Hepcidin in non-alcoholic fatty liver disease regulated by the TLR4/NF-κB signaling pathway

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Abstract. The aim of our study was to analyze the role of toll-like receptor 4 (TLR4)/nuclear factor (NF)-κB signal pathway on Hepcidin regulation in non-alcoholic fatty liver disease (NAFLD). A total of 60 male Sprague-Dawley rats were randomly divided into the control, NAFLD and intervention groups. Rats in the control group were fed with standard laboratory diet, and rats in the NAFLD and intervention groups were fed with a high-fat diet. A final volume of 2 ml of pathenolide (10 μmol/l) was administered intraperitoneally only to the rats in the intervention group. The tissue sections were stained with hematoxylin and eosin and the pathological changes in liver tissues were observed and scored. The levels of TLR4 and NF-κB in liver tissues were quantified by western blotting. NAFLD rats appeared to have typical liver fatty degeneration and the expression of TLR4/NF-κB proteins and Hepcidin mRNA was significantly higher than that in the control group ($P<0.05$). However, the pathological changes observed in the intervention group had a marked improvement with a significant reduction in the TLR4/NF-κB protein and Hepcidin mRNA expression ($P<0.05$). In conclusion, the abnormal activation of the TLR4/NF-κB signaling pathway may cause NAFLD through the overexpression of Hepcidin.

Introduction

In recent years, high-fat diet due to the improvement of standards of living have led to an increase in the morbidity of non-alcoholic fatty liver disease (NAFLD), particularly in younger patients in countries such as China (1-3). NAFLD is a medical condition characterized by a series of hepatic pathological changes including simple steatosis, non-alcoholic steatohepatitis and cirrhosis (4-6).

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Hepcidin, secreted by hepatic cells, is a key regulator of the absorption of iron into the blood circulation in human. The association between disturbances in iron metabolism and the manifestation of NAFLD is well established (7). Since Hepcidin regulates iron absorption, its increased level can accelerate the development of the disease. Nuclear factor (NF)-κB is known to play a crucial role in the transformation from simple steatosis to steatohepatitis (8). The excitation of toll-like receptor 4 (TLR4) activates NF-κB through a cascade of signal transduction, which, in turn, promotes the release of transforming growth factor-β (TGF-β), ultimately inducing the necrosis of liver cells, inflammation and the formation of fibrosis (9).

The aim of the present study was to investigate the manner in which Hepcidin in NAFLD is regulated by the TLR4/NF-κB signaling pathway by observing changes in Hepcidin expression in NAFLD rats treated with pathenolide, an established NF-κB inhibitor.

Materials and methods

Animals. Sixty male Sprague-Dawley rats (200±20 g) were obtained from the Animal Center of Wuhan University (Wuhan, China) were used for the study. Following an acclimatization period, the rats were randomly divided into the control, NAFLD and intervention groups (n=20 animals per group). The rats of the control group received standard laboratory diet, the NAFLD group received high-fat diet (standard laboratory diet + 2% cholesterol + 10% lard + 2.5% vegetable oil) and the intervention group received high-fat diet and was also administered with 10 μmmol/2 ml of pathenolide intraperitoneally. The rats were maintained and fed for 28 days. After 28 days, the rats were sacrificed by cervical dislocation under ether anesthesia and a small portion of hepatic tissue from the right lobe of the liver was excised.

Chemicals. Pathenolide was purchased from Sigma-Aldrich China, Inc. (Shanghai, China). Rabbit anti-human TLR4 antibody, goat anti-mouse NF-κB antibody and rabbit anti-rat Hepcidin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-goat immunoglobulin G (IgG) was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The quantitative polymerase chain reaction (qPCR) kit was purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China).

Table I. Primer sequences used for quantitative polymerase chain reaction.

Gene	Primer sequence	Amplicon (bp)
Hepcidin	Upstream: 5'-TGTCTCCTGCTTCTCCTCCTTG-3' Downstream: 5'-GGAGGGCAGGAATAAATAATGG-3'	291
β-actin	Upstream: 5'-CCACAGCTGAGAGGGAAATC-3' Downstream: 5'-TCTCTTCCCACTCACGGGTTGIL-3'	108

Histological analysis. The excised tissues were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm sections. The microsections were stained with hematoxylin and eosin. The stained sections were observed under a microscope (Olympus CX31-LV320; Olympus, Tokyo, Japan) and the changes recorded. Fat dying was analyzed by oil red staining in which the liver cell nucleus became hyacinthine and the lipid droplets red.

