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Review Article

Endoplasmic Reticulum Stress and Lipophagy in Nonalcoholic Steatohepatitis

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Abstract

Non-Alcoholic Fatty Liver Disease (NAFLD) is often a hepatic complication of obese, hyperlipidemia, diabetes and metabolic syndrome, and affects more than one third of general population in the US and 15% in China. It covers a spectrum of fat accumulation-associated disorders, ranging from simple fatty liver, Nonalcoholic Steatohepatitis (NASH), and End-Stage Liver Disease (ESLD). Approximately, one fifth of NAFLD patients will develop NASH, which may further progress to ESLD with various complications. Hepatocellular Carcinoma (HCC) may occur in all stages of NAFLD, and accounts for an increased incidence of HCC in the US. Despite of pandemic prevalence of NAFLD/NASH, etiology-specific treatment has not been available yet, nor is its pathogenesis fully understood. The multi-factorial feature of this disorder dictates the variance in susceptibility, possibility of disease progression and responses to therapeutics, and requires that therapeutics targeting more than single pathway should be developed, and personalized treatment implemented. To reach such goals, the delineation of Endoplasmic Reticulum (ER) stress and lipophagy, two pathophysiologic responses in addition to known pathologic alterations, such as lipotoxicity, oxidant stress, insulin resistance, would allow revealing the molecular basis for NASH initiation and progression, and point to a right direction for new therapeutic development. The present review aims to cover the current understanding of how ER stress and autophagy take a part in the initiation and progression of NASH, and what therapeutic hints exist in deep insights into these two pathophysiologic responses. The ultimate goal is to develop more effective strategies for NASH intervention.

Keywords: Nonalcoholic fatty liver disease; Nonalcoholic Steatohepatitis; Endoplasmic reticulum stress; Autophagy; Lipophagy; Insulin resistance

Abbreviations

CHOP: CCAAT/enhancer-binding Homologous Protein; CLA: Conjugated Linoleic Acid; DHA: Docosahexaenoic Acid; ER: Endoplasmic Reticulum; ESLD: End-Stage Liver Disease; HCC: Hepatocellular Carcinoma; HFC: High Fat/High Calorie diet; HSCs: Hepatic Stellate Cells; MAP1LC3: Microtubule-Associated Protein-1 Light Chain 3; MCD: Methionine/Choline-Deficient Diet; NASH: Nonalcoholic Steatohepatitis; NAFLD: Nonalcoholic Fatty Liver Disease; N-3 PUFA: N-3 Poly Non-Saturated Fatty Acids; OCA: Obeticholic Acid; PGZ: Pioglitazone; ROS: Reactive Oxygen Species; TG: Triglyceride

Introduction

As one stage of Non-Alcoholic Fatty Liver Disease (NAFLD), Nonalcoholic Steatohepatitis (NASH) possesses the characteristics of multiple factorial-etiological, and its clinical manifestation varies dramatically from patients to patients based on base conditions, such as hyperlipidemia, obese, diabetes and metabolic syndrome. It may progress to End-Stage Liver Disease (ESLD), need a medical attention, and account for an increased incidence of primary Hepatocellular Carcinoma (HCC) in the US [1]. The multi-factorial features of NASH initiation and progression dictate that complex molecular interplays play pivotal roles during these processes, and require that therapeutic strategies targeting more than single pathway be developed. At the

