Hepatocyte nuclear factor 4α in the pathogenesis of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is emerging as the most common chronic liver disease worldwide. It refers to a range of liver conditions affecting people who drink little or no alcohol. NAFLD comprises non-alcoholic fatty liver and non-alcoholic steatohepatitis (NASH), the more aggressive form of NAFLD. NASH is featured by steatosis, lobular inflammation, hepatocyte injury, and various degrees of fibrosis. Although much progress has been made over the past decades, the pathogenic mechanism of NAFLD remains to be fully elucidated. Hepatocyte nuclear factor 4α (HNF4α) is a nuclear hormone receptor that is highly expressed in hepatocytes. Hepatic HNF4α expression is markedly reduced in NAFLD patients and mouse models of NASH. HNF4α has been shown to regulate bile acid, lipid, glucose, and drug metabolism. In this review, we summarize the recent advances in the understanding of the pathogenesis of NAFLD with a focus on the regulation of HNF4α and the role of hepatic HNF4α in NAFLD. Several lines of evidence have shown that hepatic HNF4α plays a key role in the initiation and progression of NAFLD. Recent data suggest that hepatic HNF4α may be a promising target for treatment of NAFLD.

Keywords: Nonalcoholic fatty liver disease; Hepatocyte nuclear factor 4α; Lipogenesis; Inflammation; Fibrosis; Liver; Lipotoxicity; Apoptosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is emerging as the leading chronic liver disease due to the rising rates of obesity and diabetes. It refers to a range of liver conditions affecting people who drink little or no alcohol with the presence of steatosis in ≥5% hepatocytes. There are two subtypes of NAFLD, non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NASH is the more advanced subtype of NAFLD, which is characterized by liver steatosis, lobular inflammation, hepatocyte ballooning, and various degrees of fibrosis. NASH may further progress to cirrhosis, hepatocellular carcinoma (HCC), and liver failure [Figure 1]. NAFLD is often associated with diabetes, obesity, and dyslipidemia, and is considered as the hepatic manifestation of metabolic syndrome.\[1\]

Hepatocyte nuclear factor 4α (HNF4α) is a nuclear hormone receptor that is highly abundant in the liver and highly conserved across the species. In the liver, HNF4α is best known for its role as a master regulator of liverspecific gene expression and its essential role in both fetal and adult liver functions. The expression of HNF4α is markedly reduced in NAFLD patients and mouse models of NASH\[2-3\] or fibrotic livers.\[4-6\]

Dysregulation of HNF4α expression is associated with many human diseases, such as NAFLD, liver cirrhosis, HCC, ulcerative colitis, colon cancer, and maturity-onset diabetes of the young. In this review, we briefly overview the pathogenic mechanisms, diagnosis, and treatment of NAFLD, but focus on the regulation of hepatic HNF4α expression, the role of HNF4α in the pathogenesis of NAFLD, and the potential of HNF4α as a therapeutic target for NAFLD.

Pathogenic Mechanisms of NAFLD

The pathogenic mechanisms of NAFLD are yet to be fully understand. Multiple lines of evidence have indicated that the pathogenesis of NAFLD is a complicated and multifactorial process involving interactions among nutrition, metabolism, genetic predisposition, and environment [Figure 2]. Historically, a “two-hit” hypothesis is first proposed, in which fats accumulate in the liver (first hit) followed by other insults (e.g., inflammatory cytokines, oxidative stress, mitochondrial dysfunction) leading to inflammation and fibrogenesis (second hit).\[7,8\] Due to the complexity of the pathogenesis, a “multiple-hit” hypothesis is brought forward, in which multiple insults act together on genetically predisposed subjects to induce NAFLD.\[9,10\]
Dysregulation of lipid metabolism and NAFL

About 25% of the population has NAFLD worldwide. NAFLD is often associated with obesity and diabetes. Nonetheless, NAFLD is also found in non-obese or overweight children and adults, ranging from 3.3% to 21.2% of the population (with a body mass index <25 kg/m²). The prevalence of NAFLD is higher in Hispanics and whites than in Black individuals and is twice as much in men as in women. Globally, about 53.5% people with type 2 diabetes and up to 90% of obese people have NAFLD. Among people with NAFLD, cardiovascular disease is the leading cause of death, followed by cancer and liver-related death.

