Key molecular pathways in the progression of non-alcoholic steatohepatitis

J. TONG, J.-J. GUO

Department of Gastroenterology and Hepatology, Chongqing University Central Hospital (Chongqing Emergency Medical Center), Chongqing, China

Abstract. – Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide and also become an emerging risk factor for liver-related complications, such as cirrhosis and hepatocellular carcinoma (HCC). The liver-related burden of NASH is likely to increase and nonalcoholic steatohepatitis (NASH) is probably to be the leading indication for liver transplantation by 2020, as a consequence of increased disease prevalence and of the lack of an effective treatment. The first step in the NAFLD development is represented by fat accumulation in the liver, a condition that is commonly associated with features of the metabolic syndrome. Notably, it has been acknowledged that the step from nonalcoholic fatty liver (NAFL) to NASH is key step in the NASH formation, and the mechanisms behind this transition have been extensively studied. Emerging evidence indicates that innate immunity is a driving force in NAFLD progression because it directly regulates all key pathogenic features of the disease processes, including metabolic dysregulation, inflammation, and fibrosis. In this review, we summarize the currently available signaling pathways of NASH formation, including oxidative stress, NOD-like receptors (NLRs), mitochondria-associated pathways, Toll-like receptors (TLRs), nuclear receptors, and other signal pathways, for the aim of a better understanding of this disease.

Key Words: Non-alcoholic steatohepatitis, NOD-like receptors, Mitochondria.

Introduction

Nonalcoholic fatty liver disease (NAFLD), which has become the most common chronic liver disease worldwide, becomes a recognized risk factor for liver-related complications, such as cirrhosis and hepatocellular carcinoma (HCC). The histological spectrum of NAFLD encompasses simple steatosis (nonalcoholic fatty liver, NAFL) and non-alcoholic steatohepatitis (NASH), and the NASH has the probably potential to progress to cirrhosis, and even HCC. The burden of NASH is likely to increase and NASH is probably to become the liver transplantation’s leading indication in 2020, due to increased NASH prevalence and the lack of effective treatments. Besides the obesity and the metabolic syndrome, NAFLD has the potential to increase the long-term mortality from cancers and cardiovascular diseases. An increased risk for colonic adenoma and colorectal cancer has been found in the NAFLD patients, and NAFLD might play an important role in the development and progression of chronic kidney disease (CKD).

NAFLD and NASH are found not only in adults, and there is a high prevalence in children and adolescents. NAFLD is a global disease, which affects from children to adults across all ethnic backgrounds in Europe, the Middle East and Asia. The prevalence in the countries such as China, South Korea, USA, and United Kingdom generally ranges from 7.9% to 43.3%. In 2018, about 25% of the world population is suffered from NAFLD. The risk factors for the development and progression of NAFLD include metabolic syndrome, obesity, and type 2 diabetes mellitus. Most of the children and adults were observed to have low levels of physical activity, which probably due to the huge revolutions of lifestyle, such as increased sedentary leisure and more energy-efficient transport.

In 2016, the annual medical cost attributable to NAFLD is more than $100 billion in the USA, indicating that effective pharmacological interventions are badly needed to treat and prevent NAFLD. The good thing is that substantial progress has been made recently in unraveling the potential mechanisms of NAFLD. Unfortunately, to date, no effective therapies for NAFLD roved by FDA.

The first step in the development of NAFLD is characterized by fat accumulation in the liv-
er, a condition that is commonly associated with the metabolic syndrome. Notably, it has been acknowledged that the change from NAFL to NASH is a pivotal process in the NASH formation and the mechanisms behind the above process have been studied worldwide. However, even the processes that determine fat accumulation are mostly clear, and the mechanisms associated with the progression of NAFLD are not thoroughly studied. Various pathological mechanisms of NAFLD are related to insulin-resistance and metabolic syndrome, including fat accumulation, oxidative stress, lipotoxicity, and mitochondrial dysfunction.