same time, these variances also indicate that the same therapeutics may yield varied efficacy in different patients, and that personalized therapy should be implemented when specific pathways or molecular interplays are identified [2]. However, the molecular base for its initiation and progression is poorly understood, and known pathologic pathways, such as lipotoxicity, oxidant stress and insulin resistance, could not explain variations in the susceptibility, the possibility of disease progression and responses to therapeutics [3]. Particularly, how fat accumulation and its resulting oxidant stress lead to hepatocellular apoptosis and inflammatory response remains to be investigated. Endoplasmic Reticulum (ER) stress has been considered to be the molecular link between oxidant stress, apoptosis and insulin resistance. Its involvement in lipotoxicity has been extensively studied in NASH [4]. Meanwhile, autophagy is a physical response to starvation or energy depletion, and is considered to be an alternative strategy to survival under shortage of energy supplies [5]. Only very recently was it found that lipophagy is a physiologic pathway for a cell to use lipid droplets as an energy source [6]. The changes and significance of lipophagy under various conditions of abnormal lipid metabolism, such as NASH remain to be explored. Therefore, deep understanding of ER stress and lipophagy at molecular levels will greatly aid in revealing molecular mechanisms of NASH initiation and progression. The present review aims to provide a brief overview in order to delineate how ER stress and autophagy take a part in the

initiation and progression of NASH, and what therapeutic hints exist in these two pathophysiologic responses.

Basic understanding of NASH epidemiology and treatment options

Epidemiology of NAFLD/NASH: Over 45% of the US population is overweight, and the resulting insulin-resistance leads to a high prevalence of metabolic-associated disorders, such as diabetes, Nonalcoholic Fatty Liver Disease (NAFLD), metabolic syndrome, hyperlipidemia, hypertension, and vascular abnormalities in various organs [7]. NAFLD is a spectrum of fat overload-associated liver disorders ranging from simple fatty liver, Non-Alcoholic Steatohepatitis (NASH), and its resulting fibrosis/cirrhosis. NAFLD affects 30-40% of the US population and 15% in China [8], approximately one fifth of NAFLD patients will progress to NASH, and the latter has become a major factor in abnormal liver biochemical tests, and requires a medical attention. Roughly 20% of NASH patients further advance to cirrhosis or End-Stage Liver Disease (ESLD) with various complications, which confer a significant morbidity and major mortality in liver diseases. HCC may occur in various stages of NAFLD, and accounts for an increasing incidence of HCC in the US [1,9]. NAFLD is present in 70% patients with Type II Diabetes Mellitus (T2DM) and over 90% of those are obese with insulin resistance [10]. More concerning is a rapid increase of NAFLD incidence in the pediatric population worldwide over the last decade, and its incidence has been increased to 85% in obese children in the US [11,12]. Due to such a huge patient pool, NASH-associated ESLD will be the main candidate for liver transplantation in the US in the next 2-3 decades [13].

Current remedies for NASH: In spite of high prevalence of NASH, no specific etiologic treatment is available for NASH patients [2]. Dietary limit and life style changes have some positive effects on weight loss, fat accumulation and hyperlipidemia; however they are less reliable for the attenuation of necroinflammatory activity in NASH [14]. Insulin sensitizers, such as thiazolidinediones (pioglitazone, troglitazone and rosiglitazone) are used for selected NASH patients; whereas there is an increased risk of cardiovascular diseases when they are used for an extended period [15]. Pioglitazone (PGZ) has been shown to significantly improve steatosis and inflammation, but it does not halt hepatic fibrosis or progression to ESLD in NASH patients [16-18]. Anti-oxidants, such as vitamin E [19,20], Pentoxifylline (PTX) [21,22] or long chain N-3 poly non-saturated fatty acids (N-3 PUFA), such as Docosahexaenoic Acid (DHA) [23,24] are been prescribed for NASH patients without diabetes. Positive results in the improvement of steatosis and necroinflammatory activity are reported with these remedies; whereas there has been no solid evidence available yet to demonstrate their effects in blocking or reversing hepatic fibrosis. Recent clinical trials with FXR agonists, such as Obeticholic Acid (OCA) in diabetic patients demonstrated promising results in terms of its effects on insulin resistance, attenuation of steatosis and steatohepatitis [25]. A NIDDK-supported randomized multi-center trial further confirmed the benefits of improved steatohepatitis and fibrosis in histology in more patients receiving OCA for 72 weeks than those receiving placebo, although long-term benefits and increased risk for cardiovascular diseases need to be evaluated in larger clinical trials [26]. Other agents, such as pan-caspase or caspase-specific apoptosis

inhibitors (VX-166, GS-9450), Cannabinoid 1 (CB1) receptor antagonists and CB2 receptor agonists in preclinical or early clinical trials are available; and need to be further assessed in multicenter, double-blind controlled clinical trials to confirm their effectiveness and potential adverse effects [27,28].