NAFLD often starts with lipid accumulation in the liver that is not the consequence of alcohol drinking, a condition called NAFL. Triglycerides (TG), free fatty acids (FFAs), free cholesterol (FC), and cholesterol esters (CEs) may accumulate in NAFL, albeit largely in the form of TG. The accumulation of TG in the liver may result from increased de novo lipogenesis (DNL) and impaired very low-density lipoprotein (VLDL) secretion or lipolysis. Impaired fatty acid oxidation (FAO) may also lead to FA and TG accumulation in the liver.

Insulin resistance is a major risk factor for NAFLD. Under insulin resistance, more FFAs are released from adipose tissue and delivered to the liver. Hyperinsulinemia also transcriptionally induces genes that promote DNL. Sterol regulatory element-binding protein 1c (SREBP-1c) is a transcription factor that induces the lipogenic genes, such as fatty acid synthase, acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase 1. Insulin activates SREBP-1c by inducing SREBP1c mRNA levels and SREBP-1c proteolytic processing, which can be blocked by wortmannin, an

Figure 1: Progression of NAFLD. NAFLD encompasses NAFL and NASH. NASH may further progress to cirrhosis, HCC, and liver failure. Patients without cirrhosis may also develop HCC. Cardiovascular disease is the leading cause of deaths in NASH. HCC: Hepatocellular carcinoma; NAFL: Non-alcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Figure 2: Molecular mechanisms of NAFLD. NAFLD is a complex and multifac torial disease. The development and progression of NAFLD is affected by insulin resistance, genetic polymorphisms, gut microbiota, race, gender, etc. Under insulin resistance or diabetes, the influx of FFAs from adipose tissue as well as DNL is increased. FFAs, particularly saturated FFAs, can cause ER stress, oxidative stress, apoptosis, and infl ammasome activation via lipotoxic lipids (LPCs, ceramides, DAG, etc.). Cholesterol crystals also promote infl ammasome activation. The change in the gut barrier allows LPS from gut microbiota to enter the portal circulation and activate toll-like receptors or infl ammasome (pyroptosis) for induction of infl ammation. The change in other gut microbiota products (ethanol, secondary bile acids, etc.) may also contribute to the development of NAFLD. ApoB: Apolipoprotein B; BAs: Bile acids; chREBP: Carbohydrate response element-binding protein; DNL: de novo lipogenesis; DAG: Diacylglycerols; ER: Endoplasmic reticulum; FC: Freecholesterol; FAO: Fatty acid oxidation; FFAs: Free fatty acids; GCKR: Glucokinase regulatory; HSD17B13: Hydroxysteroid 17-beta dehydrogenase 13; LPS: Lipopolysaccharides; LPC: Lysophosphatidylcholine; MBOAT7: Membrane-bound O-acyltransferase domain-containing 7; NAFL: Nonalcoholic fatty liver disease; PPP1R3B: Protein phosphatase 1 regulatory subunit 3B; PNPLA3: Palatin-like phospholipase domain containing 3; SREBP-1c; Sterol regulatory element-binding protein 1c; TM6SF2: Transmembrane 6 superfamily 2.
inhibitor of phosphatidylinositol 3-kinase, and low concentrations of rapamycin, an inhibitor of the mechanistic target of rapamycin complex 1 (mTORC1). Furthermore, insulin-activated SREBP-1c proteolytic processing can be blocked by inhibition of p70 S6 kinase (S6K), suggesting that activation of the mTORC1-S6K pathway is responsible for SREBP-1c processing. Under overnutrition, endoplasmic reticulum (ER) stress promotes insulin-induced SREBP-1c cleavage. Unlike insulin, glucose promotes lipogenesis via activation of carbohydrate response element-binding protein (ChREBP). In response to increased glucose concentration, ChREBP is dephosphorylated and translocated to the nucleus, leading to induction of lipogenic genes and liver type pyruvate kinase. However, under insulin resistance or overnutrition, NAFLD is often accompanied by increased VLDL secretion and hyperlipidemia due to increased TG availability and microsomal triglyceride transfer protein (MTP) production. By contrast, the contribution of FAO to steatosis in NAFLD has been less clear. It has been shown that NAFLD patients with insulin resistance have impaired ATP production but increased hepatic FAO. Consistent with the latter finding, high fat diet (HFD) feeding increases the function of tricarboxylic acid cycle in mice. Additional studies with a larger sample size may be needed to clarify the role of FAO in fat deposition in NAFLD.

