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Original Article

The involvement of NLRX1 and NLRP3 in the development of nonalcoholic steatohepatitis in mice

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Abstract

Background: Increasing evidence suggests that innate immunity is involved in the development of nonalcoholic fatty liver disease. Nod-like receptors (NLRs) have recently been identified as key mediators of inflammatory and immune responses. The aim of this article is to explore the correlation of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)X1 and NLRP3 with nonalcoholic steatohepatitis (NASH) in mice.
Methods: In our study, a high-fat diet, lipopolysaccharides (LPSs), and normal diet were given to C57BL mice to establish high fat (HF), HF + LPS, and control groups. Thereafter, serum alanine and aspartate aminotransferase (ALT and AST) levels were measured, and NASH severity was histologically examined. We measured tumor necrosis factor (TNF)-α levels by enzyme-linked immunosorbent assay, protein expression by Western blotting, and mRNA expression by real-time fluorescent quantitative reverse transcription-polymerase chain reaction.
Results: Levels of ALT and AST were higher in HF + LPS mice than in HF mice ($p < 0.05$). NLRX1 mRNA and protein expression was lower in HF and HF + LPS mice than in control mice ($p < 0.05$). NLRP3 mRNA expression was higher in HF and HF + LPS mice than in control mice ($p < 0.05$). The mRNA and protein expression of TNF receptor-associated factor (TRAF)6, interleukin-1β, caspase-1, and apoptosis-associated speck-like protein were significantly higher in HF + LPS mice than in control and HF mice; furthermore, mRNA expression was higher in HF mice than in control mice ($p < 0.05$), but protein expression was similar.
Conclusion: NLRX1 and NLRP3 inflammasomes may be important in NASH development.

Keywords: C57BL mouse; NLRP3; NLRX1; nonalcoholic steatohepatitis

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 10–24% of the general population in various countries and is therefore an important public health problem.[1] Chronic, low-grade, systemic inflammatory responses have been associated with obesity and insulin resistance (IR), and increased levels of inflammatory cytokines can impair insulin action via antagonistic signals,[2] thereby facilitating the onset and development of nonalcoholic steatohepatitis (NASH). In recent years, great advances have been made in our understanding of the function and mechanism of innate immune responses in pathological processes such as autoimmune diseases, bacteria-free inflammation, and chronic inflammatory response. In particular, the pattern recognition mechanism and downstream regulatory networks of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) have attracted considerable attention. NLRs form a cytoplasmic protein family and were recently discovered to play a role in inflammatory responses; these molecules include important innate immune system receptors [e.g., Toll-like receptors (TLRs)] that form a defense against pathogens. NLRs actively participate in pathogen-associated molecular patterns and detect danger-associated molecular patterns, thus enabling relevant inflammatory responses.

NLRX1, a member of the NLR family, has been recently found to bind to tumor necrosis factor (TNF) receptor-

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associated factor-6 (TRAF6) to inhibit the downstream nuclear factor-kappaB (NF-κB) pathway, thus acting as a negative regulator of excessive inflammatory responses.[3] NLRP3, another typical representative of the NLR family, is a macromolecular protein complex with a molecular weight of 700 kDa; NLRP3 along with apoptosis-associated speck-like protein (ASC) and caspase-1 constitute NLRP3 inflammasomes. These function as receptors of exogenous microorganisms or endogenous danger signals in the cytoplasm. Activation of NLRP3 inflammasomes can regulate the maturation and secretion of proinflammatory cytokines, including interleukin (IL)-1β, IL-18, and IL-33.[4]

Chitturi and Farrell[5] reported that 98% of NAFLD patients had associated IR, which is an important element in the development of NASH. Inflammatory factors such as NF-κB, IL-1β, and IL-18 are closely related to IR, and the activation of these factors is regulated by NLRX1 and NLRP3. Therefore, we hypothesized that NLRX1 and NLRP3 inflammasomes are involved in the onset and development of NASH. To verify this hypothesis, we established a mouse model of NASH to investigate the correlation of NLRX1 and NLRP3 with NAFLD.

2. Methods

2.1. Animals

Fifteen specific-pathogen-free male C57BL6 mice 4 weeks of age and weighing 12–16 g were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were fed in a clean-grade barrier systems laboratory in the Laboratory Animal Centre, with the Shanghai Jiao Tong University College of Medicine. All experiments with animals were performed according to the guidelines of the Ethical Committee in China. All studies were performed with the approval of the Experimental Animal Committee of Shanghai Changning Central Hospital, Shanghai, China.

