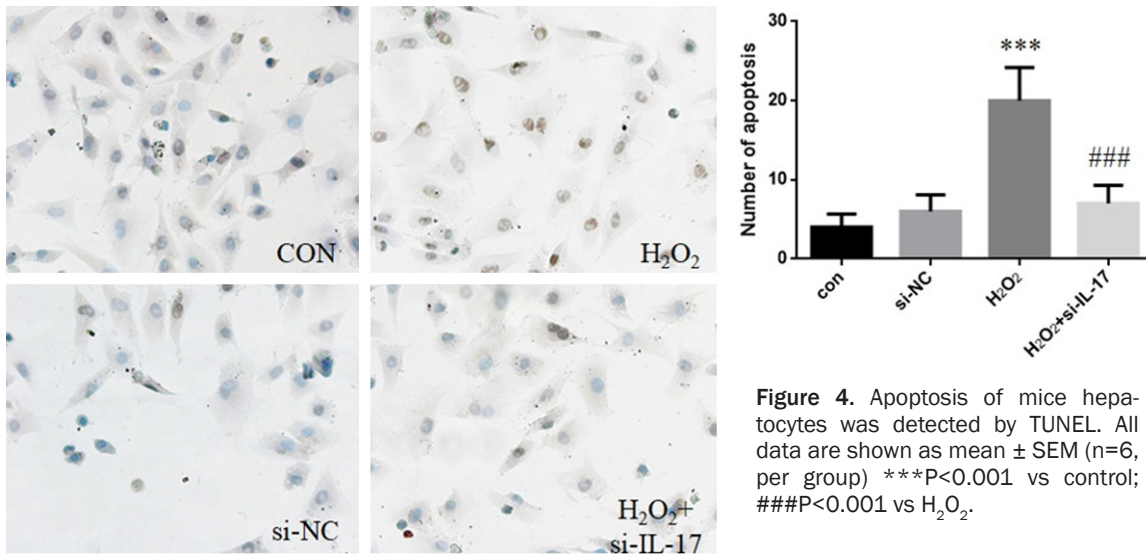


Figure 4. Apoptosis of mice hepatocytes was detected by TUNEL. All data are shown as mean ± SEM (n=6, per group) ***P<0.001 vs control; ###P<0.001 vs H₂O₂.

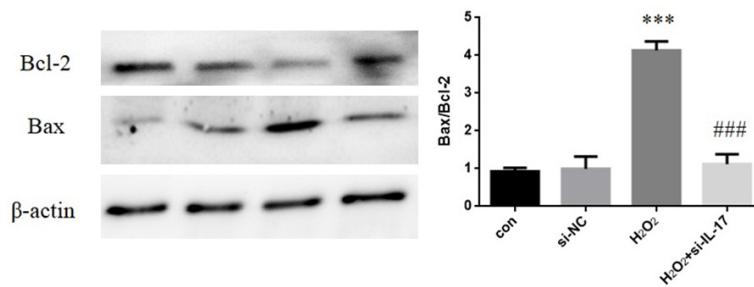


Figure 5. Expression of mice hepatocytes of apoptosis related protein Bax and Bcl-2. All data are shown as mean ± SEM (n=3, per group) ***P<0.001 vs control; ###P<0.001 vs H₂O₂.

The apoptosis related protein Bcl-2 and Bax were analyzed. In our study, the expression level of proapoptotic protein Bax (Figure 5) in H₂O₂ treatment was significantly higher than in the control and H₂O₂+si-IL-17 group, while the anti-apoptotic protein Bcl-2 (Figure 5) in the H₂O₂ treatment group did not.

IL-17 attenuated the apoptosis through the Nrf2/Keap1 pathway in mice hepatocytes