Novel insights into NASH pathogenesis

Prevailing hypotheses of NASH development: Since NASH was described during the late 70's, various hypotheses have been put forth to explain its pathogenesis. Among them the "two-hit" hypothesis and gut microbiota disturbance represent the prevailing views. In "two-hit" hypothesis, the accumulation of Triglycerides (TG) in hepatocytes is the basis (1st hit), and the occurrence of oxidant stress (2nd hit) caused by various factors, such as alcohol intake, drug metabolism or lipid metabolites, evokes lipid peroxidation, and in turn leads to necrosis/apoptosis of hepatocytes. If the injury and inflammatory response persist, fibrogenesis dominates the repair process [29], and the consequence is progressive fibrosis and cirrhosis, as well as various complications, including HCC. However, this hypothesis does not emphasize the importance of insulin resistance in NASH initiation and progression, nor does it focus on a deteriorating loop of lipid accumulation and lipotoxicity → hepatocellular injury → insulin resistance → worsened lipid metabolism [24].

Recent studies provide convincing evidence demonstrating that the disturbance of gut microbiota, such as bacterial over growth, occurrence of lipogenic or energy-producing colonies contributes to inflammation and insulin resistance in peripheral muscle, adipose and liver tissues [30,31]. The microbiome hypothesis explains increased inflammatory status, insulin resistance and steatohepatitis in obese, diabetes, metabolic syndrome and many other conditions in which multiple factors affect the progression, prognosis and therapeutic outcomes, and demands great efforts in identifying a specific colony or colonies, metabolic disturbances by systems biologic approaches and multidisciplinary collaboration [32]. This theory confers great potential in revealing mysteries of gut microbiome in normal and pathologic conditions, such as how an increased incidence of cancer, including HCC, occurs in diabetic or NASH patients [33]. The new trends of research also point to directions in developing novel therapeutic approaches, as well as gut microbiota transplants in the treatment of certain refractory diarrhea [34], or establish the rationale for the use of probiotics, prebiotics and antibodies in obese, diabetes, metabolic syndrome, NAFLD or NASH although their efficacy needs to be assessed in more convincing clinical trials [34].

Insulin resistance, oxidant stress and ES stress in NAFLD/NASH

Insulin resistance in adipocytes, muscle and hepatocytes is the hallmark of NASH pathogenesis, and the development of hepatic insulin resistance is attributed to lipid accumulation, inflammation, and ER stress [35]. The lipotoxicity due to excessive Free Fatty Acids (FFAs), especially saturated FAs (SFAs), appears to be the key factor causing the injury of hepatocytes. Excessive SFAs arise from enhanced lipolytic activity in adipose tissue, accelerated de novo synthesis or conversion of carbohydrates, an increased breakdown of lipoprotein remnants in lysosomes, and elevated lipolytic activity of lipid droplets, as well as increased FFA influx into hepatocytes [36]. It was recently shown that SFAs are released from lipid droplets