Lipolysis also plays a role in NAFLD. Adipose triglyceride lipase (ATGL; PNPLA2) is the major hepatic triglyceride lipase, although some other lipases are also reported to display triglyceride hydrolyase (TGH) in the liver, such as some of the carboxylesterase (CES) family, lysosomal acid lipase, etc. Defective lipolysis contributes to hepatic TG accumulation. Multiple observations have uncovered that the common I148M missense mutation in palatlipin-like phospholipase domain containing 3 (PNPLA3; adiponutrin) is consistently associated with NAFLD. In the presence of obesity or chronic alcohol intake, the variant is associated with hepatitis or cirrhosis. PNPLA3 (I148M) promotes steatosis by inhibition of ATGL activity through interaction with comparative gene identification-58 (CGI-58; ABHD5), a co-activator of ATGL.

In addition to PNPLA3, other genetic variants are also found to play a role in hepatic fat accumulation and inflammation. The E167K variant in transmembrane superfamily two (TM6SF2) causes fatty liver and elevates alanine aminotransferase (ALT) levels by impairing normal VLDL secretion. The membrane-bound O-acyltransferase domain-containing 7 (MOAT7; LPIAT) variant rs641738 increases risk of NAFLD, which appears to be mediated by changes in hepatic phosphatidylinositol acyl-chain remodeling. Further studies in mice show that ablation of Mcat7 causes accumulation of its substrate lysophosphatidylinositol (LPI) lipids, and that administration of LPI promotes hepatic inflammation and fibrogenesis. In contrast, the rs72613567 variant with an adenine insertion in hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13), an enzyme that is elevated in NAFLD and targets lipid droplets, is associated with a reduced risk for NASH.

The HSD17B13 rs72613567 variant is also shown to interact with PNPLA3 I148M and reduce the risk for liver disease conferred by PNPLA3 I148M. Other studies have also shown that the variants in glucokinase regulatory gene or protein phosphatase 1 regulatory subunit 3B are also associated with NAFLD.

**Progression of NAFL to NASH**

About 20% NAFL patients will develop NASH and 20% NASH patients will develop cirrhosis over time. About 1% to 2% or 20% cirrhosis patients may develop HCC or liver failure over 1 or 2 years, respectively. Inflammation is a key driver of NAFL progression to NASH. Under insulin resistance, excessive fatty acid influx from adipose tissue and increased DNL in the liver promote accumulation of lipotoxic lipids, which contribute to oxidative stress, ER stress, inflammasome activation, and apoptotic cell death, leading to inflammation and fibrogenesis.

**Lipotoxicity**

Hepatic toxic lipid species accumulate when the liver cannot handle excessive carbohydrates and fatty acids. FFAs (saturated and trans fatty acids), diacylglycerols (DAG), lysophosphatidylcholine (LPC), ceramides, and FC are considered lipotoxic species, which can mediate inflammation in NAFLD by causing ER stress, oxidative stress, and inflammasome activation, leading to apoptosis, necroptosis, release of cytokines or chemokines (tumor necrosis factor [TNF] α, interleukin [IL]-1β, IL-6, IL-18, tumor growth factor beta [TGF-β], etc.), and activation of stellate cells. Inflammasome is a cytoplasmic protein complex that responds to danger-associated molecular patterns (saturated fatty acids, cholesterol crystals, etc.) and pathogen- associated molecular proteins (e.g., products of gut microbiota). Activation of inflammasome leads to expression and release of IL-1β and IL-18, and promotes inflammation via activation of caspase-1 and induces a form of death called pyroptosis.