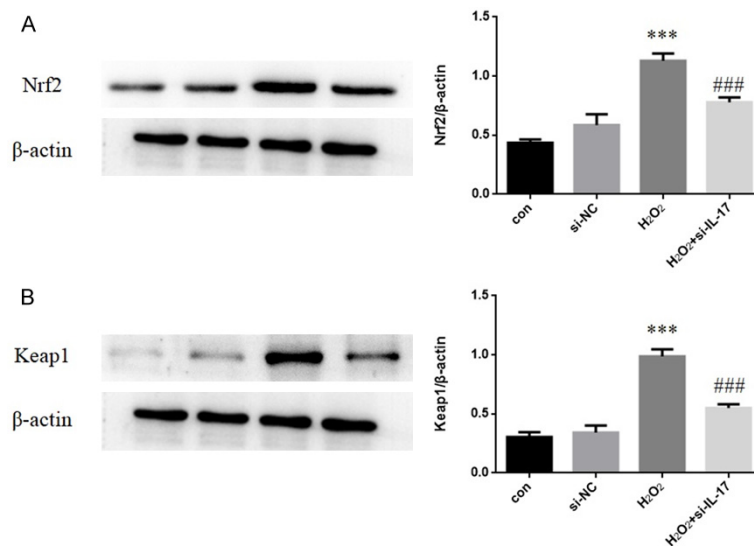


Figure 6. Expression of mice hepatocytes of Nrf2/Keap1 pathway Nrf2 and Keap1. All data are shown as mean ± SEM (n=3, per group) ***P<0.001 vs control; ###P<0.001 vs H₂O₂.

The level expressions of these proteins were evaluated by Western blotting. We have demonstrated the genes of Nrf2 and Keap1 in the H₂O₂ treatment group were increased more than those of the control group and the H₂O₂+si-IL-17 group (Figure 6).

Discussion

Hepatic diseases, such as liver cirrhosis, viral hepatitis autoimmune liver diseases, and hepatocellular carcinoma, affect billions of people worldwide [13]. These diseases are commonly associated with liver damage. The liver, as the

principal detoxifying organ in the human body, removes various pathogens, toxic chemicals and antigens from the blood, as well as metabolic waste from the circulatory system. When the liver is exposed to different types of injury, the pathological by-products lead to apoptosis or necrosis of hepatocytes. So it will become a therapeutic target for hepatic diseases to inhibit liver apoptosis.

Oxidative stress plays a critical role in hepatic diseases [14], and accumulating signs showed that a vicious cycle could be created by oxidative stress and inflammation, which promote the progression of liver diseases. Oxidative stress could elevate proinflammatory gene expression through signaling pathways such as NF- κ B, while infiltrated inflammatory cells and cytokines like IL-6 and IFN- γ could in turn produce more oxidative stress. Their tight interactions make the hepatic pathological process complicated [15]. As one of the best studied cytokines in immunology, IL-17 is involved in inflammatory pathology. An increased expression of IL-17 was found in diverse human liver diseases. We found the expression of IL-17 was increased in the H₂O₂ group.

In our research, we reduced the expression of IL-17 by RNAi to investigate the effects of down-regulated IL-17 on oxidative stress related signaling cascades. We detected the indicator of lipid peroxidation, SOD and GSH-Px by ELISA. In our research, SOD and GSH-Px were increased in the H₂O₂+si-IL-17 group.

We detected the apoptotic cells using the TUNEL assay and found an increase in the H₂O₂ group, while in the H₂O₂+si-IL-17 group, TUNEL-positive mice hepatocytes were decreased. A Western blot assay analyzed the apoptosis related protein Bax and Bcl-2. The ratio of Bax/Bcl-2 in the H₂O₂ group was significantly increased, while the expression was decreased in the H₂O₂+si-IL-17 group. It is suggested IL-17 could decrease H₂O₂ induced apoptosis.

The Nrf2/Keap1 pathway is one of pathways related to oxidative stress related apoptosis. Nuclear erythroid 2-related factor 2 (Nrf2) is a transcription factor that belongs to the Cap-n-collar basic leucine zipper family. It has a significant role in adaptive responses to oxidative stress by interacting with Kelch sample related protein-1 (Keap1) [16]. The roles of the Nrf2/

Keap1 pathway in liver diseases have been extensively investigated. In our study, Nrf2 and Keap1 were significantly up-regulated in H₂O₂ group but decreased in the H₂O₂+si-IL-17 group. These results suggest the down-regulation of IL-17 in mice hepatocytes can result in decreased oxidative stress. Therefore, the IL-17 protein affects oxidative stress related apoptosis through the Nrf2/Keap1 pathway.

In summary, we demonstrate in this study that oxidative stress related apoptosis was induced by H₂O₂ and caused mice hepatocytes apoptosis through the Nrf2/Keap1 pathway. The IL-17 protein can influence oxidative stress related apoptosis. These results provide a theoretical basis for drug therapy and pathogenesis of hepatic diseases.

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Disclosure of conflict of interest

None.

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