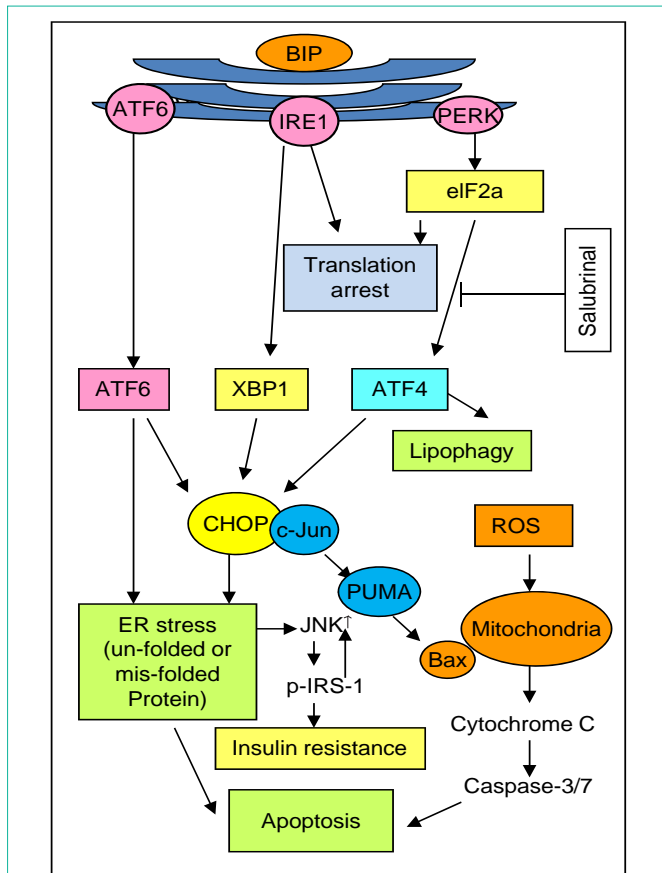


Figure 1: ER stress leads to insulin resistance and cell death through apoptosis. ATF4/6= activating transcription factor 4/6; Bax = B-cell lymphoma 2-associated X protein; BIP = Immunoglobulin heavy-chain binding protein; CHOP = CCAAT/enhancer-binding homologous protein; eIF2a = alpha subunit of the eukaryotic initiation factor 2; IRE1 = inositol-requiring enzyme 1; PERK: PKR-like endoplasmic reticulum kinase; PUMA = p53 upregulated modulator of apoptosis; XBP1 = X-box protein 1, p-IRC =phosphorylation of insulin receptor substrate (IRS). Salubrinal may inhibit the ES stress.

in the form of microlipophagy in hepatocytes as a normal route of lipid droplet clearance and energy source [5]. The increase in FFAs in hepatocytes results in lipotoxic stress in mitochondria and ER, overwhelming the production of reactive lipid intermediates (such as diacylglycerol and ceramide which may interfere insulin signaling), and Reactive Oxidant Species (ROS) from various subcellular compartments. The exposure of hepatoma cells to hydrogen peroxide (H₂O₂) led to reduced glycogen synthesis, and activation of c-Jun Kinase (JNK), and phosphorylation of Ser307 and Ser632 residues of Insulin Receptor Substrate-1 (IRS-1) in the insulin signaling pathway, thus, resulting in an interruption of normal insulin signaling through tyrosine phosphorylation and subsequent insulin resistance in these cells (Figure 1) [37]. We found that phosphorylation of AKT at threonine residue 308 was significantly decreased in the liver of mice fed diet containing Conjugated Linoleic Acid (CLA), and developed marked insulin resistance. Therefore, the reduced phosphorylation in AKT, a molecule critical for insulin signaling, may be partially responsible for insulin resistance in the mouse model of NASH [24].

A sustained increase in lipotoxic intermediates from excessive SFAs will shift normal Triglyceride (TG) synthesis towards the

induction of ER stress with the accumulation of misfolded or unfolded protein in the ER, which results in a translational arrest in protein synthesis and export. These adaptive changes are initiated with a dissociation of immunoglobulin heavy chain binding Protein (BiP), followed by the activation of three ER stress sensors: Activating Transcription Factor 6 (ATF6), Inositol Requiring Enzyme-1 (IRE1), and dsRNA-activated kinase-like ER Kinase (PERK), respectively, and are normal responses for recovery, so called the unfold protein response [38]. Failure to up-regulate these genes will lead to oxidant stress and accumulation of misfolded or unfolded proteins in the ER, which will cause hepatocellular damage by eliciting an apoptotic pathway (bax translocation into mitochondria) (Figure 1). The ER stress is linked to insulin resistance through the activation of JNK, which mediates the phosphorylation of Ser307 residue of IRS-1 [38-40]. In our previous study, we demonstrated that gene expression of proapoptotic CHOP (CCAAT/enhancer-binding homologous protein), an effector gene in the mediation of ER stress and a linking molecule between ER stress and apoptosis, was significantly up-regulated in an animal model of NASH caused by feeding diet containing CLA [24]. Whether enhanced ER stress is responsible for significant insulin resistance in this model is of great interest to investigate.