2.2. Reagents

We bought lipopolysaccharide (LPS) from Sigma-Aldrich (St. Louis, MO, USA); NLRP3, caspase-1, ASC, and β-actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); NLRX1 and TRAF6 antibodies from Abcam (Cambridge, UK); Trizol from Invitrogen (Carlsbad, CA, USA); polymerase chain reaction (PCR) mix kit and primer probe sequences from Daweike Biotechnology Co. Ltd. (Shanghai, China; Table 1); enzyme-linked immunosorbent assay (ELISA) kit and immunohistochemistry (IHC) primary anti-IL-6 antibody from Perseus Proteomics Inc. (Tokyo, Japan); secondary antibody kit [Elivision plus polymer horseradish peroxidase (Mouse/Rabbit) IHC kit] and 4-dimethylaminoazobenzene (DAB) chromogenic agent from Maixin Biology Co. Ltd. (Fuzhou, China); and a real-time fluorescent quantitative PCR device (7500 Sequence Detection System) from Applied Biosystems Inc. (Foster City, CA, USA).

Table 1
Primer probe sequences.

mRNA	Sequence (5′–3′)
NLRP3	F: TATCAAGCCCTCCTTCACCATC
	R: CCTCTGGCAGTTTCACGTTATC
ASC	F: GCTCTGCCTCCTGAGTTATG
	R: TGCCCTCTTCTGGCTTTG
Caspase-1	F: CGTCTTGCCCTCATTATC
	R: CACCTCTTTCACCATCTC
NLRX1	F: GGGTTGCAACTCAACAATCT
	R: TGTTGGAGAGCCTAAGAGTG
TRAF6	F: TCCAGGGACTGGTTTAACCC
	R: GCCGCCTGGAGCATATTTCA
IL-1β	F: TAGGCTCATCTGGGATCCTC
	R: AAAAGGTGGCATTTCACAGT
β-Actin	F: GTCGTACCACTGGCATTGTG
	R: GCTGTGGTGGTGAAGCTGTA

ASC = apoptosis-associated speck-like protein; IL-1β = interleukin-1β; NLRX1 = nucleotide-binding oligomerization domain (NOD)-like receptor X1; TRAF6 = tumor necrosis factor (TNF) receptor-associated factor-6.

2.3. Animal model and groups

The experimental animals were randomly assigned to three groups of five mice each: control group, HF (high-fat diet) group, and HF + LPS (high-fat diet + intraperitoneal LPS injections) group. The control group mice were fed a normal diet, and the HF and HF + LPS group mice were fed an HF diet for 12 weeks. In Week 12, the HF + LPS group mice were intraperitoneally injected with 10 mg/kg LPS. At the end of 12 weeks, all mice were sacrificed by cervical spine damage 12 hours after fasting. Thereafter, 1 mL blood was extracted from their eyeballs, and their liver tissues were quickly harvested. The harvested tissues were gently flushed with normal saline in an ice bath and partially fixed with 10% formaldehyde solution; a part of the tissues was rapidly stored at −70 °C.

2.4. Biochemical assays

The obtained blood was sent to the hospital laboratory for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. The liver tissues were embedded in paraffin after being fixed in formaldehyde for 24 hours and cut into 4-μm slices for hematoxylin and eosin (H&E) staining and immunohistochemical staining or stored under cryogenic conditions for Western blot and PCR examination.

2.5. Diet

Normal diet was provided by the Basic Medical School Animal Center, which is affiliated with the Shanghai Jiao Tong University School of Medicine. The HF diet (50% fats from

18% lard, 12% egg yolk, 8% sugar, and 62% basic diet) was purchased from Slack Company (Shanghai, China).

2.6. Histological examination

Liver tissue samples were taken from the same position for H&E staining, fixed with 10% formaldehyde, paraffin-embedded, H&E stained, and observed under a microscope. Hepatic steatosis was classified into the following, depending on the histological appearance of the tissue samples under a low-power lens: mild, cells with hepatic steatosis accounted for 1/3 of hepatic lobules; moderate, cells with hepatic steatosis accounted for 1/3–2/3 of hepatic lobules; and severe, cells with hepatic steatosis accounted for more than 2/3 of hepatic lobules.