Evaluation of liver histopathology. The degree of fat degeneration was evaluated by taking into account the ratio of the number of lipid droplets in the hepatic lobule/total number of cells, which was scored as: 0 (-), <1/3 (+), 1/3-2/3 (++), >2/3(+++), and =1 (++++).

Quantification of TLR4 and NF-κB. Total protein from hepatic tissue was extracted from cell lysate using TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, CA, USA). Protein concentration was determined by Coomassie Brilliant Blue Staining method. Protein (20 μg) was loaded and resolved using a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat skim milk at room temperature (25˚C). The membrane was then incubated with primary antibodies such as rabbit anti-human TLR4 polyclonal antibody and goat anti-rat NF-κB (dilution 1:500) for 12 h. Subsequently, horseradish peroxidase conjugated rabbit anti-goat IgG (dilution 1:1,000) was added and the membrane was incubated for 1 h at room temperature. Enhanced chemiluminescence reagent (Boster Biological Technology, Ltd., Wuhan, China) was used to detect hybridization signal and Quantity One® software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyze the relative amount of TLR4 and NF-κB proteins. β-actin served as the internal reference.

qPCR quantification of Hepcidin mRNA. Total RNA was extracted using TRIzol reagent. The isolated RNA was quantified spectrophotometrically at a wavelenghth of 260 nm. qPCR analysis was conducted as per the manufacturer's instructions (Takara Biomedical Technology Co., Ltd.). Obtained amplicons were subjected to agarose gel (Sigma-Aldrich Inc., Shanghai, China) electrophoresis. qPCR data analysis was performed by normalization of data to β-actin as the standard to determine the absolute quantity of starting Hepcidin gene expression. The primer sequences used to analyze the Hepcidin gene are shown in Table I.

Statistical analysis. Data were analyzed using SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA) and presented as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Table II. The relative amount of TLR4 and NK-κB proteins in the control, NAFLD and intervention groups.

Group	TLR4	NK-κB
Control	0.24±0.17	0.92±0.14
NAFLD	1.53±0.18[a]	1.63±0.12[a]
Intervention	0.31±0.11[b]	1.01±0.09[b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. NAFLD group. TLR4, toll-like receptor 4; NK-κB, nuclear factor-κB; NAFLD, non-alcoholic fatty liver disease.

Results

Visual screening of liver. The shape, texture and color of the liver in the control group appeared normal. However, in the NAFLD group, the volume of the liver had increased with an obtuse margin and the section appeared faint yellow and was slightly greasy. The appearance of liver in the intervention group was similar to the control group.

Histopathology of liver. In the control group, the structure of the hepatic lobule and hepatic cords appeared normal and the cells were arranged in an orderly manner. At the center of the cells, the nucleus was intact and the cytoplasm was well distributed without the lipid droplet (Fig. 1A). In the NAFLD group, the structure of the hepatic lobule was irregular. Diffuse and numerous round fat vacuoles of varying size with hydropic degeneration and inflammatory cell infiltration were observed. A number of cells contained large red fat vacuoles (Fig. 1B). In the intervention group, a few microvesicular fat droplets were evident (Fig. 1C).

Downregulation of TLR4 and NF-κB proteins. The levels of TLR4 and NF-κB in the NAFLD group were significantly higher when compared with the control group (P<0.05; Table II and Fig. 2). TLR4 and NF-κB levels in the intervention group were significantly decreased (P<0.05) in comparison with the NAFLD group as shown in Table II and. Fig. 2

Downregulation of Hepcidin by pathenolide. Hepcidin was found to be overexpressed in the NAFLD group as compared to the control group (P<0.05) (Fig. 3). Hepcidin was significantly

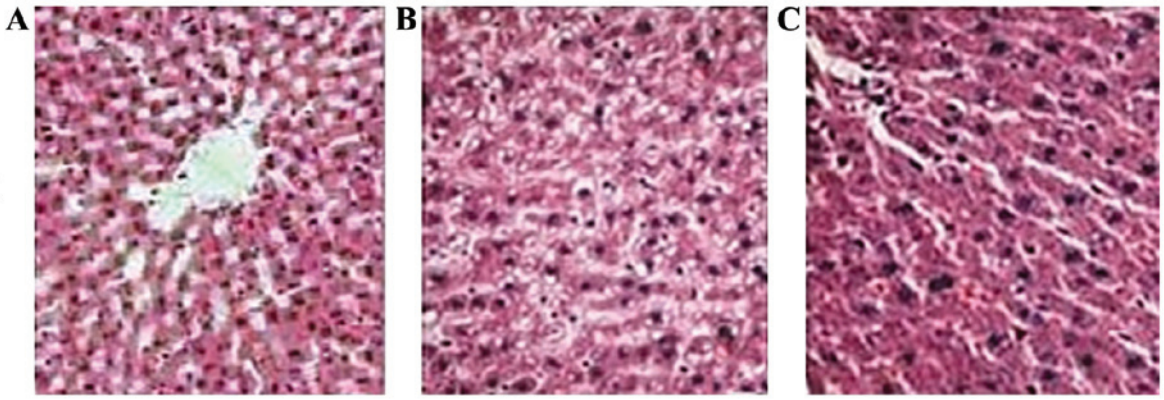

Figure 1. Histological analysis of liver biopsy. (A) Control, (B) non-alcoholic fatty liver disease and (C) intervention groups.