The consequence of lipotoxicity is cell death through apoptosis via several cascades including mitochondrial membrane permeability transition, cytochrome C release, death receptor activation, and Toll-Like Receptor-4 (TLR-4) activation [39]. The release of inflammatory signals, such as cytokines, ROS, chemokines, attractants and adipokines from various cell types in the liver provokes inflammatory responses in Kupffer cells and Hepatic Stellate Cells (HSCs). If the injury process persists, fibrotic response of HSCs exacerbates existing lipotoxic injury and worsens already-disordered lipid metabolism pathways [29], and may ultimately progress to a cirrhotic stage. This deteriorating loop as described in a former section accelerates the progression of NASH to ESLD with subsequent occurrence of life-threatening complications [41].

Lipophagy in normal liver and NASH

Autophagy is an evolutionary-conserved cellular process that mediates the degradation of bulk cytoplasm and entire organelles, including lipid droplets and mitochondria, and plays an important role in hepatic lipid homeostasis by degrading lipid droplets in the form of lipophagy [42]. Increased lipid accumulation triggers autophagy [42]. The engulfment of cytoplasmic materials will form the autophagosomes which finally merge with lysosomes for further digestion (Figure 2). During this process, Microtubule-Associated Protein-1 Light Chain 3β (MAP1LC3β) is a regulatory protein, which is present in autophagosomes until they fuse with lysosomes, and thus it has been used as a marker for autophagy. Any on-going disturbance in lipid metabolism may cause lipophagy dysfunction, presumably at the level of autophagosome-lysosome autofusion, which may promote additional lipid accumulation or increase in lipolysis by hepatic lipase in hepatocytes [39]. Lipophagy is an important pathway of lipid droplet clearance in hepatocytes, and the extent of lipophagy actually modulates the lipid content in hepatocytes, because suppression of lipophagy results in accumulation of lipid droplets. Hepatocytes are the normal site for TG storage in the liver. Unlike

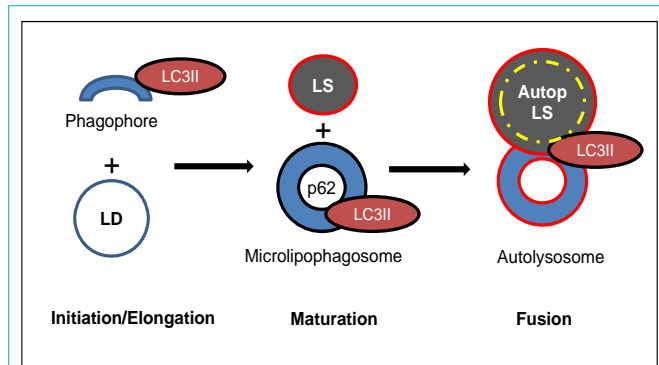


Figure 2: An assumed schematic illustration of the lipophagy process. With series of molecular activation during the initiation stage, the phagophore from an endoplasmic reticulum elongation membrane is formed, and with help of Atg7, LC3-I is converted to LC3-II, and Atg12-Atg5-Atg16L1 complex forces phagophore to elongate and integrate with LC3-II. During the maturation stage, the phagophore finally closes with lipid droplets (LD) forming a double membrane vesicle (autophagosome) containing the cargo targeted for degradation. The p62 is responsible for driving ubiquitinated proteins to the autophagosome, and its activity reflects the autophagy flux. The last step of autophagy is fusion of autophagosome with lysosome, forming the autolysosome. In this process, lysosomal hydrolytic enzymes are poured inside the microlipophagosome, degrading triglycerides into free fatty acids [48].