2.7. Real-time PCR

We determined the mRNA expression levels of NLRX1, TRAF6, IL-1β, NLRP3, caspase-1, and ASC by using real-time fluorescent quantitative PCR. Total RNA was extracted using Trizol, the purity and concentration of which were determined with an ultraviolet spectrophotometer. Reverse transcription was used to convert 2 μg total RNA to cDNA. The SYBR Green I dye method was applied. The reverse transcription products were subjected to amplification of the β-actin, NLRX1, TRAF6, IL-1β, NLRP3, caspase-1, and ASC genes. The mRNA expression levels and gray levels in all groups were compared using an automatic gel-imaging analysis system after gel electrophoresis of the PCR products. All results were normalized using the results for β-actin. The primer sequences and fragments are shown in Table 1. PCR was performed under the following conditions: initial denaturation at 50°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 30 seconds. β-Actin was used as an internal reference and was subjected to an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds; a repeat elongation was performed at 72°C for 10 minutes, with a final cycle at 4°C. Relative mRNA expression was calculated using the formula relative level $= 2 - \Delta ct \times 100\%$.

2.8. Western blot analysis

The protein expression levels of NLRX1, TRAF6, NLRP3, caspase-1, and ASC were determined with Western blotting. Liver tissues were refrigerated at −80°C immediately after being harvested, and histone extracts were prepared using conventional homogenization and centrifugation. The protein concentration was determined using the BCA protein quantitative kit (SunBio Biomedical Technology Co. Ltd., Beijing, China), according to the manufacturer's instructions. Each histone sample (50 μg loading sample) was denaturated and then subjected to reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis, with 8% polyacrylamide gels. We used a concentrated gel electrophoresis voltage of 80 V and a separating gel electrophoresis voltage of 120 V. Electrophoresis was performed with bromophenol blue until the gel bottom was visible; a semidry transmembrane (polyvinylidene difluoride membrane) was subjected to a constant current of 50 mA for 90 minutes. The membrane was sealed with 5% skim milk powder prepared using Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 hour. Next, primary antibodies were added to the membrane, which was then kept overnight in a table concentrator at 4°C. Secondary antibodies (1:3000) were added after the membrane was washed with TBST in the table concentrator. The membrane was incubated at room temperature for 1 hour, repeatedly flushed with TBST, and subjected to DAB coloration (using a DAB chromogenic agent kit, in which the reaction was terminated with water). In a dark room, the nitrocellulose membrane was brought into full contact with an illuminant and exposed using X-rays. The films were developed and fixed with conventional methods. The β-actin expression level of each membrane was determined and used as an internal standard.

2.9. ELISA

Liver tissue (1 g) was added to phosphate-buffered saline and 0.1 mmol/L phenylmethylsulfonyl fluoride, adequately grinded in a homogenizer, and centrifuged at 4°C at 100,000 rpm for 15 minutes. The supernatant was subjected to double antibody-sandwich ELISA and ELISA was performed according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analysis was performed SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Variables are expressed as $\overline{X} \pm S$. Single factors were assessed using analysis of variance, and groups were compared using the least significant difference and Student–Newman–Keuls method. All statistical tests were two-sided probability tests ($\alpha = 0.05$). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Biochemical assays

Serum ALT levels were significantly higher in the HF and HF + LPS groups than in the control group ($p < 0.05$; Fig. 1A). The serum AST level did not significantly differ between the HF and control groups, but was significantly greater in the HF + LPS group than in the control group ($p < 0.01$; Fig. 1B).

3.2. Histological examination

The samples from the control group showed clear lobular structure, no inflammatory cell infiltration of the portal area, and no proliferation of collagen fibers. The HF group showed

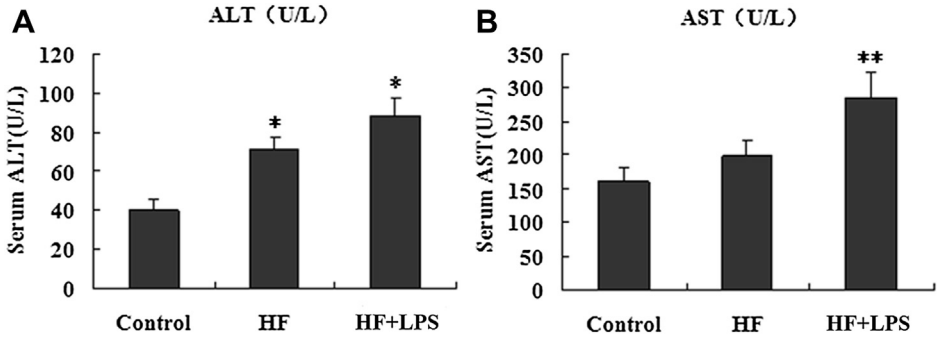

Fig. 1. (A) Serum alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) levels in HF + LPS, high-fat diet (HF), and control groups. *$p < 0.05$, compared to the control group.