Evidence indicates that innate immunity is a driving force in the development of NAFLD for the reason that it directly regulates all key pathogenic features in the processes of the disease, including fat accumulation, inflammation, and fibrosis. There is a coordinated network of resident immune cells in the liver. These include dendritic cells (DCs), Kupffer cells (KCs), natural killer cells (NKs), and invariant NKT cells (iNKTs), which constitute the first defense line against environmental challenges and invading organisms. Scholars worldwide have made great progress in exploring the mechanisms of NAFLD development and progression, which remain very limited.

In this review, we summarize the currently available signaling pathways of NASH formation, including oxidative stress, NOD-like receptors (NLRs), mitochondria associated pathways, Toll-like receptors (TLRs), nuclear receptors, and other signal pathways. A better knowledge of these mechanisms would be very helpful to the design of targeted therapies and drugs to reverse NASH progression.

Oxidative Stress

Oxidative stress, which is a common feature of chronic hepatic diseases, plays an important role in the progression of NASH. In addition, oxidative stress markers correlate with the numbers of neutrophil and the degree of liver damage in liver NASH tissues. Xu et al. showed that oxidative stress and inflammatory response in the liver caused by PM2.5 inhalation induced abnormal hepatic function and promoted lipid accumulation in the liver, which were typical characteristics of NASH. Furthermore, pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) had an inhibition effect on oxidative stress and inflammatory response in vitro, indicating that oxidative stress and inflammatory in liver tissues resulted in hepatic injury by interfering normal lipid metabolism.

Mitochondrial dysfunctions alter the balance between prooxidant and antioxidant mechanisms, leading to an increase of nonmetabolized fatty acids in the cytoplasm as a result of the block of fatty acid β-oxidation and the induction of reactive oxygen species (ROS) production.

ROS have been demonstrated to disrupt lysosomal membranes, resulting in membrane permeabilization in lysosomal and the proteases releasing from lysosomal to the cytoplasm, further triggering apoptosis and necrosis, promoting the progression of NASH. In the liver, KCs are widely acknowledged to exert an essential effect in the NASH development. KCs are the main source of ROS production, which are normally generated by NADPH oxidase. Danger-associated molecular patterns (DAMPs), such as ATP and free fatty acid (FFA), are known to activate KCs and result in production of ROS, inducing the development of NASH through ROS dependent pathways.

NOD-like Receptors

Due to the important role of innate immune response in the liver, we deem that the liver is not only an organ for metabolism but also an organ for innate immune response, filtering endogenous signaling molecules and invading pathogens. During the process of metabolic diseases, the existence of multiple insults constitutively activate the components of innate immune, inducing chronic hepatic inflammation, and further promoting steatosis and the progression from NAFL to NASH. The hepatic innate immune response is a pivotal trigger in the progression of NAFLD, with an essential role in the whole disease spectrum of NAFLD, which ranges from NAFL to cirrhosis.

The NLRs are groups of intracellular PRRs, which are able to recognize both DAMPs and PAMPs. Among all the NLRs, the NLRP3 inflammasome is the most widely studied, and has been demonstrated to be of close relationship in the progression of NASH. Several signaling pathways have been showed to activate the NLRP3 inflammasome directly and in directly, such as DAMPs and PAMPs, increased levels of extracellular ATP or Ca2+, the increased production of mitochondrial-derived ROS, mtDNA releasing, and decrease in intracellular concentration of K+.

It has also been proven that the expressions of the components of NLRP3 are all increased in both
NASH mice models and NASH human samples with\textsuperscript{23,24}. FFAs accumulation in the liver tissues possesses lipotoxicity and has the capacity to promote the progression of NASH. Lipotoxicity, which is defined as dysregulation of the lipid environment and intracellular lipid composition, has a close relationship with organelle dysfunction, cell injury, and cell death\textsuperscript{25}. Lipotoxicity is closely connected to chronic sterile inflammation, which is named as metabolically-triggered inflammation, or meta-inflammation. FFA has been proven to be a kind of DAMPs to activate NLRP3 inflammasome\textsuperscript{25}. Notably, the NLRP3 inflammasome can be detected in both innate immune cells and nonimmune cells in the liver tissues, such as hepatocytes, kupffer cells, hepatic stellate cells and endothelial cells. Cai’s study demonstrate that all components of the NLRP3 inflammasome (NL-RP3, ASC, Caspase-1) are up-regulated in the KCs from mice liver with NASH, in accompany with elevated levels of pro-inflammatory cytokines IL-1β and IL-18\textsuperscript{25}.