adipocytes, hepatocytes have much low activity of cytosolic lipase. Thus, the degradation of TG which is stored in a form of lipid droplets in hepatocytes is not as efficient as in adipocytes. Until recently, it has been found that hepatocytes break down lipid droplets through lipophagy as a pathway of endogenous lipid clearance in response to hormones or daily rhythms of nutrient supply [6]. Growing evidence exists that LC3-II, a marker of autophagosome, is co-localized with lipid droplets [43]. In another words, lipophagy may control energy homeostasis by providing Free Fatty Acids (FFAs) from breaking down of TG in lipid droplets within hepatocytes. More FFAs from breaking-down of TG drives mitochondrial production of ATP through FFA β -oxidation. The best example of hormonal control of lipophagy is the influence of Thyroidine (T3) on mitochondrial β -oxidation in the liver [44]. Interestingly, caffeine has been shown to stimulate lipophagy through increased β -oxidation and the fusion of autophagosomes with lysosomes [45].

Little is known regarding the changes of lipophagy under pathologic conditions, such as drug toxicity, Alcoholic Steatohepatitis (ASH) or NASH. Moreover, no study has analyzed the interplay of ER stress and autolipophagy under lipotoxicity, and their relationship with insulin resistance in hepatocytes during the course of NASH progression. Understanding the regulatory and signaling mechanism of lipophagy during lipid accumulation and lipotoxicity with the impact of insulin resistance is intriguing and will advance our insights into how lipid droplets are degraded in normal and pathologic conditions within hepatocytes. On one hand, excessive accumulation of lipid droplets in hepatocytes activates ATG5 in lipid droplets, and initiates a lipophagy process; on the other hands, increased influx of fatty acids in hepatocytes results in oxidant stress, ER stress and autophagy [4,24], as indicated by the fact that there is enhanced staining of LC3-II in NASH tissue [24,45]. However, lipophagy increased the susceptibility to oxidant stress-induced hepatocellular injury [46]. Autophagy flux was impaired in liver specimens of

NASH patients as indicated by increased level of p62, a substrate of autophagy (because activation of the autophagic flux leads to a decline in p62 expression), although there was enhanced staining of LC3-II in NASH tissue [47] (Figure 2). Therefore, the value of using LC3-II staining in tissue as an indication of autophagy or lipophagy is in question, and LC3-II does not precisely reflect autophagic flux [47]. In accordance with other studies, it was confirmed that increased ER stress correlates with the accumulation of p62, LC3-II and autophagosomes in liver tissue of animal models of NASH caused by feeding High Fat/high Claire (HFC) diet or Methionine/Choline-Deficient Diet (MCD). These changes were in parallel with extent of hepatic steatosis, inflammation and fibrosis [47]. Collectively, oxidant stress and ER stress initiate cellular death through apoptosis, and impaired autophagic flux in steatosis or steatohepatitis leaves the room for pharmacologic modulations, such as caffeine or rapamycin [45,48].

Activation of HSCs has been considered to be a key step for the initiation and persistence of fibrosis. The apoptosis is a critical end-point of activated HSCs, and participates in the weakening of hepatic fibrosis. Quiescent HSCs are full of fat droplets; and through activation and transition to myofibroblasts, these lipid droplets are disappeared. It has been recently confirmed that the autophagy is enhanced in activated HSCs, and autophagy inhibitors, such as bafilomycin A1, hydroxychloroquine and 3-methyladenine, suppressed HSC activation in vitro [49]. Thus, autophagy could be a potential target for inhibition of hepatic fibrosis during NASH [50]. Recently natural compounds, such as rutin, curcumin, antroquinonol and benzyl cinnamate, were found to be beneficial for suppressing fatty acid-induced HSC activation or in vivo activated HSCs isolated from thioacetamide-treated rats, probably through inhibition of autophagy in these cells [51].