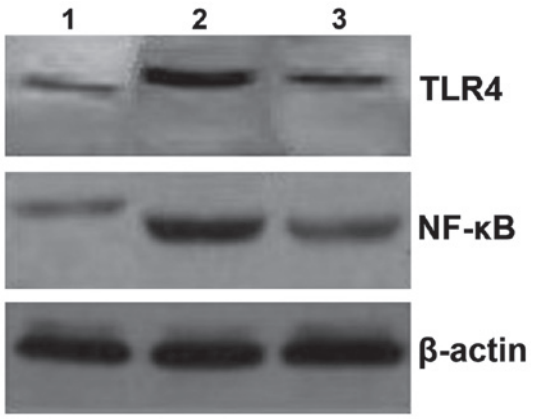

Figure 2. Western blot analysis of toll like receptor 4 (TLR4) and nuclear factor (NF)-κB. (1) Control, (2) non-alcoholic fatty liver disease and (3) intervention groups.

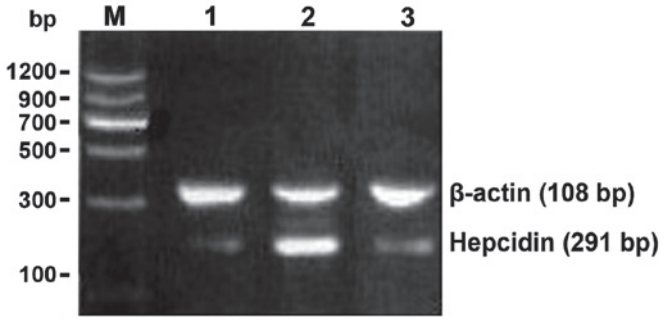

Figure 3. Quantitative polymerase chain reaction examination of Hepcidin mRNA of (1) control, (2) non-alcoholic fatty liver disease and (3) intervention groups. (M) 100 bp marker.

downregulated by pathenolide in the intervention group as compared to the NAFLD group ($P<0.05$).

Discussion

Hepcidin is a key regulatory factor of iron homeostasis (10,11) in which iron absorption and recycling is affected by the degradation of ferroprotein, thereby downregulating iron concentration in intestinal mucosa cells and macrophage cells and hepatocytes (12). Currently, iron overload has been identified in several types of chronic diseases due to imbalances in iron homeostasis. Previous findings suggested that during the transformation from simple hepatic steatosis into non-alcoholic steatohepatitis, the iron deposition in the liver gradually increases, showing a significant positive correlation with the severity of disease (13,14).

NF-κB is a crucial transcriptional regulator that regulates genes involved in the induction of inflammation. The adipose tissue is the main source of cytokines. However, the condition of liver cell steatosis promotes the release of various cytokines that have a strong proinflammatory effect such as tumor necrosis factor-α, while TGF-β plays an important role through the signaling pathway of NF-κB (15-18). Since inflammation during liver steatosis is capable of upregulating TLR4/NF-κB, which in turn leads to the overexpression of Hepcidin, this may be a possible reason for the accumulation of excess iron in the liver of NAFLD patients (19,20).

The present study has demonstrated that the NAFLD group exhibited typical steatosis. However, following intervention with the NF-κB inhibitor, Pathenolide, the pathological changes attributed to NAFLD had reversed to normal. Similarly, the expression of TLR4,/NF-κB proteins and Hepcidin mRNA in NAFLD was higher than that of the control group. However, the expression of TLR4/NF-κB proteins and Hepcidin mRNA were decreased close to the values of the control, indicating that, inhibiting the TLR4/NF-κB pathway can repress the expression of Hepcidin, thus reverting pathological characteristic changes of NAFLD.

In conclusion, inhibition of the activation of the TLR4/NF-κB pathway using pathenolide, downregulated the expression of Hepcidin. This findings is crucial in the prevention and treatment of steatohepatitis and repression of the pathological process of liver fibrosis in NAFLD.

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