A study published in 2017 demonstrated that the administration of NLRP3 inhibitor (MCC950) ameliorated the liver injury in NASH mice models\textsuperscript{26}. Active NLRP3 inflammasome particles can be released from cells during pyroptosis, indicating that pyroptosis is an important event to spread inflammasome signals to adjacent cells, including hepatocytes, hepatic stellate cells and KCs\textsuperscript{26-28}. Previous data show that NLRP3 -/- mice exhibited less severe NASH than WT mice in methionine-choline deficient diet (MCD) model. However, TXNIP (Thioredoxin-interacting protein) deficiency promoted the NLRP3 inflammasome activation and aggravated liver injury, indicating that TXNIP might exert a protective and anti-inflammatory effect in the NAFLD development by means of binding to NLRP3\textsuperscript{29}. Another study also showed that gosdermin D is involved in the process of pore formation and can be activated by various caspases. It is reported that hepatocytes from Nlrp3-overactivated mice have remarkable hepatocyte pyroptotic cell death and that persistent NLRP3 activation promotes the transition from NAFL to NASH in a high fat diet and MCD diet-induced NAFLD mouse model\textsuperscript{30,31}. Pan et al\textsuperscript{32} also found that mitochondria released mtDNA upon palmitic acid (PA) stimulation causes NLRP3 inflammasome activation, revealing a missing link between NLRP3 inflammasome activation and NASH progression, through binding of cytosolic mtDNA to the NLRP3 inflammasome.

NLRP3 inflammasome protein complex activation increases pro-inflammatory cytokines release by KCs, resulting in steatosis formation and sensitivity to endotoxin in hepatocytes, thus promoting NAFLD progression. However, due to its complex role in the tissue physiology and different pathological manifestations of NAFLD, the conclusions on the basis of experimental results from genetic deletion of NLRP3 or its components have been still controversial. Genetic manipulation strategies with better organ or cell-specificity are needed to better determine the effects of NLRP3 inflammasome at different stages of NASH development\textsuperscript{34-36}.

Other members of this family, such as NLRP2, initiate the inflammasome complexes formation instead of promoting gene transcription. One study showed that NLRP2 was remarkably decreased in the liver tissues from patients with severe steatosis. Interestingly, NLRP2 gene deletion mice showed typical metabolic syndrome and severe NAFL after HFD feeding. Moreover, NLRP2 deletion significantly aggravated HFD feeding-induced hepatic inflammation, evidenced by elevated levels of pro-inflammatory cytokines IL-1β and IL-18, and activation of nuclear factor κB (NF-κB) pathway, which indicated that suppressing the expression of NLRP2 promoted the degree of hepatic steatosis\textsuperscript{37,38}.

**Figure 1.** Key molecular pathways in the progression of non-alcoholic steatohepatitis. The progression from nonalcoholic fatty liver to non-alcoholic steatohepatitis is a key step in the NASH formation. Several signaling pathways are involved, including oxidative stress, NOD-like receptors, mitochondria associated pathways, Toll-like receptors, and nuclear receptors.

**The Role of Mitochondria in the NASH Development**

As a central feature in patients with NASH, mitochondrial dysfunction plays an essential role in the transition from NAFL to NASH\textsuperscript{39}. Mitochondria regulate the mechanisms of hepatic lipid
metabolism and oxidative stress, which are both essential cellular events in the NAFLD development. Ultrastructural mitochondrial lesions, altered mitochondrial dynamics, decreased activity of respiratory chain complexes, and impaired ability to synthesize ATP are all detected in liver tissues from patients with NASH. Mitochondrial stress and lesions would promote cell death, inflammation, and liver fibrogenesis.