The use of animal models of NASH for particular emphasis of pathologic pathways

To advance the current insights into the pathogenesis of NASH requires developing animal models to mimic the etiologic factors, pathologic process and natural histology. The most common models of NASH are mice fed MCD or HFC diet. The MCD diet causes hepatic steatosis with loss of body weight, and no insulin resistance occurs in these mice. The HFC diet causes obesity, hepatic steatosis with mild injury, fibrosis and insulin resistance. Genetic deficient ob/ob (leptin deficient) or db/db mice (defect in leptin receptor) [52] or Zucker rats [53] do not develop steatosis automatically, and often need to be fed either MCD or HFC diet [54]. More importantly, they do not represent the etiology and natural histology of NASH in humans. Therefore, more reliable animal models are needed to explore the molecular pathogenesis of NASH and to evaluate the efficacy and pharmacologic mechanisms of potential therapeutics. In our recent studies, we fed normal mice a diet containing CLA, which is transfat oil from margarine, for 8 weeks, and the mice developed severe hepatic steatosis, focal cell death, and pericellular fibrosis. At the same time, hepatic LDL-cholesterol and TG levels were increased. In contrast, circulating leptin and adiponectin levels were reduced dramatically [55]. Moreover, fasting insulin levels were much higher than in mice fed a control diet. As a result, the CLA diet-fed mice exhibited profound insulin resistance as evidenced by a marked increase in fast insulin levels and Homeostatic Model Assessment of

Insulin Resistance (HOMA-IR) [24,55]. Thus, this NASH model is superior to other NASH models currently in use, and is particularly useful in dissecting the molecular mechanisms of hepatic injury caused by lipolysis products, as well as the mobilization of lipolysis products between adipose tissue and the liver under a circumstance of insulin resistance [24]. Moreover, to explore the possible molecular link between NAFLD/NASH and an increased incidence of HCC, relevant animal models are available from PTEN knock-out mice [56] to a STAM mouse model [57]. The latter develops steatohepatitis, fibrosis and carcinoma in progression by starting treatment with streptozotocin at day 2 and feeding the HFC diet at 4 weeks, and then NASH occurs at 8 weeks and cancer at 16 weeks [57,58]. HCC may be developed in HFC diet-fed mice when the feeding is extended long enough (over 9 months).

Conclusion and Prospects

NASH affects nearly 5-10% general population, and progresses to ESLD, and accounts for an increased incidence of HCC in the US. The prevalence in pediatric patients gives rise to a critical social problem, which should draw a special attention for its prevention and improvement of therapeutic efficacy. Multi-factorial feature of this disorder requires a deep understanding of interplays of various molecular pathways, development of therapeutic strategies that could act on multiple targets, and implementation of personalized remedies in clinical practice. The known molecular pathways playing critical roles in the initiation and progression of NASH include fat accumulation, oxidant stress, lipotoxicity, inflammation, insulin resistance and fibrosis, and they may vary individually and at the different stage of progression. The intriguing hypothesis of disordered gut microbiota points to a new direction of systems biology research in metabolic disorders, and metabolomics may provide new hints to pin-point pathophysiologic role of unknown molecules in various disease processes. To understand lipotoxicity at a molecular level, and to define the link between the lipotoxicity or oxidant stress and insulin resistance or cell death through apoptosis, the delineation of the molecular basis for ER stress gains a new solution to dissect complex interactions between subcellular organelles. Understanding the physiologic role of lipophagy aids in approaching to questions, such as how lipophagy is impaired in hepatocytes; whereas it is enhanced in hepatic stellate cells in the liver with fat accumulation, and what molecular targets should be chosen when intervening the lipophagic process? Selecting a right animal model of NASH would allow to answer specific questions in pathophysiology of the disorder, and to assess the pharmacologic effects of potential therapeutics. It should be kept in mind that specific etiology treatment for NASH has not been available yet, and it is in demand that clinical trials that evaluate the efficacy of combined therapeutics can be reasonably designed and executed in addition to single candidate drug trial, although the trials in combination of multiple therapeutics are more complicated than those with single remedy. Collective efforts from multidisciplinary teams should be focused on better understanding of NASH pathogenesis and improving the treatment outcome, and block its progression to ESLD.

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