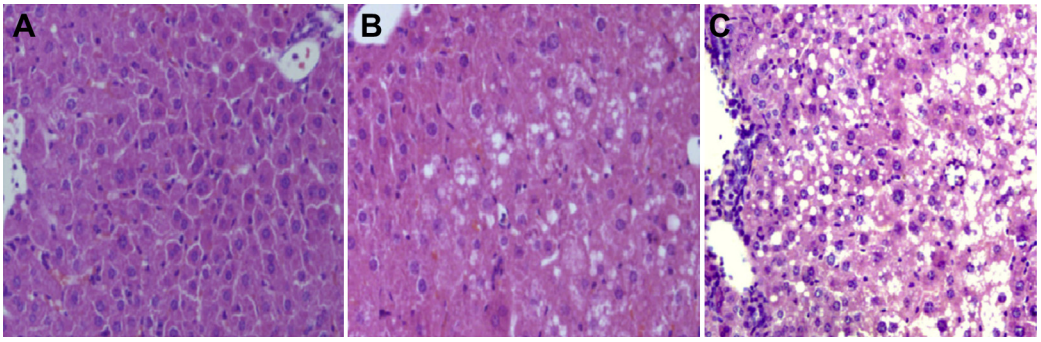

Fig. 2. Histopathological changes in mouse liver on hematoxylin and eosin staining (200×). (A) Control group, (B) HF group, and (C) HF + LPS group.

liver cell steatosis, but no inflammatory cell infiltration and collagen fiber proliferation in the portal area. The HF + LPS group showed sever liver cell steatosis with numerous inflammatory cells infiltrating the portal area. These changes were indicative of NASH (Fig. 2).

3.3. ELISA

Compared to the control group, the HF and HF + LPS groups had significantly greater TNF-α levels ($p < 0.05$; Fig. 3).

3.4. Real-time PCR

NLRX1 mRNA expression was lower in the HF and HF + LPS groups than in the control group ($p < 0.01$), whereas NLRP3 mRNA expression was higher in the HF and HF + LPS groups than in the control group ($p < 0.05$). The expression levels of TRAF6, IL-1β, caspase-1, and ASC mRNA in the NASH group were much higher than those in the control and HF groups ($p < 0.01$), and the expression levels in the HF group were higher than those in the control group ($p < 0.05$; Fig. 4). Hepatic steatosis progressively worsened with time and eventually devolved into steatohepatitis. This pathological process was associated with a significant increase in TRAF6, IL-1β, NLRP3, caspase-1, and ASC mRNA expression and an obvious decrease in NLRX1 mRNA expression.

3.5. Western blotting

NLRX1 expression levels were lower ($p < 0.05$; Fig. 5) in the HF and HF + LPS groups than in the control group. TRAF6, NLRP3, caspase-1, and ASC protein expression in the HF + LPS group was considerably higher than that in the control group ($p < 0.01$) and the HF group ($p < 0.05$); however, the expression in the HF group did not differ from that in the control group ($p > 0.05$). In HF + LPS models, the protein expression levels of NLRP3, caspase-1, and ASC increase and

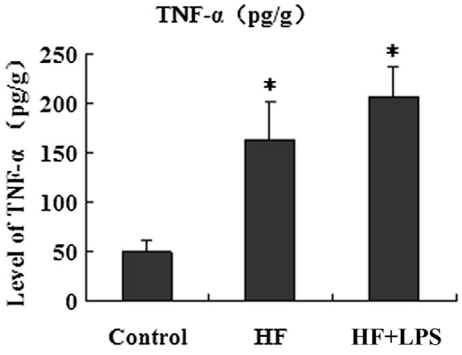

Fig. 3. Hepatic tumor necrosis factor-α (TNF-α) levels measured using enzyme-linked immunosorbent assay (ELISA). *$p < 0.05$, compared to the control group. HF = high-fat diet; HF + LPS = high-fat diet + intraperitoneal LPS injections.