**Apoptosis**

Apoptosis plays a key role in the progression of NAFLD. NASH patients have significant levels of apoptosis and caspase 3 activation. Caspase 2 appears to be an initiator caspase in multiple apoptotic pathways. Caspase 2 expression is markedly upregulated in NAFL and NASH patients and animal models of NASH, and its deficiency reduces lipid-induced hepatocyte apoptosis (lipopoptosis) and liver fibrosis. Ablation of caspase 8 in hepatocytes inhibits methionine-choline deficient diet-induced inflammation, fibrosis, and liver injury. Saturated FFAs induce c-Jun N-terminal kinase (JNK)-dependent lipopoptosis by activating the pro- apoptotic B-cell lymphoma protein 2 (Bcl-2) proteins Bim and Bad. Inhibition of apoptosis by the pan-caspase inhibitors VX-166 or Emricasan reduces inflammation or the development of fibrosis in mouse models with NASH.
### Extracellular vesicles (EVs)

EVs are non-nucleated, lipid-bound particles that include endosome-derived exosomes (30–150 nm in diameter) and plasma membrane-derived microvesicles (50–1000 nm). EVs can carry mRNAs, non-coding RNAs, lipids (cholesterol, ceramides, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine), proteins (heat shock proteins HSP70, HSP90, tubulin, actin, etc.), and mitochondrial DNA, and deliver them to other cell types. EVs are important for cell-cell communications and also act as drivers of inflammation in NAFLD. Kakazu et al. show that lipotoxic hepatocytes induced by palmitate secrete EVs enriched in C16:0 ceramide, which in turn activate macrophage chemotaxis via formation of sphingosine-1-phosphate from 16:0 ceramide. Treatment of hepatocytes with palmitate or the palmitate metabolite LPC increases the release of EVs containing TNF-related apoptosis-inducing ligand, which are capable of inducing the expression of IL-1β and IL-6 in macrophages.

### Gut microbiome

Gut microbiota is a complex ecosystem whose composition and relative abundance of species are comparable between healthy people but are affected by environmental and host-related factors, such as diets, drugs, physical activity, geographic locations, etc. A less diverse microbiota is observed in NASH patients in comparison with that of healthy subjects. Some studies have suggested a link between gut dysbiosis and the progression of NAFLD. In one study, Bacteroides and Ruminococcus have been identified as independently associated with steatohepatitis and fibrosis, respectively. The change in gut microbiota composition may regulate the development and progression of NAFLD via their metabolites (short-chain fatty acids, ethanol, etc.), endotoxemia due to increased gut permeability, and changes in hormones and bile acid signaling.

### Diagnosis and Treatment of NAFLD

#### Diagnosis of NAFLD

NAFL is histologically defined by the presence of macrovesicular steatosis in >5% of hepatocytes whereas NASH is histologically characterized by hepatic steatosis and hepatocellular injury, including hepatocyte ballooning, lobular inflammation, and various degrees of pericellular fibrosis. The majority of NAFLD patients are asymptomatic until NAFLD progresses to cirrhosis. Serum ALT and aspartate aminotransferase (AST) levels are often elevated with ALT levels higher than AST levels. Hepatic steatosis can be identified non-invasively by ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI). MRI can detect as little as 5% steatosis whereas ultrasound or CT can detect ≥20% steatosis.