Ultrastructural damage to the mitochondria was detected in liver tissues from obesity and NASH patients. It has also been found that liver tissues from early-stage NASH patients have higher mitochondrial biogenesis than liver tissues from healthy individuals. In addition, NASH patients have increased oxidative stress, increased lipid peroxidation, and oxidative DNA damage, associated with decreased antioxidant defense capacities and increased inflammatory responses. These findings demonstrate that liver adaptation and mitochondrial flexibility have been lost in the NASH patients. Alterations in mitochondrial biogenesis and damaged mitochondria accumulation might be secondary to defect in the mitophagy pathway detected in the liver tissues from NASH patients. Elevated fatty acid oxidation and lipotoxicity are principal drivers of mitochondrial deterioration in NASH. High mitochondrial ROS production and level of ROS-mediated mtDNA damage are detected in liver tissues from NASH patients. It is interesting that mtDNA, which is released into cytoplasm from the mitochondria during programmed cell death, bind to NLRP3 and induce NLRP3 inflammasome activation. All these above results provide a missing and essential link between mitochondrial dysfunction, apoptosis and NLRP3 inflammasome activation.

Nuclear Transcription Factors

Nuclear receptors, such as liver X receptor-α (LXRα) and LXRβ, peroxisome proliferator activated receptor-α (PPARα) and PPARγ, are closely associated with NASH development. Two LXR isoforms, including LXR-α and LXR-β, have the same ligand binding and considerable sequence homology, but have different tissue distribution. For example, LXR-α is highly expressed in several sites, such as the liver, macrophages and adipose tissue, while LXR-β is ubiquitously expressed.

The innate immune system has the ability to recognize immune signals through pattern recognition receptors (PRRs), which could bind to bacterial products or endogenous DAMPs liberated from cell debris and tissue damage. Under metabolic stress, it has been shown that PRRs play an essential role in the NASH pathophysiology. Of all the PRRs, TLRs are the extensively studied PRR family members and can be detected in the majority of liver cells. The activation of TLRs causes the increased levels of TNF-α, IL-1β, and interferons, which would induce proinflammatory responses. Among the thirteen TLRs identified in mammals, several TLRs, such as TLR2, TLR4, TLR5, and TLR9, have been proven to be associated with the pathogenesis of NASH.

All these receptors have been showed to regulate the progression of NASH positively or negatively. Genetic deletion of mice genes of TLR2, TLR4, and TLR9 significantly ameliorates hepatic inflammation, steatosis, and insulin resistance. On the contrast, deletion of the gene TLR5 obviously increases susceptibility to microbial dysbiosis and deteriorates the degree of NASH in mice. Of note, the multiple ingredients in the animal diet may exert an opposite impact on the intestinal barrier function, leading to some discrepancies among the published data. For example, it is reported that TLR2 deletion promotes NASH development in the animals fed with a MCD diet. However, the same TLR2 deletion has a protective effect on an NASH animal model established by choline-deficient amino acid (CDAA) diet. Upon TLR ligation, TLR2, TLR4, and TLR5 initiate signaling through MyD88 and NF-κB transcription factors to increase the expressions of proinflammatory cytokines, such as IL-1β and TNF-α. On the contrary, TLR9 starts its signaling through MyD88 and IRF7, whereas TLR4 initiates through TRIF and IRF3 to induce the expression of type I IFNs. Other TLRs may be associated with NASH, while need further exploring.

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LXR-α is a pivotal regulator of FFA and cholesterol metabolism in our whole body. After activation, LXR-α increases the level of hepatic Sterol Regulatory Element-Binding Protein (SREBP)-1c-mediated de novo lipogenesis and suppresses VLDL catabolism, promoting the pro-
gressions of hyperlipidemia and hepatic steatosis. LXR-α activation also induces cholesterol loss from the body through up-regulating ABCA1, ABCG5/G8 in hepatocytes, macrophages and enterocytes and down-regulating the expression of intestinal Niemann–Pick C1-like 1 protein, thus promoting intestinal cholesterol excretion and reverse cholesterol transport.