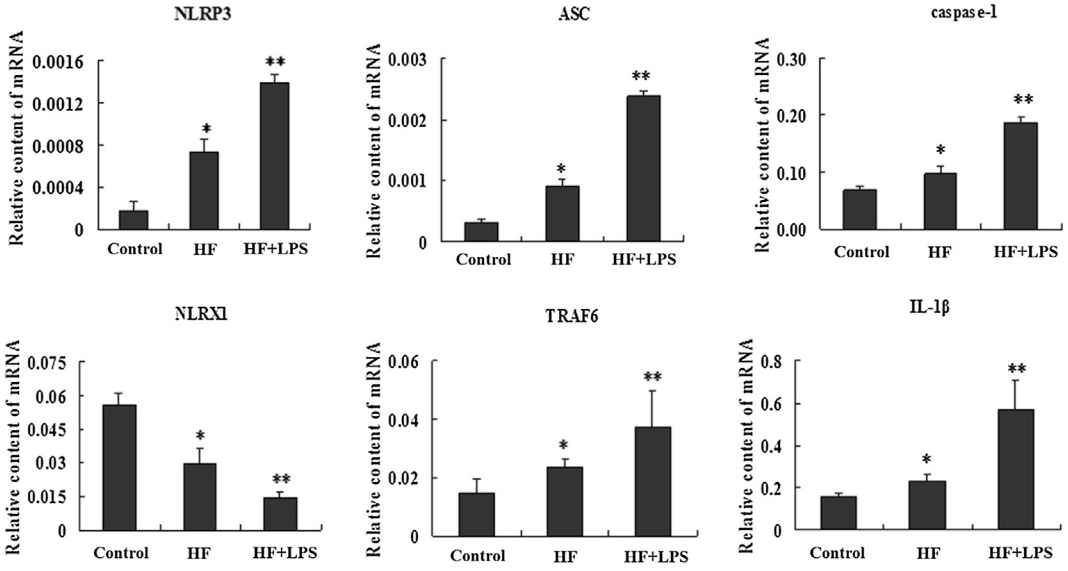

Fig. 4. Hepatic expression levels of NLRP3, caspase-1, and apoptosis-associated speck-like protein (ASC) mRNA assessed using fluorescent quantitative polymerase chain reaction (PCR). *$p < 0.05$, compared to the control group; **$p < 0.01$, compared to the control and high-fat diet (HF) groups. IL-1β = interleukin-1β; NLRX1 = nucleotide-binding oligomerization domain (NOD-like receptor X1; TRAF6 = tumor necrosis factor (TNF) receptor-associated factor-6).

exceed the expression levels in the control group and in the HF group when the hepatic steatosis deteriorates to steatohepatitis.

4. Discussion

With the recent increase in the incidence of NASH, this condition has become the most important cause of cryptogenic cirrhosis.[6] The pathogenesis of NASH is complex and involves the interaction of genetic predisposition with multiple metabolic disorders. Although the pathophysiological basis of NASH is known to be IR and oxidative stress, a satisfactory theory that explains all the clinical phenomena of NASH has yet to be found.[7] Hepatic steatosis and the associated IR are now widely believed to be major factors in the development of fatty liver diseases.[8] Therefore, all factors that affect IR may be indispensable to the development of NAFLD. Intraperitoneal LPS injections are used to promote inflammatory responses in order to successfully establish liver-damage models with typical pathological features of NASH, such as significant ALT elevation.[9] These models show ALT levels that are twice the normal value, moderate-to-severe steatosis, visible inflammatory infiltration of hepatic lobules, and partial