### Treatment of NAFLD

Lifestyle change, including a low-calorie diet (a daily reduction of 500–1000 kcal calorie intake) and 30 min of daily moderate exercise, is highly recommended. Lifestyle change-induced weight loss by ≥10% is associated with NASH resolution and fibrosis regression. For patients with NASH and obesity, bariatric surgery is associated with a significant lower risk of major adverse liver outcomes (progression to cirrhosis, HCC, liver transplantation, liver-related mortality) and major adverse cardiovascular events (coronary artery or cerebrovascular events, heart failure, cardiovascular death).

### Overview and Regulation of HNF4α

HNF4α (NR2A1) is a nuclear hormone receptor that is highly expressed in the liver, and to a lesser extent in pancreas, intestine, and kidney. In hepatocytes, HNF4α is a master regulator of many genes involved in hepatocyte differentiation and morphogenesis, drug metabolism, gluconeogenesis, lipid homeostasis, bile acid synthesis and conjugation, ureagenesis, cell proliferation and inflammation. Global Hnf4α-/- mice are embryonically lethal, highlighting the importance of HNF4α in development. Loss-of-function mutation of HNF4α causes maturity onset diabetes of the young type 1. Crystallization studies show that HNF4α has long-chain fatty acids in its ligand-binding domain. HNF4α is constitutively active as fatty acids constitute the ligand binding pocket of the ligand binding domain. HNF4α binds as a homodimer to the direct repeat 1 or DR2 sequences in the target genes to regulate gene transcription.
HNF4α is regulated at the transcriptional and post-transcriptional levels. Fasting is known to induce HNF4α mRNA expression, but the underlying mechanism is not clear. More studies have been focused on post-transcriptional regulation of HNF4α expression. Studies by liquid chromatography with tandem mass spectrometry (LC-MS/MS) have identified several phosphorylation sites (S142, T166, S167, T432, S436). Activation sites (K234, K307) and one acetylation site (K458) show that protein kinase A (PKA), AMP-activated protein kinase, steroid receptor co-activator 1 (PGC1α) to induce gluconeogenesis during fasting or to stabilize HNF4α. Interestingly, inhibition of p38 mitogen-activated protein kinase (MAPK) activity reduces the phosphorylation and nuclear translocation of HNF4α. Phosphorylation by protein kinase A, AMP-activated protein kinase, proto-oncogene tyrosine-protein kinase Src (c-Src), Smad3/Smad4, and small heterodimer partner may physically interact with forkhead box O1 (FoxO1) to increase HNF4α cytoplasmic localization and degradation. Phosphorylation by protein kinase A, AMP-activated protein kinase, proto-oncogene tyrosine-protein kinase Src (c-Src), Smad3/Smad4, and small heterodimer partner may also physically interact with FXR.

HNF4α can physically interact with forkhead box O1 (FoxO1) to reduce HNF4α stability and transcriptional activity. HNF4α may also interact with the co-activator PPARγ coactivator 1α (PGC1α) to induce gluconeogenesis during fasting or to stabilize HNF4α. Interestingly, inhibition of p38 MAPK is important for the nuclear retention of HNF4α. Acetylation at lysine residues by CREB-binding protein is reported to be crucial for the proper nuclear retention of HNF4α.

Hepatocyte-specific Hnf4α−/− (Hnf4αΔhep) mice have reduced plasma TG and cholesterol levels and increased hepatic neutral lipid accumulation. Acute ablation of hepatic HNF4α by shRNA also markedly decreases plasma TG and cholesterol levels and increases hepatic TG levels by four-fold. The drastic changes in plasma and hepatic lipid levels likely result from a profound reduction of triglyceride hydrolysis and FAO, leading to reduced hepatic TG levels. Both CES1 and CES2 are direct target genes of HNF4α. Overexpression of HNF4α in hepatocytes promotes lipolysis and FAO, whereas loss of hepatocyte HNF4α has opposite effects. Thus, CES1 and CES2 may be partly involved in the regulation of lipolysis and FAO and hepatic TG levels by HNF4α.