The elevated level of hepatic lipoperoxidation in the mice NASH model probably have abrogated the protective effects from the activation of PPARα, and could also be responsible for hepatic injury and the recruitment of inflammatory cells. Similarly, PPARγ activation attenuates hepatic inflammation through several mechanisms including suppressing NF-κB activity and reduced the synthesis of TNF-α and IL-1β in macrophages, which is owing to the special role of PPARγ: a master regulator of macrophage polarization56. It is reported that the activation of PPARγ causes a phenotype shift of macrophage polarization from an M1-predominant to an M2 phenotype, thereby alleviating hepatic inflammation in experimental mice NASH model57.

The NF-κB transcription factor, regulated by IkB kinases (IKKs), has a close connection with both inflammatory and metabolic responses. The IKK family contains both canonical and non-canonical IKKs. IKKα and IKKβ belong to prototypic canonical IKKs, which mainly regulate NF-κB transcripational activity, whereas IKKe and TBK1 are noncanonical IKKs, regulate downstream activation of NF-κB58,59. Positioned at the focus of downstream of PRRs, metabolic stress sensors, and cytokine receptors, IKKα and IKKβ are responsible for the increased production of cytokines in resident immune cells regulated by NF-κB60. Moreover, recent studies have showed persistent high expressions of non-canonical IKKs in NAFLD mice models. These novel studies provide a totally different perspective regarding the impact of intrinsic cellular metabolic processes60.

**Other Signal Pathways**

Besides the signaling pathways we mentioned above, there are some other novel pathways participated in the NASH development. Wang et al61 identified a hyperactivation of ASK1 in the livers of patients with NASH, and further found that CASP8 and FADD-like apoptosis regulator (CFLAR) and the deubiquitinase TNFα-induced protein 3 (TNFAIP3) were both essential endogenous suppressors of ASK1, which was able to ameliorate the degree of NASH62.

Caspase 2 has been previously demonstrated to be elevated in NAFLD patients, with the highest expression in NASH-induced cirrhosis patients. Interestingly, caspase 2-deficient mice were protected from obesity, metabolic syndrome and NAFLD induced by HFD. These novel findings indicated a critical role of caspase 2 in lipid-induced hepatocyte apoptosis and associated profibrogenic events63,64.

Lipotoxicity, which is defined as cellular abnormal lipid composition, leads to toxic lipid accumulation, cell injury and chronic sterile inflammation, which is the main characteristic of NASH65. SFAs are able to bind and activate the membrane receptors on the surface of hepatocyte, including death receptor TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2) and DAMP receptors. The former signaling pathway triggers the proteolytic auto-activation of caspase 8, which causes direct or indirect activation of caspases 3, 6 and 7 via Bel-2 protein family-mediated mitochondrial outer membrane permeabilization, (MOMP), eventually resulting in cell apoptosis64.

TRAFs constitute a family of adaptor proteins, and multiple studies have proved that TRAFs are involved in the regulation of NF-κB and MAPK pathways during the NASH development. Furthermore, emerging evidence indicates that TRAFs regulate the metabolic disturbances independently of their roles in inflammatory response. TRAFs take part in target protein activities by means of modulating their post-translational modifications (PTMs) that are frequently related to the changes of hepatic metabolic responses65,66.

**Conclusions**

In this review we summarize the widely acknowledged signaling pathways involved in the mechanisms of NASH development. To keep the risk of NAFLD as low as possible, healthy dietary habits and persistent physical activity should be encouraged and recommend by the government to decrease the risk of obesity and the metabolic syndrome. Weight reduction is the most established treatment for NASH. Liver biopsy remains the gold standard for diagnose the disease through histological evaluation, and non-invasive methods combining imaging and biochemical tests are warranted to pre-empt the need for liver biopsies. Increasing evidence highlights the close link between innate immunity and development...
of NASH. Consequently, understanding the nature and complexity of the innate immune signaling network provides new insights for the rational design of novel therapies for NASH. Currently, because of the complex mechanism of NASH development, there is no approved drug regimen to treat NASH. In the future, it is very necessary that further exploring the mechanistic differences in various phases of the disease, and identify the driving forces from one step to the next, which would be extremely helpful to identify novel therapeutic and preventive targets for NASH.

Conflicts of interest
The authors declare no conflicts of interest.

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