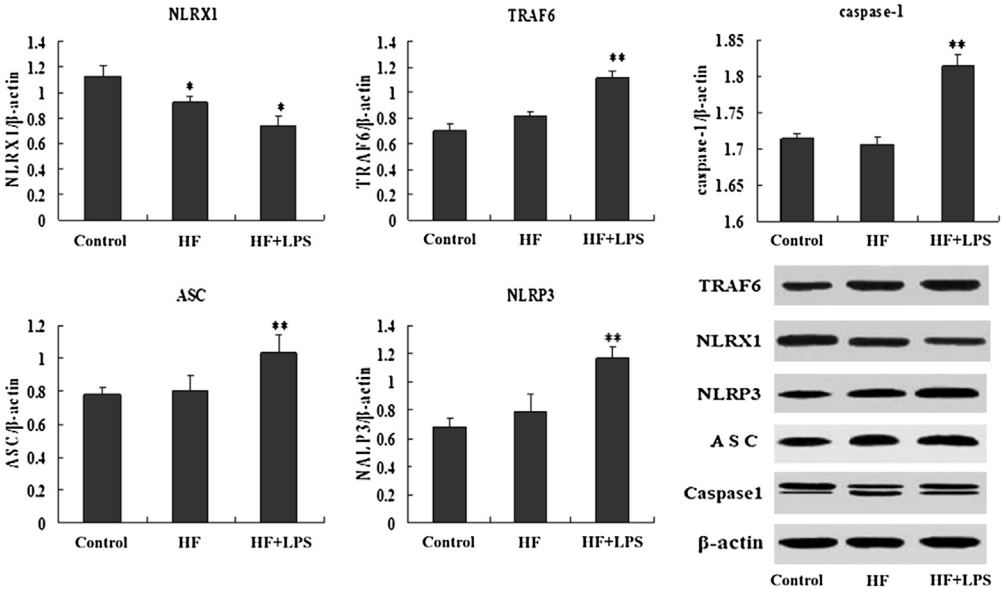

Fig. 5. NLRP3, caspase-1, and apoptosis-associated speck-like protein (ASC) protein expression assessed using Western blotting of mouse liver samples. (A) Control group, (B) HF group, and (C) HF + LPS group. *$p < 0.05$, compared to the HF group, **$p < 0.01$, compared to the control group. β-Actin was used as an internal reference. IL-1β = interleukin-1β; NLRX1 = nucleotide-binding oligomerization domain (NOD)-like receptor X1; TRAF6 = tumor necrosis factor (TNF) receptor-associated factor-6.

inflammatory infiltration of the portal area; all these changes indicate successful establishment of the NASH model. The experimental findings are similar to Ma et al's[10] research. They used an animal model of a diet-induced fatty liver: mice fed a high-fat diet develop obesity, steatosis, and IR similar to those seen in humans. Under normal, pathogen-free conditions, HF-fed mice do not develop spontaneous steatohepatitis. However, these mice are unusually susceptible to a small dose of exogenous LPS and develop liver inflammation when exposed to LPS.[10] IR and inflammatory responses, the major pathological changes in NASH,[11] modulate NF-κB-dependent signaling pathways through a number of cytokines such as TNF and IL-6.[12] Because adipose tissue is the main source of cytokines, hepatic steatosis may promote macrophage infiltration and thereby promote the release of numerous cytokines with potent proinflammatory action, which further aggravates IR.[13] Our results conform to the above theory: TNF-α and NF-κB p65 protein expression levels were higher in the HF and HF + LPS groups than in the control group.[14] The NLR family and its polyproteolytic complexes (inflammasomes),[15] as receptors of the innate immune system, can detect various danger signals and regulate inflammatory responses by inducing cell activation and releasing cytokines and inflammatory mediators. Thus far, 23 protein molecules belonging to the NLR family have been found in humans, and 35 molecules have been found in mice.[16]

NLRP3, a member of the NLR family, can attract ASC at the N-terminal pyrin domain (PYD) and attract pro-caspase-1 via ASC to form NLRP3 inflammasomes. These inflammasomes play an important role in noninfectious inflammatory diseases. Preliminary results have indicated that NLRP3 inflammasomes are involved in the onset and development of acute and chronic noninfectious inflammatory diseases such as type 2 diabetes, gout, and some kidney diseases.[17–20] Ligands of NLRP3 inflammasomes, including microbes, microbial toxins, and intracellular danger signals, trigger different inflammasome activation mechanisms. Thus far, three activation mechanisms have been discovered: outflow of potassium ions, lysosomal rupture due to ligands, and generation of ligand-mediated reactive oxygen species (ROS).[16,21] NLRP3 inflammasomes precisely regulate caspase-1 activation, which is a crucial step in the production and secretion of important proinflammatory cytokines such as IL-1β, IL-18, and IL-33.[22] IL-1β can induce its own expression as well as the expression of other proinflammatory cytokines, adhesion molecules, and chemokines such as IL-6, IL-8, and TNF-α. Pro-IL-1β synthesized from inactive precursors must undergo caspase-1-mediated digestion to form biologically active mature IL-1β.[23] Studies have shown that NLRP3 inflammasomes regulate the activation of IL-1β and IL-18, which are closely related to IR.[24,25] IL-1α and IL-1β gene knockout significantly inhibited the progression of simple steatosis to steatohepatitis in a diet-induced, wild-type mouse NASH model; thus, liver IL-1α and/or IL-1β may be therapeutic targets to inhibit the progression of simple steatosis to NASH.[25] The expression levels of NLRP3, caspase-1, and ASC mRNA greatly increase with time, and the corresponding protein expression levels are significantly higher in the HF + LPS group than in the HF and control groups when hepatic steatosis progresses to steatohepatitis, indicating that NLRP3 inflammasomes influence NASH onset and development.