AAV8-mediated overexpression of human HNF4α in hepatocytes protects against HFCF diet-induced steatohepatitis, whereas loss of hepatocyte HNF4α has an opposite effect. P53 is a tumor suppressor and a primary stress sensor that is induced in the liver of NAFLD patients and experimental NASH. Overexpression of hepatocyte HNF4α promotes lipolysis and FAO, whereas loss of hepatocyte HNF4α has opposite effects. Thus, CES1 and CES2 may be partly involved in the regulation of lipolysis and FAO and hepatic TG levels by HNF4α.
and NASH development. In addition, HNF4α is shown to inhibit the expression and nuclear translocation of RelA (p65) and NF-κB activation via induction of miR-7 and miR-124.\(^\text{146}\) NASH is a risk factor for HCC. Over-expression of HNF4α inhibits the development of HCC likely by inhibiting β-catenin activation.\(^\text{147,148}\)

**HNF4α as a therapeutic target**

Since hepatic HNF4α is markedly repressed in NASH and liver fibrosis,\(^\text{12-6}\) HNF4α may be a therapeutic target for treatment of NAFLD. Adenovirus-mediated overexpression of HNF4α is shown to attenuate liver fibrosis induced by dimethylsulfoxide or bile duct ligation.\(^\text{5}\) AAV8-mediated overexpression of HNF4α under the control of an albumin promoter is shown to attenuate HFCF diet-induced NAFL and NASH.\(^\text{137}\) Yang et al\(^\text{6}\) show that delivery of HNF4α mRNA in lipid nanoparticles to four different mouse models protects against hepatotoxic- and cholestatic-induced liver fibrosis. Compounds that can induce HNF4α expression or activation have also been investigated. Lee et al\(^\text{149}\) show that N-trans caffeoyltyramine (NCT) is an HNF4α activator, and administration of this compound can prevent HFD-induced hepatosteatosis, although its role in NASH needs to be evaluated. These promising findings suggest that HNF4α may be a good candidate for treatment of NASH.

**Conclusion and Future Perspectives**

NAFLD is the most common chronic liver disease in developed countries. So far, the pathogenic mechanisms of NAFLD remain to be fully elucidated. No drugs have been approved for NASH treatment. As one of the most abundantly expressed genes in the liver, HNF4α appears to be a key player in the pathogenesis of NAFLD, which is supported by several lines of evidence. First, the expression of hepatic HNF4α is markedly reduced in NAFLD patients, diabetic or HFD-fed mice, and fibrotic livers. Second, ablation of hepatocyte HNF4α promotes the development and progression of NAFLD in a mouse model of NASH. Third, AAV8-mediated overexpression of HNF4α in hepatocytes attenuates steatohepatitis in mice. Delivery of HNF4α by adenovirus or lipid nanoparticles-embedded mRNA inhibits liver fibrogenesis. Administration of a compound that induces HNF4α expression prevents HFD from inducing hepatosteatosis. These findings highlight the importance of HNF4α in the pathogenesis of NAFLD and suggest that hepatic HNF4α may be targeted for treatment of NAFLD.

Hepatic HNF4α inhibits the development and progression of NAFLD via regulation of multiple pathways, including VLDL secretion, lipolysis, FAO, apoptosis, lipotoxicity, and inflammation. P53 and bile acid signaling pathways play an important role in the progression of NAFL to NASH mediated by HNF4α. Although increased hepatic HNF4α expression may cause hyperlipidemia via increased VLDL secretion, Huang et al\(^\text{130}\) report that delivery of small activating RNA specific for upregulating HNF4α to rats improves FAO and liver steatosis, and lowers plasma TG levels, suggesting that raising hepatic HNF4α expression may even improve dyslipidemia. Hepatic HNF4α can increase TG hydrolysis, FAO, and the conversion of cholesterol to bile acids via inducing CYP7A1 and CYP8B1 expression, which may help to reduce VLDL-TG or VLDL-cholesterol levels. Considering the factors discussed above, it is plausible to summarize that hepatic HNF4α is a promising therapeutic target for NASH.

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**Conflicts of interest**

None.

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