Injection of IL-1 receptor blockers or IL-Trap, a new-generation IL-1β antagonist,[26] can inhibit excessive IL-1β secretion caused by NLRP3 imbalance, and may thus be used to treat related genetic or acquired diseases, including arthritis,[27] cryopyrin-related periodic fever syndrome, gout, and type 2 diabetes.[28] This finding suggests that NLRP3 inflammasomes/IL-1β could be used as a new target for treating NASH. In addition to its involvement in the inflammasome signaling pathway, NLRP3 can also activate NF-κB and induce the classic NF-κB signaling pathway.[29] Lamkanfi et al[30] found that caspase-1 can activate NF-κB synchronously with IL-1β maturation. Negative regulatory mechanisms prevent innate immune responses that seriously damage tissues and somatic cells. A number of NLR molecules have been found to be involved in such immune regulation,[31] and NLRX1 is one important member of the family. Recent studies have shown that NLRXI combined with TRAF6 inhibits the downstream NF-κB pathway, as a negative regulator of excessive inflammatory responses.[3] NLRX1 is expressed on the mitochondrial outer membrane. The N-terminal of the nucleotide-binding domain can combine with the caspase activation and recruitment domain area of the mitochondrial antiviral signaling (MAVS) protein.[32] Increased cellular NLRX1 can reduce the activity of the retinoic acid-inducible gene-1 (RIG-I) and melanoma differentiation-associated gene-5 (MDA5)-mediated interferon (IFN) pathway. Similarly, NLRX1 inhibition by, for example, vesicular stomatitis virus infection can increase the levels of type I IFN, but a lack of the leucin-rich repeat area at the C-terminal of NLRX1C can lead to a failure of this function.[32]

Xia et al[33] reported that LPS stimulation can rapidly induce NLRX1 protein ubiquitination, NLRX1 dissociation from TRAF6, and interaction with inhibitor κB kinase complexes to ultimately inhibit TLR-triggered NF-κB activation. Allen et al[3] reported that NLRX1 not only negatively regulates LPS-induced TRAF6-mediated activation of the NF-κB signaling pathway, but also regulates the influenza virus-induced RIG-I–MAVS pathway, thereby inhibiting the type I IFN-mediated antiviral immune response. Our results show that NLRX1 mRNA expression is significantly reduced in steatohepatitis, and thus, NLRX1 protein expression was lower in the HF + LPS group than in the HF and control groups. This finding indicates that the onset and development of NASH is associated with significantly decreased NLRX1 mRNA and protein expression and inhibition of the negative regulatory function of NLRX1.

TRAF, a genetically conservative, cytoplasmic signal-transduction molecule, directly combines with the intracellular domains of cell surface receptors, affecting cell survival, proliferation, differentiation, and death, and participates in the regulation of multiple biological processes. TRAF6, as a regulator of the LPS/TLR4 downstream signaling pathways, can modulate several signaling pathways [TNF-α, IL-1, and receptor activator of nuclear factor-κB ligand (RANKL)

pathways],[34] and activate transcription factors such as NF-κB, activating protein-1, and protein kinase B/Akt, thus mediating innate/acquired immune and inflammatory responses.[35−37] We found that NLRX1 mRNA and protein expression were significantly reduced, the negative regulatory function of NLRX1 was inhibited, and TRAF6 mRNA and protein expression were significantly increased in the HF + LPS group, compared to the HF and control groups. No report has been found on the correlation of NLRX1 and NLRP3 with NASH in the existing literature. This experiment indicates that NLR family members (NLRX1 and NLRP3 inflammasomes) may be important factors affecting the onset and development of NASH. This study provided an experimental basis for further investigation into the role of NLR molecules in NASH. NLRX1 and NLRP3 inflammasome regulation are potential new targets of NAFLD treatment, worthy of further study.

Acknowledgments

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References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;**346**:1221−31.
2. Maedler K, Dharmadhikari G, Schumann DM, Størling J. Interleukin-1 beta targeted therapy for type 2 diabetes. *Expert Opin Biol Ther* 2009;**9**:1177−88.
3. Allen IC, Moore CB, Schneider M, Lei Y, Davis BK, Scull MA, et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I−MAVS and TRAF6−NF-κB signaling pathways. *Immunity* 2011;**34**:854−65.
4. Leemans JC, Cassel SL, Sutterwala FS. Sensing damage by the NLRP3 inflammasome. *Immunol Rev* 2011;**243**:152−62.
5. Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin Liver Dis* 2001;**21**:27−41.
6. Kallwitz ER, McLachlan A, Cotler SJ. Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. *World J Gastroenterol* 2008;**14**:22−8.
7. Adams LA, Angulo P, Lindor KD. Nonalcoholic fatty liver disease. *CMAJ* 2005;**172**:899−905.
8. Duvnjak M, Lerotić I, Barsić N, Tomasić V, Virović Jukić L, Velagić V. Pathogenesis and management issues for non-alcoholic fatty liver disease. *World J Gastroenterol* 2007;**13**:4539−50.
9. Csak T, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology* 2011;**54**:133−44.
10. Ma X, Hua J, Mohamood AR, Hamad AR, Ravi R, Li Z. A high-fat diet and regulatory T cells influence susceptibility to endotoxin-induced liver injury. *Hepatology* 2007;**46**:1519−29.
11. Arkan MC, Hevener AL, Greten FR. IKK-beta links inflammation to obesity induced insulin resistance. *Nat Med* 2005;**11**:191−8.
12. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;**214**:199−210.
13. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003;**112**:1821−30.
14. Wei J, Shi M, Wu WQ, Xu H, Wang T, Wang N, et al. IκB kinase-beta inhibitor attenuates hepatic fibrosis in mice. *World J Gastroenterol* 2011;**17**:5203−13.
15. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular plat form triggering activation of inflammatory caspases and processing of pro-IL-beta. *Mol Cell* 2002;**10**:417−26.
16. Schroder K, Tschopp J. The inflammasomes. *Cell* 2010;**140**:821−32.
17. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 2006;**440**:237−41.
18. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 2010;**11**:136−40.
19. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 2010;**327**:296−300.
20. Iyer SS, Pulskens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, et al. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci USA* 2009;**106**:20388−93.
21. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* 2010;**10**:210−5.
22. Mariathasan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol* 2007;**7**:31−40.
23. Fantuzzi G, Dinarello CA. Interleukin-18 and interleukin-1 beta: two cytokine substrates for ICE (caspase-1). *J Clin Immunol* 1999;**19**:1−11.
24. Netea MG, Joosten LA, Lewis E, Jensen DR, Voshol PJ, Kullberg BJ, et al. Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nat Med* 2006;**12**:650−6.
25. Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, Arbel Y, et al. Lack of interleukin-1alpha or interleukin-1beta inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. *J Hepatol* 2011;**55**:1086−94.
26. Kalliolias GD, Liossis SN. The future of the IL-1 receptor antagonist anakinra: from rheumatoid arthritis to adult-onset Still's disease and systemic-onset juvenile idiopathic arthritis. *Expert Opin Investig Drugs* 2008;**17**:349−59.
27. Verma D, Lerm M, Blomgran Julinder R, Eriksson P, Söderkvist P, Särndahl E. Gene polymorphisms in the NALP3 inflammasome are associated with interleukin-1 production and severe inflammation: relation to common inflammatory diseases? *Arthritis Rheum* 2008;**58**:888−94.
28. Hoffman HM. Rilonacept for the treatment of cryopyrin-associated periodic syndromes (CAPS). *Expert Opin Biol Ther* 2009;**9**:519−31.
29. O'Connor Jr W, Harton JA, Zhu X, Linhoff MW, Ting JP. Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-kappa B suppressive properties. *J Immunol* 2003;**171**:6329−33.
30. Lamkanfi M, Kalai M, Saelens X, Declercq W, Vandenabeele P. Caspase-1 activates nuclear factor of the kappa-enhancer in B cells independently of its enzymatic activity. *J Biol Chem* 2004;**279**:24785−93.
31. Pinheiro AS, Eibl C, Ekman-Vural Z, Schwarzenbacher R, Peti W. The NLRP12 pyrin domain: structure, dynamics, and functional insights. *J Mol Biol* 2011;**413**:790−803.
32. Moore CB, Bergstralh DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, et al. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature* 2008;**451**:573−7.
33. Xia X, Cui J, Wang HY, Zhu L, Matsueda S, Wang Q, et al. NLRX1 negatively regulates TLR-induced NF-κB signaling by targeting TRAF6 and IKK. *Immunity* 2011;**34**:843−53.
34. Doyle SL, O'Neill LA. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem Pharmacol* 2006;**72**:1102−13.
35. Wu H, Arron JR. TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology. *Bioessays* 2003;**25**:1096−105.
36. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. *Bone* 2007;**40**:251−64.
37. Wang C, Deng L, Hong H, Akkaraju GR, Inoue J, Chen ZJ. TAKI is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 2001;**412**:346−51.